Archiv für die gesamte Virusforschung 26, 97—104 (1969)

Virology Section, University Department of Microbiology,
Belvidere Hospital, Glasgow, E. 1., Scotland

A Rapid and Reliable Immunofluorescent Method for the Identification of Common Virus Infections of the Skin

By

R. G. Sommerville

With 2 Figures

(Received July 25, 1968)

In establishing the laboratory diagnosis of infection with a "pox" virus (variola, vaccinia, cowpox), the two major pre-requisites are speed and accuracy.

Electron microscopy (1—3) offers reasonable prospect of success on both points, although electron microscopic facilities are not widely available in many countries. The immunofluorescent technique applied directly to skin scrapings or exudate obtained from the clinical suspect should, in theory, offer excellent prospects for success. In practice, (4), the results are not reliable because of the frequent occurrence of "false negatives" and "false positives".

"False negatives" are most often caused by insufficient antigenic material in the clinical specimen. This means that, although the correct immunological reaction has taken place on the microscope slide, fluorescent particles are too few in number to be recognised. "False positives" are most often caused by a non-specific reaction due to material on the slide which reacts with fluorescein. Both false reactions produce confusion in the often dramatic circumstances attending small-pox diagnosis.

The diagnosis by immunofluorescent staining is therefore regarded with scepticism and its great advantage of speed is potentially lost while confirmatory, conventional virus laboratory tests are performed.

In an effort not to lose the entire advantage of speed, while improving accuracy to acceptable levels, the following technique has been evolved. It is based on a short incubation of suspect clinical material in tissue cultures of mixed cell type and the method also incorporates a number of modifications on the standard patient-to-slide technique. The details are critical and the technique is therefore described in full.

Method

Clinical Specimens

These usually consist of vesicle fluid in capillary glass tubing, crusts or skin scrapings. The specimen should be gently homogenized in 1—2 ml Hanks' or Eagle's tissue culture medium containing penicillin and streptomycin at 200 U. and 100 μg/ml, respectively. If unfixed smears are submitted for examination, part of the material can be eluted from the slides by gentle washing with tissue culture medium.

Tissue Culture

These are prepared in flat-based screw-capped half-drachm vials. To ensure even better growth of the tissue culture cells a sterile 12 mm circular coverslip may be placed in the vial, to form a false bottom before the cells are inoculated. Each vial receives an inoculum of 5×10^4 cells each of the following types: (1) secondary rhesus or cynomolgus monkey kidney, (2) secondary human amnion, (3) HEp-2 cells. The total cell inoculum is suspended in 1.0 ml Eagle's basal medium supplemented with 10% fetal bovine serum and containing penicillin and streptomycin. The cells are allowed to settle on the plane surface at the base of the vial for 4—24 hours. Monolayer formation is neither necessary nor desirable.

Inoculation of Specimen

The medium is removed from a single vial and 1.0 ml of the clinical specimen suspended in Eagle's medium is introduced. The re-capped vial is then centrifuged at $500 \times g$ for 20 minutes at room temperature. The supernatant is replaced by 1.0 ml Eagle's medium supplemented with 10% fetal bovine serum and the vial is incubated for 16 hours, stationary and upright.

Preparation for Fluorescence Microscopy

After 16 hours incubation, the medium is removed from the culture, and may be retained. 1.0 ml of 1:20,000 versene solution (ethylene-diamine-tetra-acetic acid) is added to the vial and left for 5 minutes with

occasional gentle agitation at room temperature. During this time the cell-sheet is dispersed into single cells. The suspended cells are deposited by low-speed centrifugation (500× *g* for 5 minutes) and are re-suspended in 0.2 ml of phosphate buffered saline pH 7.2 (PBS).

Four replicate 5 mm spots of cells are placed on a standard 3″×1″ microscope slide, and air dried. The slide is fixed in acetone for 2 minutes at room temperature and may be exposed, after drying, to ultraviolet light (2857 Å) at 5 cm from the UV source for 10 minutes.

Fluorescent Staining

The "spots" are stained by the indirect immunofluorescent technique as follows:

One spot each is covered by rabbit hyperimmune serum at appropriate dilution to Pox group (Vaccinia), Herpes simplex and Varicella-zoster viruses. The fourth spot, which serves as a negative control for the others, is covered by non-immune rabbit serum. The slide is incubated in a humidified chamber at 37°C for 20 minutes. It is then washed in PBS for 10 minutes with continual agitation from a magnetic stirrer, and with a minimum of two changes of PBS.

Anti-rabbit globulin, conjugated with fluorescein isothiocyanate is applied to all spots. In the author's laboratory this globulin is prepared from mouse ascitic fluid which, in turn, is prepared by an ascites-inducing technique, in albino mice (5, 6). It routinely has naphthalene black TS powder added at a concentration of 1 mg per ml of undiluted conjugate, as counterstain. The fluorescent globulin:counterstain solution is incubated on the slide for 20 minutes at 37°C, before washing in PBS as above. The slide is rinsed finally in de-ionized water and is airdried. It is examined unmounted.

Fluorescence Microscopy

At this stage of the technique either mercury vapor (HBO-200) or quartz-iodine illumination can be used. The individual cells in the "spots' are examined ideally with a ×6 eyepiece; ×50 fluorite oil-immersion objective; dark-ground oil-immersed condenser.

Optimal conditions for microscopy are achieved with the following filters:

Exciter filters: Kodak Wratten gelatin filters — 32 and 38A,

Agfa-Gevaert gelatin filter R 438 E 10.
(The three filters should be bound together between cover-glasses.)

Barrier filter: Kodak Wratten gelatin filter —12.

Results

Using conditions precisely as detailed above, virus infected cells can be identified easily by the green-coloured specific fluorescence which is invariably contained in the cytoplasm. If a pox-virus is present the infected cells in the "spot" treated with antivaccinial serum show a distinct perinuclear halo and may also contain discrete aggregates of bright fluorescent material. If the virus is Herpes simplex or a Varicella-zoster virus, the appropriate "spot" will contain fluorescent-staining cells. In these instances, the fluorescence is usually more diffuse and lies in the

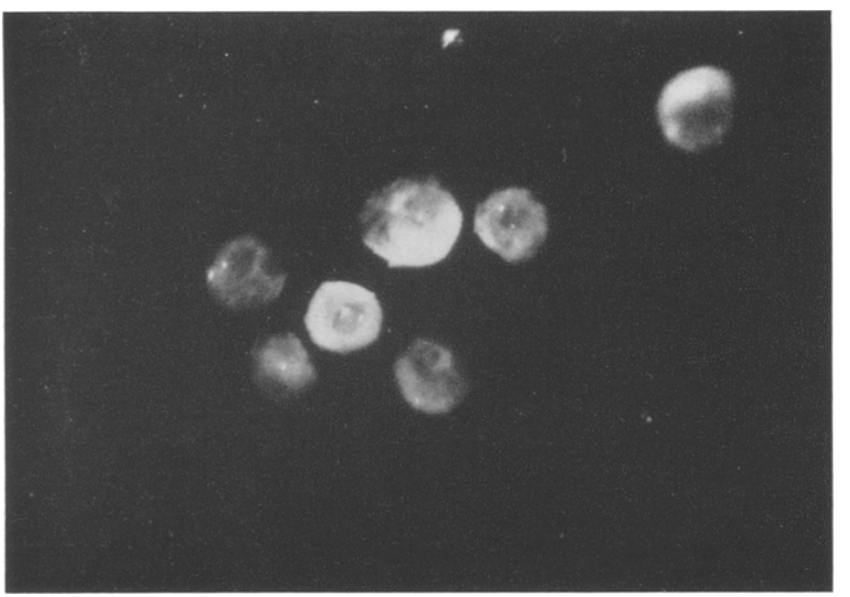

Fig. 1. "Mixed cell" tissue culture showing positive immunofluorescence staining pattern with rabbit hyperimmune antivaccinia serum (×560).

peripheral zone of the cytoplasm. In either case the fluorescent cells can be distinguished with ease because the filter combination described above, when used with the counterstain, produces a red colour in the cytoplasm of non-infected cells, with a brown colour in the nucleus. This colour contrast enhances the ease of identification of positive specimens.

The appearances which may be expected are shown in Figs. 1 and 2.

The opportunity has presented to examine this system in clinical practice in the following instances: Vaccinia —6, Varicella-zoster —4, Herpes simplex —25. In every instance a positive result was obtained

easily and within 24 hours. Each positive was subsequently confirmed by virus isolation from the supernatant tissue culture fluid after re-incubation in mixed cell tissue cultures.

In the immunofluorescent test cross-reactions between Pox group and Herpes-Varicella-zoster viruses were not encountered. Non-specific fluorescence was not encountered.

Discussion

The object in describing this method has been to present a system by means of which the immunofluorescent technique, while losing some

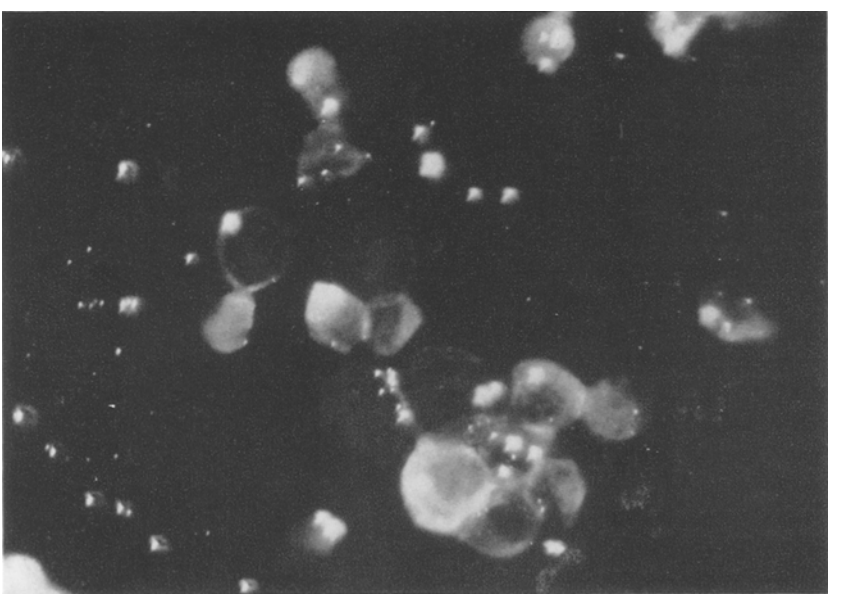

Fig. 2. The appearance of "mixed cell" tissue culture showing typical immunofluorescence pattern of Herpes simplex after staining with rabbit hyperimmune serum (× 560).

of its speed, gains appreciably in specificity and accuracy in the diagnosis of the common virus infections of the skin.

The basic problem in applying immunofluorescent methods directly to skin exudate has always been the potential presence of non-specific fluorescence. This problem arises because the exudative material contains, or is coated with, protein. Fluorescein isothiocyanate has a marked affinity for protein, if the former is present in a free state. In a properly prepared immunofluorescent-globulin the ratio of fluorescein to globulin must be

kept to a minimum to avoid the possibility of free (*i.e.* uncoupled) fluorescein being present in the globulin solution. Passage of the fluorescein-globulin solution, after conjugation, through columns packed with Sephadex (6) will remove uncoupled fluorescein, but even under ideal conditions and after free fluorescein removal, dissociation may take place in the solution when the temperature is raised to 37°C (7). If this happens, free fluorescein then becomes available for combination with other proteins present in the exudative material.

It thus seems unlikely that the problem of non-specific fluorescence can be overcome completely in attempts to utilize immunofluorescence for diagnostic purposes, directly on material obtained from lesions of the skin. The incorporation of a counterstain into the fluorescent layer of the sandwich for the indirect technique offers some practical help in the diminution of the non-specific fluorescence problem, but even this alone will not provide a complete guarantee of removal; nor will it help the "false-negative" problem.

The complete answer lies in allowing a short incubation period in tissue culture and also in aiding the "hit-potential" between virus and tissue culture by using centrifugal force (7). Any serum which may be present in the tissue culture medium is to a great extent removed by the versene resuspension of the tissue culture cells. The laboratory worker is presented with a specimen for immunofluorescent examination which contains known cellular forms only, and an additional control for the presence of non-specific fluorescence is introduced by treating one "spot" of identical cells on the slide with non-immune rabbit serum before staining with the fluorescent globulin: counterstain solution.

It may, at first, appear strange that the tissue cultures recommended should consist of a mixture of three cell types. This technique is based on a recommendation by *Hayflick* (8) and is now standard practice in this laboratory. The cells grow well together in tubes and, in mixture as described, offer the advantage of optimal sensitivity for the viruses which are likely to be encountered from the common skin lesions. It may be argued that inclusion of a human diploid cell in the mixture would offer advantages over HEp-2 cells. Preliminary experiments have not indicated a significant advantage, however, and the inclusion of human diploid cells in the mixture adds the potential complication of altered growth and maintenance medium requirements for this type of cell.

As to the time required for the method of examination described here, it should be remembered that immunofluorescence, applied directly to scrapings from skin lesions, at the moment requires confirmatory laboratory testing — usually complement fixation tests and often egg inoculation. These confirmatory techniques require a minimum of 24 and 48

hours respectively. The technique described here will give a clear-cut result in 20 hours and has the advantage that additional material is available, in the form of the supernatant fluid from the overnight incubation of the tissue culture phase of the method. This can be used for further virus characterization studies in tissue culture or in fertile eggs.

Summary

Improvements to the immunofluorescent identification of common virus infections of the skin are described. These include preliminary incubation of the clinical specimen in tissue cultures of mixed cell type for 16 hours; transfer of the cells from the original container to microscope slides; incorporation of a counterstain into the immunofluorescent serum; modifications to the standard exciter filters on the fluorescence microscope.

These changes in technique produce excellent, easily read specimens within 24 hours in which the cellular elements have normal morphology and in which the common pathogenic viruses of the skin can easily be identified. The virus-infected cells are recognized from their green immunofluorescent staining, and contrast well against a background of red-brown negative cells. The method eliminates the dangers which are inherent in the direct immunofluorescent examination of skin-scrapings.

Acknowledgement

The work described here was performed with the help of a grant from "Action for the Crippled Child", London. The generosity of the Fund in providing this grant is gratefully acknowledged.

References

1. *Nagington, J.*, and *A. D. Macrae:* Smallpox diagnosis and electron microscopy. Mth. Bull. Minist. Hlth Lab.-Serv. **24,** 382—385 (1965).
2. *Gordon, C. W., J. D. Donnelly, R. Fothergill, F. L. Ker, E. L. Millar, T. H. Flewett, H. S. Bedson,* and *J. G. Cruickshank:* Variola minor. Lancet **i,** 1311—1313 (1966).
3. *Cruickshank, J. G., H. S. Bedson,* and *D. H. Watson:* Electron microscopy in the rapid diagnosis of smallpox. Lancet **ii,** 527—530 (1966).
4. *El-Ganzoury, A. L. A.:* Evaluation of the fluorescent antibody technique for the diagnosis of smallpox. J. clin. Path. **20,** 879—882 (1967).
5. *Sommerville, R. G.:* The production of fluorescent antibody reagents for virus diagnosis in the albino mouse. I. Hyperimmune anti-species serum. Arch. ges. Virusforsch. **20,** 445—451 (1967).
6. *Sommerville, R. G.:* The production of fluorescent antibody reagents for virus diagnosis in the albino mouse. II. Coupling of mouse immune globulin with fluorescein isothiocyanate (FITC). Arch. ges. Virusforsch. **20,** 452—458 (1967).

7. *Hahon, N.:* Assay of variola virus by the fluorescent cell-counting technique. Appl. Microbiol. **13,** 865—871 (1965).
8. *Hayflick, L.:* Personal communication.

Author's address: *R. G. Sommerville,* M. D., M. C. Path., Belvidere Hospital, Glasgow, E. 1., Scotland.