References

Adlercreutz, H. and Luukkainen, T. (1970). Identification and determination of oestrogens in various biological materials in pregnancy. *Ann. clin. Res.*, **2**, 365-380.

Catt, K. J. and Tregear, G. W. (1967). Solid-phase radioimmunoassay in antibody-coated tubes. *Science*, **158**, 1570-1572.

Dickey, R. P., Carter, W. T., Besch, P. K., and Ullery, J. C. (1966). Effect of posture on estrogen excretion during pregnancy. *Amer. J. Obstet. Gynec.*, **96**, 127-130.

Goebel, R. and Kuss, E. (1974). Circadian rhythm of serum unconjugated estriol in late pregnancy. *J. clin. Endocr.*, **39**, 969-972.

Goebelsmann, U., Katagiri, H., Stanczyk, F. Z., Cetrulo, C. L., and Freeman, R. K. (1975). Estriol assays in obstetrics. *J. Steroid. Biochem.*, **6**, 703-709.

Hunter, W. M. and Greenwood, F. C. (1962). Preparation of iodine-131 labelled growth hormone of high specific activity. *Nature (Lond.)*, **194**, 495-496.

Katagiri, H., Stanczyk, F. Z., and Goebelsmann, U. (1974). Estriol in pregnancy. III. Development, comparison and use of specific antisera for rapid RIA of unconjugated estriol in pregnancy plasma. *Steroids*, **24**, 225-238.

Macourt, D., Corker, C. S., and Naftolin, F. (1971). Plasma oestriol in pregnancy. *J. Obstet. Gynaec. Brit. Cwlth.*, **78**, 335-340.

Masson, G. M. and Wilson, G. R. (1972). Variability of total plasma oestriol in late human pregnancy. *J. Endocr.*, **54**, 245-250.

Oakey, R. E., Bradshaw, L. R. A., Eccles, S. S., Stitch, S. R., and Heys, R. F. (1967). The rapid estimation of oestrogens in pregnancy to monitor foetal risk. *Clin. chim. Acta*, **15**, 35-45.

Stafford, J. E H. (1973). A versatile radioimmunoassay for the determination of serum oestriol in pregnancy. *Ann. clin. Biochem.*, **10**, 159-166.

Townsley, J. D., Dubin, N. H., Grannis, G. F., Gartman, L. J., and Crystle, C. D. (1973). Circadian rhythms of serum and urinary estrogens in pregnancy. *J. clin. Endocr.*, **36**, 289-295.

Tulchinsky, D., Frigoletto, F. D., Jr., Ryan, K. J., and Fishman, J. (1975). Plasma as an index of fetal well-being. *J. clin. Endocr.*, **40**, 560-567.

Morphological methods in the study of the gut immune system in man

J. M. SKINNER[1] and R. WHITEHEAD[1] *Department of Pathology, University of Oxford*

The gut associated lymphoid system is arguably the most important part of the body's peripheral immune mechanism.

Almost exclusively only indirect methods have been used in evaluating its status. There is a massive literature concerning the circulating antibody formation to various food, bacterial, and even epithelial antigens in association with a variety of gastrointestinal diseases, and similar studies have also been made on the antibodies secreted into the gut lumen. The peripheral blood lymphocyte function has also been investigated. Transformation to blastoid forms in response to a variety of stimuli and their cytotoxicity for elements of the gut epithelium have been studied intensively. Much of the information produced by these studies has been largely unhelpful in increasing our understanding of gastrointestinal disease and some has been contradictory.

Little attention has been given to those aspects of the morphological features of the lymphoid system which might affect function but Cottier *et al* (1972), for example, have shown that the histological appearance of a lymph node can often give information on immunological function. Using similar principles, Skinner and Whitehead (1974) examined the mesenteric nodes in Crohn's disease and ulcerative colitis. However, of potentially greater importance is the application of immunohistochemical methods with which the exact secretory function of gut immunocytes can be defined in terms of antibody production and immunoglobulin type. Using morphometric methods the number of immunocytes can be accurately counted and their changes monitored on sequential studies. The present progress in the latter type of methodology is the subject of this report.

Methods for Identification of Cell Type

From the above it is clear that the normal morphological parameters have yet to be defined and much has still to be done in this field. Abnormalities are best shown by objective techniques, for example the presence or absence of a single feature, but when changes involve similar cells or tissues to a different

[1]Present address: Flinders University School of Medicine, Pathology Department, Bedford Park, Adelaide, South Australia 5042.

Received for publication 15 December 1975

degree then quantitative methods are required. Most work to date has concentrated on plasma cells rather than lymphocytes because it is this cell which produces immunoglobulin, making a positive identification of the cell easy in tissue sections.

There are a number of histochemical methods that can be used to identify plasma cells, for example, the methyl green/pyronin technique, but they are nonspecific and give positive results with any cell which is actively synthesizing protein, eg, the erythroblast. More specific methods have been developed which utilize the fact that plasma cells produce immunoglobulin and this large molecule is itself antigenic. The immunofluorescence method of Coons *et al* (1942) and Coons (1958) can thus be applied. Antibodies raised in other animals against the specific immunoglobulin classes (A, G, M, D, E) can be conjugated with fluorescein isothiocyanate and then can be used to identify specifically the plasma cells in the tissue which contain the immunoglobulin provided that suitable control experiments are incorporated in the tests (Nairn, 1969). This method, known as the direct immunofluorescence method, is easy to control but gives a less intense fluorescence in general than the recently more widely used indirect method. Both techniques are usually applied to frozen unfixed tissue sections but have the defect that cell outlines are indistinct because of diffusion of the proteins in the aqueous solutions employed. Counting of positive cells thus becomes difficult and tedious as Crabbé *et al* (1965) noted in their original study. The use of cold alcohol fixation (4°C) followed by a cold paraffin embedding technique (Sainte-Marie, 1962) was an improvement in that cell outlines were better preserved and surrounding structures more easily identified, and until recently this has been the method of choice. For the purpose of quantitation, however, fluorescence techniques have a further drawback. Fluorescence fades rapidly and the time available for accurate cell counting is limited. It is thus necessary to photograph many different fields so that permanent preparations, in photographic form, can be used for quantitation. This is in itself time consuming and expensive, making large-scale surveys difficult. More recently an alternative immunohistochemical method using the enzyme horse-radish peroxidase as a label rather than fluorescein has been available (Nakane and Pierce, 1966) and has been further developed (Burns, 1975).

The technique depends on the same principle as the fluorescence method but the site of the immunological reaction is identified by developing the peroxidase activity using a suitable substrate (eg, diaminobenzidine which gives a brown precipitate). The preparation is permanent and can be visualized with a routine light microscope, and because the surrounding tissues are clearly outlined the positive cells can be accurately quantified. There is a further advantage in that routine formalin-fixed paraffin embedded sections can be used (Burns, 1975) and, provided it is not too old, material collected in previous years can be examined. The criticism of using fixed tissues is that complement is said to be destroyed and thus cannot be demonstrated. However, even in frozen material complement is difficult to identify unless it lies within the blood vessels of gut tissues.

Quantitative Methods

The first attempt to quantify the plasma cells of the gut wall was by Crabbé *et al* (1965) using what was basically a planimetric method. This was extremely time consuming and the authors noted a pronounced fatigue which has an inherent tendency to cause considerable observer error. A second method and one which is still widely favoured is based upon the use of counting grids incorporated into the microscope eyepiece (Binder, 1970). An area of lamina propria is estimated by counting the number of small squares of known size which lie on it, and in this area the number of plasma cells is evaluated and expressed as number per unit area or, if the section thickness is known, as number per unit volume. Because of the structure of the gut mucosa difficulty is met owing to squares falling partially on epithelium, particularly in the normal situation, and errors arise because of the need to guess the proportion of the small square which lies on epithelium rather than lamina propria. This can be overcome in two ways, first, by examining only squares which fall on lamina propria, but this imposes limitations of sampling. Secondly, it can be overcome by using extremely small squares but this then introduces the same drawback as planimetry, namely, observer fatigue owing to the necessity to count large numbers of squares.

The method of choice is based upon the principle of point counting (Weibel, 1963). A grid composed of a number of regularly dispersed points is cast at random onto the tissue to be assessed. The number of points falling on any component of the tissue is proportional to its volume. It follows that if a particular number of points is counted this will always correspond to a fixed tissue volume. In this volume it is then possible to count the number of plasma cells, the results being expressed as cells per unit volume (for example, number of cells per 1000 points of lamina propria). This method clearly is not attempting to derive absolute figures but does allow an accurate and reproducible comparison between

specimens. If, in a given study, there is a gross alteration of a particular histological component by a disease process, a simple assessment of cell density may be misleading. For example, if, in a comparison of ulcerative colitis cases, the mucosa is extremely thin and atrophic in one end of normal thickness or thickened in another, a measurement of cell density may be the same but it is clear that the number of cells will be different. In order to account for this it is necessary to include a further morphometric principle, that is, the linear intercept. In this instance the aim is to count the number of cells overlying a given surface area of muscularis mucosae or other histological structure not affected by the disease process. This area is determined by counting the number of intercepts through the surface of the muscularis or similar structure made by randomly placed lines of known length (Weibel, 1963). Thus a given area is defined by a fixed number of intercepts and cell counts are related to this. The point or line density used in the grid is determined empirically choosing a density which gives the greatest accuracy within the amount of tissue available for examination. The accuracy is easily tested using the principle of summation averages (Chalkley, 1943).

It can be usefully stressed again that these methods do not purport to give absolute values. To assign absolute values to the measurements obtained by any of these techniques requires a knowledge of section thickness, which must be of low order in comparison to the size of cells counted, the exact area of the grid used, and the precise magnification. Most of all the tissue shrinkage between the time of biopsy and examination on the slide must be known. The requirement is almost impossible to meet. Sections have to be cut at random angles throughout the block (Hennig, 1956), a factor which of itself is wasteful of the tissue to be examined. Consequently non-absolute techniques are the choice methods for any studies involving more than a few specimens. Abnormal tissue should ideally always be compared with normal control material. Sometimes, as already stated, there will be some difficulty in the choice of method because of alteration of the histological structure by the disease process. When choosing a reference point for comparison of cell density counts it is imperative, therefore, that the reference point itself is not involved in the disease process. The counting of lymphocytes in small bowel epithelium can be used as an example to illustrate this point. In the accompanying figure it is plain that, using a given number of epithelial cells as reference point, the ratio of lymphocytes to epithelial cells, in b as opposed to a, has increased. However, the area of epithelium has decreased, and a fixed area of epithelium as defined by linear intercepts would be a better fixed point for comparison (Skinner *et al*, 1971) as no assumption has been made that epithelial cells are of uniform size in both cases. Better still would be to use a fixed point which is not subject to variation, in this case the muscularis mucosae. In the above example, using this reference point, the number of cells does not alter. In fact only decreases in number or enormous increases would be meaningful using epithelial cell numbers as a reference point. Much of the recent literature concerning lymphocyte numbers in the superficial epithelium in relation to coeliac disease can be criticized from this point of view.

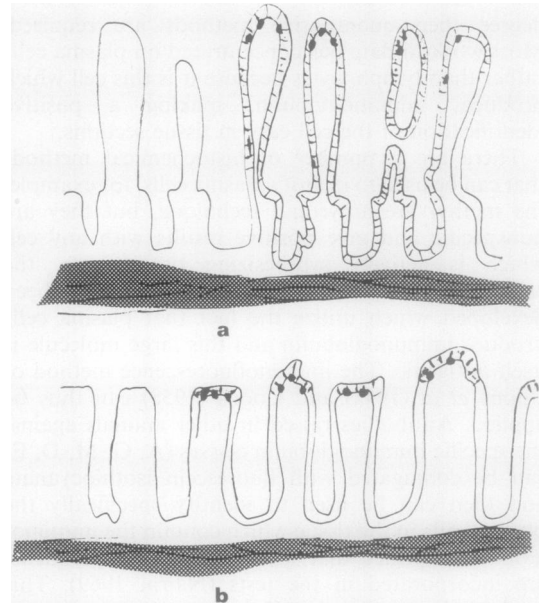

Figure *Counting intraepithelial lymphocytes.*

The normal levels for plasma cells in the gut were recorded by Crabbé and Heremans (1966), and their findings have been confirmed in subsequent work (Gelzayd *et al*, 1968; Søltoft, 1969; Chen and Tobé, 1974). Crabbé *et al* (1965) showed that in the jejunal mucosa IgA cells numbered 181 000 (± 19 000)/mm³, IgM cells 30 000 (± 4500)/mm³, and IgG 18 000 (± 3000)/mm³. As stated above, the use of absolute figures in this way is questionable, and the most important point is the ratio of cells IgA:IgM:IgG which is 10:1·6:1. Most workers have shown that IgA cells compose 80% or slightly more of the plasma cell population throughout the gut, and the IgD and IgE cells are even fewer than IgG cells.

Many of the problems set by the broad spectrum of diseases in which the gut associated lymphoid tissue may be involved have yet to be investigated using the methods outlined above.

Attempts have already been made using less complete or less accurate methods which have produced encouraging information concerning the alteration of gut responses in several disease states. These include the infections (Søltoft and Seberg, 1972; Davidson *et al*, 1975), pernicious anaemia (Odgers and Wangel, 1968), chronic gastritis unassociated with pernicious anaemia, coeliac disease (Douglas *et al*, 1970), tropical and temperate sprue (Swanson and Thomassen, 1965; Drummond and Montgomery, 1970), ulcerative colitis and Crohn's disease (Skinner and Whitehead, 1974), and alpha chain disease (Manousos *et al*, 1974). There is sufficient evidence from these studies that further investigation using the more elaborate and accurate methods outlined above will yield much more valuable data. Moreover the techniques are simple, require no complicated mathematics, and are within the capabilities of everyday laboratories equipped with a microscope and the ability to perform straightforward histological methods. Tissues do not require special processing and large-scale studies can be attempted both prospectively and retrospectively.

References

Binder, V. (1970). Cell density in lamina propria of the colon: a quantitative method applied to normal subjects and ulcerative colitis patients. *Scand. J. Gastroent.*, **5**, 485-490.

Burns, J. (1975). Background staining and sensitivity of the unlabelled antibody enzyme (P.A.P.) method. Comparison with the peroxidase labelled antibody sandwich method using formalin fixed paraffin embedded material. *Histochemistry*, **43**, 291-294.

Chalkley, H. W. (1943). Method for the quantitative morphologic analysis of tissues. *J. nat. Cancer Inst.*, **4**, 47-53.

Chen, S. T. and Tobé, T. (1974). Cellular sites of immunoglobulins. IV. Studies of antral mucosa of human stomachs. *Digestion*, **10**, 177-183.

Coons, A. H. (1958). In *General Cytochemical Methods*, edited by J. F. Danielli. Vol. 1, pp. 400-422. Academic Press, New York.

Coons, A. H., Creech, H. J., Jones, R. N., and Berliner, E. (1942). The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. *J. Immunol.*, **45**, 159-170.

Cottier, H., Turk, J., and Sobin, L. (1972). A proposal for a standardised system of reporting human lymph node morphology in relation to immunological function. *Bull. Wld Hlth Org.*, **47**, 375-408.

Crabbé, P. A., Carbonara, A. O., and Heremans, J. F. (1965). The normal human intestinal mucosa as a major source of plasma cells containing γA immunoglobulin. *Lab. Invest*, **14**, 235-248.

Crabbé, P. A. and Heremans, J. F. (1966). The distribution of immunoglobulin-containing cells along the human gastrointestinal tract. *Gastroenterology*, **51**, 341-347.

Davidson, G. P., Bishop, R. F., Townley, R. R. W., Holmes, I. H., and Ruck, B. J. (1975). Importance of a new virus in sporadic enteritis in children. *Lancet*, **1**, 242-245.

Douglas, A. P., Crabbé, P. A., and Hobbs, J. R. (1970). Immunochemical studies of the serum, intestinal secretions and intestinal mucosa in patients with adult celiac disease and other forms of the celiac syndrome. *Gastroenterology*, **59**, 414-425.

Drummond, M. B. and Montgomery, R. D. (1970). Acute sprue in Britain. *Brit. med. J.*, **2**, 340-341.

Gelzayd, E. A., Kraft, S. C., Fitch, F. W., and Kirsner, J. B. (1968). Distribution of immunoglobulins in human rectal mucosa. II. Ulcerative colitis and abnormal mucosal control subjects. *Gastroenterology*, **54**, 341-347.

Hennig, A. (1956). Inhalt einer aus Papillen oder Zotten gebildeten Fläche. *Mikroskopie*, **11**, 206-213.

Manousos, O. N., Economidou, J. C., Georgiadou, D. E., Pratisika-Ougourloglou, K. G., Hadziyannis, S. J., Merikas, G. E., Henry, K., and Doe, W. F. (1974). Alpha-chain disease with clinical, immunological, and histological recovery. *Brit. med. J.*, **1**, 409-412.

Nairn, R. C. (1969). *Fluorescent Protein Tracing*, Livingstone, Edinburgh.

Nakane, P. K. and Pierce, G. B., Jr. (1966). Enzyme-labeled antibodies: preparation and application for the localisation of antigens. *J. Histochem. Cytochem.*, **14**, 929-931.

Odgers, R. J. and Wangel, A. G. (1968). Abnormalities in IgA-containing mononuclear cells in the gastric lesion of pernicious anaemia. *Lancet*, **2**, 846-849.

Sainte-Marie, G. (1962). A paraffin embedding technique for studies employing immunofluorescence. *J. Histochem. Cytochem.*, **10**, 250-256.

Skinner, J. M. and Whitehead, R. (1974). The plasma cells in inflammatory disease of the colon: a quantitative study. *J. clin. Path.*, **27**, 643-646

Skinner, J. M., Whitehead, R., and Piris, J. (1971). Argentaffin cells in ulcerative colitis. *Gut*, **12**, 636-638.

Søltoft, J. (1969). Immunoglobulin-containing cells in normal jejunal mucosa and in ulcerative colitis and regional enteritis. *Scand. J. Gastroent.*, **4**, 353-360.

Søltoft, J. and Seberg, B. (1972). Immunoglobulin-containing cells in small intestine during acute enteritis. *Gut*, **13**, 535-538.

Swanson, V. L. and Thomassen, R. W. (1965). Pathology of the jejunal mucosa in tropical sprue. *Amer. J. Path.*, **46**, 511-537.

Weibel, E. R. (1963). Principles and methods for the morphometric study of the lung and other organs. *Lab. Invest.*, **12**, 131-155.