Technical method

A simple procedure for the preparation of rosetted cells for electron microscopy

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Examination of surface markers on lymphocytes from the normal peripheral circulation has revealed a heterogeneity of lymphocyte populations. Many of the surface marker tests performed in a routine clinical immunology and pathology laboratory utilise specific marker particles which adhere to receptors on the cell surface, forming a rosette which is visible by light microscopy. The ultrastructural detail of the central rosette-forming cell (CRFC) and the mode of interaction between the CRFC and its marker particles have been investigated in only a limited number of situations. In addition, the necessity for examining lymphocytes at the ultrastructural level has received new impetus after Payne and Glasser determined that lymphocytes which contain specific organelles called parallel tubular arrays represent a distinct subpopulation of lymphocytes.

We have examined many rosetted preparations at the ultrastructural level and have encountered two major technical difficulties in preparing them for transmission electron microscopy. The first involved the undesirable packing of unrosetted cells around the rosettes after centrifugation. The second involved the dissolution of the pellet during subsequent dehydration steps, often resulting in an inadequate specimen for evaluation. A survey of the literature revealed that most authors did not discuss these problems in their methodology sections. Most authors simply state that 'cell pellets were fixed, dehydrated, and embedded' for electron microscopy. We therefore present a simple technique for routinely preparing rosettes for electron microscopy which overcomes these two major technical difficulties and may be incorporated into any clinical pathology laboratory set-up.

Material and methods

Rosettes were prepared by mixing a suspension of white blood cells (1 x 10^6 cells/ml) with an equal volume of a red blood cell suspension (1-5 x 10^7 cells/ml). The volume of the final rosetted preparation for electron microscopy varied between 0.2 and 4 ml. An equal volume of 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) was then added to the rosetted cell suspension and gently mixed by drawing the suspension up and down in a glass pipette. Any pellet present was likewise resuspended. The rosetted preparations were allowed to pre-fix in the diluted glutaraldehyde for 1 hour at room temperature. The suspension was then gently centrifuged at 1000 rpm in a Sorvall GLC-1 swinging bucket clinical centrifuge for 5 minutes. The pellet was resuspended in a small aliquot of human plasma and transferred to a large (17 x 100 mm) round-bottomed plastic centrifuge tube (No. 2059 tube; Falcon, Oxnard, Calif). The suspension was again centrifuged at 1000 rpm in the swinging bucket clinical centrifuge for 5 minutes.

The supernatant was removed and replaced with cold 3% glutaraldehyde in 0.1 M phosphate buffer. The pellet was allowed to fix for 1 hour at 4°C. The fixative was then decanted and replaced with 0.1 M phosphate buffer. After two changes of buffer (15 minutes each), the pellet was post-fixed for 1¼ hours in 1% osmium tetroxide in 0.1 M phosphate buffer and dehydrated through a graded series of ethanol. The pellet was then loosened from the bottom of the plastic tube with a thin wedge-shaped wooden stick. The ethanol was replaced with a 1:1 mixture of 100% ethanol and Spurr's epoxy and allowed to infiltrate for 2-4 hours. The pellet was broken up into smaller pieces, transferred to a new vial of pure epoxy after blotting on filter paper, and allowed to infiltrate for approximately 20-24 hours at room temperature. The small pieces were then transferred to Beem capsules containing freshly mixed Spurr's epoxy and allowed to polymerise in a 70°C oven for approximately 20 hours.

One-micron sections were cut and stained with toluidine blue and examined with the light microscope. Those blocks containing numerous rosettes were selected, and ultrathin sections were cut with a diamond knife on a Sorvall MT2-B ultramicrotome and mounted on uncoated 200-mesh copper grids. The ultrathin sections, each of which almost covered the entire grid surface, were stained with 5% aqueous uranyl acetate and lead citrate and then lightly carbon-coated before examination with a Hitachi HU-12 electron microscope.

The above procedure was used with (1) human lymphocytes which form spontaneous rosettes with
sheep erythrocytes (‘E’ rosettes), (2) human mononuclear cells which form rosettes with human Rh(+) erythrocytes reacted with anti-D antibody (‘EA’ rosettes), and (3) human mononuclear cells which form rosettes with sheep erythrocytes reacted with sheep cell hemolysin and fresh human AB, Rh(−) serum as a source of complement (‘EAC’ rosettes).

Results

In every attempt to prepare ‘E’, ‘EA’, and ‘EAC’ rosettes for electron microscopy using the method described herein, the pellets remained intact during the dehydration and infiltration steps.

In all preparations examined, rosetted cells could be easily distinguished from unrosetted cells since there was no packing of red cells around the rosettes. Three rosettes can be seen in the field. The homogeneous, slightly electron dense material which surrounds the cells represents the plasma in which the cells were resuspended. (uranyl acetate, lead citrate × 2900)

Low-power electron micrograph of an ‘EA’ rosette preparation. Rosetted cells can be easily distinguished from unrosetted cells since there is no packing of red cells around the rosettes. Three rosettes can be seen in the field. The homogeneous, slightly electron dense material which surrounds the cells represents the plasma in which the cells were resuspended. (uranyl acetate, lead citrate × 2900)
surface and allowed one to examine numerous rosettes under the electron microscope at one time.

Discussion

The procedure outlined in this paper represents a simple technique which we use routinely when preparing rosettes for electron microscopy. One major technical difficulty which was overcome was the undesirable packing of unrosetted cells around the rosetted cells after centrifugation. This was accomplished by centrifuging the cell suspensions, which were resuspended in plasma, in large, round-bottomed plastic centrifuge tubes in a swinging bucket centrifuge which allowed the cells to spread out in a near monolayer in some areas at the bottom of the tube. A comparison of the area formed by the pellet of cells at the bottom of the tube revealed a larger area in the rosette preparations that were resuspended in plasma than in those without. Cells which are surrounded by a protein solution apparently do not pack as closely as those which are surrounded by buffer or fixative. Spinning the cells in smaller, round-bottomed tubes or in conical tubes caused unrosetted cells to pack too closely around the rosettes, thereby interfering with the visualisation of the true rosetted cells. The rosettes were prefixed before centrifugation to stabilise them during the centrifugation steps. In an ultrastructural study of human lymphocyte-sheep erythrocyte rosettes, Kataoka et al. \textsuperscript{14} lighted agitated their tubes before fixation to minimise the mechanical contact of the cells. This is an undesirable step since fragile rosettes could easily be disrupted. Reyes et al. \textsuperscript{15} used the technique of micromanipulation to separate the rosettes from unrosetted cells, which is very time consuming. Lay et al. \textsuperscript{16} used chamber-slides and inverted gelatin capsules of epon directly on to the slides. Chamber slides are not routinely used in a clinical immunology laboratory, and, additionally, the technique involves washing off non-adherent cells which would also result in washing off some rosettes. The inverted capsule technique is also time-consuming and is not applicable to the low-viscosity epoxy resin used here. \textsuperscript{18}

The second major technical difficulty that was overcome was the dissolution of the cell pellet during the dehydration and infiltration steps which could result in an inadequate specimen for evaluation. The object was to replace the medium surrounding the cells with a protein-rich matrix which would cross-link upon contact with glutaraldehyde and behave as a piece of tissue. Three different sources of protein were utilised including human plasma, a 7% bovine serum albumin (BSA) solution, and a 1% agar solution. Human plasma was superior to the other solutions and formed a firm gel upon contact with 3% glutaraldehyde, thereby binding the cells to each other while at the same time maintaining their spatial orientation. The 7% BSA solution, although a similar protein concentration to human plasma, \textsuperscript{17} did not form as firm a gel with glutaraldehyde as the plasma and, in addition, was difficult initially to get into solution; it is also relatively expensive. The 1% agar solution is a solid at room temperature and must be heated in order to resuspend the cells. Care must also be exercised to prevent premature cooling and trapping of cells in the gel before they can be spread at the bottom of the centrifuge tube. Because of these difficulties in working with BSA and agar, human plasma was preferred.

Abramson et al. \textsuperscript{18} used a technique developed by Anderson and Doane \textsuperscript{19} for the ultrastructural examination of rosettes which employs a commercially available vinyl cup. No details were given for preventing loss of material during the fixation and dehydration steps. In addition, Anderson and Doane state that difficulty in stripping the vinyl cup from the polymerised plastic was encountered when the temperature of polymerisation exceeded 60°C. The Spurr's epoxy resin we use requires polymerisation at 70°C. Therefore, our technique for preparing rosettes is simple and reproducible and can be used with any epoxy or other plastic resin. One advantage, however, in using Spurr's epoxy is the large block face which can be thin-sectioned, enabling many rosettes to be examined under the electron microscope at the same time. Embedding the thin cell pellet in Beem capsules as opposed to flat embedding moulds is recommended in order to obtain large surface areas for cutting.

We feel that this technique can be easily adapted to any clinical or research laboratory setting and will enable valuable material to be consistently preserved for ultrastructural evaluation.

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**Letters to the Editor**

**Use of Intralactam for detection of β-lactamase production by Neisseria gonorrhoeae**

In your Journal of July 1979 (p 738), DB Wheldon and Mary PE Slack reported an evaluation of Intralactam for the detection of β-lactamase production by *Haemophilus influenzae*. At the end of their paper they commented that it might also be used to detect gonococcal β-lactamase.

We have used this acridometric method to detect β-lactamase production in primary isolates of *Neisseria gonorrhoeae* for the past year. Initially we prepared paper strips impregnated with penicillin and indicator, as described by Slack et al., but then began using the commercially available Intralactam.

All confirmed isolates of *N. gonorrhoeae* have minimum inhibitory concentrations (MIC) determined to penicillin, spectinomycin, and tetracycline. At first, testing for β-lactamase production was limited to isolates having an MIC to penicillin of > 2.0 mg/l. (Exact quantification of the MIC value above 2 mg/l was not performed.) Of 2357 *N. gonorrhoeae* isolated in this laboratory from clinical specimens in 1978-79, 38 organisms (1.6%) had an MIC to penicillin of > 2.0 mg/l. These 38 organisms had a β-lactamase determination performed, the majority using Intralactam strips, but a small number using our own prepared strips with penicillin and neutral red incorporated, as described by Slack et al. Of these 38 organisms, 36 were identified as β-lactamase producers. However, as MIC results and identification of β-lactamase production were not usually available to the clinicians until four days after primary isolation, we decided to perform β-lactamase determination on all isolates using Intralactam strips at the same time as a presumptive diagnosis of *N. gonorrhoeae* was made.

We now provide clinicians with a presumptive diagnosis of *N. gonorrhoeae* if it is oxidase-positive, Gram-negative diplococci are present on primary isolation plates after 18-24 hours incubation, and at the same time report the presence or absence of β-lactamase production. The primary isolation medium used is modified New York City Medium. We have used Intralactam strips to...