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$CD4^+$ T cells, but not $CD8^+$ T cells, are required for the development of experimental autoimmune gastritis

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SUMMARY

Murine autoimmune gastritis, induced by neonatal thymectomy, is characterized by a mononuclear infiltrate within the gastric mucosa, loss of parietal and zymogenic cells and circulating autoantibodies to the gastric H/K ATPase. The infiltrate contains both $CD4^+$ and $CD8^+$ T cells. Here we have investigated the roles of $CD4^+$ and $CD8^+$ T cells in the development of gastritis by *in vivo* treatment with depleting rat anti-CD4 and anti-CD8 monoclonal antibodies. Depletion of $CD4^+$ T cells decreased the incidence of gastric mononuclear infiltrates from 63% (5/8), observed in normal rat immunoglobulin G (IgG)-injected mice, to 8% (1/12) and also abolished the production of antigastric autoantibodies. In contrast, depletion of $CD8^+$ T cells did not reduce the incidence of gastritis. The absence of $CD8^+$ T cells in the infiltrate of the stomach of anti-$CD8^+$-treated mice was confirmed by immunocytochemistry. These results argue that neonatal thymectomy-induced autoimmune gastritis is mediated by $CD4^+$ T cells and that $CD8^+$ T cells do not play a significant role in the development of the gastric lesion.

INTRODUCTION

Experimental autoimmune gastritis is an organ-specific autoimmune disease induced by thymectomy of genetically susceptible strains of mice, such as BALB/c, 2–4 days after birth.[1,2] Murine gastritis is characterized by mononuclear cell infiltrates in the gastric mucosa and circulating autoantibodies directed to the α- and the β-subunits of the gastric parietal cell H/K ATPase, the enzyme responsible for acid secretion into the stomach.[1–7] Experimental autoimmune gastritis is a cell-mediated disease, as the mononuclear cell invasion of the gastric mucosa occurs prior to the appearance of circulating autoantibodies to the gastric H/K ATPase[8] and adoptive transfer studies have demonstrated that the disease is mediated by T cells and not by the antiparietal-cell autoantibodies.[9,10] Furthermore, using transgenic strategies our previous studies demonstrated that a T-cell response to the H/K ATPase β-subunit is required for disease onset, indicating that this protein is an initiating autoantigen.[11]

Autoimmune gastritis has the hallmarks of an inflammatory autoimmune disease. We have demonstrated that the gastric mononuclear infiltrate contains both $CD4^+$ and $CD8^+$ T cells, as well as macrophages and B cells.[8] Furthermore, the early lesion is characterized by an influx of $CD4^+$ T cells and macrophages into the stomach mucosa.[8] Previous adoptive transfer studies suggest that $CD4^+$ T cells, but not $CD8^+$ T cells, play a major role in the induction of autoimmune gastritis.[10] However, the role of $CD8^+$ T cells in the development of gastritis following neonatal thymectomy has not previously been investigated. To define the contribution of $CD4^+$ and $CD8^+$ T cells to the development of autoimmune gastritis induced by neonatal thymectomy, we have carried out *in vivo* depletion studies of T-cell subsets. The results presented here clearly demonstrate that autoimmune gastritis is initiated by $CD4^+$ T cells and, furthermore, strongly suggest that $CD8^+$ T cells are not required for development of the gastric lesion.

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MATERIALS AND METHODS

Mice

Inbred BALB/c mice were supplied from the Monash University Animal Facilities and were maintained at the Monash Medical School Animal House.

Monoclonal antibodies

The depleting antibodies used in this study were GK1·5, a rat immunoglobulin G2b (IgG2b) antimouse CD4 monoclonal antibody[12] and YTS 169·4, a rat IgG2b antimouse CD8 monoclonal antibody.[13] The hybridomas were cultured in Dulbecco's modified Eagles medium (DMEM), containing 10% fetal calf serum (FCS), 2 mM L-glutamine and 100 U/ml penicillin–streptomycin, in 2 l roller bottles. Cultures were grown to stationary phase when the supernatant was harvested by centrifugation at 400 *g* and monoclonal antibodies purified by protein G–sepharose chromatography as described.[14]

Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). The specificity of the purified GK1·5 and YTS 169·4 monoconal antibodies was confirmed by flow cytometric analysis using defined $CD4^+$ (obtained from Dr F. Carbone) and $CD8^+$ (BW lyt 2·4)[15] cell lines. Polyclonal rat IgG (Sigma, Castle Hill, NSW, Australia) was used as the isotype-matched control.

Neonatal thymectomy

Mice were thymectomised on day 3 after birth under cold anaesthesia as previously described.[11]

In vivo *administration of antibodies to mice*

Mice were injected intraperitoneally from day 7 after birth and then weekly until 10 weeks of age. For the first 4 weeks, mice were injected with 0·3 mg of monoclonal GK1·5, YTS169·4 or polyclonal rat IgG (Sigma Chemicals), prepared in phosphate-buffered saline (PBS). After that time, mice received either 0·5 mg of GK1·5 or rat IgG, or 0·3 mg of YTS169·4. These doses were chosen based upon preliminary *in vivo* depletion experiments with these monoclonal antibodies.

Analysis of peripheral blood and splenocytes by flow cytometry

Peripheral blood (0·3 ml) was collected from the tail vein into heparinised tubes and red cells were lysed by mixing with water for 5 seconds. Splenocyte suspensions were prepared in PBS/10% FCS by gently teasing whole spleens though a wire mesh (pore size 200 μm). Spleen cells were collected by centrifugation and resuspended in 0·01 M Tris-HCl, pH 7·2 (containing 0·16 M NH_4Cl) for 10 min to lyse red blood cells. Cell suspensions (1×10^6 cells) were dually labelled with rat antimouse CD8-fluorescein isothiocyanate (FITC) (Becton Dickinson Franklin Lakes, NJ; diluted 1:100 in PBS containing 1% FCS, 0·02% azide) and rat antimouse CD4-phycoerythrin (Becton Dickinson; diluted 1:100 in PBS containing 1% FCS, 0·02% azide) for 30 min on ice. The cells were then washed once and resuspended in 0·5 ml of PBS (containing 1% FCS and 0·02% azide) and analysed using a fluorescence-activated cell sorter (FACScan) (Becton Dickinson). To detect the presence of injected monoclonal antibodies bound to T cells, splenic cell suspensions were directly labelled with (mouse adsorbed) sheep antirat IgG-FITC (diluted 1:100 in PBS containing 1% FCS and 0·02% azide; Silenus, Melbourne, Australia). Non-viable cells were excluded from data acquisition on the basis of forward and side light scatter and propridium iodide gating. Analyses were performed using LYSIS II research software (Beckon Dickinson).

Immunohistochemistry

Mice were killed by CO_2 asphyxiation and the stomachs removed. Each stomach was opened, rinsed in PBS, and divided in half through the body of the stomach. One half was fixed in formalin, embedded in paraffin and sections were stained with haematoxylin and eosin as described.[11] The other half of the stomach was mounted in Tissue-Tek II OCT embedding compound and snap frozen in isopentane cooled by liquid N_2. Serial 4 μm cryosections were air dried onto gelatinized slides for 30 min at room temperature, fixed in ice-cold acetone for 10 min and then air dried for 5 min. Sections were incubated in methanol containing 0·3% H_2O_2 for 10 min at room temperature to inactivate endogenous peroxidase activity. Sections were incubated with either GK1·5 or YTS169·4 hybridoma supernatant for 45 min at room temperature, washed twice in PBS/0·02% Tween 20, and incubated with biotinylated rabbit antirat immunoglobulin (diluted 1:50 in PBS; Vector Laboratories Inc., Burlingame, CA) for 45 min. After washing as above, sections were incubated in streptavidin-biotinylated horseradish peroxidase (diluted 1:100 in PBS; Amersham Life Sciences, Little Chalfont, Bucks, UK) for 45 min, and washed. Bound peroxidase was detected by a 10–20 min incubation with PBS/diaminobenzidine (2 mg/ml) nickel chloride (7 mg/ml)/H_2O_2 (0·03%). The reactions were stopped in tap water and sections counterstained with haematoxylin.

Immunofluorescence

Sera were tested for antiparietal cell autoantibodies by indirect immunofluorescence using paraffin-embedded mouse stomach sections as described.[16]

Gastric H/K ATPase enzyme-linked immunosorbent assay (ELISA)

An ELISA to detect antibodies directed to the gastric H/K ATPase was performed as previously described,[11] using purified pig H/K ATPase[17] and goat antimouse IgG-biotin (diluted 1:500 in PBS; Southern Biotechnology Birmingham, AL).

RESULTS

Depletion of CD4 and $CD8^+$ T cells by *in vivo* administration of monoclonal antibodies

To ensure that our purified preparations of GK1·5 and YTS 169·4 were able to deplete T cells *in vivo*, BALB/c mice were injected intraperitoneally with 0·5 mg of monoclonal antibody GK1·5 or 0·3 mg of YTS 169·4 and lymphocyte populations were analysed over a 2–3-week period. Three days after administration of GK1·5 there was a >99% reduction in the $CD4^+$ T-cell population whereas the $CD8^+$ T-cell population was unaffected (data not shown). Only a small number of splenocytes (<1%) were directly labelled with the sheep antirat IgG-FITC, excluding the possibility of a blocking effect by the injected antibody. Likewise, analysis of peripheral blood lymphocytes 2 days after administration of the YTS 169·4-treated mice showed a specific reduction of >99% of the $CD8^+$ T-cell population (data not shown). In both cases maximum reduction was observed for up to 8 days after administration of the respective monoclonal antibody.

To determine the roles of $CD4^+$ and $CD8^+$ T cells in the development of autoimmune gastritis, neonatally thymectomised BALB/c mice were treated with either GK1·5 or YTS 169·4 from day 7 after birth until 10 weeks of age. A control group of thymectomised mice were treated with an equivalent mass of normal rat IgG. At 12 weeks after thymectomy, mice were killed and splenic T cells analysed and mice assessed for gastric autoimmunity.

Analysis of splenic T cells at completion of the experiment (i.e. 2 weeks after the final antibody treatment) showed that all mice treated with the anti-CD4 monoclonal antibody had a greatly reduced number (10-fold reduction) of $CD4^+$ T cells compared with mice treated with rat immunoglobulin (Fig. 1),

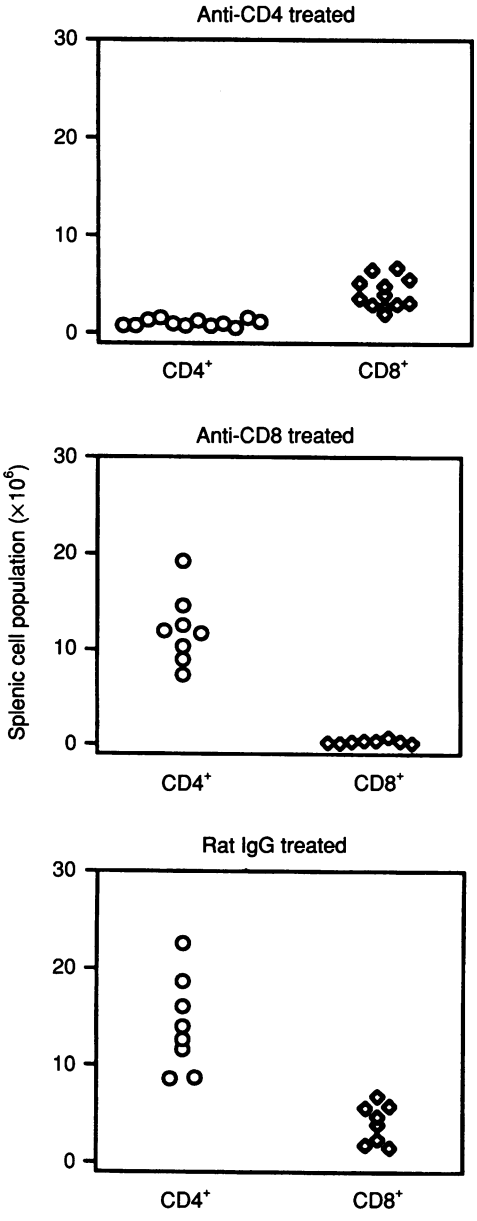

Figure 1. Analysis of splenic T cells from mice treated with depleting antibodies. BALB/c mice were neonatally thymectomised and injected weekly with either anti-CD4 monoclonal antibody, anti-CD8 monoclonal antibody or polyclonal rat IgG for 10 weeks as described in the Materials and methods. Two weeks after the final antibody treatment mice were killed and splenocytes were analysed by flow cytometry for $CD4^+$ and $CD8^+$ cells. Each symbol represents data from an individual mouse.

whereas the number of $CD8^+$ T cells in these treated mice was the same as in the control group.

Mice treated with the anti-CD8 antibody had a 15-fold reduction in the $CD8^+$ T-cell population compared with mice treated with rat IgG (Fig. 1), whereas the number of $CD4^+$ T cells was normal.

Of the eight rat-IgG-injected mice, five (63%) had circulating antiparietal cell antibodies detected by indirect immunofluorescence staining of stomach sections (Fig. 2) and autoantibodies specific for the H/K ATPase detected by

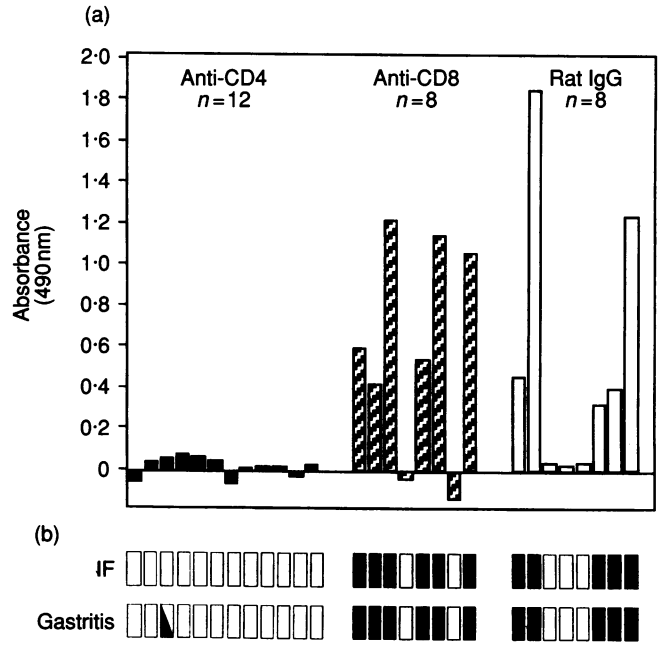

Figure 2. H/K ATPase autoantibodies and gastritis in neonatally thymectomised mice treated with anti-CD4 and anti-CD8 monoclonal antibodies. BALB/c mice were neonatally thymectomised and injected weekly with either anti-CD4 monoclonal antibody, anti-CD8 monoclonal antibody or polyclonal rat IgG for 10 weeks. Two weeks after the final antibody treatment, sera were collected and mice killed for histological examination. (a) Anti-H/K ATPase antibodies were detected by ELISA; filled bars indicate injection with anti-CD4, stripped bars indicate injection with anti-CD8 and empty bars indicate injection with rat IgG. (b) Immunofluorescence (IF) to detect antiparietal cell antibodies and histological examination of stomachs of mice for gastritis. Mice are represented in the same order as in (a) and are shown directly below the corresponding ELISA readings. The presence of parietal cell antibodies is indicated by filled bars. The half-filled bar indicates that the infiltrate was mild, dispersed and contained many polymorphonuclear cells typical of an acute inflammatory event.

ELISA. All five mice with autoantibodies in this group had gastritis. This incidence of gastritis is within the range of disease that we normally observe in BALB/c mice.

In the group of mice treated with anti-CD4 antibody, no autoantibodies were detected by either assay. Histological examination of stomachs revealed that only one of 12 mice (8%) had a cellular infiltrate; furthermore, the infiltrate was mild and contained both mononuclear and polymorphonuclear cells. On the other hand, of the eight mice treated with anti-CD8 antibody, six (75%) were positive for both antigastric H/K ATPase autoantibodies by ELISA and antiparietal cell antibodies detected by indirect immunofluorescence staining of stomach sections. All six autoantibody-positive mice had gastritis (Fig. 2).

To ensure that the elimination of $CD8^+$ T cells was effective, frozen sections of stomachs from mice with autoimmune gastritis were stained for $CD4^+$ and $CD8^+$ T cells. No $CD8^+$ cells were observed in anti-CD8 treated mice, whereas $CD8^+$ T cells were detected in the gastric mucosa of thymectomised mice treated with normal rat IgG (not shown). Therefore, these results show that gastritis can develop in the absence of $CD8^+$ T cells.

DISCUSSION

Induction of organ-specific autoimmune diseases by neonatal thymectomy provides an effective experimental model to investigate the immunopathogenesis of autoimmunity, as disease induction does not rely on immunisation of the autoantigen but rather on perturbation of the immune system where the natural tissue autoantigens, rather than a surrogate model antigen, is immunologically targeted. We have recently analysed the infiltrates in the gastric mucosa of mice with autoimmune gastritis and shown that both $CD4^+$ and $CD8^+$ T cells are present.[8] Adoptive transfer studies have shown that $CD4^+$ T cells, but not $CD8^+$ T cells, from mice with preexisting gastritis or oophoritis induced by neonatal thymectomy are able to transfer pathology to immunocompromised recipients.[9,10] These results suggest that gastritis is induced by $CD4^+$ and not $CD8^+$ T cells. However, the role of $CD4^+$ and $CD8^+$ T cells in the development of autoimmune gastritis following neonatal thymectomy had not been previously examined. The results presented in this study clearly show that autoimmune gastritis is mediated by $CD4^+$ T cells, as disease was very effectively prevented by depletion of $CD4^+$ T cells. Furthermore, as depletion of $CD8^+$ T cells has no apparent effect on the development of gastritis, cytotoxic T cells do not appear to play a significant role in disease development. The $CD8^+$ T cells found in the gastritic infiltrate[8] may be T cells of irrelevant specificities that have migrated into the gastric mucosa as a consequence of $CD4^+$ T-cell-mediated inflammation.

Chronic inflammatory infiltrates associated with autoimmune lesions often contain both $CD4^+$ and $CD8^+$ T cells. However, in contrast to autoimmune gastritis, in a number of cases $CD8^+$ T cells have been shown to contribute to the development of a particular lesion. For example, in the non-obese diabetic mouse strain, both T-cell subsets are considered to play a role in destruction of islet β-cells[18] and both $CD4^+$ and $CD8^+$ T cells are important in the development of experimental autoimmune myasthenia gravis.[19] Thus, the nature of T-cell responses associated with organ-specific autoimmune diseases are likely to vary depending on the organ or tissue, the genetic background of the animal and the procedure used to trigger the development of the disease.

The results presented here show that experimental autoimmune gastritis is mediated by a $CD4^+$ T-cell response. The gastric lesion is likely to be mediated by cytokines released by the $CD4^+$ T cells and the macrophages that are recruited to this site. Indeed, we have previously detected a range of cytokines in gastritic stomachs, including interferon-γ and tumour necrosis factor-α.[8] Cytokine-mediated cell damage would explain the loss of not only parietal cells but also zymogenic cells, as a convincing demonstration of an autoimmune response to zymogenic cells in experimental autoimmune gastritis has not been demonstrated.

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