Title: Does necrosis have any role in gastric epithelial cell biology? Evidence to support a gastric holocrine epithelial cell secretion in *Helicobacter pylori* infection

Author Howard W. Steer

Institution Southampton General Hospital,
Southampton University Hospital NHS Trust,
University of Southampton School of Medicine,
Southampton, SO16 6YD United Kingdom.

Abstract

Gastric biopsies have been examined by immunohistochemistry and by electron microscopy in normal patients and patients with *Helicobacter pylori* infection. Epithelial necrosis of gastric glands resulting in the shedding of these necrosing epithelial cells is found in patients with *Helicobacter pylori* infection.

There is upregulation of interleukin 2, interleukin 4, interferon gamma and tumour necrosis factor alpha in *Helicobacter pylori* infection. Evidence is presented that these upregulated cytokines lead to the development of the cytocidal lymphocytes responsible for inducing the epithelial necrosis. The necrosing chief cells with their secretions, being shed into the gastric gland lumen would constitute holocrine secretion.

Keywords: Stomach, epithelial necrosis, *Helicobacter pylori*, cytotoxic lymphocyte, cytokines, holocrine secretion.

Introduction

The epithelial cells of the gastric glands, including chief cells and parietal cells, are produced in the isthmus/neck region of the gastric glands – the so-called proliferative area. Cells of the proliferative area can be readily visualized in human tissue using a monoclonal antibody specifically targeting these cells. Ki 67 is a mouse monoclonal antibody that recognises an epitope on a nuclear antigen which is expressed by cells in all stages of the cell cycle except Go. A correlation has been shown between Ki 67 staining and other markers of cell proliferation demonstrating the reliability of Ki 67 as a proliferation marker (Yu et al 1992). Subsequently, antibodies were raised against recombinant parts of Ki 67 antigen and these antibodies included MiB 1 (Cattoretti et al 1992). MiB 1 has been shown to be equivalent to Ki 67 as a marker of cell proliferation. Using MiB 1 the proliferative area of human gastric gland can be easily identified (figure 1). The downward migration of some cells from the proliferative area into the neck region of the gastric glands has been concluded from the work of Kaku (1966), Willems et al (1972) and Hattori and Fujita (1976).

Mucus neck cells transform into chief cells and parietal cells in the isthmus/neck region of the gastric glands (Hattori and Fujita 1976). Interestingly, both the mucous neck cells and the chief cells produce pepsinogen 11. The downward migration of chief cells is not a simple 'pipe line flow' with the cells migrating straight down the gastric glands but may also involve horizontal or oblique movements of the cells, referred to as a Stochastic flow system (Hattori and Fujita 1976). Conventionally the cells of the gastric glands are considered to be produced in the proliferative area, migrate down the gastric glands and are shed from the depth of the gastric glands into the gland lumen (see discussion between Wright and Code in Wright 1984). The principle cells of the gastric glands survive for a significant length of time with parietal cells and chief cells calculated to take 200 days to migrate from the proliferative area to the lower end of the gastric gland in the golden hamster (Hattori and Fujita 1976).

In vitro studies using bromodeoxyuridine have shown that *Helicobacter pylori* infection of the human stomach is associated with a significant increase in the percentage of cells in the proliferative area (Lynch et al 1995). This increase in the proliferative area in *Helicobacter pylori* infection has been noted in humans when examining gastrectomy specimens excised for gastric malignancy (Harvard 1998).

Infrequently, shed cells can be seen in the lumen of the gastric glands in the normal human gastric fundus but the number of these shed cells in the lumen of the gastric glands is significantly increased when the stomach is infected with *Helicobacter pylori*. *Helicobacter pylori* are seen lying between unshed epithelial cells and epithelial cells shed into the gastric lumen in figure 2. The shed epithelial cells contain numerous cytoplasmic secretory granules (figure 3). The shedding of cells into the gastric glands and the increased size of the proliferative area of these gastric glands in *Helicobacter pylori* infection suggests that the survival of these parietal cells and chief cells is reduced in *Helicobacter pylori* infection.

If those chief cells shed into the lumen of the gastric gland are examined immunohistochemically they not only contain pepsinogen 11 but also IL-1β, IL-8, INFδ, TGFα and TGFβ (Steer 2005; Steer 2007). The shedding of chief cells with these cytokines is an example of holocrine secretion.

The present study investigates the changes to the gastric epithelium resulting from *Helicobacter pylori* infection. The necrosis of gastric epithelial cells, the shedding of these cells into the lumen of the gastric glands and the causes for this necrosis have been evaluated.

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 (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 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 then 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**
Interleukin 2 (IL-2)	mouse monoclonal (5355.11)	R & D Systems
Interleukin 4 (IL-4)	mouse monoclonal (3H4)	A.M.S. Biotechnology
Interferon gamma (INFδ)	mouse monoclonal (25718)	R & D Systems
Tumour necrosis factor alpha (TNFα)	mouse monoclonal (4H31)	Celltech Therapeutics
MHC Class 11	mouse monoclonal (CR3/43)	Abcam (ab7856)
Helicobacter pylori	rabbit polyclonal	DakoCytomation

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

Ultrastructurally, those chief cells and parietal cells which are in the process of being shed into the lumen of the gastric gland (figure 3 and 4) in *Helicobacter pylori* infection of the stomach are undergoing necrosis rather than apoptosis. This conclusion results from the electron microscopic observations of the chief cells and the parietal cells which are undergoing chromatin clumping with preservation of the basic chromatin pattern (figure 5), swelling of the mitochondria (figure 6) with flocculation of the mitochondria (figure 6) and cell membrane breakdown. These are the ultrastructural characteristics of necrosis. The majority of these cells visualised are undergoing necrosis

but are not necrotic. They will ultimately become necrotic. These changes would lead to disintegration of the shed cells and release of their intracellular content into the gastric lumen. There is no ultrastructural evidence for these shed chief cells having any of the typical apoptotic changes of nuclear fragmentation, blebbing, the development of apoptotic bodies or phagocytosis (Wyllie et al 1980). These results are at variance with the interpretation of Stachura et al (1993) who reported the changes to the parietal and chief cells in the gastric glands as indicative of apoptosis. Their observations were not confirmed by the TUNEL technique. If human gastric biopsies are examined by the TUNEL technique the epithelial apoptosis is principally related to the superficial gastric epithelium (Jones et al 1997).

What is the mechanism responsible for inducing the necrosis of the gastric epithelial cells?

This question cannot be answered with certainty but evidence will be presented supporting the conclusion that the shedding of these cells is due to the action of a subpopulation of lymphocytes. Amongst these necrotic cells in the gastric gland epithelium it is common to find not only viable chief cells (figure 4) and viable parietal cells but also viable intraepithelial lymphocytes (figure 4).These lymphocytes are in intimate contact with the necrotic epithelial cells. These lymphocytes show no signs of necrosis. It has already been reported that Major Histocompatibility Complex Class 11 (MHC Class 11) is upregulated at the surface epithelium and pit/isthmus of the gastric glands in *Helicobacter pylori* infection (Steer 2005). This is also seen in parietal cells and chief cells. These cells are not stained for MHC Class 11 in the normal gastric mucosa (figure 7) but in patients with *Helicobacter pylori* infection there is a variable degree of membranous staining (figure 8) of the parietal cells and cytoplasmic staining of the chief cells (figure 8). When examining those gastric glands containing necrotic chief cells or necrotic parietal cells, there are numerous clumps of necrotic cells or occasionally whole gastric glands with necrotic chief cells and necrotic parietal cells. With conventional light microscopic fixation and processing the gastric epithelial necrosis could easily be mistaken for an artifact resulting from the time of biopsy and time to fixation.

Does apoptosis have a role in the shedding of chief cells and parietal cells?

Activated T lymphocytes are known to be a source of CD 95 ligand (Fas ligand) which is intimately involved in the initiation of apoptosis. The CD 95 ligand, which can be membrane bound on activated T lymphocytes, binds to Fas (CD 95) a membrane protein receptor on a cell about to undergo a sequence of events leading to apoptosis. It is the cross linking of Fas by its ligand which induces this sequence of events. Two vital components of this sequence are CD 95 ligand and CD 95 (Fas). The expression of CD 95 (Fas) in gastric mucosa has been examined (Steer 2005).

When the gastric mucosa in the normal stomach is examined the CD 95 expression is limited to some staining of the basement membrane of epithelial cells in the region of the pit/isthmus of the gastric glands. There is some upregulation of CD 95 expression in the gastric mucosa of those patients infected with *Helicobacter pylori* (Steer 2005).

The expression of CD 95 does not necessarily imply apoptosis as there has to be cell signaling from the CD 95 ligand. Ultrastructure has been regarded as one of the 'gold standards' for apoptosis. There is no ultrastructural evidence for apoptosis in the body of the gastric glands of the fundus of the stomach. Gastric epithelial apoptosis is said to be increased in *Helicobacter pylori* infection but the apoptotic cells visualized by the TUNEL technique are present in the superficial gastric mucosa and no comment has been made with respect to observations in the body of the gastric glands presumably because of the absence of apoptosis at that site (Jones et al 1997).

The lack of evidence supporting a role for apoptosis in the shedding of these gastric epithelial cells into the lumen of the gastric glands has resulted in the consideration of alternative mechanisms for the shedding of these cells from the depth of the gastric glands. The shed epithelial cells have features of necrosis but are not necrotic. Are there any factors which would facilitate necrosis of these epithelial cells? The mucosal intraepithelial lymphocytes are T lymphocytes. Certain types of T lymphocytes can be cytotoxic and some lymphocytes can be induced to become lymphocyte-activated killer cells (LAK cells) and natural killer (NK) cells exist.

Are any subtypes of lymphocytes responsible for the necrosis of gastric epithelial cells?

The majority of cytotoxic T lymphocytes (CTL) are CD 8 positive cells and the majority of intraepithelial lymphocytes are CD 8 positive lymphocytes. Cytotoxic T lymphocyte (CTL) activation requires IL-2, INFδ and IL-4 as well as other cytokines (Mizel 1989). Lymphokine-activated killer cells (LAK cells) are generated *in vitro* by incubation with IL-2. INFδ and TNFα may be involved in this activation process.

Those cytokines implicated in T lymphocyte activation resulting in the generation of cytotoxic cells are upregulated in *Helicobacter pylori* infection. This data will now be presented.

1. **Interleukin 2 (IL-2)**

In the normal gastric mucosa, there is coarse granular IL-2 staining in the blood vessels (figure 9, 10 and 11) and occasional staining of the epithelial cell basement membrane (figure 9, 10 and 11). Could the intravascular coarse granular IL-2 staining be related to platelets? When the gastric mucosa is infected with *Helicobacter pylori* there is significant upregulation of the IL-2 expression. IL-2 staining is again found as coarse granules in the blood vessel lumen but the epithelial basement membrane staining is increased and is more complete (figure 10 and 11). The connective tissue is stained for IL-2 particularly in the region of the pit of the gastric gland (figure 10 and 11). Some connective tissue cells are positively stained for IL-2 in *Helicobacter pylori* infection.

2. **Interferon gamma (INFδ)**

The expression of INFδ is upregulated in *Helicobacter pylori* infection of the stomach (figure 12 and 13). There is weak or no INFδ expression in the normal gastric mucosa with slight staining in the region of the epithelial mucus and slight granular staining in the mucosal blood vessels. The connective tissue expression is greatly upregulated paricularly in the pit/isthmus regions of the gastric glands (figure 13). The chief cells are not stained in the normal stomach (figure 14) but there is strong

expression of INFδ in the chief cells in *Helicobacter pylori* infection (figure 15). There is strong INFδ expression in those chief cells shed into the lumen of the gastric glands.

3. Interleukin 4 (IL-4)

In the mucosal biopsies from the normal stomach there is very weak or no expression of epithelial IL-4 (figure 16). However, in those gastric biopsies from patients infected with *Helicobacter pylori* there is marked upregulation of IL-4. There is intense staining of the mucus (figure 17), connective tissue cells and connective tissue stroma particularly in the region of the pit/isthmus of the gastric glands. There is occasional granular staining in the mucosal blood vessels.

4. Tumour necrosis factor alpha (TNFα)

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 the basement membrane of the endothelium in the pit/isthmus region of the gastric glands (figure 18). There are very occasional weakly stained connective tissue cells (figure 19) 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 endothelial cells present throughout the gastric mucosa (figure 19 and 20). There is a significant increase in the TNFα expression of the connective tissue particularly in the pit/isthmus area of the gastric glands. Some of the positively stained connective tissue cells have the morphological characteristics of plasma cells. 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*.

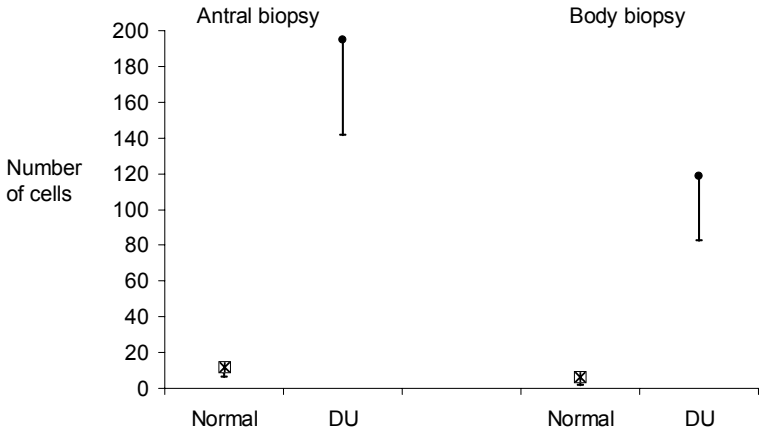

Figure 21. Number of cells positive for cytoplasmic TNFα in the mucosal connective tissue per 0.783 square millimetre of connective tissue in patients with a normal stomach and patients with benign duodenal ulceration. Mean – Standard Error of the Mean.

The present results indicate that those factors which have been shown to induce T lymphocytes to become cytotoxic are upregulated in *Helicobacter pylori* infection. Thus, T lymphocytes which are migrating to the gastric epithelium would be passing through a connective tissue environment containing significant IL-2, IL-4, INFδ and TNFα. Such a situation would mimick the *in vitro* experimental situation which has been shown to induce specific subpopulation of lymphocytes to become cytotoxic. This process is diagrammatically depicted in figure 22.

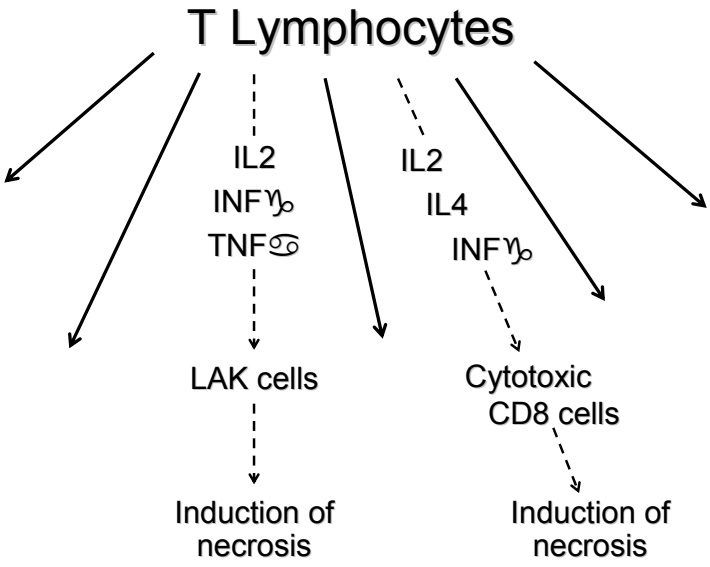

Figure 22. Diagrammatic representation of the upregulation of cytokines in *Helicobacter pylori* infection influencing the development of cytotoxic cells.

Acknowledgements

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Figure 1.

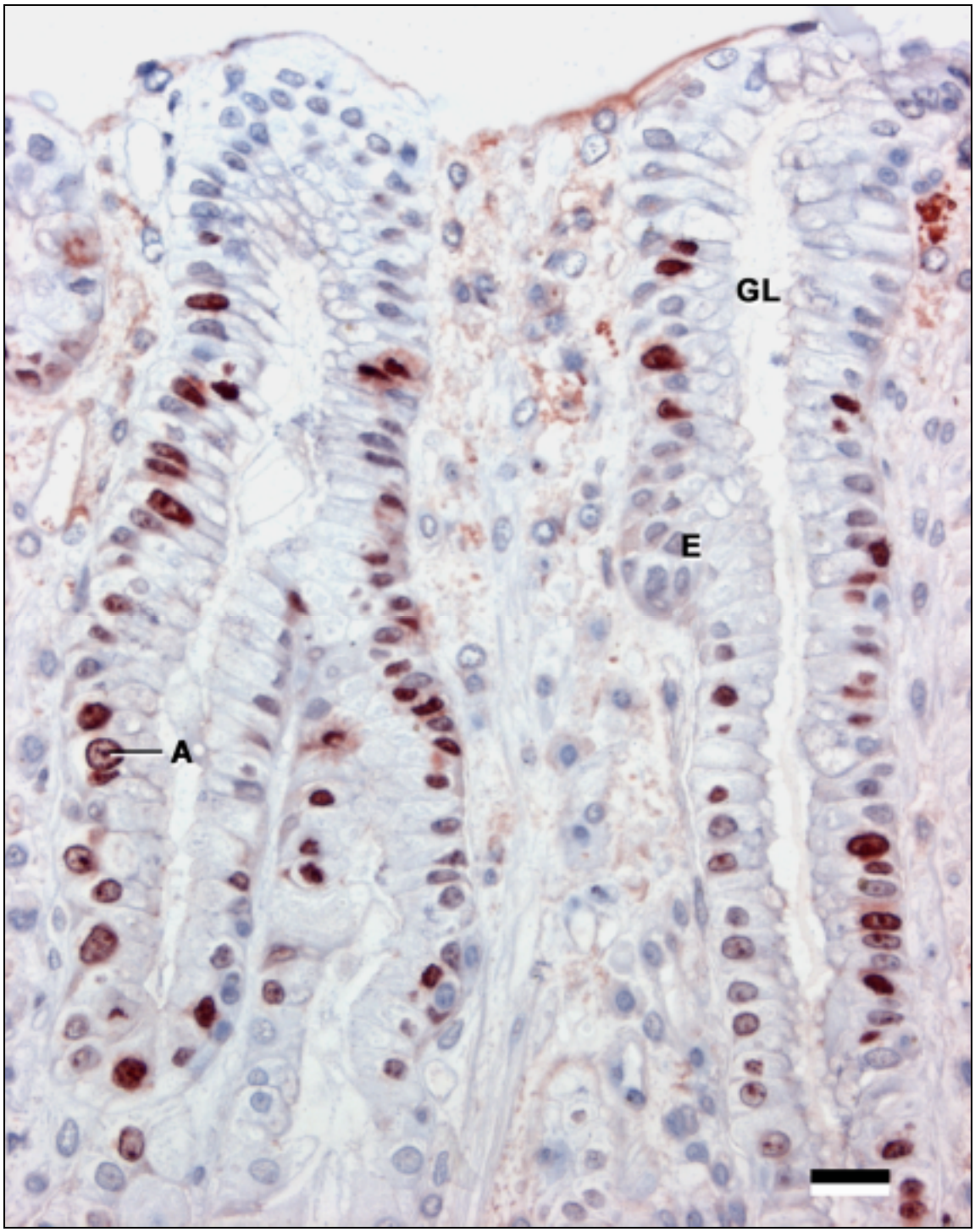

Figure 1. Ki 67 (Mib1). (DAB substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. The gastric epithelium (E), the lumen (GL) of a gastric gland and an epithelial cell nucleus (A) labelled with Mib1 are shown. Scale bar is 20µm.

Figure 2.

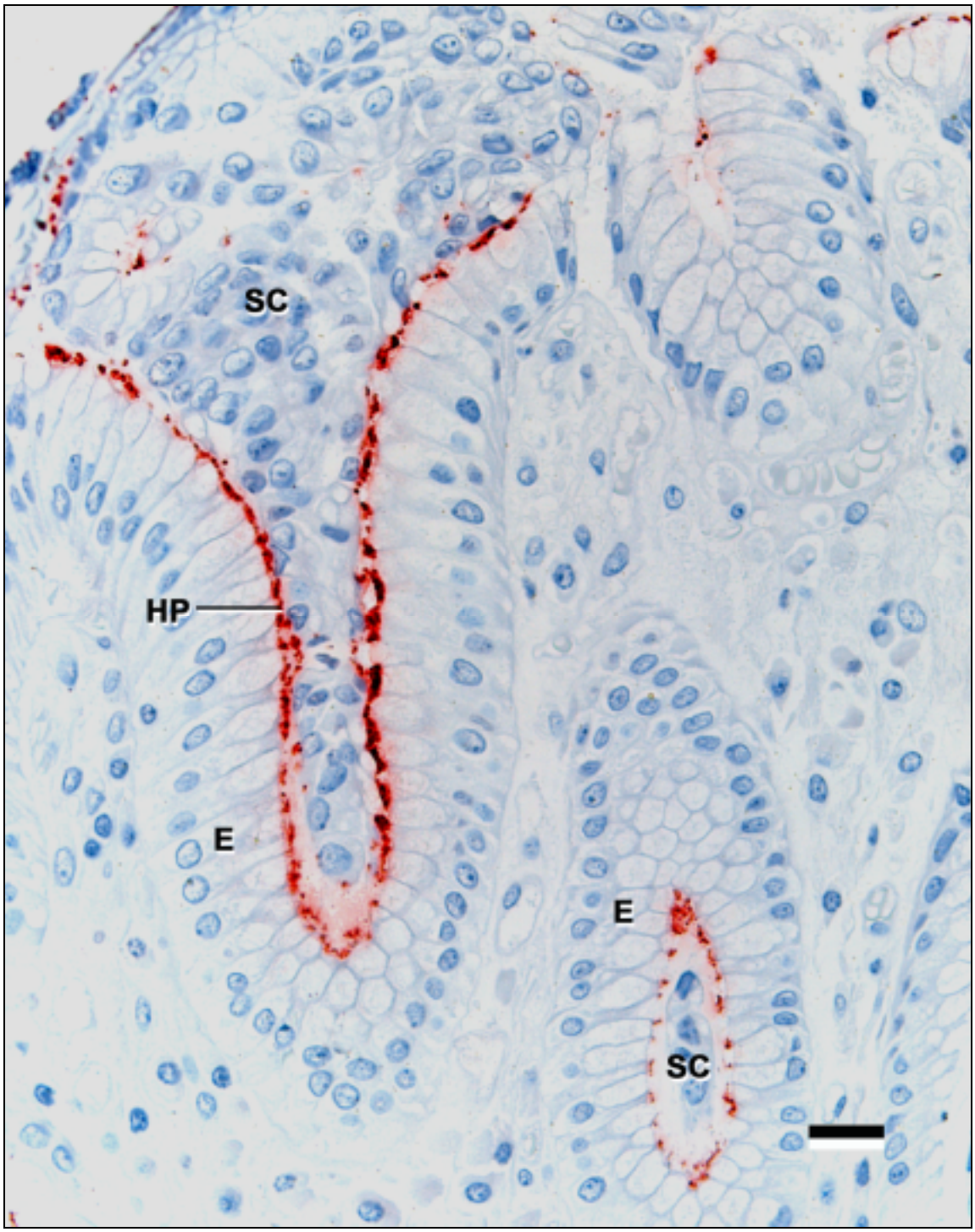

Figure 2. *Helicobacter pylori*. (Chromogen substrate).

Mucosal biopsy from the antrum of a stomach infected with *Helicobacter pylori* (HP). The gastric epithelial cells (E) and cells shed (SC) into the lumen of a gastric gland are shown. Scale bar is 20µm.

Figure 3.

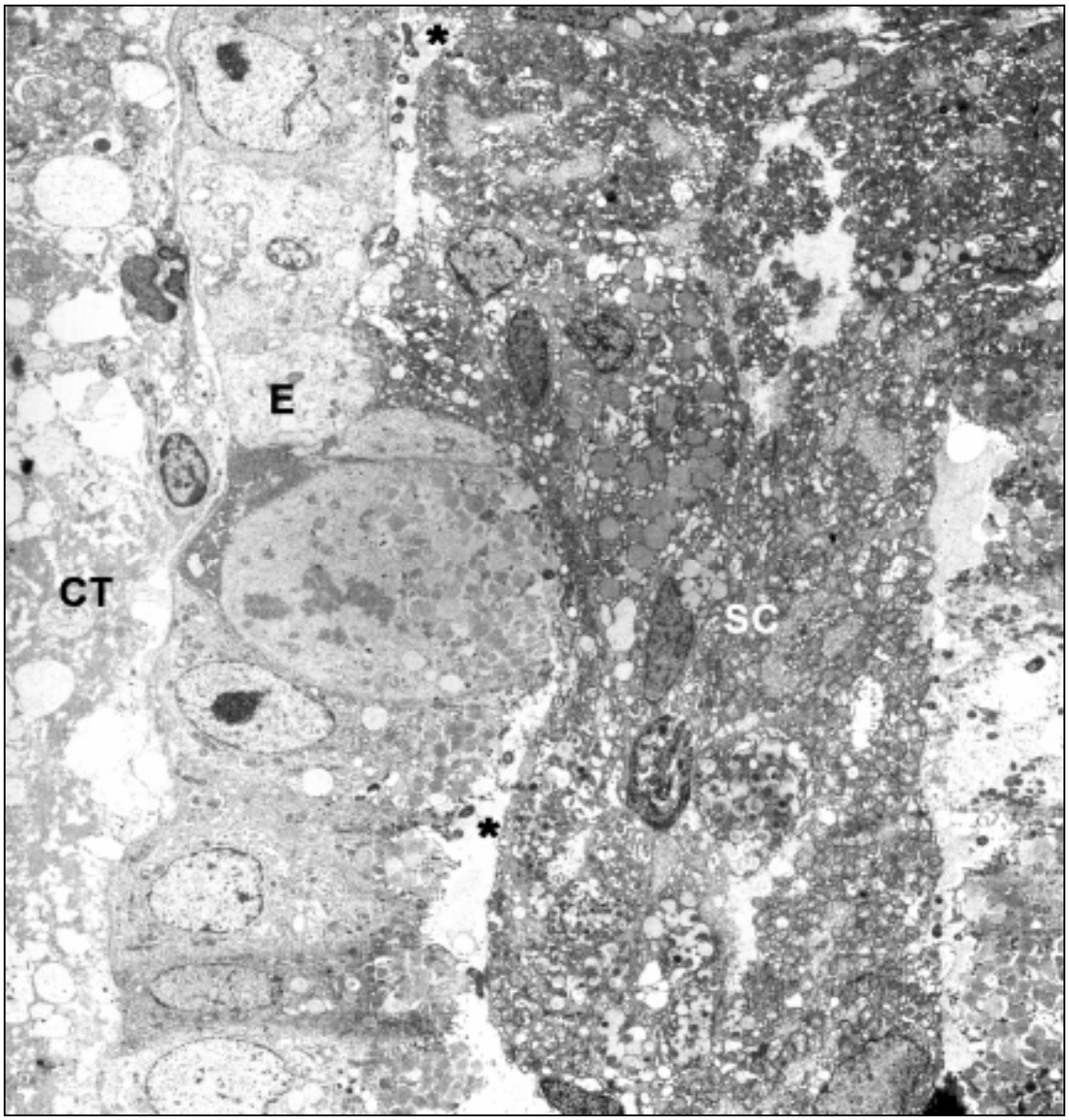

Figure 3. Transmission electron micrograph.

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

Mucus secreting epithelial cells (E) lining a gastric gland. The lumen of the gland contains numerous shed cells (SC) in various stages of necrosis. Numerous *Helicobacter pylori* (*) bacteria are present between the surface of the epithelial cells and the shed cells. Connective tissue (CT) abuts the basal surface of the epithelial cells.
Magnification x 2,430.

Figure 4.

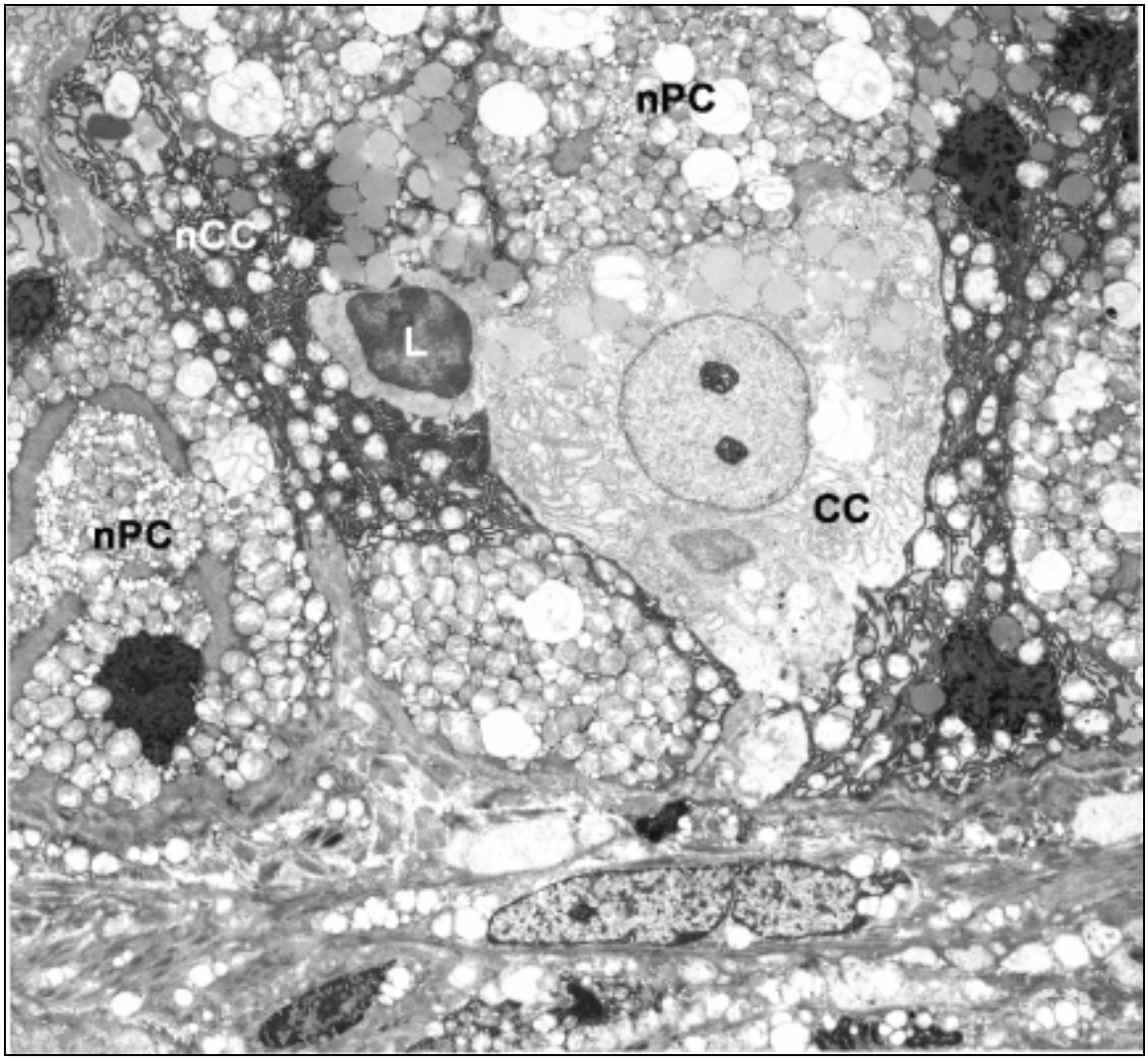

Figure 4. Transmission electron micrograph.

Male aged 59 years.
Helicobacter pylori infection.
Mucosal biopsy from high on the lesser curve of the stomach.

Gastric epithelial cells with the underlying connective tissue (CT). A large viable chief cell (CC) is surrounded by numerous necrosing chief cells (nCC) and necrosing parietal cells (nPC). A viable intraepithelial lymphocyte (L) is related to both the viable cell and the necrosing cells.
Magnification x 3,285.

Figure 5.

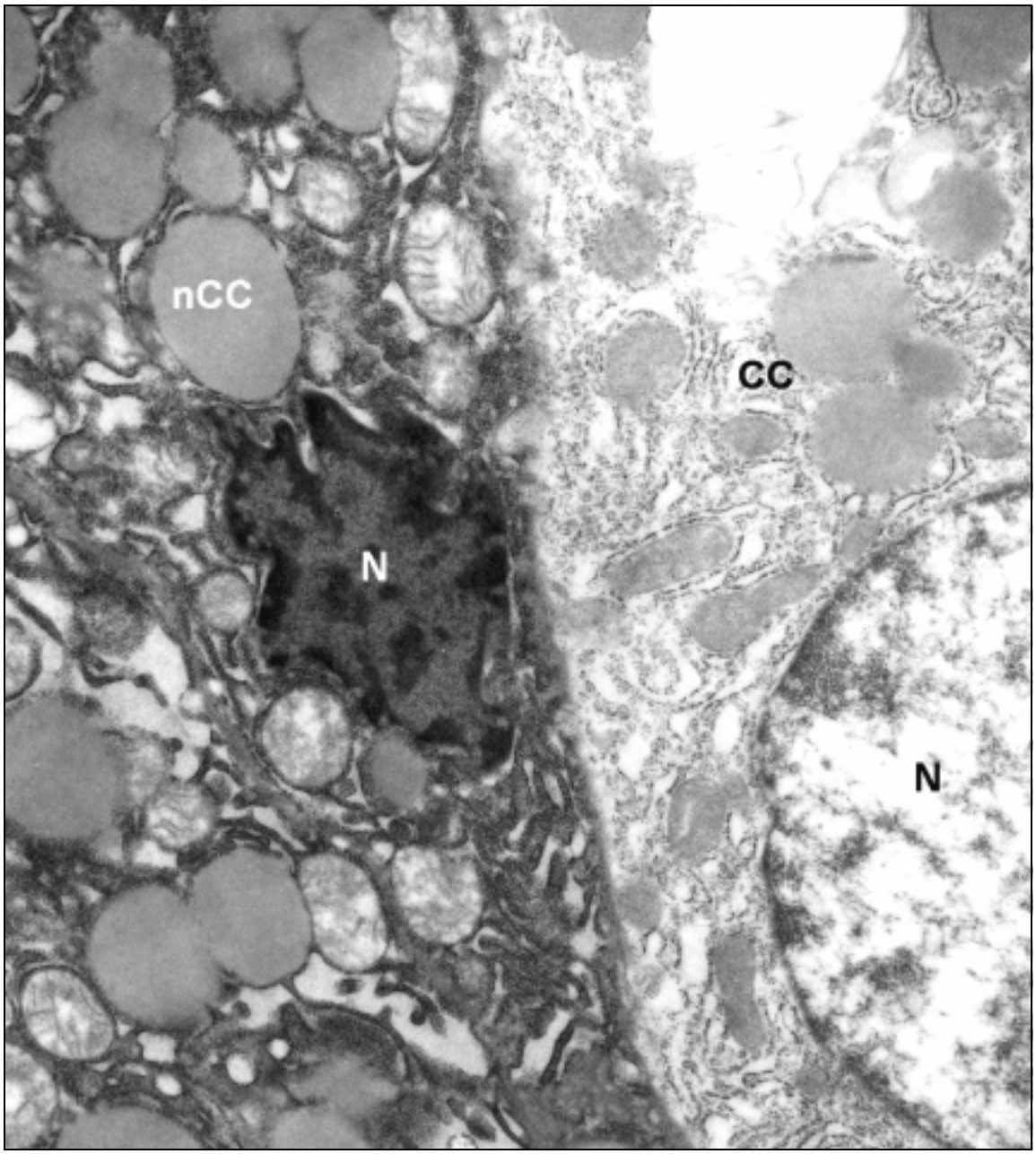

Figure 5. Transmission electron micrograph.

Male aged 63 years.
Helicobacter pylori infection.
Mucosal biopsy from high on the greater curve of the stomach.

The junction between two adjacent epithelial cells is shown. One epithelial cell is a viable chief cell (CC) and the other is a necrosing chief cell (nCC). The nucleus (N) of each cell is indicated.
Magnification x 17,890.

Figure 6.

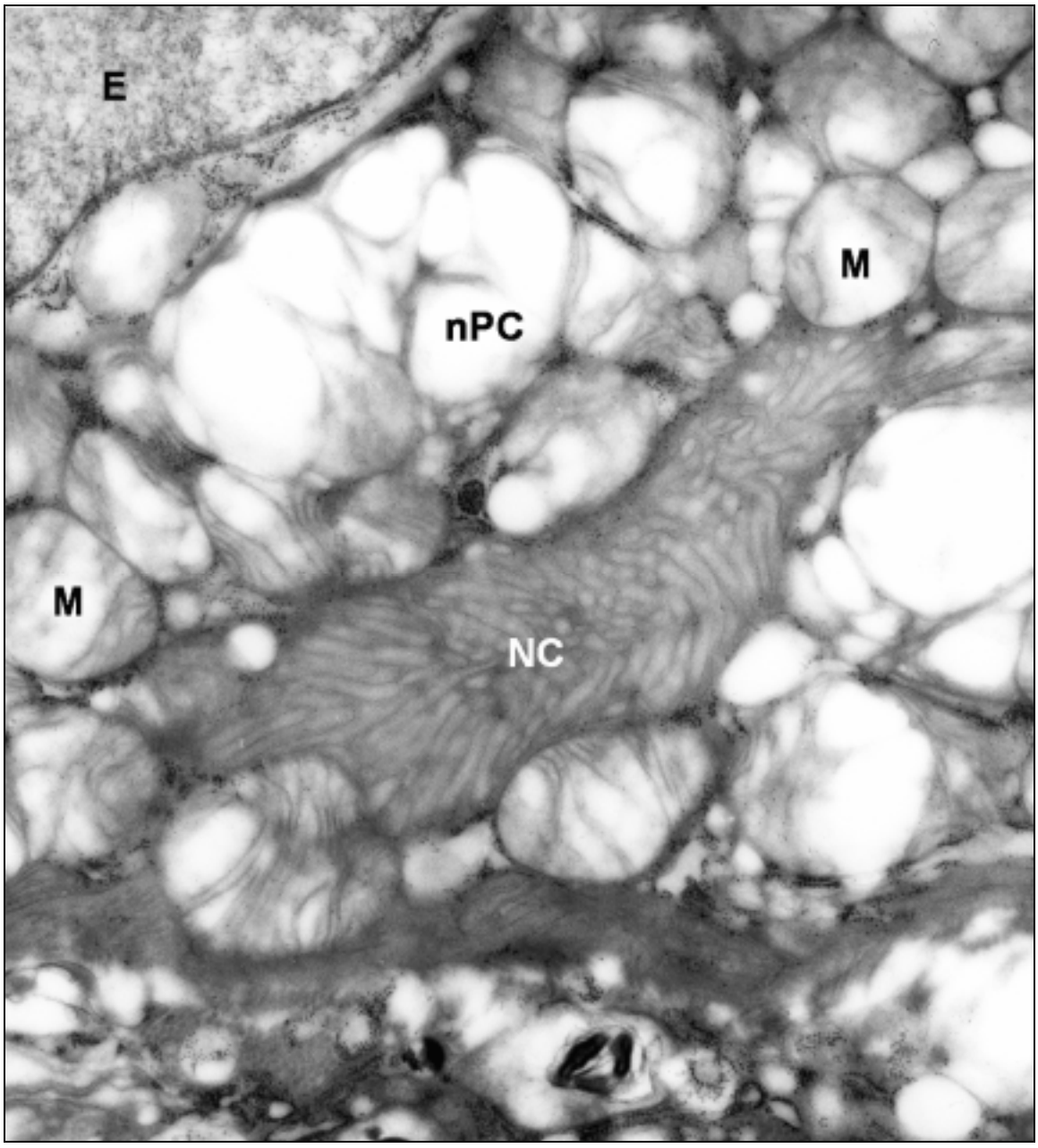

Figure 6. Transmission electron micrograph.

Male aged 59 years.
Helicobacter pylori infection.
Mucosal biopsy from high on the lesser curve of the stomach.

A necrosing parietal cell (nPC) is adjacent to a viable epithelial cell (E). A necrosing canaliculus (NC) is present as well as numerous swollen mitochondria (M) with flocculent material present in these mitochondria.
Magnification x 22,310.

Figure 7.

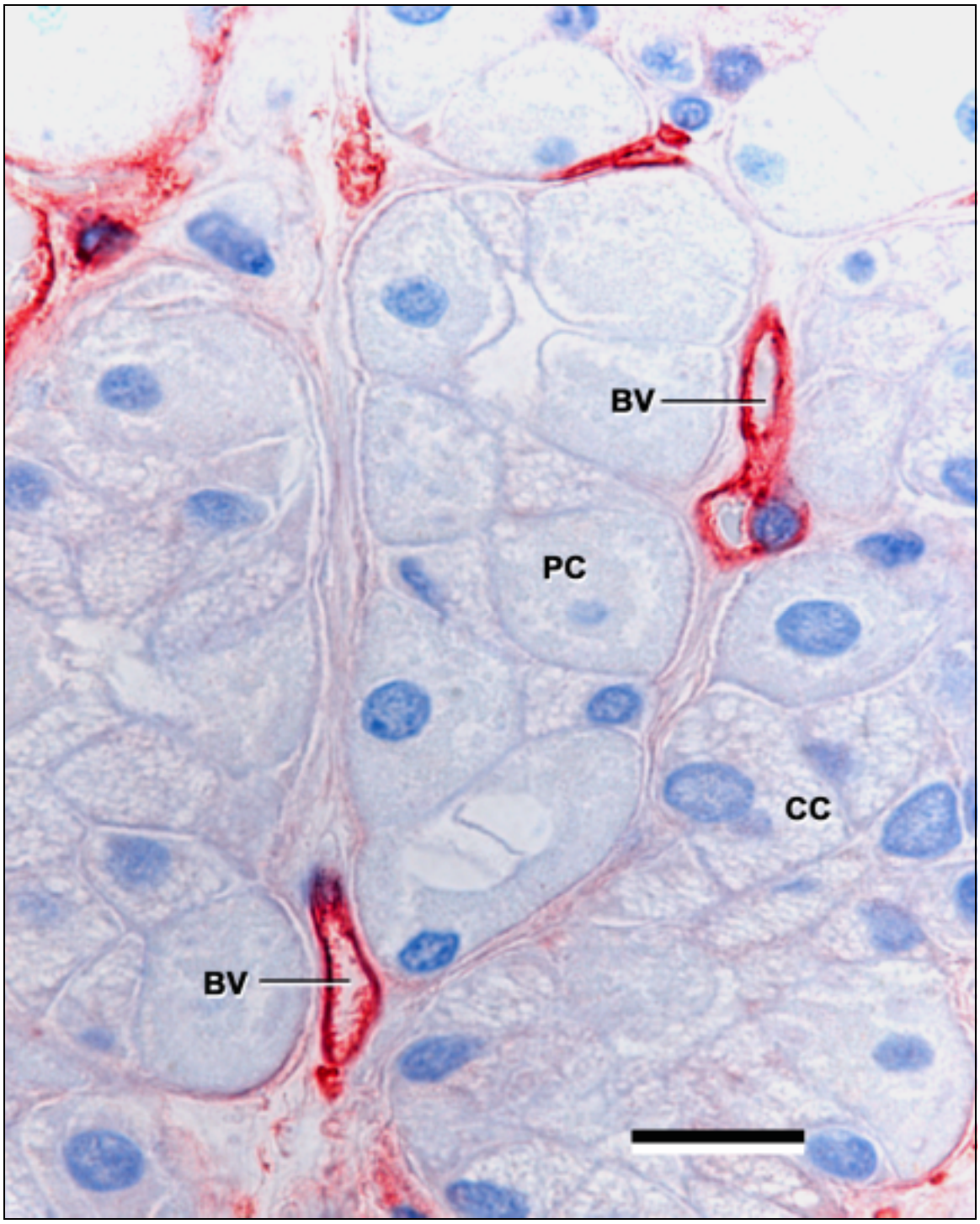

Figure 7. Major Histocompatibility Complex Class 11. (Chromogen substrate).

Mucosal biopsy from the body of the normal stomach. The chief cells (CC), parietal cells (PC) and mucosal blood vessels (BV) are shown. Scale bar is 20µm.

Figure 8.

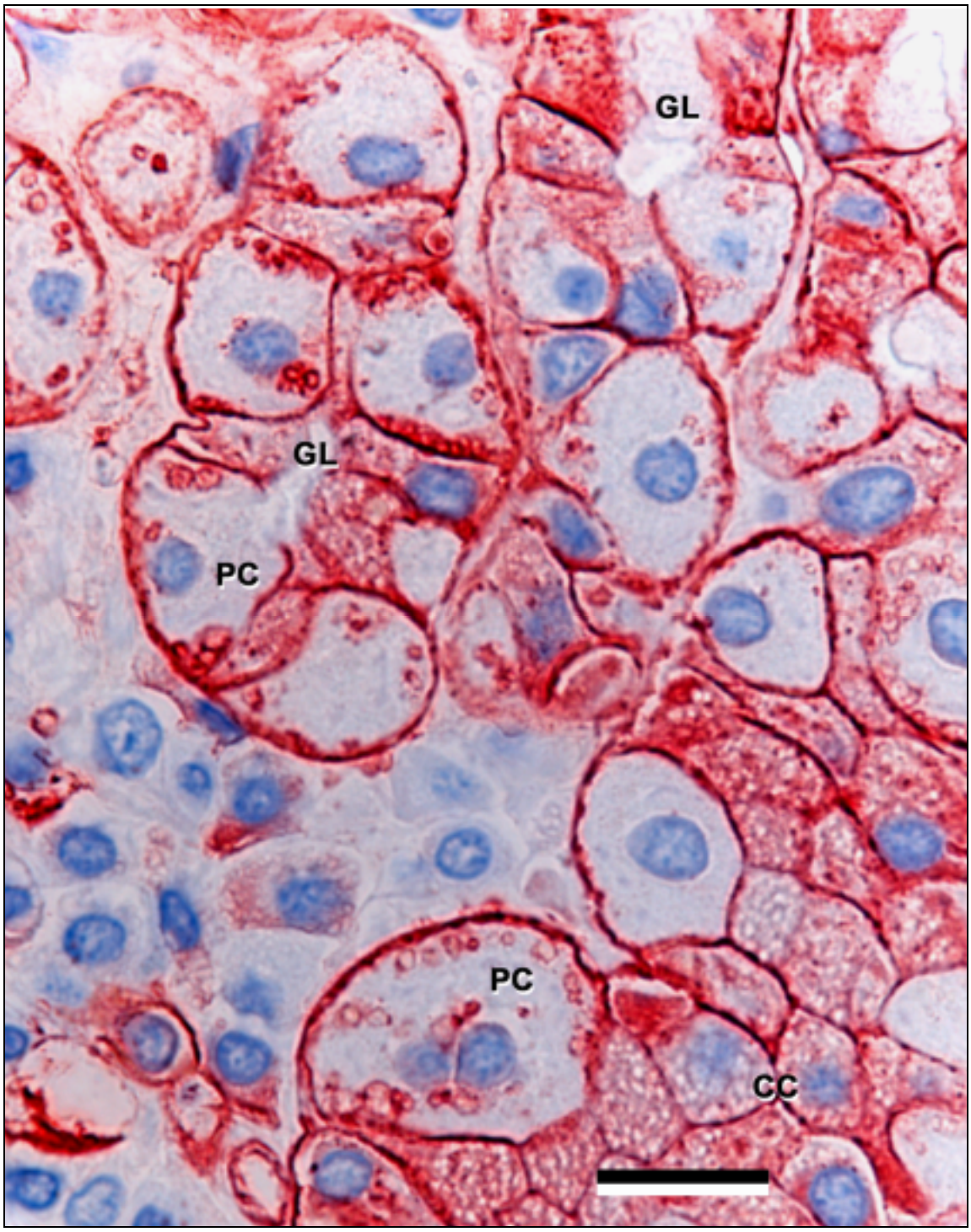

Figure 8. Major Histocompatibility Complex Class 11. (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. The chief cells (CC), parietal cells (PC) and lumen of a gastric gland (GL) are indicated. A parietal cell is seen opening into the lumen of a gastric gland. Scale bar is 20µm.

Figure 9.

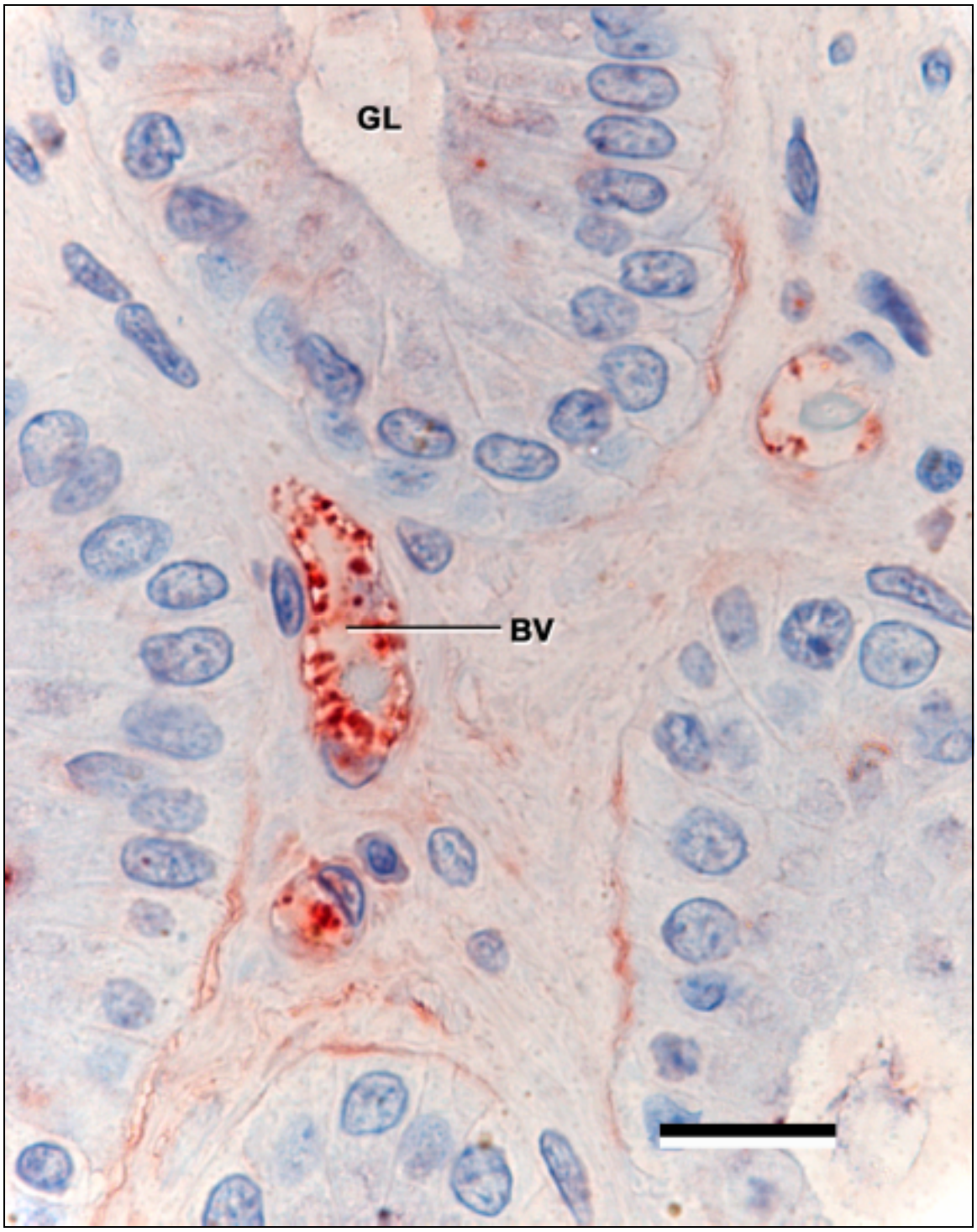

Figure 9. Interleukin 2. (Chromogen substrate).

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

Figure 10.

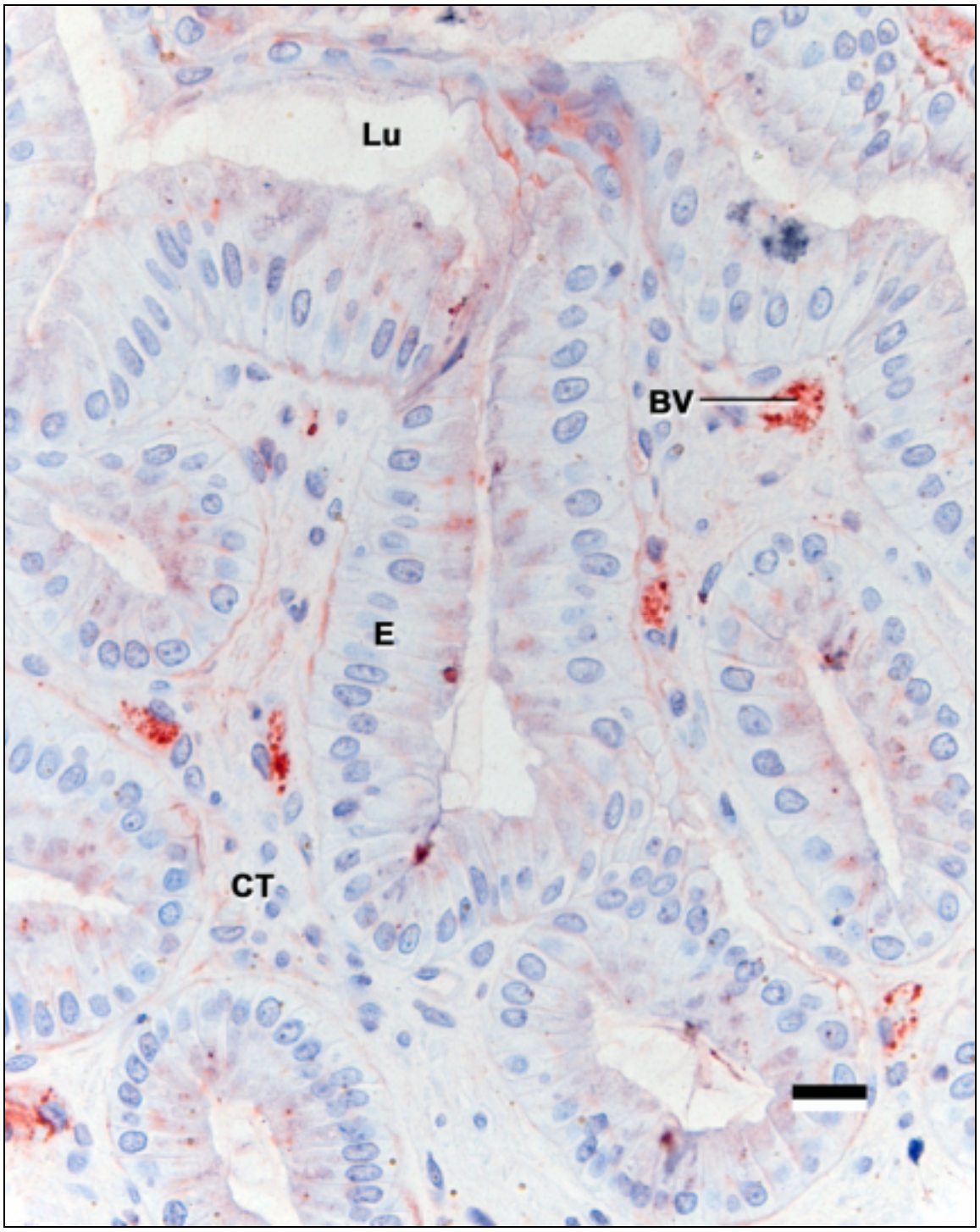

Figure 10. Interleukin 2. (Chromogen substrate).

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

Figure 11.

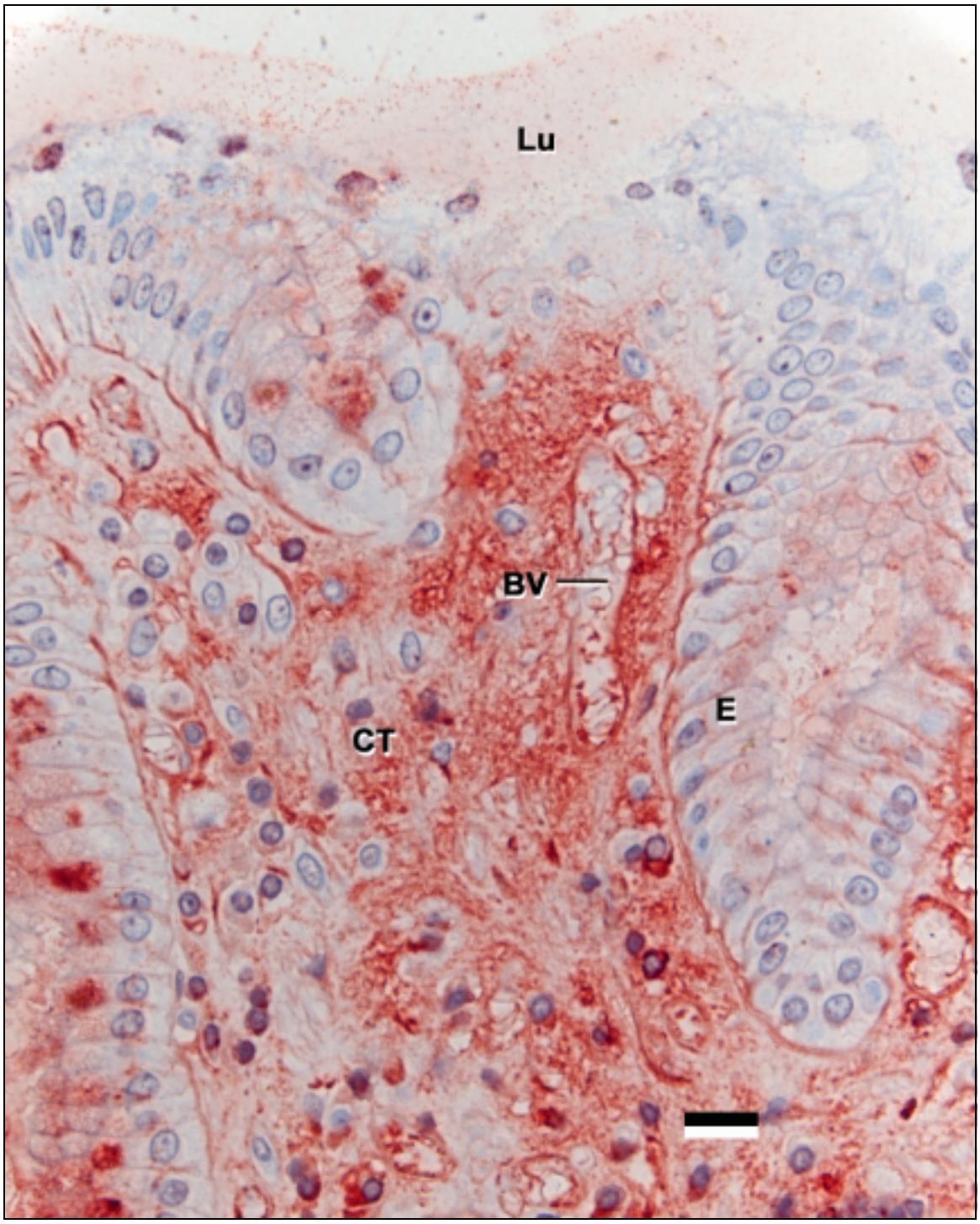

Figure 11. Interleukin 2. (Chromogen substrate).

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

Figure 12.

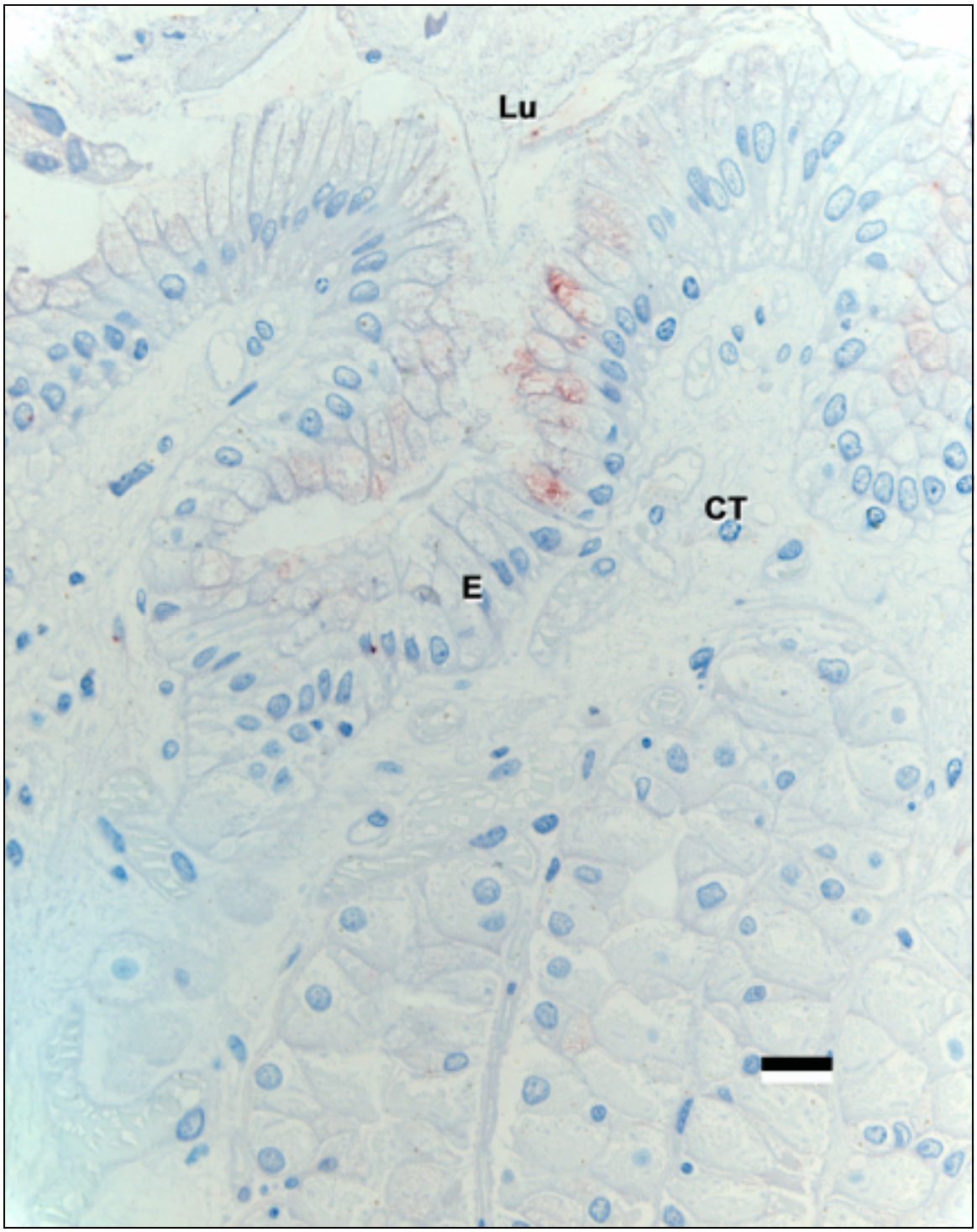

Figure 12. Interferon δ. (Chromogen substrate).

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

Figure 13.

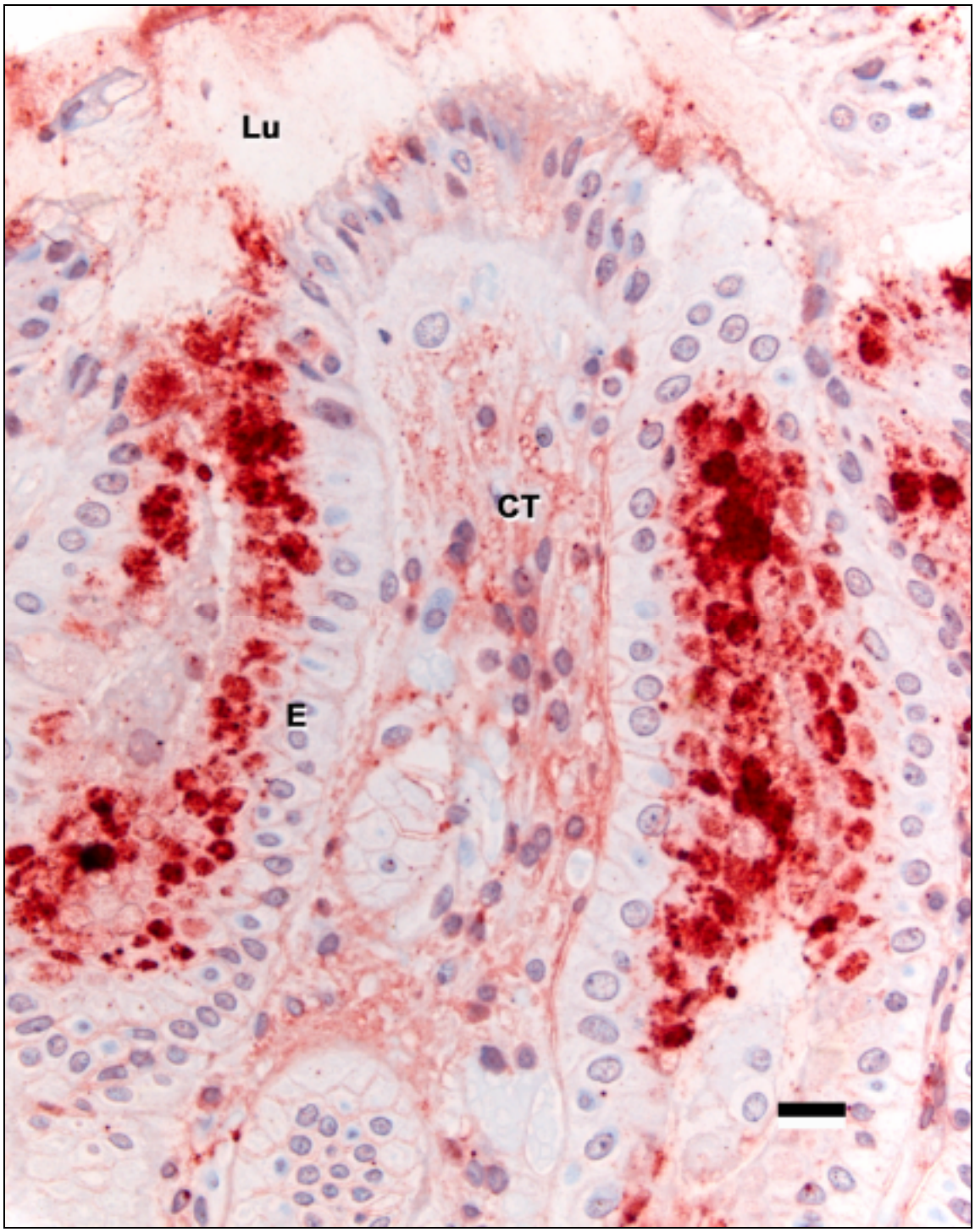

Figure 13. Interferon δ. (Chromogen substrate).

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

Figure 14.

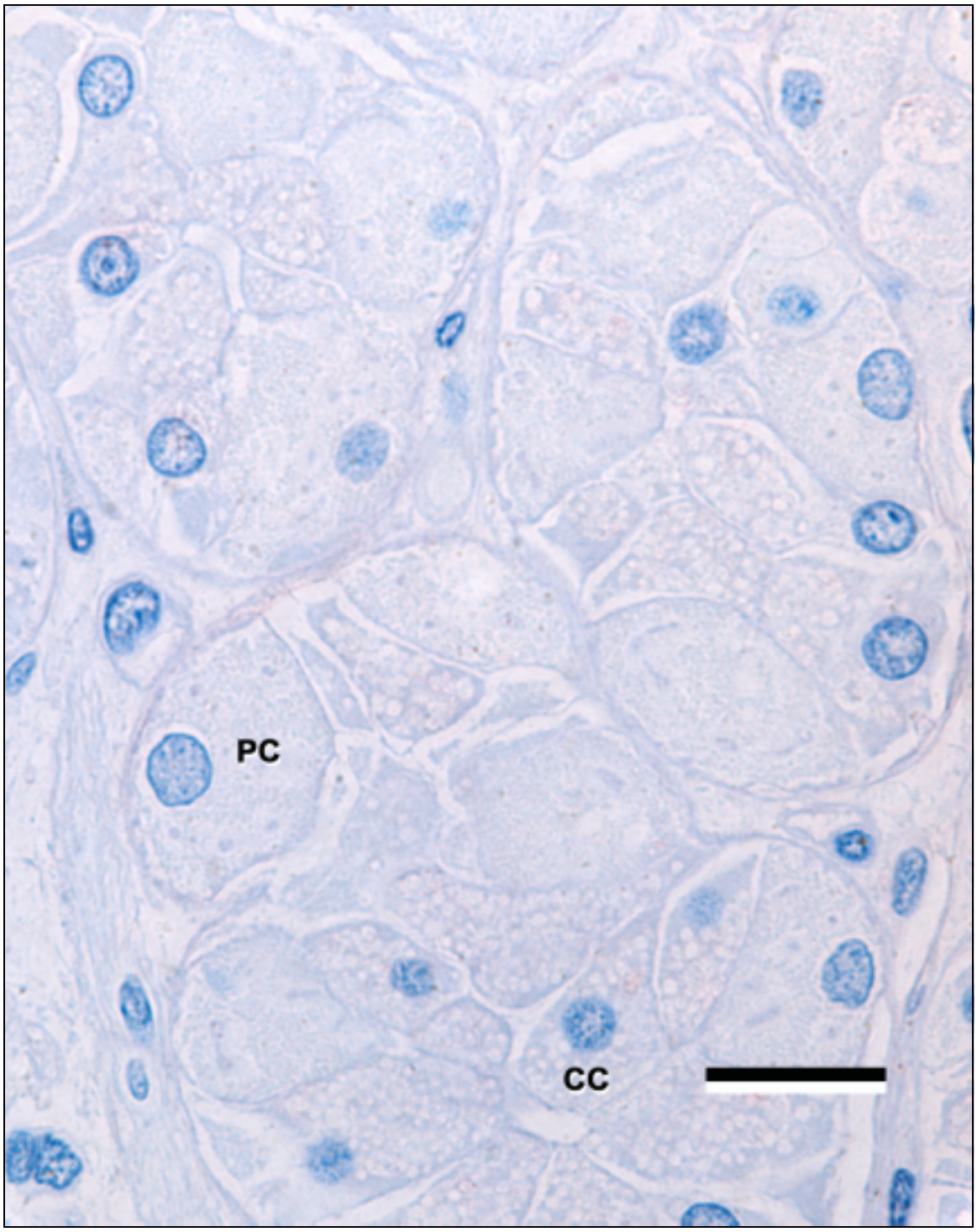

Figure 14. Interferon δ. (Chromogen substrate).

Mucosal biopsy from the body of the normal stomach. The chief cells (CC) and parietal cells (PC) are shown. Scale bar is 20µm.

Figure 15.

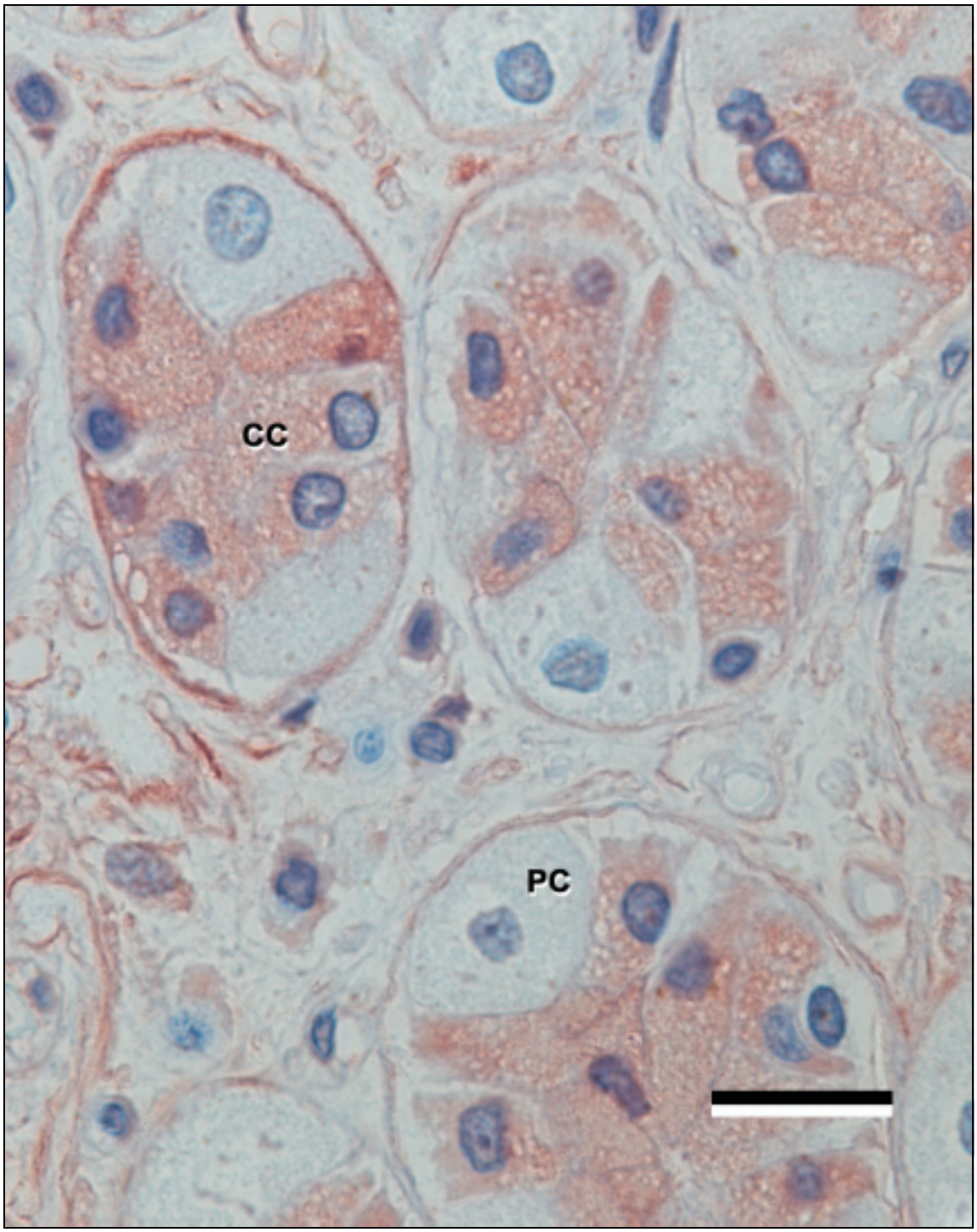

Figure 15. Interferon δ. (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. The chief cells (CC) and parietal cells (PC) are shown. Scale bar is 20µm.

Figure 16.

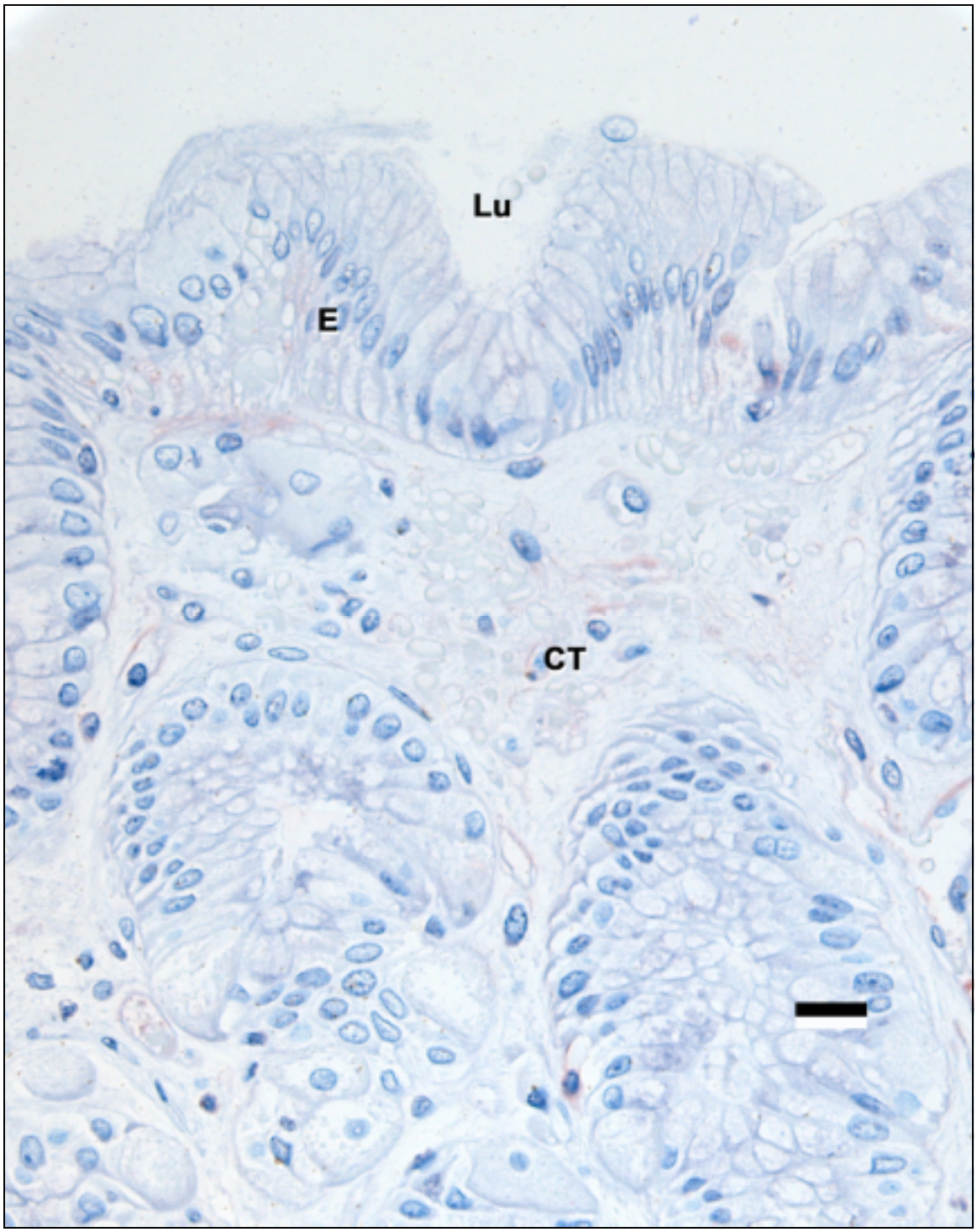

Figure 16. Interleukin 4 (3H4). (Chromogen substrate).

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 17.

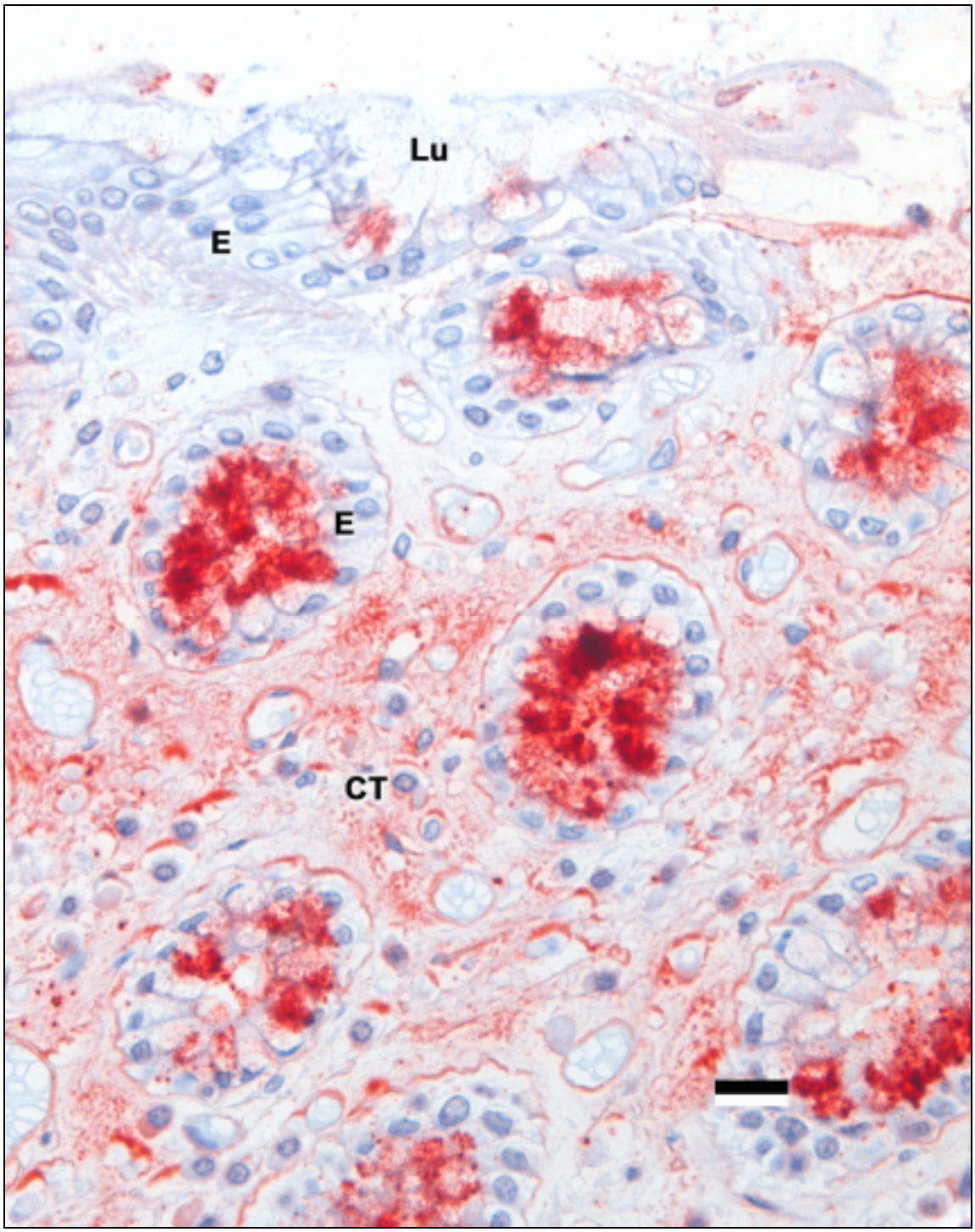

Figure 17. Interleukin 4 (3H4). (Chromogen substrate).

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 18.

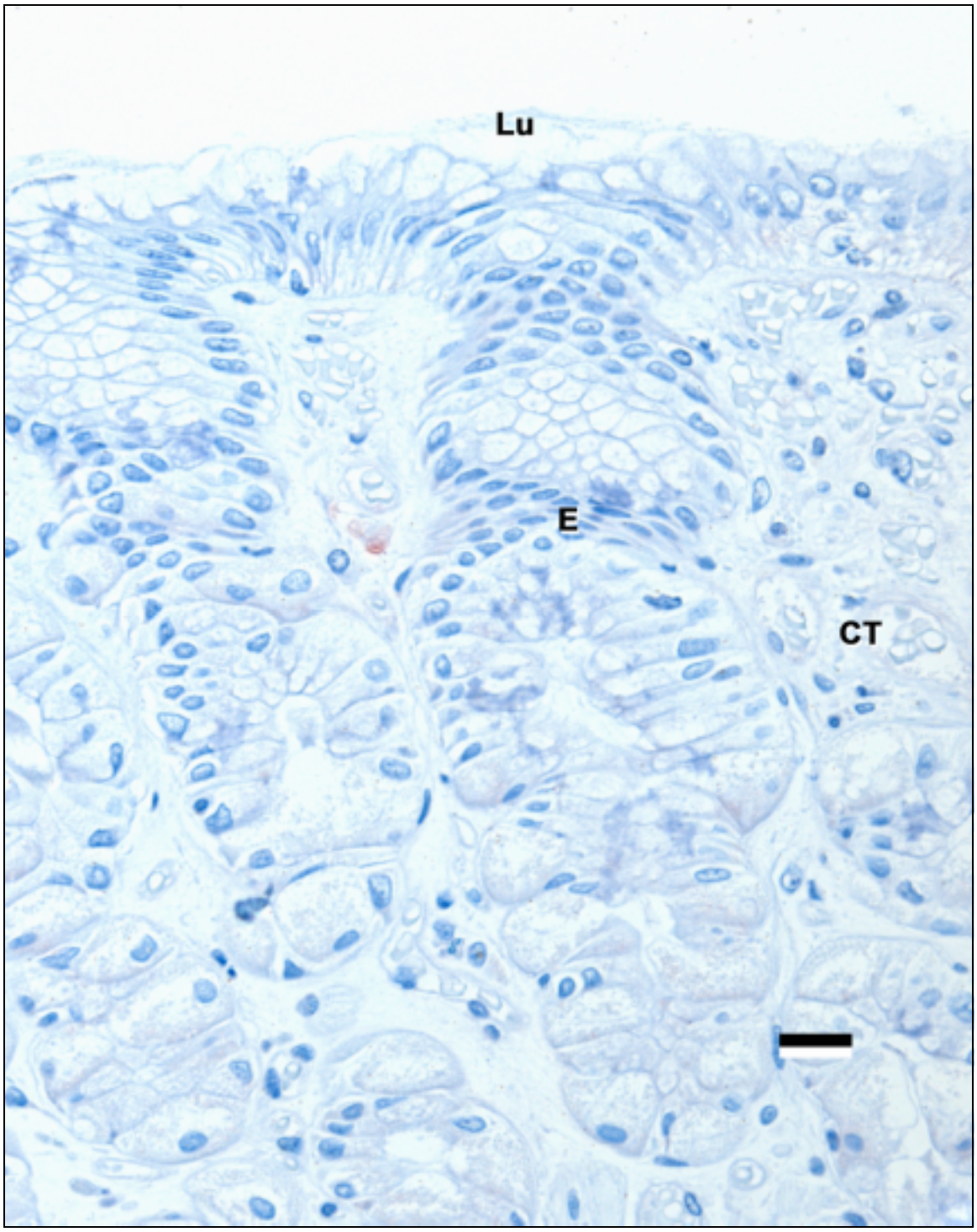

Figure 18. Tumour Necrosis Factor α. (Chromogen substrate).

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.

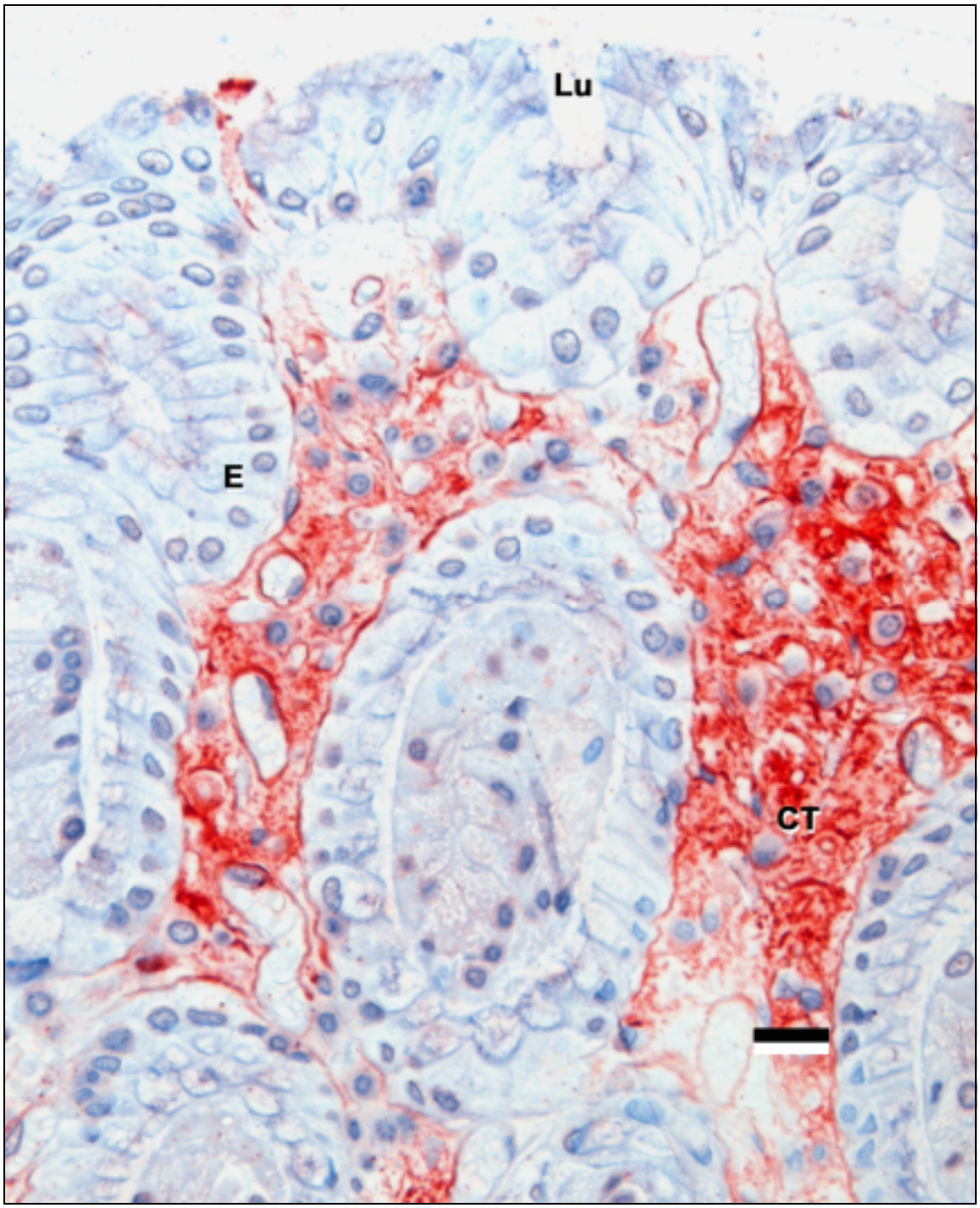

Figure 19. Tumour Necrosis Factor α. (Chromogen substrate).

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

Figure 20.

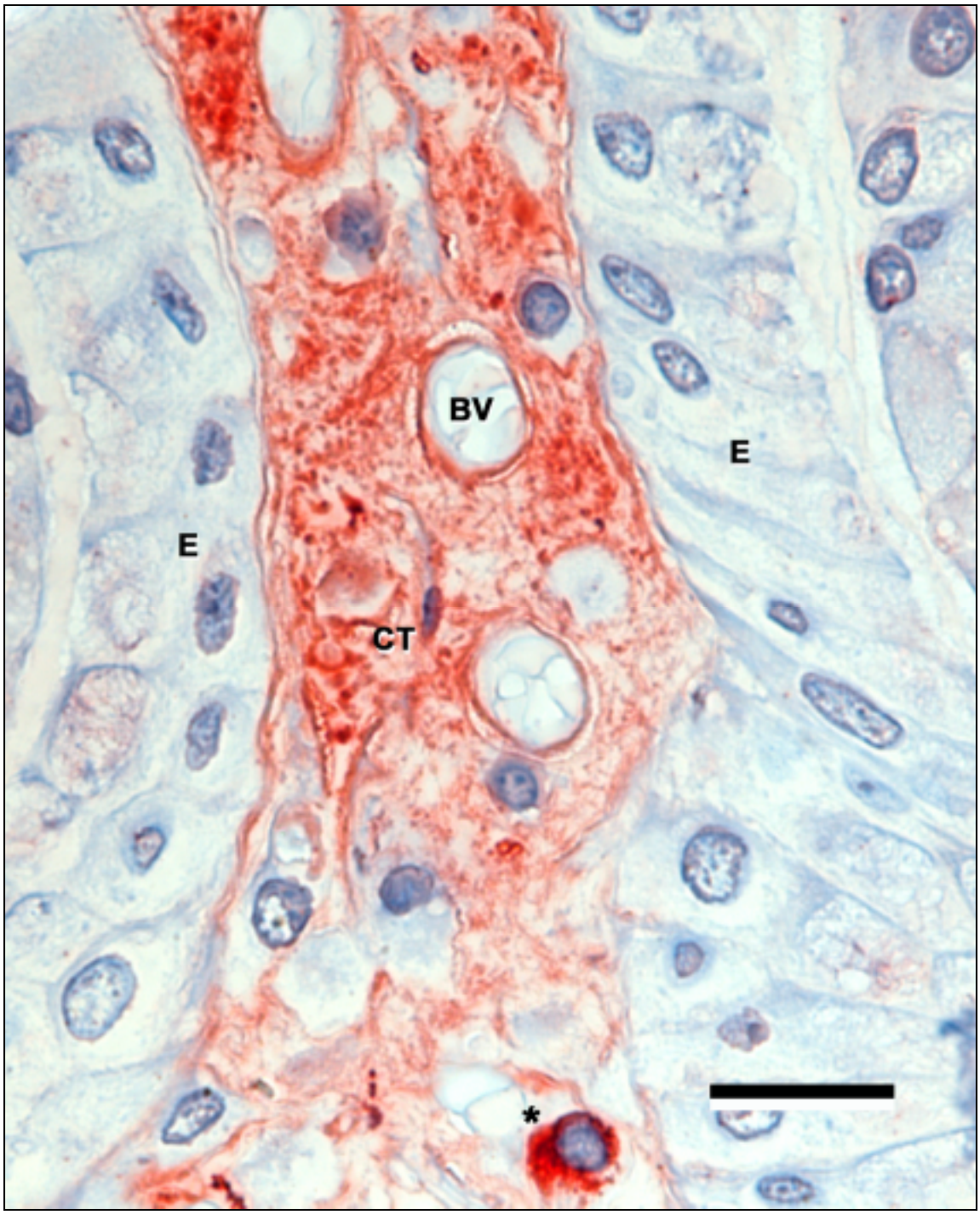

Figure 20. Tumour Necrosis Factor α. (Chromogen substrate).

Mucosal biopsy from the body of a stomach infected with *Helicobacter pylori*. The gastric epithelium (E), mucosal blood vessel (BV) and subepithelial connective tissue (CT) are shown. A connective tissue cell with cytoplasmic TNFα (∗) is shown. Scale bar is 20μm.