what these declines in tuberculin sensitivity mean in terms of protection against tuberculosis.

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X HUMAN ORF X

ISOLATION OF THE VIRUS BY TISSUE CULTURE

BY

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[WITH SPECIAL PLATE]

Orf, or contagious pustular dermatitis (C.P.D.), is a virus disease of sheep which mainly affects lambs and has a seasonal incidence in the spring months. The oral mucosa is most commonly affected, though spread may occur to other mucosal surfaces and the skin. The lesion begins as a vesicle, which becomes encrusted and heals after several weeks. Sometimes secondary infection produces ulceration and it may be fatal, though the mortality rarely exceeds 1% (National Veterinary Medical Association, 1944).

In man orf is considered rare and is confined to those in direct contact with sheep or their carcasses (Hodgson-Jones, 1951). Human infections were described by Hansen in 1879, but not until 1937 were they reported in Great Britain (Peterkin, 1937). Characteristically, a single lesion occurs on a finger, and less frequently multiple lesions occur. Little discomfort is produced in the absence of secondary infection, general symptoms are mild or absent, and resolution takes place in four to eight weeks. Transmission from person to person has not been recorded.

Diagnosis has usually been based on the clinical picture and the history of contact with sheep, because of the lack of a readily available laboratory test. The virus is inconstant in the production of change in the chick embryo (Lyell and Miles, 1950; Greig, 1956; Abdussalam, 1957); rabbits, mice, and guinea-pigs (Bennett *et al.*, 1944; Greig, 1956; Abdussalam, 1957) have proved of little value for its isolation, and serological methods such as complement fixation may be of only limited value (MacDonald, 1951). Though the laboratory virus strains can be grown in tissue cultures of sheep, goat, and ox cells (Plowright *et al.*, 1959), isolation has usually required the inoculation of sheep with the infectious material (Glover, 1928; Lloyd *et al.*, 1951). As this is not a method likely to be used in laboratories concerned with the diagnosis of human

infections, the following description of the isolation of the virus from two patients and its identification may be of interest. It was found that the virus can be grown in primary human amnion tissue cultures and that it produces a characteristic cytopathic change, one result of which is to provide a means of titration of neutralizing antibody.

Clinical Details

Case 1

The first patient was a woman who helped to look after a flock of about 100 sheep during the spring of 1961. The flock suffered a severe outbreak of orf immediately after the introduction of several fresh animals, and sheep up to 2 years old were affected as well as the lambs. The death of at least two lambs was attributed to it. The patient had bottle-fed these and other lambs with infected mouths

FIG. A.—Case 1. Right thumb of patient showing paronychia type of lesion of four weeks' duration. (Normal size.)

for some weeks before she noticed that a painless inflammatory paronychia of the right thumb was developing (Fig. A). The condition remained painless, and she did not seek medical advice until the fourth week, when the lesion was incised and a small quantity of serous exudate removed for culture. The lesion did not progress further and gradually resolved.

Case 2

The second patient was a trainee farmer aged 16 years whose duties in the spring of 1961 included handling sheep when they were "dipped." Though this flock was not known to be infected with orf, a number of the lambs were suffering from an ulcerated condition of the mouth. Several weeks after first coming into contact with the sheep he noticed a blister on the lateral surface of the left little finger. It remained unbroken and gradually became yellow and indurated, until after three weeks it was a firm, yellow, flat-topped, painless circular granulomatous swelling about 1 cm. across (Fig. B). There was practically no adjacent inflammation, though at the end of the second week it had been painful for a few days and there had been a generalized reaction with slight pyrexia and malaise accompanied by

FIG. B.—Case 2. Left little finger of patient showing rounded raised lesion of three weeks' duration. Note absence of surrounding inflammation. (×2.)

painful lymph nodes at the elbow and axilla of that side. The lesion was incised at this time, but though it looked pustular it yielded only a little serous exudate for culture. After incision resolution took place rapidly.

Materials and Methods

Tissue Culture

Primary human amnion cultures were prepared by trypsinization of fresh amnion by the method described by Ferguson and Tobin (1958). When confluent sheets of cells had grown they were washed with phosphate-buffered saline and maintained in 5% horse serum in Gey's salt solution (Paul, 1959), with the addition of 100 μ g. each of penicillin and streptomycin per ml. and 25 μ g. of nystatin per ml. For primary isolation several tubes were each inoculated with a drop of serous fluid from the lesion and incubated at 37° C.

Secondary monkey kidney cultures were prepared as described by Mair and Tobin (1960) and maintained in the same way as the amnion.

Continuous cell lines used included two strains of HeLa, MK2, and Am9. The last-mentioned is a transformed human amnion line which was developed in this laboratory.

Neutralization Tests

The production of infected foci in the form of plaques or holes in the cell sheet after incubation for three to four days enabled neutralizing antibody titration to be based on 50% plaque reduction. Dilutions of serum, after inactivation at 56° C. for 30 minutes, were made in maintenance medium, and infective tissue-culture fluid, centrifuged free from the larger debris, was added so as to yield 200–500 plaque-forming units per ml. Virus-serum mixtures were stood at room temperature for one hour before addition to tubes of washed cells, then incubated at 37° C. for three to four days, when counts were made.

Virus Strains

The strain of C.P.D. virus used was the same as Plowright *et al.* (1959) described, and was kindly supplied by Dr. Montgomerie, of the Wellcome Laboratories, Beckenham. The vaccinia virus was the calf-lymph strain of the Lister Institute adapted to growth in the continuous cell lines above.

Results

Tissue Cultures

1. *Macroscopic Appearances.*—Amnion-cell sheets inoculated with material from Case 1 showed a few scattered rounded granular cells after 18 hours and no further change for 15 days, when a few holes were noted in the sheet near the edge. They differed from the holes seen occasionally in uninoculated ageing amnion sheets in that the marginal cells were rounded and refractile and in some the cytoplasm was conspicuously granular. Passage to a fresh amnion culture produced a similar cytopathic change after six days, and the holes increased in size until there was gross sheet destruction. Subsequent passage gave the same cytopathic change after three to four days in fresh cells (see Special Plate, Figs. 1 and 2), and took longer when the cells were old. Cells inoculated in their third week or later were found to be unreliable in their capacity to show cytopathic change.

Material from Case 2 gave a few typical holes and plaques in the cell sheets after only five days, and in subsequent passage the changes were identical to those described above.

The virus grew in secondary monkey kidney cells and gave a similar picture to that produced in amnion, but the tendency for hole formation was not so marked and rounded cells with granular cytoplasm were more conspicuous. These changes, in both types of cell, were indistinguishable from those produced by a strain of C.P.D. virus of ovine origin.

Attempts to grow the virus in continuous cell lines were made, but without success. Infective fluids harvested from amnion cultures when diluted tenfold produced what appeared to be toxic changes in HeLa, MK2, and Am9 after 18 hours. But no further changes occurred, and there was no virus multiplication, although up to five passages have been tried in each cell line.

2. *Microscopical Appearances.*—The earliest changes in infected cell sheets stained with haematoxylin and eosin or Giemsa are seen at 18 hours, when groups of two or three adjacent cells appear more intensely stained. This is mainly due to the cytoplasm, which becomes increasingly eosinophilic. After three to four days some of these cells have completely degenerated and become detached from the glass to produce a hole in the sheet. They remain attached to the less affected cells adjoining them, which produces the characteristic margin of rounded cells, which continues to retract as cells infected later become detached from the glass (Special Plate, Figs. 2, 3, and 4).

The series of changes which take place in the cell seem to begin with the appearance of the eosinophilic "matrix" in the cytoplasm, and this may become markedly granular. It usually surrounds the nucleus and is seldom paranuclear. Unequivocal inclusion bodies were rarely seen. The cell becomes rounded, and at the same time the nucleus, usually elongated, becomes rounded and shows margination of the chromatin. It continues to shrink and stains more deeply until pyknotic (Special Plate, Fig. 5). When the matrix is paranuclear it seems to be associated with fragmentation of the nucleus; when perinuclear, the peripheral cytoplasm loses its staining properties, becomes vacuolated, and eventually disintegrates to leave behind the pyknotic nuclear residue, which usually retains much of the matrix attached (Special Plate, Fig. 6).

Serological Findings

Limited serological investigations only have so far been made, but they are of interest in that they show that convalescent sheep which were severely infected during the epidemic in the flock with which Case 1 was concerned had neutralizing antibodies. Uninfected sheep from a clean flock had no detectable neutralizing antibodies (Table I).

The first patient had some neutralizing antibody, but only a convalescent serum could be titrated. It may be significant, as such antibody is not likely to be normally present in human sera and we have not found it in a small number of sera titrated to date. The second

TABLE I.—*Neutralizing Antibody Titrations* (Uninfected and Convalescent Sheep and Patients' Sera)*

Virus Strain	Sheep Sera				Patients' Sera		
	Uninfected		Convalescent		Case 1 Convalescent	Case 2	
	1	2	1	2		Acute	Convalescent
"A.M."	<5	<5	60	60	40		
C.P.D.	<10	<10	40	40	80	5	15
						10	40

* In Tables I and II titre is expressed as the reciprocal of the dilution which gives 50% reduction in plaque count.

TABLE II.—*Neutralizing Antibody Titrations (Antivaccinia and Convalescent Sheep Sera)*

Virus Strain	Antivaccinia Sera		Convalescent Sheep Sera	
	Rabbit Serum	Human Convalescent Serum	1	2
"A.M." C.P.D. Vaccinia	<5 <10 5,000	<10 Not done 500	60 40 10	60 40 10

patient showed only a slight rise in titre, which is of doubtful significance.

As the virus of orf has the properties of a pox virus, some titrations were made to see if these antibodies had any action on vaccinia. It can be seen from Table II that they did not neutralize vaccinia, nor did antivaccinia sera neutralize these strains or C.P.D. virus, which confirms the absence of serological overlap that MacDonald found by complement-fixation titrations (MacDonald, 1951).

Chorio-allantoic Inoculations

Orf virus does not readily grow on the chorio-allantoic membrane of the chick embryo. Some freshly isolated strains have produced small pocks on the chorio-allantoic membrane and others have failed to produce any. Those which grew died out after three or four consecutive passages (Lyell and Miles, 1950; Greig, 1956; Abdussalam, 1957).

For comparison, sixth-passage tissue-culture fluid from Case 1 (strain "A.M.") was titrated in parallel with C.P.D. virus on the chorio-allantoic membranes of 12-day-old chick embryos. Membranes harvested at four days showed a clearer picture than at two days. Both strains gave discrete opaque lesions up to 0.5 mm. in diameter which were macroscopically indistinguishable. Strain "A.M." in a dilution of 1 in 10 gave some oedema of the membrane as well as discrete pocks, and higher dilutions produced pocks equivalent to a titre of about 10^3 pock-forming units per ml. Titrated in amnion the same fluid had a titre of at least 5×10^4 plaque-forming units per ml. The C.P.D. strain had a titre of about 5×10^4 pock-forming units per ml. and about 1.5×10^5 plaque-forming units per ml. As it seemed unlikely that the strains would propagate successfully by this method no subsequent passages were attempted.

Electron Microscopy

Observations on the morphology of the virus were made by a modification of the phosphotungstate negative staining method (Brenner and Horne, 1959) already described (Horne and Nagington, 1959). It is hoped to publish details of the technique in full in a separate communication.

The particles often show a regular criss-cross or woven pattern which has not been previously described. It is hoped to discuss the significance of this elsewhere (Nagington and Horne, in the press).

The usual form of virus particle seen is a symmetrical cylindrical structure which is convex at each end. It is uniform within a narrow range of size, except for particles which are apparently imperfect, and measures approximately $260 \times 160 \mu\mu$. This gives a length/breadth ratio of 1.62 and is in close agreement with the finding of Abdussalam and Cosslett (1957) that metal-shadowed particles of two C.P.D. strains measured $252 \times 158 \mu\mu$ with an axial ratio of 1.6, which was the highest figure they could discover among the pox viruses.

Discussion

The lesion in Case 1 is an uncommon type of orf infection and has some resemblance to the herpetic whitlow described by Stern *et al.* (1959), which also begins as a vesicle and then assumes a pustular appearance, yet on incision yields no pus. But herpetic whitlow is characteristically painful, whereas the lesions of orf are not except when accompanied by secondary infection.

The virus which was isolated in human amnion tissue cultures from the two patients would appear to be a strain of orf or C.P.D. It has been shown to produce a characteristic cytopathic change which in many ways resembles that of ovine C.P.D. virus in tissue cultures of sheep cells examined by Greig (1956) and Plowright *et al.* (1959). It is neutralized by the sera of convalescent but not uninfected sheep, and there seems to be a low level of neutralizing antibody detectable in the serum of convalescent patients. The lesions on the chorio-allantoic membranes of chick embryos are consistent with those of C.P.D. as described by Lyell and Miles (1950) and Abdussalam (1957), and the morphology as seen by the electron microscope is compatible with that described by Abdussalam and Cosslett (1957) for two C.P.D. strains, one ovine and one of human origin, but maintained in sheep, which were examined by a metal-shadowing technique.

In addition to this an attempt was made to infect sheep with virus which had had several passages in human amnion, but, though some lesions were produced on the skin of the inside of the thigh, they regressed after seven to eight days, and it was thought that the virus might have lost some of its virulence for sheep after serial passage in human cells. This may be a similar phenomenon to the rapid loss of pock-forming ability which occurs in chorio-allantoic-membrane culture. It remains to be seen whether the virus will continue to propagate in human tissue. So far seven serial passages have been made in human amnion without any apparent reduction in infective yield, but it may eventually require to be passed through sheep cells.

Summary

A virus identical with that of orf or contagious pustular dermatitis was isolated from two patients with lesions on the fingers who had been in contact with sheep. The presence of neutralizing antibodies in convalescent sheep and patients was demonstrated, and studies have been made of the morphology of the virus by electron microscopy which have revealed for the first time some details of the structure.

We would like to express our gratitude to Dr. J. Seed for the phase-contrast photographs; to Dr. A. O. Betts for the provision of sheep and their inoculation; to Dr. C. Kaplan for the gift of antivaccinia serum; to Mr. A. J. King and Mr. S. W. Patman for the stained-preparation photographs; and to Miss M. Lawrence for technical assistance.

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J. NAGINGTON AND C. H. WHITTLE: HUMAN ORF

FIG. 1.—Human amnion cell sheet: uninfected control. (Phase-contrast photograph of unfixed preparation. $\times 170$.)

FIG. 2.—Human amnion cell sheet. Infected seven days earlier with virus from Case 1 (strain "A.M."). There is gross destruction of the cell sheet, in which many holes have formed, the edges of which consist of highly refractile rounded cells. (Phase-contrast photograph of unfixed preparation. $\times 170$.)

FIG. 3.—Human amnion cell sheet: uninfected control. (Haematoxylin and eosin. $\times 40$.)

FIG. 4.—Human amnion cell sheet seven days after inoculation with virus "A.M." Characteristic cytopathic change with hole formation and deeply staining marginal cells. (Haematoxylin and eosin. $\times 40$.)

FIG. 5.—Human amnion showing early lesion four days after inoculation with virus "A.M." Cells are becoming detached from glass surface, cytoplasm of infected cells stains more deeply, and nuclei of some have become pyknotic (Haematoxylin and eosin. $\times 400$.)

FIG. 6.—Human amnion. Two infected cells which show pyknotic nuclei, condensation of perinuclear cytoplasm, and vacuolation of peripheral cytoplasm. (Haematoxylin and eosin. $\times 1,000$.)

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PNEUMOCYSTIS PNEUMONIA

REPORT OF THREE CASES IN ADULTS AND ONE IN A CHILD WITH A DISCUSSION OF THE RADIOLOGICAL APPEARANCES AND PREDISPOSING FACTORS

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[WITH SPECIAL PLATE]

In recent years, as the treatment of certain chronic diseases has become more effective, a number of agents previously considered as innocuous have been found to behave as pathogens. They flourish especially in tissues freed from competing organisms or deprived of their normal power of resistance by the actions, often combined, of radiotherapy, cytotoxic drugs, and long-term administration of antibiotics and corticosteroids. The marked increase in the incidence of fungal infections such as moniliasis and aspergillosis is an example of the development of this kind of "new" disease. A less common disease which may arise under similar conditions is *Pneumocystis carinii* pneumonia. We here report three adult cases and one case in a child of 2 years. Though it is not always certain to what extent the organism has behaved as a pathogen, there is little doubt that its multiplication in the lungs in these cases is associated with debilitating disease and corticosteroid therapy.

Pathological Methods

Five or more blocks from the lungs representing at least one block from each lobe were examined in every case. All sections were stained with haematoxylin and eosin, periodic-acid-Schiff (P.A.S.) stain, and Gömöri's methenamine silver method; Humberstone's modification of Le Tan's method (Symmers, 1960) was also used, but was less successful than Gömöri's technique in our hands. Gram stains and stains for fibrin were used as required. In Case 1, where pneumocystic infection

was suspected before death, the right lung was fixed after distension (Gough and Wentworth, 1960) and, in addition to routine sectioning, a giant section was prepared for us by Professor Gough. In the other three cases the lungs were not artificially distended.

Case Reports

Case 1

A man aged 57 was diagnosed as having lymphosarcoma in 1954 following biopsy of a lymph node from the right supraclavicular fossa. A chest radiograph showed pronounced enlargement of hilar lymph nodes. Both sides of the neck and both axillae were irradiated, and he also received 43 mg of nitrogen mustard in 14 days. Later in 1954 the chest and both groins were irradiated, after which the swelling of the hilar nodes regressed and he remained well until 1956, when he was found to have an enlarged spleen and enlarged lymph nodes in the neck, axillae, and groins. He received deep x-ray therapy to the abdomen, and in February, 1957, treatment with prednisone, 20 mg. daily, was started. He remained fairly well until November, 1959, when he became orthopnoeic and developed a severe cough. In January, 1960, his chest symptoms worsened and he became febrile. He was given tetracycline, later changed to erythromycin and novobiocin, with temporary clinical improvement. Lung biopsy was then performed and a provisional diagnosis of pulmonary alveolar proteinosis made. Following this he had a persistent left pneumothorax, which developed increasing tension necessitating decompression 17 days after biopsy; this resulted in some surgical emphysema. After temporary improvement his condition again worsened, and he died in February, 1960. He had been on corticosteroid treatment for three years.

Radiological Findings.—A chest radiograph taken on February 13, 1959, before his final illness revealed only slight enlargement of the right hilum. On December 30, 1959, a radiograph showed a bilateral "batwing" type of shadow more marked in the upper lobes and with some drawing up of the lesser fissure (Special Plate, Fig. 1). This shadowing had an over-all homogeneity, particularly on the "softer" films, but small translucencies, both rounded and tubular, could be seen within it. Many of these were obviously small air-filled bronchi; others presumably represented emphysematous air spaces. A penetrated film showed this more clearly. Though the consolidation was mainly central, producing a "hilar flare" appearance, there was no clear-cut margin and peripherally the lungs had a "ground-glass" appearance extending to the chest wall in the upper lobes. No pleural reaction was present. The heart size was normal. By January 15, 1960, the consolidation was denser and more extensive, and penetrated films showed definite "cyst" formation in a number of areas, the largest cyst measuring about 1.5 cm. in diameter. A film taken after lung biopsy on January 22 showed extension of cyst formation in both upper lobes. There was a small left pneumothorax with a little basal fluid. The pneumothorax gradually increased until by February 8 there was a definite tension pneumothorax in the left upper zone with adherence of the lower lobe to the chest wall (Special Plate, Fig. 2). Further cystic change had occurred and a very large cyst was present in the collapsed left upper lobe. At this stage the pneumothorax was decompressed and the lung re-expanded.

The final film taken on February 14 showed multiple upper-lobe cysts, some with fluid levels (Special Plate, Fig. 3). There was fluid at the left base and widespread surgical emphysema following the decompression.

Necropsy Findings.—The trachea and main bronchi contained blood-stained mucus. In the right lung (examined after distension and fixation) were a number of irregular cavities radiating out from the hilum, and having a multi-locular appearance attributed in part to the breaking-down of intervening lung tissue, as shown in the accompanying illustration. The walls of these cavities were predominantly