Title: Mast cells, *Helicobacter pylori* infection and gastric erosions

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Abstract

Immunohistochemistry and ultrastructure have been used to examine changes in gastric mucosal mast cells associated with *Helicobacter pylori* infection. There is significant activation of these mast cells. This activation, together with the exposure of the epithelium to urea, can result in the zona occludens of the surface epithelium at the gastric lumen becoming 'leaky' for antigenic molecules from the gastric lumen. The extracellular exposure of these epithelial cells in the intercellular space to antigen results in the loss of the superficial epithelium causing the development of gastric erosions.

Keywords: Stomach, mast cells, *Helicobacter pylori*, gastric erosions.

Introduction

Mast cells are widely distributed in the body. If the protease enzyme content is evaluated, mast cells can be subdivided into those containing tryptase and chymase (MCtc) and those containing tryptase alone (MCt). Tryptase is found in all human mast cells. MCtc cells are found predominantly in the skin and intestinal submucosa whereas MCt cells are found in the alveolar septa of the lung and in the intestinal mucosa. If the mast cells of the human small intestinal mucosa are examined, approximately 80% contain the protease enzyme tryptase alone whereas approximately 20% contain tryptase and chymase (Metcalfe et al 1997). Tryptase has multiple functions which include the cleaving of fibrinogen, activation of collagenase (Gruber et al 1989) and the hydrolysing of neuropeptide proteins.

The protease content of mast cells has been related to the ultrastructure of mast cell granules. The granules of those mast cells containing tryptase alone have a scroll-like configuration and their diameter is less than one-half the diameter of the granules in mast cells containing both tryptase and chymase enzymes. The granules of mast cells with tryptase and chymase have a crystalline or grating/lattice configuration (Craig et al 1989).

The present study has been undertaken to determine whether there are any mast cell changes associated with *Helicobacter pylori* infection. This has been evaluated by

immunohistochemistry and by electron microscopy. The immunohistochemical study has involved resin embedded specimens (Britten, Howarth and Roche 1993) using a mouse monoclonal antibody to mast cell tryptase.

Material 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 (Kimberly-Clark, Ballard Medical Products, Utah, USA) and immunohistochemical detection of the *Helicobacter pylori* in the biopsies.

The biopsies are processed for transmission electron microscopic study, for scanning 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.

The scanning electron microscopic study has involved the endoscopic biopsies being fixed in cacodylate buffered 5% glutaraldehyde (pH 7·3) for 16 hours at 4°C. The biopsies are then rinsed in cacodylate buffered 10% sucrose (pH 7·3) for one to five days at 4°C prior to being postfixed in 2% osmium tetroxide (pH 7·3) for 2 hours at 4°C. The dehydration is carried out in a graded series of acetone solutions. The specimens are then critically point dried from the acetone in a Polaron E3000 system using liquid carbon dioxide as exchange medium and mounted onto stubs using quick drying silver paint. The specimens are coated with gold-palladium to a thickness of approximately 25nm. in a Polaron E5100 sputter coated before being examined with a JOEL JSM-35 scanning electron microscope at 25kV.

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 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
Mast cell tryptase	mouse monoclonal (AA1)	DakoCytomation
Helicobacter pylori	rabbit polyclonal	DakoCytomation.
MHC Class 11	mouse monoclonal (CR3/43)	Abcam (ab7856)

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

Immunohistochemical analyses of the human gastric mucosa have confirmed the presence of tryptase in the lamina propria mast cells (figure 1) and in the intraepithelial mast cells (figure 2). Mast cells are distributed throughout the gastric mucosa and related to all parts of the gastric glands. The mast cells are most frequently related to the body of the gastric glands and more numerous in the body of the stomach than in the antrum of the stomach. Some connective tissue mast cells have granules up to 1µm diameter with a scroll-like substructure (figure 3). This substructure is consistent with tryptase only mast cells. Other mast cells have a predominance of granules (up to 1·2µm diameter) which have a granular/membranous configuration (figure 4). Mast cell degranulation with the development of cytoplasmic vacuoles has been observed (present study; Steer 1976) in those mast cells entering and in an intraepithelial positions (so-called globular leucocytes). Degranulation has also been observed with the shedding of intact mast cell granules into the connective tissue surrounding lamina propria mast cells (Steer 1976).

In the majority of those biopsies from patients with an endoscopically and histologically normal gastric mucosa, the immunohistochemical staining for tryptase and the mast cell granules are confined to the mast cell or to its immediate vicinity (figure 1). Some lamina propria mast cells have a larger area of tryptase staining with some staining of the basement membrane and basal cell membrane of the epithelial cells in the immediate vicinity of these mast cells. However, in *Helicobacter pylori* infection there is a more diffuse staining (figure 5) with granules outside mast cells in the surrounding connective tissue. The staining of the basement membrane and basal cell membrane of local epithelial cells are more likely to be observed in patients infected with *Helicobacter pylori* (figure 6). Such changes are more readily related to the neck/base of the gastric gland rather than the pit/isthmus region of the gastric gland. In *Helicobacter pylori* infection the major change as far as the non-cellular component of the connective tissue of the lamina propria is concerned relates to that part in the region of the pit/isthmus of the gastric gland where there is a more generalized staining of the connective tissue for mast cell tryptase (figure 7). This is most intense in severe *Helicobacter pylori* infection.

The generalized release of tryptase in the pit/isthmus region of the gastric glands in *Helicobacter pylori* infection would result in cleaving of fibrinogen and activation of collagenase (Gruber et al 1989) and the probability of the associated release of heparin into the adjacent connective tissue. The biochemical response in the connective tissue at the site of severe *Helicobacter pylori* infection would enable a more effective

inflammatory and immune reaction to occur by reducing the natural restrictive measures present in the non-infected gastric mucosa.

There is no significant quantitative difference between the numbers of lamina propria mast cells in the normal stomach compared to the stomach infected with *Helicobacter pylori*. However, *Helicobacter pylori* infection is associated with signs of increased activation of mast cells as indicated by mast cell tryptase activity.

The magnitude and complexity of mast cell heterogeneity is illustrated by considering the potential functions of these cells. The biochemical products of mast cells are dependent upon the stimulus given to these cells. Thus, human intestinal mast cells respond *in vitro* to stem cell factor (SCF) and interleukin 4 (IL-4) (see Lorentz et al 2001). SCF is a growth factor specific to human mast cells and, in addition to being involved in the development, survival and maturation of mast cells, results in the production of a number of proinflammatory cytokines by mast cells (TNFα, IL-1β, IL-6, IL-8, IL-16 and IL-18). However, if the mast cells are cultured with both IL-4 and SCF the cytokine profile is quite different with the production of Th 2 type cytokines (IL-3, IL-5 and IL-13). If mast cells are simply activated with IgE receptor cross linking or gram negative bacteria there is an upregulation of TNFα whereas high affinity IgE receptor cross linking results in the production of the Th 2 type cytokines.

IL-4 induces a selective increase in the MCt cell number whereas the number of MCtc cells remains almost unchanged or decreases (Befus et al 1987; Irani et al 1989). MCt are the predominant mast cell type found in the gastric lamina propria. Although IL-4 is markedly upregulated in *Helicobacter pylori* infection of the stomach (Steer 2005) the number of lamina propria mast cells has not been increased. The present study has not separately evaluated MCtc and MCt cell density.

Mast cells have been implicated in antigen-induced enhancement of epithelial permeability and such action would have a role in *Helicobacter pylori* infection of the stomach. The involvement of mast cells with epithelial permeability was first suggested by Crowe et al (1993) following intra-luminal antigen challenge of sensitized rats which resulted in a dramatic increase in jejunal permeability for small molecules (five fold) and macromolecules (fourteen fold). This increased permeability also applied to the antigen itself (Crowe et al 1990; Berin et al 1997).

Following experiments with horse radish peroxidase (HRP), two phases have been described with respect to the changes in epithelial permeability. Phase 1 involves the uptake of antigen which was rapid, specific and exclusively transcellular. This uptake from the surface was into cytoplasmic endosomes. Phase 1 was followed by Phase 2 when the transport of HRP was increased by enabling the HRP to pass through the epithelial tight junctions directly into the intercellular space (Berin et al 1997; Berin et al 1998). Phase 2 is totally absent in mast cell deficient rats demonstrating the involvement of mast cells in Phase 2.

The Phase 2 phenomenon of epithelial permeability change has implications as far as antigen presentation is concerned. Antigen passing directly into the intercellular space would not be coupled with MHC Class11 in the epithelial cell cytoplasm but presented at the plasma membrane of that antigen presenting cell (Steer 2005). The antigen in the intercellular space during phase 2 could pass directly into the lamina propria and if presented to the immune system would have to have this function performed by other antigen presenting cells such as macrophages/dendritic cells. It must also be mentioned that extracellular presentation of antigen to epithelial cell lines has resulted in apoptosis of those epithelial cells (Fan et al 2000).

Are there any other causes of a 'leaky' cell junction?

Exposure of the surface epithelium to urea has been shown to cause the tight junctions (zona occludens) to become 'leaky' (Eastwood and Kirchner 1974). *Helicobacter pylori* possess the enzyme arginase which enables the hydrolysis of arginine to take place with the formation of ornithine and urea. Arginine can be ingested in food but activation segment of pepsinogen has a high concentration of arginine (Kageyama and Takahashi 1980) and could act as a source of arginine. The urea formed by the action of *Helicobacter pylori* arginase could then act on the tight junction to make this junction 'leaky'. An examination of the surface of the stomach infected with *Helicobacter pylori* has revealed intermittent gaps between epithelial cells (figure 8) where antigens could gain access to the MHC Class 11 molecules from an extracellular route (Steer 2005).

It is possible that the exposure of the epithelium to urea causing a 'leaky' tight junction is manifest by way of phase 2 of the mast cell response. This possibility has yet to be investigated.

The diagrammatic representation of the development of 'leaky' tight junctions is shown in figure 9.

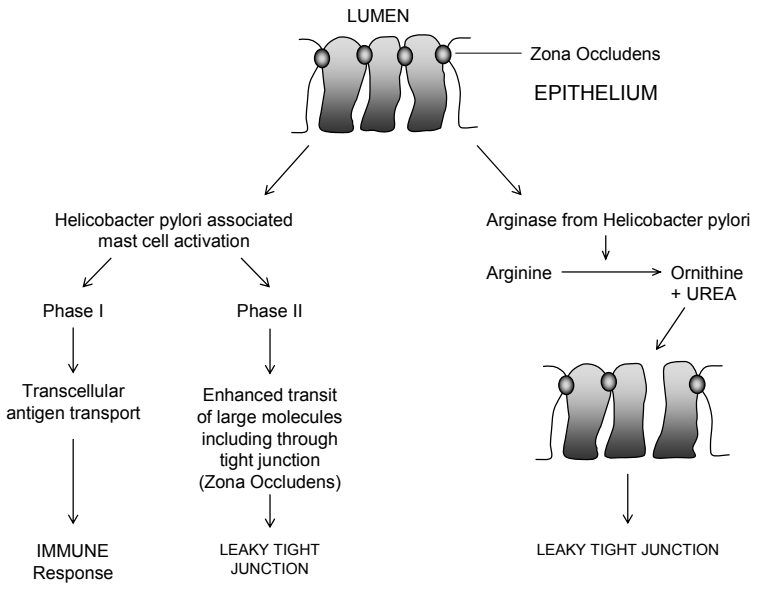

Figure 9. The causes of a 'leaky' tight junction.

Transmission electron microscopic examination of the surface/pit epithelium of the human stomach in *Helicobacter pylori* infection has revealed areas where the continuity of the basement membrane of the epithelium is disrupted. This may be a localised disruption enabling a cytoplasmic process from the epithelial cell to pass into the underlying connective tissue (figure 10) or a large area (figure 11 and 12) where the homogeneous material of the intercellular space is in continuity with ultrastructurally similar material beneath the epithelium (figure 11) or beneath the epithelial basement membrane (figure 12). The break in the continuity of the basement membrane underlying the gastric epithelium is apparent in figure 13. Such appearances sometimes extend over relatively large areas covering many epithelial cells. Under such circumstances the

shedding of epithelial cells separated from their basement membrane would result in the endoscopic and histological appearance of erosions.

Phase 2 changes in epithelial permeability as a result of mast cell activity have implications with respect to the presence of antigen at the non-luminal cell membrane and the potential for apoptosis (see Fan et al 2000). This apoptosis is facilitated by the extracellular binding of antigen to Major Histocompatibility Complex Class 11 (MHC Class 11) molecules at the cell membrane. The MHC Class 11 expression at the cell membrane is upregulated in *Helicobacter pylori* infection (figure 14 and 15; Steer 2005). The gastric epithelium in the normal stomach lacks any obvious MHC Class 11 expression (figure 14) but the gastric epithelium of patients infected with *Helicobacter pylori* often has MHC Class 11 expression on the lateral cell membrane and the basal cell membrane but not on the surface cell membrane of these epithelial cells (figure 15). The tight junction (zona occludens) is sited at the luminal aspect of the lateral cell membrane. If the tight junction is functional the MHC class 11 molecules will not be exposed to external antigen from the lumen but if the tight junction is 'leaky' the antigens will have ready exposure to the MHC Class 11 molecules. Interestingly, gastric epithelial apoptosis is said to be increased in *Helicobacter pylori* infection. The apoptotic cells visualized by the TUNEL technique are present in the surface epithelium of the gastric mucosa (Jones et al 1997) at the site of the currently described changes of the 'leaky tight junctions' and not in the gastric glands where *Helicobacter pylori* infection is associated with the changes of necrosis (Steer 2005; Steer 2007). This apoptosis at the surface epithelium would increase the susceptibility of the *Helicobacter pylori* infected stomach to develop erosions by the loss of this surface epithelium. The exposure of MHC Class 11 molecules to external antigen and the apoptosis of epithelial cells are diagrammatically depicted in figure 16.

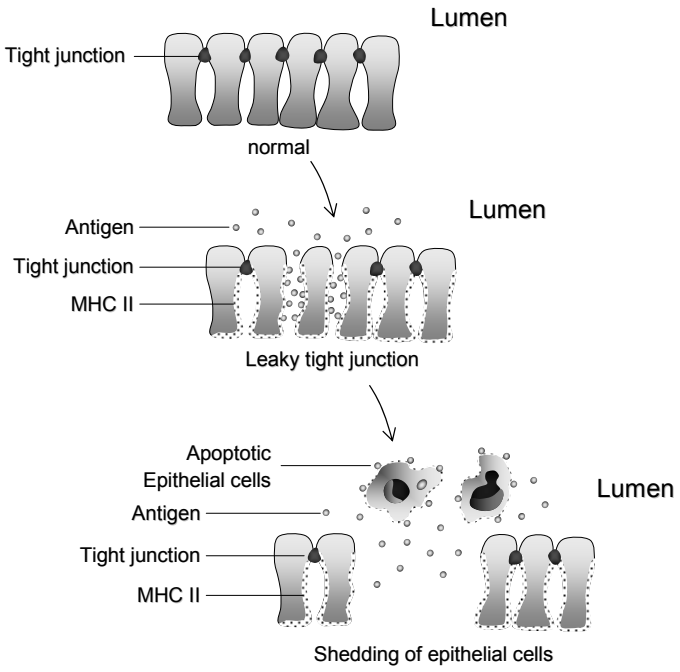

Figure 16. Extracellular exposure of the MHC Class 11 molecules at the intercellular membrane to antigen and the development of apoptosis.

A diagram indicating the changes leading to the development of gastric epithelial erosions is shown in figure 17.

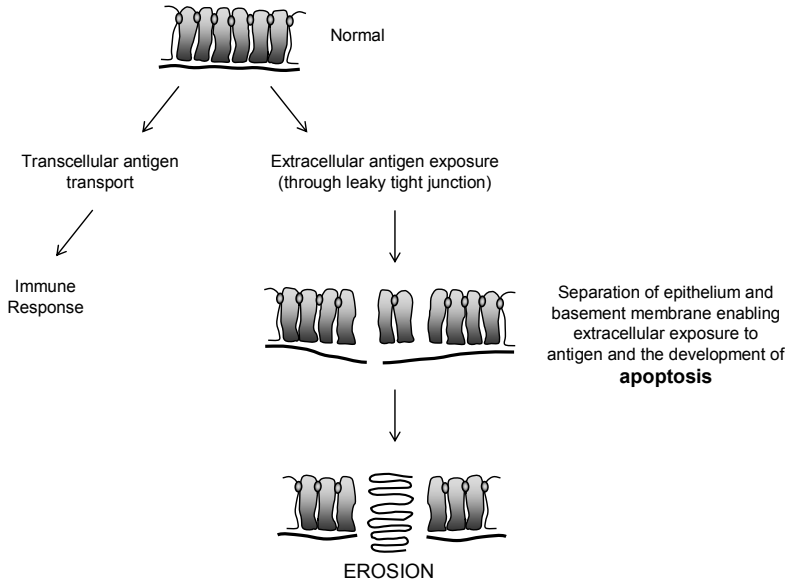

Figure 17. The development of gastric erosions.

Acknowledgements

Grateful acknowledgement is made for the help received from Dr. Susan Wilson, Linda Jackson, Helen Rigden and Jon Ward of the Histochemistry Research Unit, University of Southampton School of Medicine, Anton Page, Nick Barnett and Sue Cox of the Biomedical Imaging Unit, University of Southampton School of Medicine / Southampton University Hospital NHS Trust and Adie Falcinelli of the Learning Media, Southampton University Hospital NHS trust.

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The Stomach, *Helicobacter pylori* and acid secretion.
Pub. by Howard W. Steer 2005 (ISBN 0 9550009)
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Steer HW.
2007. www.howardsteer.co.uk/papers/003.
Does necrosis have any role in gastric epithelial cell biology? Evidence to support a gastric holocrine epithelial cell secretion in *Helicobacter pylori* infection.

Figure 1.

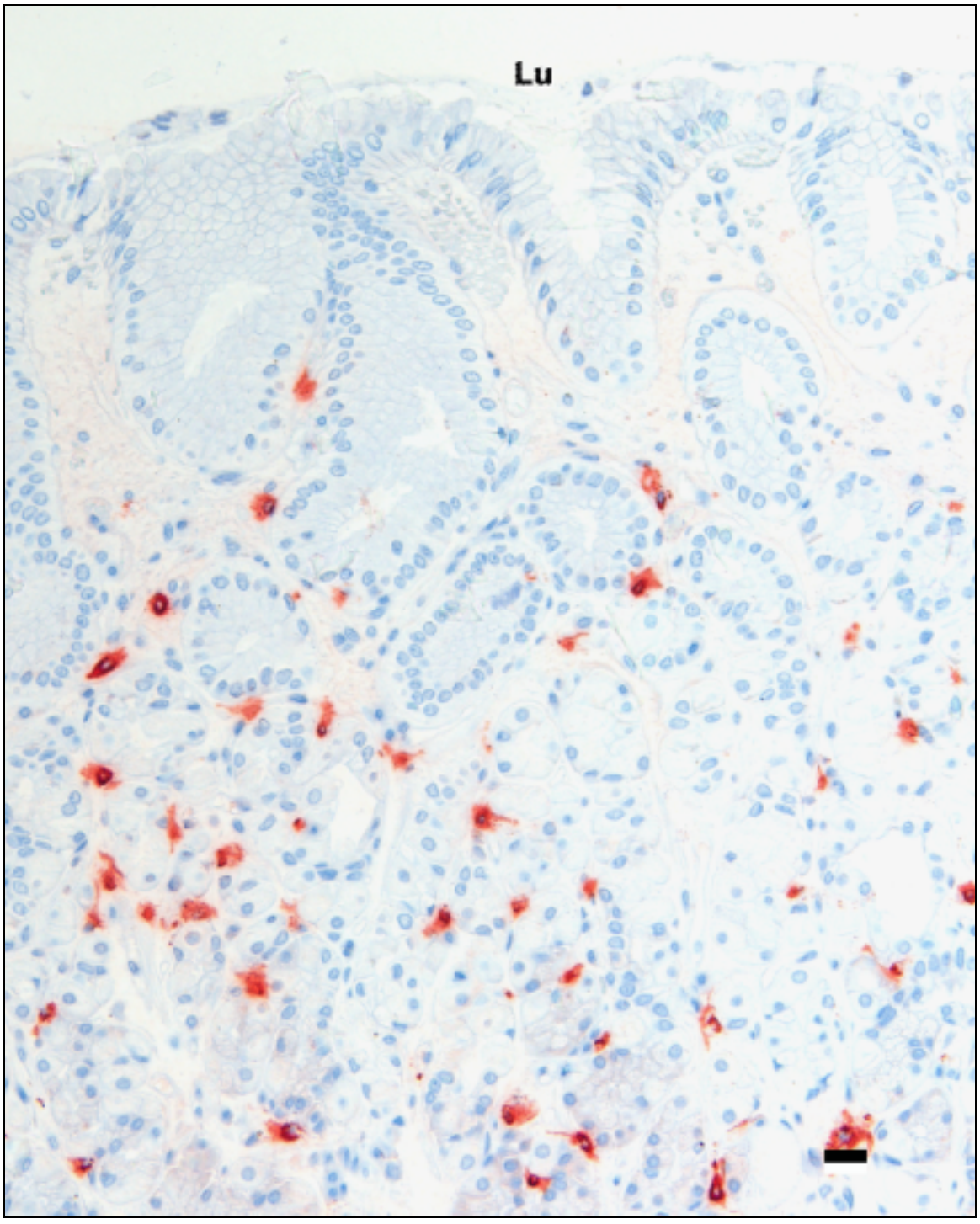

Figure 1. Mast cell tryptase. (Chromogen substrate).

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

Figure 2.

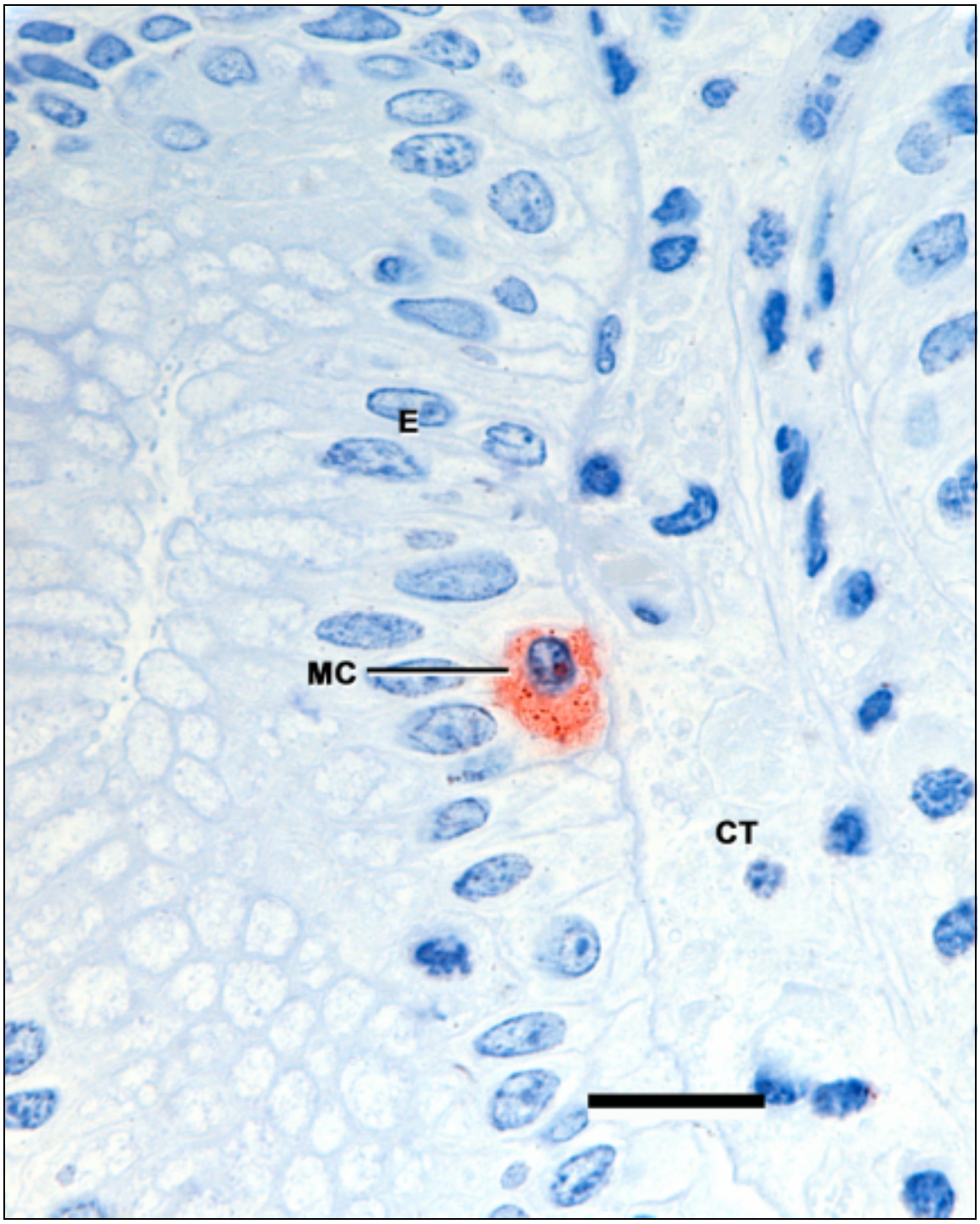

Figure 2. Mast cell tryptase. (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. The gastric epithelium (E) and subepithelial connective tissue (CT) are shown. An intraepithelial cell containing mast cell tryptase (MC) is indicated. Scale bar is 20µm.

Figure 3.

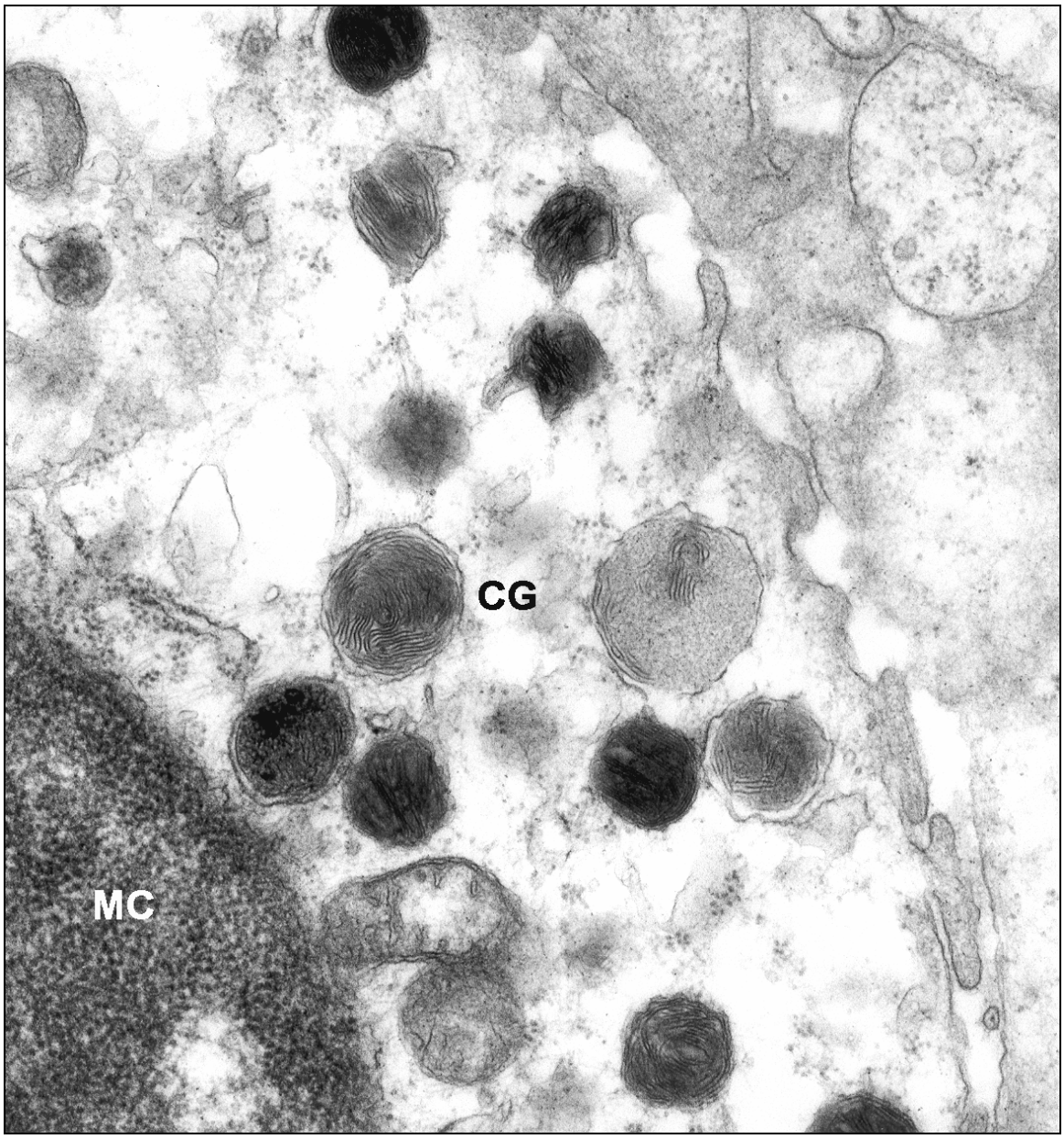

Figure 3. Transmission electron micrograph.

Male aged 62 years.
Helicobacter pylori infection.
Mucosal biopsy from high on the greater curve of the stomach.

A subepithelial connective tissue mast cell (MC) with numerous cytoplasmic granules (CG) undergoing degranulation. The cytoplasmic granules have various scroll-like forms. Magnification x 47,990.

Figure 4.

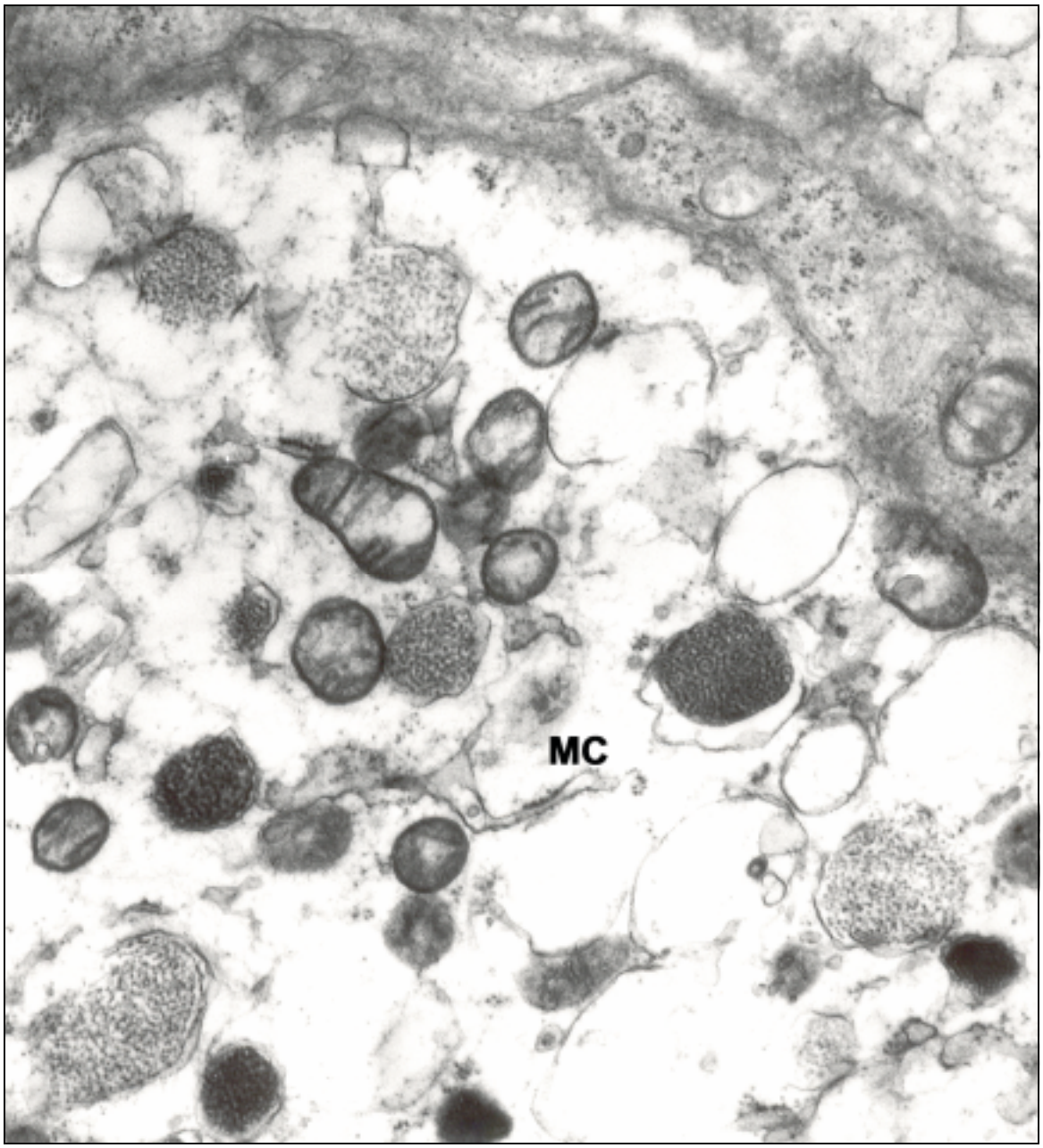

Figure 4. Transmission electron micrograph.

Male aged 62 years.
Helicobacter pylori infection.
Mucosal biopsy from high on the lesser curve of the stomach.

A degranulating intraepithelial mast cell (MC) is shown.
Magnification x 33,040.

Figure 5.

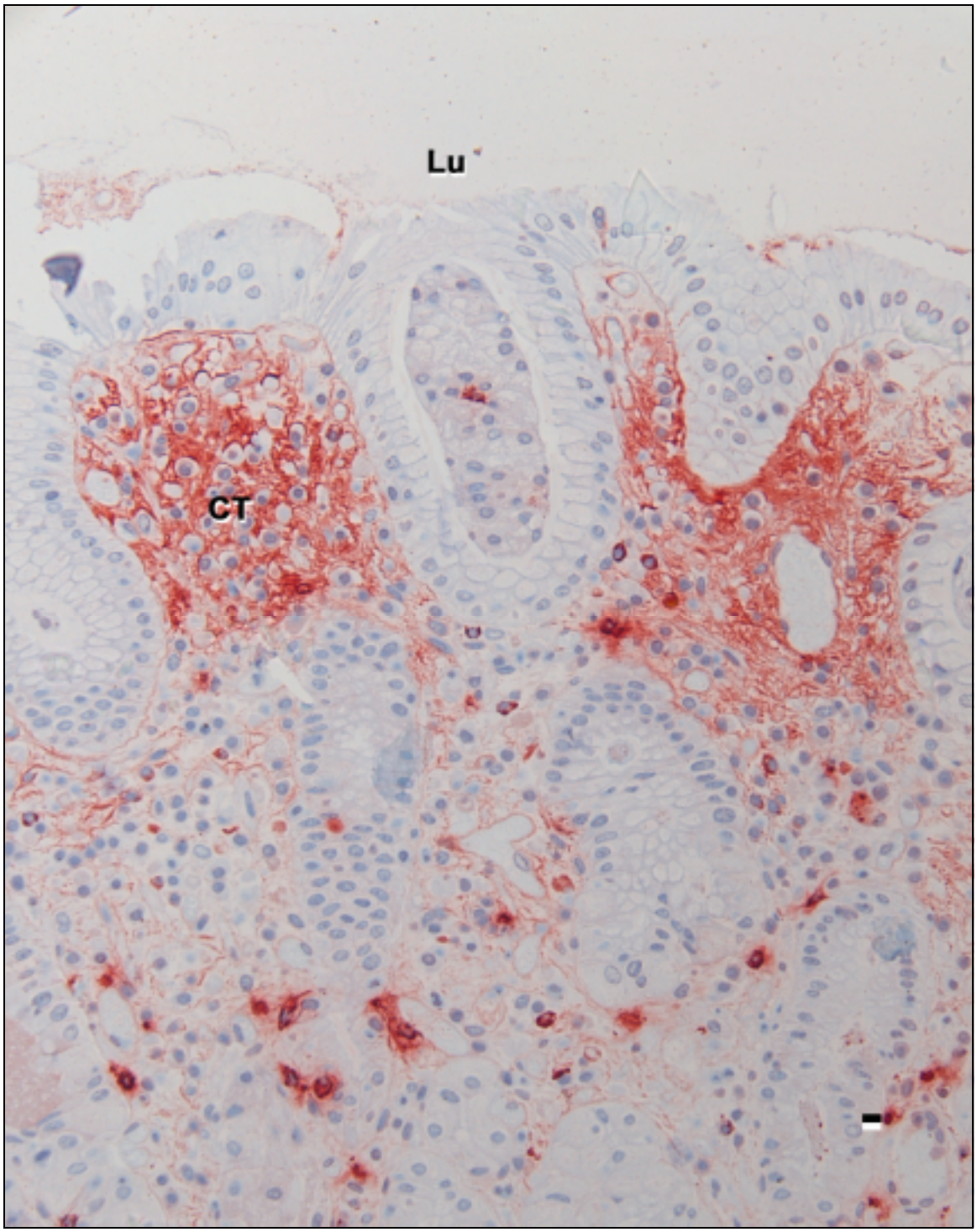

Figure 5. Mast cell tryptase. (Chromogen substrate).

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

Figure 6.

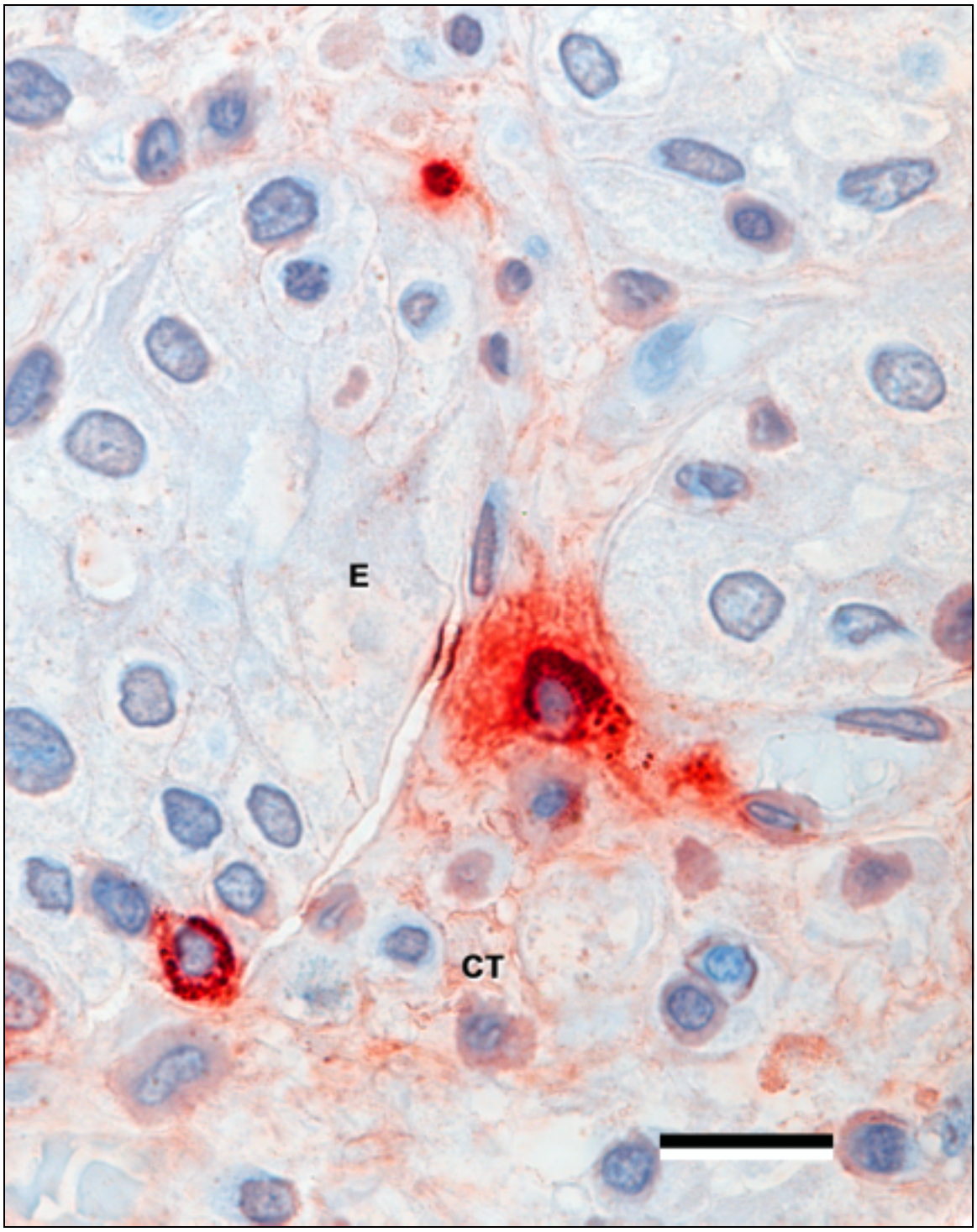

Figure 6. Mast cell tryptase. (Chromogen substrate).

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

Figure 7.

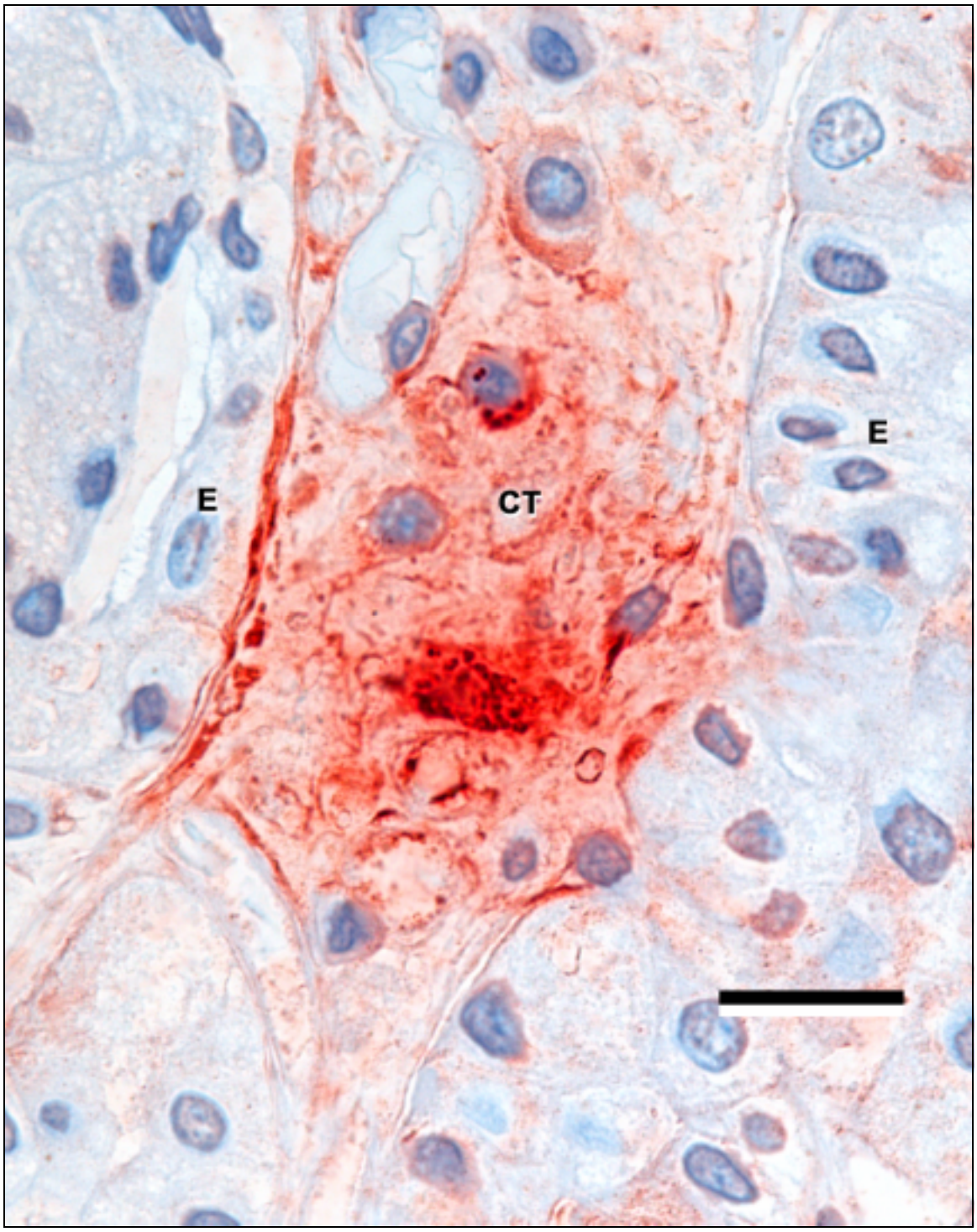

Figure 7. Mast cell tryptase. (Chromogen substrate).

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

Figure 8.

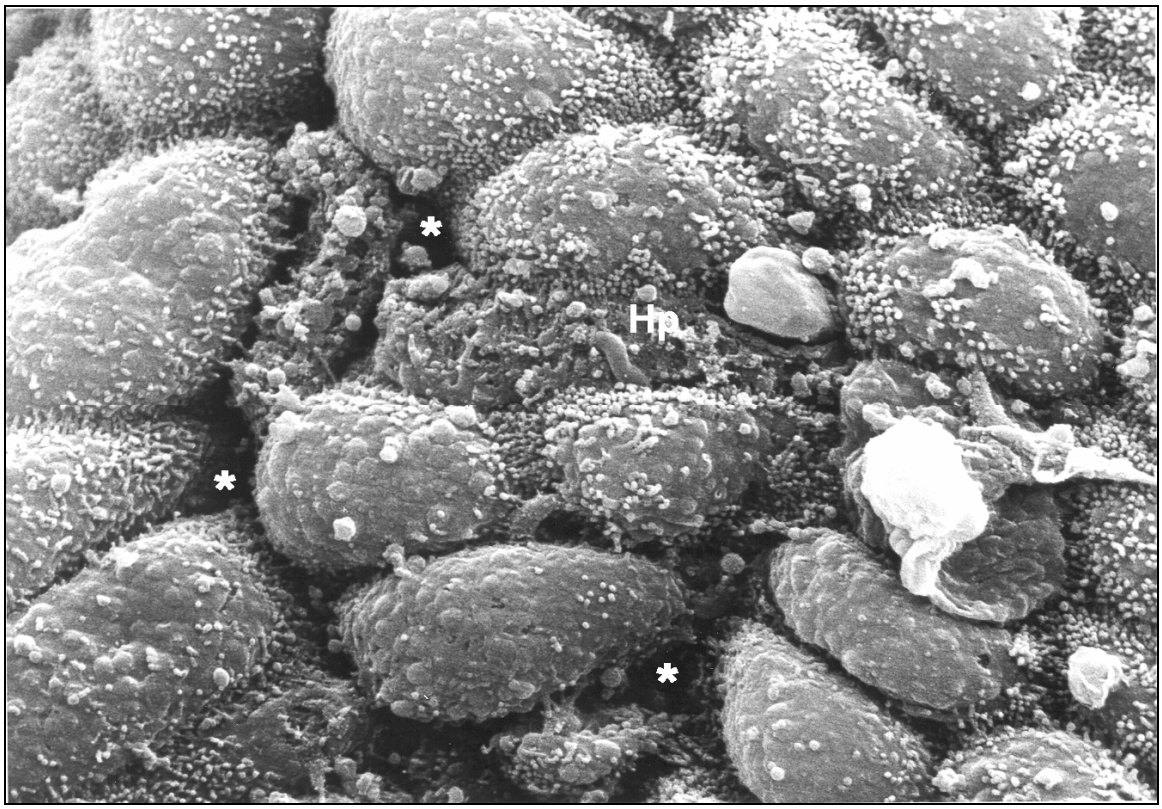

Figure 8. Scanning electron micrograph.

The luminal surface of the gastric epithelial cells showing gaps between epithelial cells (∗) at sites where the antigens can approach the MHC Class 11 molecules from an extracellular route. A *Helicobacter pylori* bacterium (Hp) is shown.
Magnification x 4,570.

Figure 10.

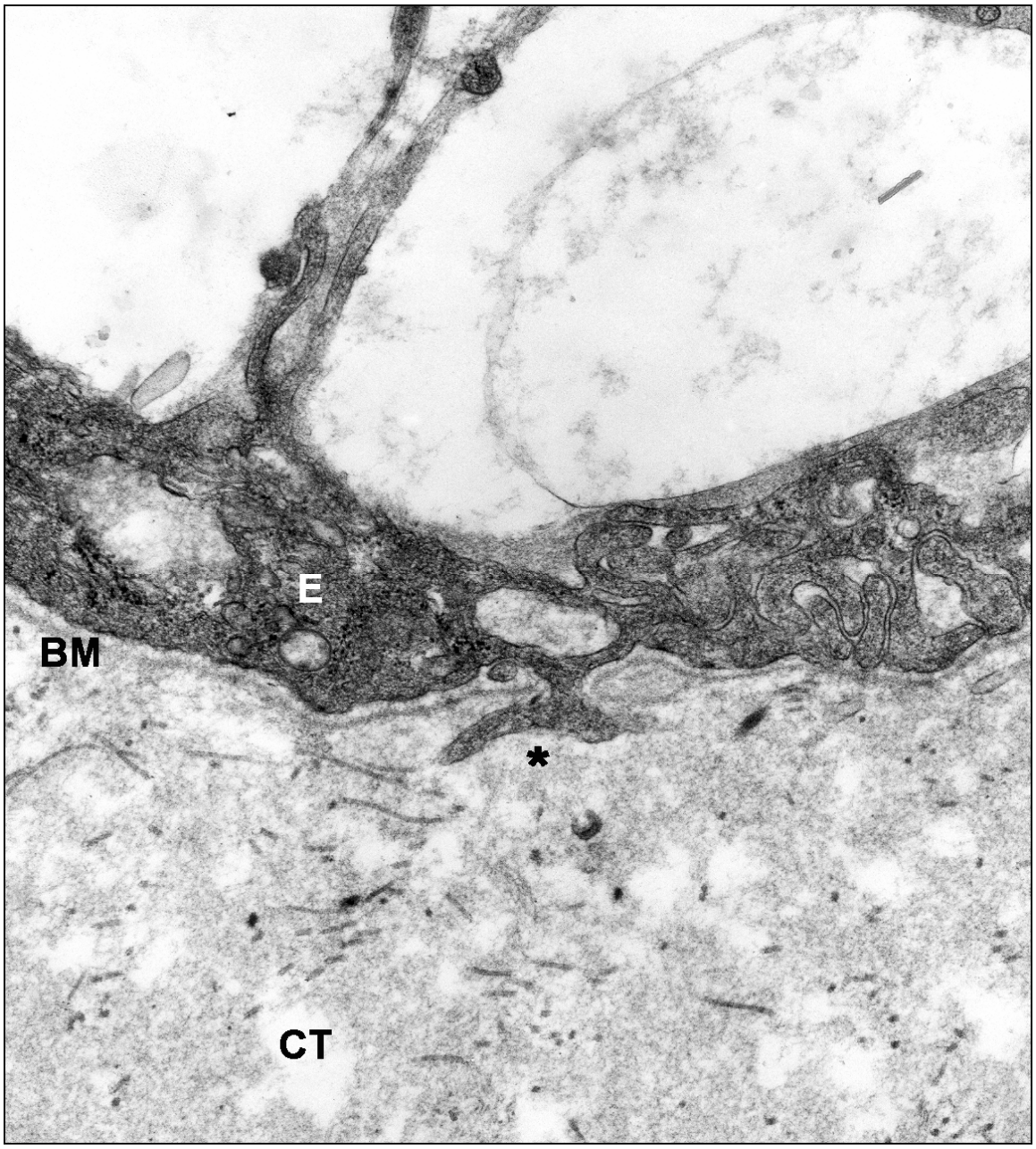

Figure 10. Transmission electron micrograph.

Male aged 62 years.
Helicobacter pylori infection.
Mucosal biopsy from the incisura angularis of the stomach.

Base of a mucus secreting epithelial cell (E) with the underlying basement membrane (BM). A cytoplasmic process (∗) passes through the basement membrane into the connective tissue (CT).
Magnification x 31,920.

Figure 11.

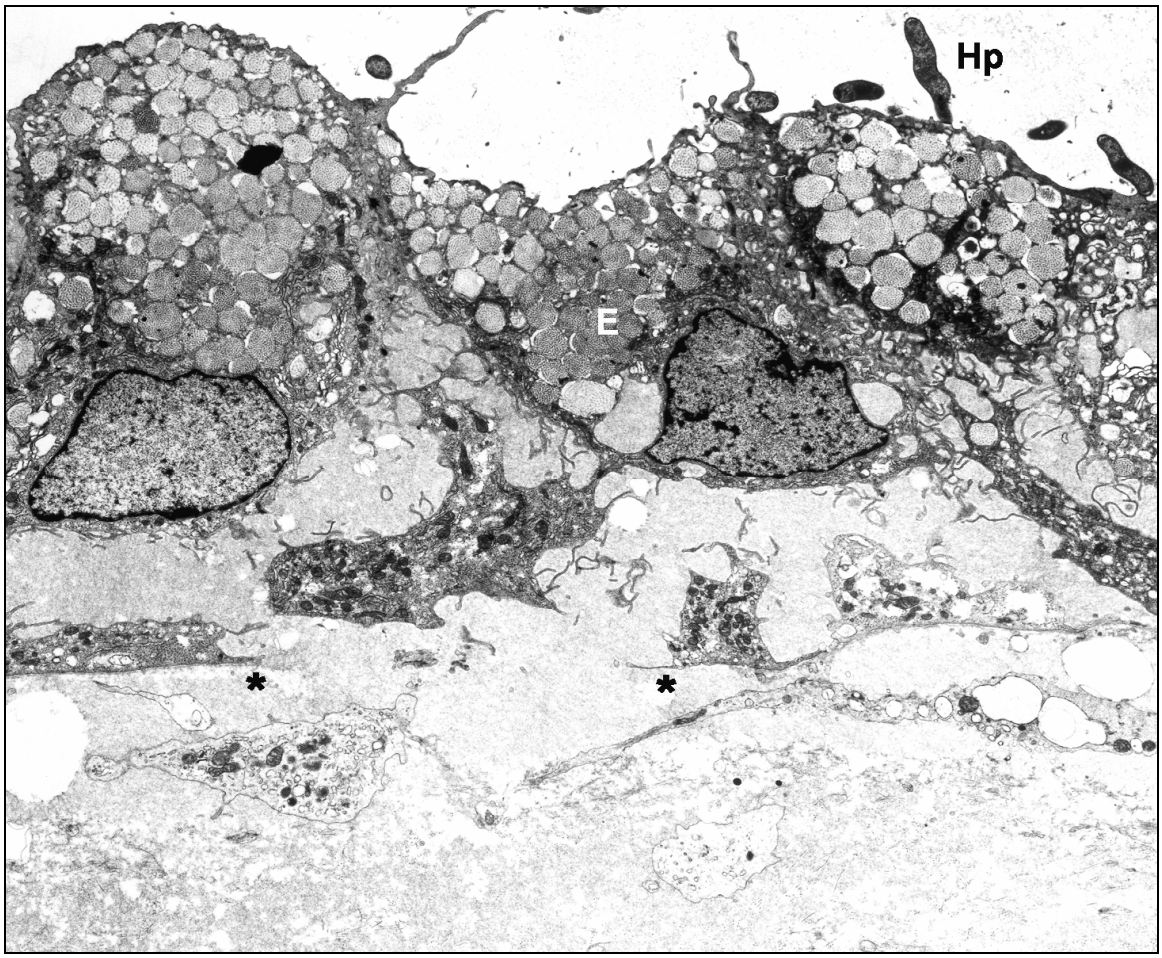

Figure 11. Transmission electron micrograph.

Male aged 62 years.
Helicobacter pylori infection.
Mucosal biopsy from the incisura angularis of the stomach.

Mucus secreting epithelial cells (E) with *Helicobacter pylori* (Hp) at the luminal surface of the stomach. The basement membrane underlying the epithelial cells has a break in its continuity with the edges of the break indicated by (∗).
Magnification x 4,050.

Figure 12.

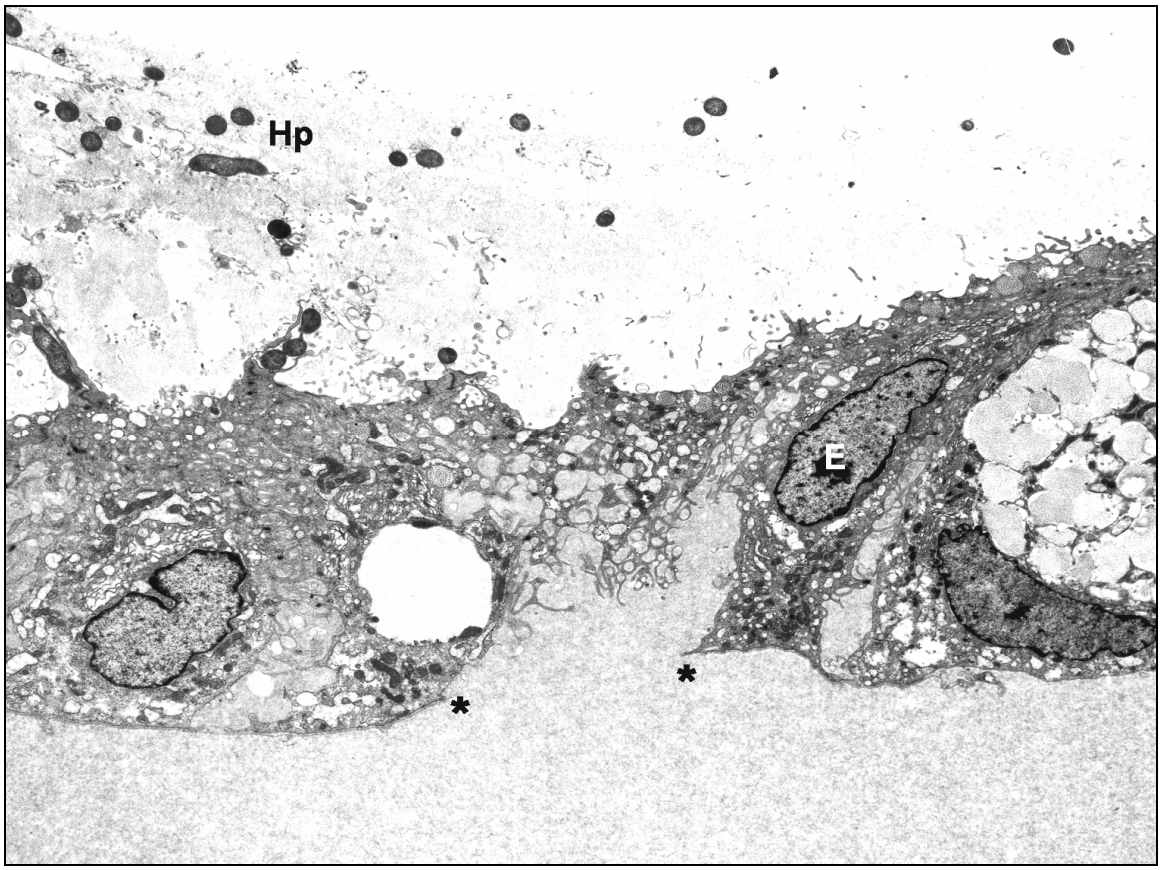

Figure 12. Transmission electron micrograph.

Male aged 62 years.
Helicobacter pylori infection.
Mucosal biopsy from the incisura angularis of the stomach.

Mucus secreting epithelial cells (E) with *Helicobacter pylori* (Hp) in the mucus overlying these cells. Beneath these epithelial cells is the basement membrane. A gap in the basement membrane is marked (∗).
Magnification x 1,780.

Figure 13.

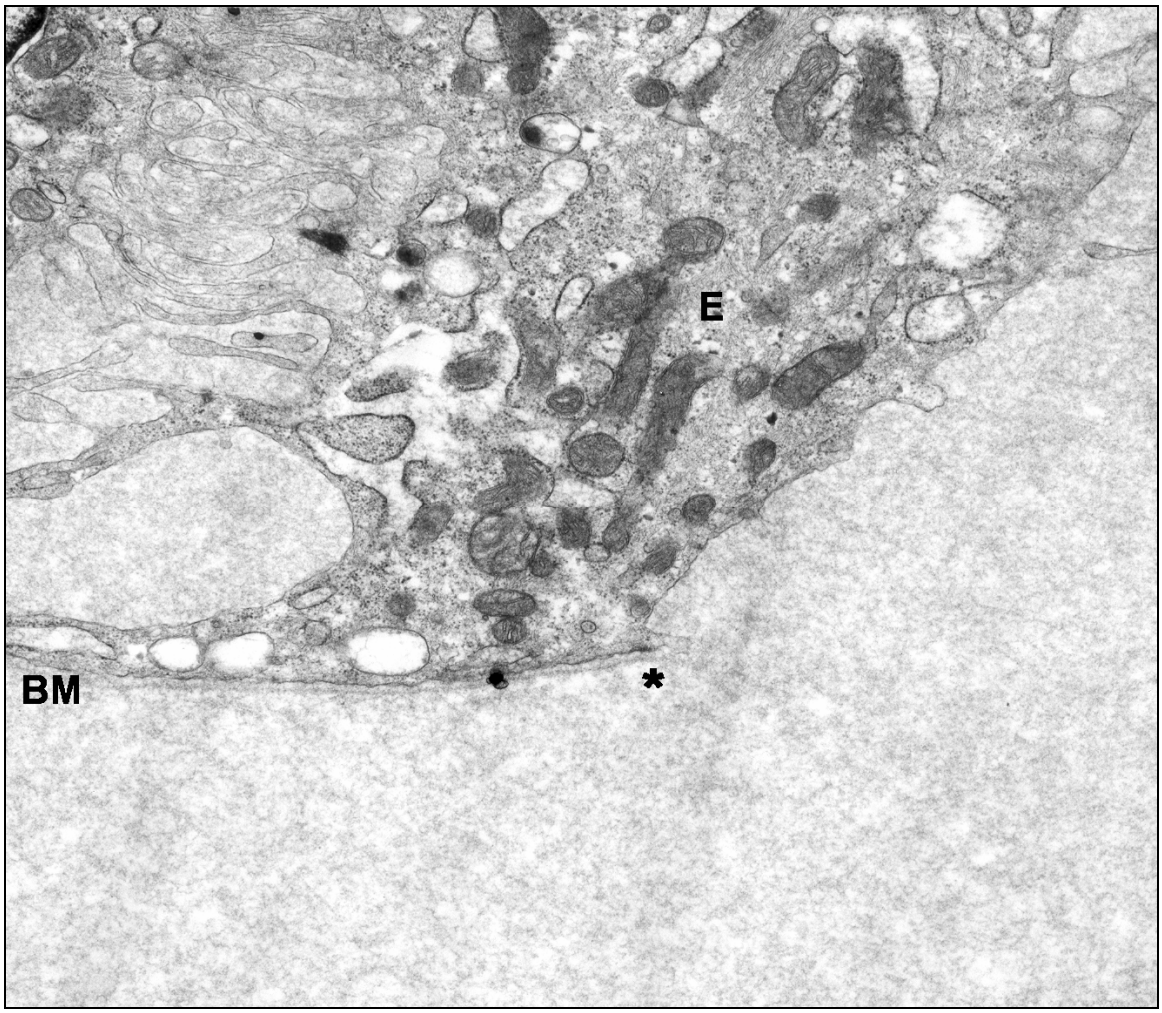

Figure 13. Transmission electron micrograph.

Male aged 62 years.
Helicobacter pylori infection.
Mucosal biopsy from the incisura angularis of the stomach.

Basal region of the mucus secreting epithelial cell (E) with the underlying basement membrane (BM). The break in the continuity of the basement membrane is marked (∗) with the basement membrane absent from the right side of the electron micrograph.
Magnification x 16,015.

Figure 14.

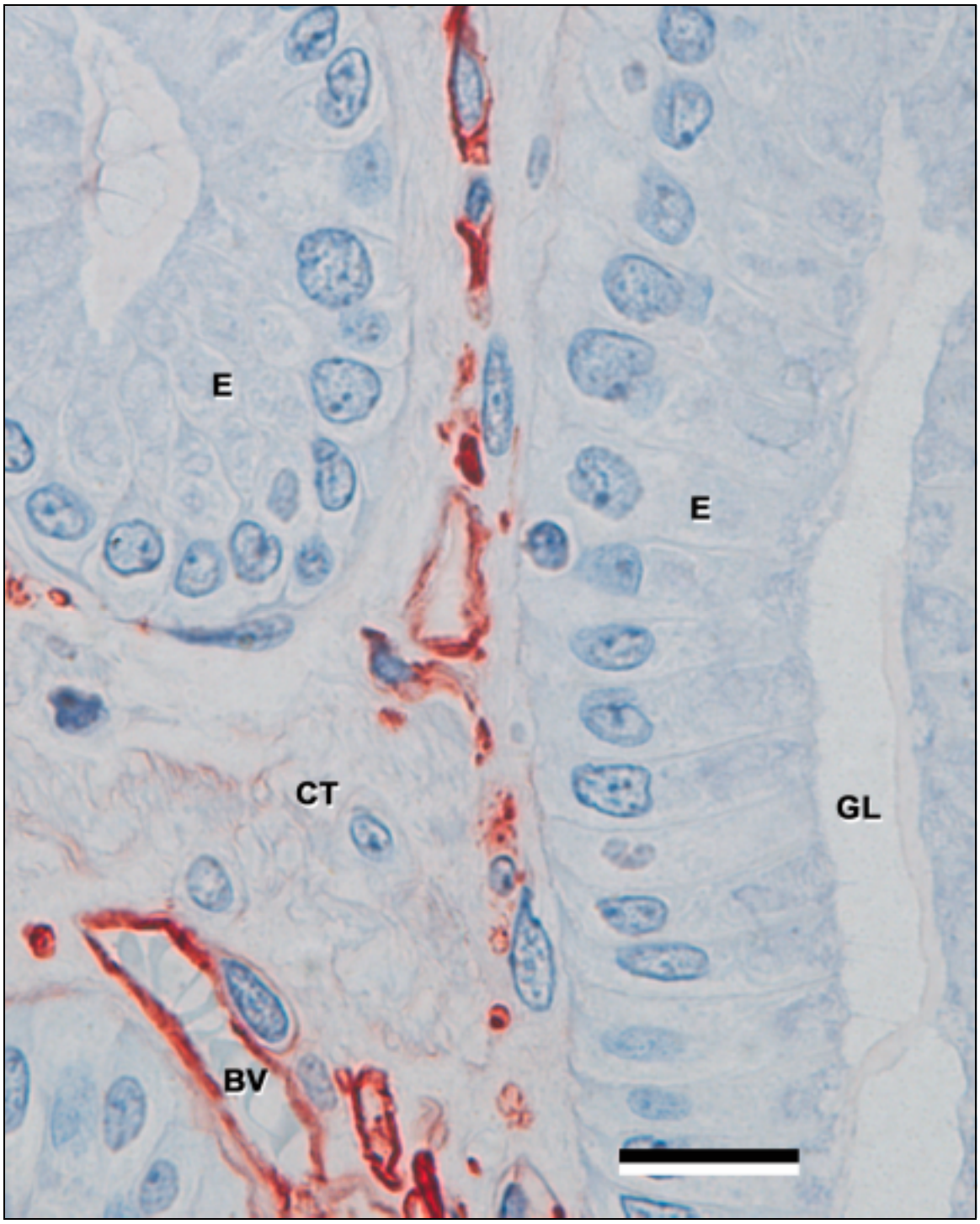

Figure 14. Major Histocompatibility Complex Class 11. (Chromogen substrate).

Antral biopsy from the normal stomach. The gastric epithelium (E), lumen of a gastric gland (GL), subepithelial connective tissue (CT) and a mucosal blood vessel (BV) are shown. Scale bar is 20µm.

Figure 15.

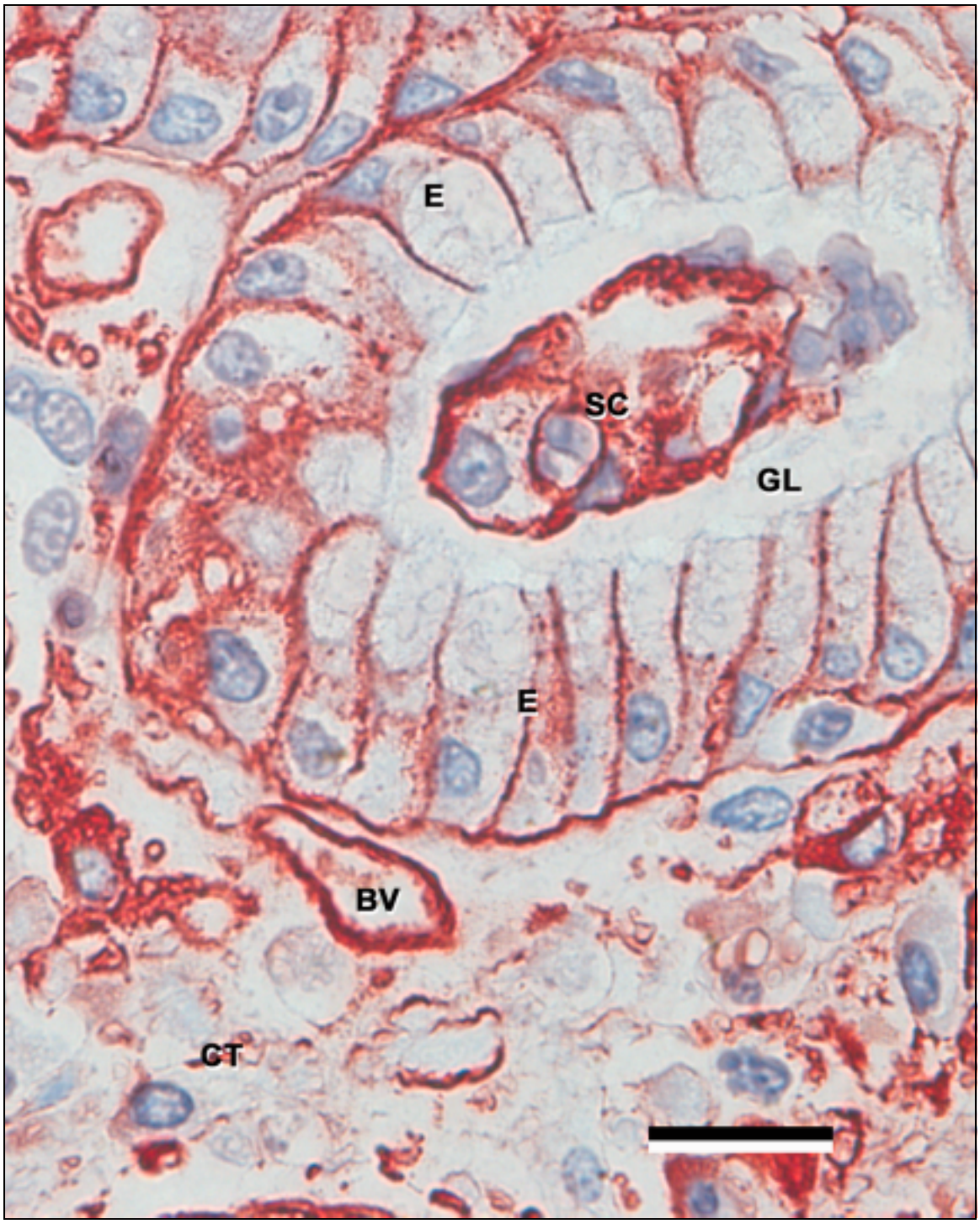

Figure 15. Major Histocompatibility Complex Class 11. (Chromogen substrate).

Antral biopsy from a stomach infected with *Helicobacter pylori*. The gastric epithelium (E), lumen of a gastric gland (GL), cells shed into the gland lumen (SC), subepithelial connective tissue (CT) and a mucosal blood vessel (BV) are shown. Scale bar is 20µm.