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Murine experimental autoimmune gastritis models refractive to development of intrinsic factor autoantibodies, cobalamin deficiency and pernicious anemia

Deanne L.V. Greenwood[a,*,1], **John W. Sentry**[a,b]

[a]*Department of Medicine, Monash University Central and Eastern Clinical School, Alfred Medical Research and Education Precinct (AMREP), Commercial Road Prahran, Victoria, 3181, Australia*
[b]*Alfred Pathology Service, Alfred Hospital, Commercial Road, Melbourne, Victoria, 2005, Australia*

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Abstract Researchers have developed murine lymphopenic, non-lymphopenic, transgenic, spontaneous and infectious agent based models to induce an experimental autoimmune gastritis (EAG) for the study of human organ-specific autoimmune disease. These models result in a chronic inflammatory mononuclear cell infiltrate in the gastric mucosa, destruction of parietal and zymogenic cells with autoantibodies reactive to the gastric parietal cells and the gastric H^+/K^+ ATPase (ATP4), arguably hallmarks of a human autoimmune gastritis (AIG). In the case of AIG, it is well documented that, in addition to parietal cell antibodies being detected in up to 90% of patients, up to 70% have intrinsic factor antibodies with the later antibodies considered highly specific to patients with pernicious anemia. This is the first report specifically investigating the occurrence of intrinsic factor antibodies, cobalamin deficiency and pernicious anemia in EAG models. We conclude, in contrast to AIG, that, in the three EAG models examined, intrinsic factor is not selected as a critical autoantigen.

Introduction

Cobalamin (vitamin B_{12}) deficiency is a common cause of macrocytic anemia and has been implicated in a spectrum of neuropsychiatric disorders [1]. Pernicious anemia is generally the cause of cobalamin deficiency in Western populations with an incidence of around 1.9% in persons over 60 years of age [2]. Human autoimmune gastritis (type A chronic atrophic gastritis) is considered the underlying pathological lesion of pernicious anemia [3,4]. The gastric lesion, confined to the fundus and body of the stomach, is characterized by an inflammatory infiltrate within the gastric mucosa, and the

* Corresponding author. Centre for Animal Biotechnology, The University of Melbourne, Cnr Park Dve and Flemington Rd, Parkville, Victoria, 3004, Australia. Fax: +61 3 9347 4083.
E-mail address: deanneg@unimelb.edu.au (D.L.V. Greenwood).
[1] Current address: Centre for Animal Biotechnology, Department of Veterinary Science, The University of Melbourne, Parkville, Victoria 3010, Australia.

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loss of parietal and zymogenic cells from gastric glands [4]. Most patients with pernicious anemia have circulating autoantibodies reactive to ATP4 (the proton pump), present on and in parietal cells (~85%) and to intrinsic factor (~60%) [5]. Intrinsic factor, secreted by stomach parietal cells in humans, promotes absorption of cobalamin in the ileum via a specific receptor-mediated endocytosis [6].

There have been many reports on murine experimental autoimmune gastritis (EAG) models arguing their relevance to the study of human autoimmune gastritis (AIG) and pernicious anemia [7,8]. The histopathological features of gastric lesions in patients with chronic atrophic gastritis and murine EAG are similar [9,10]. It is also well documented that all murine EAG models can result in the development of circulating antibodies reactive to parietal cells and the proton pump (ATP4A and B). However, there are no reports as to whether murine EAG results in circulating antibodies reactive to intrinsic factor, or the development of a cobalamin deficiency and subsequent macrocytic anemia.

Here we report on the incidence of circulating antibodies reactive to intrinsic factor, levels of cobalamin in blood and evidence of anemia in mice subjected to the immunization (imm:EAG), neonatal thymectomy (nTx:EAG) and a transgenic model carrying a granulocyte macrophage colony stimulating factor (GMCSF) transgene whose expression is controlled by the ATP4 β subunit promoter (PC-GMCSF:EAG).

Methods

Mice

BALB/cCrSlc mice were immunized intraperitoneally at 24 h, 72 h and between 20 to 21 days after birth with 7.5 μg crude gastric membranes [11] ($n=39$). Neonatal thymectomy (nTx) ($n=59$) was performed on 3-day old BALB/cCrSlc mice under cold anesthesia, as previously described in Alderuccio *et al*. [12]. Transgenic PC-GMCSF mice were identified by PCR of genomic DNA isolated from tails, using the method previously described by Biondo *et al*. [13] ($n=31$). In addition, normal age matched BALB/cCrSlc mice ($n=63$) were also included in this study. All mice were maintained at Monash University, Central and Eastern Clinical School animal facility (Victoria, Australia). Animal experimentation received prior approval from the relevant institutional ethics committee (ALFR 1999/13 and 2000/011).

Preparation of mouse and pig ATP4 and validation by immunoblotting

Gastric mucosal membranes from 200 BALB/c mice and one pig were prepared as described previously [14]. Protein concentration of gastric and liver membranes was determined using a micro-BCA protein assay (Pierce, Rockford, IL). Pig gastric ATP4 was further purified from gastric membranes by tomato-lectin affinity chromatography [14]. Purity of ATP4 preparations was assessed by staining with Commassie Brilliant Blue (National Diagnostics, USA) after SDS-PAGE [15] carried out under reduced (not-heated) and non-reduced (heated) conditions. The presence of circulating antibodies reactive to ATP4 was examined by ELISA using purified pig gastric ATP4 as a substrate, as described previously in Greenwood *et al*. [11].

Histology

Mice were killed by CO_2 asphyxiation, and stomachs removed at 52 weeks of age. Stomachs were opened, rinsed in phosphate buffered saline (PBS: 145 mM NaCl, 7.5 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, pH 7.2), divided in half through the body of the stomach, fixed in 10% (v/v) formalin in PBS, dehydrated in ethanol and embedded in paraffin wax. Sections cut (4 μm) were dewaxed and stained with Hematoxylin and Eosin (H&E) [16] and by a modification of Maxwell staining [17]. The scoring regiment described in Greenwood *et al*. [11] was employed to define gastritic stomach sections.

Blood analysis

At the time mice were killed, peripheral blood was collected by cardiac heart puncture and before clotting 5 μl was spread onto a clean slide with a glass spreader and allowed to air dry. Blood film slides were fixed in 100% methanol for 5 min and stained using the Romanowsky's method [18], by the Alfred Hospital Pathology Service. Mouse blood (~250 μl) was collected into heparinized collection tubes and sent to Gribbles Pathology for a full blood analysis. Blood was examined using a Cell-Dyn 3500 Automated Analyser (Abbott Diagnostics) for parameters: mean corpuscular volume, hematocrit, hemoglobulin and platelets. Manual examination of slides was performed to determine the morphology of red blood cells and presence of hyper-segmented neutrophils. Approximately, 63 high powered fields were examined for each specimen using light microscopy.

Measurement of cobalamin (Vitamin B_{12})

Serum samples were processed using the IMMUNLITE 2000 automated analyzer programmed for the IMMUNLITE B_{12} competitive assay (Abbott Diagnostics). The strategy used for determination of cobalamin levels involved the initial release of all cobalamin forms from binding proteins in serum using a treatment of either: dithiothreitol, sodium hydroxide or potassium cyanide solution followed by a competitive assay. The pre-treatment of serum converted unstable hydroxy-, methyl- and adenosyl-forms of cobalamin to stable cyanocobalamin which was then measured utilizing intrinsic factor as a capture agent as per manufacturer's instructions. Initially, a range of cobalamin levels for male and female BALB/cCrSlc mice, relative to age (3, 6 and 12 months) were determined. Dilution factors of 1:200 were selected for use with all serum samples to maintain the cobalamin concentrations of the samples within the reportable range of the analyzer. Samples were reported as $pmol/l \times 10^2$. Data were analyzed for statistical significance utilizing a generalized linear model (SAS Version 8.2, Sas Institute Inc., Cary, NC, USA).

Construction of murine intrinsic factor expression vector (ptac-M$(H)_6$mrGIF)

A DNA fragment encoding the open reading frame of murine intrinsic factor was generated to exclude the signal peptide sequence. Forward primer 5′ CCGGAATTCAGC ACCCGTGCCC-AGAGC 3′ including an *Eco*RI endonuclease restriction site