Evaluation of the fluorescent antibody technique for the diagnosis of smallpox¹

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SYNOPSIS Smears on slides taken from 44 patients suspected of having smallpox were examined for the presence of smallpox antigens and from 15 of them for chickenpox antigens also, by the fluorescent antibody method, using the conventional 'sandwich' and the complement techniques. In seven, very strong non-specific fluorescence made the result unreadable. When staining for smallpox antigen results agreeing with the diagnoses established by other methods were obtained in 36 of 37, and when staining for chickenpox in 12 of 13 specimens examined. One false positive diagnosis of smallpox and one false negative of chickenpox were made. Because of intense non-specific staining no diagnosis could be made from other smears stored for three years. It was not possible to reach a diagnosis by examination of crust suspensions spread on slides. In five fresh specimens examined during the outbreak there was hardly any non-specific fluorescence and results could more easily be read. Morphological features as seen by fluorescence microscopy are described. The value of the method in the diagnosis of smallpox is discussed.

It was shown by Avakyan, Al-Tshtein, Kirillova, and Bykovskii (1961) and by Murray (1963) that smallpox antigen and elementary bodies could be specifically stained by the fluorescent antibody method using either the direct or 'sandwich' techniques. However, the occurrence elsewhere of at least one false-positive result which caused the erroneous diagnosis of smallpox in a patient eventually shown to have chickenpox has cast doubt on the value of the method for use in clinical diagnosis. Material from an outbreak of variola minor in the Midlands in the summer of 1966 provided an opportunity for further evaluation of fluorescent antibody methods. Both the indirect 'sandwich' and the more recently introduced complement technique have been investigated. The results are reported in this paper.

MATERIALS AND METHODS

SPECIMENS Smears from five patients were made at the time of the Midlands outbreak and examined within 48 hours. Three of these patients had smallpox and two were suffering from eczema vaccinatum.

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Smears from 44 smallpox suspects were made during the outbreak but not examined until afterwards: these were smears surplus to the requirements for other diagnostic tests and had been stored unfixed first at 4°C. and then at room temperature for periods varying from one to seven months. The final diagnoses of these patients were known with reasonable certainty, those of chickenpox and smallpox having been established as described elsewhere (Gordon, Donnelly, Fothergill, Ker, Millar, Flewett, Bedson, and Cruickshank, 1966; Cruickshank, Bedson, and Watson, 1966).

A collection of unfixed slides made in East Africa three or more years ago from patients diagnosed clinically as variola major or minor was available: the diagnosis had been confirmed by egg culture in nine of the 11 cases examined. These slides were made available through the kindness of Professor K. R. Dumbell.

A series of six smears had been made from extracts of crusts from four patients with alastrim and crusts from two patients with chickenpox: the crusts had been stored at +4°C. for periods of up to eight months.

In each case the slides were labelled either by number or with the patient's name, and fluorescent antibody tests were carried out and read before the diagnosis was revealed. This enabled an objective assessment of the reliability of the technique to be made.

FIXATION

FIXATION Slides were disinfected by ultraviolet irradiation

tion for two minutes at a distance of 2 to 3 cm. from a Hanovia Chromatolite model no. 2015 and were then fixed in acetone for 10 minutes at -20°C .

Preliminary studies had shown that acetone was ineffective in inactivating variola virus and that ultraviolet irradiation as specified was satisfactory.

SERA Serum from rabbits hyperimmunized with cowpox virus was used at a dilution of 1:5 in phosphate-buffered saline. This serum at a dilution of 1:10 gave a strong specific staining when used in preliminary studies with smears made from a preparation of variola virus.

Human convalescent serum from a case of herpes zoster was used at a dilution of 1:4 in phosphate-buffered saline for testing the presence of chickenpox antigen. Preliminary experiments with smears from known cases of chickenpox had proved its potency at a dilution of 1:8.

'Normal' rabbit and human sera were used as controls at a dilution of 1:5 in phosphate-buffered saline.

CONJUGATES Fluorescein isothiocyanate conjugated goat anti-rabbit globulin and rabbit antihuman globulin (Burroughs Wellcome) were used for indirect staining at a dilution of 1:8.

Rabbit anti- β 1C guinea-pig globulin (Stratton 1966) was conjugated with fluorescein-isothiocyanate according to the method described by Lee, Davidsohn, and Takahashi (1965) and used at a dilution of 1:4 for complement staining. Before use both sera were absorbed once with acetone-extracted human placenta powder.

STAINING The number of smears from each patient was not as a rule sufficient to carry out the different stainings and controls on separate slides. Smears were therefore divided into separate areas by walls of Glyceel (G. T. Gurr Ltd.). This also helped to create hollow compartments which contained the antisera used for staining.

The indirect method was applied as described by Weller and Coons (1954) and the complement method as described by Hinuma, Miyamoto, Ohta, and Ishida, (1963).

MICROSCOPY AND PHOTOMICROGRAPHY The specimens were examined with the aid of a Vickers Patholux microscope fitted with a 100 w quartz-iodine lamp. The illuminating light was filtered through a 30/063 (Gillett and Sibert) excitation filter 1.5 mm. thick, and focused by an immersion dark ground condenser; a 10/285 (Gillett and Sibert) yellow orange filter was fitted above the objectives. An EEL microphotometer was used to help in determination of photographic exposures for recording the results on a Kodak high speed Ektachrome daylight film.

RESULTS

All the smears which had been taken from the 11 patients in East Africa three years previously gave such intense non-specific fluorescence that no specific staining could be detected. Non-specifically

stained fluorescent fragments were found in both the test and control smears made from crusts; no specific staining could be identified in any specimen.

The results of staining for antigens in the vaccinia-variola group with the stored smears from 44 patients in England are shown in Table I. There was no

TABLE I
RESULTS OF FLUORESCENT ANTIBODY TESTS
WITH COWPOX ANTISERUM ON
44 STORED SPECIMENS

Diagnosis	No. of Patients	Positive	Negative	Non-specific
Smallpox	13	11	0	2
Chickenpox	14	0	11	3
Others	17	1	14	2
Total	44	12	25	7

difference in the results given by the indirect and complement methods. In seven of the specimens there was again intense non-specific fluorescence and no diagnosis could be made. Two of these were from patients with smallpox. Of the remaining 37, 12 were positive by both methods; 11 of these had been confirmed by electron microscopy and egg inoculation. The twelfth case had not been so confirmed; no definite pathogen had been identified and no firm clinical diagnosis had been made, but it is extremely unlikely that it was a case of smallpox. This must therefore be regarded as a false-positive result. In the remaining 25 specimens, results were negative with both methods. None of these came from patients with smallpox. The controls were consistently negative.

From only 15 of the 44 patients was sufficient material left for tests to be made with zoster serum. Of these, two gave such intense non-specific fluorescence that a diagnosis could not be made. Six were found positive and seven negative. With the exception of one false-negative result these findings (Table II) were in accordance with the diagnoses previously established by electron microscopy.

TABLE II
RESULTS OF FLUORESCENT ANTIBODY TESTS WITH
ZOSTER SERUM ON SPECIMENS FROM 15 PATIENTS

Diagnosis	No. of Patients	Positive	Negative	Non-specific
Smallpox	4	0	3	1
Chickenpox	8	6	1	1
Others	3	0	3	0
Total	15	6	7	2

The fresh smears taken and stained during the outbreak were examined only for antigen of the vaccinia-variola group. All five were diagnosed correctly, strong specific staining being observed with both indirect and complement techniques.

In addition to the specific fluorescence the morphological characteristics seen after fluorescent antibody staining may be helpful as a guide to the correct diagnosis. In smallpox specimens irregular areas of bright specific fluorescence could usually be seen, and also numerous points of bright fluorescence, probably elementary bodies, which did not appear in the control preparations. In chickenpox strongly fluorescent granular cellular elements may be of assistance in arriving at a correct diagnosis. These very characteristic objects have also been observed in similarly stained scrapings from cases of herpes zoster. Small points of faint fluorescence, possibly individual virus particles, were seen in chickenpox smears, but were not as bright as the small particles seen in the smallpox smears.

DISCUSSION

Acetone fixation, at any rate for the brief periods used for fixation of smears for fluorescent antibody tests, failed to kill smallpox virus. Infectivity could not be detected after ultraviolet irradiation as specified above. Nevertheless care should be taken with slides so treated in case an occasional particle may have been shielded from irradiation. The investigation described here was therefore carried out with all precautions necessary for the safe handling of live smallpox virus.

Considerable difficulty was caused by non-specific fluorescence which was to some extent a problem with all specimens except the five examined when fresh. The intensity of fluorescence was such as to render useless all 11 East African smears examined and seven of the 44 smears saved from the Midlands outbreak. Old preparations are known to fluoresce much more than fresh ones. Nairn (1964) and Kopp (1963) found that the cytoplasm of dried, but not of fresh, leucocytes produced non-specific fluorescence. Tissues stored unfixed and at room temperature are more likely to give this reaction than those kept frozen at -20°C . (R. G. Sommerville, personal communication, 1965). It may be, therefore, that much of the difficulty encountered in the present investigation was due to the age of the specimens and their condition of storage. However, non-specific staining has been reported even with fresh smears prepared from pustular or scabbing lesions (Kirsh and Kissling, 1963) and in the present series no definite relationship could be established between length of storage and the non-specific fluorescence observed.

Several authors have claimed from controlled experimental studies that the complement method is more sensitive than the indirect technique (Goldwasser and Shepard, 1958; Carski, 1960;

Hinuma and Hummeler, 1961) and this difference has been confirmed with other antigen-antibody systems (El-Ganzoury, 1967, unpublished). Nevertheless, there was no demonstrable advantage to the complement method in the present series; identical results were obtained with the two methods throughout. The specimens examined were, however, to some extent selected in that they came only from those patients of a series of 90 where the residual material was sufficient to permit fluorescent antibody tests. These specimens may well have been taken, therefore, from the more florid cases. This point has also to be borne in mind when assessing the results of the trial. The agreement between the results of fluorescent antibody tests and the established diagnoses was in general very reasonable. Correct results were obtained with the anti-cowpox serum in 41 of 42 tests and with the zoster serum in 12 of 13 tests. The occurrence of an occasional false-negative result is of little significance, but the finding of a false-positive result for smallpox is much more serious and emphasizes the danger of placing reliance on fluorescent antibody tests for the rapid diagnosis of smallpox. Hearsay has it that such false-positive results have occurred in other hands, but unfortunately no record of them has been published. The technique has the further failing that it is useless with crusts, at any rate those which have been stored for any length of time.

Egg inoculation still remains the most reliable technique for the diagnosis of smallpox but it takes at least two to three days to provide an answer. The advantages of electron microscopy for rapid diagnosis in this situation have been clearly demonstrated (Peters, Nielsen, and Bayer 1962; Cruickshank *et al.*, 1966), but the availability of this method may be limited by the expense of the equipment involved and by the requirement for an experienced microscopist. Fluorescence microscopy does not suffer from these limitations and the results presented here suggest that it is a very sensitive technique for the diagnosis of smallpox and chickenpox. For the moment, however, the unexplained occurrence of false-positive results precludes its universal adoption for this purpose.

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