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## H<sup>+</sup>,K<sup>+</sup>-ATPase (Proton Pump) Is the Target Autoantigen of Th1-Type Cytotoxic T Cells in Autoimmune Gastritis

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**Background & Aims:** The proton pump H<sup>+</sup>,K<sup>+</sup>-adenosine triphosphatase (H<sup>+</sup>,K<sup>+</sup>-ATPase) of parietal cells is the major humoral autoantigen in both human and experimental autoimmune gastritis (AIG) characterized by an inflammatory infiltrate in the gastric mucosa and loss of parietal cells. The aim of this study was to detect H<sup>+</sup>,K<sup>+</sup>-ATPase-specific T cells in the gastric mucosa of patients with AIG and to define their functional properties.

**Methods:** In vivo-activated T cells from the infiltrates of the gastric mucosa of 5 patients with AIG were isolated and cloned. The ability of gastric T-cell clones to proliferate and to produce cytokines in response to H<sup>+</sup>,K<sup>+</sup>-ATPase, as well as their expression of B-cell help, perforin-mediated cytotoxicity, and Fas-Fas ligand-mediated apoptosis in target cells, were assessed. **Results:** A proportion (25%) of the CD4<sup>+</sup> clones from the gastric corpus of AIG patients proliferated in response to porcine H<sup>+</sup>,K<sup>+</sup>-ATPase. Most of these clones (88%) showed a Th1 profile, whereas a few secreted both Th1 and Th2 cytokines. Virtually all of the H<sup>+</sup>,K<sup>+</sup>-ATPase-specific clones produced tumor necrosis factor  $\alpha$  and provided substantial help for B-cell immunoglobulin production, and most of them expressed perforin-mediated cytotoxicity against antigen-presenting cells and induced Fas-Fas ligand-mediated apoptosis in target cells. **Conclusions:** Activation of proton pump-specific Th1 cytotoxic/proapoptotic T cells in the gastric mucosa can represent an effector mechanism for the target cell destruction in AIG.

Chronic autoimmune gastritis (AIG) is an inflammatory disorder of the gastric corpus that does not usually result in overt symptoms until development of mucosal atrophy and malabsorption of cobalamin and iron. Subclinical AIG is often suggested by the detection of anti-parietal cell autoantibodies (PCA) in the serum of patients with suspected autoimmune endocrine disease.<sup>1</sup> Thyroid disorders, such as Graves disease and Hashimoto

thyroiditis, show a striking association with pernicious anemia and/or AIG, particularly in women older than 40 years.<sup>2,3</sup> AIG and PCA were found in a proportion of women with postpartum thyroiditis, and examination of biopsy specimens from the gastric corpus of these patients showed moderate corpus atrophy, mucosal infiltration by CD4<sup>+</sup> T cells and macrophages, and abundant epithelial expression of HLA-DR.<sup>4</sup>

In AIG patients, with or without pernicious anemia, H<sup>+</sup>,K<sup>+</sup>-adenosine triphosphatase (ATPase), the proton pump of parietal cells, is the key autoantigen recognized by PCA.<sup>5-9</sup> Gastric H<sup>+</sup>,K<sup>+</sup>-ATPase is also the major autoantigen in experimental autoimmune gastritis (EAIG), an organ-specific autoimmune disease that can be elicited in nonthymectomized animals by immunization with either gastric mucosal extracts or purified gastric H<sup>+</sup>,K<sup>+</sup>-ATPase<sup>10-15</sup> or by neonatal thymectomy.<sup>16,17</sup> These models of AIG are characterized by an inflammatory infiltrate in the gastric mucosa, subsequent loss of acid-secreting parietal cells and zymogenic cells, and late appearance of circulating autoantibodies directed against the  $\alpha$  and  $\beta$  subunits of parietal cell H<sup>+</sup>,K<sup>+</sup>-ATPase.<sup>13-15</sup> The gastric mononuclear infiltrate contains both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, macrophages, and B cells,<sup>18</sup> and the histopathologic lesions are similar to those observed in humans affected with chronic AIG and pernicious anemia.<sup>18,19</sup> Although many studies on human AIG and pernicious anemia have examined autoan-

**Abbreviations used in this paper:** AIG, autoimmune gastritis; APC, antigen-presenting cell; EAIG, experimental autoimmune gastritis; EBV, Epstein-Barr virus; H<sup>+</sup>,K<sup>+</sup>-ATPase, H<sup>+</sup>,K<sup>+</sup>-adenosine triphosphatase; Hp-CG, *Helicobacter pylori*-induced chronic gastritis; IFN, interferon; mAb, monoclonal antibody; PCA, parietal cell autoantibody; PMA, phorbol-12-myristate 13-acetate; TNF, tumor necrosis factor.

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tibody response to  $H^+,K^+$ -ATPase, no information is available on the possible pathogenic mechanisms mediated by T cells, eventually leading to parietal cell destruction.

The aims of this study were (1) to provide evidence for the presence of  $H^+,K^+$ -ATPase-specific T cells in the gastric mucosa of patients with AIG, (2) to define these cells' cytokine pattern and mode of delivering help for B-cell antibody production, and (3) to assess the cytolytic and proapoptotic potential of  $H^+,K^+$ -ATPase-specific effector T cells.

## Materials and Methods

### Patients

Five women (mean age, 48; range, 33–56 years) from Tuscany with type A chronic AIG and 5 women (mean age, 51; range, 40–59 years) with *Helicobacter pylori*-induced uncomplicated type B chronic gastritis without atrophy (Hp-CG) provided their informed consent for this study, which was performed after the approval by the local Ethical Committee. All AIG patients had serum gastric PCA, as assessed by indirect immunofluorescence. These autoantibodies proved to be specific for gastric  $H^+,K^+$ -ATPase, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting with the purified antigen.<sup>20</sup> None of the AIG patients had intrinsic factor autoantibodies or hematologic abnormalities, and their vitamin B<sub>12</sub> serum levels ranged between 345 and 480 pg/mL. One patient (G.A.) was undergoing treatment with low-dose methimazole for concomitant Graves disease, and 2 patients (C.A. and S.M.) were receiving treatment with levothyroxine for chronic thyroiditis and hypothyroidism. These 3 patients also had thyroid peroxidase autoantibodies in their serum. All of the AIG patients had negative results on both serology (Helicoblot 2.0; Genelabs Diagnostic, Geneva, Switzerland) and <sup>13</sup>C-urea breath test, which assess previous and/or actual *H. pylori* infection. In contrast, the 5 control patients with Hp-CG were infected with CagA<sup>+</sup>VacA<sup>+</sup> *H. pylori* type I strains and were positive for anti-CagA serum immunoglobulin (Ig) G antibodies but negative for PCA or other organ-specific autoantibodies. Biopsy specimens obtained from the gastric antrum and corpus of AIG and HpCG patients were used for diagnosis (histology and typing of *H. pylori* strain) and culture of mucosa-infiltrating T lymphocytes.

### Generation of Gastric T-Cell Clones

Biopsy specimens were cultured for 7 days in RPMI 1640 medium supplemented with human interleukin (IL)-2 (50 U/mL; Eurocetus, Milan, Italy) to expand in vivo-activated T cells.<sup>21</sup> Mucosal specimens were then disrupted, and single T-cell blasts were cloned under limiting dilution (0.3 cells/well), as described previously.<sup>21,22</sup> Each clone was screened for responsiveness to porcine  $H^+,K^+$ -ATPase or albumin (Sigma-Aldrich, Milan, Italy) and *H. pylori* by measuring [<sup>3</sup>H]thymidine uptake after 60 hours of stimulation with medium,

different concentrations of  $H^+,K^+$ -ATPase (0.3 mg/mL being optimal), porcine albumin (5 μg/mL), or *H. pylori* lysate (aqueous extract of NCTC11637 strain, 0.5–50 μg/mL; kindly provided by Dr. John L. Telford, Chiron Vaccines, Siena, Italy) in the presence of irradiated autologous mononuclear cells as antigen-presenting cells (APCs).<sup>21</sup> Gastric  $H^+,K^+$ -ATPase was purified from pig gastric mucosa as previously reported.<sup>23</sup> The major histocompatibility complex (MHC) class II restriction of  $H^+,K^+$ -ATPase recognition by T-cell clones was determined with either a murine monoclonal antibody (mAb) reacting with all major histocompatibility class II HLA-DR and -DP antigens and most DQ molecules (clone TU39; Pharmingen, San Diego, CA) or allogeneic irradiated APCs (HLA mismatched with the T-cell donors). Autologous irradiated APCs ( $5 \times 10^4$ ) were incubated first with 5 μg/mL anti-MHC class II mAb or isotype (IgG2a) control for 1 hour at 37°C, then with 0.3 μg/mL  $H^+,K^+$ -ATPase, and finally with  $5 \times 10^4/0.2$  mL responder clonal T-cell blasts in triplicate cultures; [<sup>3</sup>H]thymidine uptake was measured after 60 hours. Virtually all  $H^+,K^+$ -ATPase- or *H. pylori*-specific clones showed individual patterns of response to superantigens (4 staphylococcal enterotoxins: SEA, SEB, SED, and SEE in the presence of allogeneic APCs), suggesting a difference in their T cell-reactive Vβ-chain expression.

### Cytokine Profile of Gastric T-Cell Clones

To assess the cytokine production of  $H^+,K^+$ -ATPase- or *H. pylori*-specific Th clones,  $10^6$  T-cell blasts of each clone were cocultured in duplicate cultures for 48 hours in 1 mL medium with  $5 \times 10^5$  irradiated autologous peripheral blood mononuclear cells as APCs and  $H^+,K^+$ -ATPase or *H. pylori* lysate.<sup>21</sup> The  $H^+,K^+$ -ATPase (0.3 μg/mL) and *H. pylori* lysate (10 μg/mL) found to be optimal for proliferation were also optimal for induction of cytokine production. To induce cytokine production by gastric T-cell clones in the absence of APCs, T-cell blasts were stimulated for 36 hours with phorbol-12-myristate 13-acetate (PMA, 10 ng/mL) in microwells coated with anti-CD3 mAb, as previously reported.<sup>22</sup> Duplicate samples of each supernatant were assayed for interferon (IFN)-γ, IL-4, IL-5, and tumor necrosis factor [TNF]-α; BioSource International, Camarillo, CA). Supernatants showing IFN-γ, IL-4, IL-5, and TNF-α levels 5 SD over the mean levels in control supernatants derived from irradiated feeder cells alone were regarded as positive. T-cell clones able to produce IFN-γ but not IL-4 or IL-5 were categorized as Th1; clones able to produce IL-4 and/or IL-5 but not IFN-γ as Th2; and clones producing both IFN-γ and IL-4 or IL-5 as Th0.

### Assay for Helper Function to B Cells by Gastric T-Cell Clones

B cell-enriched suspensions were prepared by a double-step rosetting with neuraminidase-treated sheep red blood cells, as previously described.<sup>24</sup> Peripheral blood B cell-enriched suspensions usually consisted of 68%–87% B cells, 9%–21% monocytes, and <1% T cells. They are referred to as B cells. The cell culture system used to assess the ability of

antigen-stimulated T-cell clones to induce polyclonal B-cell activation and Ig synthesis was performed in duplicate tubes containing complete medium supplemented with 10% fetal calf serum. B cells ( $5 \times 10^4$ ) were cultured alone or with autologous clonal T-cell blasts ( $5 \times 10^4$ ) in the absence or presence of H<sup>+</sup>,K<sup>+</sup>-ATPase (0.3  $\mu$ g/mL) or porcine albumin (5  $\mu$ g/mL). After 10 days, culture supernatants were collected and assayed for their IgM, IgG, and IgA content by immunoradiometric assays, as previously described.<sup>24</sup>

### Perforin-Mediated Cytolytic Activity

Perforin-mediated cytolytic activity of T-cell clones was assessed as previously reported.<sup>21</sup> T-cell blasts of H<sup>+</sup>,K<sup>+</sup>-ATPase-specific clones were incubated at ratios of 10, 5, and 2.5 to 1 with <sup>51</sup>Cr-labeled autologous Epstein-Barr virus (EBV)-transformed lymphoblastoid B cells (EBV-B cells) preincubated with H<sup>+</sup>,K<sup>+</sup>-ATPase or porcine albumin or *H. pylori* lysate (control antigens). After centrifugation to favor cell-to-cell contact, microplates were incubated for 8 hours at 37°C, and 0.1 mL of supernatant was removed for measurement of <sup>51</sup>Cr release. Maximum release (MR) was obtained by treating target cells with 0.1 mL of 1 mol/L HCl. Spontaneous release (SR) was determined in microcultures without T cells. Specific lysis was calculated according to the formula: Percent Specific Lysis =  $100 \times (\text{Experimental Release} - \text{SR}) / (\text{MR} - \text{SR})$ . Cultures in which <sup>51</sup>Cr release exceeded the mean SR by more than 5 SD were considered positive for cytolytic activity. The ability of H<sup>+</sup>,K<sup>+</sup>-ATPase-specific T-cell clones to express perforin-mediated cytotoxicity was confirmed in a lectin-dependent assay against <sup>51</sup>Cr-labeled P815 murine mastocytoma cells at effector-to-target ratios of 10, 5, and 2.5 to 1 in the presence of phytohemagglutinin (1%, vol/vol), as previously described.<sup>21</sup>

### Fas-Fas Ligand-Mediated Apoptotic Killing

The ability of H<sup>+</sup>,K<sup>+</sup>-ATPase-specific gastric T-cell clones to induce Fas-Fas ligand-mediated apoptosis was assessed using Fas<sup>+</sup> Jurkat cells as target.<sup>25</sup> T-cell blasts from each clone were cocultured with <sup>51</sup>Cr-labeled Jurkat cells at an effector-to-target ratio of 10, 5, and 2.5 to 1 for 18 hours in the presence of PMA (10 ng/mL) and ionomycin (1 mmol/L). Specific lysis was calculated according to the formula reported above. To block Fas-Fas ligand interaction, the anti-Fas antagonistic mAb M3 (Immunex Corp., Seattle, WA)<sup>26</sup> was used at 5  $\mu$ g/mL final concentration in a 30-minute pretreatment of <sup>51</sup>Cr-labeled Jurkat cells. The anti-Fas or its isotype (IgG1) control was also added during the cytolytic assay at 2  $\mu$ g/mL final concentration.

## Results

### Reactivity to H<sup>+</sup>,K<sup>+</sup>-ATPase and Cytokine Profile of Gastric T-Cell Clones

In vivo-activated T cells present in the lymphocytic infiltrates of the gastric antrum and corpus of 5 PCA-positive patients with chronic AIG without evidence of previous or actual infection with *H. pylori* were

isolated and cloned. Likewise, control T-cell clones were generated from in vivo-activated T cells isolated from the gastric antrum and corpus of 5 PCA-negative, age- and sex-matched patients with uncomplicated Hp-CG. A total number of 175 CD4<sup>+</sup> and 55 CD8<sup>+</sup> clones were obtained from the biopsy specimens of the gastric corpus of AIG patients, whereas a total of 105 CD4<sup>+</sup> and 38 CD8<sup>+</sup> T-cell clones were generated from specimens of the gastric antrum of the same patients with AIG. The biopsies of corpus and antrum in patients with Hp-CG yielded 73 CD4<sup>+</sup> plus 31 CD8<sup>+</sup> and 186 CD4<sup>+</sup> plus 22 CD8<sup>+</sup> T-cell clones, respectively. When assessed for their ability to proliferate in response to H<sup>+</sup>,K<sup>+</sup>-ATPase or *H. pylori* lysate in the presence of irradiated autologous APCs, none of the CD8<sup>+</sup> T-cell clones from patients with either AIG or Hp-CG showed significant reactivity to either of the antigens. Likewise, although 49 of 186 CD4<sup>+</sup> clones from the antrum (26%) and 1 of 73 CD4<sup>+</sup> clones (1.3%) from the corpus of patients with Hp-CG showed significant proliferation to *H. pylori* lysate (Table 1), none of these gastric T-cell clones proliferated in response to H<sup>+</sup>,K<sup>+</sup>-ATPase at different concentrations. In contrast, 43 of the 175 CD4<sup>+</sup> clones from the corpus (25%) and 3 of the 105 CD4<sup>+</sup> clones from the antrum (2.8%) of patients with AIG showed substantial proliferation in response to porcine H<sup>+</sup>,K<sup>+</sup>-ATPase (Figure 1), with a peak response to 0.3  $\mu$ g/mL of this antigen preparation (Figure 2). In the 5 AIG patients, the proportion of H<sup>+</sup>,K<sup>+</sup>-ATPase-specific CD4<sup>+</sup> clones isolated from the corpus ranged between 13% and 32%, whereas few H<sup>+</sup>,K<sup>+</sup>-ATPase-specific CD4<sup>+</sup> clones could be isolated from the antrum of only 1 patient (C.A.). Control proliferation experiments with porcine albumin showed that such T-cell clones responded only to porcine gastric H<sup>+</sup>,K<sup>+</sup>-ATPase (Figure 1). Blocking experiments with anti-MHC class II mAb and antigen presentation by allogeneic APCs showed that H<sup>+</sup>,K<sup>+</sup>-ATPase was recognized by reactive T-cell clones under MHC class II-restricted conditions (Figure 3).

Upon stimulation with the specific antigen, 38 of the 43 H<sup>+</sup>,K<sup>+</sup>-ATPase-specific clones (88%) from the corpus and the 3 H<sup>+</sup>,K<sup>+</sup>-ATPase-specific clones from the antrum of patients with AIG produced IFN- $\gamma$  but neither IL-4 nor IL-5 (Th1 cytokine profile), whereas 5 H<sup>+</sup>,K<sup>+</sup>-ATPase-specific clones from the corpus (12%) secreted both Th1- and Th2-type cytokines (Th0 profile; Table 1 and Figure 4). In agreement with the results of our previous studies<sup>21,27</sup> in the series of *H. pylori*-specific CD4<sup>+</sup> clones from the antrum of patients with Hp-CG, 61% expressed a Th0 profile and 39% a Th1 profile upon stimulation with *H. pylori* lysate (Figure 4), the unique *H.*

**Table 1.** Antigen Specificity and Cytokine Secretion Profile of T-Cell Clones Derived From the Gastric Mucosa of Patients With AIG and Patients With Hp-CG

Patients and source of T-cell clones	No. of CD4 <sup>+</sup> clones obtained	No. (%) of CD4 <sup>+</sup> clones reactive to		% of H <sup>+</sup> ,K <sup>+</sup> -ATPase-specific T-cell clones with the indicated profile		
		H <sup>+</sup> ,K <sup>+</sup> -ATPase	<i>H. pylori</i> lysate	Th1	Th0	Th2
G.A.						
Corpus	33	9 (27) <sup>a</sup>	0	100 <sup>b</sup>	0	0
Antrum	22	0	0	0	0	0
C.A.						
Corpus	34	8 (24)	0	88	12	0
Antrum	27	3 (11)	0	100	0	0
N.G.						
Corpus	30	4 (13)	0	75	25	0
Antrum	19	0	0	0	0	0
S.M.						
Corpus	41	13 (32)	0	77	23	0
Antrum	21	0	0	0	0	0
F.F.						
Corpus	37	9 (24)	0	100	0	0
Antrum	16	0	0	0	0	0
All AIG cases						
Corpus	175	43 (25)	0	88	12	0
Antrum	105	3 (3)	0	100	0	0
Hp-CG cases (n = 5)						
Corpus	73	0	1 (1)			
Antrum	186	0	49 (26)			

<sup>a</sup>T-cell blasts from each clone ( $5 \times 10^4$ ) were seeded in triplicate cultures with irradiated autologous APCs ( $5 \times 10^4$ ) in the presence of medium alone, H<sup>+</sup>,K<sup>+</sup>-ATPase (0.3 μg/mL), or *H. pylori* lysate (10 μg/mL). After 60 hours, [<sup>3</sup>H]thymidine uptake was measured and expressed as mitogenic index.

<sup>b</sup>T-cell blasts from each clone ( $10^6$ ) were cocultured with irradiated autologous APCs ( $5 \times 10^5$ ) in 1 mL in the presence of medium alone, H<sup>+</sup>,K<sup>+</sup>-ATPase (0.3 μg/mL), or *H. pylori* lysate (10 μg/mL). After 48 hours, cell-free supernatants were assayed for their IFN-γ, IL-4, and IL-5 content. Mean cytokine levels in culture supernatants of irradiated APCs alone were <0.1 ng/mL IFN-γ, <0.03 ng/mL IL-4, and <0.06 ng/mL IL-5. T-cell clones able to produce IFN-γ but not IL-4 or IL-5 were categorized as Th1; clones able to produce IL-4 and/or IL-5 but not IFN-γ were categorized as Th2; and clones producing both IFN-γ and IL-4 or IL-5 were categorized as Th0.

*pylori*-specific clone derived from the corpus also showing a Th1 profile (Table 1). Of note, all H<sup>+</sup>,K<sup>+</sup>-ATPase-specific clones produced high concentrations of TNF-α, particularly upon stimulation with PMA plus insoluble anti-CD3 antibody in the absence of APCs (mean ± SE,  $5.1 \pm 0.6$  ng/mL per  $10^6$  T cells). This suggests that high TNF-α production was a peculiar property of autoreactive gastric T cells of patients with AIG because under the same experimental conditions, in the series of *H. pylori*-specific T-cell clones from patients with Hp-CG TNF-α-producing clones were 78%, with a mean (±SE) production of  $2.7 \pm 1.8$  ng/mL per  $10^6$  T cells ( $t$ , -8.12;  $P < 0.05$ ).

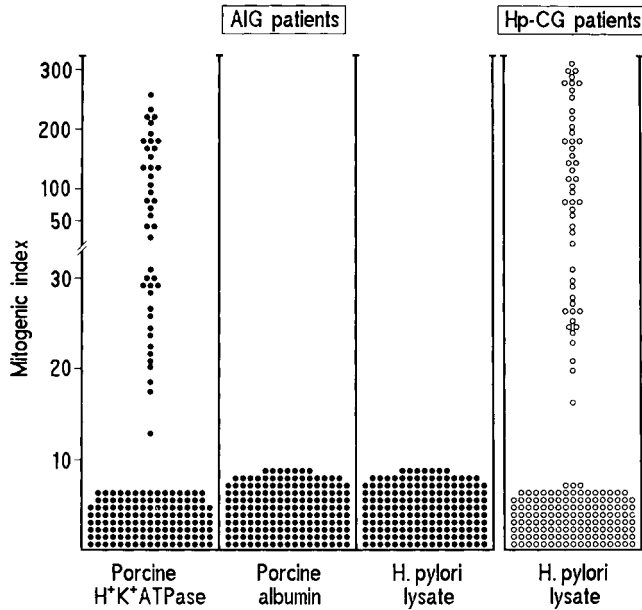
#### H<sup>+</sup>,K<sup>+</sup>-ATPase-Specific T-Cell Clones Express Antigen-Dependent Help to Autologous B Cells for Ig Production

The ability of H<sup>+</sup>,K<sup>+</sup>-ATPase-specific T-cell clones to provide B-cell help for Ig synthesis was then investigated. To this end, T-cell blasts of each clone were cocultured at ratios of 0.2, 1, and 5 to 1 with autologous peripheral blood B cells in the absence or presence of H<sup>+</sup>,K<sup>+</sup>-ATPase or porcine albumin, and IgM, IgG, and IgA levels in cell-free

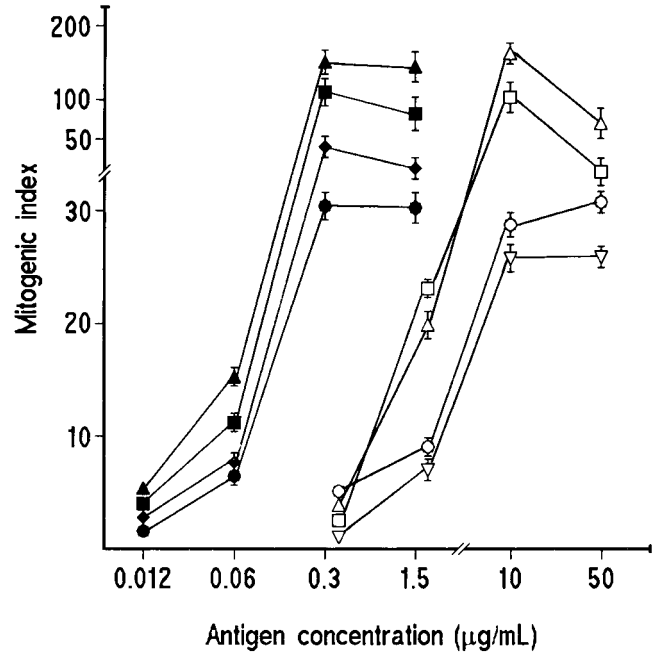
culture supernatants on day 10 were measured. In the absence of the specific antigen, no increase in IgM, IgG, or IgA production above the spontaneous levels measured in cultures containing B cells alone was observed. In the presence of H<sup>+</sup>,K<sup>+</sup>-ATPase and at a T-to-B cell ratio of 0.2 to 1, all of the H<sup>+</sup>,K<sup>+</sup>-ATPase-specific T-cell clones (from either corpus or antrum) provided substantial help for Ig production. At a 1-to-1 T-to-B cell ratio, H<sup>+</sup>,K<sup>+</sup>-ATPase-dependent T-cell help for IgM, IgG, and IgA production by B cells was remarkably higher (Figure 5). However, at a 5-to-1 T-to-B cell ratio, coculturing B cells with autologous H<sup>+</sup>,K<sup>+</sup>-ATPase-specific T-cell clones in the presence of H<sup>+</sup>,K<sup>+</sup>-ATPase resulted in much lower Ig synthesis (Figure 5).

#### H<sup>+</sup>,K<sup>+</sup>-ATPase-Specific T-Cell Clones Can Induce Death in Target Cells via Both Perforin- and Fas-Fas Ligand-Mediated Pathways

Because most antigen-activated Th1 and Th0 clones express perforin-mediated cytotoxicity against autologous APCs (e.g., antigen-pulsed B cells),<sup>24</sup> the cyto-

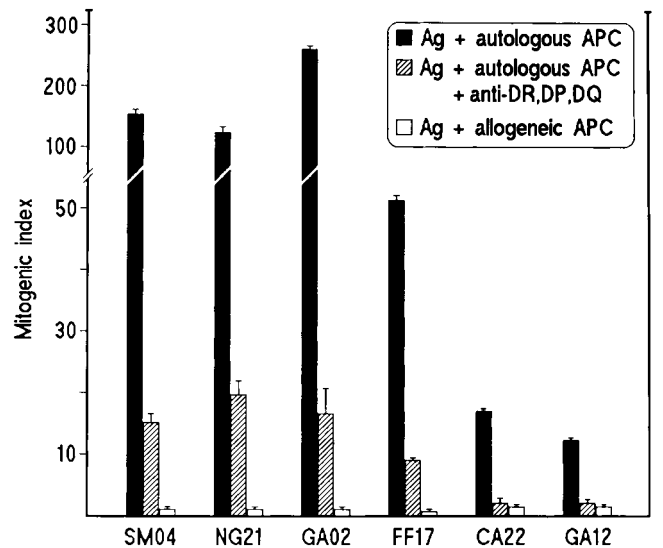


**Figure 1.** Proliferative response to H<sup>+</sup>,K<sup>+</sup>-ATPase by gastric T-cell clones derived from patients with AIG. In vivo-activated T cells were recovered from biopsy specimens of gastric mucosa of patients with AIG or Hp-CG and cloned by limiting dilution. T-cell blasts from each clone were seeded in triplicate cultures with irradiated autologous mononuclear cells (as APCs) in the presence of medium alone, porcine H<sup>+</sup>,K<sup>+</sup>-ATPase (0.3 μg/mL), porcine albumin (5 μg/mL), or *H. pylori* lysate (5 μg/mL). After 60 hours, [<sup>3</sup>H]thymidine uptake was measured and expressed as mitogenic index.



**Figure 2.** Dose-response curves of proliferative response to H<sup>+</sup>,K<sup>+</sup>-ATPase by representative gastric T-cell clones derived from patients with AIG (closed symbols) and proliferative response to *H. pylori* lysate by representative gastric T-cell clones derived from patients with Hp-CG (open symbols). T-cell blasts from each clone were seeded in triplicate cultures with irradiated autologous mononuclear cells (as APCs) in the presence of medium alone or graded concentrations of porcine H<sup>+</sup>,K<sup>+</sup>-ATPase or *H. pylori* lysate. After 60 hours, [<sup>3</sup>H]thymidine uptake was measured and expressed as mitogenic index.

lytic potential of H<sup>+</sup>,K<sup>+</sup>-ATPase-specific T-cell clones from patients with AIG was assessed by using H<sup>+</sup>,K<sup>+</sup>-ATPase-pulsed <sup>51</sup>Cr-labeled autologous EBV-B cells as targets. At the effector-to-target ratio of 10 to 1, all 41 Th1 and 3 of 5 Th0 H<sup>+</sup>,K<sup>+</sup>-ATPase-specific clones lysed H<sup>+</sup>,K<sup>+</sup>-ATPase-presenting autologous EBV-B cells, whereas autologous EBV-B cells pulsed with porcine albumin (Figure 6) or *H. pylori* lysate (data not shown) and cocultured with the same clones were not lysed. The ability of H<sup>+</sup>,K<sup>+</sup>-ATPase-specific clones to express perforin-mediated cytotoxicity was confirmed by a lectin-dependent cytolytic assay against <sup>51</sup>Cr-labeled P815 cells, in which only the same 2 Th0 clones failed to lyse their targets (data not shown). Because activated effector T cells can also kill their targets by inducing apoptosis through Fas-Fas ligand interaction,<sup>25,28-30</sup> the ability of activated H<sup>+</sup>,K<sup>+</sup>-ATPase-specific T-cell clones to induce <sup>51</sup>Cr-release by Fas<sup>+</sup> Jurkat cells undergoing apoptosis was evaluated. Upon mitogen activation, 35 of 41 Th1 (85%) and 3 of 5 Th0 H<sup>+</sup>,K<sup>+</sup>-ATPase-specific T-cell clones were able to induce apoptosis in target cells. The role of Fas-Fas ligand interaction in this <sup>51</sup>Cr release was confirmed by its substantial inhibition (>50%) by a blocking anti-Fas antibody (Figure 7).

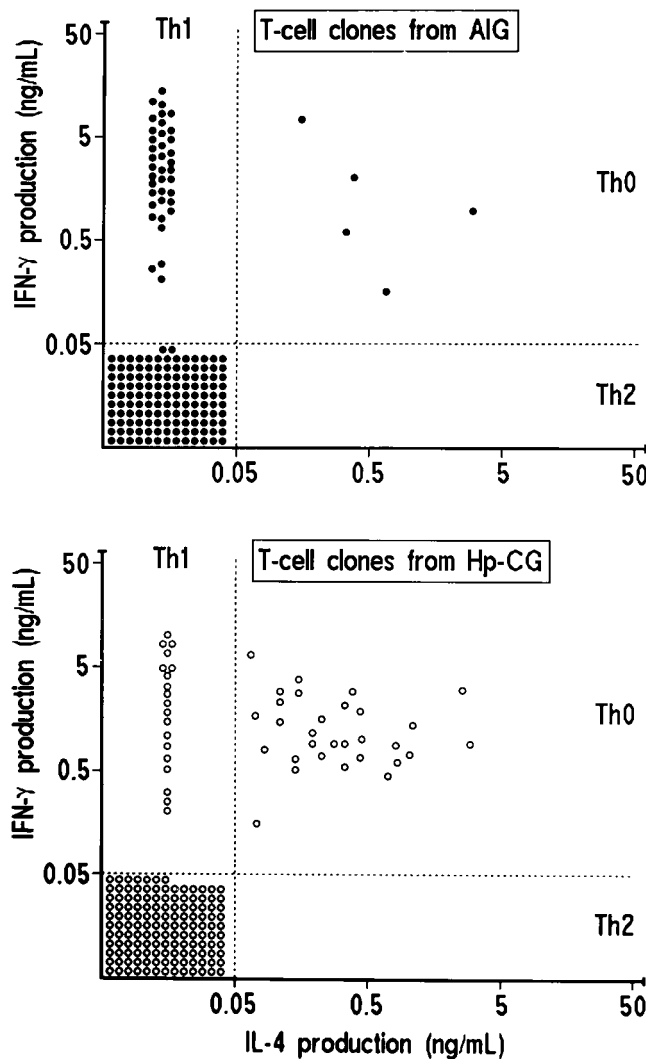


**Figure 3.** HLA class II restriction of H<sup>+</sup>,K<sup>+</sup>-ATPase recognition by gastric T-cell clones. HLA class II restriction was assessed either by using irradiated HLA class II mismatched allogeneic APCs or by adding in culture an mAb reacting with all major histocompatibility class II HLA-DR and -DP antigens and most DQ molecules. Results obtained with 6 representative H<sup>+</sup>,K<sup>+</sup>-ATPase-specific gastric T-cell clones from AIG patients are shown. Experimental conditions are reported in Figure 1.

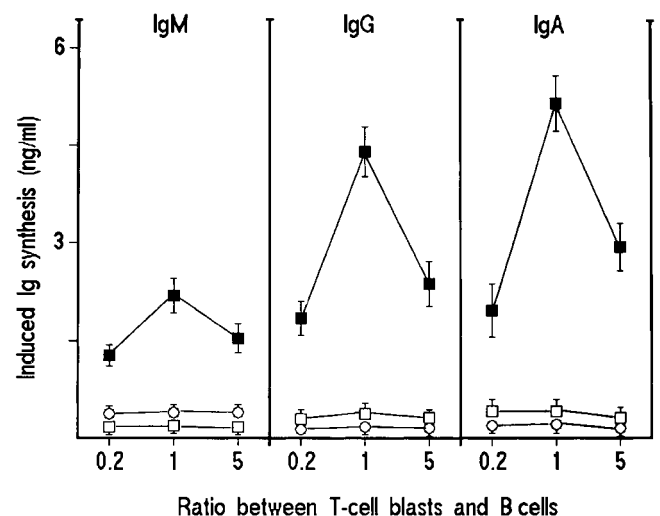
## Discussion

We have investigated the pathogenic mechanisms of human AIG in this study. Data showed for the first time that T cells specific for  $H^+,K^+$ -ATPase of parietal cells are present in the gastric mucosa of patients with AIG. These autoreactive T cells were found to be Th1 effector cells with cytolytic potential through both perforins and Fas–Fas ligand interaction, and we propose that parietal cell loss in AIG proceeds through an auto-

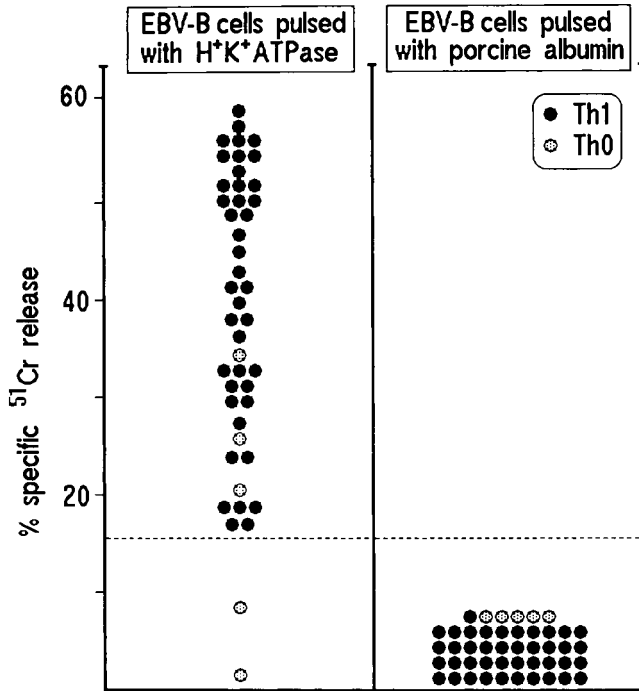
immune T-cell attack. In vivo-activated T cells present in the lymphocytic infiltrates of the lesional gastric mucosa of 5 patients with chronic AIG were expanded in vitro and efficiently cloned to assess their reactivity to  $H^+,K^+$ -ATPase, as well as their functional profile. This cloning procedure has proved useful and accurate for in vitro studies of tissue-infiltrating T cells in various diseases.<sup>31</sup> In the clonal progenies of T cells from the gastric corpus of all AIG patients, a noticeable proportion of  $CD4^+$  T-cell clones were reactive to porcine  $H^+,K^+$ -ATPase under MHC-restricted conditions. Each patient with AIG contributed an almost equal number of gastric T-cell clones, whose functional features were consistently shared by each patient. A few clones with the same reactivity were also isolated from the antral mucosa in a patient with AIG, suggesting that the gastric corpus, in which disturbed parietal cell function and reduced acid production usually occur, is the major target of autoreactive  $H^+,K^+$ -ATPase-specific gastric T cells. A reasonable objection may be that the high proportion of  $H^+,K^+$ -ATPase-specific T-cell clones merely reflects the IL-2-induced selective expansion of 1 or few  $H^+,K^+$ -ATPase-specific gastric T cells. However, such a possibility is unlikely because the majority of  $H^+,K^+$ -ATPase-specific T-cell clones expressed different T-cell receptor  $V\beta$  rearrangements, as indicated by their almost individual patterns of response to 4 different staphylo-



**Figure 4.** IFN- $\gamma$  and IL-4 production induced by  $H^+,K^+$ -ATPase in gastric T-cell clones derived from patients with AIG. To assess the cytokine production of  $H^+,K^+$ -ATPase-specific Th clones from patients with AIG (●) or *H. pylori*-specific Th clones from patients with Hp-CG (○),  $10^6$  T-cell blasts of each clone were cocultured in duplicate cultures for 48 hours in 1 mL medium with  $5 \times 10^5$  irradiated autologous peripheral blood mononuclear cells as APCs and  $H^+,K^+$ -ATPase (0.3  $\mu$ g/mL) or *H. pylori* lysate (10 mg/mL). Duplicate samples of each supernatant were assayed for IFN- $\gamma$  and IL-4 by appropriate assays. Dotted lines indicate 5 SD over the mean cytokine levels in control supernatants derived from irradiated feeder cells alone.



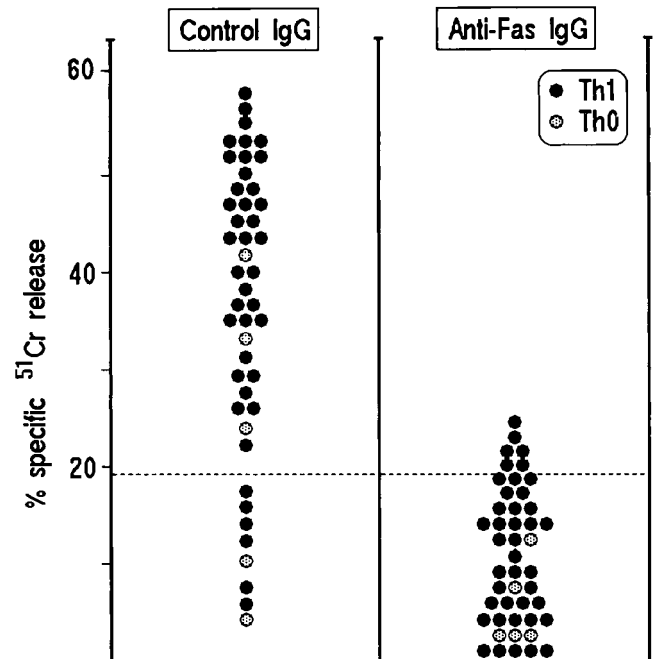
**Figure 5.** In vitro synthesis of IgM, IgG, and IgA induced in autologous B cells by  $H^+,K^+$ -ATPase-specific gastric T-cell clones stimulated with the specific antigen. Autologous peripheral blood B cells ( $5 \times 10^4$ ) were cultured with T-cell blasts of each  $H^+,K^+$ -ATPase-specific gastric clone at T-to-B cell ratios of 0.2, 1, and 5 to 1 in the absence (□) or presence of  $H^+,K^+$ -ATPase (■) or porcine albumin (○). After 10 days, cell-free culture supernatants were assayed for their IgM, IgG, and IgA content using appropriate immunoradiometric assays. The results represent the mean ( $\pm$ SE) Ig levels induced by T-cell clones over the spontaneous Ig production in cultures of B cells alone.



**Figure 6.** Ability of H<sup>+</sup>,K<sup>+</sup>-ATPase-specific gastric T-cell clones from patients with AIG to express antigen-induced perforin-mediated cytotoxicity against antigen-presenting autologous EBV-B cells. Cytolytic activity of each H<sup>+</sup>,K<sup>+</sup>-ATPase-specific clone was tested in triplicate at an effector-to-target ratio of 10 to 1 against <sup>51</sup>Cr-labeled autologous EBV-B cells pulsed with H<sup>+</sup>,K<sup>+</sup>-ATPase (3 μg/mL) or with porcine albumin (50 μg/mL). Percent of specific <sup>51</sup>Cr release was calculated as described in Materials and Methods. The dotted lines indicate 5 SD above the mean spontaneous release of target cells alone.

coccal enterotoxin superantigens (data not shown). Thus, it is reasonable to conclude that gastric autoimmunity elicits a powerful local inflammatory response that recruits and activates a large number of H<sup>+</sup>,K<sup>+</sup>-ATPase-specific effector T cells. As expected on the basis of their negative serology, <sup>13</sup>C-urea breath test, and histology, no patient with AIG had gastric T-cell reactivity to *H. pylori* antigens, whereas in control patients affected with *H. pylori*-induced chronic gastritis a number of CD4<sup>+</sup> T-cell clones reactive to *H. pylori* antigens could be isolated from the gastric antrum.<sup>21,27</sup> On the other hand, in none of the 5 patients with *H. pylori*-induced chronic gastritis selected for their negative serology for organ-specific autoimmunity did the same cloning procedure yield any T-cell clones reactive to porcine H<sup>+</sup>,K<sup>+</sup>-ATPase, suggesting that such a T-cell reactivity does not simply result from an inflammatory process at the gastric level, allowing foreign antigens (e.g., porcine food antigens) to cross the mucosa and to be recognized by local T cells. Rather, T-cell recognition of porcine H<sup>+</sup>,K<sup>+</sup>-ATPase at the gastric level is specific for patients with gastric autoimmunity and reasonably results from reactivity against T-cell epitopes shared by both human and por-

cine H<sup>+</sup>,K<sup>+</sup>-ATPase. Previous studies have shown that H<sup>+</sup>,K<sup>+</sup>-ATPase prepared from vesicular membranes isolated from porcine gastric mucosa as a 114-kilodalton protein is specifically recognized by parietal cell autoantibodies present in patients with AIG,<sup>5-9</sup> in some patients with gastritis associated with postpartum thyroiditis,<sup>4</sup> and in a subgroup of *H. pylori*-infected patients who develop parietal cell autoantibodies and mucosal atrophy of the gastric corpus.<sup>32,33</sup> Because human H<sup>+</sup>,K<sup>+</sup>-ATPase (natural or cloned) was not available, the porcine enzyme that can be purified in large amounts and is at least 95% identical was used. The results of this study suggest that human and porcine H<sup>+</sup>,K<sup>+</sup>-ATPase share not only a number of B-cell epitopes recognized by autoantibodies, but also T-cell epitopes that require their coupling to MHC class II molecules on APCs to be recognized by specific autologous T cells. The possibility that our porcine H<sup>+</sup>,K<sup>+</sup>-ATPase preparation is contaminated by superantigens can be excluded on the basis of the lack of response by gastric T-cell clones to H<sup>+</sup>,K<sup>+</sup>-ATPase presented by allogeneic APCs and on the lack of response by gastric T-cell clones isolated from *H. pylori*-infected patients.



**Figure 7.** Ability of H<sup>+</sup>,K<sup>+</sup>-ATPase-specific gastric T-cell clones from patients with AIG to induce Fas-Fas ligand-mediated apoptosis and lysis in Jurkat cells in the presence of control mouse Ig or anti-Fas mAb. Proapoptotic activity of each H<sup>+</sup>,K<sup>+</sup>-ATPase-specific clone was tested in triplicate at an effector-to-target ratio of 10 to 1 against <sup>51</sup>Cr-labeled Jurkat cells in the absence or presence of 10 ng/mL PMA and 1 mmol/L ionomycin. Percent of specific <sup>51</sup>Cr release was calculated as described in Materials and Methods. The dotted lines indicate 5 SD above the mean spontaneous release of target cells alone.



When assessed for their cytokine secretion profile, most  $H^+,K^+$ -ATPase-specific T-cell clones generated from the gastric mucosa of patients with AIG produced IFN- $\gamma$  and TNF- $\alpha$  but neither IL-4 nor IL-5, thus exhibiting a clear-cut Th1 pattern. The possibility that this outcome does not reflect the real functional attitude of the gastric T-cell counterparts in vivo, but is the result of in vitro artifacts caused by the cloning procedure, was considered. However, *H. pylori*-specific clones with predominant Th0 profile were generated from the gastric mucosa of control patients with *H. pylori*-induced chronic gastritis using the same cloning protocol. The present results, showing that both Th1 and Th0  $H^+,K^+$ -ATPase-specific gastric T-cell clones are able to help B-cell Ig production, suggest that chronic autoantigen-induced T cell-dependent B-cell activation is probably responsible for the local synthesis of  $H^+,K^+$ -ATPase autoantibodies found in the sera.

T-B-cell interaction is a multistep process resulting in B-cell help and/or B-cell death, depending on the functional commitment of the helper T cells involved. In vitro, a decrease in antigen-induced B-cell help at high T-to-B cell ratios is peculiar to all Th1, and most Th0, clones because of their concomitant expression of cytolytic killing of antigen-presenting autologous B cells.<sup>24</sup> Two complementary lytic pathways mediate T-cell cytotoxicity: the exocytosis of perforin-containing granules on cognate target cells and the engagement of Fas on cognate or neighboring target cells by Fas ligand,<sup>34,35</sup> activities that are usually both expressed by Th1 clones.<sup>24,25,36</sup> The ability of Th1 effector cells present in the gastric mucosa to express cytolytic and proapoptotic activity against autoantigen-presenting cells may represent a key mechanism in the mucosal atrophy of AIG. Indeed, local IFN- $\gamma$  production by Th1 cells (possibly potentiated by concomitant TNF- $\alpha$  production) can increase the expression of MHC class II molecules by gastric cells,<sup>37</sup> which in turn would enable these cells to present peptides of gastric autoantigens, including  $H^+,K^+$ -ATPase, to T cells that differentiate into Th1 effector cells. Because gastric epithelial cells were also found to express either B7-1 (CD80) or B7-2 (CD86) costimulatory molecules, which are required for T-cell activation, and are up-regulated after cross-linking of MHC class II molecules and exposure to IFN- $\gamma$ ,<sup>38</sup> the possibility that gastric epithelial cells act as antigen- or autoantigen-presenting cells is real.

There is strong analogy between human chronic atrophic AIG and mouse postthymectomy EAIG.<sup>16</sup> Susceptible mice undergoing neonatal thymectomy spontaneously develop chronic atrophic gastritis and circulating

parietal cell autoantibodies that recognize the  $\alpha$  and  $\beta$  subunits of  $H^+,K^+$ -ATPase.<sup>39</sup> Although autoantibodies can appear at different stages of the disease,<sup>14,19</sup> several studies suggest that  $H^+,K^+$ -ATPase subunits are important T-cell targets involved either in the development or in the maintenance of EAIG in thymectomized mice.<sup>17,40,41</sup> A gastritogenic CD4<sup>+</sup> T-cell clone, established from an EAIG thymectomized mouse, that recognizes a peptide of the  $\alpha$  subunit of  $H^+,K^+$ -ATPase in a MHC class II-restricted manner was found to express the Th1 cytokine profile. Adoptive transfer into syngeneic *nu/nu* mice of this Th1 clone able to express Fas ligand and to induce apoptosis in APCs resulted in gastritis associated with loss of parietal cells.<sup>41</sup> More recently, 2 gastritogenic  $H^+,K^+$ -ATPase  $\alpha$  chain-specific T-cell lines showing opposite Th1/Th2 profiles, both able to induce severe destructive gastritis with parietal cell loss in *nu/nu* mice, were established from thymectomized mice. However, the Th2 line recruited a gastric infiltrate composed by polymorphonuclear leukocytes and eosinophils, whereas the Th1-induced gastric infiltrate consisted primarily of lymphocytes and monocytes, i.e., they showed the typical pattern found in human AIG.<sup>17</sup>

In conclusion, the demonstration of  $H^+,K^+$ -ATPase-specific autoreactive Th1 cytotoxic effector cells in the target organ of AIG is consistent with previous data showing predominance of Th1 responses in human thyroid autoimmune disorders,<sup>42,43</sup> which are frequently associated with AIG, and multiple sclerosis.<sup>44</sup> Our present data reinforce evidence accumulated in animal models suggesting that experimental organ-specific autoimmune diseases, such as encephalomyelitis, thyroiditis, gastritis, insulin-dependent diabetes mellitus, and myasthenia gravis, are mainly mediated by IFN- $\gamma$ -secreting Th1 cells.<sup>45,46</sup> Our interpretation of the data is that AIG is caused by parietal cell destruction by CD4 Th1 cells that recognize  $H^+,K^+$ -ATPase presented in the context of MHC class II up-regulated by IFN- $\gamma$  on the surface of parietal cells, whose destiny is death induced by cytotoxic mediators and apoptosis.

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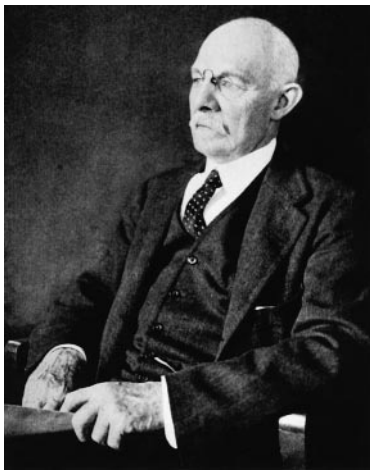
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## ***Halsted of the Halsted Suture***



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William Stewart Halsted (1852–1922) was born in New York City, the scion of a prominent, affluent family. As an undergraduate at Yale, he was more proficient at athletics than scholarship, yet in 1877 he graduated first in his class at the College of Physicians and Surgeons of Columbia University. From a diligent tour of the major medical centers in Europe, he acquired a passionate interest in clinical and laboratory investigation. Once back in New York, he gained esteem as a practitioner and teacher but his career seemed cut short when he was invalidated by addiction to cocaine, acquired in seeking relief from painful neuritis that had complicated a finger infection. Nevertheless, at the urging of the pathologist William Welch he moved to Baltimore and joined the staff of the Johns Hopkins Hospital where he became surgeon-in-chief and established a pre-eminently influential school of surgery. There, too, his affliction was compassionately tended by his medical colleague William Osler. In addition to a method of suture long used in gastrointestinal anastomoses, he introduced the use of rubber gloves and devised a variety of innovative surgical instruments. Meticulous in the handling of tissues, he was equally fastidious in his personal attire and social conduct.

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