Title: The stomach, eosinophils and *Helicobacter pylori* infection

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Abstract

Immunohistochemical studies on resin embedded gastric biopsies have been performed to investigate the changes to eosinophils in the gastric mucosa as a result of *Helicobacter pylori* infection. There is an increase in the number of eosinophils and an increased activation of eosinophils as measured by the amount of major basic protein in patients with *Helicobacter pylori* infection. *Helicobacter pylori* infection is associated with upregulation of IL-4, IL-5, TNFα, ICAM-1, Eotaxin and IgE. This upregulation would result in the greater release of eosinophil progenitor cells and an increased recruitment of eosinophils into the tissues. The implications of these changes are discussed.

Keywords: Stomach, eosinophils, cytokines, *Helicobacter pylori*.

Introduction

Gastritis is associated with the presence of eosinophils in the gastric mucosa but their role is uncertain. In acute gastritis there is an increase in the numbers of eosinophils (Morris and Nicholson 1987; Frommer et al 1988) and in chronic gastritis there is a significant increase in eosinophil numbers and extracellular major basic protein in chronic gastritis (McGovern et al 1991).

The present study has been undertaken to examine eosinophils and the relationship of any eosinophilic changes with *Helicobacter pylori* infection.

This study has utilized the considerable knowledge available on the biology of eosinophils which has resulted from the research carried out by workers involved in respiratory diseases.

The study has examined endoscopic biopsies with the transmission electron microscope and immunohistochemical analyses using resin embedding with the biopsies processed into glycol methacrylate (Britten, Howarth and Roche 1993).

Materials and methods

Endoscopic biopsies have been taken from specific sites in the stomach of 38 normal patients and 54 patients whose stomach has been infected with *Helicobacter pylori*. This infection has been substantiated by a positive CLO test (Kimberley-Clark, Ballard Medical Products, Utah, USA) and immunohistochemical detection of the *Helicobacter pylori* in the biopsies.

The biopsies are processed either for transmission electron microscopic study or for immunohistochemical studies. The transmission electron microscopic study has involved fixing the biopsies in 3% cacodylate buffered glutaraldehyde (pH 7·3) at 4°C for four to twenty four hours. The biopsies are then rinsed in cacodylate buffered 10% sucrose (pH 7·3) at 4°C for twenty four hours. Following postfixing in veronal acetate buffered 1% osmium tetroxide (pH 7·3) at 4°C for two hours, the biopsies are rinsed in chilled tap water at 4°C. Dehydration is carried out in a graded series of ethyl alcohol and the biopsies are cleared in propylene oxide. The biopsies are embedded in epoxy resin. Sections are cut 25nm thick and mounted on copper grids prior to being stained with 1% uranyl acetate and Reynolds lead citrate. The sections are examined with a Philips 7000 electron microscope.

Those biopsies used for immunohistochemical studies have been resin embedded by the technique of Britten, Howarth and Roche (1993). Endoscopic biopsies are immediately placed into ice acetone containing 2mM phenyl methyl sulphonyl fluoride and 20mM iodoacetamide and fixed overnight at -20°C. The fixative is replaced with acetone at room temperature for 15 minutes followed by methyl benzoate at room temperature for 15 minutes. The biopsies are then infiltrated with processing solution consisting of 5% methyl benzoate in glycol methacrylate (GMA solution A) at 4°C with three changes of GMA solution A with two hours in each change of solution. The embedding solution consists of 10 millilitres GMA solution A and 70 millilitres benzoyl peroxide. The embedding solution is freshly prepared by dissolving the benzoyl peroxide in solution A by gently shaking. When dissolved add GMA solution B (250µls). The processed biopsies are embedded in the embedding solution, polymerized at 4°C for 48 hours and stored in airtight boxes at -20°C.

The immunohistochemical studies have been performed using the following antibodies:

Antibody	Clone	Source
EG2	mouse monoclonal (EG2)	Pharmacia
Major Basic Protein	mouse monoclonal (BMK13)	Biogenesis
Eotaxin	mouse monoclonal (43911.11)	R & D Systems
TNFα	mouse monoclonal (4H31)	Celltech.Therapeutics
IgE	mouse monoclonal (CIA-E-7.12)	DakoCytomation
IL-1β	mouse monoclonal (2805)	R & D Systems
IL4	mouse monoclonal (3H4)	AMS Biotechnology
IL5	mouse monoclonal (MAB7)	gift
Helicobacter pylori	rabbit polyclonal	DakoCytomation
ICAM-1 (CD54)	mouse monoclonal (RR1/1)	Biosource International

The immunochemical staining for monoclonal and polyclonal antibodies has been carried out as described in Steer (2005).Ethical approval for the study has been obtained. Permission to obtain the endoscopic biopsies as well as perform the cytochemical

analyses have been obtained from the patients. The patients have been undergoing endoscopic examinations as part of the investigation of their presenting symptoms.

Results and discussion

1. Eosinophils and their activation

The presence of eosinophils in the normal gastric mucosa has been confirmed in the present study using the monoclonal antibody EG2 to identify the eosinophils (figure 1) and by transmission electron microscopy. If the number of eosinophils per unit area is calculated, the eosinophils are more numerous in the deep part of the lamina propria related to the neck and base of the gastric glands (figure 2),

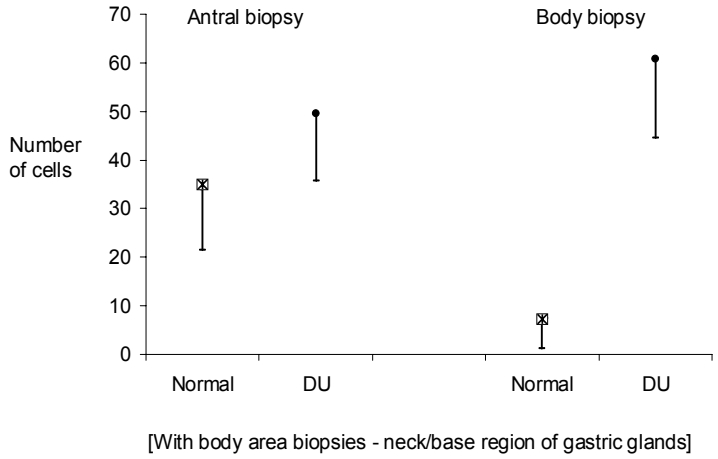

Figure 2. Number of eosinophils per 0.783 square millimetre in the deep part of the mucosa. Mean – Standard Error of the Mean.

as compared with the pit and isthmus areas of the gastric glands (figure 3).

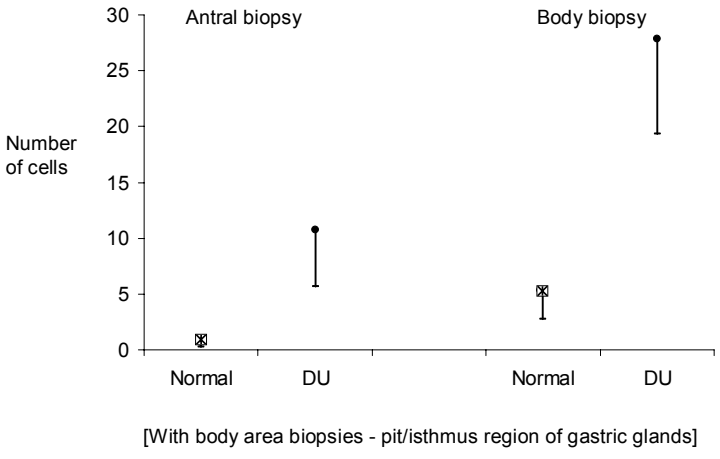

Figure 3. Number of eosinophils per 0.783 square millimetre in the superficial part of the mucosa. Mean – Standard Error of the Mean.

There is a positive correlation between the number of eosinophils in the lamina propria of the antrum and the body of the stomach. This positive correlation is observed when comparing the whole of the mucosa (correlation coefficient 0.702, $p<0.01$), the pit/isthmus area of the gastric glands (correlation coefficient 0.931, $p<0.01$) and the neck/basal area of the gastric glands (correlation coefficient 0.634, $p<0.05$). These significant correlations between the antrum and body of the stomach would suggest that the eosinophil numbers in the stomach at these different sites are influenced by related factors.

Immunohistochemical staining with the monoclonal antibody EG2 has revealed positively stained granules free in the connective tissue matrix (figure 4). This is particularly apparent in the region of the pit/isthmus of the gastric glands in some patients whose stomach is infected with *Helicobacter pylori*. These are the so-called 'clusters of free eosinophilic granules' and confirmation of this finding is made when specimens are examined with the transmission electron microscope (figure 5).

Helicobacter pylori infection is associated with an increase in the number of eosinophils in the lamina propria (figure 6) with this increase being most marked in those biopsies from the body of the stomach.

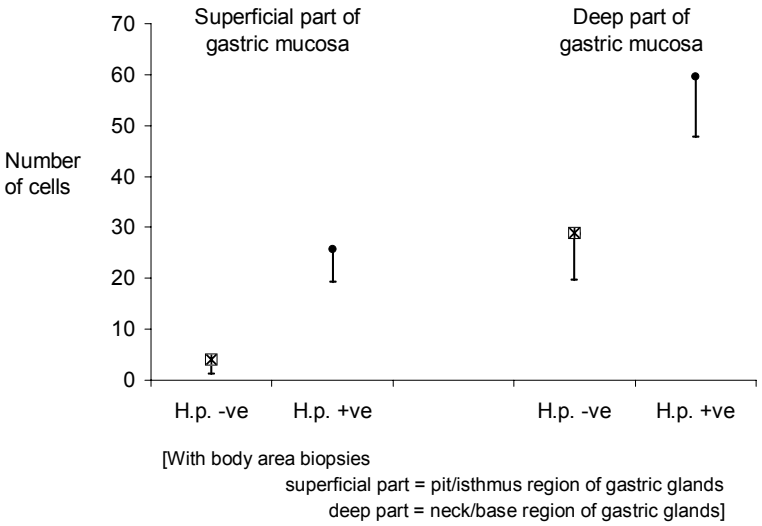

Figure 6. The influence of *Helicobacter pylori* status on the number of eosinophils per 0.783 square millimetre of gastric mucosa. Mean – Standard Error of the Mean.

Eosinophils in normal patients have the classical ultrastructural appearance of a well-defined cell membrane and characteristic cytoplasmic granules with an electron dense core and an electron lucent matrix which is more peripherally sited. In patients with *Helicobacter pylori* infection, when eosinophils in the region of the pit area of the gastric glands are examined they have the ultrastructural appearance of activated eosinophils. The cell membrane is less distinct and there are well-defined changes to the eosinophil cytoplasmic granules (figure 7). The central core of the eosinophilic granules develops electron lucent areas (figure 7) which may involve the whole of the central core

(figure 8). These activated eosinophils are in close contact with other connective tissue cells such as plasma cells (figure 5) and mast cells. The plasma cells in the proximity of activated eosinophils not only have the characteristic well-defined rough endoplasmic reticulum but this endoplasmic reticulum is distended. Free in the connective tissue of the lamina propria of patients infected with *Helicobacter pylori* are cluster of eosinophilic granules, the so-called 'clusters of free eosinophilic granules'(cfegs). The eosinophilic granules in these cfegs have the ultrastructural appearance of 'non-activated' eosinophilic granules so that the central core is electron dense and the granule matrix is electron lucent. These cfegs frequently have a close relationship with plasma cells with some cfegs appearing to have been phagocytosed by the plasma cells.

Eosinophilic granules contain a number of basic proteins with significant biological activity. The crystalline core of the granule contains major basic protein (MBP) (Gleich et al 1973; Lewis et al 1978) and the matrix contains eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO). Major basic protein is strongly cationic with an isoelectric point (pI) of 10.9 (Wasmoen et al 1988) and is rich in arginine. MBP has been used as a marker for eosinophils, the degranulation of eosinophils and the activation of eosinophils. *In vitro*, MBP has been shown to be toxic for parasites (Butterworth et al 1979) and mammalian cells (Gleich et al 1979; Frigas et al 1980).

In the normal human stomach MBP is localized to or in the immediate vicinity of the mucosal eosinophils (figure 9). Approximately 40% of the biopsies from a normal stomach have MBP staining beyond the cell membrane of the eosinophil extending into the connective tissue adjacent to the eosinophil. The sub-epithelial connective tissue has more intense staining in the area of the basement membrane underlying these epithelial cells.

In those patients infected with *Helicobacter pylori*, the distribution of MBP in the gastric mucosa is more diffuse (figure 10). MBP is not localised to the immediate area of the eosinophil as in the normal gastric mucosa but extends into a large area of connective tissue with this staining being particularly marked in the area of the pit/isthmus of the gastric glands. A greater area of the basement membrane underlying the epithelial cells is more intensely stained (figure 10 and 11) in *Helicobacter pylori* infection. In approximately 25% of patients with *Helicobacter pylori* infection of the stomach the infranuclear cytoplasm of the epithelial cells in the vicinity of activated eosinophils is stained (figure 11). In addition, the nuclei of epithelial cells in the vicinity of activated eosinophils in patients infected with *Helicobacter pylori* sometimes contain MBP (figure 11). This nuclear staining is most frequently observed in the neck/basal areas of the gastric glands in the body of the stomach. The MBP appears to be more concentrated in the nuclei of these epithelial cells rather than in the cytoplasm (figure 11). The epithelial cells whose nuclei stain for MBP do not appear to be morphologically different to the adjacent epithelial cells. MBP is known to be cytotoxic (Gleich et al 1979; Frigas et al 1980) but the epithelial cells whose nuclei stain for MBP show no morphological evidence of cellular toxicity. It must therefore be asked "What are the functional implications of the presence of eosinophilic granule proteins in epithelial cells?" Eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) are both eosinophilic granule proteins whose terminal amino acid sequences have significant homology to ribonuclease especially in those regions of the ribonuclease molecule

involved in ligand binding (Gleich et al 1986). This may indicate that ECP and EDN have ribonuclease-like activity. If these molecules have such activity their presence in epithelial cells particularly in *Helicobacter pylori* infection may indicate a role in changing the epithelial cell ribonucleic acid configuration so as to adapt to the infection or they may function in removing unwanted ribonucleic acid from these epithelial cells.

The results support the evidence that eosinophils are not only present in the stomach but are activated in *Helicobacter pylori* infection (figure 35).

2. What causes the activation of these eosinophils?

Eosinophils have surface receptors to immunoglobulins, cytokines and complement. The engagement of these receptors has been shown to result in eosinophil activation.

Fc receptors for immunoglobulin E (IgE (FceR)) are present on the surface membrane of eosinophils (Capron et al 1981). In the present study the lamina propria contains cells with cytoplasmic IgE and membranous IgE (figure 12). These cells are found in both the normal stomach and the stomach infected with *Helicobacter pylori*. The lamina propria contains more cells with membranous IgE than cytoplasmic IgE whether examining biopsies from the antrum or body of both the normal stomach and the stomach infected with *Helicobacter pylori*. The principle IgE change seen in *Helicobacter pylori* infection occurs in the number of cells with membranous IgE in the body of the stomach. The maximum number of cells with membranous IgE occurs in the body of the stomach in patients infected with *Helicobacter pylori* (figure 13).

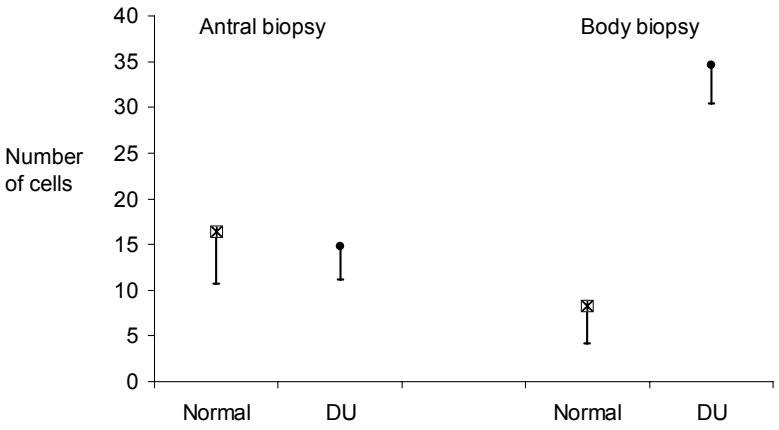

Figure 13. The number of lamina propria cells with membranous IgE per 0.783 square millimetre of gastric mucosa. Mean – Standard Error of the Mean.

Infection of the stomach with *Helicobacter pylori* is also associated with an increase in those cells with cytoplasmic IgE particularly in the body of the stomach. Generally, the body of the stomach contains more cells with cytoplasmic IgE than the antrum of the stomach. The cells with cytoplasmic IgE have the general morphology of plasma cells. Some of those cells with membranous IgE have the morphology of mast cells.

Eosinophils have surface receptors for immunoglobulins other than IgE. Thus, IgG antibodies to *Schistosoma mansoni* are able to induce eosinophil degranulation in a helminth targeting assay (Butterworth et al 1977). In an erythrocyte resetting assay approximately 35% of eosinophils have IgG receptors. In addition, IgA and secretory IgA are potent signals for eosinophilic degranulation with evidence that this is a Fc receptor mediated activity (Abu-Ghazaleh et al 1989). There is no evidence for any synergism between IgA receptors and IgG receptors (Abu-Ghazaleh et al 1989) suggesting that different receptors are involved with these immunoglobulins. Similarly, pre-incubation of eosinophils with anti-receptor antibodies to FceR caused significantly more inhibition to IgE rosettes than IgG rosettes suggesting different receptors (Capron et al 1984).

In the gastric mucosa it is not known whether eosinophilic surface receptors to IgE, IgG, IgA, sIgA or cytokines are responsible for the activation seen in eosinophils in the mucosa of patients infected with *Helicobacter pylori*. It has already been shown that the number of lamina propria cells with both cytoplasmic IgE and membranous IgE are increased in *Helicobacter pylori* infection.

Helicobacter pylori infection of the stomach results in a significant serum antibody response in patients with duodenal ulceration when compared with control patients (Steer, Hawtin and Newell 1987). Duodenal ulceration is associated with a significant increase in anti-*helicobacter pylori* serum IgG, IgG1, IgG3, IgG4 and IgA antibodies. Whether these IgG or IgA antibodies to *Helicobacter pylori* are implicated in eosinophil activation has yet to be demonstrated.

It is possible to diagrammatically represent the situation with respect to the activation of eosinophils in the stomach as shown in figure 35.

3. What factors are implicated in eosinophil recruitment?

Numerous factors control eosinophil recruitment. The eosinophils have to migrate from the vascular lumen through the endothelial cell lining of the blood vessels into the connective tissue in order to be recruited into the tissues.

Adhesion molecules

Adhesion molecules have an important role in eosinophil recruitment. Eosinophils are known to bind to adhesion molecules. Eosinophils bind to the intercellular adhesion molecule-1 (ICAM-1, CD54) by means of lymphocyte function-associated antigen-1 (LFA-1, CD11α/CD18) (Weller et al 1991; Dobrina et al 1991; Bochner et al 1991; Kyan-Aung et al 1991).

If ICAM-1 expression is evaluated in the normal gastric mucosa (figure 13) the expression of this molecule is confined to the endothelial cells of the vascular channels

where the ICAM-1 is strongly expressed. However, when gastric mucosal biopsies from patients infected with *Helicobacter pylori* are examined the expression of ICAM-1 is upregulated. ICAM-1 is not only strongly expressed in the endothelial cells (figure 14) but is now expressed in the connective tissue particularly around the connective tissue cells (figure 15). There is very weak expression of ICAM-1 in the region of the epithelial basement membrane. Thus, the ICAM-1 expression in the normal mucosa would facilitate migration of cells with appropriate receptors from the blood vessels. The upregulation of ICAM-1 would assist in any increased demand for this migration.

Expression of adhesion molecules is controlled by, amongst other processes, inflammatory cytokines such as IL-1β and TNFα (see Pober and Cotran 1990; Ebisawa et al 1992). If another adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1) is considered, the upregulation of VCAM-1 in the pulmonary vasculature of allergic airways eosinophilia requires IL-4 in addition to TNFα (Lei et al 1998).

ICAM-1 expression is upregulated by IL-1 and TNF (Dustin et al 1986) with the upregulation by TNF being the greater. This effect of TNF has been calculated as resulting in an eight fold increase of ICAM-1. TNFα upregulation of ICAM-1 has been noted in *Helicobacter pylori* associated gastritis (Hatz et al 1997). The upregulation of IL-1β (figure 16 and 17), TNFα (figure 18 and 19) and ICAM-1 (figure 13 and 14) in *Helicobacter pylori* infection has been noted in the present study.

The expression of ICAM-1 surrounding the mucosal connective tissue cells (figure 15) in *Helicobacter pylori* infection of the stomach may have implications with respect to lymphocyte trafficking, lymphocyte activation and lymphocyte proliferation in the gastric mucosa. Fibroblast expression of ICAM-1 is upregulated by various cytokines resulting in greater T lymphocyte binding (Dustin et al 1986; Krzesicki et al 1991; Meng et al 1995). Intestinal fibroblasts can bind T lymphocytes (Ebert et al 1996; Ina et al 1996) with ICAM-1 being essential for this binding (Musso et al 1999). This binding of T lymphocytes to fibroblasts provides costimulatory signals leading to further activation of T lymphocytes (Van Seventer et al 1990; Damle et al 1992). In addition, human intestinal fibroblasts modulate mucosal T lymphocyte proliferation and apoptosis (Ina et al 1995). With such interactions between these biochemical molecules of the lamina propria fibroblasts and T lymphocytes, the importance of these molecules and mucosal changes in *Helicobacter pylori* infection can be appreciated.

Interleukin 1β (IL-1β)

IL-1β production is significantly increased *in vitro* in the gastric mucosal biopsies from patients with *Helicobacter pylori* infection when compared with the normal gastric mucosa (Noach et al 1994; Peek et al 1995). In the present study IL-1β is found in the mucosa of the normal stomach (figure 16). There is significant staining for IL-1β in the mucus of the supranuclear portion of the epithelial cells at the luminal surface of the mucosa and at the pit/isthmus of the gastric glands. There is a generalised but weak expression of IL-1β in the connective tissue of the mucosa and patchy staining of the epithelial basement membrane/basal cell membrane in the region of the pit/isthmus of the gastric glands. There is some coarse granular staining in the lumen of mucosal blood vessels and positive staining of the lining of these blood vessels. In addition, in the body of the stomach there is positive granular staining of the cytoplasm of the chief cells.

An examination of the gastric mucosa of patients with *Helicobacter pylori* infection reveals that there is a significant upregulation of IL-1β expression in the mucus of the epithelial cells at the gastric surface and the pit/isthmus of the gastric glands (figure 17). In addition, some of the connective tissue cells are positively stained for IL-1β and there is more intense and generalised staining for IL-1β of the basal cell membrane/basement membrane of the epithelial cells in the region of the pit/isthmus of the gastric glands (figure 20) as well as the luminal epithelium of the stomach. The IL-1β staining in the mucosal blood vessels is similarly distributed to that of normal mucosal biopsies but is upregulated (figure 20).

The effect of *Helicobacter pylori* on the IL-1β expression *in vivo* supports the previous *in vitro* evidence and is consistent with the conclusion that this proinflammatory cytokine is associated with the upregulation of IL-8 expression in *Helicobacter pylori* infection (Steer 2005).

Tumour necrosis factor α (TNFα)

Eotaxin is an eosinophil specific chemokine. TNFα can stimulate monocytes *in vitro* to produce eotaxin (Nakamura et al 1998). This eotaxin production does not involve protein kinase C or protein synthesis which indicates that the expression of eotaxin can be regulated by more than one mechanism.

Cell lines other than monocytes can produce eotaxin when stimulated by TNFα. Thus, eotaxin mRNA is rapidly and transiently induced by TNFα in respiratory epithelial cell lines (Lilly et al 1997). Lung fibroblasts produce both eotaxin and RANTES on TNFα stimulation (Teran et al 1999) with the production of eotaxin being enhanced by synergism with IL-4.

In the present study, the normal gastric mucosa contains little identifiable TNFα. There is a trace of staining of the basement membrane of the epithelium and blood vessels in the pit/isthmus region of the gastric glands (figure 18). There are very occasional weakly stained connective tissue cells (figure 18) and no other positively stained material.

In those biopsies from patients infected with *Helicobacter pylori* there is considerable upregulation of TNFα expression. There is strongly positive TNFα expression of the basement membrane of the blood vessels and endothelial cells present throughout the gastric mucosa (figure 19). There is a significant increase in the TNFα expression of the connective tissue particularly in the pit/isthmus area of the gastric glands. There is no staining of any of the various types of epithelial cells in the normal biopsies or those biopsies from patients infected with *Helicobacter pylori*.

If the number of connective tissue cells expressing TNFα is determined (figure 21), there is a significant increase in the number of these cells in the antral area of the stomach ($p<0.05$) and in the body of the stomach ($p<0.04$) when the stomach is infected with *Helicobacter pylori*.

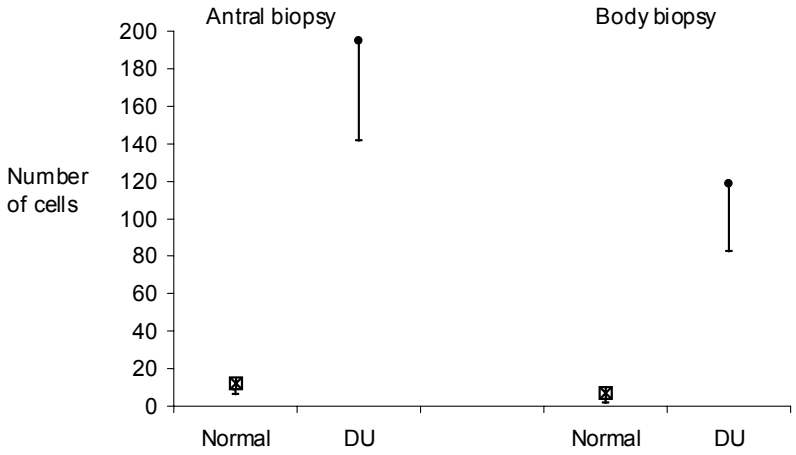

Figure 21. Number of cells positive for cytoplasmic TNFα in the mucosal connective tissue per 0.783 square millimetre of connective tissue in patients with a normal stomach and patients with duodenal ulceration. Mean – Standard Error of the Mean.

Interleukin 4 (IL-4)

Interleukin-4 is a T helper 2 (Th 2) lymphocyte specific cytokine which promotes eosinophil tissue recruitment (Rothenberg et al 1995; Lukacs et al 1997; Mueller et al 1997). IL-4 has been implicated in eotaxin production (Rothenberg et al 1995) and it has been demonstrated that eotaxin mRNA expression is induced by IL-4 in epithelial and endothelial cells (Garcia-Zepeda et al 1996; Stellato et al 1997), in dermal fibroblasts (Sticherling et al 1995) and in lung fibroblasts (Teran et al 1999).

Mochizuki et al (1998) have been able to recover eotaxin from dermal fibroblast cell line cultures following stimulation with IL-4. The ability of IL-4 to induce eotaxin production in airway inflammation in asthma has been confirmed by Li et al (1999) but they found that IL-13 was significantly more potent in stimulating eotaxin production.

In the present study IL-4 has been identified using the monoclonal antibody 3H4. In the mucosal biopsies from the normal stomach there is very weak or no expression of epithelial IL-4 (figure 22). However, in those gastric biopsies from patients infected with *Helicobacter pylori* there is marked upregulation of IL-4 with intense staining of the mucus (figure 22), connective tissue cells and connective tissue particularly in the region of the pit/isthmus of the gastric glands. There is occasional granular staining in the mucosal blood vessels.

IL-4 has also been shown to facilitate eosinophil migration into tissues as it induces VCAM expression on endothelial cells *in vitro* and induces VLA-4/VCAM dependent adherence of eosinophils and basophils but not neutrophils to endothelium (Schleimer et al 1992). The *in vitro* upregulation of endothelial cell VLA-4/VCAM and LFA-1/ICAM-1 by IL-4 has been confirmed by Moser and colleagues (Moser et al 1992A; Moser et al 1992B).

Eotaxin

Eotaxin is an eosinophil specific chemokine which was originally identified in the bronchoalveolar lavage (BAL) of guinea pigs with experimental allergic airways disease (Jose et al 1994). Eotaxin has been studied in respiratory tract diseases and has been shown to be upregulated in asthma and allergic rhinitis. Eotaxin mRNA is constitutively expressed in the small intestine and colon of the normal human gastro-intestinal tract (Garcia-Zepeda et al 1996) and is upregulated in inflammatory bowel disease. Previously, studies relating to any possible role for eotaxin in gastric diseases have been lacking.

Numerous cell types have been identified as a source of eotaxin with most of this information resulting from studies of respiratory diseases.

Epithelial cells

In vitro experiments using a number of different immunological techniques have revealed that the respiratory epithelium is one source of eotaxin (Conroy et al 1997; Mattoli et al 1997; Lilly et al 1997; Cook et al 1998; Ying et al 1997; Lankhioued et al 1997; Garcia-Zepeda et al 1996).

The present study supports an origin from epithelial cells with the epithelial cells at the pit/isthmus areas of the gastric glands having dense eotaxin staining in the supranuclear cytoplasm of those patients infected with *Helicobacter pylori* (figure 24). The luminal epithelial cells are stained for eotaxin to a lesser degree (figure 24) and the epithelium in the neck of the gastric glands does not stain for eotaxin. In the normal gastric mucosa, the epithelial cells lack any eotaxin expression but there are occasional small eotaxin positive granules in the gastric mucus at the pit region of the gastric glands (figure 25). The expression of eotaxin in the gastric mucosa can be divided into four broad categories (grades 0,1,2 and 3). These grades are defined as follows:

Grade 0	No eotaxin.
Grade 1	Occasional epithelial cells in the region of the pit/isthmus of the gastric glands possess 1 to 3 small eotaxin positive granules in the supranuclear cytoplasm (figure 25). Such granules are occasionally seen as clumps in the mucus that has already been shed into the gland lumen.
Grade 2	Most of the epithelial cells in the region of the pit/isthmus of the gastric glands have small eotaxin positive supranuclear cytoplasmic granules (figure 26). The whole of the supranuclear cytoplasm of the epithelial cells at the pit/isthmus of the glands is ‘tinged’ red (eotaxin positive). Occasional epithelial cells at the luminal surface of the stomach have a small number of eotaxin positive supranuclear cytoplasmic granules.
Grade 3	(a) Epithelial cells at the pit/isthmus of the gastric glands have dense eotaxin granular staining in the supranuclear cytoplasm (figure 24). The luminal epithelial cells are stained to a lesser degree than at the pit/isthmus of the gland and the epithelium in the neck/base of the glands is not stained for eotaxin.

(b) Granular eotaxin positive staining of the connective tissue in the region of the pit/isthmus of the gastric glands (figure 24).

(c) Linear eotaxin staining in the region of the basement membrane of the epithelial cells, the outlining of connective tissue cells and the outlining of the mucosal blood vessels. All this connective tissue staining is in the region of the pit/isthmus of the gastric glands. The basement membrane related to the chief cells and the parietal cells (figure 28) is stained for eotaxin as well as the endothelial cell basement membrane.

If these grades of eotaxin expression are used to evaluate biopsies from the normal stomach and the stomach of patients with benign duodenal ulceration the eotaxinexpression is increased in both the antral area (figure 29),

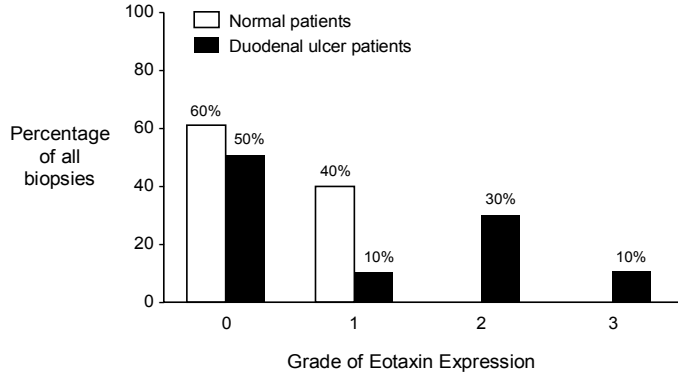

Figure 29. Grade of eotaxin expression in antral biopsies from the normal stomach and from patients with benign duodenal ulceration.

and the body area of the stomach (figure 30).

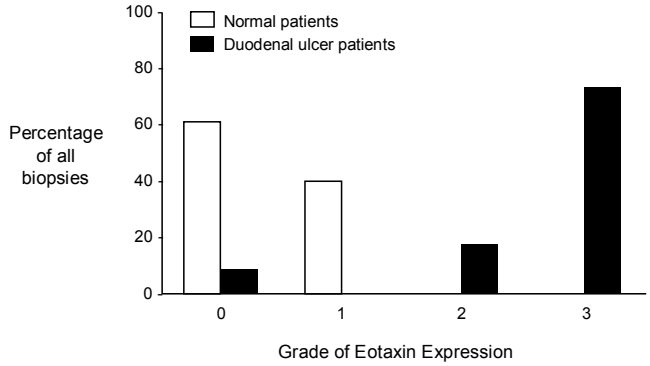

Figure 30. Grade of eotaxin expression in biopsies from the body of the normal stomach and from patients with benign duodenal ulceration.

When eotaxin expression is compared with *Helicobacter pylori* infection there is a greater proportion of *Helicobacter pylori* positive patients expressing the Grade 2 and Grade 3 distribution of eotaxin (figure 31).

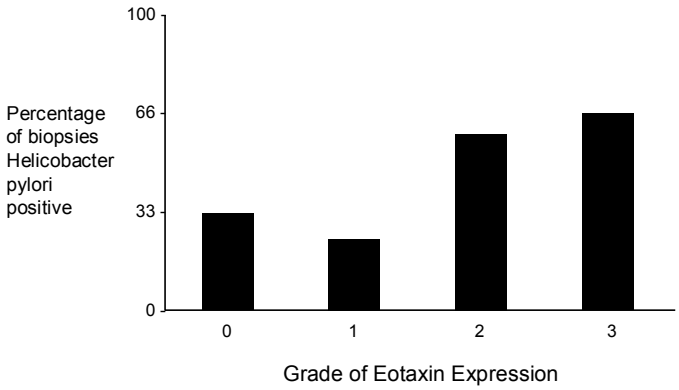

Figure 31. The percentage biopsies in each grade of eotaxin expression which are *Helicobacter pylori* positive.

Endothelial cells

Endothelial cells as well as the respiratory epithelium are major sources of eotaxin in atopic asthmatics (Ying et al 1997). Endothelial cells are also stained for eotaxin in those cases of renal interstitial nephritis which are heavily infiltrated with eosinophils (Wada et al 1999). Garcia-Zepeda et al (1996) showed that human umbilical cord endothelial cells expressed eotaxin mRNA *in vitro* after stimulation with IL-1α, TNFα and, to a lesser extent, INFδ.

The present work widens the distribution of endothelial cells with the potential for eotaxin production from the lung, kidney and umbilical cord to the stomach. Eotaxin staining is related to gastric mucosal blood vessels (figure 28) in those patients infected with *Helicobacter pylori* and is seen to surround those mucosal endothelial cells. In the normal gastric mucosa, endothelial cells of blood vessels in the pit/isthmus areas of the gastric glands are sometimes positive for eotaxin. Eotaxin stained material is also found as coarse granules in the lumen of the mucosal blood vessels.

Connective tissue cells

Eotaxin is produced by fibroblasts *in vitro* after cytokine stimulation with TNFα (Noso et al 1998) and IL-4 (Teran etal 1999; Mochizuki 1998). The potential for fibroblasts to produce eotaxin after IL-4 stimulation has also been demonstrated for dermal fibroblasts (Sticherling et al 1995). With fibroblasts being an essential component of the connective tissue environment it is interesting and important to observe the effect of *Helicobacter pylori* infection on the expression of eotaxin in the lamina propria connective tissue of the stomach. The normal gastric mucosa contains a small number of eotaxin expressing connective tissue cells. These positive lamina propria cells appear to

be equally distributed in the body and antrum of the normal stomach. *Helicobacter pylori* infection is associated with a general increase in the expression of eotaxin in the lamina propria (figure 24) and in the connective tissue cells (figure 24) with this increase being particularly marked in the antral area of the stomach at the site of maximal *Helicobacter pylori* infection.

It has been shown that many factors influence eosinophil recruitment. Some of these factors are shown diagrammatically in figure 35.

4. Are there any factors influencing the conversion of eosinophil progenitor cells and the release of these cells from the bone marrow?

Interleukin 5 is a cytokine with restricted biological activity which primarily affects eosinophils and their progenitor cells. Eosinophil recruitment from the bone marrow is dependent upon IL-5 (Collins et al 1995). The mobilization of progenitor eosinophils from the bone marrow is dependent upon circulating rather than local interleukin 5 (Wang et al 1998). Thus, the IL-5 needs to be available to the bone marrow and initiate the cell signalling release of these progenitor cells. It has been demonstrated *in vitro* that IL-5 stimulates the differentiation of eosinophil progenitor cells (Clutterbuck and Sanderson 1988; Lee et al 1997) and it is possible that IL-5 may have this effect *in vivo* either before release of progenitor cells from the bone marrow or after their release from the bone marrow.

Both asthmatic patients and experimental models of asthma-like inflammation are associated with an increase in eosinophil numbers. Such inflammatory conditions of the respiratory tract are associated with increased levels of IL-5 in the peripheral blood and in the local lung tissue (Hamid et al 1991; Robinson et al 1992; Walker et al 1992; Alexander et al 1994; Yamaguchi et al 1994; Ohkawara et al 1997).

In the normal human stomach, IL-5 is present in the lumen of mucosal blood vessels (figure 32). This IL-5 takes the form of coarse granular staining which is frequently located near the periphery of the blood vessel lumen. IL-5 is located in the region of the basement membrane of the endothelial cells of these blood vessels and is patchily located in the basement membrane of the gastric epithelial cells (figure 33). There is very occasional positive granular staining of the gastric mucus that has been shed into the gastric lumen (figure 33).

When the stomach is infected with *Helicobacter pylori* there is a significant upregulation of IL-5 expression. There is increased mucus staining of epithelial cells at the gastric surface and pit area of the gastric glands. This staining is in the supranuclear part of these epithelial cells (figure 34). This increased staining of the mucus is located at the sites where *Helicobacter pylori* colonization occurs.

The staining of the basement membrane of the epithelial and endothelial cells is significantly increased in *Helicobacter pylori* infection. This basement membrane staining is more continuous in the pit/isthmus region of the gastric gland rather than the patchy minimal staining found in the normal stomach (figure 34).

IL-5 is present in the connective tissue (figure 34) and in some connective tissue cells of the lamina propria in *Helicobacter pylori* infection with some of these connective tissue cells having the morphology of plasma cells.

There is persistence of the coarse granular IL-5 staining in the lumen of mucosal blood vessels in *Helicobacter pylori* infection.

The presence of IL-5 in the mucosal blood vessels of the stomach is comparable to the circulatory IL-5 which has been shown to promote the release of eosinophil progenitor cells from the bone marrow.

The eosinophils have to migrate from the vascular lumen through the endothelial cell lining of the blood vessels into the connective tissue in order to be recruited into the tissues.This migration of eosinophils is facilitated by adhesion molecules.

The role of the biochemical signalling molecules from the conversion of eosinophil progenitor cells to the functioning of eosinophils in the gastric mucosa in *Helicobacter pylori* infection is diagrammatically shown in figure 35.

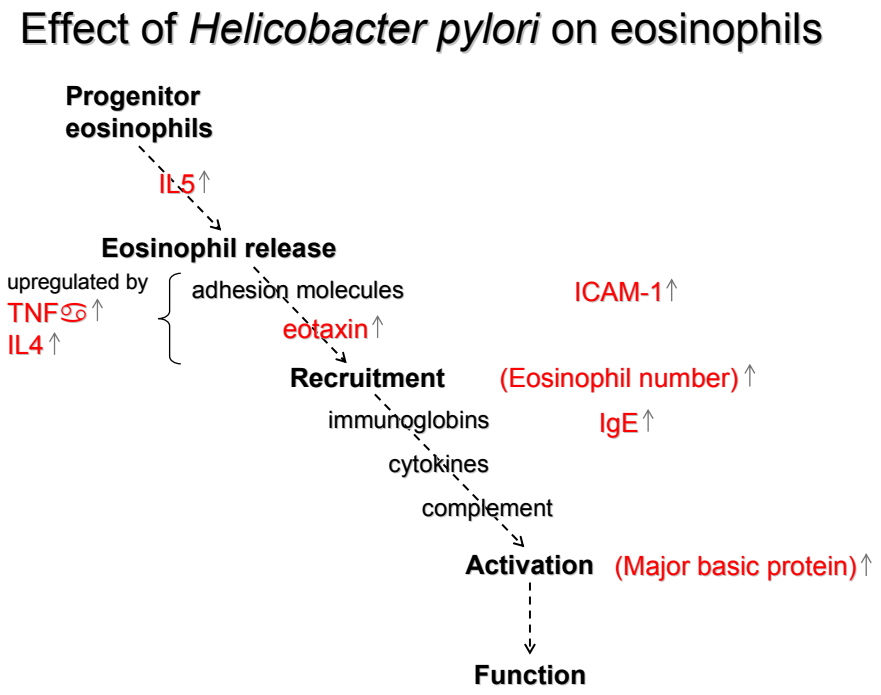

Figure 35. Schematic effect of *Helicobacter pylori* infection on eosinophils with the parts investigated in this study in red.

Acknowledgements

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Figure 1.

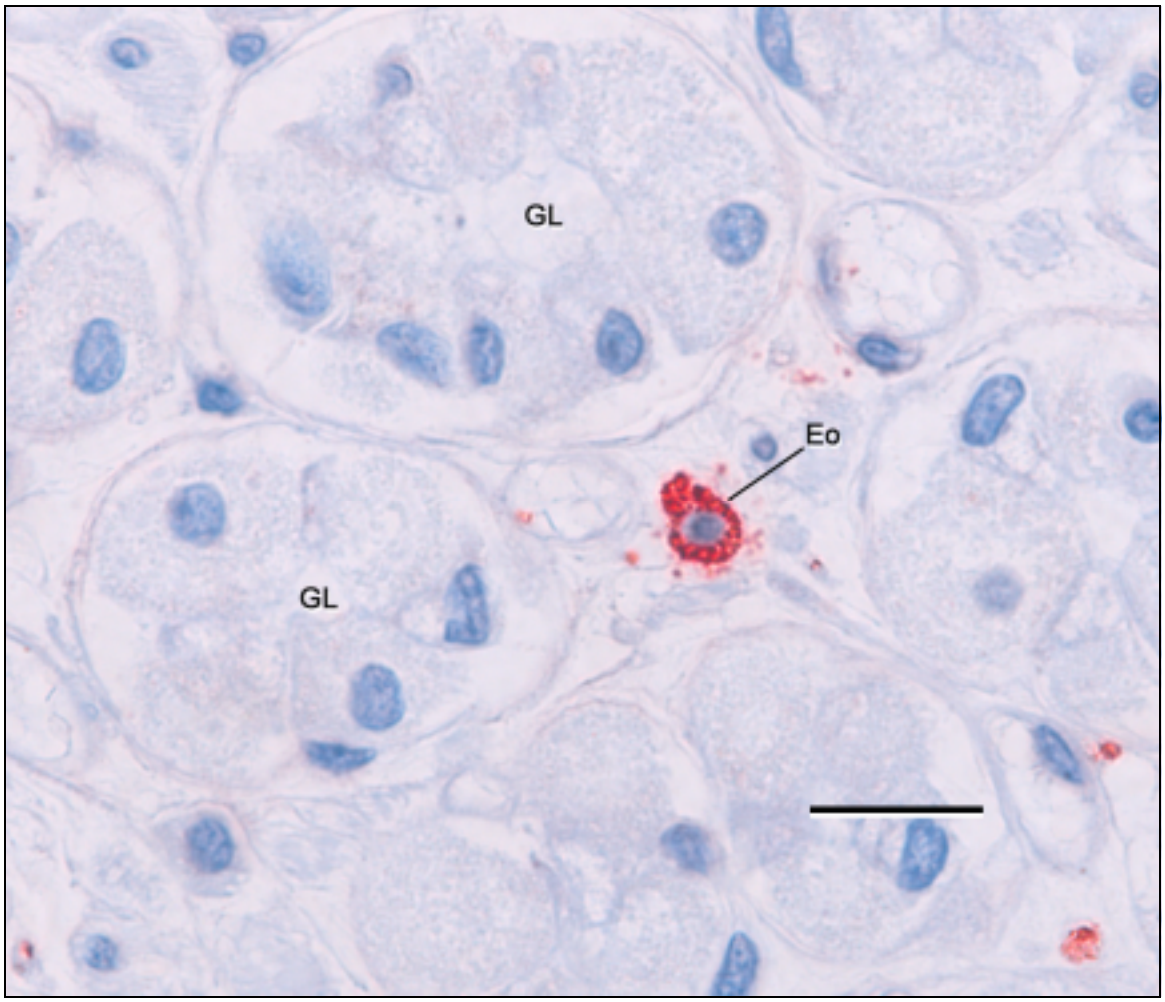

Figure 1. EG2. (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. The lumen (GL) of gastric glands and an eosinophil (Eo) are shown. Scale bar is 20µm.

Figure 4.

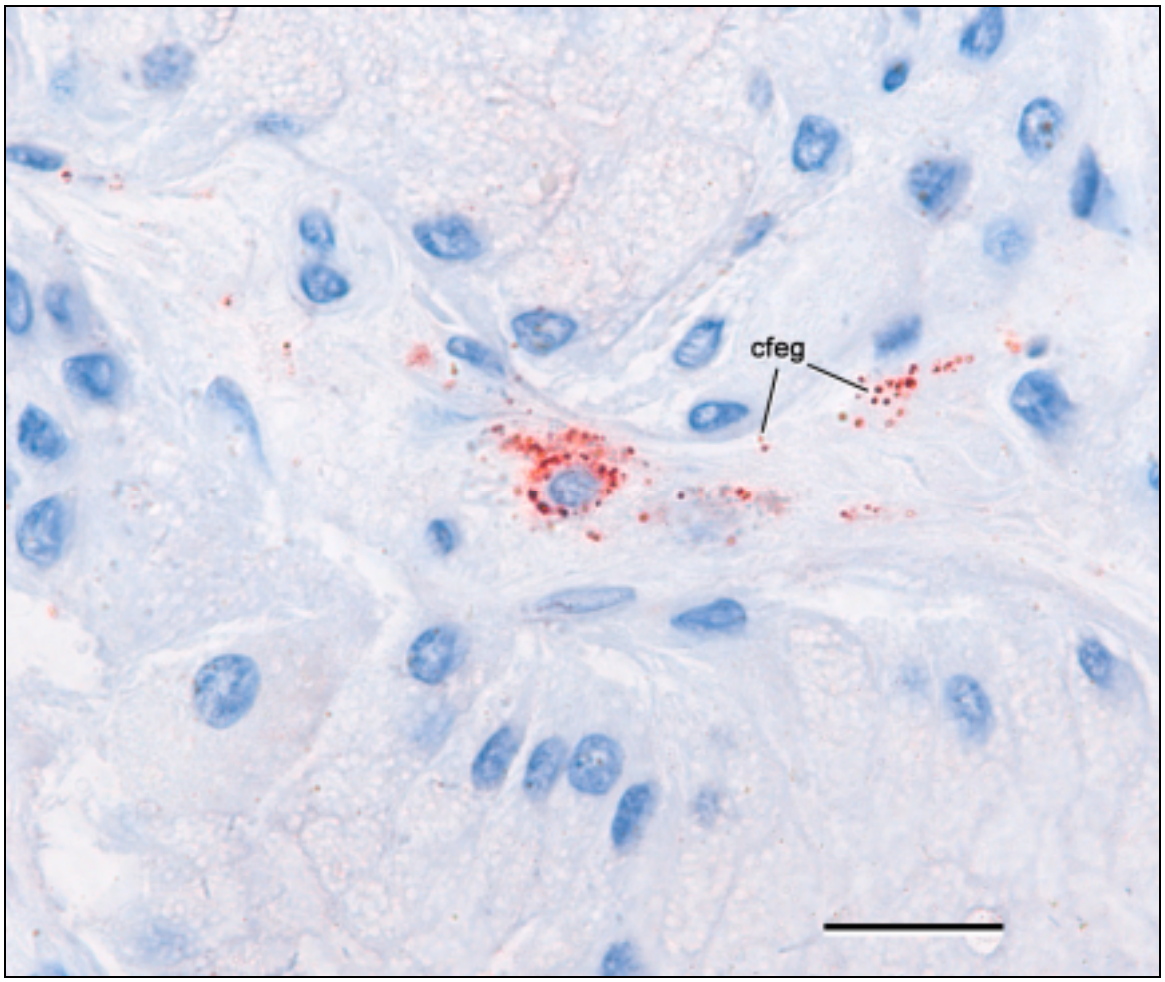

Figure 4. EG2. (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. Numerous clusters of free eosinophilic granules (cfeg) are shown. Scale bar is 20µm.

Figure 5.

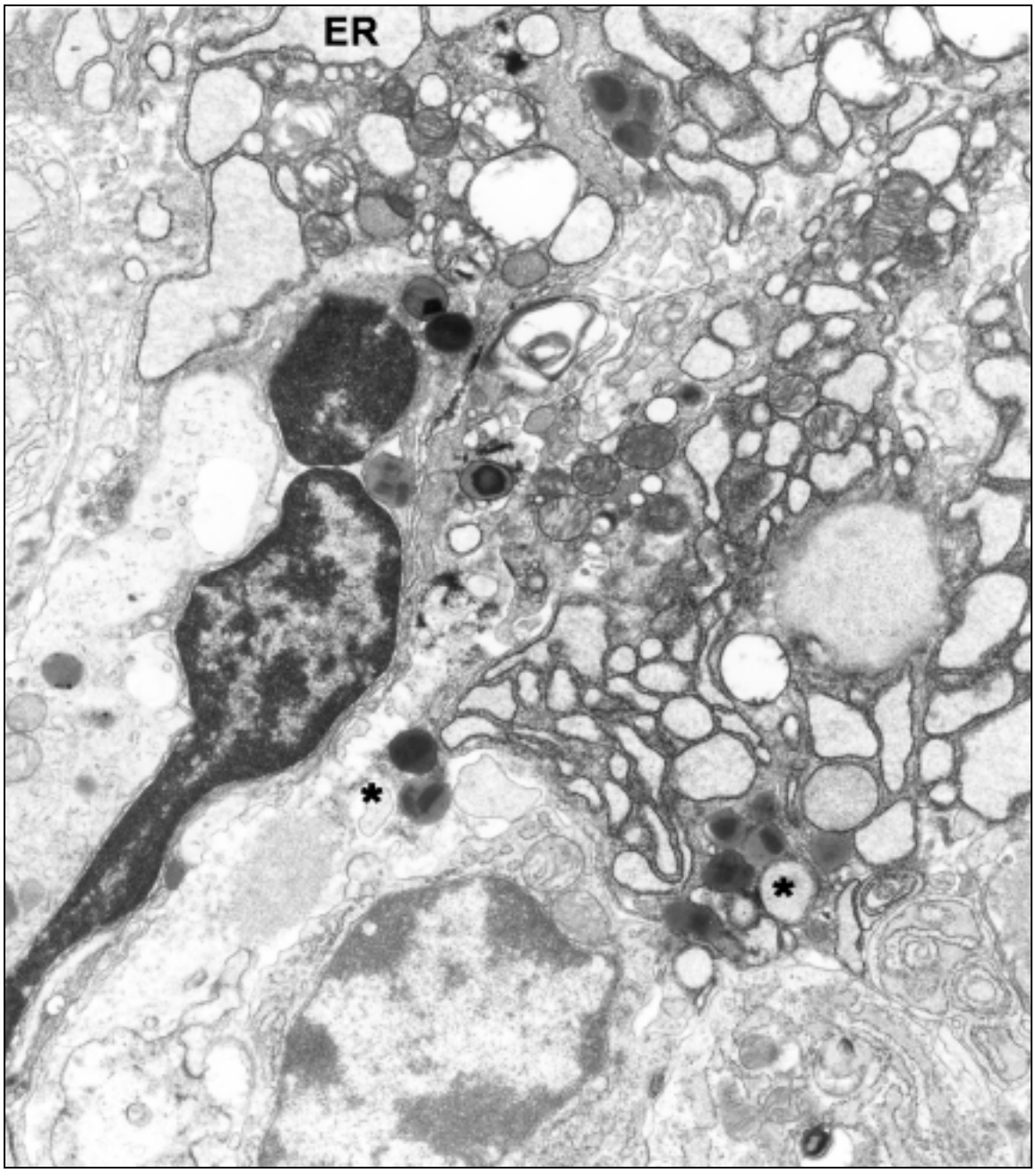

Figure 5. Transmission electron micrograph.

Male aged 51 years.
Helicobacter pylori infection.
Mucosal biopsy from the antrum of the stomach.

Mucosal connective tissue eosinophil and numerous clumps of free eosinophilic granules (∗) together with the dilated rough endoplasmic reticulum (ER) of plasma cells.
Magnification x 12,703.

Figure 7.

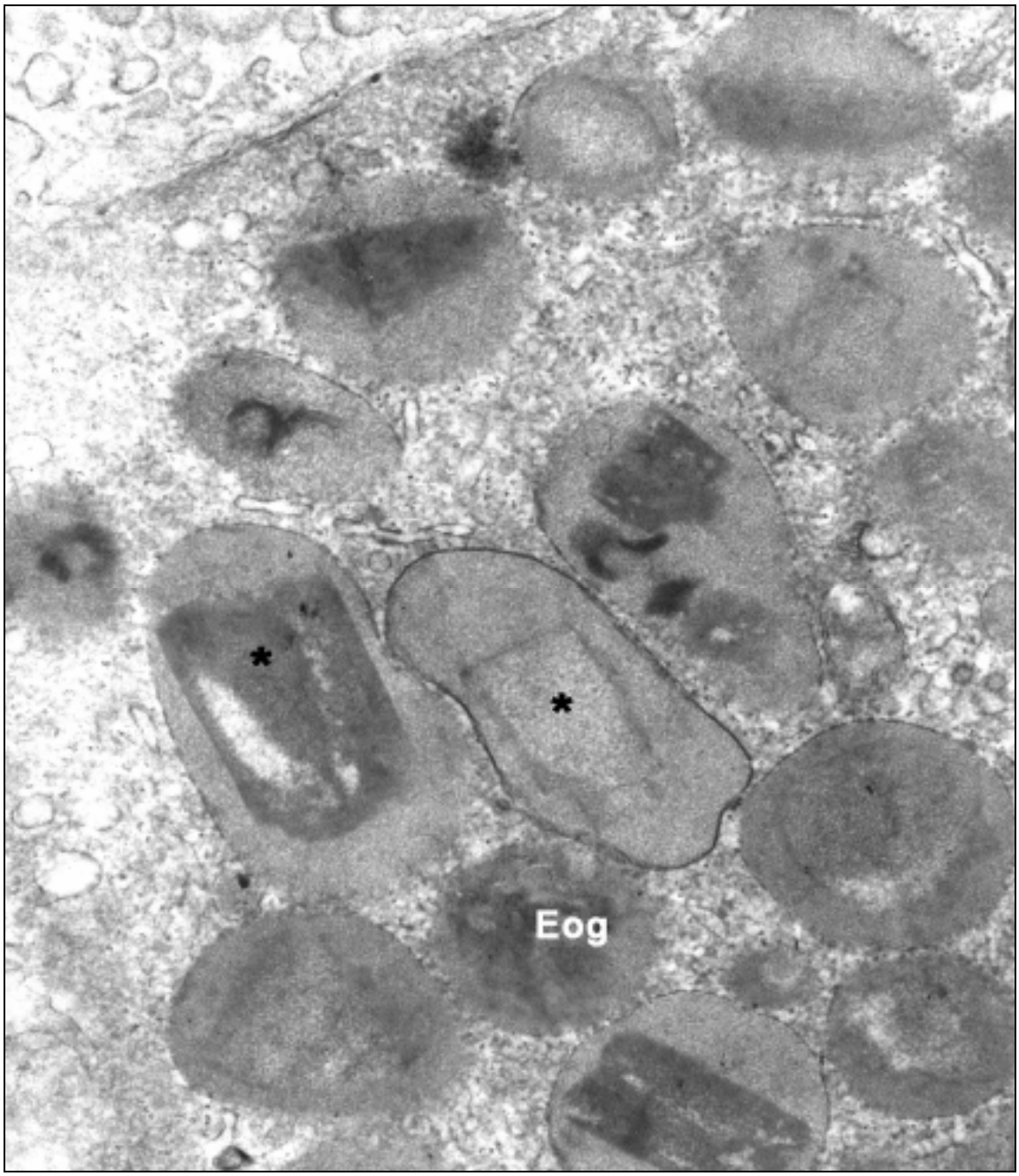

Figure 7. Transmission electron micrograph.

Male aged 29 years.
Helicobacter pylori infection.
Mucosal biopsy from the incisura angularis of the stomach.

Cytoplasmic granules (Eog) present in an activated eosinophil. The dense central core (∗) of a number of granules show radiolucent areas characteristic of degranulation.
Magnification x 51,933.

Figure 8.

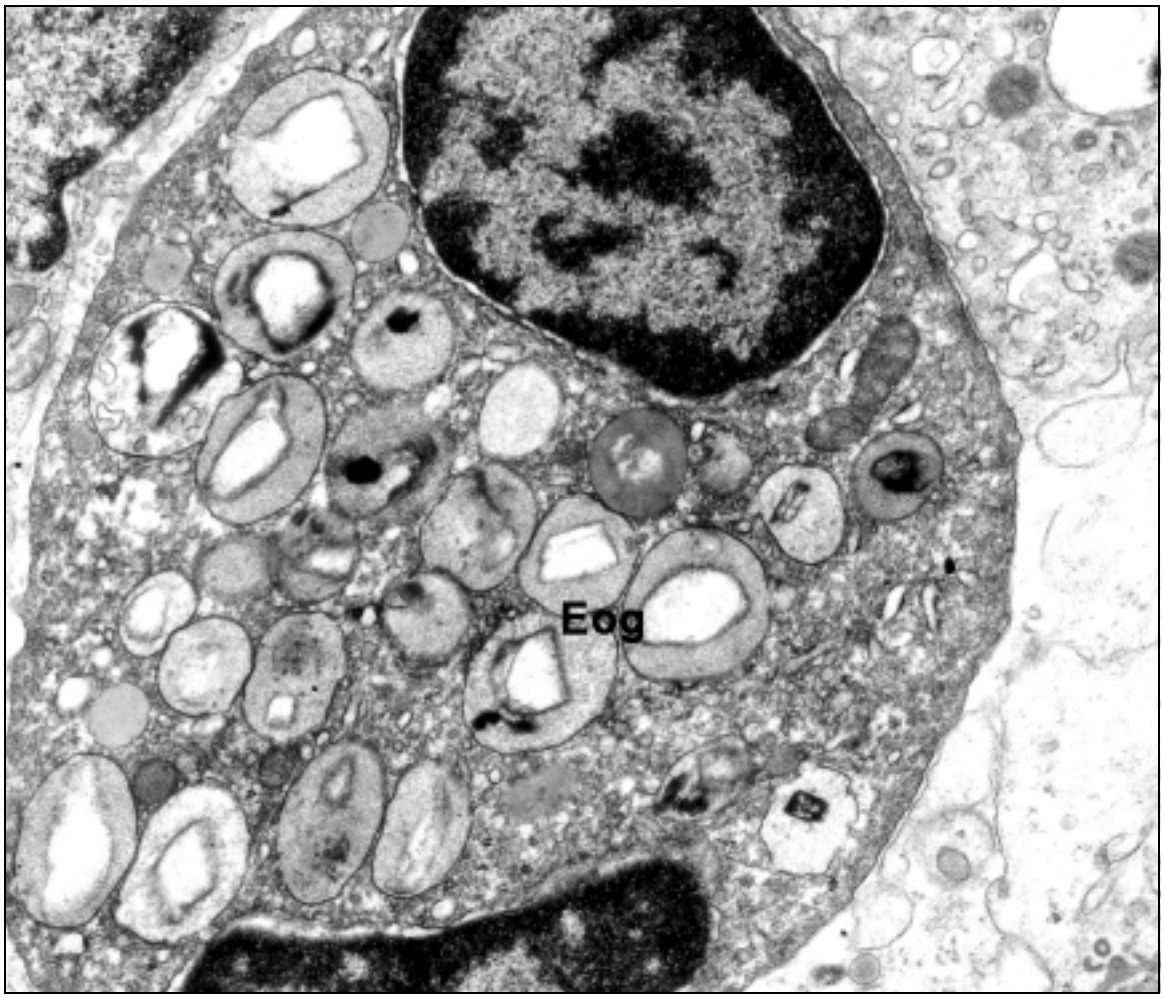

Figure 8. Transmission electron micrograph.

Male aged 63 years.
Helicobacter pylori infection.
Mucosal biopsy from the prepyloric area of the stomach.

An activated eosinophil in the mucosal connective tissue near the surface of the mucosa with the eosinophil granules (Eog) devoid of the electron dense central core.
Magnification x 21,168.

Figure 9.

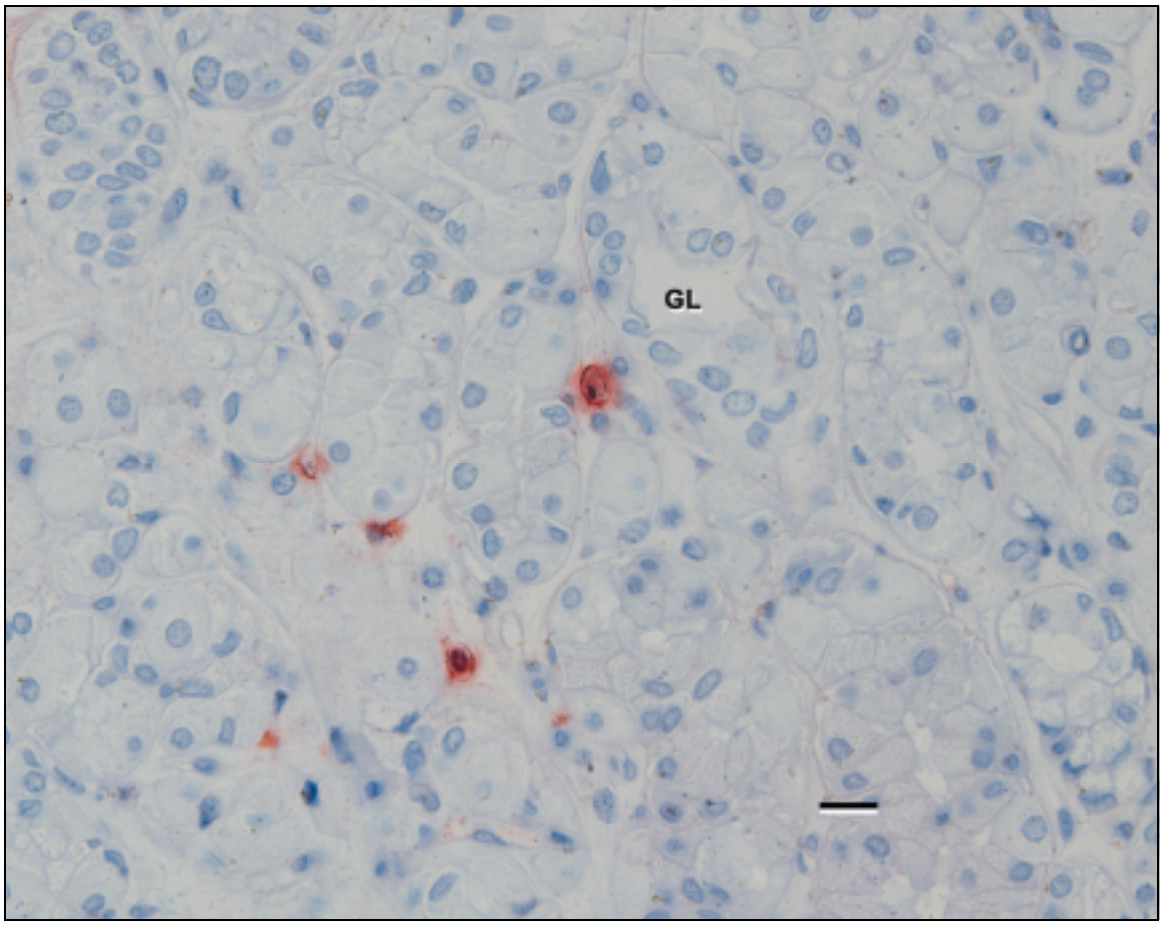

Figure 9. Major Basic Protein. (Chromogen substrate).

Mucosal biopsy from the body of the normal stomach. The lumen (GL) of a gastric gland is shown. Scale bar is 20µm.

Figure 10.

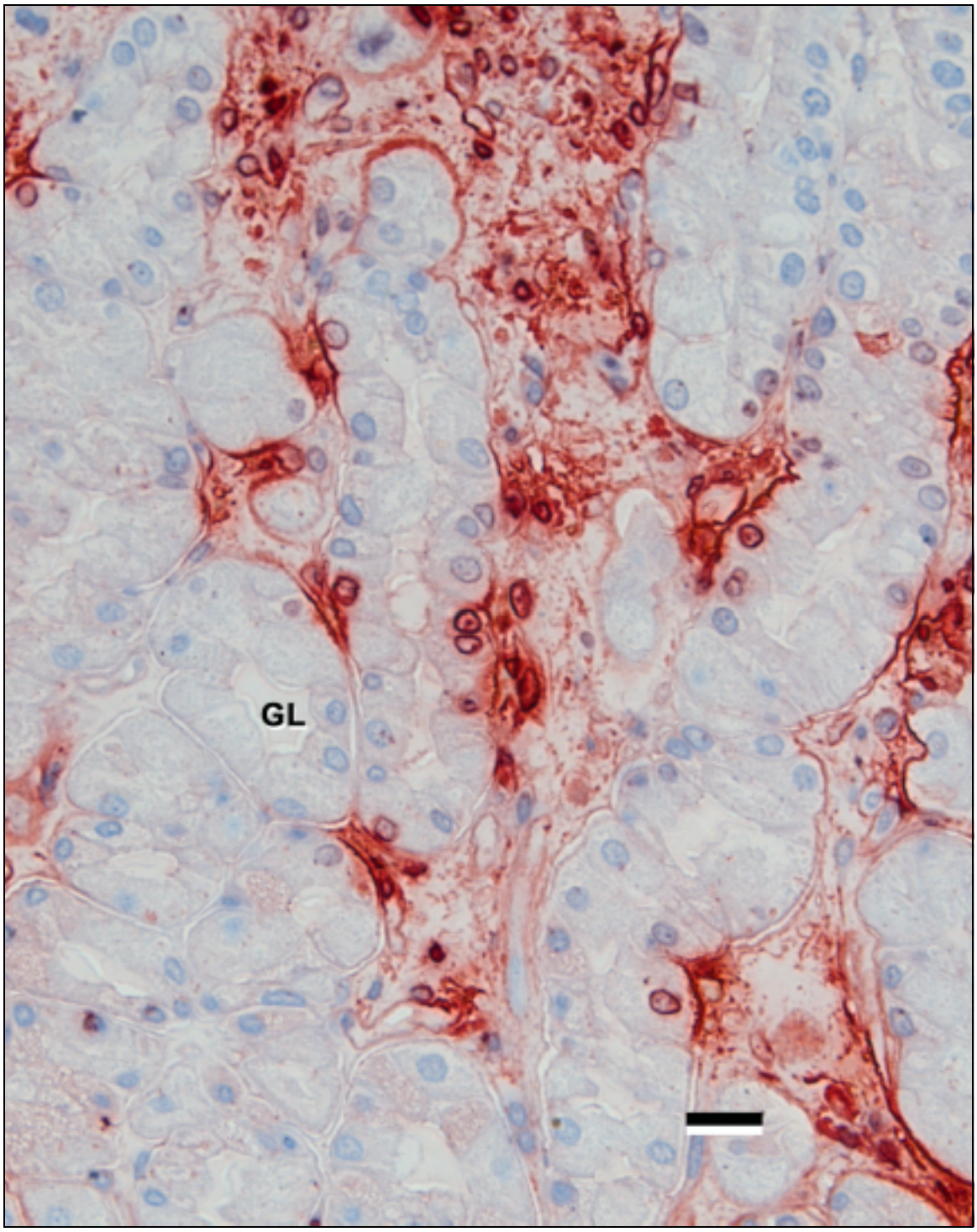

Figure 10. Major basic protein. (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*.
Scale bar is 20µm.

Figure 11.

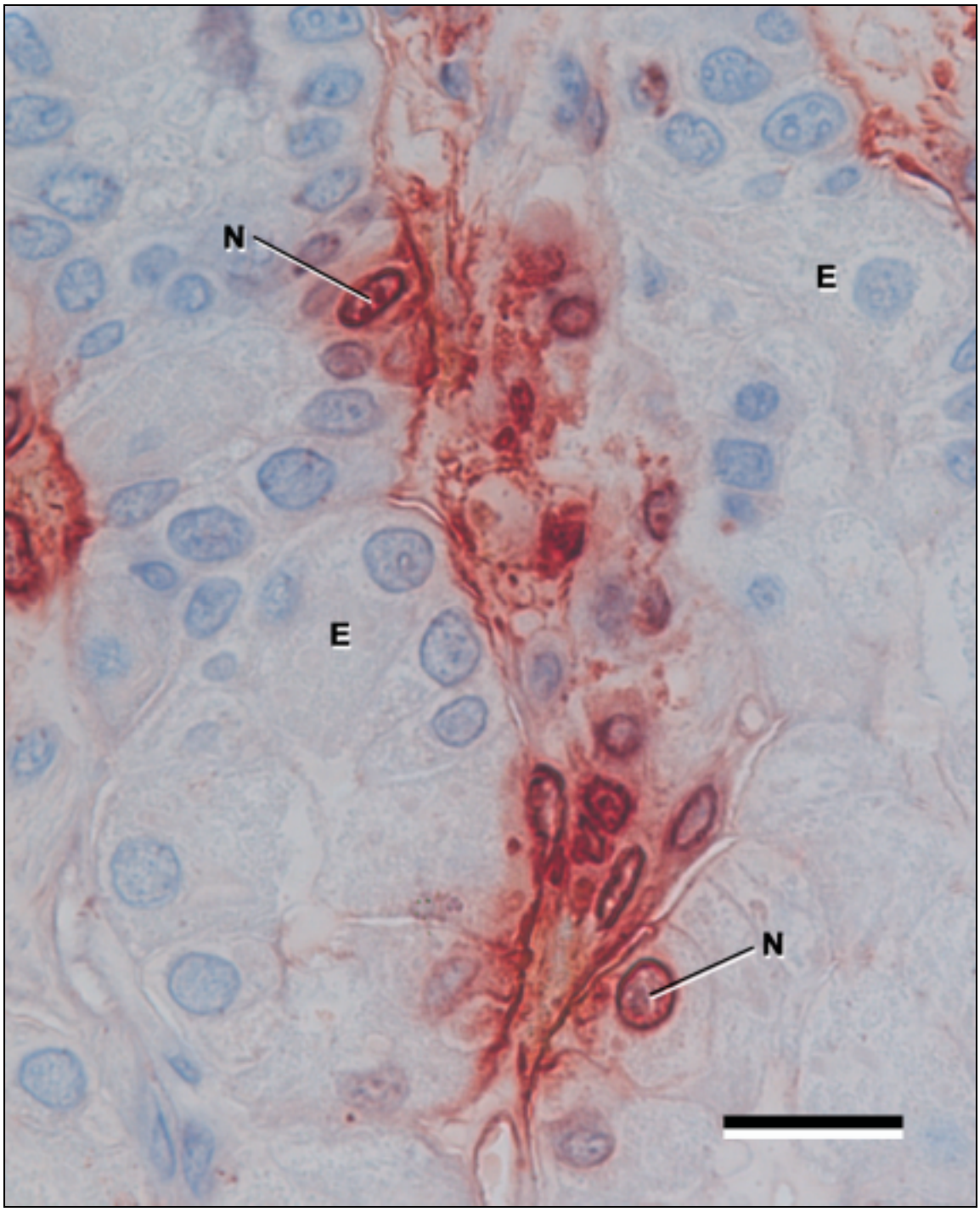

Figure 11. Major basic protein. (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. The gastric epithelium (E) and positively stained epithelial cell nuclei (N) are indicated. Scale bar is 20µm.

Figure 12.

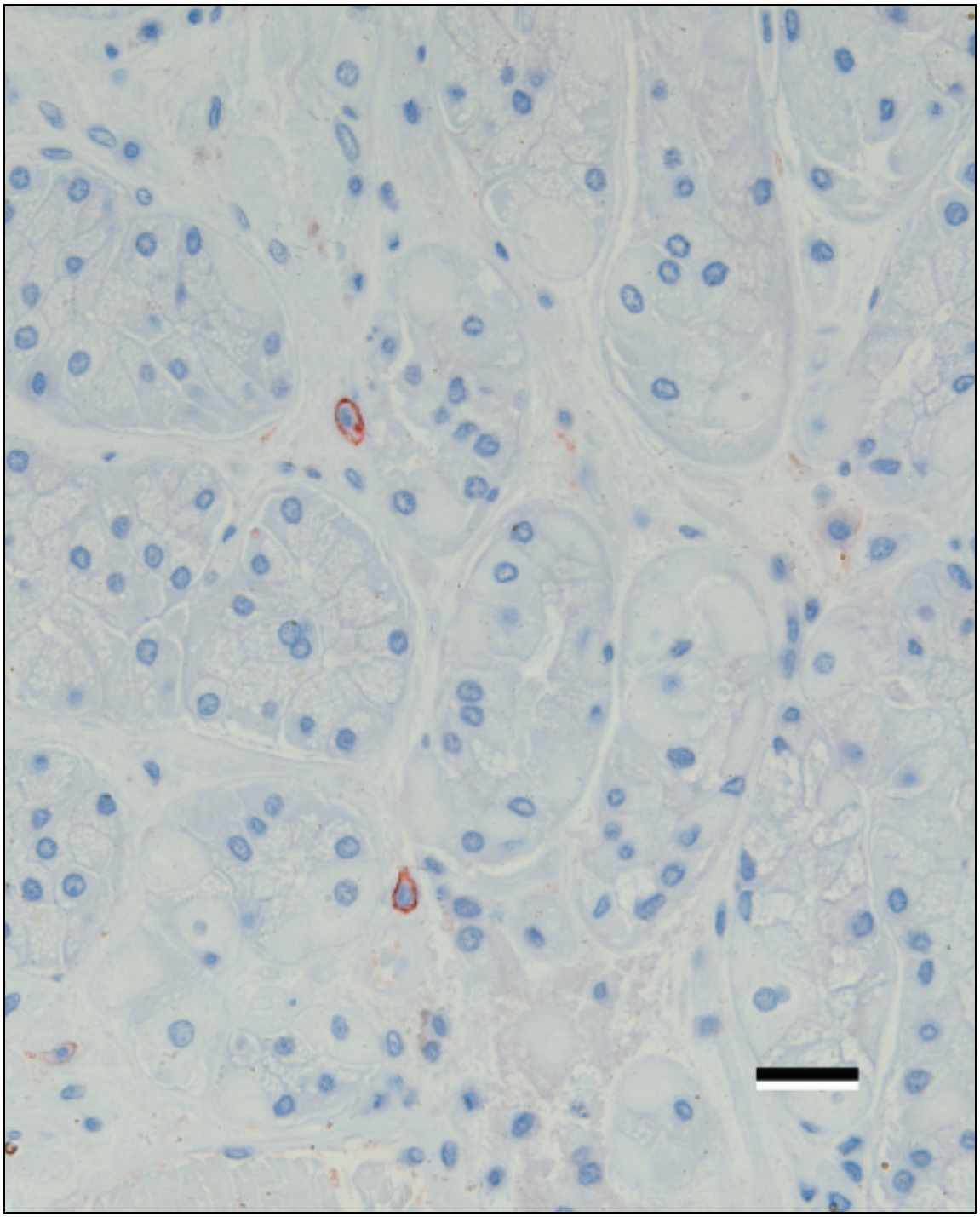

Figure 12. Immunoglobulin E. (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. Two connective tissue cells with membranous IgE are seen. Scale bar is 20µm.

Figure 13.

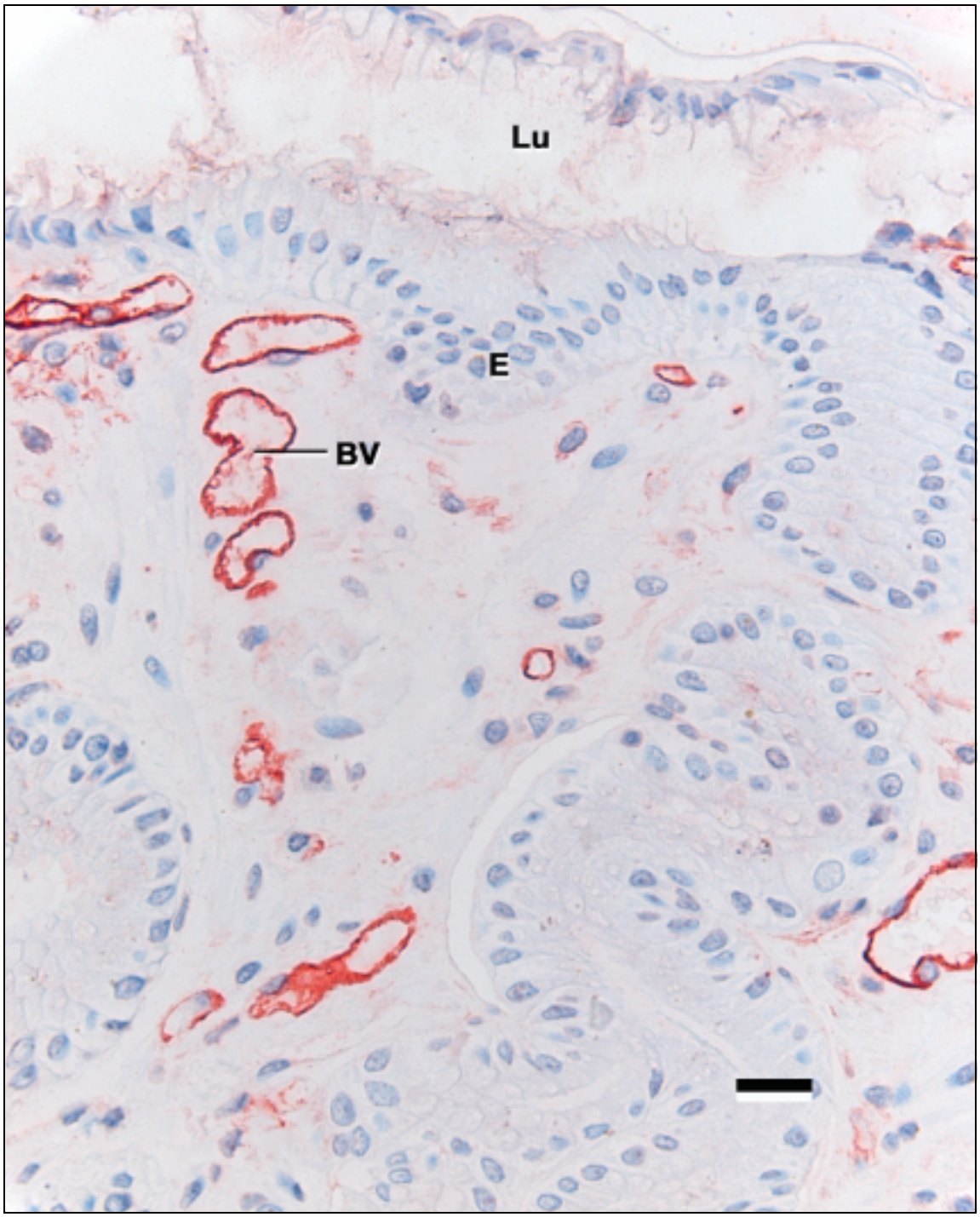

Figure 13. Intercellular adhesion molecule – 1. (Chromogen substrate).

Mucosal biopsy from the antrum of the normal stomach. The lumen (Lu) of the stomach, gastric epithelium (E) and mucosal blood vessels (BV) are shown.
Scale bar is 20µm.

Figure 14.

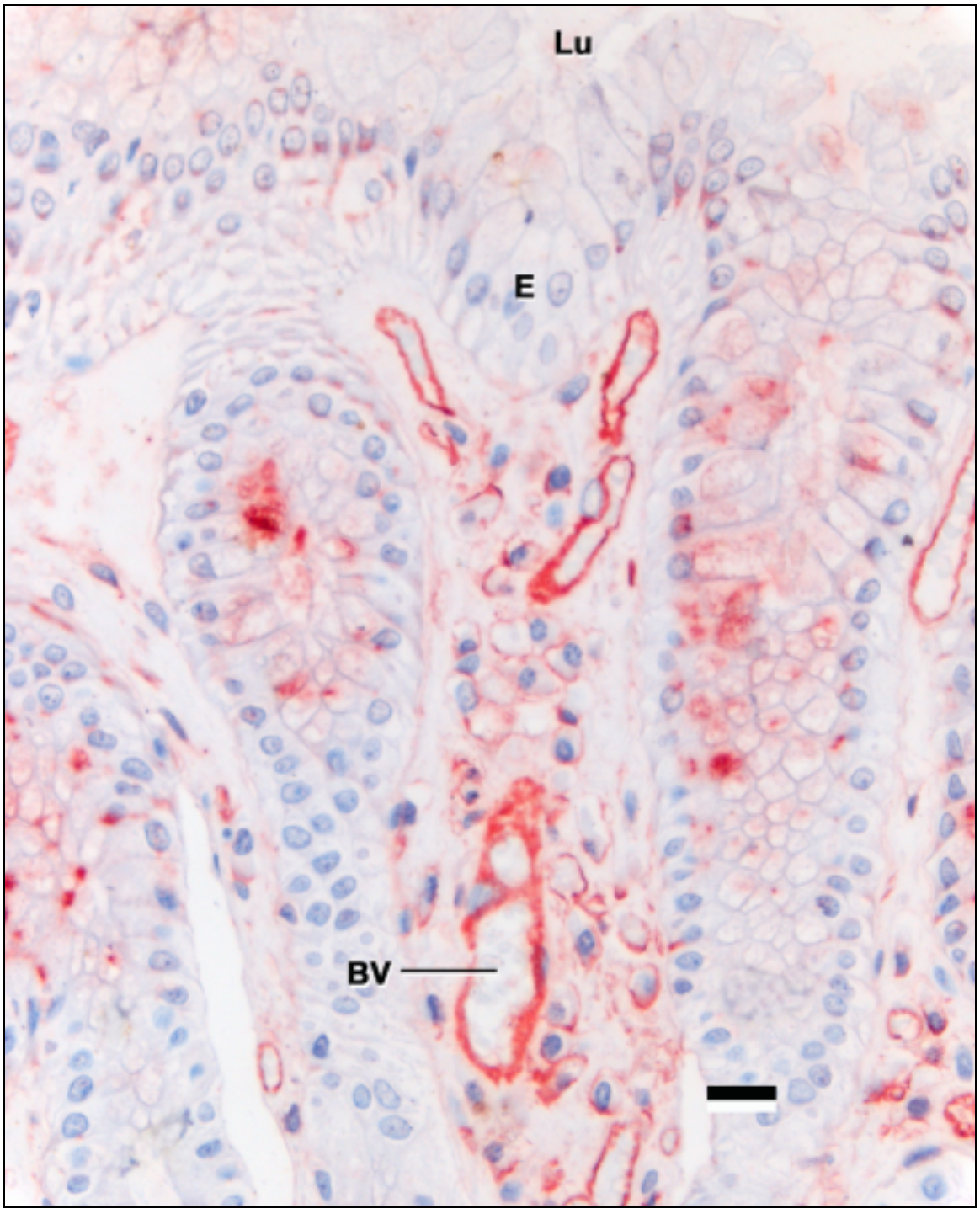

Figure 14. Intercellular adhesion molecule – 1. (Chromogen substrate).

Mucosal biopsy from the antrum of a stomach infected with *Helicobacter pylori*.
Scale bar is 20µm.

Figure 15.

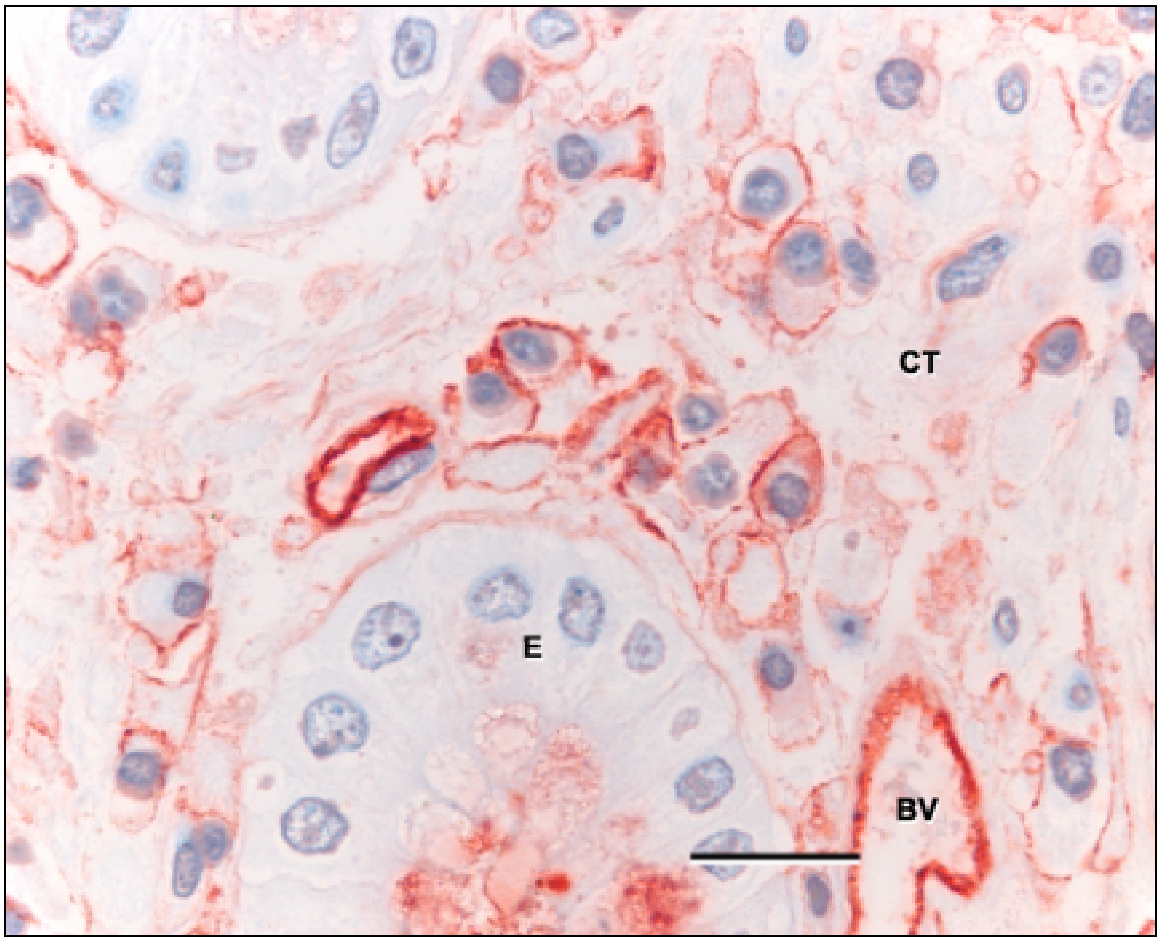

Figure 15. Intercellular adhesion molecule – 1. (Chromogen substrate).

Mucosal biopsy from the antrum of a stomach infected with *Helicobacter pylori*. The gastric epithelium (E), mucosal blood vessel (BV) and connective tissue (CT) are indicated. Scale bar is 20µm.

Figure 16.

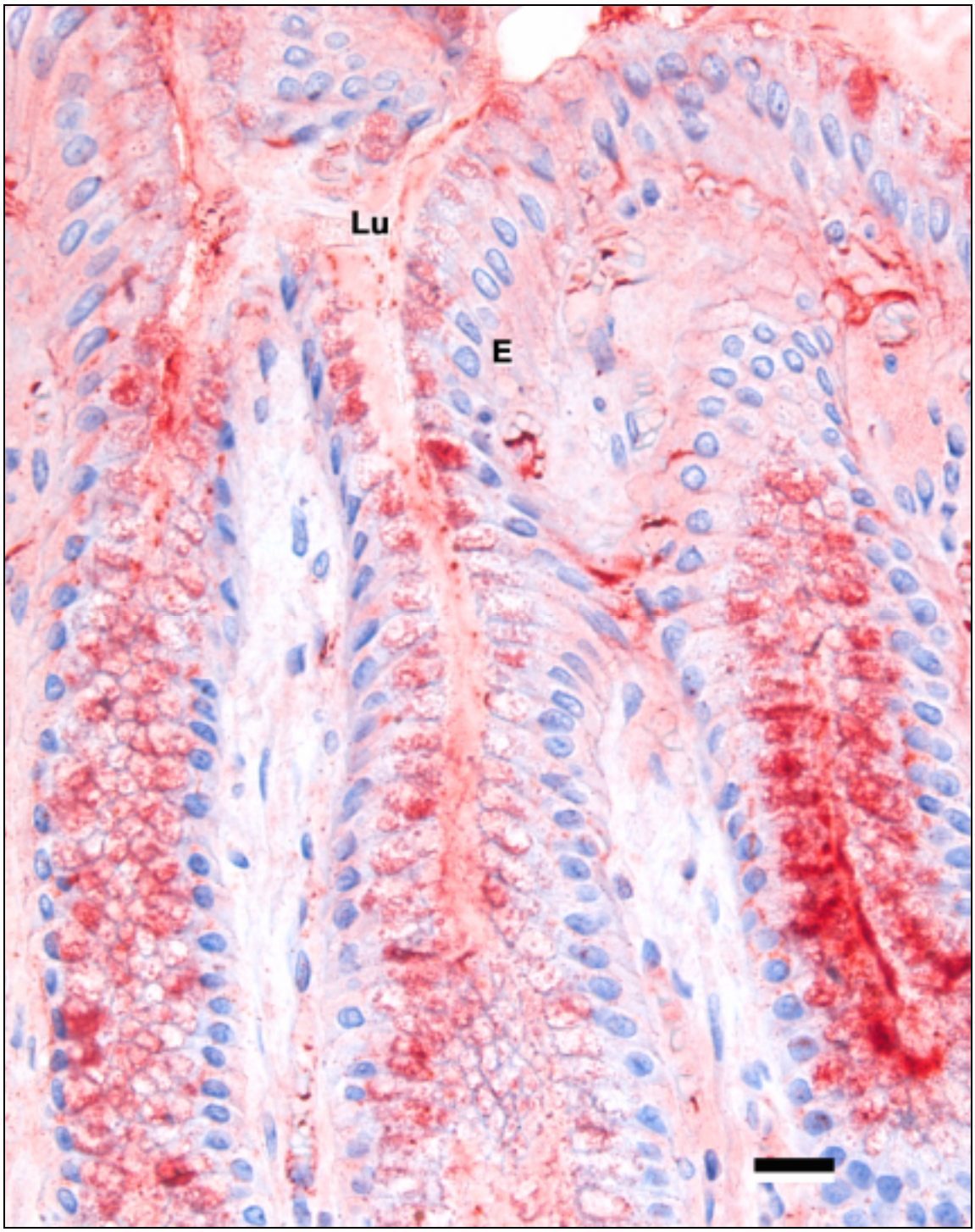

Figure 16. Interleukin 1β. (Chromogen substrate).

Mucosal biopsy from the antrum of the normal stomach. The gastric lumen (Lu) and gastric epithelium (E) are shown. Scale bar is 20µm.

Figure 17.

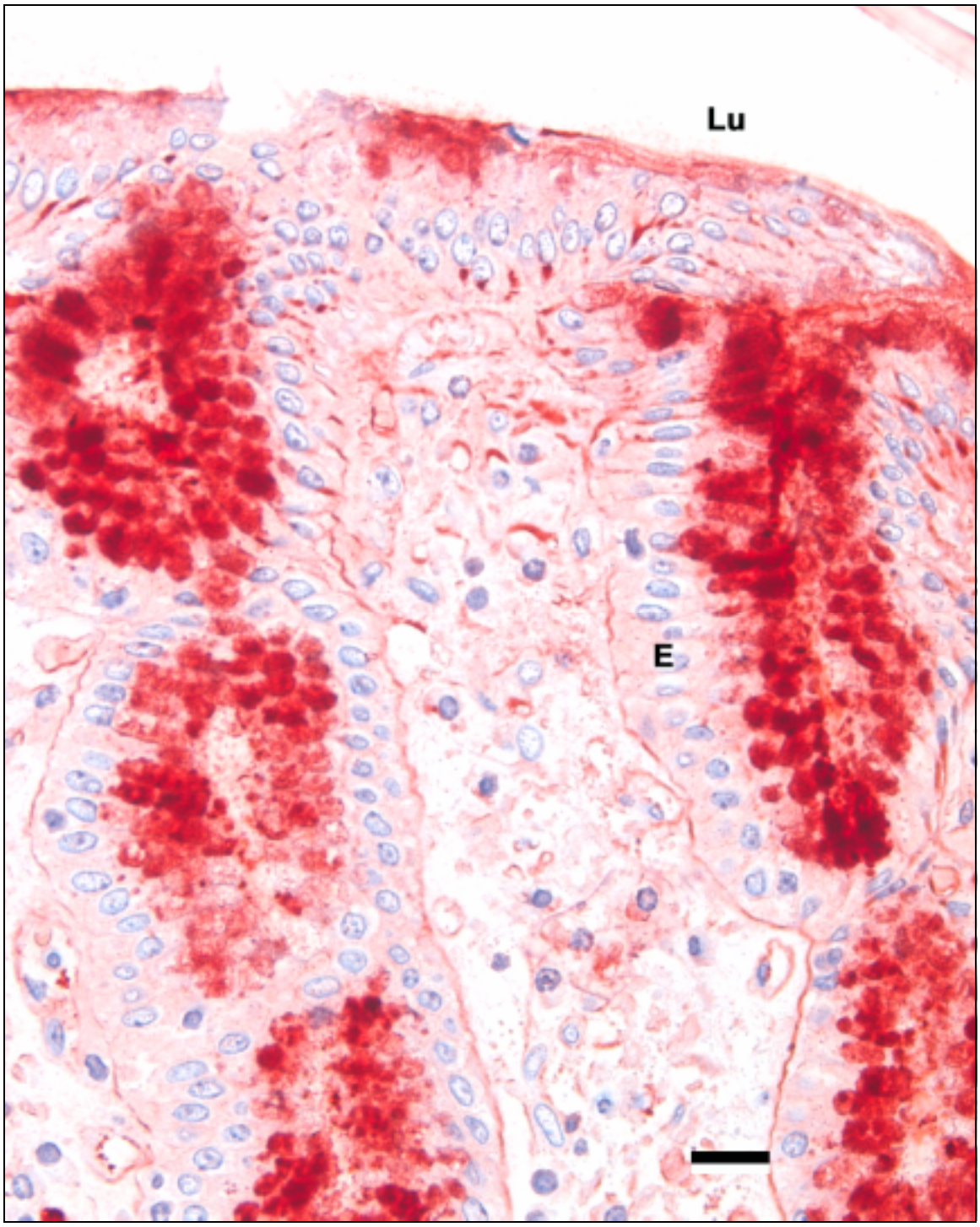

Figure 17. Interleukin 1β. (Chromogen substrate).

Mucosal biopsy from the antrum of a stomach infected with *Helicobacter pylori*.
Scale bar is 20µm.

Figure 18.

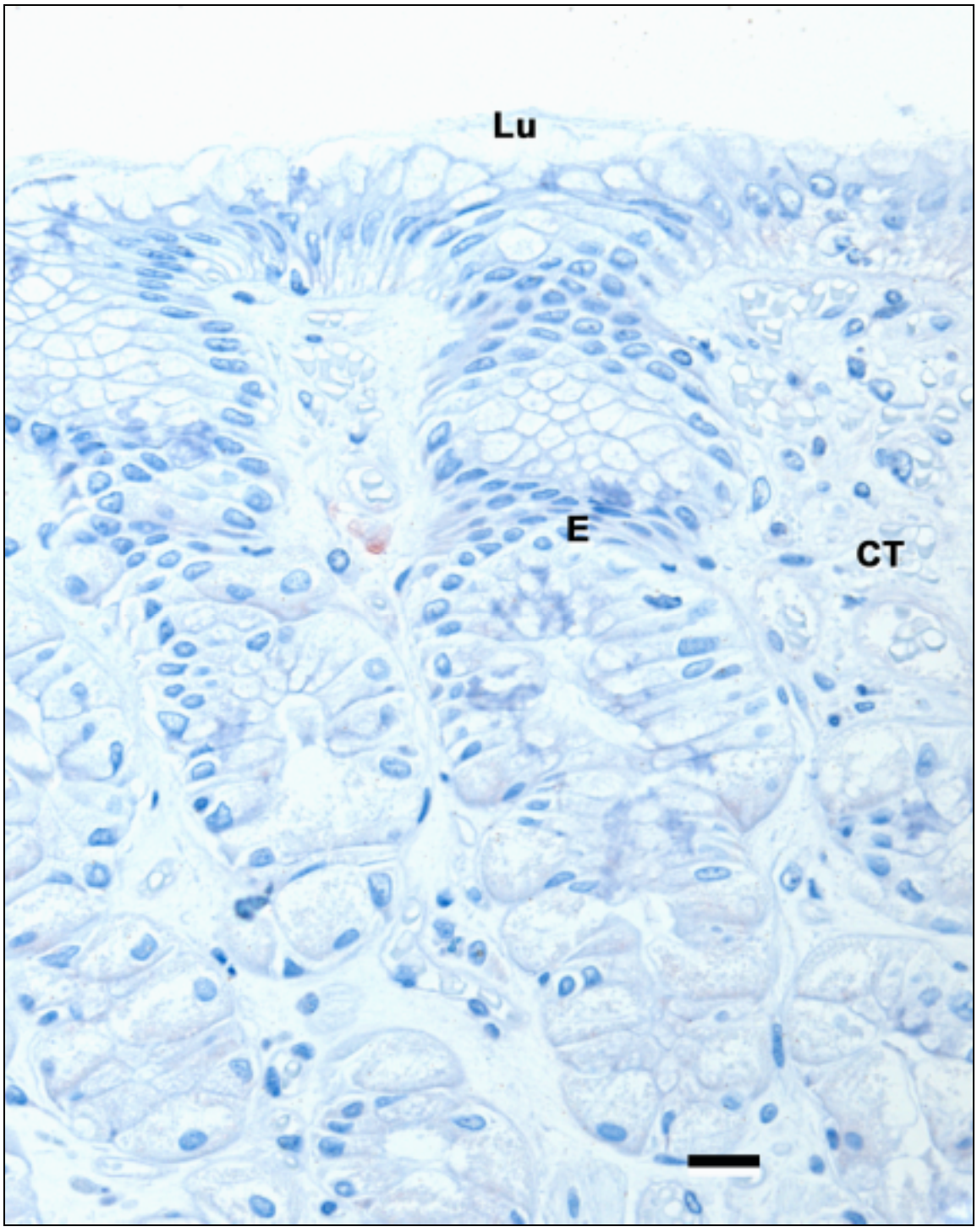

Figure 18. Tumour necrosis factor α. (Chromogen substrate).

Mucosal biopsy from the body of the normal stomach. The gastric lumen (Lu), gastric epithelium (E) and subepithelial connective tissue (CT) are shown.
Scale bar is 20µm.

Figure 19.

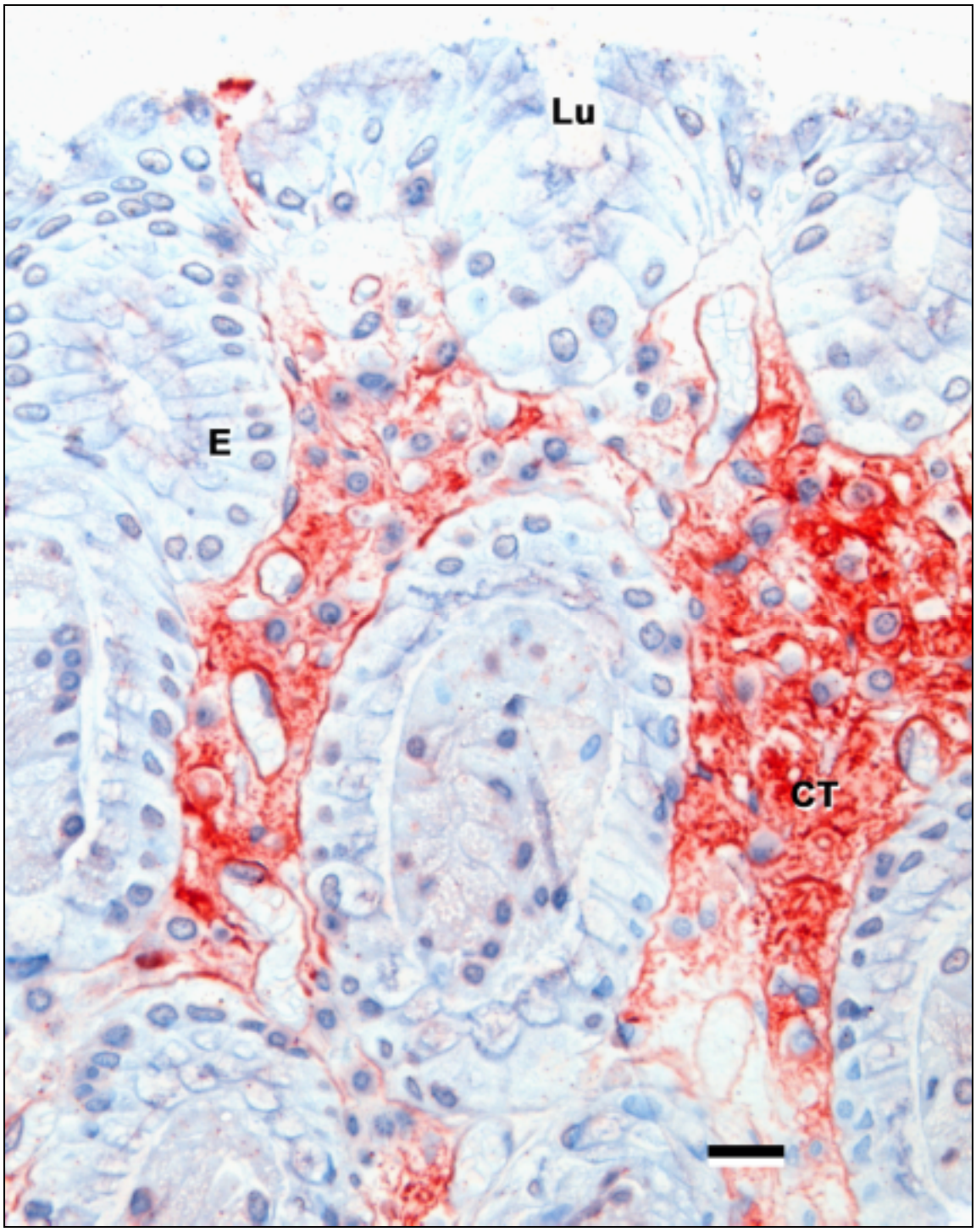

Figure 19. Tumour necrosis factor α. (Chromogen).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*.
Scale bar 20µm.

Figure 20.

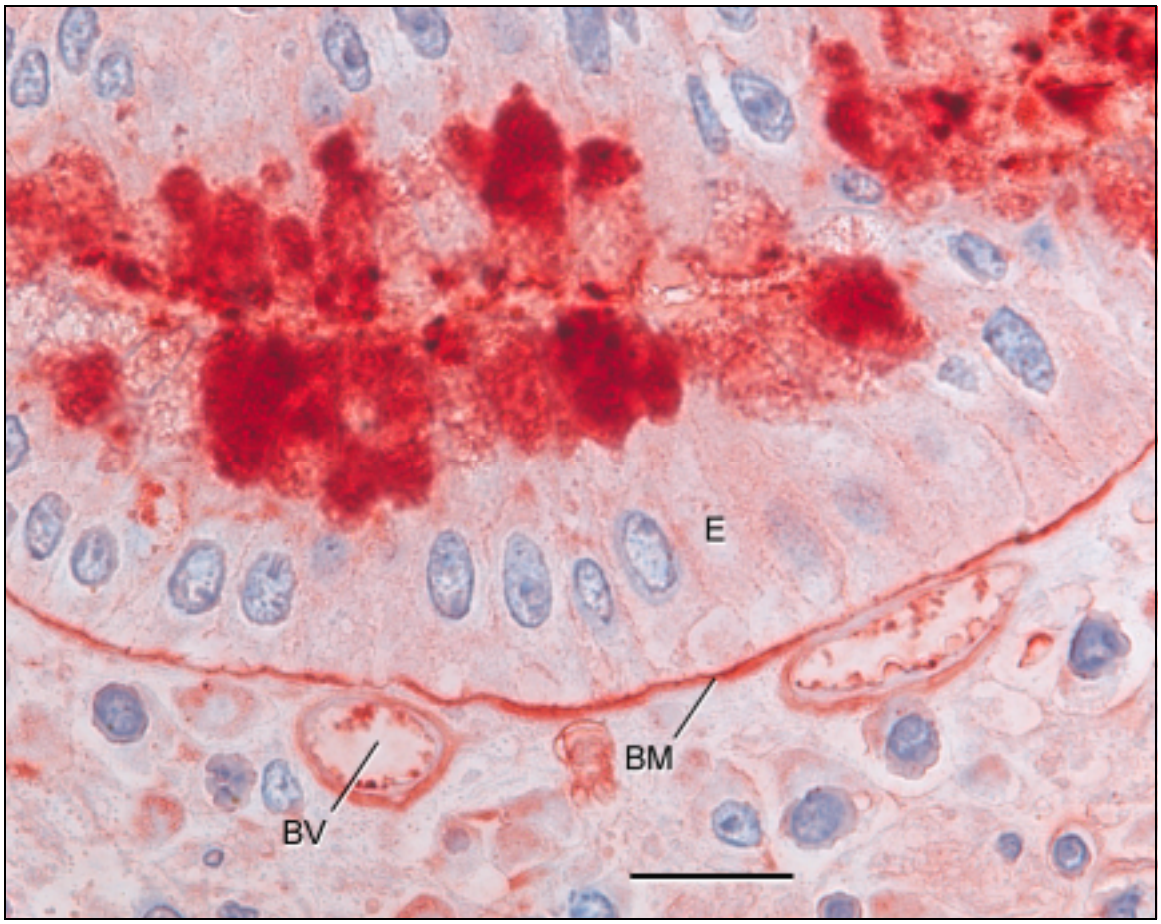

Figure 20. Interleukin 1β. (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. The gastric epithelium (E), the epithelial basement membrane (BM) and a mucosal blood vessel (BV) are shown. Scale bar is 20µm.

Figure 22.

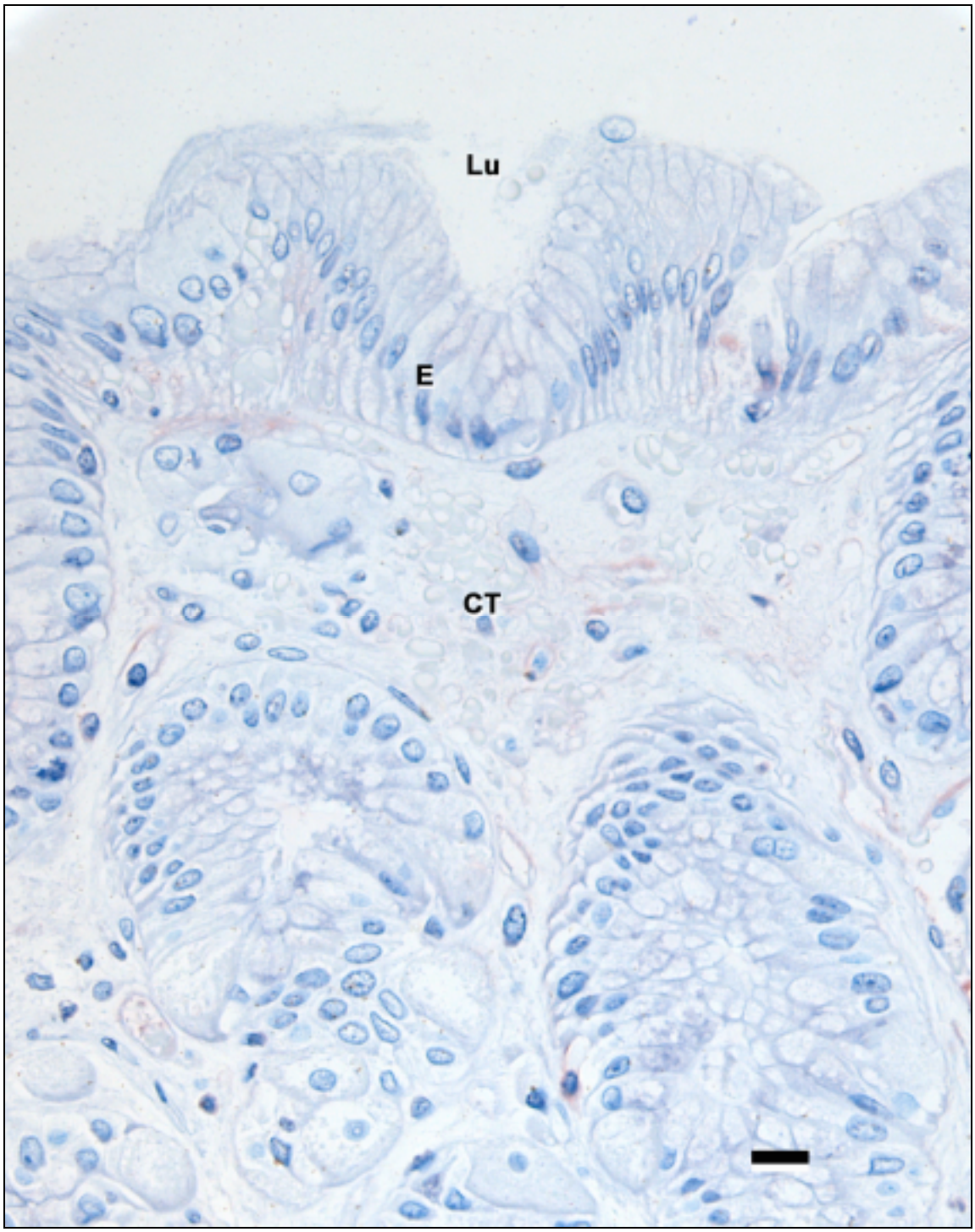

Figure 22. Interleukin 4 (3H4). (Chromogen substrate).

Mucosal biopsy from the body of the normal stomach. The lumen (Lu) of the stomach, gastric epithelium (E) and subepithelial connective tissue (CT) are shown. Scale bar is 20µm.

Figure 23.

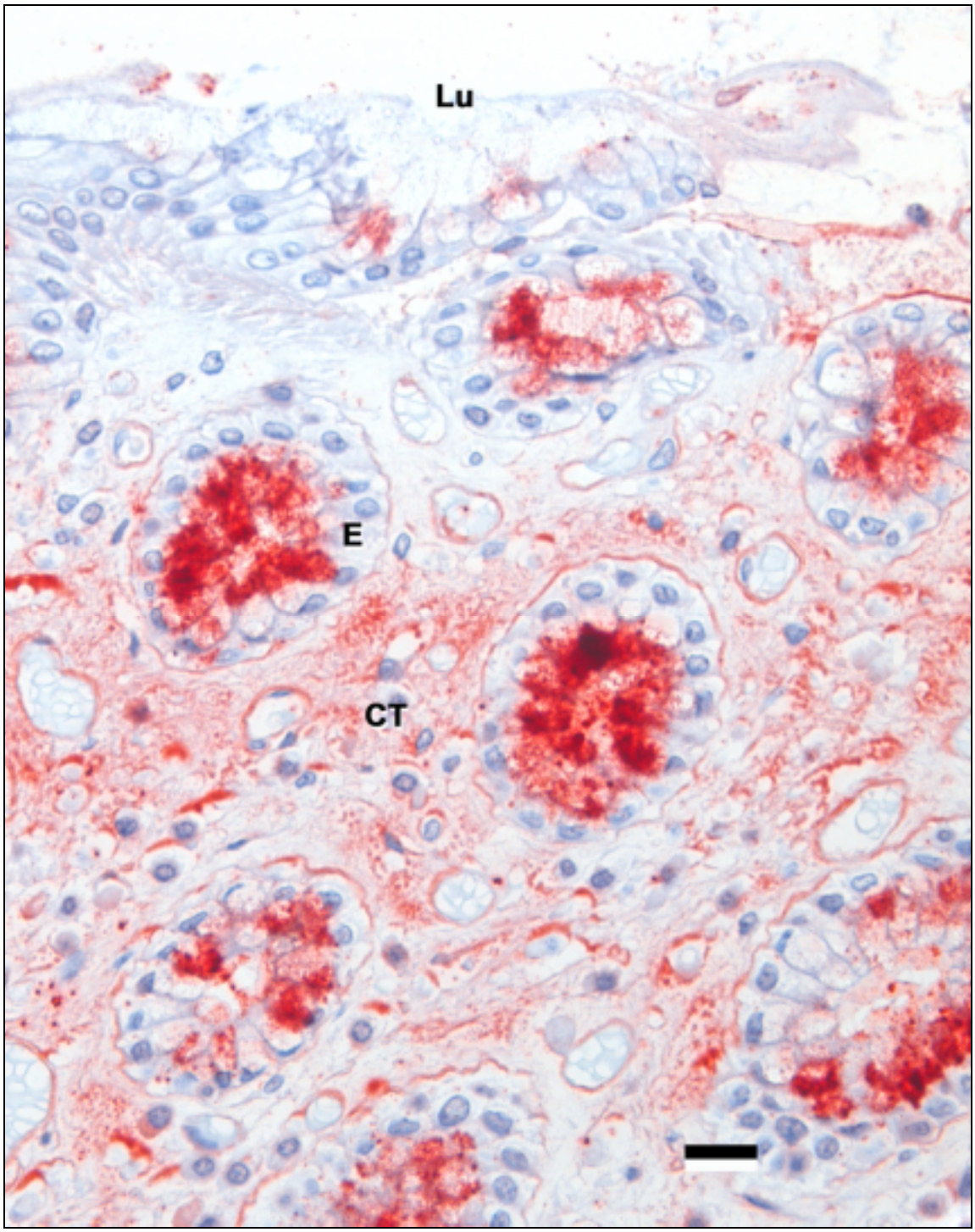

Figure 23. Interleukin 4 (3H4). (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. The lumen (Lu) of the stomach, gastric epithelium (E) and subepithelial connective tissue (CT) are shown. Scale bar is 20µm.

Figure 24.

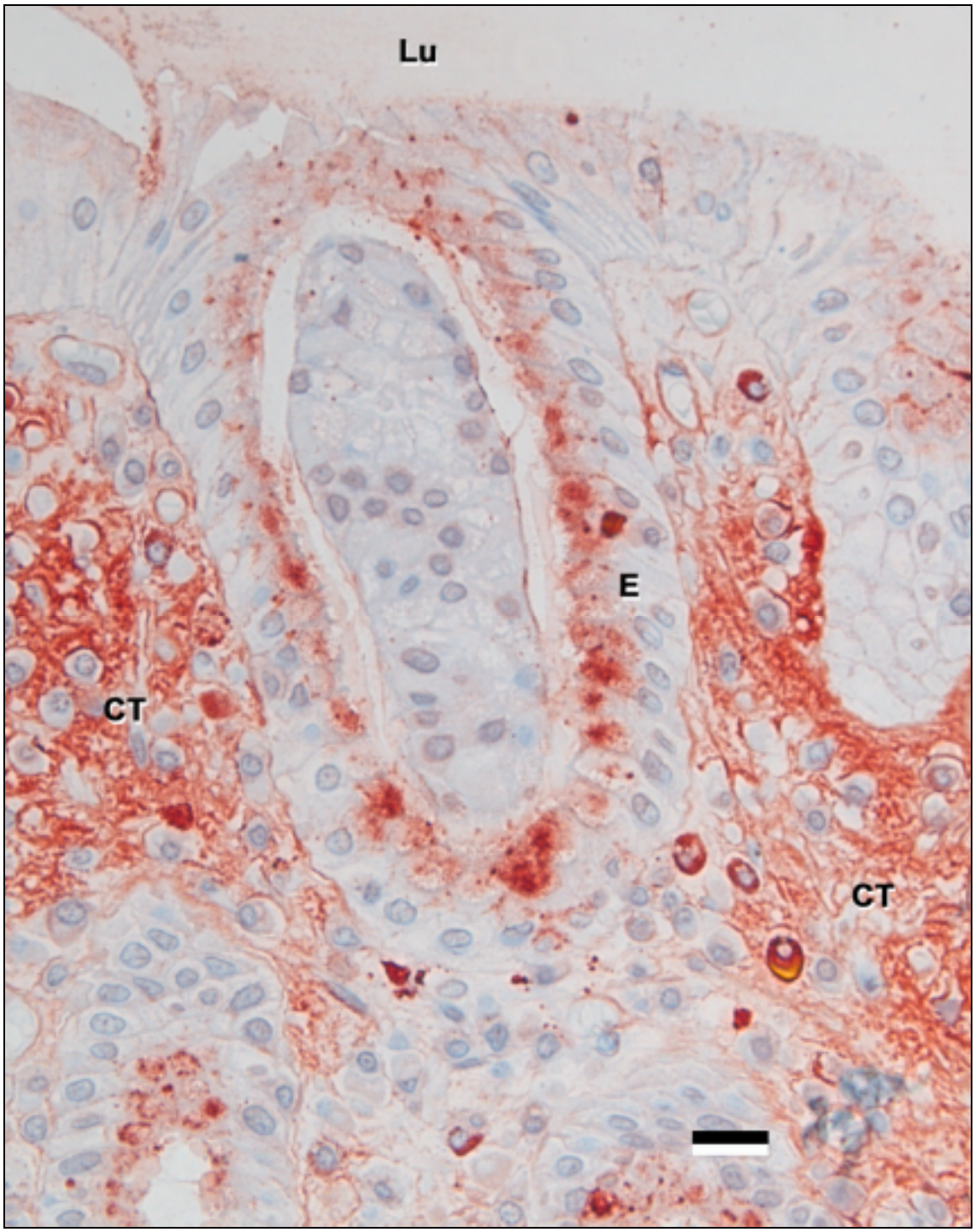

Figure 24. Eotaxin. (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. The lumen (Lu) of the stomach, gastric epithelium (E) and subepithelial connective tissue (CT) are shown. Scale bar is 20µm.

Figure 25.

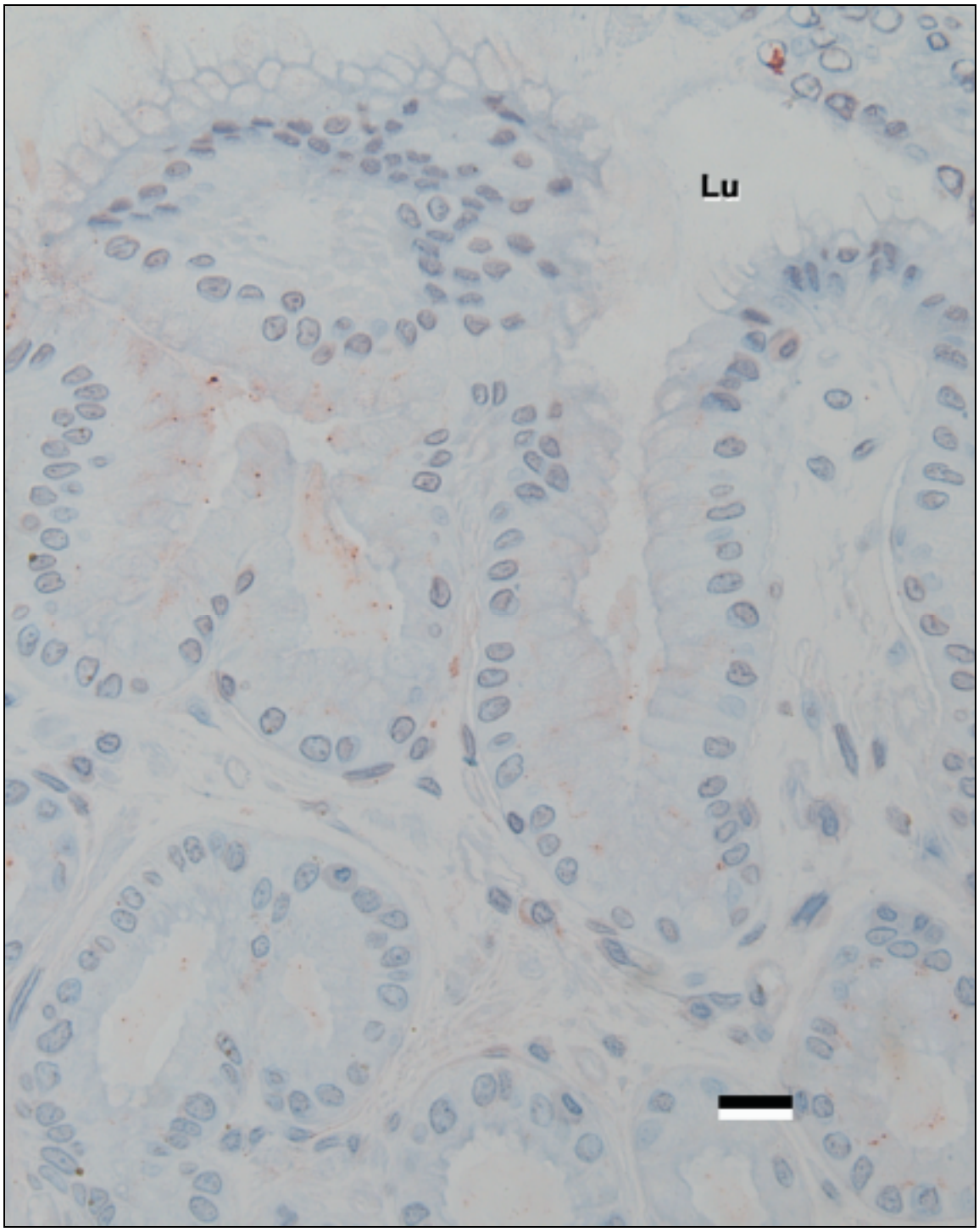

Figure 25. Eotaxin. (Chromogen substrate).

Mucosal biopsy from the body of the normal stomach. The lumen (Lu) of the stomach is indicated. Scale bar is 20µm.

Figure 26.

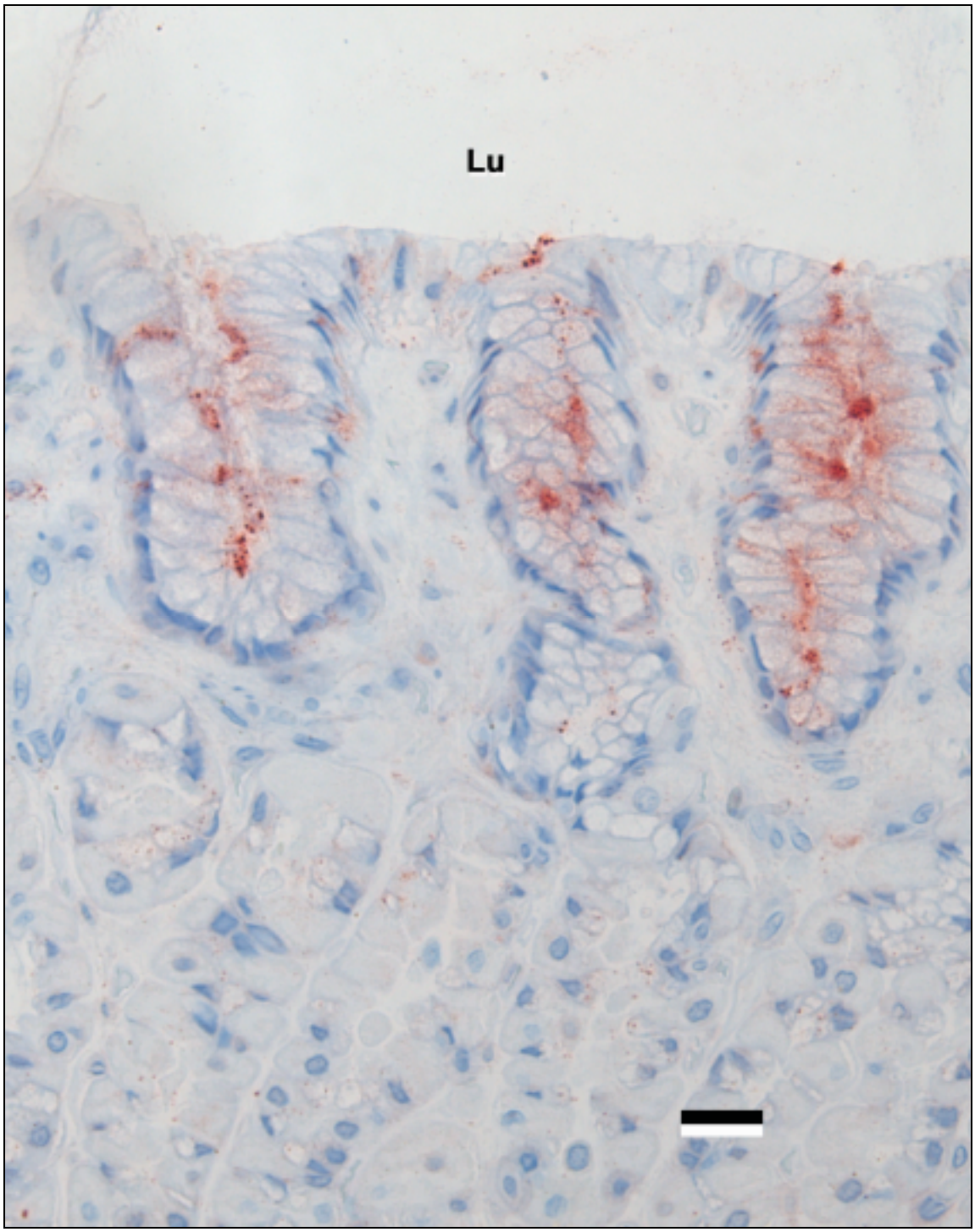

Figure 26. Eotaxin. (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. The lumen (Lu) of the stomach is indicated. Scale bar is 20µm.

Figure 28.

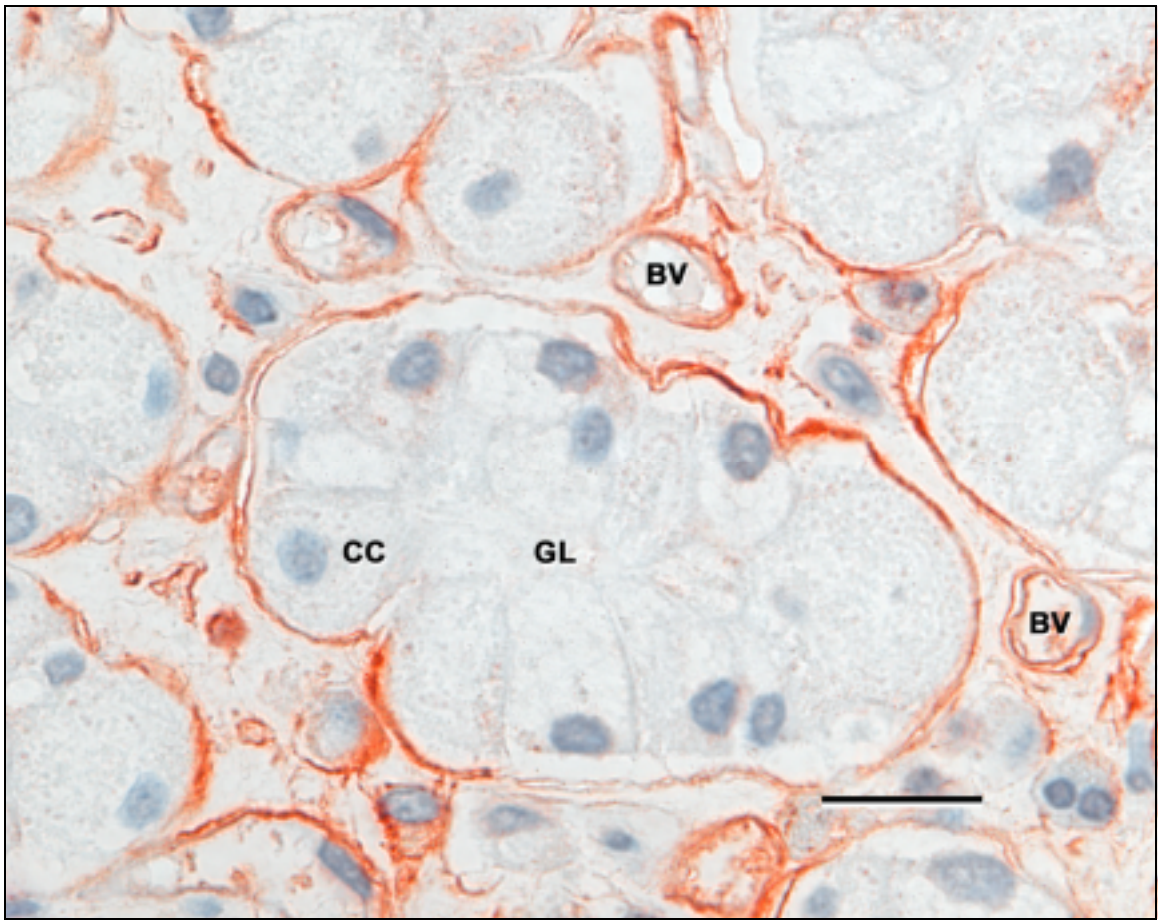

Figure 28. Eotaxin. (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. The lumen (GL) of a gastric gland is shown. Chief cells (CC) and mucosal blood vessels (BV) are seen. Scale bar 20µm.

Figure 32.

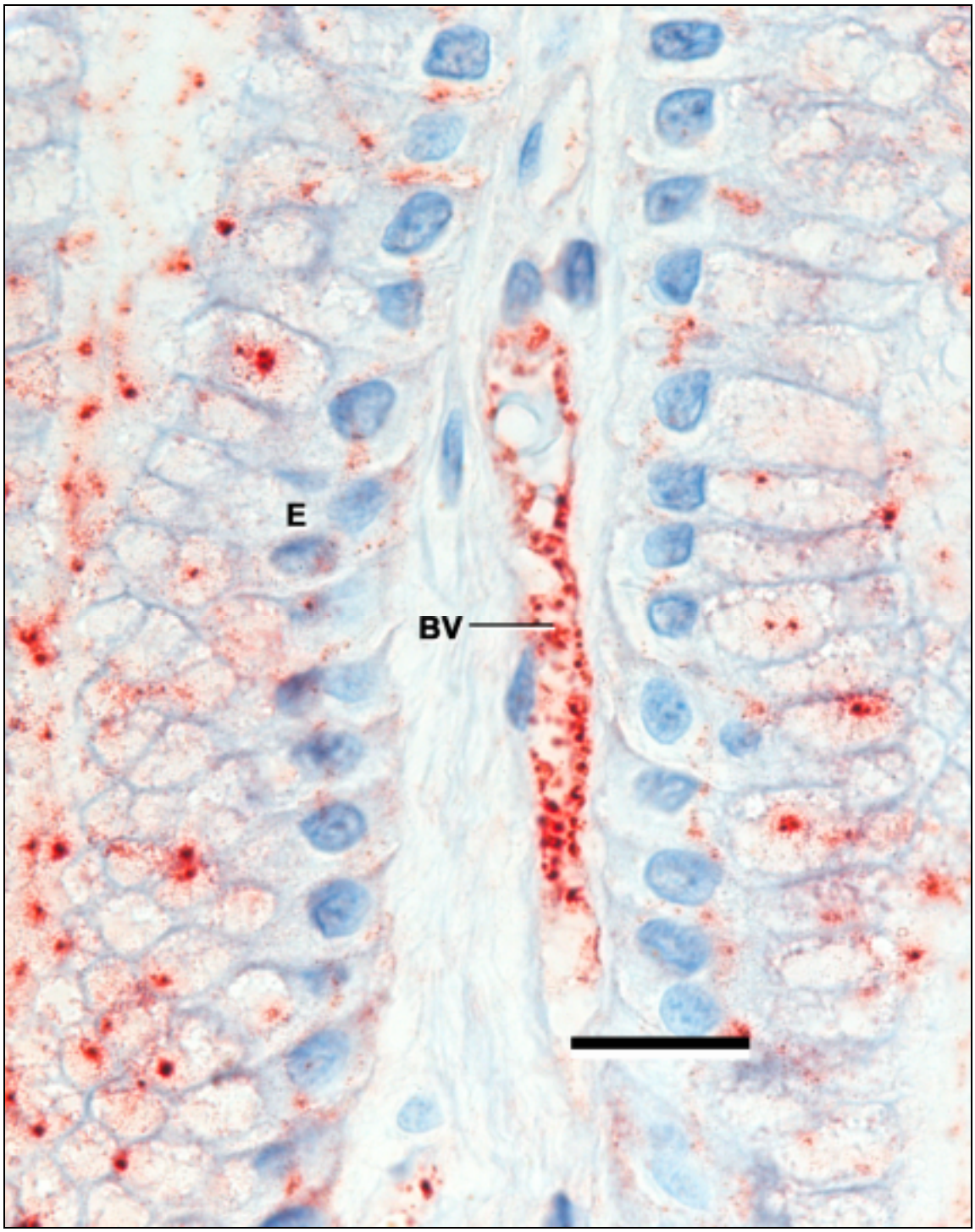

Figure 32. Interleukin 5. (Chromogen substrate).

Mucosal biopsy from the antrum of the normal stomach. The gastric epithelium (E) and mucosal blood vessel (BV) are shown. Scale bar is 20µm.

Figure 33.

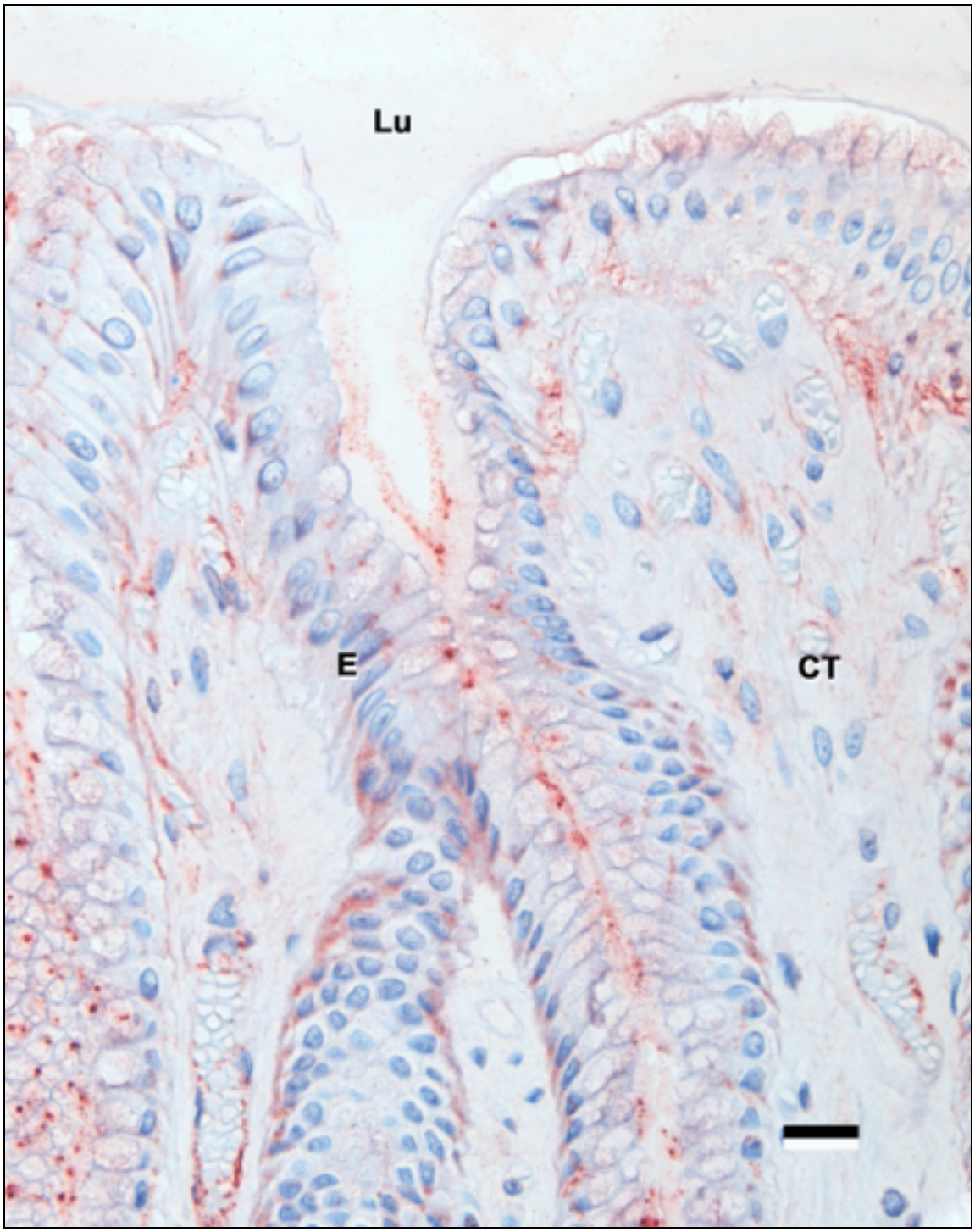

Figure 33. Interleukin 5. (Chromogen substrate).

Mucosal biopsy from the antrum of the normal stomach. The lumen (Lu) of the stomach, gastric epithelium (E) and subepithelial connective tissue (CT) are shown. Scale bar is 20µm.

Figure 34.

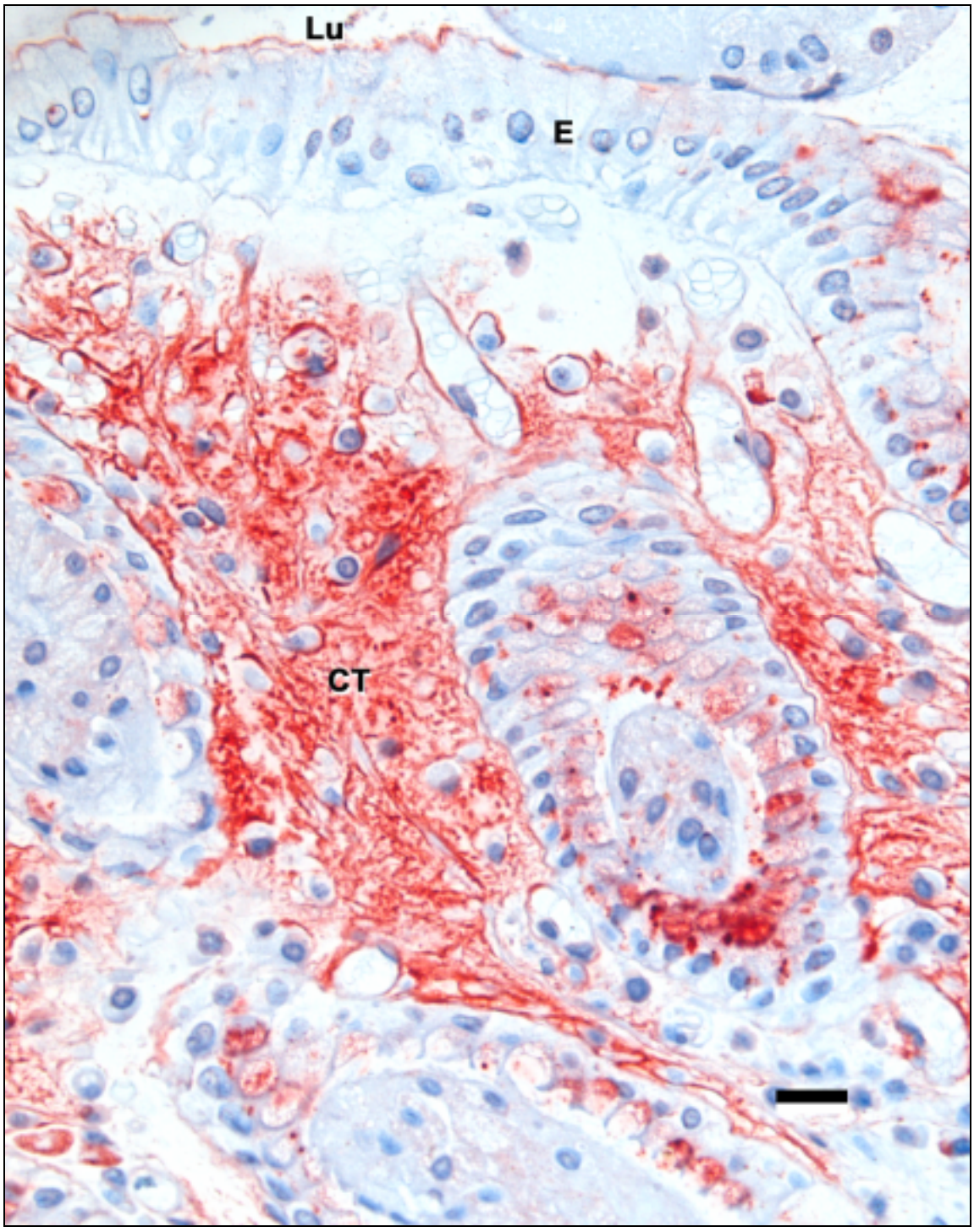

Figure 34. Interleukin 5. (Chromogen substrate).

Mucosal biopsy from the antrum of a stomach infected with *Helicobacter pylori*. The lumen (Lu) of the stomach, gastric epithelium (E) and subepithelial connective tissue (CT) are shown. Scale bar is 20µm.