PROCEDURE

Of the test blood, 0.1 ml is mixed with an equal volume of an endotoxin solution. The original method (Park and Good, 1970) recommends the commercial preparations, and these will give the best results; we have found, however, that a crude filtrate of a broth culture of *Escherichia coli* or *Pseudomonas pyocyanea* will work sufficiently well. It is vital to perform the procedure in parallel on a known normal blood sample. The blood-endotoxin mixtures are incubated at 37°C for 30 minutes, and then, using 0.1 ml aliquots the direct test is performed, exactly as described earlier. The normal control will be stimulated to a level well above the normal range, ie, will rise from less than 11 to 25-30%, and the test sample is assessed against this. A temporarily suppressed neutrophil population will reveal some stimulation, though it may not be of the magnitude of the normal control, but the neutrophils in chronic granulomatous disease will remain completely unreactive.

Comment

If the early promise of the NBT test is realized, it will quickly and justifiably become a standard test in many of our hospitals. Our concern is that it should not suffer any unjustified setbacks simply because it uses techniques which are unfamiliar to many. We hope that this guide, culled from our short experience and that of others, will help to promote an important addition to laboratory investigation.

We thank those of our clinical colleagues who allowed us such generous access to their records. This has been invaluable in trying to evaluate our techniques.

References

- Davidsohn, I., and Wells, B. B. (Eds.) (1962). *Todd-Sanford Clinical Diagnosis by Laboratory Methods*, 13th ed., p. 938. Saunders, Philadelphia.
- Feigin, R. D., Shackelford, P. G., Choi, S. C., Flake, K. K., Franklin, F. A., Jr., and Eisenberg, C. S. (1971). Nitroblue tetrazolium dye test as an aid in the differential diagnosis of febrile disorders. *J. Pediatr.*, **78**, 230-237.
- Freeman, R., and King, B. (1971). A modification to the N.B.T. test. *Lancet*, **2**, 1154.
- Johnston, R. B., Jr., and Baehner, R. L. (1971). Chronic granulomatous disease: correlation between pathogenesis and clinical findings. *Pediatrics*, **48**, 730-739.
- Lancet* (1971). Nitroblue tetrazolium: a routine test? (Editorial). *Lancet*, **2**, 909-910.
- Matula, G., and Paterson, P. Y. (1971). Spontaneous *in vitro* reduction of nitroblue tetrazolium by neutrophils of adult patients with bacterial infection. *New Engl. J. Med.*, **285**, 311-317.
- Park, B. H. (1971). The use and limitations of the nitroblue tetrazolium test as a diagnostic aid. *J. Pediatr.*, **78**, 376-378.
- Park, B. H., and Good, R. A. (1970). N.B.T. test stimulated. *Lancet*, **2**, 616.

Reprocessing of wax-embedded tissue blocks for the electron microscope applied to Crohn's disease

T. WATSON, M. J. FULKER, AND M. G. COOK *From the Department of Experimental Pathology and Cancer Research, Leeds University, and the University Department of Surgery, The General Infirmary at Leeds*

The reprocessing of tissue from wax blocks for electron microscopy provides a means of observing ultrastructural detail at the expense of some resolution (Kobernick and Thomas, 1970; Hallowes and Streek, 1970). Much ultrastructural information can, however, be obtained at medium magnifications. This is of considerable value in certain pathological situations where the sparsity of significant lesions makes conventional electron microscopy uneconomic. In this case we have applied the technique to the study of giant cells in Crohn's disease (Albot, Parturier-Albot, Camilleri, and Diebold, 1970).

Materials and Method

Tissue blocks (approx $2 \times 2 \times 0.5$ cm) were removed from fresh proctocolectomy specimens. The specimens were fixed for four hours in 4% glutaraldehyde in sodium cacodylate buffer (pH 7) and subsequently dehydrated with graded alcohols, immersed in chloroform, and embedded in paraffin wax following the conventional histological procedure. Sections, 3-4 μ m, were cut and stained with haematoxylin and eosin and the areas thought worthy of further study with the electron microscope were selected by light microscopy. The required parts of the wax block were then cut out, to a size of approximately $2 \times 3 \times 2$ mm and immersed in benzene at 45°C for one hour and rehydrated through graded alcohols. The tissue was washed for one hour in two changes of sodium cacodylate buffer, postfixed for one hour in sodium cacodylate-buffered 1% osmium tetroxide. This was followed by dehydration through graded alcohols to propylene oxide and embedding in Araldite. Sections, 0.5 μ m, were stained with toluidine blue. Thin sections approximately 80 nm thick cut with an LKB Ultratome III, mounted on plain copper grids, and stained with lead tartrate, were examined on a Philips EM 300 electron micro-

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scope. Where giant cells were present, these were most easily located on thin sections by using the lowest magnification available on the microscope ($\times 250$). The whole giant cell could be seen with good contrast so that the context of ultrastructural detail could be established.

Results

The reprocessing technique was used to examine ultrastructural features of lesions first seen in the wax sections. Figure 1 shows a giant cell diameter

Fig. 1 Paraffin wax section showing the giant cell from which the electron micrographs Figs. 2 and 3 were taken ($\times 270$).

Fig. 2 Giant cell edge with marked folding of the outer membrane ($\times 11\,500$).

Fig. 3 Tangential section through the giant cell plasma membrane showing the complex arrangement of folds ($\times 15\,000$).

about $130\ \mu\text{m}$ in the lamina propria of a colon with Crohn's disease. The nuclei are arranged peripherally and some cytoplasmic vacuolation is seen in the centre. The surrounding cells are epithelioid cells and lymphocytes. Figure 2 shows some of the ultrastructural aspects of the same giant cell after reprocessing, for example, the outer edge of the giant cell, numerous mitochondria, and some rough endoplasmic reticulum. The cytoplasmic vesicles are believed to correspond to the vacuolation visible in the thick section in Figure 1. It is often difficult to resolve the vesicle membrane, possibly because

of the sacrifice of some resolution as a result of the reprocessing.

The complex folding of the giant cell outer membrane (Fig. 2) was a characteristic feature. The tangential section (Fig. 3) also shows these folded membranes.

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Letters to the Editor

Automated Antibody (anti-D) Quantitation Technique

We were interested to read the article by Gunson, Philips, and Stratton (1972) concerning inherent errors within the automated antibody (anti-D) quantitation technique using the Technicon AutoAnalyzer. However, we are unable to agree with the authors' statement that non-linearity of graphs, obtained by plotting reciprocal of dilution against optical density, is due to loss of antigen excess (antibody excess).

The following calculations are based on the conditions occurring during the reaction phase using the technique described by us (Judd and Jenkins, 1970), and show conclusively that one is always operating with a vast excess of antigen: Assuming, (1) the molecular weight of IgG anti-D is 150 000, (2) the number of D sites per red cell (R_1R_1) is 20 000, a 20% red cell suspension will contain 2×10^9 red cells/ml.

According to Avogadro's number, 1 gram molecule of any substance will contain 6×10^{23} molecules, hence 6×10^{23} molecules of anti-D will weight 150 000 grams. Therefore, $1 \mu\text{g}$ of anti-D will contain

$$\frac{6 \times 10^{23}}{1.5 \times 10^5 \times 10^6} \text{ molecules}$$

ie, 4×10^{12} antibody molecules.

A 20% red cell suspension will contain $2 \times 10^9 \times 20\,000$ D antigen sites per ml, ie, 4×10^{13} D sites per ml.

In our experience an anti-D of $1 \mu\text{g}$ per ml requires to be diluted 1:100 for automated assay and this dilution will, therefore, contain 4×10^{10} antibody molecules per ml.

As equal volumes of red cell suspension

and serum are used in the AutoAnalyzer 4×10^{10} anti-D molecules per ml will be distributed amongst 4×10^{13} D antigen sites, that is to say 1/1000th of the total number of D sites may be combined with antibody molecules assuming that all antibody is bound. In order to obtain antibody excess within the AutoAnalyzer one would require more than 4×10^{13} molecules/ml, ie, $10 \mu\text{g}$ of anti-D/ml to be run through the system undiluted. If one postulates two antigen-combining sites for each antibody molecule then this figure can be halved, but at no time does one use this technique with such vast amounts of antibody.

We find a satisfactory standard graph can be obtained when operating within the range of 1:1000-1:4000 for a $20 \mu\text{g}/\text{ml}$ standard, ie, between 0.02 and 0.005 $\mu\text{g}/\text{ml}$. Under these conditions any non-linearity of the graph cannot be due to antibody excess.

W. J. JUDD AND W. J. JENKINS
North-East Metropolitan Regional Blood
Transfusion Centre, Crescent Drive,
Brentwood, Essex

References

- Gunson, H. H., Philips, P. K., and Stratton, F. (1972). Manipulative and inherent errors in anti-D quantitation using the AutoAnalyzer. *J. clin. Path.*, 25, 198-205.
Judd, W. J., and Jenkins, W. J. (1970). Assay of anti-D using the Technicon AutoAnalyzer and the International Standard anti-D typing serum. *J. clin. Path.*, 23, 801-804.

Our calculations show that almost complete agglutination is achieved in this system with approximately 25 antibody molecules per erythrocyte; an antibody concentration of about 0.018 μg per ml. Further increase in antibody concentration

leads to a proportional increase in bound antibody but does not materially affect the degree of agglutination. We note the authors now quote a range for their standard graph of 0.005 to 0.02 μg per ml whereas our comments related to their previous graph of 0.0115 to 0.115 μg per ml (Judd and Jenkins, 1970).

A large antigen site excess is required to maintain sufficient erythrocytes so that the number of bound antibody molecules per erythrocyte remains small. 'Loss of antigen excess', in this context, arises when the number of bound antibody molecules per erythrocyte is such that the degree of agglutination is no longer directly proportional to anti-D concentration and does not imply excess of antibody molecules over antigen sites.

H. H. GUNSON
P. K. PHILLIPS
F. STRATTON
Regional Transfusion Service,
Manchester

Reference

- Judd, W. J., and Jenkins, W. J. (1970). Assay of anti-D using the Technicon AutoAnalyzer and the International Standard anti-D typing serum. *J. clin. Path.*, 23, 801-804.