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In situ identification of immune competent cells in gastrointestinal mucosa: an evaluation by immunoelectronmicroscopy

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Summary. The *in situ* identification of lymphocyte subpopulations by means of immunopathological techniques using specific monoclonal antibodies provides a tool for the study of the gastrointestinal-associated lymphoid tissue (GALT) in health and disease. In this field, monoclonal antibodies have been applied previously using light microscopy and either immunofluorescence or immunoperoxidase; however, these techniques are not sensitive enough to allow precise evaluation of localization of labelling. We describe an immunoelectronmicroscopic method, which defines labelling specificity, since it allows the identification of cells by immunophenotype labelling and ultrastructural markers simultaneously. This in turn allows a better evaluation of the labelled cells and of the relationship between labelled and unlabelled cells.

The main features of the method are the use of fresh tissue samples, fixing in paraformaldehyde $CaCl_2$, and the coupling of the immune reaction to an amplification system (avidin-biotin-peroxidase complex). The technique yields a good preservation of cellular ultrastructure, together with a strong and specific immunolabelling.

Our results confirm the high specificity of monoclonal antibodies when applied to immunopathology techniques. We confirm the pattern of distribution of various lymphocyte subsets in the jejunal mucosa described by other authors by light microscopy.

Key words: Immunoelectronmicroscopy – Lymphocytes – Monoclonal antibodies – Jejunum

Introduction

The widespread distribution of immunocompetent cells along the gastrointestinal tract and the increasing recognition of their functional importance, has led many authors to investigate the gastrointestinal associated lymphoid

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tissue (GALT) and its changes in the course of digestive diseases (Ferguson 1977; Ferguson and Mowat 1980; Mowat and Ferguson 1981; Ferguson and Strobel 1983b).

The available data on this matter, however, are far from conclusive; this is probably related to differences in study designs and to technical problems. In fact, currently available methods to study gastrointestinal immune cells require the isolation of such cells from tissue samples. The isolation procedure itself may be a source of error (Bland et al. 1979; Chiba et al. 1981; Selby et al. 1984), since the efficiency of extraction from tissue may be different for different cell types and cells with short life-span may not survive the time-consuming extraction procedures. Moreover, methods devised to study peripheral blood mononuclear cells might require modifications for the study of tissue-derived lymphoid cells (Ferguson 1977).

The recent development of monoclonal antibodies which specifically identify cell membrane phenotypes could make a direct evaluation of inflammatory infiltrates possible. This has already been accomplished in the skin (Taylor et al. 1983), in the liver (Colucci et al. 1983) and in other tissues (Janossy et al. 1981).

In the study of the GALT, monoclonal antibodies have so far been applied only to light-microscopy, using either immunofluorescence or immunoperoxidase (Selby et al. 1981; Ljunghall et al. 1982; Selby et al. 1983). An immunoelectronmicroscopic (IEM) method could offer advantages over the above techniques, particularly in defining the labelling specificity, since it might allow the simultaneous identification of cells by immunophenotype and ultrastructural markers. This might lead to a better definition of the labelled cells and of the relationships between labelled and unlabelled cells.

The application of IEM techniques to tissue sections is technically very difficult, however, the main problem being to obtain good tissue preservation without losing cellular antigens. The present paper deals with the development of an IEM technique for the *in situ* identification of immune cell populations and subpopulations in the gastrointestinal mucosa. This method id suitable for application on routine suction and endoscopic biopsy specimens from all portions of the GI tract. Its main applications are in confirming the specificity of simpler immunopathology methods by detailed structural correlations, and in testing the specificity of newly developed monoclonal antibodies.

The preliminary results of the application of this method to jejunal biopsies from normal subjects are reported here.

Material and methods

Mucosal samples. Jejunal biopsies were performed, under fluoroscopic control, by a directable catheter (Medi-Tech), in five subjects undergoing evaluation for chronic diarrhoea. In all five patients jejunal histology was classified as normal. The final diagnosis for all patients was the irritable bowel syndrome.

Monoclonal antibodies. Anti-Leu 1 (Becton Dickinson) (Engleman et al. 1981; Martin et al. 1981) was used to detect T-lymphocytes. Suppressor-cytotoxic type T lymphocytes were identi-

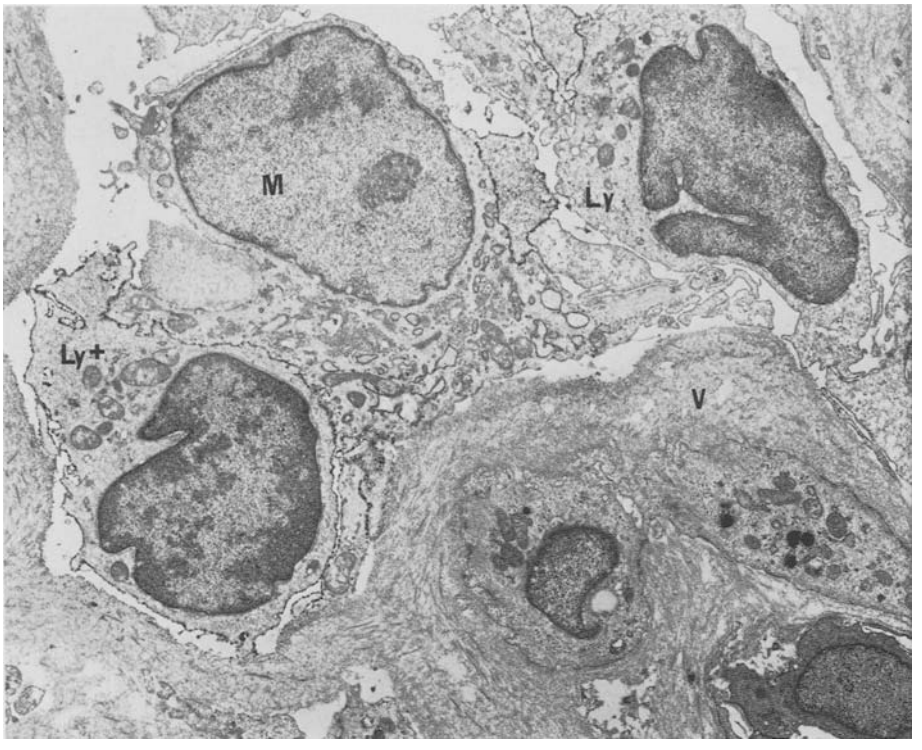

Fig. 1. Leu 1 positive lymphocyte (*Ly+*) in lamina propria, close to a macrophagic cell (*M*), a vascular structure and a Leu 1 negative lymphocyte (*Ly*) (× 3,000)

fied using OKT8 (Ortho) (Reinherz and Schlossman 1980) and Anti-Leu 2a (Becton Dickinson) (Engleman et al. 1982). OKT4 (Ortho) (Reinherz and Schlossman 1980) and Anti-Leu 3a (Becton Dickinson) (Engleman et al. 1982) were used to identify helper type T lymphocytes. Anti-Leu 7 (Becton Dickinson) was used to detect NK and K lymphocyte subpopulations (Abo and Balck 1981).

Method. Preliminary experiments were carried out to identify the best combination of reagents for each step of the method. For fixation, 2% paraformaldehyde $CaCl_2$ Hanau et al. 1983) was compared with periodidelysine-paraformaldehyde (PLP) prepared according to McClean (1974) and with Glutaraldehyde 0.5–3% in PBS, pH 7.2. For immunolabelling the Avidin-Biotin-Peroxidase complex (ABC) was compared with other amplification systems, such as peroxidized avidin and free avidin followed by peroxidized biotin. In post-fixation, various incubation times (from 20 to 90 min) in Osmium Tetroxide were evaluated, with and without previous incubation in Karnovsky fixative (Karnovsky 1965).

From these experiments, the following procedure emerged as the best: jejunal mucosal samples were immediately fixed in 2% paraformaldehyde $CaCl_2$ for 15 min at room temperature, embedded in 7% agarose and cut into 40 μm sections using a tissue chopper (Sorvall).

Sections were again fixed for 30 min in 2% paraformaldehyde $CaCl_2$ at room temperature and rinsed in PBS 7% sucrose, 1% bovine serum albumen (BSA) for 30 min at +4° C. Specific monoclonal antibodies diluted in PBS 3% BSA were then added for 60 min at 4° C. Sections were rinsed in PBS 7% sucrose 1% BSA and incubated with biotinilated horse antimouse IgG diluted in PBS for 30 min at +4° C. After rinsing in PBS, sections were incubated with avidin-biotin-peroxidase complex (ABC kit) for 60 min at +4° C. Reactions were then revealed by incubating sections in tris HCl buffer 0.05 M, pH 7.6 with diaminobenzidine 0.05% and H_2O_2. 0.1% for 10 min at room temperature. Sections were fixed in Karnowsky fixative for 30 min, rinsed in Tris-HCl, then fixed again in osmium-tetroxide for 60 min. After dehydration,

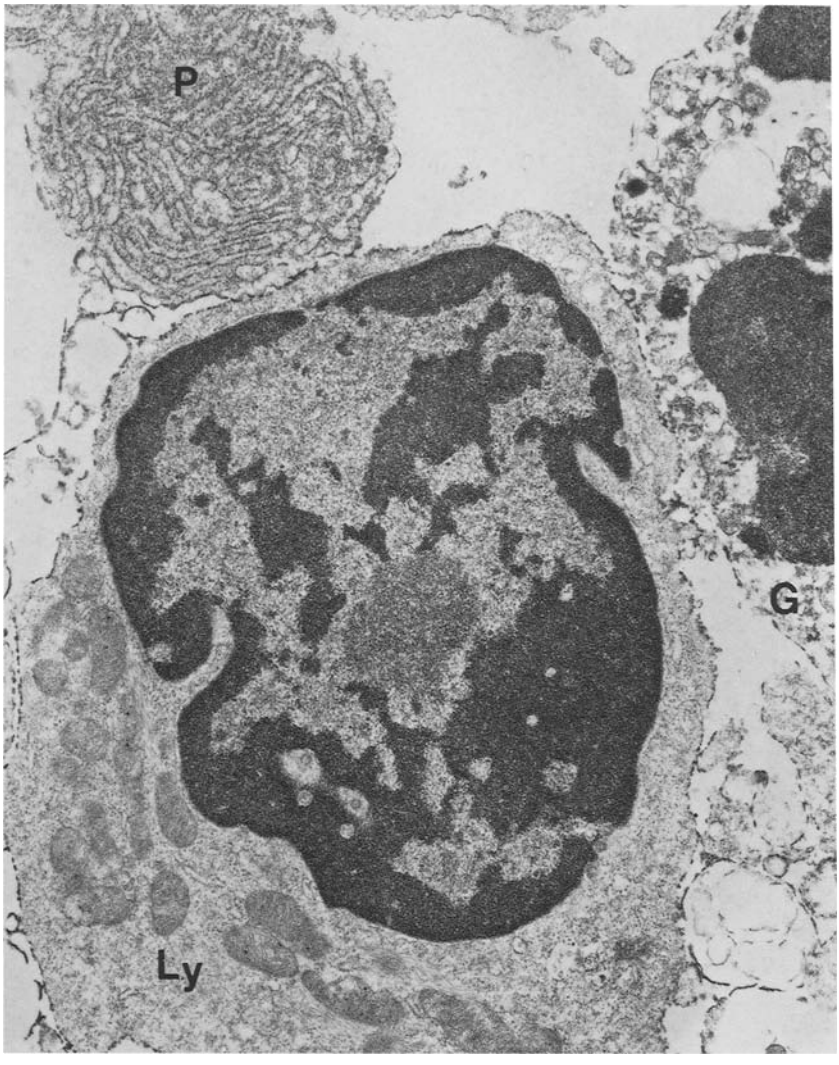

Fig. 2. Helper type (*Leu3a+*) lymphocyte (*Ly*) in lamina propria, close to a plasmocytoid cell (*P*) and to a granulocyte (*G*) (×14,500)

sections were included in Epon. Semi-thin sections, 1 μm in thickness, counterstained with toluidine blue, were observed under the light microscope, to identify areas of interest for EM examination. Thin sections (500 Å), counterstained with uranyl acetate and Pb Citrate, were observed by electron microscopy (Philips EM 400).

Negative controls: in each experiment, some sections were incubated with PBS in the first step and used as negative controls.

Results

Our method gave a strong and uniformly distributed cell membrane labelling (Fig. 1). In most sections, the preservation of plasma membranes, cytoplas-

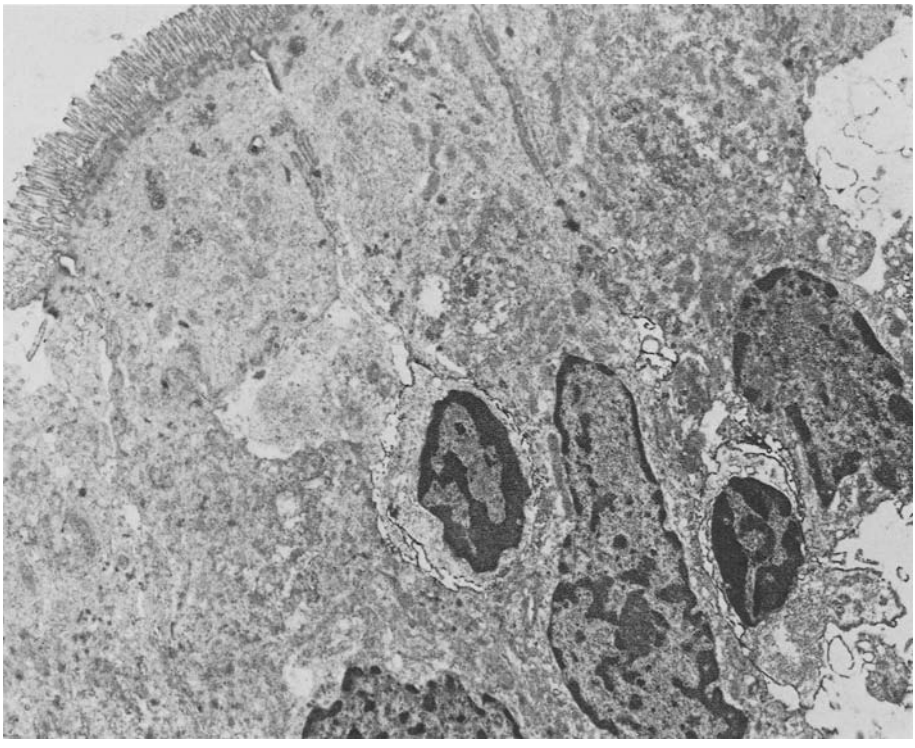

Fig. 3. Two suppressor-cytotoxic (*Leu2a*+) intraepithelial lymphocytes (×9,300)

mic organules and nuclei was nearly as good as that obtained in normal transmission EM. For this reason, some immunocompetent cells such as macrophages, plasmocytes and polymorphonuclear leucocytes were directly identified by ultrastructural cytoplasmic markers (Fig. 2). Furthermore, the identification of suppressor-cytotoxic, helper and natural killer lymphocyte subpopulations was possible at the same time, due to a strong labelling of specific membrane antigens by monoclonal antibodies. Almost all intraepithelial lymphocytes (IEL) expressed the suppressor-cytotoxic antigens (OKT8+, OKT4−). IEL were observed at various levels along epithelial cells, from basal membrane to apex, either isolated (Fig. 3) or in clusters (Fig. 4). In the lamina propria, most lymphoid cells were of helper type (Anti-Leu1+ OKT8-OKT4+), sometimes in clusters and close to plasmocytoid cells (Fig. 5). A smaller proportion (30–40%) of LP lymphocytes were OKT8+ OKT4−. No differences in ultrastructural findings between OKT8+ and OKT4+ cell populations or between IEL and LPL were noted.

"Natural killer" and "Killer" cells (Anti-Leu7+) were rarely seen, most of them being observed in lamina propria, often containing one or more electrondense cytoplasmic structures. No labelling of cells was seen when the incubation with monoclonal antibody was omitted, no cells other than lymphocytes showed diffuse membrane labelling whenever the first step-incubation with specific antibody was done.

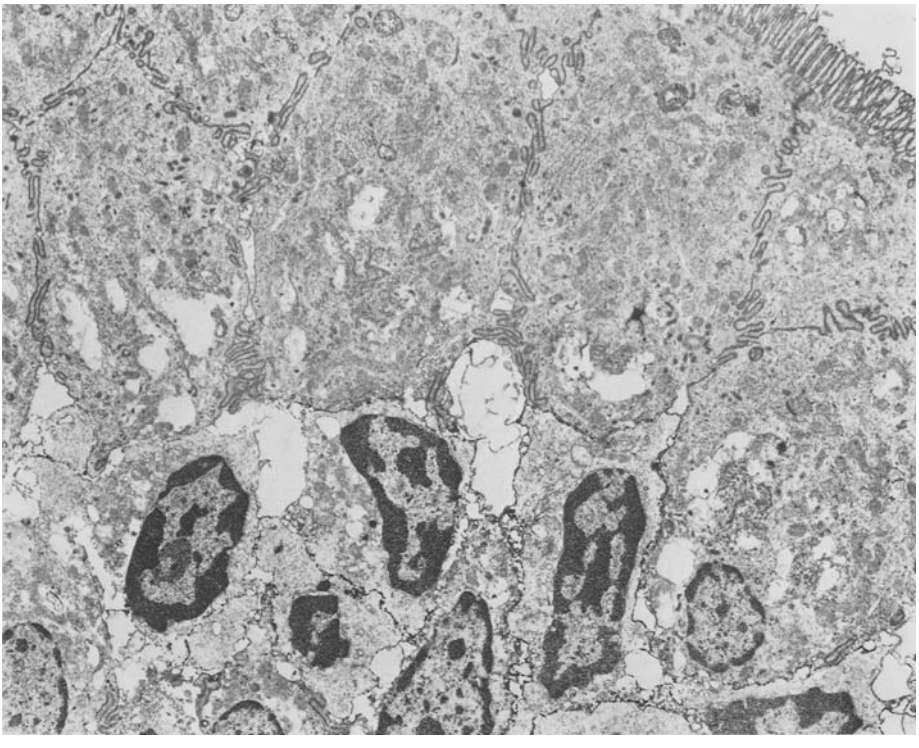

Fig. 4. Cluster of suppressor-cytotoxic (*OKT8+*) intraepithelial lymphocytes (× 6,750)

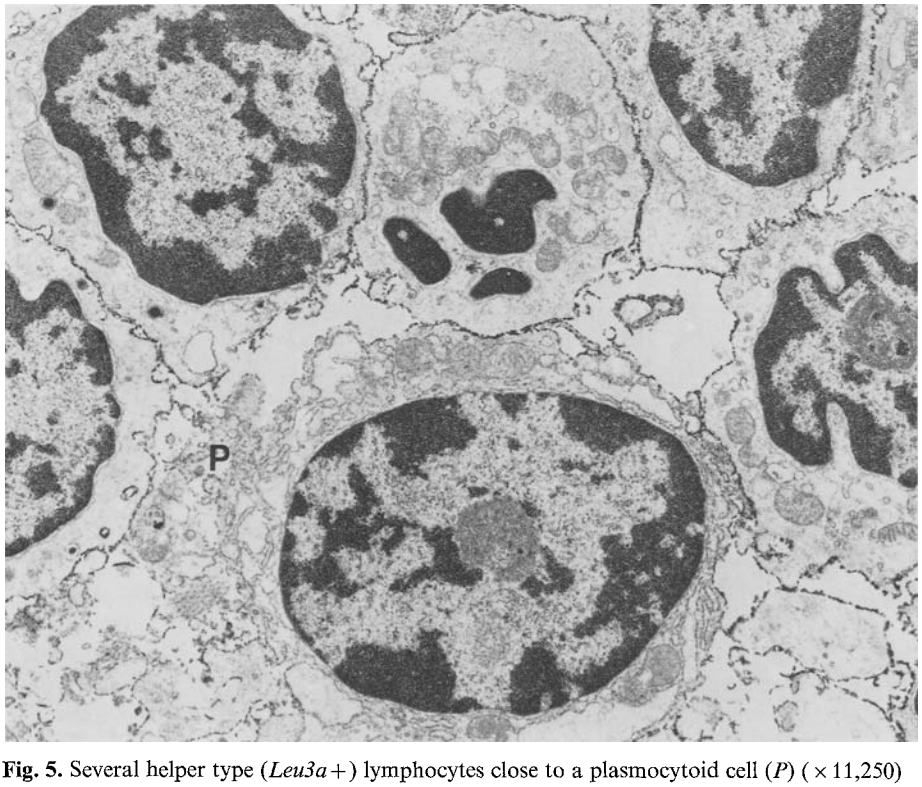

Fig. 5. Several helper type (*Leu3a+*) lymphocytes close to a plasmocytoid cell (*P*) (× 11,250)

Discussion

The *in situ* identification of immune cell populations and subpopulations in the gastrointestinal mucosa may help in understanding the physiology of the GI immune system and its alterations in pathological conditions. Good results have already been obtained both with immunofluorescence and immunoperoxidase light microscopy techniques, the latter permitting a characterization of infiltrates together with a reliable evaluation of histological pictures (Selby et al. 1981a and 1981b, Ljunghall et al. 1982; Selby et al. 1983; Selby et al. 1984). However, some doubts may arise about the specificity of these techniques and the interpretation of results, owing to the co-occurrence of cells of different lineages in the same microscopic field. Such doubts might be solved by IEM methods which allow the identification of ultrastructural characteristics of labelled cells.

IEM techniques without counterstaining of sections, while giving a specific labelling, do not allow a precise identification of specific ultrastructural markers of different cell lineages. By contrast our IEM method, which includes uranium and lead counterstaining, seems to be particularly interesting because cells can be characterized both by immunolabelling and by their ultrastructural markers, so that labelling specificity and localization together with ultrastructural markers of labelled cells may be best evaluated. To achieve this, immunolabelling procedures which precede the usual sample handling for EM, should be capable of giving a strong and specific membrane labelling without damaging cell structures. This is particularly important when the intestinal mucosa is studied, since its structure is far more sensitive to damaging agents than that of other tissues (Berti et al. 1983).

In our experience, the following criteria are of paramount importance in order to obtain satisfactory results:

1) fresh tissue samples should be used, because freezing causes severe membrane alterations.

2) The fixative solution used should not alter cellular antigens, while allowing a good preservation of cytoplasmic ultrastructure. In this respect, paraformaldehyde $CaCl_2$ proved far superior to glutaraldehyde or PLP.

3) The use of an amplifications system is advisable, althought it prolongs the incubation time, since it provides a very strong enhancement of labelling, without decreasing specificity. In fact, the labelling of portions of membranes of non-lymphocytic cells, which is always incomplete and confined to portions of cell membranes in close contact with labelled lymphocytes (Fig. 1) is an usual occurrence in IEM. This phenomenon is related to tissue shrinking during the dehydration and fixation steps which follow immunolabelling. Among the different amplifications systems tested in this study, the avidin-biotin-peroxydase complex gave the best results. When the above criteria are used, two goals can be achieved at the same time, namely the identification of several cell strains by their ultrastructural markers (owing to a good preservation of cellular structure) and of further immunocompetent cells by immunolabelling.

As far as the characterization and localization of infiltrates in the jejunal mucosa are concerned, our preliminary data seem to be in agreement with

those from other authors in light microscopy studies (Selby et al. 1981a; Selby et al. 1981b; Ljunghall et al. 1982; Selby et al. 1983; Selby et al. 1984): the great majority of IEL are cytotoxic-suppresor. The presence of such lymphocytes at various levels along epithelial cells, from the basal membrane up to the luminal surface, and the ultrastructural identity between these lymphocytes and OKT8+ lymphocytes in the lamina propria supports the hypothesis that part of the latter lymphocytes are "in transit" toward the epithelium (Selby et al. 1984). In the lamina propria, most lymphocytes are OKT4+. Morphologically, they are identical to OK8+ lymphocytes. Rare lymphocytes in the LP show the membrane antigen H-NK1, typical of killer and natural killer lymphocytes. This data, together with the finding of Leu7+ cells within vascular spaces, supports the hypothesis proposed by Gibson, that such lymphocytes, lacking the receptors which allow migration (Gibson et al. 1984), are not able to leave the mesenteric vasculature.

In conclusion, our data confirm the high specificity of monoclonal antibodies immune reactions and the reliability of the application of this technique to GI immunopathology studies.

We believe that immunopathology methods utilizing monoclonal antibodies are a suitable tool for the study of the GALT in health and disease.

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