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Electron Microscopic, Ultracytochemical and Immunohistological Observations in Crohn's Disease of the Ileum and Colon* **

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Summary. Immunohisto- and ultracytochemical studies were carried out on surgical and biopsy specimens from 27 patients suffering from Crohn's disease of the ileum or colon. Control specimens were obtained from 16 patients with nonspecific proctitis or neoplastic disorders of the caecum or rectum. Our results suggest that the initial lesions in Crohn's disease are associated with a typical humoral immune response. In non-ulcerated mucosa a uniform increase of IgA-, IgG- and IgM-cells was found (numbers of IgA-cells: IgG-cells ~14.4), whereas disproportional increases of IgG- and IgE-cells were observed in ulcerated mucosa (IgA:IgG ~0.7). The IgE-cell multiplication in ulcerated areas suggests the possibility of local hypersensitivity reactions. Macrophages and granulocytes contained IgG, which was also demonstrated in multinucleated giant cells. The granulomas contained extracellular IgG, acid phosphatase and peroxidase. The finding of potentially harmful extracellular lysosomal enzymes may be of pathogenetic significance in view of the hypothesis of Weissmann (1964). Micro-ulcerations of the dome epithelium of hyperplastic Peyer's patches were seen by electron microscopy a finding which can be interpreted as an early lesion through which luminal antigens gain uncontrolled access to Peyer's patches. This could lead to (1.) overstimulation of the local immune system, (2.) disturbance of local immune homeostasis, (3.) imbalanced Ig-production with disproportional increases in IgG and IgE. We were not able to detect Clq or C3 bound to epithelial or vascular basement membranes, and no electron dense deposits were found. Viral particles or bacteria in any of the specimens were not demonstrated by electron microscopy. The type of immune response in Crohn's disease and its pathogenetic significance with remain unclear until more is known about the specificity of the locally produced antibodies.

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** Dedicated to Professor Dr. G. Seifert on the occasion of his 60th birthday

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Introduction

Recently, a transmissible, cytopathogenic agent has been found in filtered homogenates of small intestine from some patients with Crohn's disease. The size of this agent is that of a virus (Aronson et al. 1975; Whorwell et al. 1976; Gitnick et al. 1976 and 1979; Donnelly et al. 1977; Cave et al. 1978), or perhaps of a cell wall-defective bacterium (Orr 1975; Parent and Mitchell 1978; Burnham et al. 1978). The aetiologic importance of this agent, however, is unknown (Review: Sachar et al. 1980) and the pathogenesis of Crohn's disease is still uncertain. A role for faulty phagocytosis by granulocytes and macrophages and for a primary immunodeficiency have both been postulated but not proven (Segal and Loewi 1976; Ward 1977; Auer et al. 1978a, b).

There is an increasing acceptance that immunological mechanisms are implicated in the pathogenesis of Crohn's disease. This is derived from clinical observations and from findings of altered reactivity of the systemic and gut-associated immune apparatus. Immunohistological studies have yielded controversial results (Strickland et al. 1975; Brown et al. 1975; Green and Fox 1975; Lloyd et al. 1975; Goodman et al. 1976; Baklien and Brandtzaeg 1975 and 1976; Meuwissen et al. 1976; Meijer et al. 1979; O'Donoghue and Kumar 1979; Chiba et al. 1979; Otto et al. 1980). We have studied the local inflammatory process and correlated our findings with disease activity. Immunohistological and ultracytochemical investigations were done on the same material, looking for indications of local involvement of the complement system, at the degree and patterns of the local B-cell response and the functional activities of macrophages, granulocytes, and granuloma associated cells. This was an attempt to characterise the local immune phenomena connected with the inflammatory response.

Material and Methods

Surgical and biopsy specimens from 27 patients (mean age 31.2 years, range 13–37, 16 females and 11 males) suffering from Crohn's disease were studied. The disease was confined to the terminal ileum in seven, ileocolitis was diagnosed in 11, and the diagnosis of Crohn's disease of the colon was established in nine cases, according to criteria described by Korelitz et al. (1972), Morson (1971), and by Whitehead (1973). Colono- and rectoscopically obtained biopsies of a selected group of 63 young patients with ulcerative colitis (ranging in age from 16 to 34 years) were also studied (Gebbers and Otto 1977 and 1978). Finally, 16 biopsies from patients with nonspecific proctitis, colonic adenomas and tissues from uninvolved mucosa of resection specimens with colorectal cancer served as controls.

We used the unlabelled antibody enzyme technique (indirect immunoperoxidase (PAP) method) as well as the indirect and direct immunofluorescence method on paraffin wax embedded sections of Bouin- or sublimate-formaldehyde-fixed tissue. IgA, IgG, IgM, IgE, lysozyme, secretory component, Clq and C3 were demonstrated in two laboratories (Lucerne and Hamburg) on the same material.

IgA-, IgG-, IgM-antisera were obtained from Nordic Immunological Laboratories b.v., Tilburg, Netherlands, anti-Clq and anti-C3 from Behringwerke AG, Marburg/Lahn, and anti-IgE, anti-

secretory component, anti-lysozyme and the PAP immune complex (made by Sternberger's method (1970)) were obtained from Dakopatts A/S, Copenhagen.

The class pattern of immunoglobulin containing cells (IgA, IgG, IgM) were determined quantitatively. For quantification of the plasma cell reaction, systematic counts of labelled immunocytes were based on the method described by Brandtzaeg et al. (1974) and Baklien (1977) in individually defined "mucosal tissue units" constituting a 6 µm thick and 500 µm wide block of tissue, including the mucosa at full height from the muscularis mucosae. Quantitative evaluations of immunoglobulin-containing cells in the deeper layers of the bowel wall was done by using a modified method described by Skinner and Whitehead (1974).

Parallel electron microscopy and ultracytochemistry were performed for the demonstration of acid phosphatase and peroxidase. Details of the method together with appropriate controls have been described elsewhere (Gebbers and Otto 1978).

Results

The degree of the inflammatory changes varies greatly within individual intestines; this makes difficult the correlation of ultrastructural findings with the overall activity of the disease.

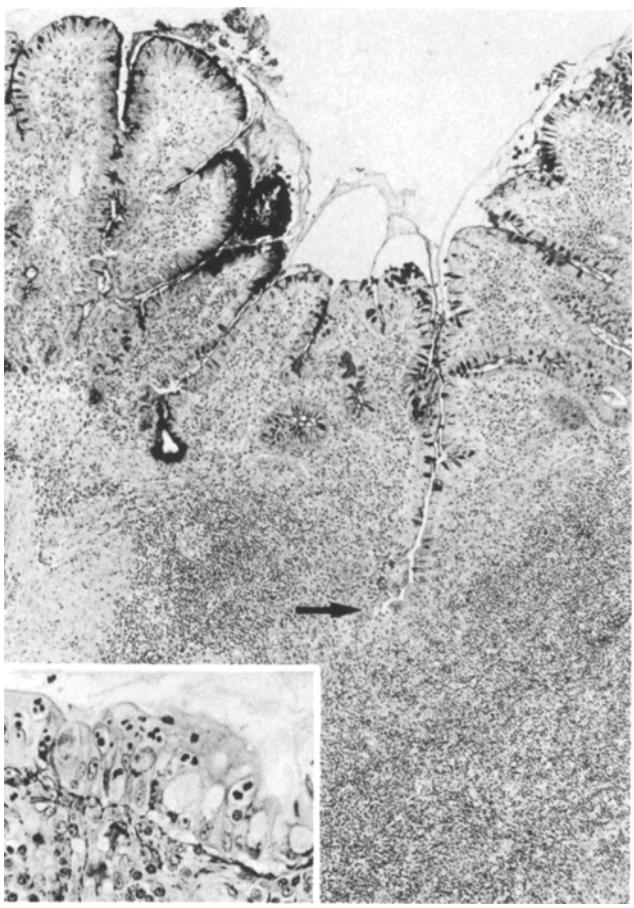

Fig. 1. "Early" Crohn's disease of the terminal ileum: Irregular shaped villi with hyperplasia of lymphoid tissue of the lamina propria mucosae. Small fissuring ulceration in the basal part of the mucous membrane (*arrow*). PAS, ×80. *Inset:* Surface epithelium with infiltrated granulocytes and lymphocytes. Gomori, ×350. (J. No. 17583/80; Institute of Pathology, Hamburg University)

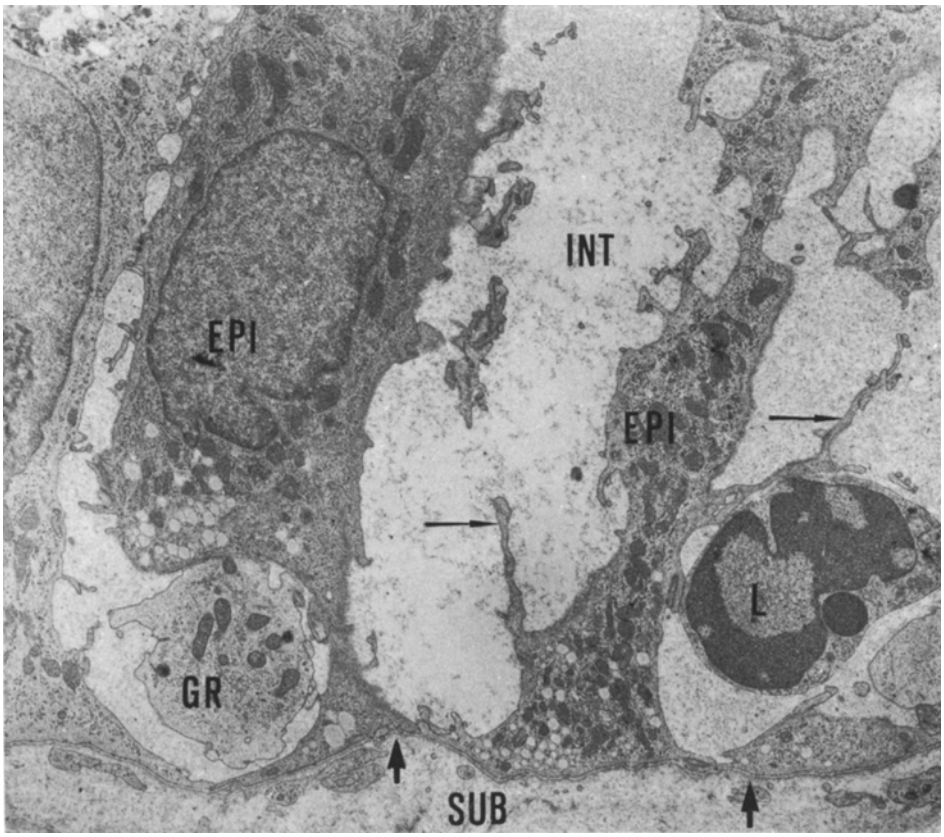

Fig. 2. Surface epithelium (*EPI*) in "early" Crohn's disease (same case as in Fig. 1) with enlarged intercellular spaces (*INT*) and infiltrated granulocytes (*GR*) and lymphocytes (*L*). Delicate cell projections are remnants of the interdigitations of the epithelial cells (*long arrows*). Epithelial basement membrane (*short arrows*). Subepithelial oedema (*SUB*). Uranyl acetate and lead citrate, ×6,000

The earliest macroscopical changes of Crohn's disease of the small intestine consist of a marked hyperplasia of Peyer's patches and aphthous ulcers. Microscopic examination shows that the aphthous ulcers are either ulcerating lymphoid follicles or a focal accumulation of lymphocytes in the basal part of the mucous membrane (Fig. 1). They may contain granulomas and multinucleated giant cells of the Langhans or foreign body type (see Fig. 9).

Systematic examination of the intestinal epithelium away from ulcerated areas show a marked leukopedesis at the surface epithelium (Fig. 1, Inset and Fig. 2). Granulocytes (mostly neutrophilic, some eosinophils) and lymphocytes are lying within the epithelium and form microabscesses.

At the site of this infiltration destruction of the basement membrane and of the periepithelial fibroblast sheath occurs (Fig. 2). The granulocytes are situated within the oedematous, dilated interepithelial spaces. These granulocytes are often seen in a state of degranulation. The release of their lysosomal enzymes into the interstitial tissues during degranulation is demonstrated in Fig. 3. This

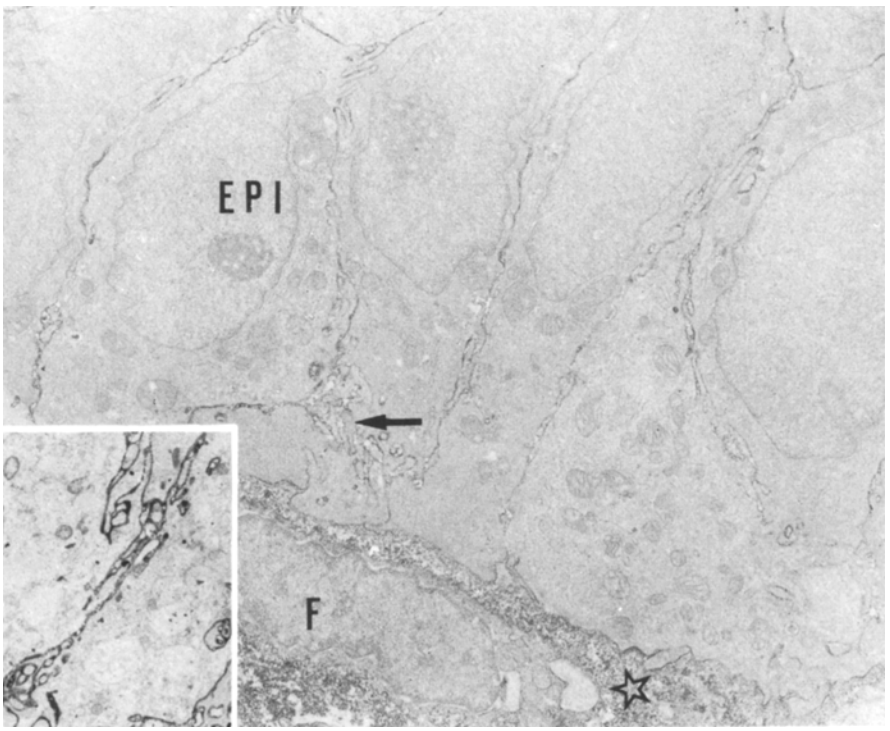

Fig. 3. Ultracytochemical demonstration of peroxidase in the epithelial (*EPI*) interstice (*arrow*) and in the subepithelial layer (*asterisk*). Periepithelial fibroblast (*F*). Same case as in Fig. 1. Unstained, ×4,500. Inset: ×11,000

finding is not specific for Crohn's disease, since similar observations are described in ulcerative colitis (Gebbers and Otto 1978). Of course, one would expect the occurrence of extracellular lysosomal enzymes in chronic inflammatory processes from theoretical conclusions based on *in vitro* findings (Weissmann et al. 1980) but this has not been previously demonstrated in situ.

Epithelial defects and microulceration are often seen at the dome of Peyer's patches (compare: Otto et al. 1980; Gebbers and Otto 1980). In addition to small lymphocytes, lymphoid cells with cerebriform nuclei and plasmacytic cells are frequently seen in the vicinity of the so-called microfold cells, described by Owen (1977).

In the active state of the disease the non-ulcerated area shows massive oedema of the submucosal layer with lymphatic dilatation (Fig. 4). The oedematous submucosa contains only a small number of inflammatory cells, preponderantly mast cells, neutrophilic granulocytes and some macrophages. Non-degranulated and degranulated mast cells are present in all specimens in the acute state of the disease. Numerous mast cells, both non-degranulated and degranulated and few granulocytes are preferentially located in the various oedematous muscle layers a similar cellular infiltrate is seen adjacent to and within the autonomic nervous system elements of the gut. In the deeper layers of the

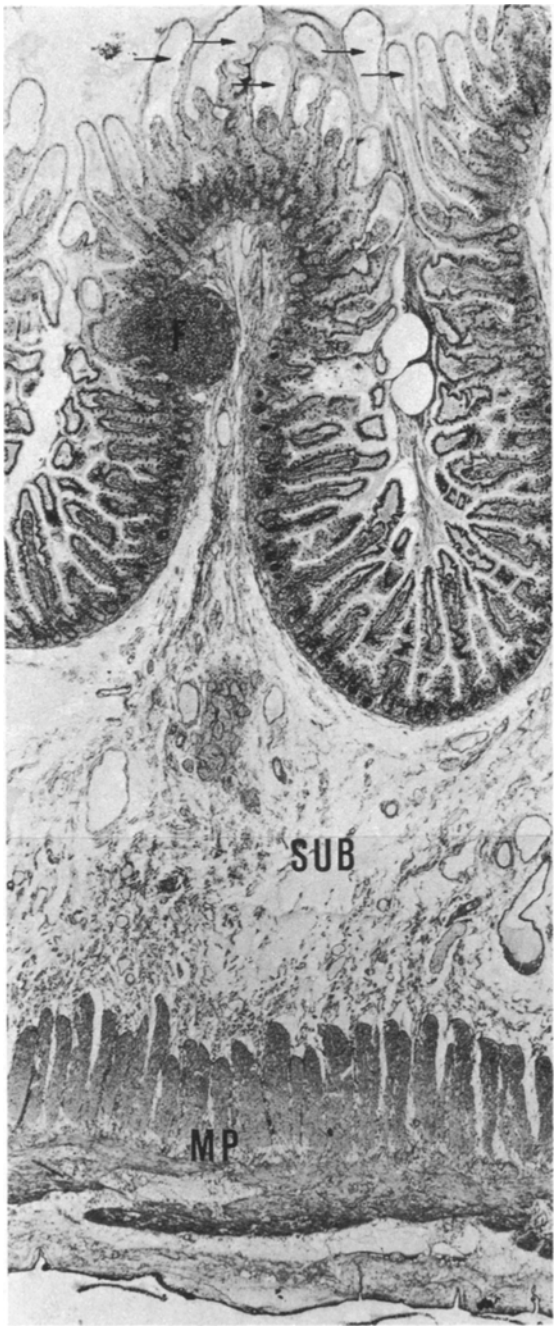

Fig. 4. "Early" Crohn's disease of the terminal ileum. Massive edema of the submucosal layer (*SUB*) with lymphatic dilatation. Note the edematous dilatation of Gruenhagen-Mingazzini's spaces (*arrows*). Lymphoid follicle (*F*). Muscularis propria (*MP*). Gomori, ×60 (J. No. 14509/80; Institute of Pathology, Hamburg University)

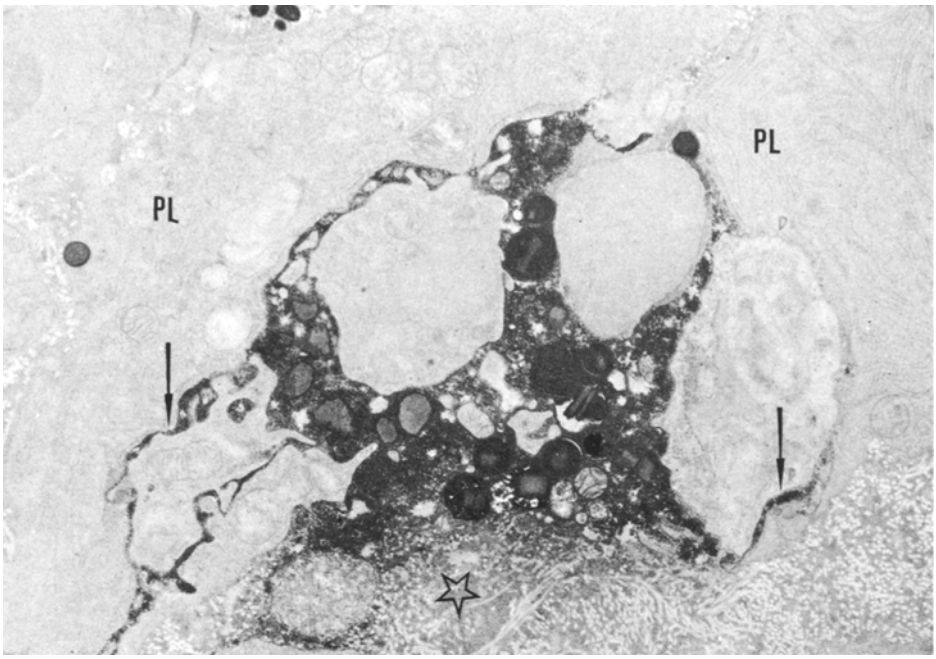

Fig. 5. Ultracytochemical demonstration of peroxidase. Degranulated granulocyte releasing its lysosomal peroxidase into the interstice (*arrows*). Note the stained breakdown products of collagenous fibres (*asterisk*). Plasma cells (*PL*). Unstained, × 11,000 (J. No. 01/76; Institute of Pathology, Hamburg University)

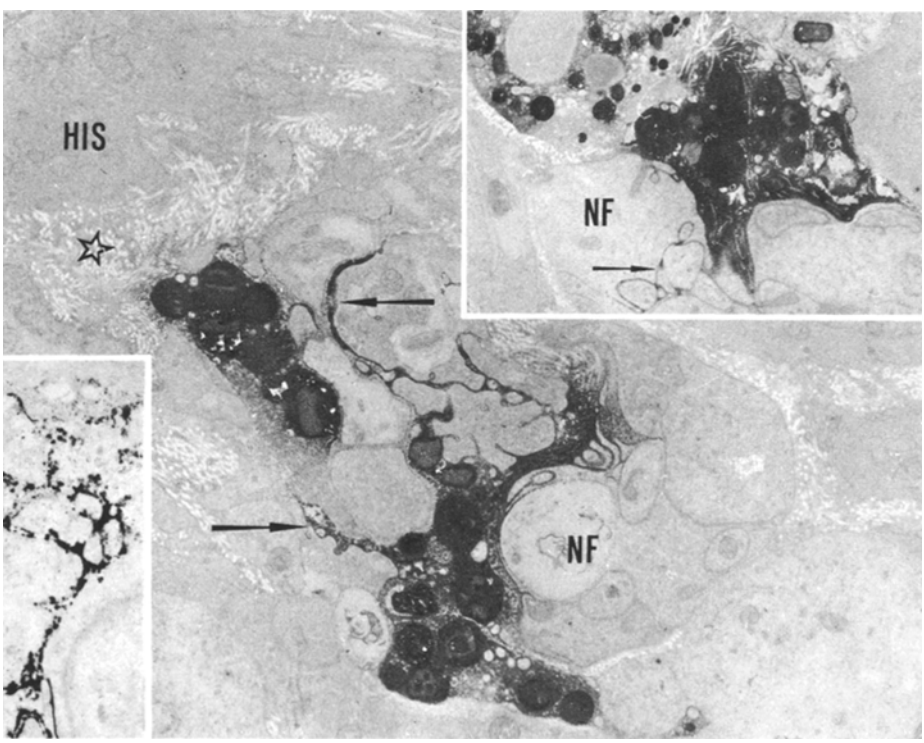

Fig. 6. Ultracytochemical demonstration of peroxidase and acid phosphatase (*Inset, left below*). Degranulated granulocyte. Free deposits of peroxidase (*arrows*) and acid phosphatase are seen in the interstices of nerve fibres (*NF*) and Schwann's cells. Histiocytic cells (*HIS*). Collagenous fibres (*asterisk*). Unstained, × 11,000 (J. No. 014/75; Institute of Pathology, Hamburg University)

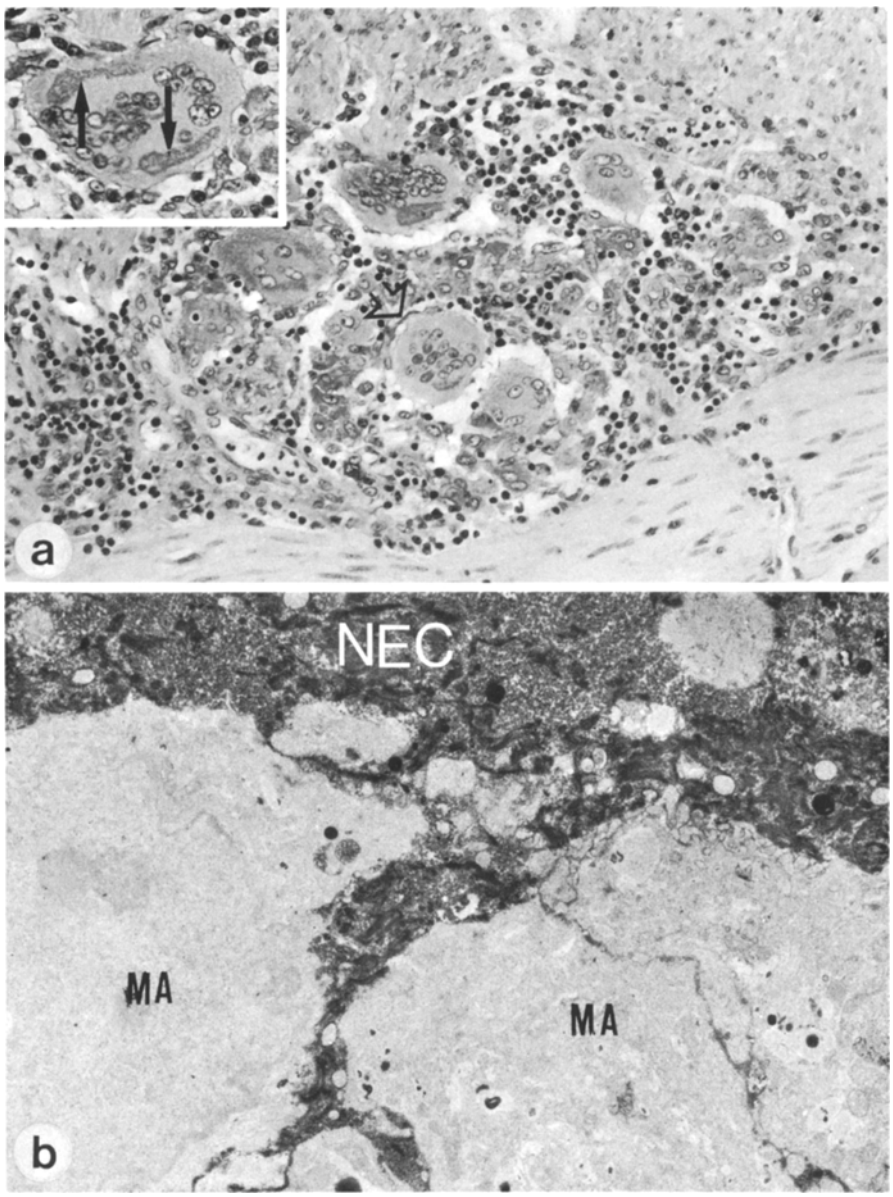

Fig. 7. a Photomicrograph of a granuloma in Crohn's disease with multinucleated giant cells and membrane-associated IgG (*broad arrow*). *Inset:* Intracellular IgG (*arrows*) in a giant cell. Immunoperoxidase-Diaminobenzidine, ×320. *Inset:* ×380 (J. No. 20369/79; Institute of Pathology, Hamburg University). **b** Ultracytochemical demonstration of peroxidase in the interstice between two macrophages (*MA*) and around necrotic material (*NEC*=core of the granuloma). Unstained, ×5,400 (same case as in Fig. 7a)

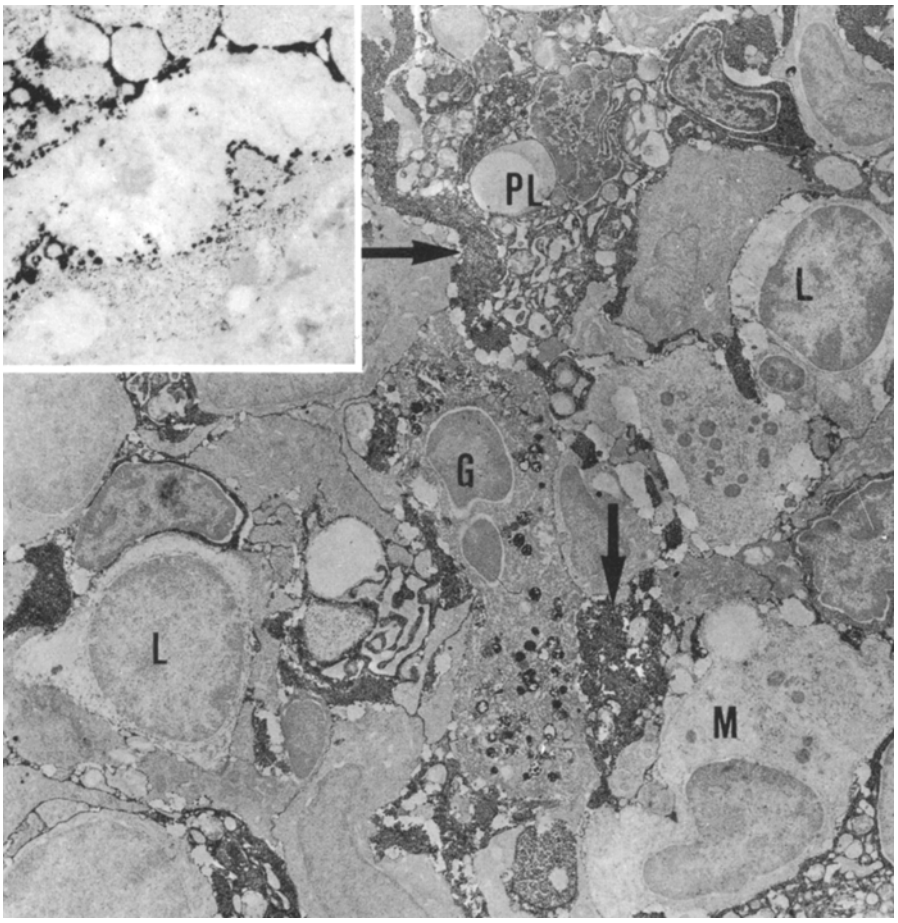

Fig. 8. Ultracytochemical demonstration of free deposits of peroxidase (*arrows*) and acid phosphatase (*Inset*) in a granuloma in Crohn's disease. Plasma cells (*PL*), granulocytes (*G*), lymphocytes (*L*), macrophages (*M*). Unstained, ×7,000. *Inset:* ×8,500 (J. No. 025/75; Institute of Pathology, Hamburg University)

bowel wall the granulocytes are also often seen in a state of degranulation (Figs. 5 and 6). These pictures show free deposits of peroxidase and acid phosphatase interstitially around nerve fibres and ganglia, Schwann cells, and adjacent to the breakdown products of collagen fibres.

The development of the heteromorphic "Crohn granulomas" is related to the degree of inflammatory changes. The core of the granulomas contains intercellular (free) IgG (see: Otto et al. 1980; Gebbers and Otto 1980), peroxidase and acid phosphatase (Figs. 7 and 8). Electron microscopic examination of the granulomas reveals them to be highly cellular, the cells closely packed with occasional protrusions and interdigitations. The granulomas are composed of epithelioid and multinucleated giant cells, small lymphocytes and their larger activated forms, monocytes, macrophages, neutrophilic and eosinophilic granulo-

Table 1. Distribution of the immunoglobulin-containing cells in a defined "mucosal tissue unit"[a] in controls ($n=11$) and patients with Crohn's disease ($n=15$) of the terminal ileum

Class distribution of Ig-cells	Normal controls	Crohn's disease: inflammatory activity		
		Light	Severe (non-ulcerated area)	Severe (ulcerated area)
IgA	43 (81%)	127 (79%)	341 (57%)	367 (36%)
IgM	7 (13%)	23 (14%)	67 (11%)	111 (11%)
IgG	3 (6%)	11 (7%)	187 (32%)	553 (53%)
Total	53	161	595	1031
IgA/IgG ratio	14.3	11.5	1.8	0.7

[a] For quantification of immunoglobulin-containing cells, systematic counts of labelled cells were based on the method described by Brandtzaeg et al. (1974) in individually defined "mucosal tissue units" constituting a 6 µm thick and 500 µm wide block of tissue, including the mucosa at full height from the muscularis mucosae (compare: Gebbers and Otto 1978)

Table 2. Distribution of the immunoglobulin-containing cells in a defined "mucosal tissue unit" in controls ($n=16$) and in patients with Crohn's disease ($n=12$) of the colon and in patients with ulcerative colitis ($n=21$)

Class distribution of Ig-cells	Normal controls	Crohn's disease Severely inflamed mucosa	Ulcerative colitis in acute states
IgA	95 (92%)	190 (50%)	213 (51%)
IgM	4 (4%)	27 (7%)	17 (4%)
IgG	5 (4%)	167 (43%)	190 (45%)
Total	114	384	420
IgA/IgG ratio	19	1.1	1.1

cytes, plasma cells and some mast cells. Membrane-associated and intracellular IgG is found in the multinucleated giant cells and in some granulocytes (see: Gebbers and Otto 1980). Lysozyme is demonstrable in many monocytes and macrophages but not in all granulocytes. Ultrastructurally, there is a conspicious positivity for extracellular peroxidase within the granulomas (Figs. 7 and 8). Since peroxidase is a marker enzyme for lysosomal enzymes the positivity is presumably related to the presence of acid phosphatase. Controversely, peroxidase is virtually absent from the macrophages, multinucleated giant cells and epithelioid cells which are associated with granulomas. In contrast to observations by Klockars et al. (1977) and Yamashita et al. (1978), no lysozyme was detected in the giant cells.

The early B-cell response is characterized by a balanced augmentation of the number of plasma cells with IgA, IgG and IgM (Table 1 and 2). This corresponds to a typical humoral immune response (Figs. 9 and 10). In the course of the disease and in direct relationship to the degree of inflammatory

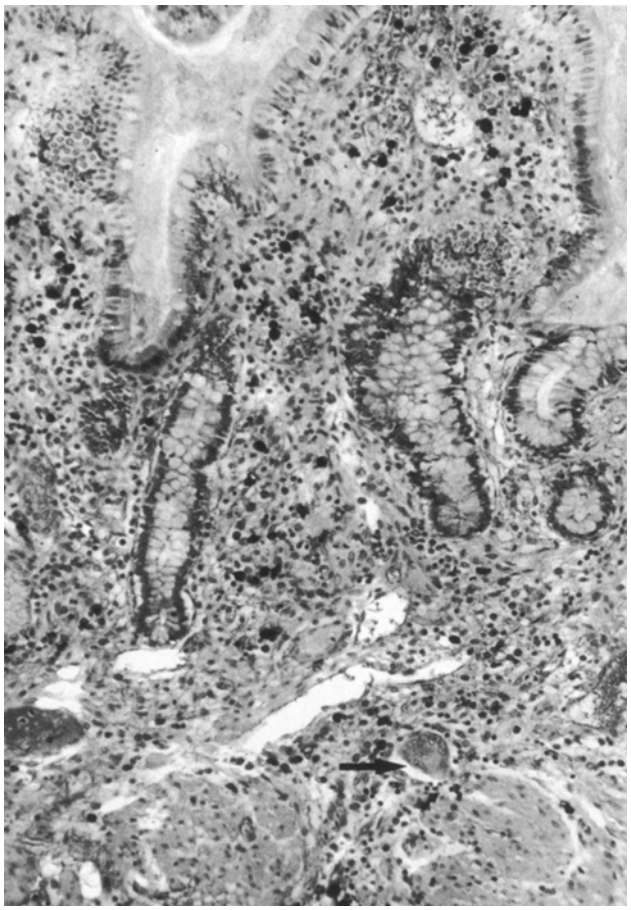

Fig. 9. Crohn's disease of the terminal ileum. Non-ulcerated area with specific demonstration of IgA-containing immunocytes (*black cells*). Irregular shaped villi. An IgA-negative giant cell in the basal part of the mucous membrane (*arrow*). Immunoperoxidase-Diaminobenzidine, ×210 (J. No. 15010/79; Institute of Pathology, Hamburg University)

changes, there is a progressive increase of IgG-positive cells (Fig. 11). The ratio of the cell-number IgA:IgG decreases from the normal value of 14 to 0.7 in severely inflamed mucosa. Similar changes are found in diseased segments of the large intestine, akin to those seen in ulcerative colitis (Gebbers and Otto 1978; Otto and Gebbers 1979). Signs of transmural inflammation are typically seen in advanced stages of Crohn's disease. In the deeper layers, i.e. the tunica muscularis and the serosa, we found increased proportions of IgG-positive plasma cells (Fig. 12). Here they constitute about 90% of all Ig-positive cells. There is a considerable accumulation of IgE-cells around ulcerations (Fig. 11c).

We were not able to detect Clq or C3 bound to epithelial or vascular basement membranes, and no electron dense deposits were found. No viral particles nor bacteria were demonstrated in any of the specimens by electron

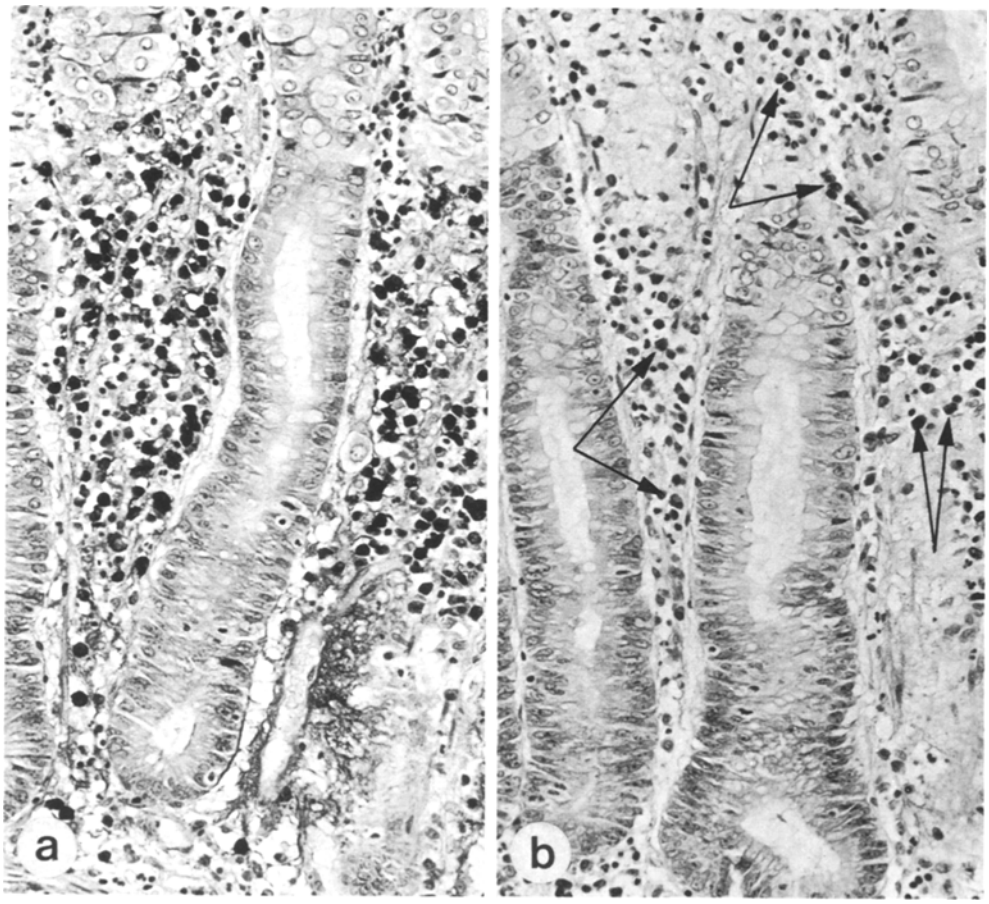

Fig. 10a, b. Crohn's disease of the colon. Non-ulcerated area with specific demonstration of IgA- (**a**) and IgG- (**b**) containing immunocytes (*black cells* and *arrows*). Note the proportional increase of the two types of plasma cells. Immunoperoxidase-Diaminobenzidine, ×320 (J. No. 8528/80; Institute of Pathology, Hamburg University)

microscopy. This does not rule out the presence or participation of an infectious agent in the disease.

Discussion

Several reports have indicated a possible role for immunological mechanisms in the pathogenesis of Crohn's disease (Review: Sachar et al. 1980). Immunohistological studies with different methods have yielded controversial results (e.g. Strickland et al. 1975; Brown et al. 1975; Baklien and Brandtzaeg 1975 and 1976; Meuwissen et al. 1976; Meijer et al. 1979; O'Donoghue and Kumar 1979). Whether T- or B-lymphocytes predominate in the inflammatory infiltrates of Crohn's disease remains a topic of discussion. Breucha and Riethmüller (1975) have found a relative increase of T-lymphocytes in cell suspensions prepared

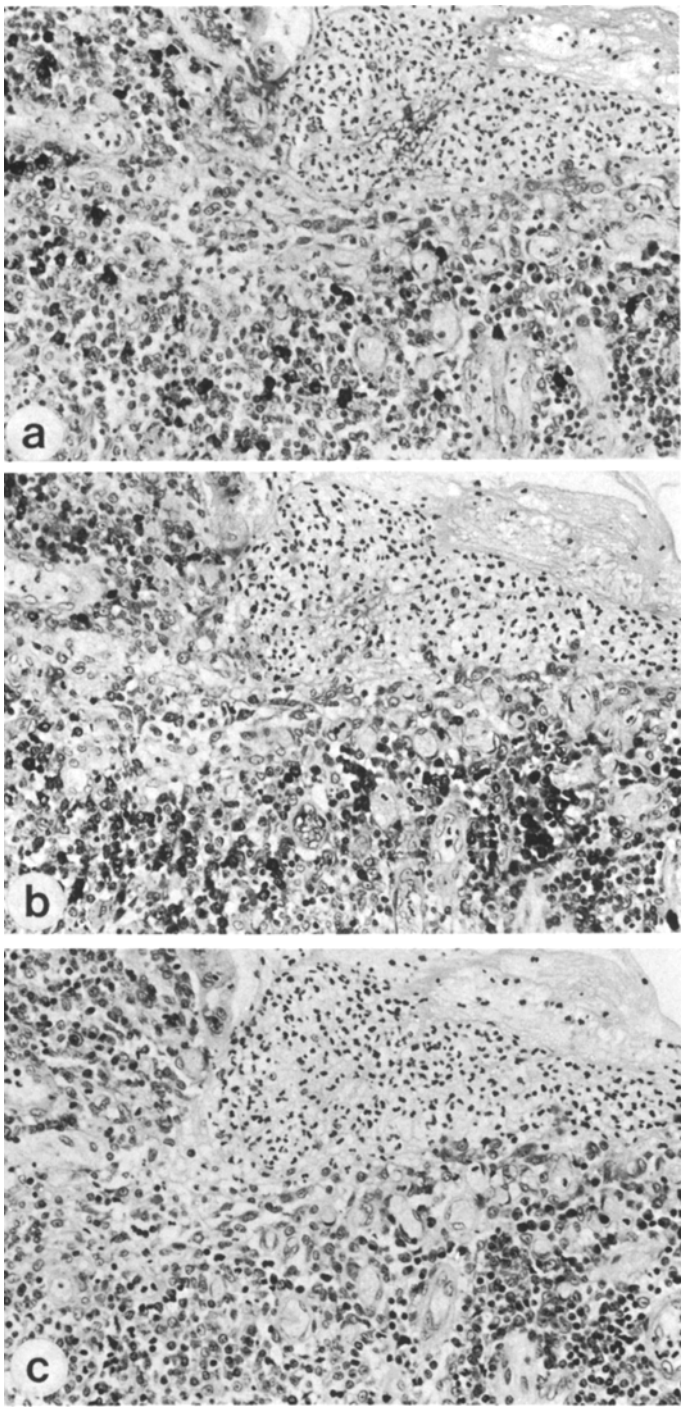

Fig. 11a–c. Crohn's disease of the terminal ileum. Ulcerated area with specific demonstration of IgA- (**a**), IgG- (**b**) and IgE- (**c**) containing immunocytes (*black cells*) on serial sections. Note the marked increase of IgG- and IgE-positive cells. Immunoperoxidase-Diaminobenzidine, ×360 (J. No. 15010/79; Institute of Pathology, Hamburg University)

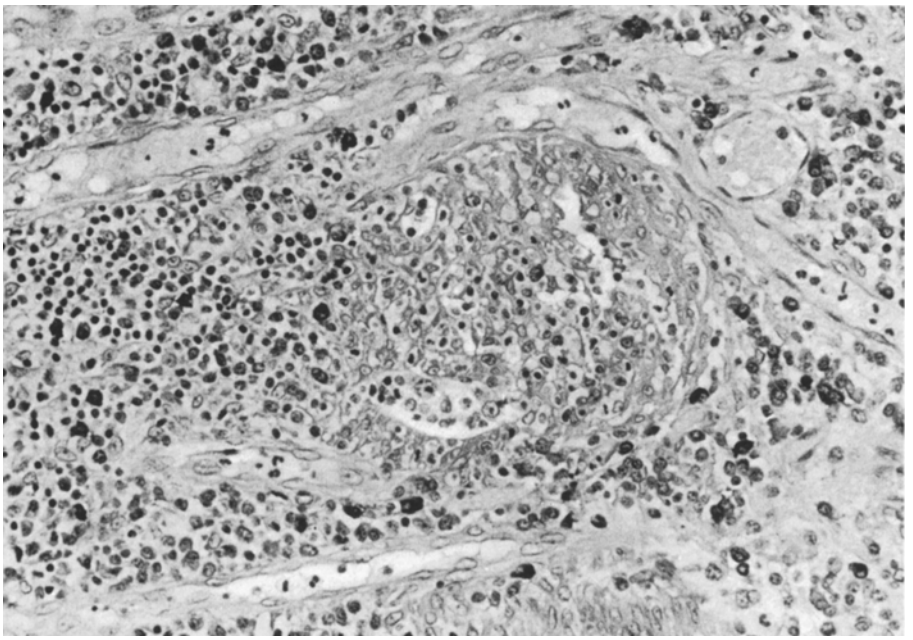

Fig. 12. Crohn's disease of the terminal ileum (same case as in Fig. 11). Specific demonstration of IgG-containing cells (*black cells*) in deeper layers of the affected bowel. Immunoperoxidase-Diaminobenzidine, ×360

from resected ileum affected by Crohn's disease. Strickland et al. (1975) and Meuwissen et al. (1976), using immunofluorescence and immunoperoxidase techniques, reported increasing numbers of T-lymphocytes in the deeper layers of resected colon and ileum in patients with Crohn's disease. On the basis of these immunohistological data the authors concluded that the inflammatory process in Crohn's disease involves mainly T-cellular immune reactions.

In contrast, the immunohistological observations by Baklien and Brandtzaeg (1975 and 1976) and by Meijer et al. (1979) indicate an intense B-cell response in the diseased tissue. However, since Strickland et al. (1975) and Meuwissen et al. (1976) found T-cells in close relation to B-lymphocytes, it is possible that T-cells play a modulating role on the B-cell response in Crohn's disease.

Our findings are in accordance with results by Baklien and Brandtzaeg (1975 and 1976) and Meijer et al. (1979). They indicate an intense B-cell response in the diseased tissue. In the beginning there is a proportional increase of IgA-, IgG- and IgM-cells. This initial balanced augmentation of the number of plasma cells corresponds to a typical humoral immune response. In the course of the disease and in direct relationship to the degree of the inflammatory changes there occurs a progressive increase of IgG-containing immunocytes. This points to a defect of autoregulation with disturbances of the local immune homeostasis. The imbalance of the immunoglobulin class pattern with IGg overproduction could be responsible for the formation of granulomas, because there is evidence that the tissue response which produces granulomas is sometimes

dependant on the presence of antigen-antibody complexes in antibody excess (Spector and Heesom 1969). Membrane associated and intracellular IgG in multinucleated giant cells and in some granulocytes and macrophages indicate the phagocytosis of IgG-containing immune complexes, which are probably soluble, since electron dense deposits or Clq and C3 were not detected. The simultaneous occurrence of coli-antigen (demonstrated by polyvalent anti-OK-coli sera, Behring Institute, Marburg/Lahn) (Gebbers, unpublished data) and IgG in granulocytes provides evidence for the phagocytosis of immune complexes. This process could induce the release of lysosomal enzymes by granulocytes and macrophages. The detection of extracellular lysosomal enzymes (peroxidase, acid phosphatase) could be of pathogenetic significance in connection with the hypothesis of Weissmann (1964 and 1980) who suggested that lysosomal enzymes damage native constituents of cells and/or of connective tissue. The breakdown products of this reaction might induce the production of antibodies. These act not only against the denatured constituents but cross-react with the antigenetically-related normal tissues. Thereby, an autoimmune process might be established.

Lloyd et al. (1975), using immunofluorescence techniques, reported decreased numbers of IgE-containing immunocytes, together with an almost total absence of stainable mast cells in affected areas of the bowel. But there is ultrastructural evidence of mast cell degranulation (Dvorak et al. 1978). This finding suggests a role for released mast cell mediators (histamine, platelet activation factor, slow-reacting substance of anaphylaxis, eosinophil chemotactic factor) in the pathogenesis of Crohn's disease. In contrast to findings by Lloyd et al. (1975), Green and Fox (1975) and Baklien and Brandtzaeg (1975, 1976) but consistant with observations by Brown et al. (1975) and O'Donoghue and Kumar (1979), we found that the number of IgE-positive plasma cells is increased in ulcerated and severely inflamed mucosa.

It seems likely that a primary defect of the secretory immunoglobulin system cannot explain the development of the disease; definite conclusions need further characterization of the quality of the local IgA response. In the course of the disease and related to the degree of inflammatory changes, the increase of IgG- and IgE-cells indicate an ineffective IgA response, which allows ingress of antigens and the development of IgG- and IgE-reactions (Taylor et al. 1973). The type of local immune reaction and its pathogenetic significance remains unclear, but the IgG- and IgE-multiplications in ulcerated areas point to the possible induction of any of the various hypersensitivity reactions.

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