Technical methods

Demonstration of lymphocyte nucleoli

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The presence of nucleoli in the nuclei of mature human lymphocytes has long been a question of controversy probably arising from the difficulty of rendering them visible. Electron microscope studies confirm that nucleoli are present in lymphocytes (Huhn and Stich, 1969). The fact that they contain a high proportion of RNA is the basis of the methods for demonstrating them by staining.

Methods giving a negative picture of the nucleoli, such as the Feulgen reaction (Gardikas and Israels, 1948) and breakdown of RNA by enzymes (quoted by Smetana, Lejnar, and Potměšil, 1969), whilst helping to localize them in more immature cells, are not so useful for mature cells where the nucleoli are usually smaller and difficult to distinguish from spaces in the denser chromatin structure.

The most satisfactory methods are those which give a positive picture of the nucleoli. These methods have been compared and the most suitable one tested under varying conditions to assess the optimal method of use on peripheral blood films and buffy layer preparations.

Staining methods were carried out on blood films and buffy layer preparations of normal blood and blood from patients with chronic lymphatic leukaemia when available.

The following methods were used:

After 'wet' and 'dry' fixation in Heidenhain's Susa fixative or in 10% formaldehyde in absolute methanol: (1) Feulgen (Bauer, 1937); (2) Feulgen-substitute staining technique (Grieg, 1959); (3) Unna-Pappenheim for varying lengths of time.

After fixation in methanol, Giemsa-Chromotrope 2R (Gillis and Baikie, 1964): without fixation; (1) toluidine blue from two sources (Eastman and G. T. Gurr) by the method of Smetana *et al* (1969) and (2) Azure C from Eastman by the same authors' method in which a 0.5% solution of the dye is made in 10% methanol. This stock solution will keep for at least three years and from it is made a 1 in 100 dilution in McIlvain's buffer pH 5 (0.21% citric acid 48.5 ml and 0.356% disodium hydrogen phosphate

dihydrate, 51.5 ml). Unfixed blood films are stained in this working solution for 10 minutes at room temperature and the stain is then tipped off and the film allowed to dry without washing. RNA is stained dark blue. The specificity of the reaction at pH 5 for RNA has been demonstrated by ribonuclease digestion (Smetana *et al*, 1969).

Of all these techniques the method of choice was found to be the Azure C which demonstrated the nucleoli in normal mature lymphocytes very well, and experiments were carried out to determine what effects varying the conditions had upon this method.

1 It is possible to fix blood films before staining in either methanol or 50% ether/methanol mixture for up to one minute but it is suggested that this is not usually necessary as blood films will keep unstained (see below). Any fixative containing formaldehyde inhibits staining by this method.

2 When films from capillary blood, EDTA, and heparinized blood were made, stored unfixed at room temperature, and one of each stained with Azure C at daily intervals for two weeks, it was found that there was no difference in staining for up to one week but after that time it was less easy to distinguish the nucleoli from the chromatin and this was not possible at all after two weeks.

3 It was found that blood could be collected into either heparin or EDTA and stored in the refrigerator for up to five days before there was any loss of nucleolar staining in comparison with freshly made films.

4 The working solution of stain in McIlvain's buffer will keep for four days, after which time it also stains the chromatin structure in the nucleus to a varying degree.

It is of interest that neither of the commercially available toluidine blue stains used was satisfactory but that a sample obtained from Professor Smetana gave good results.

The use of Azure C for the demonstration of nucleoli in mature lymphocytes is a simple, quick method which can be carried out in any laboratory. The nucleoli and azurophilic granules in the cells are stained and the fact that red cells are not fixed make the blood films or buffy layer preparations easy to examine. It is also useful for demonstrating the nucleoli in immature cells in marrow films and leukaemic blood.

The nucleolus in the Azure C preparation appears as a small, round, ring-like structure. In some of the large lymphocytes it is larger, with a thicker and darker staining appearance and in abnormal cells it

Fig. 1

Fig 1 *Small lymphocytes stained with Azure C × 2500.*

Fig 2

Fig 2 *Lymphocytes after 72 hours' culture with phytohaemagglutinin stained with Azure C. × 1500.*

may appear solid, but this type is practically never seen in normal blood though occasionally a small double ring form can be found.

In addition, the Azure C demonstrated azurophilic granules and sometimes lines appearing to divide the lymphocyte nucleus into lobes. No nucleoli are seen in polymorph nuclei and monocytes have two to four minute structures much smaller than the lymphocytic nucleoli.

Usually most normal lymphocytes are shown to have a single small nucleolus but a proportion have two or more and it is possible to produce a nucleolar coefficient:

$$\text{NC} = \frac{\text{number of nucleoli in 100 lymphocytes}}{100}$$

This coefficient has been reported in several clinical states including Hodgkin's disease (Potměšil, Smetana, and Wienerová, 1965) and in breast carcinoma (Smetana, Janele, and Malinský, 1966) in both of which conditions the coefficient is raised.

The morphology of the nucleoli can perhaps be related to cellular nucleoprotein metabolism, as Studzinaks and Gierthy (1972) were able to show that after treatment with inhibitors of RNA synthesis the nucleoli of HeLa cells became ring shaped. The presence of ring-shaped nucleoli in the majority of normal lymphocytes would thus be consistent with the observation that they have only a small amount of active ribonucleic acid synthesis. This has in fact been demonstrated by autoradiographic studies (Cooper and Rubin, 1965). However, metabolic activation of the cell, as by mitogens, is accompanied by RNA synthesis shown morphologically by an increase in size of the nucleolus and change in its appearance from ring form to a solid structure all of which shows positive staining for RNA (fig 2).

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Letter to the Editor

Candida Isolates

Regarding Dr R. C. Bartlett's letter commenting on our paper (Stieritz, Law and Holder, *J. clin. Path.*, 1973, 26, 405-408), we did not mean to imply all *Candida* isolates from any patient should be speciated. Our paper concerned burned patients infected with *Candida* and in the context of *Candida* isolated from the wounds, urine, and blood of compromised hosts we still feel that speciation is important.

While I concur with Dr Bartlett's comment that 'the decision to treat should be based on clinical evidence of infection, not the species isolated', we have found this not to be the case when dealing with *Candida* infections. Because of the highly toxic nature of Amphotericin B, the decision to treat *Candida* infections is frequently delayed until the clinician is forced to treat or lose the patient. As we pointed out, many clinicians consider *C. albicans* the only *Candida* which is of clinical concern. When they receive a culture report stating that *Candida* other than *albicans* has been isolated from an infected patient, they may be lulled into a false sense of security and then finally be forced to treat with too little too late.

Our data showed that while all of the Amphotericin B MICs for *C. albicans* were 0.16 µg/ml or below, most of the MICs for *C. tropicalis* were at least one dilution higher, and in some cases two and three dilutions higher. This may imply that *C. tropicalis* infection could be a treatment problem, whereas *C. albicans* infection may not be. Support for this idea may be found in the fact that the first report, to my knowledge, of clinical isolates of *Candida* being resistant to Amphotericin B has recently been

published (Woods, R. A., *et al*, 1974). These resistant isolates were *C. tropicalis* not *C. albicans*.

While we all would like to keep the costs of health care to a minimum, the attitude that we should only do that clinical microbiology which is 'essential to diagnosis and treatment', I believe to be incorrect. In the evolution of infectious diseases and in defining infection problems, the microbiology that was 'essential to diagnosis and treatment' yesterday is not the same as the microbiology that is 'essential to diagnosis and treatment' today; nor will today's microbiology suffice tomorrow. We, the clinical microbiologists, must be alert to identifying new infection problems; the latest 'emerging' pathogens, and it must be our responsibility to warn the medical fraternity of these new dangers. If we don't, who then?

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Book reviews

Transplacental Carcinogenesis Proceedings of a meeting held at the Medizinische Hochschule, Hanover, 1971 (IARC Scientific Publications No. 4). Edited by L. Tomatis and U. Mohr. (Pp. xiii + 181; illustrated. £5.00; \$12.00; Sw. fr. 40.-) Geneva: World Health Organization. International Agency for Research on Cancer. 1973. Available from HMSO, London.

Interest in the transplacental action of certain carcinogens was widely stimulated by the report from Boston in 1971 of a cluster of eight cases of adenocarcinoma of the vagina in young women; the mothers of all but one of them had received stilboestrol during their pregnancy. Although the transplacental carcinogenic activity of stilboestrol has not yet been reproduced in experimental animals, the general subject of transplacental carcinogenesis has been extensively investigated. A useful summary of current knowledge is presented in this book. Particular attention has been paid to the nitrosamines, and one group of them—the nitrosoalkylureas—has been clearly shown to act as transplacental carcinogens with the brain as the main target organ. The dose of the compound given and the stage of foetal development both appear to be crucial in determining the outcome—in particular, death or induction of malformations rather than tumours. Predictably, no tidy relationship appears to exist between teratogenic and carcinogenic activities, and many details of the actual mechanisms of transplacental carcinogenesis are still quite unknown.

The subject is, however, one of considerable potential importance. Tumours are now a major cause of death among children in advanced societies, and the