Original Article

Lymphocytic Infiltration and Immune Activation in Metallothionein Promoter–Exendin-4 (MT-Exendin) Transgenic Mice

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Glucagon-like peptide 1 (GLP-1) exhibits considerable potential for the treatment of type 2 diabetes because of its effects on stimulation of insulin secretion and the inhibition of gastric emptying, appetite, and glucagon secretion. However, native GLP-1 undergoes rapid enzymatic inactivation, prompting development of long-acting degradation-resistant GLP-1 receptor agonists such as exendin-4 (Ex-4). To study the consequences of sustained exposure to Ex-4, we generated metallothionein promoter–exendin-4 (MT-Exendin) mice that continuously express a proexendin-4 transgene in multiple murine tissues. We now report that MT-Exendin mice develop extensive tissue lymphocytic infiltration with increased numbers of $CD4^+$ and $CD8a^+$ cells in the liver and/or kidney and increased numbers of $B220^+$ cells present in the pancreas and liver. MT-Exendin mice generate antibodies directed against Ex-4, exendin NH_2-terminal peptide (ENTP), and proexendin-4 as well as antibodies that cross-react with native GLP-1. Furthermore, lymphocytes isolated from MT-Exendin mice proliferate in response to proexendin-4 but not after exposure to Ex-4 or ENTP. These findings demonstrate that expression of a proexendin-4 transgene may be associated with activation of humoral and cellular immune responses in mice. *Diabetes* 55:1562–1570, 2006

Glucagon-like peptide 1 (GLP-1) lowers blood glucose through several distinct mechanisms that include amplification of glucose-stimulated insulin secretion and inhibition of both glucagon secretion and gastric emptying. In diabetic rodents, GLP-1 increases β-cell mass via proliferative, neogenic, and antiapoptotic actions (1). GLP-1 also has anorectic effects, and short-term infusions of GLP-1 promote satiety and reduce food intake in normal, obese, and diabetic humans (2). Consequently, there is considerable interest in the therapeutic use of GLP-1 for the treatment of diabetes.

Despite the efficacy of continuous GLP-1 administration for the treatment of type 2 diabetic patients (3), the clinical potential of the native peptide is hampered by its very short plasma half-life, due to rapid inactivation of GLP-1 by the ubiquitous protease dipeptidyl peptidase-4 (DPP-4) (4) and by renal clearance. Hence, current GLP-1–based therapies are focused on the use of GLP-1 mimetics with protracted action, including the lizard peptide exendin-4 (Ex-4), a DPP-4–resistant GLP-1 receptor agonist that can reduce both fasting and postprandial glucose levels in type 2 diabetic patients (5). Complementary strategies aimed at prolonging the half-life and reducing the renal clearance of GLP-1 include the development of GLP-1–based analogs such as liraglutide, a DPP-4–resistant fatty-acylated GLP-1 molecule that binds noncovalently to serum albumin and exhibits more potent and sustained glucose-lowering effects compared with native GLP-1 (6).

Although clinical studies illustrate the beneficial effects of GLP-1 receptor agonists for blood glucose regulation in diabetic patients (3,7–9), the requirement for continuous infusion or repeated injections to maintain the efficacy of these agents has stimulated interest in the development of alternative approaches to achieve sustained GLP-1 receptor activation. For example, cell-based delivery using cells genetically engineered to produce a DPP-4–resistant GLP-1 analog has demonstrated persistent improvements in glucose control after implantation of GLP-1–producing cells into diabetic mice (10). Alternatively, administration of GLP-1 analogs via gene therapy approaches provides additional means to achieve increased circulating levels of GLP-1 receptor agonists for the treatment of diabetes.

To assess the efficacy of continuous Ex-4 delivery for the control of glucose homeostasis, we previously generated and analyzed metallothionein promoter–exendin-4 (MT-Exendin) transgenic mice (11,12). These mice express the lizard proexendin-4 cDNA (including exendin NH_2-terminal peptide [ENTP] linked to Ex-4) under the control of the mouse metallothionein-I promoter (Fig. 1) (11). MT-Exendin mice express proexendin-4 mRNA in multiple tissues and process the proexendin-4 precursor to Ex-4, resulting in detectable circulating levels of bioactive Ex-4 (11). After induction of transgene expression, MT-Exendin mice exhibit reduced glycemic excursion and increased plasma insulin levels in response to a glucose challenge (11).

MT-Exendin mice have also proven useful for studies of proexendin-4 processing (12) and elucidation of the consequences of sustained Ex-4 production on GLP-1 receptor

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ConA, concanavalin A; DPP-4, dipeptidyl peptidase-4; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; ENTP, exendin NH_2-terminal peptide; Ex-4, exendin-4; GLP-1, glucagon-like peptide 1; GST, glutathione S-transferase; MT-Exendin, metallothionein promoter–exendin-4; ssDNA, single-stranded DNA; TBS-T, Tris-buffered saline containing 0.1% Tween-20.

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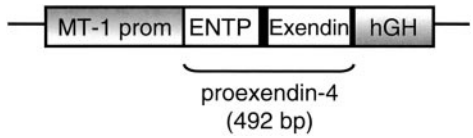

FIG. 1. MT-Exendin transgene structure. The *Heloderma suspectum* proexendin-4 cDNA (492 bp) was cloned into the pEV142 expression vector, downstream of the mouse metallothionein-1 promoter (MT-1) and upstream of 3′-flanking sequences from the human growth hormone (hGH) gene (11).

activation and desensitization (13). In the course of our studies using MT-Exendin mice, we have periodically observed the development of nonspecific illness in association with tissue lymphocytic infiltration detected at autopsy. These findings raised the possibility that MT-Exendin mice are prone to the development of immune abnormalities, possibly as a result of expression of a foreign transgene protein. To elucidate the mechanisms responsible for the sporadic illness and abnormal pathology in MT-Exendin mice, we have carried out a histological and immunological characterization of MT-Exendin and control mice from 2 to 12 months of age. Our findings suggest that transgenic expression of proexendin-4 is associated with immune system activation and mononuclear cell infiltration in MT-Exendin mice as young as 2 months of age.

RESEARCH DESIGN AND METHODS

Reagents. Ex-4 and GLP-1 were purchased from California Peptide Research (Napa, CA). ENTP was provided by NPS Pharmaceuticals (Mississauga, ON, Canada). Recombinant proexendin-4 was expressed and purified as a glutathione S-transferase (GST)–proexendin-4 fusion protein by cloning a 492-bp cDNA encoding lizard proexendin-4 (14) into the bacterial expression vector pGEX-4T-2 (Amersham Biosciences, Piscataway, NJ). Normal mouse serum, concanavalin A (ConA), and mouse CD4, CD8a, CD11b (Mac-1), and B220 monoclonal antibodies were purchased from Cedarlane Laboratories (Hornby, ON, Canada). Goat anti-mouse horseradish peroxidase conjugate, 3′,3′,5′,5′-tetramethylbenzidine substrate, and stop solution were from Alpha Diagnostic International (San Antonio, TX). Dulbecco's modified Eagle's medium, GST, and rabbit anti-GST antibody were obtained from Sigma (St. Louis, MO).

Mice. MT-Exendin transgenic mice express the lizard proexendin-4 cDNA under the control of an inducible mouse metallothionein-I promoter (Fig. 1). The generation and characterization of two independent lines (L18 and L19) of MT-Exendin transgenic mice on a C57BL/6 × SJL genetic background has been described previously (11). In these animals, proexendin-4 mRNA is expressed in multiple tissues, and basal circulating plasma levels of Ex-4 range between 250–475 and 50–200 pg/ml for L19 and L18 mice, respectively (11). However, the SJL genetic background is associated with increased susceptibility to experimental autoimmune encephalomyelitis (15), reduced natural killer cell activity, deficits in natural killer T-cell number and function (16,17), and a high incidence of both spontaneous malignant B-cell lymphoma and myositis (18,19). Thus, to avoid confounding issues related to genetic background, immunological studies were carried out using MT-Exendin transgenic mice that were backcrossed onto the C57BL/6 genetic background for six generations. In MT-Exendin mice maintained on the C57BL/6 genetic background, plasma levels of circulating Ex-4 range from 12 to 18 pg/ml in L19 mice (13), whereas plasma levels of Ex-4 in L18 MT-Exendin mice are generally below the lower limit of detection using an assay with a detection limit of 10 pg/ml. All studies were carried out using male and female mice from both lines of MT-Exendin transgenic mice, unless otherwise specified. Control animals included age- and sex-matched transgene-negative mice from the same litter or family or age- and sex-matched wild-type C57BL/6 mice purchased from Charles River Laboratories (Montreal, PQ, Canada). Wild-type mice were allowed to acclimatize to the animal facility for a minimum of 1 week before analysis. All mice were housed under specific pathogen-free conditions in microisolator cages and maintained on a 12-h light/dark cycle with free access to standard rodent diet and water. All experiments were carried out in accordance with protocols and guidelines approved by the Toronto General Hospital Animal Care Committee.

Immunohistochemistry. Tissues were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A., Torrance, CA), frozen rapidly in a dry ice/ethanol bath, and then stored at −80°C. Frozen tissues were cut into 5-μm sections and stained with hematoxylin and eosin using standard techniques. Frozen sections were acetone fixed and immunostained with mouse CD4 (1:100), CD8a (1:100), CD11b (Mac-1; 1:100), or B220 (1:150) monoclonal antibodies using a conventional avidin-biotin-peroxidase method. The number of immunopositive cells within each section was counted and expressed as number of immunopositive cells per square millimeter tissue. For each tissue, the number of immunopositive cells per square millimeter was averaged from two different sections that were obtained from the same block of tissue sectioned at two different levels.

Measurement of microalbumin and creatinine levels. Urine samples were collected from individual mice, and microalbumin and creatinine levels were measured using a DCA 2000+ Analyzer (Bayer, Toronto, ON, Canada).

Detection of anti–double-stranded DNA and anti–single-stranded DNA antibodies. The presence of anti–double-stranded DNA (anti-dsDNA) or anti–single-stranded DNA (anti-ssDNA) antibodies was assessed in mouse serum using specific commercially available enzyme-linked immunosorbent assay (ELISA) kits (Alpha Diagnostic International, San Antonio, TX).

Detection of antibodies to Ex-4, ENTP, GLP-1, or proexendin-4 in mouse serum. Control or MT-Exendin transgenic mouse serum was analyzed for antibodies to Ex-4, ENTP, GLP-1, or proexendin-4 by ELISA. Microtest 96-well ELISA plates (Becton Dickinson, Franklin Lakes, NJ) were coated with 100 μl buffer or peptide (10 μg/ml in 0.05 mol/l sodium carbonate buffer, pH 9.6) at 4°C overnight. For detection of serum anti–proexendin-4 antibodies, ELISA plates were coated with GST–proexendin-4. To rule out nonspecific binding of mouse serum to the GST moiety of GST–proexendin-4, control wells were coated with GST alone and included for each sample. Excess peptide was removed by washing wells three times with Tris-buffered saline containing 0.1% Tween-20 (TBS-T), and unbound sites were blocked for 1 h at room temperature using 2.5% skim milk dissolved in TBS-T. Mouse serum was diluted 1:100 with blocking buffer, 100 μl diluted serum was added to each well, and plates were incubated overnight at 4°C. Wells were washed with TBS-T to remove unbound antibodies, and 100 μl goat anti-mouse horseradish peroxidase conjugate, diluted 1:5,000 in TBS-T, was added to each well. Plates were incubated for 3 h at 37°C and then washed with TBS-T. Bound antibody was visualized by incubating with 50 μl 3′,3′,5′,5′-tetramethylbenzidine chromogenic substrate for 5 min at room temperature, followed by termination of the reaction by the addition of 50 μl stop solution and then measuring the absorbance at 450 nm.

Peptide immunization experiments. Peptides (Ex-4, ENTP, or GLP-1) were dissolved in PBS, and 8-week-old male and female wild-type C57BL/6 mice were immunized by intraperitoneal injection with 0.l ml PBS or peptide (50 μg) emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI). Subsequent immunizations consisted of 0.1-ml i.p. injections of PBS or 50 μg peptide emulsified with an equal volume of incomplete Freund's adjuvant (Difco Laboratories). Mice were immunized biweekly for 1 month and then once per month until 6 months of age. In a second study, 8-week-old wild-type C57BL/6 male and female mice were immunized via intraperitoneal injection of PBS or 1 μg Ex-4 or GLP-1 in the absence of Freund's adjuvant. Mice were repeatedly injected with the peptides according to the above schedule, except that immunizations were carried out until mice were 8 months old. Immediately before and throughout both immunization studies, serum was prepared from blood samples collected from the tail vein, and the presence of antibodies was determined by ELISA as described above.

Cell proliferation assay. Spleens were excised from 4-month-old control C57BL/6 mice, dispersed into single-cell suspensions, and then treated with Gey's solution to lyse erythrocytes. Splenocytes were irradiated with 3,000 rads using a Gammacell 3000 Elan (Nordion International, Vancouver, British Columbia, Canada). Mesenteric, inguinal, and axillary lymph nodes were removed from 4-month-old control or MT-Exendin transgenic mice, and single-cell suspensions were prepared in Dulbecco's modified Eagle's medium (lacking phenol red) supplemented with 0.5% normal mouse serum. Lymphocytes were seeded into 96-well flat-bottom tissue culture plates at a density of 5×10^5cells/well and cultured with 5×10^5 cells/well irradiated splenocytes. One hundred microliters medium alone or medium containing Ex-4 (10 μg/ml), ENTP (10 μg/ml), GST–proexendin-4 (1 or 10 μg/ml), or ConA (1 μg/ml) was then added, and cells were incubated for 72 h at 37°C. Cell proliferation rates were determined using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI), and stimulation indexes were calculated by dividing the proliferation rate of treated cells by the proliferation rate of the medium-only control sample.

Statistical analysis. Statistical significance was determined by χ^2 test for independence or one-way ANOVA and Bonferonni's post hoc test. All statistical analyses were carried out using Prism Version 3.03 software (GraphPAD

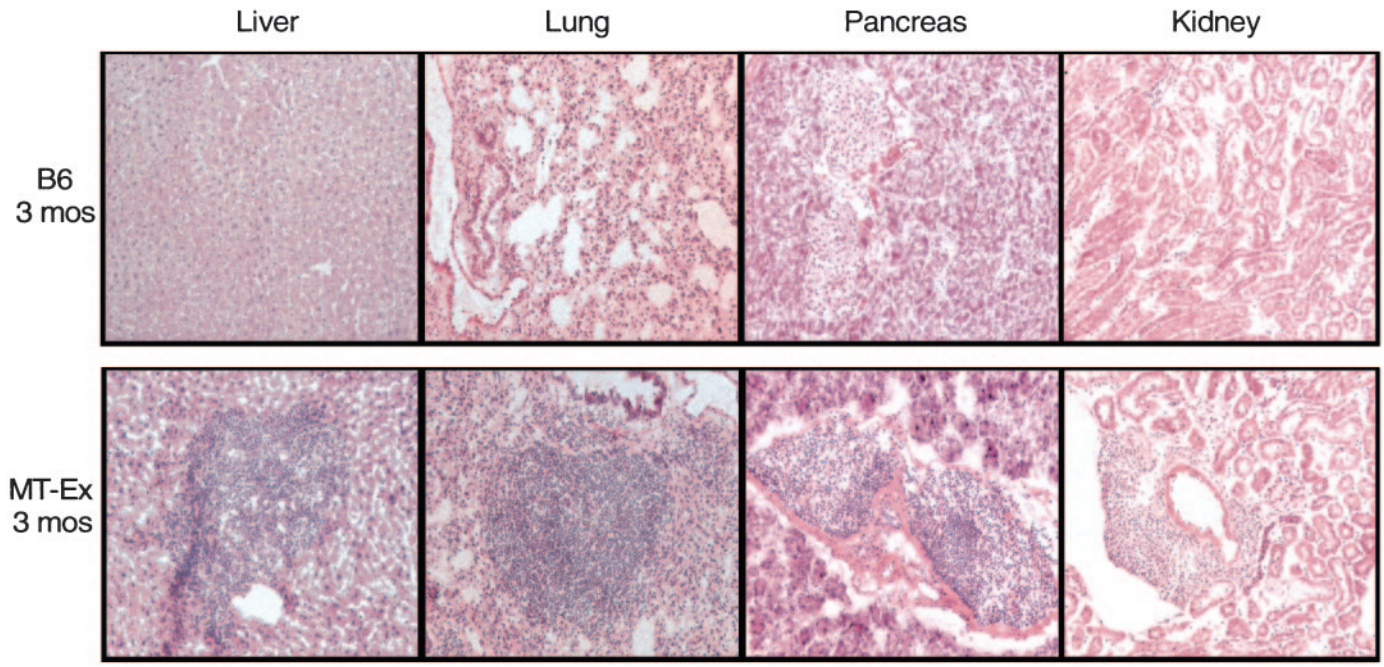

FIG. 2. Lymphocytic infiltrates are found in several tissues from MT-Exendin transgenic mice. Representative photomicrographs of tissues from 3-month-old L19 MT-Exendin (MT-Ex) and C57BL/6 (B6) wild-type control female mice stained with hematoxylin and eosin. Original magnification ×20.

Software, San Diego, CA) and a P value <0.05 was considered to be statistically significant.

RESULTS

MT-Exendin transgenic mice exhibit increased tissue lymphocytic infiltration. Preliminary observations of histopathological abnormalities and severe lymphocytic infiltrations in multiple tissues from MT-Exendin transgenic mice on the SJL genetic background (data not shown) prompted us to examine the phenotype of these mice on the C57BL/6 genetic background. Immunohistochemical analysis demonstrated that MT-Exendin transgenic mice on the C57BL/6 background exhibited increased lymphocytic infiltration in several tissues when compared with both wild-type C57BL/6 and transgene-negative littermate control mice (Fig. 2). We subsequently quantified the number of tissues exhibiting lymphocytic infiltrates in male and female mice from each group (L18 and L19 MT-Exendin, transgene-negative littermate controls, and wild-type C57BL/6 mice) on a monthly basis, from 2 to 10 months of age, with a focus on pancreas, kidney, lung, and liver. Although all groups of mice displayed at least some detectable tissue infiltration by 10 months of age, both male and female MT-Exendin mice exhibited a significantly greater cumulative incidence of lymphocytic infiltrates, relative to either group of control mice, with infiltrating lymphocytes appearing at an earlier age in the transgenic mice (Table 1; data not shown).

To identify the nature of the infiltrating lymphocytes, tissue sections from 3- and 4-month-old L19 MT-Exendin and control mice were stained with T-cell–, B-cell–, and macrophage-specific markers (Fig. 3*A*–*D*), and the number of immunopositive cells per square millimeter tissue was determined. Significantly greater numbers of CD11b(Mac-1)–immunopositive (macrophage marker) cells were detected in the pancreas, liver, and kidney of 3- and 4-month-old MT-Exendin transgenic mice compared with wild-type and transgene-negative littermate controls (online appendix Table 1 [available at http://diabetes.diabetesjournals.org]). Relative to control mice, increased numbers of $CD4^+$ and $CD8a^+$ (T-cell marker) cells were found in the liver and/or kidney, whereas greater numbers of $B220^+$ (B-cell marker) cells were present in the pancreas and liver of 3- or 4-month-old MT-Exendin mice (online appendix Table 1). Extensive lymphocytic infiltrates, comprising a mixture of CD4-, CD8a-, B220-, and Mac-1–immunopositive cells, were also observed in the lungs of some but not all MT-Exendin mice (Figs. 2 and 3; online appendix Table 1).

TABLE 1
Cumulative incidence of tissue lymphocytic infiltrates in male and female control and MT-Exendin mice from 2 to 4 months of age

	Incidence		
Genotype	2 months	3 months*	4 months*
Males			
B6	0/6 (0)	0/12 (0)	3/18 (17)
Tg. Neg.	0/5 (0)	0/12 (0)	1/18 (6)
MT-Ex L19	2/11 (18)	5/22 (23)†	9/32 (28)
MT-Ex L18	0/7 (0)	3/16 (19)	6/26 (23)
Females			
B6	0/6 (0)	1/12 (8)	2/18 (11)
Tg. Neg.	1/7 (14)	1/13 (8)	2/18 (11)
MT-Ex L19	3/10 (30)	9/21 (43)†	17/33 (52)‡
MT-Ex L18	1/7 (14)	3/14 (15)	8/23 (35)

Data are n (%). Liver, pancreas, lung, and kidney sections from L19 and L18 MT-Exendin (MT-Ex L19 and MT-Ex L18), C57BL/6 (B6), and transgene-negative (Tg. Neg.) mice were stained with hematoxylin and eosin, and the percentage of mice with lymphocytic infiltrates in one or more tissues was determined. *Data at 3 and 4 months corresponds to the combined data from 2- and 3-month-old and 2-, 3-, and 4-month-old mice, respectively. †,‡$P < 0.05$ and 0.01 for MT-Ex L19 vs. B6 and Tg. Neg. control mice, respectively.

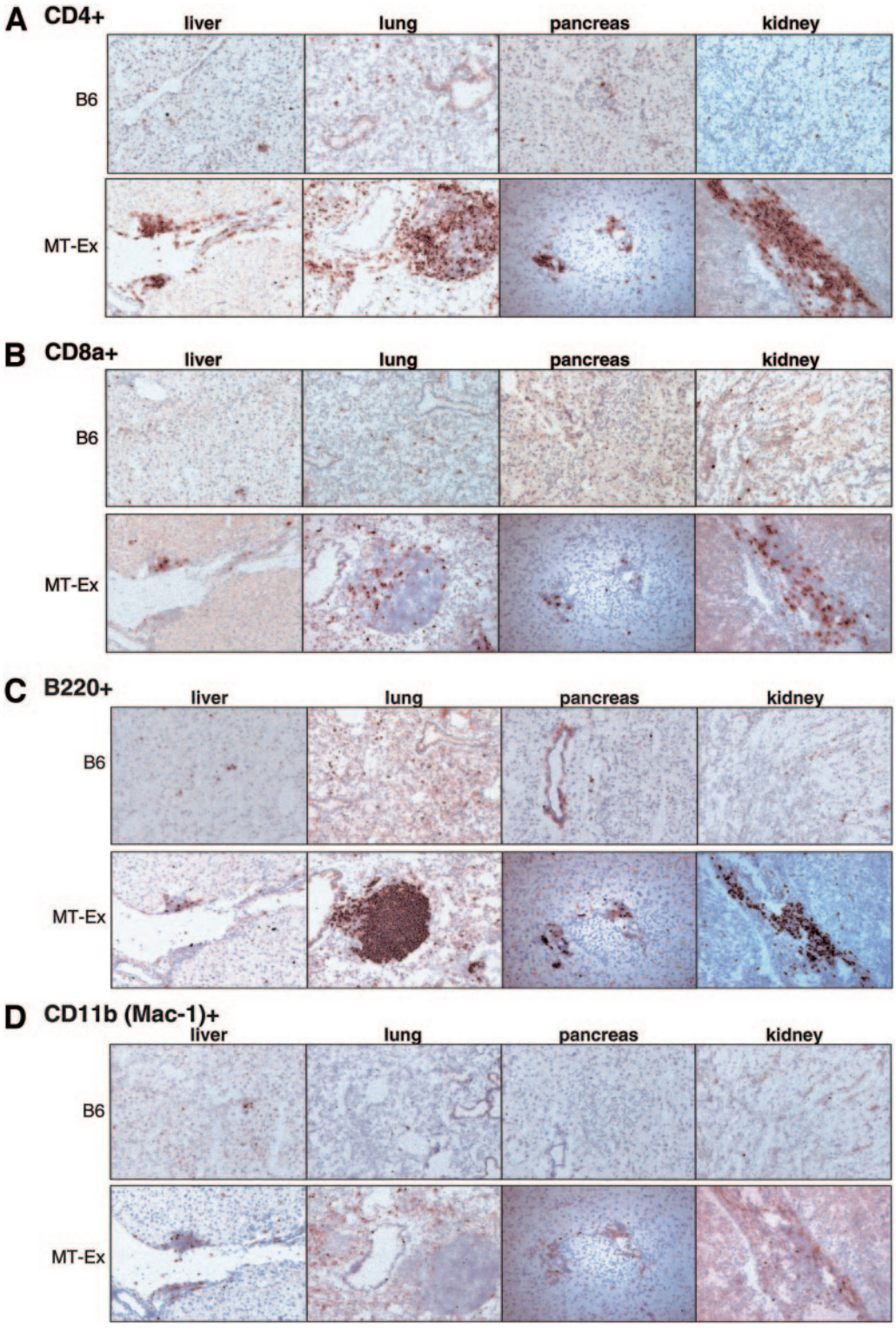

FIG. 3. MT-Exendin tissues have greater numbers of infiltrating T-cells, B-cells, and macrophages relative to control mice. Representative photomicrographs of tissues from 3-month-old L19 MT-Exendin (MT-Ex) and C57BL/6 (B6) wild-type control female mice stained with monoclonal antibodies specific for $CD4^+$ (*A*) and $CD8a^+$ (*B*) T-cells, $B220^+$ B-cells (*C*), or $CD11b\ (Mac\text{-}1)^+$ (macrophage) cells (*D*). Original magnification ×20.

MT-Exendin transgenic mice do not produce antibodies to ssDNA or dsDNA. To determine whether MT-Exendin transgenic mice show evidence of systemic autoimmunity, we looked for the presence of anti-ssDNA or anti-dsDNA antibodies in the serum of control and L19 MT-Exendin mice at different ages. There was no significant difference in the number of male or female MT-Exendin transgenic mice with detectable serum ssDNA or dsDNA antibodies relative to either C57BL/6 or transgene-negative control mice (data not shown).

MT-Exendin mice produce antibodies to Ex-4, ENTP, proexendin-4, and GLP-1. In previous studies of MT-Exendin mice, using a combination of high-performance liquid chromatography and radioimmunoassay analyses, we demonstrated that proexendin-4 is processed to mature bioactive Ex-4 in some, but not all, tissues, whereas the majority of circulating Ex-4–like immunoreactivity corresponds to Ex-4 (12). To determine whether MT-Exendin transgenic mice generate antibodies to proexendin-4–derived peptides, we used ELISAs to detect the

presence of Ex-4, ENTP, or proexendin-4 antibodies (defined as >2 SDs above the mean value obtained for C57BL/6 control mice at each age) in sera from both male and female control and MT-Exendin transgenic mice at different ages (Fig. 4*A* and *B*), and data from across the entire age range studied (2–10 months) was combined. Anti–Ex-4 antibodies were detected in a significantly greater number of MT-Exendin male and female transgenic mice relative to sex-matched control animals (0% of C57BL/6 males vs. 16% of L19 MT-Exendin males, $P < 0.001$; 0% of C57BL/6 females vs. 29% of L19 and 13% of L18 MT-Exendin females, $P < 0.001$ and 0.05 for L19 and L18 MT-Exendin females, respectively).

The prevalence of anti-ENTP antibodies was also greater in MT-Exendin mice compared with controls (0% of C57BL/6 males vs. 15% of L19 MT-Exendin males, $P < 0.01$; 0% of C57BL/6 females vs. 27% of L19 and 11% of L18 MT-Exendin females, $P < 0.001$ and 0.05 for L19 and L18 MT-Exendin females, respectively). In addition, relative to control mice, a comparatively greater proportion of both male and female MT-Exendin transgenic mice also exhibited anti–proexendin-4 antibodies (0% of C57BL/6 males and 5% of transgene-negative males vs. 19% of L19 MT-Exendin males, $P < 0.05$ for both; 0% of C57BL/6 females and 7% of transgene-negative females vs. 13% of L19 and 17% of L18 MT-Exendin females, $P < 0.05$ for L19 MT-Exendin vs. C57BL/6 females; $P < 0.001$ and 0.01 for L18 MT-Exendin vs. C57BL/6 and transgene-negative females, respectively).

To determine whether MT-Exendin mice produce antibodies that cross-react with GLP-1, we looked for the presence of anti–GLP-1 antibodies in the serum of MT-Exendin transgenic and control mice at 2 and 10 months of age (Fig. 4*A* and *B*). The combined data from both age-groups indicated that a significant number of MT-Exendin mice generate antibodies that recognize GLP-1 compared with control mice (0% of C57BL/6 males vs. 35% of L19 and 54% of L18 MT-Exendin males, $P < 0.05$ and 0.01 for L19 and L18 MT-Exendin males, respectively; 0% of C57BL/6 females vs. 43% of L19 MT-Exendin females, $P < 0.01$).

The nature of the antibody response in individual MT-Exendin mice was variable; some mice produced antibodies to only one of the tested antigens (Ex-4, ENTP, proexendin-4, or GLP-1), whereas other mice produced antibodies to more than one or to all of the tested antigens.

MT-Exendin transgenic mice have normal parameters of kidney function. Despite the presence of lymphocytic infiltrates in several tissues, MT-Exendin mice appeared healthy, even up to 10 months of age. Thus, to assess the physiological impact of the immune abnormality in MT-Exendin mice, we measured urine albumin and creatinine levels in male and female MT-Exendin, transgene-negative, and wild-type C57BL/6 control mice at different ages to evaluate renal function. No significant differences in albumin or creatinine levels were observed in MT-Exendin versus either group of control mice (data not shown).

MT-Exendin lymphocytes proliferate in response to proexendin-4 but not Ex-4 or ENTP. To determine whether lymphocytes from MT-Exendin mice are sensitized to Ex-4, ENTP, or proexendin-4, the proliferative response to these peptides was evaluated using isolated lymphocytes from L19 MT-Exendin and control mice. MT-Exendin transgenic lymphocytes, isolated from either male or female mice, were able to proliferate in response to proexendin-4 but not in response to either Ex-4 or ENTP treatment (Fig. 5; data not shown). Thus, despite the formation of antibodies to Ex-4, ENTP, and proexendin-4, activated lymphocytes from MT-Exendin mice appeared to be reactive against proexendin-4 only (Fig. 5). Surprisingly, proliferative responses to proexendin-4 were also observed in lymphocytes from both male and female transgene-negative littermate control mice (Fig. 5; data not shown).

Immunization with Ex-4 or ENTP in the presence of Freund's adjuvant induces antibody formation in wild-type mice. To determine whether antibody production against proexendin-4–derived peptides could be triggered in wild-type animals, we immunized male and female C57BL/6 control mice with Ex-4, ENTP, or GLP-1 and looked for the presence of specific antibodies using ELISAs. Antibodies were considered to be present when ELISA absorbance values at 450 nm were >2 SDs above the mean value obtained for PBS-immunized mice at each age. In the first experiment, immunizations (PBS, Ex-4, ENTP, or GLP-1) were carried out in the presence of Freund's adjuvant. In a second experiment, immunizations (PBS, Ex-4, or GLP-1) were performed using peptide or vehicle alone. In the presence of Freund's adjuvant, immunization of female mice with Ex-4 resulted in transient production of antibodies to Ex-4 (Fig. 6; $P < 0.05$ at 2.5 and 4 months and $P < 0.01$ at 5 months for Ex-4– vs. PBS-immunized females), whereas immunization of males with ENTP, but not Ex-4, lead to the transient generation of antibodies to Ex-4 (Fig. 6; data not shown; $P < 0.01$ at 4 months for ENTP- vs. PBS-immunized males). Although mice immunized with Ex-4 or ENTP did not develop antibodies to GLP-1, a significant number of male and female mice immunized with GLP-1 developed antibodies to GLP-1 (data not shown; $P < 0.01$ at 4 and 6 months and $P < 0.01$ at 6 months for GLP-1– vs. PBS-immunized males and females, respectively). In contrast, repeated injections of Ex-4 or GLP-1 in the absence of Freund's adjuvant did not lead to antibody formation in either male or female mice (data not shown).

DISCUSSION

We have demonstrated that transgenic expression of proexendin-4 is associated with the development of lymphocytic infiltration and immune system activation in MT-Exendin transgenic mice. Moreover, the immune response in MT-Exendin mice appears to be T-cell mediated, as lymphocytic infiltrates were found in multiple tissues. However, despite the occurrence of lymphocytic infiltrates, MT-Exendin mice do not appear to have a general breakdown in tolerance as evidenced by a lack of anti-ssDNA or anti-dsDNA antibody production. Furthermore, the development of lymphocytic infiltrates was sporadic and varied considerably in mice of different ages.

MT-Exendin transgenic mice develop antibodies to Ex-4; however, whether or not these antibodies are neutralizing was not determined. We also found that antibody production against proexendin-4–derived peptides can be triggered in wild-type mice upon repeated immunization with Ex-4 or ENTP in the presence, but not in the absence, of Freund's adjuvant. Ex-4 was originally isolated from the venom of the *Heloderma suspectum* lizard, and evidence to date indicates that mice and humans do not contain a gene encoding for an Ex-4–related peptide (14,20). Consequently, it is not surprising that Ex-4 is recognized as a

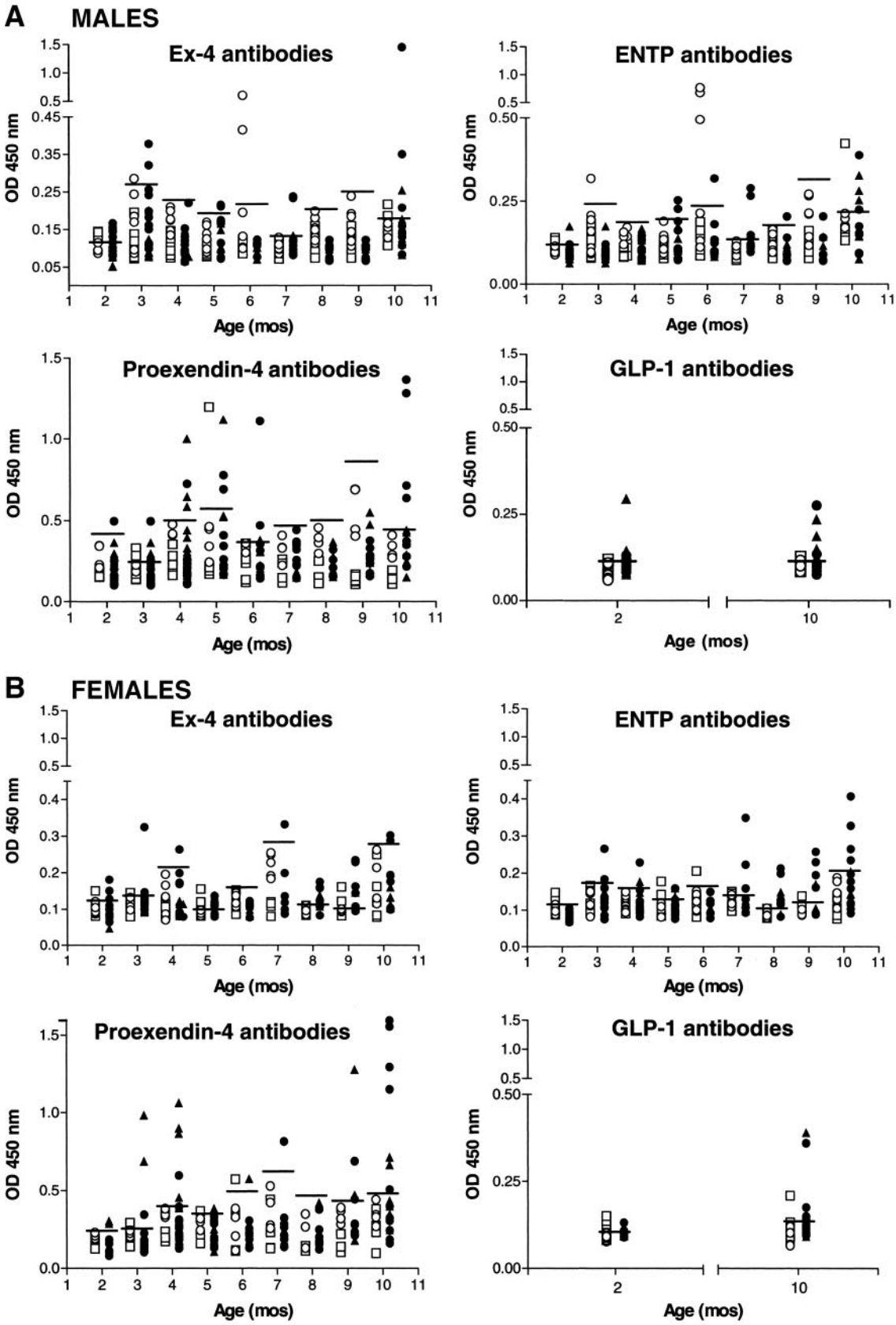

FIG. 4. MT-Exendin mice produce antibodies to Ex-4, ENTP, proexendin-4, and GLP-1. Absorbance values at 450 nm for detection of antibodies to Ex-4, ENTP, proexendin-4, and GLP-1 in male and female MT-Exendin (L19 [●] and L18 [▲]), C57BL/6 (○), and transgene-negative (Tg−) (□) mice at different ages. For each antibody (Ex-4, ENTP, proexendin-4, or GLP-1), the combined total of antibody-positive MT-Exendin transgenic mice across the entire range of age-groups was compared with wild-type C57BL/6 or transgene-negative controls. Each symbol represents an absorbance value for a single mouse. n = 2–15 mice per age-group. The horizontal line at each age represents 2 SDs above the mean value for C57BL/6 control mice. OD, optical density.

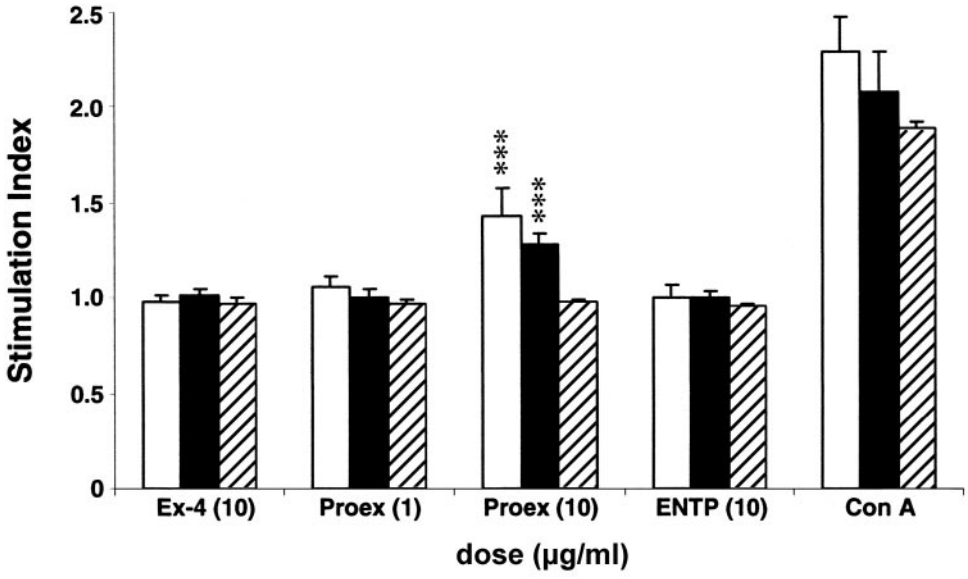

FIG. 5. Lymphocytes from MT-Exendin mice proliferate in response to proexendin-4 but not Ex-4 or ENTP. Lymphocytes from 4-month-old L19 MT-Exendin (L19; ■), transgene-negative (Tg−; □), or wild-type C57BL/6 (B6; ▨) male mice were incubated in medium alone or in the presence of 10 μg/ml Ex-4 (10), 1 μg/ml (1) or 10 μg/ml (10) proexendin-4 (Proex), or 1 μg/ml ConA. Cell proliferation rate was determined, and the stimulation index relative to control (medium alone) was calculated. n = 6–8 mice per group per treatment.

foreign peptide by the murine immune system. Similarly, human diabetic subjects treated with twice-daily Ex-4 develop anti–Ex-4 antibodies in 41–49% of treated patients after 30 weeks of therapy (7–9). However, no adverse consequences attributable to antibody formation have been reported in human patients treated with Ex-4. Furthermore, in contrast to human subjects that are treated only with Ex-4, MT-Exendin transgenic mice generate ENTP and incompletely processed proexendin-4, in addition to processed 39–amino acid Ex-4 (12). Hence, the potential for immune activation in MT-Exendin mice is markedly different relative to the situation arising in human subjects treated with Ex-4.

MT-Exendin transgenic mice also produce antibodies that cross-react with GLP-1, likely as a result of the 53% amino acid identity shared by Ex-4 and mammalian GLP-1 (14). Furthermore, immunization with GLP-1, but not Ex-4 or ENTP, was associated with the production of anti–GLP-1 antibodies in wild-type mice. Whether Ex-4 treatment of human diabetic subjects is associated with the development of antibodies that cross-react against native human GLP-1 has not yet been reported.

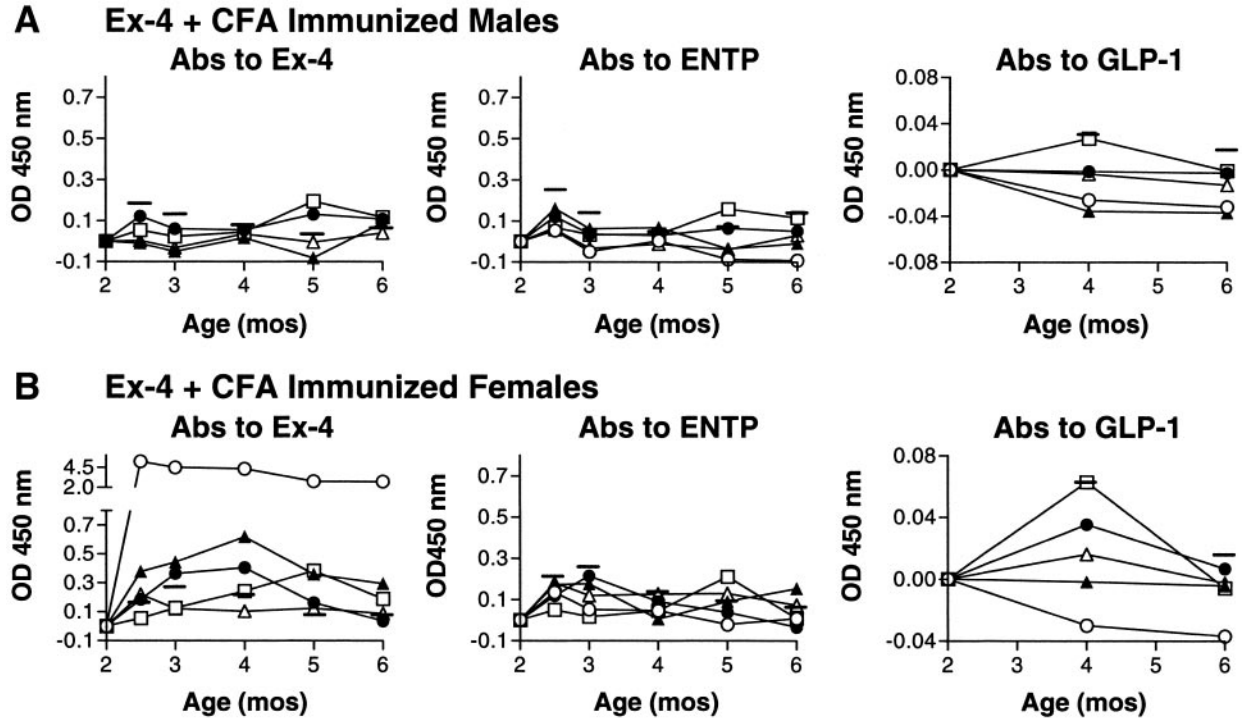

FIG. 6. Immunization with Ex-4 in the presence of Freund's adjuvant can induce antibody formation in wild-type mice. C57BL/6 male (*A*) and female (*B*) mice were given repeated intraperitoneal injections of 50 μg Ex-4 in the presence of Freund's adjuvant (CFA), and the development of antibodies to Ex-4, ENTP, or GLP-1 was assessed. Absorbance values at 450 nm were determined, and the baseline value (2 months) for each mouse was subtracted in all age-groups. Each line represents the antibody response of a single mouse over time. n = 4–6 mice per age. The horizontal line at each age represents 2 SDs above the mean value for PBS-immunized mice. OD, optical density.

To ascertain the potential basis for the development of lymphocytic infiltration in MT-Exendin mice, we determined whether transgenic lymphocytes were sensitized to one or more proexendin-4–derived peptides. Although we did not detect significant proliferation after exposure of isolated lymphocytes to Ex-4 or ENTP, lymphocytes derived from both male and female mice exhibit enhanced proliferation after exposure to proexendin-4, suggesting that sustained expression of proexendin-4 is not sufficient to establish tolerance in MT-Exendin transgenic mice. Remarkably, proexendin-4 also stimulated cell proliferation in lymphocytes isolated from transgene-negative littermate control mice. Because all transgene-negative mice used in these studies were the progeny of hemizygous proexendin-4 transgenic females, it is possible that small amounts of proexendin-4 are capable of crossing the placenta and sensitizing the immune system of transgene-negative progeny. In keeping with this possibility, we also found that some transgene-negative mice produced antibodies to Ex-4, ENTP, and GLP-1 (data not shown). These results imply that transgene-negative littermates may not be a perfect negative control for studies examining the potential immune response to transgenic proexendin-4 expression.

The molecular basis for the inability of MT-Exendin mice to develop tolerance to proexendin-4 or its processing products is not known. MT-Exendin mice express the proexendin-4 transgene in several tissues, with correct processing to mature bioactive Ex-4 detectable in the adrenals and testis (12). However, the processing profile of proexendin-4 in the thymus of MT-Exendin mice has not been determined. Because deletion or inactivation of potentially autoreactive T-cells in the thymus is believed to play a major role in preventing autoimmunity (21), it is possible that the profile of proexendin-4 processing in the thymus is not sufficient to induce tolerance to proexendin-4 or its products in other tissues and hence can lead to the development of tissue lymphocytic infiltrates.

The effectiveness of GLP-1 receptor agonists for the treatment of type 2 diabetes requires continuous infusion or repeated injections of these agents and, thus, has prompted the development of newer molecules with more prolonged pharmacokinetic profiles. Nevertheless, these agents may exhibit considerable differences in sequence relative to native GLP-1, as is the case with Ex-4, or may contain one or more modifications in the native GLP-1 sequence, as exemplified by the acylated human GLP-1 analog liraglutide (22). Moreover, several strategies for the development of long-acting GLP-1R agonists involve conjugation of peptides to albumin (23) or the generation of recombinant albumin–GLP-1 proteins (24). Alternatively, continuously elevated levels of circulating GLP-1 receptor agonists could be achieved using GLP-1–based gene therapy. Such approaches have been used successfully to generate bioactive GLP-1 or a GLP-1 analog using rodent insulinoma and HepG2 cells (25–27). Although the use of a proexendin-4 transgene as a means to achieve sustained GLP-1R agonist levels is clearly different from current clinical strategies that deliver GLP-1R agonists via exogenous injection, our studies in MT-Exendin transgenic mice highlight the potential for a foreign proexendin-4 protein to be associated with the development of antibody formation, lymphocyte activation, and tissue lymphocytic infiltration in vivo. Hence, further attention to and characterization of the potential immunogenicity related to therapeutic administration of structurally distinct GLP-1 receptor agonists, via delivery of exogenous proteins or potentially through gene therapy approaches, seems warranted.

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