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Methyl green-pyronin stain for the diagnosis of trachoma

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The clinical diagnosis of TRIC-agent infection of the conjunctiva is not difficult for ophthalmologists in endemic areas such as Egypt, but the similarity of the disease to other types of follicular conjunctivitis makes laboratory investigations especially important (Thygeson, 1960; Duke-Elder, 1965). These include the staining of conjunctival scrapings with Lugol's iodine or Giemsa stain for demonstration of the inclusion bodies of Halberstaedter and von Prowazek (1907) in the cytoplasm of infected cells (Rice, 1936; Gilkes, Smith, and Sowa, 1958; Dunlop, Jones, and Al-Hussaini, 1964; Al Hussaini, Jones and Dunlop, 1964), and the use of the fluorescent antibody technique has recently attracted interest (Nichols, Bobb, Haddad, and McComb, 1967; Surman, Hardy, and Howarth, 1967). Isolating the TRIC-agent or finding the inclusions are the most conclusive means of diagnosis; complement-fixation tests give variable results (Barwell, Dunlop, and Race, 1967), and intradermal tests are usually negative (Barwell and others, 1967).

All these methods require an experienced investigator familiar with virological methods and very well equipped laboratories, which are not always available in developing countries. The demonstration of the inclusions by Giemsa stain is time-consuming and does not differentiate the colours. To isolate the TRIC-agent two or three egg passages are sometimes required, and this may take about a month. Moreover, Giemsa-stained smears from infected yolk sacs sometimes present artefacts of fine and coarse deposits which make it difficult to read the slides.

It is obvious, therefore, that easier and quicker methods of laboratory diagnosis are needed.

Histochemical stains have been successfully used to detect viral particles in other conditions, such as rabies (Shokeir and Elbagoury, 1966). Pollard and Tanami (1962) have used acridine orange to study the replication of TRIC-agent in tissue cultures.

Since TRIC-agent contains both desoxyribonucleic acid and ribonucleic acid (Jawetz, 1964), infected cells containing inclusion bodies at any stage of development may be expected to react with stains specific for nucleic acids. For this reason we selected methyl green-pyronin stain as described by Brachet (1942, 1953) and Gurr (1958) to demonstrate TRIC-agent in conjunctival scrapings and yolk-sac smears.

Material and Methods

Active trachoma was clinically diagnosed in 129 patients at the Assiut University Eye Clinic during 1967. Scrapings were obtained from the upper palpebral conjunctiva of both eyes under local anaesthesia, and each specimen was spread on four to six microscopic slides which were then stained

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with Lugol's iodine, Giemsa, and methyl green-pyronin. A specimen was also placed in sucrose potassium glutamate solution (SPG) for isolation of the TRIC-agent by egg inoculation (Litwin, 1962).

Fixation Slides to be stained with Giemsa or by Brachet's method were fixed before staining with a drop of absolute ethyl alcohol and left to dry in air.

Staining with iodine A drop of Lugol's iodine was poured on the slide and left for 5 min., after which the preparation was blotted with filter paper and examined.

Staining with Giemsa Slides were immersed in a 5 per cent. solution of Giemsa stain (Merck) in 0·01 M phosphate buffer pH 6·8 for 24 hrs, washed with the same buffer, blotted with filter paper, and examined.

Staining with methyl green-pyronin The staining solution was prepared by taking 0·5 g. methyl green (BDH) which had been washed repeatedly with chloroform to remove the traces of methyl violet which form spontaneously when it has been in contact with air for any length of time. After the residual methyl green had been filtered and dried, 0·15 g. was taken and mixed with 0·25 g. pyronin Y, and dissolved in 2·5 ml. 95 per cent. alcohol and 97·5 ml acetate buffer pH 4·7 (Gurr, 1958). This solution is stable at room temperature when kept in a clean glass bottle and can be used repeatedly for several months.

After fixation with absolute alcohol, conjunctival scrapings or yolk-sac smears were immersed in the stain for 20 min., rinsed quickly with distilled water, differentiated in 95 per cent. alcohol for 1 min., blotted with filter paper, and examined. Over-differentiation with alcohol should be avoided to prevent the colours fading.

Results

Iodine stain Cap-shaped granular inclusions, mahogany brown in colour on a yellow ground, indicate a positive reaction for glycogen. Only well-formed inclusions can be demonstrated by iodine stain, as it is the matrix of the inclusion body which contains glycogen. Also this method does not permit the demonstration of the cell boundaries of either epithelial or inflammatory cells.

Giemsa stain The nuclei of epithelial conjunctival cells appear violet, while the inclusion bodies appear as bluish cytoplasmic cap-shaped formations. In some instances other forms of these inclusions can be seen, and pseudo-inclusions may be mistaken for true ones (Al-Hussaini and others, 1964). Smears prepared from infected yolk sacs show fine violet granules representing free elementary bodies, but artefacts from yolk-sac granules and stain deposits may confuse the picture.

Methyl green-pyronin stain Conjunctival scrapings show a sharply contrasting picture; the cytoplasm of epithelial and inflammatory cells is red (pyroninophilic), while the nucleus takes the green to violet colour. The inclusions are easily differentiated (Figs 1 and 2), as they appear as bright deep pink to brick-red granular cytoplasmic bodies. Initial bodies and free elementary bodies are thus easily distinguished. The inflammatory exudate, formed of lymphocytes, plasma cells, polymorphs, and Leber cells, is also clearly seen. Such formations are not observed in scrapings from control conjunctivae from trachoma-free subjects.

Infected yolk-sac smears show many fine granules stained bright deep pink to violet (Fig. 3). Such formations are not observed in smears from uninfected control embryos of

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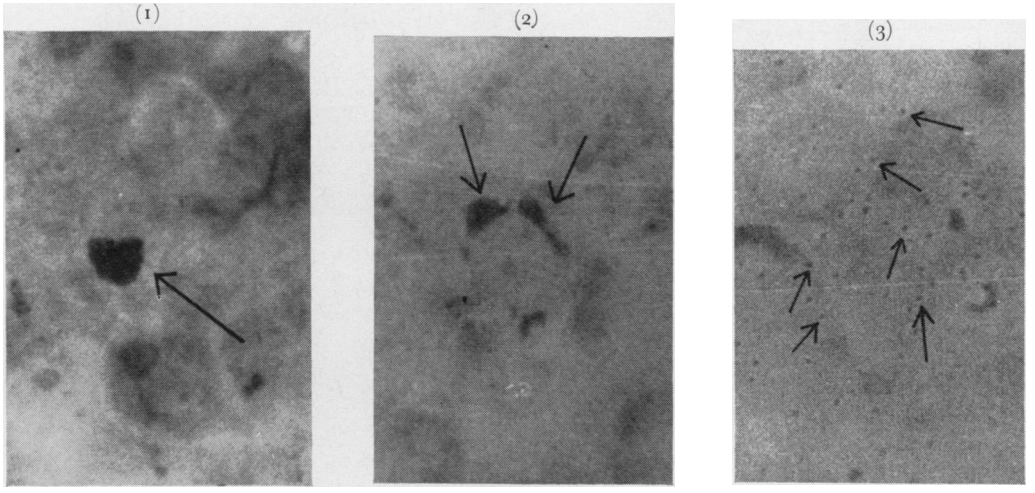

FIGS 1 and 2 *Conjunctival scrapings from trachoma patients, stained with the methyl green-pyronin stain, showing Halberstaedter-Prowazek inclusion bodies.* ×1,600

FIG. 3 *Smear from yolk-sac of egg embryo infected with trachoma, stained with the methyl green-pyronin stain, showing free elementary bodies.* ×1,600

the same age. This finding suggests that the fine granules seen in infected preparations are the free elementary bodies.

For this procedure good fixation is necessary, otherwise the preparation will not take the stain readily, and over-differentiation in alcohol or long exposure to day-light may cause the colours to fade.

Discussion

Iodine stain demonstrates mature inclusion bodies, but is not so useful for detecting earlier stages in the life cycle of the organism, and fails to show the cytological pattern of the scraping. In scrapings stained by Giemsa, positive cases may be missed, and microscopic reading of the stained preparation may take as long as half an hour. The whole procedure may occupy 24 hours. Methyl green-pyronin stain quickly demonstrates inclusion bodies in conjunctival scrapings with bright contrasting colours, and also avoids artefacts in yolk-sac smears. The procedure takes only 20 to 25 min., and the solution can be used repeatedly for several months and is stable at room temperature if kept in a properly stoppered glass container.

Summary

Methyl green-pyronin stain may be used routinely for the diagnosis of TRIC-agent infection in busy ophthalmic clinics and does not require very highly trained staff or elaborately equipped laboratories. It enables inclusion bodies to be clearly seen in conjunctival scrapings from trachoma patients, and infected yolk-sac smears show deep pink to violet elementary bodies. The procedure has proved to be simpler and much quicker than the iodine or Giemsa staining previously employed.

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