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, i.e., 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

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

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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 × 2 × 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 µm, 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 × 3 × 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 µm, 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 microscope.

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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 about 130 $\mu$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 epitheloid 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 reproprocessing.

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.

The authors wish to thank Professor J. C. Goligher for access to the pathological material, and Professor E. H. Cooper for the use of laboratory facilities.

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 Auto-Analyzer. 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, R1) is 20 000, a 20% red cell suspension will contain $2 \times 10^8$ red cells/ml.

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

$$6 \times 10^{23}$$

$$1.5 \times 10^4$$

molecules

ie, $4 \times 10^{13}$ antibody molecules.

A 20% red cell suspension will contain $2 \times 10^8 \times 20 000$ D antigen sites per ml, ie, $4 \times 10^{13}$ D sites per ml.

In our experience an anti-D of 1 µg per ml requires to be diluted 1:100 for automated assay and this dilution will, therefore, contain $4 \times 10^{13}$ antibody molecules per ml.

As equal volumes of red cell suspension and serum are used in the AutoAnalyzer, $4 \times 10^{13}$ anti-D molecules per ml will be distributed amongst $4 \times 10^{13}$ 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 $4 \times 10^{13}$ molecules/ml, ie, 10 µg 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 µg/ml standard, ie, between 0.02 and 0.005 µg/ml. Under these conditions any non-linearity of the graph cannot be due to antibody excess.

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References


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 therefore, the authors now quote a range for the standard graph of 0.005 to 0.02 µg/ml whereas our comments related to the previous graph of 0.015 to 0.15 µg/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 antibody' or 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.

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Reference