DIAGNOSTIC PROCEDURES FOR VIRAL AND RICKETTSIAL INFECTIONS

FOURTH EDITION

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This volume was prepared by the Subcommittee on Viral and Rickettsial Infections. It has been reviewed by the Coordinating Committee on Laboratory Methods and recommended for publication. Publication has been authorized by the Committee on Evaluation and Standards of the American Public Health Association.

Published by the

American Public Health Association, Inc.
1740 Broadway, New York, N. Y. 10019

1969

CHAPTER 7

POXVIRUSES

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I. INTRODUCTION

Variola (smallpox), cowpox, and vaccinia are exanthematous infections caused by immunologically closely related but distinct viruses. Smallpox is essentially a disease of man with no known animal reservoir; it is most prevalent in South East Asia, Central Africa, and Central and South America. It occurs in two clinically and epidemiologically distinct forms; the classical severe form—variola major with an overall mortality of 15–30%, and the milder form—variola minor or alastrim, with a mortality of less than 1%. Variola major is endemic in South East Asia and Africa, while variola minor is predominant in Brazil but occurs also in Africa and Indonesia. Cowpox is an infection of cattle, endemic in several countries of Europe and apparently is less common in the Americas; in man it is an occupational disease among those handling infected cows. Vaccinia strains are laboratory strains, probably derived from cowpox and skin-adapted by repeated propagation in the skin of calves, sheep and rabbits. Such strains are universally used for active immunization against smallpox. In the course of routine vaccination serious complications occasionally occur with extensive involvement of the skin, such as eczema vaccinatum, generalized vaccinia and progressive vaccinia (vaccinia necrosum).

Orf or contagious pustular dermatitis (see Chapter 22, III) is a world wide infection of sheep and goats. Infection in man occurs in those handling infected animals and carcasses and usually takes the form of one or more lesions on the hands, forearm or face. Milker's nodules is an infection contracted usually by milkers who acquire the disease from cows suffering from warty lesions of the udder. Molluscum contagiosum (Chapter 22, X) is a mild, common skin infection only slightly contagious, caused by a pox virus which affects man only.

A. Historical Note

Smallpox is believed to have been present in Asia before the Christian era. It was introduced into Europe in the 12th or 13th centuries and was first clearly described in England early in the 16th century and introduced into North America shortly thereafter. From this period onwards it appears to have been widespread throughout the world. Only in the present century has the disease ceased to be endemic in most countries of Europe and North America. Although a milder type of smallpox was recorded in England in the 18th century (*11*), it was only in the present

century that variola minor was recognized as a clinically distinct disease. This form of the disease became prevalent in England and in the Americas during the 1920's and remained endemic for some years thereafter. It is still prevalent in Brazil and some areas of Africa. Cowpox was first clearly described by Jenner in 1798 (*21*) although it appears to have been an occupational hazard of milkers for many years before. The practice of vaccination, inoculation of cowpox, introduced by Jenner as a safer immunizing procedure than variolation, was at first performed by arm to arm vaccination. In the 1860's the practice of using calves for the propagation of cowpox virus spread throughout Europe and these strains, somewhat altered by repeated passage by dermal inoculation of animals, constitute the vaccinia virus.

B. Clinical Aspects

1. **Variola**—Smallpox is usually spread by direct contact with a clinical case although it may occur indirectly by handling of clothing, bedclothes or utensils soiled by the infectious discharges of patients. It is believed that the site of entry of the virus is in the upper respiratory tract. The incubation period is most commonly 12 or 13 days but occasionally it may be as short as 8 or as long as 17 days. The clinical illness begins with fever, headache, pains in the limbs and prostration. During this preeruptive-phase, lasting 2 to 4 days, transient erythematous or petechial rash over the groins, axillae and flanks is seen in about 10% of cases. The focal eruption appears usually on the 3rd or 4th day and soon afterwards the temperature drops and the patient feels much better. The focal rash usually appears first on the oral and pharyngeal mucosa, on the face or forearms and hands and then spreads to the trunk and legs. The eruption on the skin evolves through the stages of macule, papule, vesicle and pustule over a period of 5 or 6 days. In all except the mildest cases the temperature usually rises again as the lesions become pustular. In the fully developed pustular eruption—about the 8th to 10th day of illness—the distribution is usually characteristic, the face and extremities being more extensively involved than the trunk, the back more than the front of the trunk and the chest more than the abdomen. Flexures such as the groins, axillae, the front of the elbow and popliteal skin tend to be spared. Although the eruption on the face and arms may be a day in advance of those on the legs, the eruption on any part of the body is homogeneous in that all elements are at the same stage of development. In patients who are to recover, the lesions begin to dry up about the 10th to 12th day of illness and after 3 weeks most of the scabs have been shed, with the exception of the deep brown seeds in the palms of the hands and soles of the feet.

In severe cases, the eruption on the face may be confluent and

when this confluent eruption evolves slowly it feels soft and velvety, with umbilication and is associated with hemorrhages in the base of the lesions; in malignant confluent cases the prognosis is bad. In the most severe cases, the fulminant or hemorrhagic, petechiae appear in the skin on the 2nd or 3rd day; there may be bleeding into the conjunctiva, from the mouth, nose, bladder, rectum or vagina and the patient may die in 4 or 5 days before the focal eruption can develop. In these cases the illness may be erroneously diagnosed as purpura, acute leukemia or meningococcal septicemia. In mild cases, especially where the disease is modified by previous vaccination, the eruption may be scanty, more superficial and there may be no secondary fever. Such cases are liable to be mistaken for chickenpox. Usually a leukopenia develops early in the disease followed by leukocytosis when the eruption becomes pustular. In hemorrhagic cases normoblasts and atypical white cells of the lymphocyte or lymphoblast type may appear in the peripheral blood and suggest a diagnosis of acute leukemia. Except in the acute fulminant cases most deaths occur towards the end of the 2nd week of illness.

Patients are most infectious from about the 3rd to the 10th day of illness and infection is spread at this time chiefly from the heavily infected secretions from the mouth and upper respiratory tract. However, virus is present in the crusts and the patient cannot be regarded as free from infection until all crusts have separated. The commonest complications are abscesses in the skin or subcutaneous tissues, and variolous infection of bones. Encephalitis is a rare complication; since the introduction of antibiotics secondary bacterial bronchopneumonia, once common in severe cases, is now rarely seen.

Variola minor or alastrim shows a clinical picture similar to that of variola major with discrete eruptions. The incubation period is of the same duration, there is the same preeruptive illness, but confluent rash is relatively uncommon and hemorrhagic cases are very rare. Secondary fever during the pustular stage is often absent and the eruption tends to run its course more quickly.

a. Pathogenesis—The virus probably enters the body through the mucosa of the upper respiratory tract. No lesion has been observed at the site of entry and as patients are not infectious during the incubation period, no virus is shed from the respiratory passages at this time. It seems likely, as has been demonstrated in ectromelia (*33*) and in rabbit pox (*3*), that the virus passes through the mucosa and is carried possibly by phagocytic cells to the local lymph nodes where primary multiplication occurs. From this site virus probably seeps into the bloodstream from which it is removed by cells of the reticuloendothelial system. In these tissues—spleen, lymph nodes, liver and bone marrow—the virus undergoes further multiplication and passes to the bloodstream at, or just before the onset of

clinical illness. Virus can be detected in the blood at this time and from this viremia the epithelial cells of the mucosa of the mouth, pharynx, etc., and of the skin become infected. The early histologic changes in the skin suggest that the virus infects the capillary endothelium in the corium. In hemorrhagic cases, extravasation occurs around small vessels in this area. In average cases of smallpox, virus is not found in the blood after the first 2 days from the onset of illness, but in more severe and particularly in fulminant cases virus may be present in increasing amounts up to the time of death (*14*). In such cases, the infection may be so intense and widespread that virus antigen may be detected in the blood serum by precipitation or complement fixation tests with an immune serum. The presence of virus after the first 2 days of illness, or the demonstrable presence of virus antigen in the blood, usually portends a fatal outcome.

Neutralizing antibody usually appears in the blood about the 5th or 6th day of illness when the temperature has dropped near to normal, but in severe infections the appearance of antibody may be delayed and in acutely fatal infections may not be detectable. The appearance of antibody in the blood does not, however, prevent the evolution of the skin lesions from the vesicular to the pustular stage. Virus within cells is protected from the antibody and the destruction of infected cells leads to inflammatory infiltration of polymorphonuclear leukocytes which are responsible for the pustulation. Bacteria are not usually found in unbroken pustules although secondary bacterial infection, especially of the bronchioles and pulmonary tissue, may contribute to death in a small proportion of cases; secondary bronchopneumonia has not been a common complication since the widespread use of antibiotics. The mortality rate in variola major has not been greatly reduced since the introduction of antibiotic treatment to prevent secondary bacterial infections.

b. Pathology—The earliest changes in the focal lesions of the skin are dilatation of the capillaries in the corium, swelling of the endothelial cells and accumulation of lymphocytes around the vessel. By the time macules are visible in the skin the virus has infected epithelial cells and suitably stained smears of macular and early papular scrapings will usually show enormous numbers of virus particles. There is marked thickening of the epithelial layer and, as the infected cells undergo ballooning degeneration, fluid appears between the cells and its accumulation results in vesicle formation. In the fully formed vesicle or pustule in the skin, the roof is formed by a thin layer of keratinized cells, and the base by the degenerating cells in the lower malpighian layer or by the corium. Because in the lesions of the mucosa of the mouth and pharynx there is no keratin layer the virus is discharged earlier from the surface of these lesions and it is from this source that most of the infections are spread. Postmortem studies of fatal cases show that the liver and spleen are generally enlarged, but apart from occasional small hemorrhages gross changes are not obvious (*5*). Large basophilic mononuclear cells are common in liver, spleen, and bone marrow. Focal degenerative changes may be found in the kidneys and testes. In fulminant cases of smallpox hemorrhages may be found in the internal organs, especially in the mucosa of

the gastrointestinal tract. It is not known whether in those patients who recover focal lesions occur in the internal organs.

2. **Cowpox**—This infection is seen in milkers or farm workers handling infected cows. Lesions are generally seen on the hands, especially on the fingers and in the first interdigital cleft, but may occur on the skin of the forearms or face. The lesions are similar in appearance and development to the lesions of primary vaccination, although at the vesicular stage the contents may be blood stained. The development of the lesions may be accompanied by some fever and regional lymphadenitis although in well vaccinated individuals these features may not be prominent. The lesions usually heal in 2 to 4 weeks. Occasionally generalized eruptions occur. In the acute stage, histologic examination shows similar appearance to that of a vaccinial or variola lesion except that there are present numerous large strongly acidophilic inclusions in the infected epithelium, such as are not seen in the other two poxvirus infections.

3. **Vaccinia**—*Primary vaccinia* is usually the result of vaccination against smallpox. It may occur accidentally in laboratory workers, especially among those engaged in the production of calf lymph vaccine when the fingers, eyes, or lips may be affected, or occasionally in family contacts of a recently vaccinated child. On the 3rd or 4th day after inoculation a small red papule appears which increases in size, becomes vesicular on the 6th or 7th day and pustular very soon thereafter. The peak of the reaction occurs between the 8th and 10th day when there is usually regional lymphadenitis, slight malaise and a moderate degree of fever. The lesion dries up and a scab forms which usually separates within 3 weeks, leaving a scar.

Occasionally a lesion may occur elsewhere on the skin due to mechanical transfer of virus from the site of inoculation to another area of the skin, usually the face. Apart from this, some extension of infection may occur, sometimes by the bloodstream, and may be manifested in three different forms (a) generalized vaccinia, (b) eczema vaccinatum, (c) progressive vaccinia or vaccinia necrosum. Generalized vaccinia occurs as a complication in children or adults who have no predisposing skin lesions, usually at the height of the primary vaccinial take. There may be an extensive vesicular eruption affecting the trunk and limbs but without the marked centrifugal distribution of smallpox; usually recovery is complete with high titers of antibodies. This condition is presumably caused by a massive viremia perhaps associated with a delayed immunological response.

In *eczema vaccinatum* innumerable vesicular lesions are found over the areas of skin affected by preexistent dermatitis. But lesions

often occur in previously normal areas of skin and all lesions tend to appear at the same time, suggesting infection by blood-borne virus. This condition is commonly seen in children who have not themselves been vaccinated but have had contact with a recently vaccinated sibling. The mortality rate in eczema vaccinatum patients under 1 year of age is high.

Progressive vaccinia (vaccinia necrosum) is fortunately a rare complication. It is characterized by progression and extension of the initial vaccinial lesion over a period of 3–5 months. As the lesion extends deeply and laterally, fresh vesicles appear around the edge of the necrotic ulcer and secondary lesions occur in other areas of skin, mucous membrane and bone, as well as in other organs. There may be no obvious reactive inflammation around the lesions and histologic examination shows little cellular infiltration around the necrotic tissue. Systemic reaction is initially mild but death generally occurs after some months in the absence of specific treatment. The condition results from reduced or absent capacity to develop delayed hypersensitivity mediated via small lymphocytes.

Some cases of progressive eczema vaccinatum and vaccinia necrosum may respond to large doses of vaccinia immune gamma globulin. In a proportion of those who do not respond to treatment with antibody, methisazone (1-methylisatin-β-thiosemicarbazone)—200 mg/kg daily in divided doses—may produce clinical and virologic cure, although death may sometimes occur from complications of the underlying disease. In some cases treatment with methisazone fails, possibly due to defective absorption of the drug as shown by low blood levels, drug binding by the large vaccinial mass or the presence of an inducer enzyme in these patients. The great immunologic variability in the clinical syndrome of vaccinia necrosum demands stringent immunologic studies before treatment is instituted. Results to date suggest that patients with acquired immunologic defects (lymphoma, leukemia) and those who have single defects (Bruton type agammaglobulinemia) do better than those with multiple defects (Swiss type agammaglobulinemia). When immune gamma globulin or methisazone therapy fail, multiple exchange transfusions with high antibody titer blood may be life-saving, provided the patient has an intact lymphoid system and is not immunologically tolerant. However, in the absence of small lymphocytes and of delayed hypersensitivity to various antigens (monilia, mumps), the use of competent white blood cells in even a single transfusion of whole blood has led to acute and fatal graft-versus-host reaction (GVH disease).

4. Milker's Nodules (Melkerknoten)—This disease is contracted from cows which show papular or warty lesions on the udders and probably represents the spurious cowpox of Jenner. It has been reported from North America and various European countries. The lesions appear on the fingers or hands of those engaged in milking infected cows. They may resemble the lesions of Orf (*29*). Papular

lesions, slowly increasing in size over a period of 1 to 2 weeks, become painless bluish-red nodules without vesiculation. Lymphadenitis and fever are usually absent although there may be associated allergic skin rashes. Lesions disappear without scar formation after 4 to 6 weeks. Histologically, the lesions have the appearance of vascular granulomata with little involvement of the epithelial layer of the skin.

II. NATURE OF AGENTS

A. Common Characteristics

Poxviruses are large, chemically more complex than smaller viruses. All appear to be oval when examined in thin sections of infected cells or tissue but are brick-shaped when dried for electron microscopy. Most appear to be 250–300 $m\mu \times 200–250$ $m\mu$ in size. There is a central electron dense pepsin resistant body—the nucleoid—and the nucleic acid is DNA. They are relatively resistant viruses and most grow in the cytoplasm of host cells with the formation of inclusion bodies. Closely related viruses form hybrids when simultaneously infecting the cells, and heat-inactivated poxviruses may be reactivated in cells superinfected with another viable poxvirus but not by other viruses. Poxviruses infect a wide variety of animals but only variola and molluscum viruses are strictly human pathogens.

B. Size and Shape

Vaccinia virus has been studied more intensively than any other poxvirus but thus far no differences have been determined in morphology, chemical composition and resistance for vaccinia, variola and cowpox viruses.

The size of vaccinia virus determined by electron microscopy is larger than the size deduced from centrifugation and filtration studies, being approximately 250–300 $\times$ 150–200 $\times$ 100 $m\mu$. Various methods of preparative treatment of purified particles combined with electron microscopy have demonstrated some of the structure of the particles. Most workers agree that there is a double-layered outer envelope covering a layer of soluble protein antigens which may be removed without affecting the infectivity of the virus (*37*). Immediately underneath is the capsular layer in which seem to be embedded tubular strands of protein or lipoprotein (Fig 1). The capsule encloses the biconcave nucleoid and the lateral bodies centrally placed over the concavities of the nucleoid (Fig 2). The nucleoid contains protein and DNA which is double-stranded of 80 or 160 million mol wt (*23*).

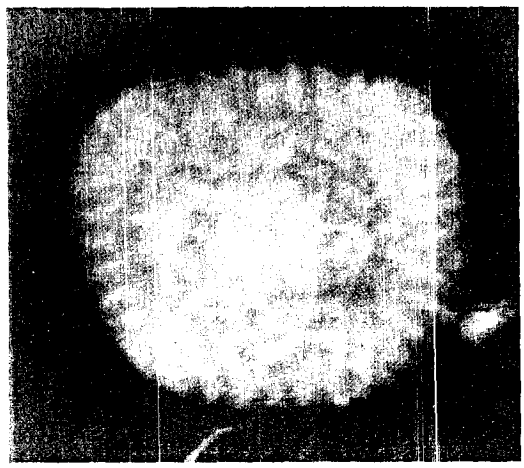

Figure 1—Vaccinia virus stained with phosphotungstic acid. ×170,000. (*Source:* Westwood et al in *J. Gen. Microbiol.* 34:67–78, 1964.)

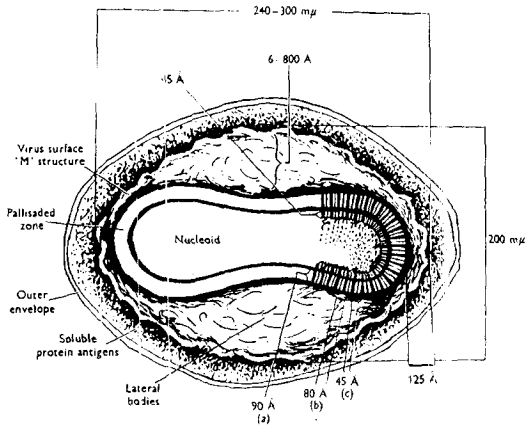

Diagram of the side elevation of a virus particle showing the various layers identifiable with their suggested structure and principal dimensions.

Figure 2—Diagram of side elevation of a vaccinia virus particle. (*Source*: Westwood et al in *J. Gen. Microbiol.* 34:67–68, 1964.)

C. Chemical Composition

Recent chemical analyses of vaccinia virus have given values similar to those of Smadel and Hoagland (*35*). In addition to DNA (3–5%) and protein (86%) the virus contains cholesterol (1.4%), phospholipid (2.2%), neutral fat (2.2%), carbohydrate, copper, flavin and biotin. Zwartouw (*40*) regards the lipids as essential components of the virus and suggests that the small amounts of copper, biotin and flavin were impurities. His finding

of 3.2% DNA is rather less than the figure of 5.6% determined by Smadel and Hoagland and by Joklik (22) and his value for carbohydrate was also less (0.2% instead of 2.8%).

D. Resistance

The viruses resist drying and desiccation. Smallpox virus may be recovered from crusts kept at room temperature for many months and from dried exudate on fabric or glass for many weeks. In fluid suspension these viruses are destroyed by heating at 60 C for 10 min, but dried vaccine lymph may withstand heating at 100 C for 10 min. Ultraviolet light, alpha rays, x-rays, and gamma rays all have a rapidly lethal effect, and the virus is sensitive to the photodynamic action of various dyes in the presence of light. The effect of various chemicals varies with the nature and reaction of the suspending medium, the amount of protective protein present, and the temperature at which tests are made. Vaccinia virus is inactivated at pH 3 within 1 hr. Alcohol, methyl-alcohol and acetone in 50% concentration inactivate vaccine lymph within 1 hr. The virus is resistant to the action of ethyl-ether (20%) at 4 C. Similarly, it withstands the action of 1.0% phenol for weeks at 4 C but may be inactivated at 37 C in 24 hr. The virus is not destroyed by certain disinfectants which are rapidly lethal to many bacteria, e.g., 1:10,000 brilliant green, but is inactivated by oxidizing disinfectants such as hypochlorites or by potassium permanganate in a concentration of 1:10,000. Formaldehyde in a concentration of 0.2% destroys infectivity in 24 hr at room temperature.

E. Classification

Variola, vaccinia and cowpox viruses with the viruses of rabbit pox, monkey pox and ectromelia, have been placed in Group I of six poxvirus groups because of similar morphology and close antigenic relationship. The viruses of contagious pustular dermatitis (Orf) and of milker's nodules have been placed in Group II, while the virus of molluscum contagiosum has tentatively been placed in an unclassified Group VI (*1*). Recent work indicates that the viruses of milker's nodules, Orf and bovine papular stomatitis might be grouped together under paravaccinia viruses (*4, 18, 29*).

F. Antigenic Composition

The poxviruses in Group I, which includes variola major, variola minor, vaccinia and cowpox, are antigenically very similar; immunologic diversity among strains of variola major and minor, of vaccinia and of cowpox has not been detected. All of these viruses contain soluble antigens—extractable from virus infected cells and separable from the virus particles—viral antigens, hemagglutinin and nucleoprotein antigens.

1. Soluble antigens—Virus-free extracts of vaccinia infected cells contain antigens which react specifically with antivaccinial serum in precipitation and in complement fixation (CF) tests. Immunization of rabbits by repeated injection of these soluble antigens produces neutralizing antibodies and to some degree an immunity to infection with living virus. By isoelectric precipitation a fraction was separated from soluble antigen by Craigie and Wishart (*10*) which they labelled L.S. because it had heat labile and heat stable antigenic determinants. The soluble antigens have in recent years been subjected to detailed examination by precipitation in agar gel, immuno-electrophoresis, column fractionation on Sephadex, acid precipitation, etc. Precipitation in agar gel with vaccinia immune serum shows at least 8 separate precipitation lines, and as many as 17 lines have been described (*38*). L.S. antigen prepared by the method of Craigie and Wishart (*10*) gives rise to three or more lines. By filtration of soluble antigen through Sephadex columns, two classes of antigens may be separated, those (HMW) with 200,000–300,000 mol wt and those (LMW) with 50,000–100,000 mol wt. The HMW-antigens produced neutralizing antibody in rabbits whereas the LMW-antigens did not (*8*). The relation of the immunizing antigen of Appleyard and Westwood (*2*) to the various antigens revealed by precipitation in agar gel is not quite clear. At least 5 of the components of soluble antigen which produced 17 lines were labile when heated to 60 C (*38*). It has been suggested that not all of the components producing precipitation lines with immune serum in agar gel represent structural proteins and that some may represent specific substances (perhaps enzymes) required for virus replication but are not incorporated into the virus. It is clear that "soluble antigen" contains a mixture of serologically reactive components and that more work on separation and characterization of these components is required (*8, 26*). So far, no qualitative difference in the soluble antigens of variola, cowpox and vaccinia have been determined. There may be quantitative differences in the amount of individual antigens produced by cowpox and vaccinia (*34*).

2. Viral antigens—Purified suspensions of virus particles are specifically agglutinated by vaccinial immune serum and from the particles antigens elute slowly and can be demonstrated by CF and precipitation tests with immune serum. After treatment with alkali or trypsin, virus-free extracts of purified suspensions produce eight (*38*) or nine (*27*) precipitation lines with immune serum in agar gel. Seven (*38*) or eight (*27*) of these appear to be identical with lines produced by soluble antigen (*27, 38*). The nucleoprotein antigen obtained in earlier studies by extraction with alkali and believed to be common to many poxviruses (*39*) is probably a mixture, as it gives several precipitation lines in immunodiffusion tests. Purified virus suspensions contain no hemagglutinin.

3. Hemagglutinin—Suitable extracts of vaccinia infected tissue agglutinate the red cells of a number of normal fowls. This hemagglutinin is a relatively large particle (65 mμ) which does not diffuse in agar in Ouchterlony plates (*7*). Purified virus suspensions have no hemagglutinating activity. Hemagglutinin is inhibited by immune sera, but the titer of

antihemagglutinin in such sera may be unrelated to the neutralizing titer. This is especially true of human serum after revaccination. Immunization of rabbits with suspensions of inactivated virus particles may produce high levels of neutralizing antibody but no antihemagglutinin. Fowl red cells may be adsorbed to tissue culture cells infected with the vaccinia-variola group of viruses, but it is not certain that this hemadsorption phenomenon is due to the action of hemagglutinin. Hemagglutinin may be readily obtained from saline extracts of vaccinia infected chorioallantois or tissue cultures. The hemagglutinins produced by variola, vaccinia and cowpox appear to be identical; more potent preparations are obtained from vaccinia-infected tissue.

G. Pathogenicity for Animals

Apart from man, variola virus will produce mild disease in monkeys inoculated by various routes, and the virus may be passed in such animals in series. There is, however, no proof that infection of these animals occurs in nature. Inoculation of variola virus into the rabbit skin produces well marked lesions locally but transfer to further rabbits fails. By intratesticular inoculation in the rabbit the virus may be passed through a number of animals without evident enhancement of virulence. Lethal infection of infant mice is produced by intracerebral inoculation, and the virus can be passaged regularly by this route in day-old mice. Variola virus grows readily on the chick chorioallantois (CAM) and produces visible lesions in 2 days. After 3 days the lesions, when discrete, are white, convex, with a smooth shiny surface and measure about 1 mm diam (Fig 3).

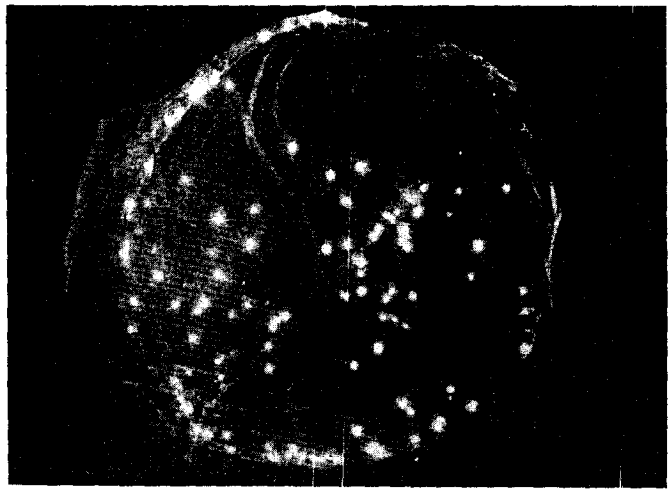

Figure 3—Variola. Pocks on CAM after 3 days. About 1½ times original size. (*Source:* Downie and MacDonald in *Brit. Med. Bull.* 9:191–195, 1953.)

Vaccinia and cowpox viruses have a much wider host-range. They will produce local skin infections in many laboratory and domestic animals and lethal infections are induced if large doses are inoculated by the intravenous, intraperitoneal or intratesticular routes. Some adaptation to special tissue may be evident with vaccinia strains; a virus passed repeatedly by skin scarification will give higher titers by this route than a strain that has been passed by subcutaneous or intratesticular inoculation. On the chick CAM vaccinia virus produces much larger lesions than does variola; after 3 days they may measure 3–4 mm diam and may be ulcerated (Fig 4). Some strains—e.g., neurovaccinia—produce lesions with a hemorrhagic center. Cowpox virus produces typical hemorrhagic lesions which after 3 days measure 2–3 mm diam. After CAM inoculation both cowpox and vaccinia strains will kill the chick embryo after 3 or 4 days even with relatively small inocula—100 to 1000 pock forming units (PFU). The lethal dose of variola major virus for the chick embryo is 100,000 PFU or more, and with variola minor strains doses 100 times larger may be necessary (*16*).

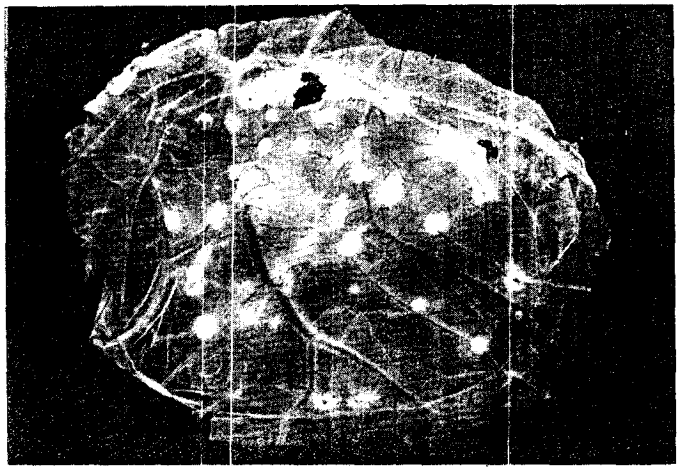

Figure 4—Vaccinia. Pocks on CAM after 3 days. About 1½ times original size. (*Source:* Downie and MacDonald in *Brit. Med. Bull.* 9 191–195, 1953.)

H. Growth in Tissue Culture

Variola virus will grow and produce cytopathic effects (CPE) in tissue cultures from various animal sources including chick embryo, bovine skin, human and monkey tissues. The time of appearance of CPE varies with the size of the inoculum and nature of the tissue. In human embryo tissue cultures inoculated with a large dose of virus, CPE may be visible in 24 hr, but with small inocula this may

not appear for 3 or 4 days. In cultures derived from malignant cells, such as HeLa, proliferative foci appear before obvious degenerative changes set in. These proliferative foci are not seen in cultures from normal tissue nor are they produced by vaccinia or cowpox viruses. Cowpox and vaccinia grow readily in tissue cultures derived from many animal sources. CPE appears more readily and spreads more rapidly than in cultures infected with variola virus. With vaccinia virus the formation of multinucleated giant cells or of syncytia is common. Cowpox infection is characterized by the appearance of large strongly acidophilic cytoplasmic inclusions in infected cells which are not a feature of vaccinia and variola infected cultures (*13*); in the latter, the inclusions are less strongly acidophilic and tend to appear as granular masses in the cytoplasm.

I. Milker's Nodule Virus

This virus is morphologically similar to the virus of Orf (*18*), showing the same pattern of regular tubular fibrils on the surface of the particle. The virus is inactivated by chloroform in 10 min. It appears to have little serologic relation to vaccinia virus, is nonpathogenic to suckling mice by intracerebral or intraperitoneal inoculation and cannot be passed on the skin of the rabbit. The strain studied by Friedman-Kien et al (*18*) was isolated in tissue cultures of bovine embryonic kidney from a lesion on the hand of a 17 year old male. After several passages, CPE appeared in 1–3 days. This culture virus produced CPE in cultures of rabbit kidney, primary human embryo kidney or fibroblasts and rhesus monkey kidney, but could not be passed in these tissues. In tissue cultures of bovine embryonic kidney cells eosinophilic cytoplasmic inclusions of a granular type were produced. In tissue culture the virus behaves like the virus of Orf (*29*). The virus does not grow on the CAM and hemagglutinin has not been demonstrated.

III. PREPARATION OF IMMUNE SERUM

For diagnostic tests in smallpox, convalescent-phase smallpox sera are suitable although immune sera are usually prepared in laboratory animals. In vaccine lymph institutes, such immune sera are prepared in quantity by repeated injections of large doses of living vaccinia virus into calves or sheep that have recovered from vaccinial dermal infection. However, in many laboratories vaccinia immune sera are prepared in rabbits and the method found successful for the production of high titer immune sera in these animals is given below.

For immunization a virus suspension prepared from infected rabbit skin is used; a strain of vaccinia virus passed repeatedly in rabbit skin is de-

sirable. If this is not available, one may pass the virus by rubbing the vaccine lymph into the shaved scarified skin on the back of a rabbit, or the lymph may be spread on the skin and inoculated by light scarification with the cut edge of a piece of copper gauze. Four days later the animal is exsanguinated by cutting the large blood vessels in the neck. The inflamed vaccinia infected skin is scraped with a metal spoon or a blunt scalpel to remove the infected epithelium. This is suspended in 10–20 ml buffered distilled water containing penicillin (100 units/ml), and streptomycin (100 μg/ml) in a screw capped bottle and shaken vigorously by hand or by mechanical shaker for 5–10 min. The suspension is spun at 1500 rpm for 15 min to deposit cellular debris. The supernatant fluid is spun in a Spinco centrifuge at 10,000 rpm for 30 min. The virus deposit is resuspended in 1 ml of buffered distilled water: 0.5 ml of sterile glycerol is added and the mixture is used to inoculate a 2nd rabbit on the shaved skin of the back. The virus may be passed in this way in 3 or 4 rabbits to increase the yield of virus. From the last rabbit in the series, the supernatant fluid from the first high-speed centrifugation is preserved for use as antigen for CF and precipitation in agar gel tests. The virus deposit from the high-speed centrifugation is resuspended and washed twice by centrifugation at 10,000 rpm. The final deposit is resuspended in 5 ml of buffered distilled water and spun at 1500 rpm for 15 min to deposit remaining cellular debris and aggregated virus. The supernatant fluid will have a virus titer of 10^9 to 10^{10} PFU/ml and, while not highly purified, this stock virus suspension will serve for the later intravenous immunizing injections in the rabbits. If the suspension is mixed with 40% glycerol and stored at −20 C it will retain its titer for months.

For the immunization of rabbits 4 to 6 intradermal injections, 0.1 ml each are made on the back of the rabbit with the stock virus suspension diluted 1:100. After 14 to 21 days when the lesions have healed, 3 or 4 intravenous injections of 2.0 ml of diluted virus suspension are given at intervals of 6 days. The suspension for these intravenous injections should be faintly turbid and have a titer of about 10^8 PFU/ml (about 1:20 to 1:40 dilution of the stock virus suspension). Five or 6 days after the last intravenous injection, the rabbits are bled and the serum is separated from the blood clot. Several rabbits should be immunized; the sera are tested individually for precipitating and CF activity against vaccinial antigen, and the best serum is set aside for diagnostic tests. This serum may be distributed into ampules and kept frozen without preservative at −20 to −40 C.

IV. COLLECTION AND PREPARATION OF SPECIMENS FOR LABORATORY DIAGNOSIS

A. Specimens to be Collected

The selection of laboratory diagnostic tests depends on the materials which can be collected at various stages of the disease as shown in Table 1.

Table 1—Laboratory Tests at Various Stages of the Disease

Stage of Disease	Material Submitted	Electron Microscopy	Stained Smears	Antigen in Lesions Ppt or CF	Culture CAM or TC	Antibody in Serum Ppt, CF, or HI
Preeruptive illness	Blood			±	±	−
Macular and Papular	Smears on slides	+	+	±	+	−
	Blood serum					−
Vesicular	Smears	+	+	±	+	
	Vesicle Fluid	+	±	+	+	
	Blood serum					±
Pustular	Smears	+	±	±	+	
	Pustule Fluid	+	−	+	+	
	Blood serum					+
Crusting	Crusts	+	−	+	+	
	Blood serum					+
Later	Blood serum					+
Time Required for Test:		1 hr	1 hr	3–20 hr	1–3 days	3–20 hr

Ppt. = Precipitation in agar gel
HI = Hemagglutination inhibition
TC = Tissue culture
CF = Complement fixation
CAM = Chorioallantoic membrane

Laboratory diagnostic procedures include: (1) Direct examination of material from lesions on the skin or mucous membranes for virus elementary bodies by a) light-microscopy of stained smears, b) electron microscopy, c) immunofluorescent examination. (2) Examination of material from skin lesions and blood serum from fulminant cases for virus antigen by precipitation in agar gel and by CF test. (3) Culture of virus from the blood in the preeruptive-phase of the disease, from saliva and from material from skin lesions by inoculation of CAM of embryonated eggs, and by inoculation of tissue culture. (4) Detection of antibody or a rise in antibody in the patient's serum by a) precipitation in agar gel, b) CF test, c) hemagglutination-inhibition (HI), d) neutralization (Nt) tests.

B. Precautions for Handling Infectious Material

All laboratory workers, janitors and night watchman, should be vaccinated yearly. All material from suspected cases of smallpox, including smears on microscope slides, should be regarded as in-

fectious. The handling and preparation of specimens are carried out in glass cabinets which can be sterilized by ultraviolet light, or on benches covered by cloths soaked in disinfectant such as 10% chlorox. All wrappers, containers, contaminated instruments, infected eggs, etc., are immersed in strong disinfectant before being taken out of the room for burning or sterilizing in the autoclave.

C. Collection of Material

Blood: 10 ml of venous blood are collected in a dry sterile tube and held to clot if serum is required for antibody studies. For detection of virus in the early preeruptive-phase of illness, blood is collected into heparin—10 units/ml of blood.

Saliva: In the early eruptive-phase of illness 1.0 ml is collected in a sterile glass bottle for isolation of virus by culture.

Smears: Clean the surface of 6 or more lesions with a cotton applicator soaked in alcohol or ether. In the maculopapular stage the lesions are scraped gently with a scalpel or needle with a cutting edge to obtain material from the lower epithelial layers of the skin. This material is spread thinly on clean slides. When the lesions are vesicular or pustular, the fluid is aspirated in capillary glass tubes or with a tuberculin syringe, and kept for cultural or serologic examination. The floor of the vesicles or pustules is scraped with a needle or scalpel to obtain material for the preparation of smears on slides. The smears are allowed to dry in air but should not be fixed. The slides are held apart at their ends by match sticks or by rubber bands before being placed in a box for dispatch to the laboratory.

Vesicular or pustular fluid: The contents of lesions collected in open-ended capillary glass tubes or by capillary pipette are placed in a screw capped bottle or rubber stoppered tube. Alternatively, the fluid may be aspirated with a tuberculin syringe which is placed in a sterile rubber stoppered tube and sent to the laboratory.

Scabs: At least 12 crusts should be placed in a sterile screw capped bottle.

D. Packing and Shipping of Specimens

All specimens are packed in a metal container with a screw-on-top or in a strong wooden box. The package must be labelled *"Highly infectious material from suspected smallpox"* so that proper precautions will be taken in the laboratory in handling the specimens. As examination of the specimens is usually an urgent matter they should be sent or taken to the laboratory as quickly as possible, and the laboratory should be warned by telephone of their arrival. The following information is supplied on the history sheet accompanying the specimen.

Name and age of patient
Brief clinical history with dates of onset of illness and of rash
Date of last successful vaccination of patient
Known exposure to smallpox or vaccinia, or recent travel abroad
Known exposure to chickenpox or history of previous chickenpox, or both.

Specimens need not be refrigerated in transit to the laboratory unless it is likely to take several days. Specimens not to be examined immediately, e.g., serum for antibody studies, are frozen. Blood specimens should only be frozen after separation of serum from the clot.

V. LABORATORY DIAGNOSIS

A. Direct Microscopic Examination for Virus

1. **Light-microscopy of stained smears**—Only 1 or 2 of the available smears are stained, the others are reserved as a source of material for electron microscopy or for tissue culture. A number of suitable staining methods are available but Gutstein's methyl violet and Gispen's silver method are simple and satisfactory.

a. **Gutstein's Method** (*20*)

Solutions required: 1.0% methyl violet 6 B or crystal violet in distilled water.
2.0% sodium bicarbonate ($NaHCO_3$) in distilled water.

If the air-dried smears are thick they may be rinsed with saline solution and then with distilled water. This removes protein but also some of the virus. If smears are thin, which is desirable, this step may be omitted. Smears are fixed with methyl alcohol for 30 min. This is washed off with water; equal parts of methyl violet and sodium bicarbonate are mixed, and filtered through filter paper to run under the inverted slide supported on capillary glass tubing in a petri dish. Stain for 20–30 min in an incubator at 37 C. Staining may be accelerated by filtering the stain onto the slide and heating it gently until steam rises (stain must not be boiled). The heating is repeated twice during the staining period of 4–5 min. The stain is washed off the slide under tap water, dried with filter paper and mounted in liquid paraffin for examination. In good preparations, numerous round virus particles of uniform size (0.3–0.4 mμ) stained very dark-purple will be seen in suitable areas of the smear (Fig 5).

b. **Gispen's Modification of Morosow's Silver Method** (*19*)

Solutions required: **Solution A** (fixative): glacial acetic acid 1 ml; formalin 2 ml; distilled water 100 ml.
Xylol in a cylindrical glass staining jar with ground glass stopper.

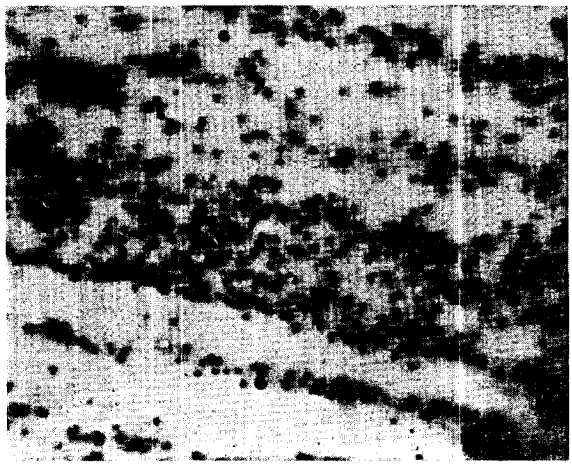

Figure 5—Virus particles in a smear from skin lesions of a smallpox patient stained by Gutstein's method. ×1500.

Solution B (mordant): Carbolic acid 1 ml; tannin 5 g; distilled water 100 ml.
Ammonia: 25% solution and 5% solution (the latter is preferable to 1% recommended by Gispen).
Silver nitrate: 10% solution.

Preparation of silver solution: Wash clean Erlenmeyer flask with distilled water. Place a loopful of 25% ammonia solution into remaining drop of water. Add 0.5 ml of 10% silver nitrate. Add 20 ml of distilled water. Add 5% ammonia solution drop by drop until ammoniacal silver precipitate redissolves (about 0.4 ml). One drop of 10% silver nitrate is added producing a faint bluish opalescence. This solution must be freshly made.

Air-dried smears are treated with solution A for 1 min. Rinse in tap water and dry in air. Immerse slide in xylol for 5 min and dry in air. Treat with solution B and heat until steam rises for 1 min. Rinse thoroughly with tap water and wash with distilled water. Apply freshly prepared silver solution and heat till steam rises for 2 min. Rinse in tap water and dry in air. Mount in liquid paraffin for examination. Virus particles, uniform in size, are stained brownish-black against a pale yellow background.

Gispen's method, although more involved, gives positive results from pustular lesions and with thicker smears. Until experience has been gained with these methods it is wise to have a control preparation of vaccinia virus stained for comparison.

c. Interpretation—The finding of numerous typical elementary bodies as shown in Fig 3 will often permit a tentative diagnosis of smallpox or vaccinia. However, it does not distinguish between variola and other poxviruses, but it does virtually rule out varicella

or herpes virus. A negative finding in stained smears does not exclude a diagnosis of smallpox for much depends on the stage of the disease and the care with which the smears are made. A positive finding in smears should always be confirmed by tests for virus antigen by serologic methods and by isolation and identification of the virus in culture (see below).

2. **Electron microscopy** (*12*)—In the hands of experienced workers electron microscopy provides a reliable and rapid identification of poxvirus particles. Material from skin lesions at any stage in the eruptive period is suitable.

a. **Procedure**—Smears on slides should be rubbed up with a tiny drop of distilled water. Vesicle and pustule fluids require no treatment. Crusts are ground in a small volume of distilled water. A small drop of specimen is placed on a Formvar (polyvinyl formal) coated grid. When almost dry, the grid is washed 3 times by touching it successively to a drop of distilled water on a slide and to the edge of a piece of filter paper. The grid is then touched to a drop of 1% glutaraldehyde and left in contact for 30 sec. (Variola virus is inactivated by such contact in 10 sec—H. S. Bedson, personal communication.) The grid is washed as before in a drop of distilled water and then placed in contact with a drop of 2% potassium phosphotungstate (brought to pH 7.0 with 1% potassium hydroxide). After 5 sec excess phosphotungstate is removed with filter paper and the grid is allowed to dry. It is then examined at 20,000 to 40,000×. Virus particles may be seen within one minute but grids should be scanned for at least 10 min before being considered negative.

b. **Interpretation**—The appearance of smallpox and varicella viruses as revealed by this negative staining technic is shown in Fig 6 and 7. With this technic the viruses of the varicella and herpes virus group are quite different in appearance from poxvirus particles. Poxvirus particles have a characteristic size and shape which is common to the variola-vaccinia group. Isolation of the virus in culture and further study are required for their separate identification.

3. **Immunofluorescence**—Smears from skin lesions, prepared as for examination by light-microscopy, and vesicle or pustule fluid may be used for FA microscopy. The technic is also suitable for examination of infected monolayers on cover slips in tissue culture tubes.

Two methods may be employed, direct and indirect. In the first method, fluorescein isothiocyanate is directly conjugated with the immune serum—antivaccinial serum prepared in the rabbit, or with variola convalescent-phase serum or gamma-globulin prepared from them. In the indirect method the same immune sera, unconjugated, are used to treat the specimen and this is followed by treatment

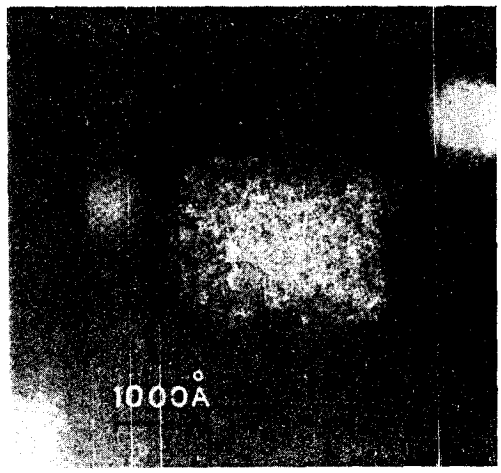

Figure 6—Electron micrograph of variola virus from skin lesions of a variola minor patient. (*Source:* Cruickshank, Bedson and Watson in *Lancet* 2:527–530, 1966.)

with an antirabbit serum, or antihuman serum, conjugated with fluorescein isothiocyanate. The method of preparing antivaccinial serum in the rabbit is given in section III. The methods used in preparing gamma-globulin, conjugation with fluorescent dye and absorption to remove free dye are described in Chapter 4.

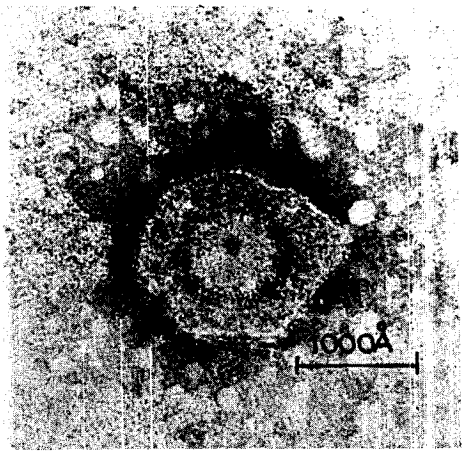

Figure 7—Electron micrograph of varicella virus from skin lesions of a chickenpox patient. (*Source:* Cruickshank, Bedson and Watson in *Lancet* 2:527–530, 1966.)

a. Staining procedure—direct method

1. Fix material on slide with acetone for 10 min at 4 C.
2. Circle smear with instant drying paint.
3. Cover smear with 0.1 ml of diluted conjugate. Incubate at 37 C for 30 min in a closed petri dish containing wet filter paper to prevent drying.
4. Wash twice with PBS pH 7.2 for 10 min on each occasion.
5. Allow slide to dry in air, mount in 10% glycerol with cover slip and examine under the FA microscope (see Chapter 4).

b. Staining procedure—indirect method—After fixation in acetone, 0.1 ml of rabbit antivaccinial serum or smallpox convalescent serum is applied to the smear and allowed to act for 30 min at 37 C, as in the direct method. The serum is removed by washing with buffered saline solution, as before. The smear is then treated with fluorescein-conjugated goat antirabbit serum or, if smallpox convalescent serum has first been used, with fluorescein-conjugated rabbit antihuman serum. The slide is held in moist chamber for 10 min at 37 C and then washed as above. Mount the preparation with a cover slip and examine as before.

c. Interpretation—The interpretation of appearances seen under the FA microscope requires care and experience. Nonspecific fluorescence to some extent can be avoided by careful preparation and absorption of the fluorescein-conjugated serum. The most suitable dilution of antiserum and conjugate to give specific staining is determined by preliminary tests with known positive smears. It is advisable to use for control purposes (a) a known positive smear, (b) a smear made from other skin lesions, e.g., varicella, (c) a normal serum or serum conjugate to treat one-half of the smear or a duplicate of the specimen under test. A positive result will not distinguish a case of generalized vaccinia or cowpox from variola. Good results have been claimed for the indirect method (*17, 28, 31*) but it seems little superior to stained smears of clinical specimens (*28*) and false positive results have been reported. Cover slip preparations of infected tissue culture monolayers are more suitable for examination by this technic and are reported to give good results 24 hr after infection of the tissue culture (*6, 25*).

B. Examinations for Virus Antigen

Antigen may be detected by the specific reaction of extracts of infected tissue or exudates with an immune serum in precipitation and CF tests. Precipitation tests in agar gel have for this purpose replaced the older type of test in tubes. Both the precipitation and CF tests provide evidence of poxvirus infection within 24 hr of receipt in the laboratory of suitable specimens.

1. Precipitation in agar gel to demonstrate antigen (*15*)—This method gives satisfactory results with vesicle or pustule fluid or extracts of crusts. Several thick smears on glass slides sometimes provide enough material to give a positive result. The material on the slides is suspended in not more than 0.1 ml of saline solution. Vesicle or pustule fluid is used undiluted, or diluted no more than 1:5 in saline solution and crusts are ground in no more than ten times their weight of saline solution. It is unnecessary to clarify crust extract or pustule fluid for testing. Sera from about one-third of acute fulminant cases have enough antigen to show precipitation when tested undiluted. Sera from ordinary cases do not give positive reactions. The material to be tested should not be heated to inactivate the virus; heating at 60 C greatly decreases the precipitating capacity of antigen. A good antivaccinial rabbit serum when diluted 1:2 or 1:4 with saline solution is suitable for testing clinical material.

a. Procedure—A plastic template is made in the dimensions of an ordinary microscope slide. Holes are drilled just large enough to admit a thin metal tube with a lower cutting edge of 4 mm internal diameter. The holes in the template should be cut in 3 rows with the centers of the holes 5.5 mm apart; 1% agar (Oxiod Ionagar No. 2 is suitable) in physiologic saline solution or distilled water is melted and pipetted between 2 glass slides held apart at their ends by oblong pieces of glass slide about 1 mm thick. When the agar has solidified, one of the glass slides is slid off the agar layer. With the template supported just above the agar by pieces of glass slide or perspex under its ends, the hollow metal tube is used to cut 4 mm diam reservoirs in the agar layer, with the centers of contiguous holes 5.5 mm apart. The template is removed and the agar disks are lifted out with the point of a thin knife blade. The antivaccinial rabbit serum is placed in one, with the test materials in neighboring reservoirs. A known positive antigen—extract of vaccinia-infected CAM or from rabbit skin, liquid smallpox vaccine or extract of smallpox crusts—is always included in the tests; normal rabbit serum is also tested against the extracts as an additional control. The slide is held at room temperature in a petri dish or in a plastic box containing moist filter paper or cotton wool to prevent drying of the agar. If the extract is from a case of smallpox or vaccinia, lines of precipitate will appear in the agar between the well containing the extract and the well with the immune serum. The lines will appear in 2 or 3 hr and link up in 4–5 hr with those formed between the known positive extract and the immune serum (Fig 8). The test may be further enlarged by including an extra reservoir containing a zoster convalescent-phase serum, found by previous testing to react with chickenpox or zoster vesicle fluid. Extracts of chickenpox crusts, however, will frequently fail to react with such a serum.

b. Interpretation—The occurrence of precipitation lines between the test extract and immune serum linking with those between the known positive extract and immune serum is evidence

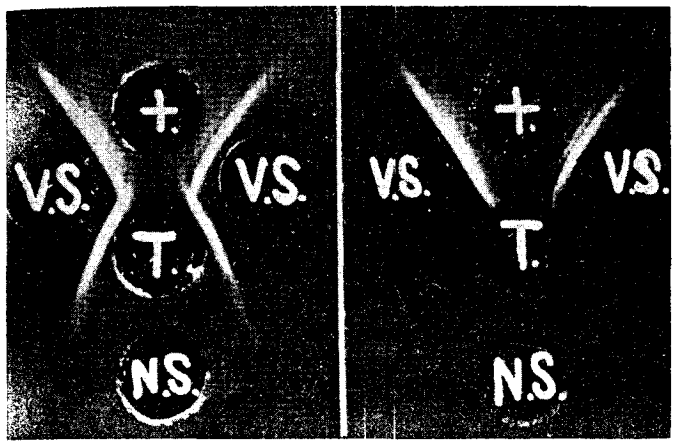

Figure 8—Precipitation test in agar gel for variola-vaccinia antigen. Left, positive reaction; right, negative reaction. T = test extract; + = positive control extract of variola crusts; V.S. = antivaccinial rabbit serum (undiluted and 1:2); N.S. = normal rabbit serum. (*Source:* Dumbell and Nizamuddin in *Lancet* 1:916–917, 1959.)

of the presence of variola, vaccinia or cowpox antigen in the test material. The test should not be considered negative until the preparation has been allowed to stand 24 hr. In cases of smallpox the test may be negative if the available material is inadequate to prepare the extract.

2. Complement fixation test for detection of antigen in clinical specimens (*9*)—CF test is more sensitive than the precipitation technic but requires more technical experience and 18–24 hr for completion. The same materials may be examined as in the precipitation test but more care is required in the preparation of extracts.

The following may be tested for antigen:

a. **Blood serum** from hemorrhagic or fulminant cases—This is heated at 58 C for 30 min and 2-fold dilutions are tested.

b. **Smears from papules, vesicles and pustules**—The material on 2 or 3 slides is suspended in 1.0 ml of saline solution by scraping with the end of a clean slide. The suspension is held at room temperature for 1 hr; 0.2 ml is removed and diluted with saline solution containing antibiotic for virus isolation in eggs or in tissue culture. The remainder of the suspension is centrifuged at 1500 rpm for 15 min; the supernatant fluid is heated at 58 C for 30 min and used for the CF test.

c. **Vesicle and pustule fluids**—If sent in capillary tubes these are ground up in the test tube with a glass rod; 1.0 ml of saline solution is added and the material is held at room temperature for 1 hr; 0.2 ml is removed

and treated with antibiotics for virus isolation; the remainder is spun at low speed. The clear supernatant fluid is removed and heated at 58 C for 30 min before testing.

d. Scabs or crusts—If these are wet or moist they are dried in a phosphorus pentoxide (or calcium chloride) desiccator for 4 hr. The dried scabs are weighed, ground up with glass powder in an enclosed tube and 4–5 ml of ether are added. After about 30 min the ether is pipetted off and the remainder removed by warming the tube in a waterbath at 37–50 C. The ground crusts are then extracted with saline solution to make 1:100 dilution, held for 2 hr at room temperature and shaken frequently. The suspension is spun at 1500 rpm for 15 min; the clear supernatant fluid is removed and heated at 58 C for 30 min. The treatment with ether tends to remove anticomplementary activity from the final extract. The heating of extracts at 58 C does not materially affect their CF activity and renders them safer for manipulation.

The antivaccinial rabbit serum is tested beforehand in doubling dilutions in a block titration with dilutions of vaccinial antigen—such as that prepared from infected rabbit skin (section III). The dilution chosen for use in testing extracts of clinical material is the highest dilution which gives complete fixation with the highest reacting dilution of antigen. The example shown in Table 2 was a dilution of 1:100.

e. Procedure—In setting up a tube test it is convenient to use 0.1 ml volumes of extract dilutions, antiserum and complement. The extracts are also tested against normal rabbit serum diluted as the immune serum. A known positive antigen in suitable dilutions is always included. The tubes, including appropriate antigen, complement and serum controls, are held overnight at 4 C. Next morning the rack is placed in a waterbath at 37 C for 10 min; then sensitized cells are added to the tubes and replaced in the waterbath.

Table 2—Complement Fixation: Block Titration of Dilutions of Vaccinia Immune Rabbit Serum and Rabbit Vaccinial Antigen

Rabbit Vaccinial Antigen Dilutions	Dilutions of Antivaccinial Rabbit Serum								Antigen Controls
	1:25	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	
1:25	++++	++++	++++	++++	++++	++++	+++	–	–
1:50	++++	++++	++++	++++	++++	++++	+++	–	–
1:100	++++	++++	++++	++++	++++	++++	+++	–	–
1:200	++++	++++	++++	++++	++++	++++	++	–	–
1:400	++++	++++	++++	++++	++++	++++	++	–	–
1:800	++++	++++	++++	++++	++++	++++	+	–	–
1:1600	++++	++++	++++	++++	++++	–	–	–	–
1:3200	++	++	+++	+	–	–	–	–	–
Serum Controls	±	–	–	–	–	–			

++++ Complete fixation, +++, ++, + and ± Diminishing degrees of fixation,
– No fixation (complete hemolysis).

f. Interpretation—If 2 or more tubes show complete fixation of complement while the antigen dilutions by themselves and those mixed with normal rabbit serum are completely hemolysed, the result can be considered positive for variola-vaccinia antigen. Approximately two-thirds of sera from hemorrhagic cases of smallpox will give a positive test for antigen by CF test when diluted 1:10 and sometimes even in dilutions up to 1:160 or 1:320. The serum from ordinary smallpox cases will be negative. Extracts of crusts will usually show fixation up to a dilution of 1:3000 or 1:5000 and vesicle and pustule fluids up to 1:300 or 1:500. A thin smear submitted on one slide will frequently not provide sufficient material to give a positive test. A positive result for antigen by precipitation or CF tests does not differentiate between variola and vaccinia but conclusively rules out varicella and herpes simplex which might be considered in the differential diagnosis.

C. Isolation of Virus

The tests described above will not distinguish between the virus of vaccinia and that of variola. It is only by isolation of the virus in the laboratory that the differentiation can be made with certainty. This also applies to the identification of a smallpox virus as variola major or variola minor. Isolation of variola virus by inoculation of animals is not a practical procedure. The methods used are inoculation on CAM of 11–13 day chick embryos or into tissue culture. The chick embryo technic depends on the availability of incubated fertile hens' eggs of the appropriate age. In the laboratory concerned with smallpox diagnosis this involves incubating eggs twice weekly to ensure that embryos of the right age are always available.

1. Isolation of virus on the chick chorioallantois—Preparation of material for inoculation: For making suspensions and dilutions of material for inoculation, PBS, Hanks' BSS or Earle's saline solution or physiologic saline solution with nutrient broth added to make 10% may be used. To all these fluids penicillin (500 units/ml) and streptomycin (500 μg/ml) are added.

a. Blood—If a heparinized specimen is available it is held at room temperature for 1 hr to allow the red cells to settle. The supernatant plasma is removed and centrifuged to deposit the white cells. The supernatant plasma and resuspended white cells are inoculated separately in 0.1 ml amounts into eggs. If only clotted blood has been submitted, serum and the ground up clot are inoculated. Virus can be isolated from the blood of most fulminant cases, but only in the first 2 days of fever from ordinary cases of smallpox.

b. Saliva is examined for virus in the early stages of disease, but in the eruptive-phase, material from skin lesions is more suitable. Saliva is mixed with an equal volume of diluent before inoculation.

c. Smears on slides—from lesions at any stage of eruption should yield virus on culture. The material is suspended in 1.0 ml of fluid and also at 1:100 dilution of this suspension should be used for inoculation.

d. Vesicle and pustule fluids are suspended in 1.0 ml of diluent and also a 1:100 or 1:1000 dilution of this suspension are inoculated to ensure that discrete lesions may be observed later.

e. Crusts are ground in 1.0 to 2.0 ml of fluid. The suspension is held for 10–15 min or lightly centrifuged to deposit gross particulate material. The supernatant fluid and a 1:100 dilution of it are used for inoculation.

f. Procedure—The technic for CAM inoculation of embryonated eggs is described in Chapter 1. A spring loaded punch (*24*) or some similar instrument is used to make holes in the shell over the air space and over the chorioallantois. After inoculation the eggs are incubated at 36 C. Some of the inoculated eggs are opened after 2 days, the others after 3 days of incubation. The egg shell over the new air space is removed with a pair of sterile forceps and the exposed chorioallantois is excised with scissors, care being taken to cut a few millimeters beyond the point of attachment of the CAM to the shell membrane. The membranes are examined against a dark background in a good light.

g. Interpretation—The viruses of variola major and minor produce after 48 hr tiny convex shiny greyish-white lesions 0.5 to 1 mm diam; at 48 hr the lesions produced by vaccinia are opaque white with a smooth surface about 1 to 2 mm diam. After 3 days the difference is even more obvious; the lesions of variola are opaque white raised lesions about 1 mm diam with a smooth shiny surface (Fig 3) whereas the pocks of vaccinia are larger (3–4 mm diam), flatter and often have a dull ulcerated surface (Fig 4). The lesions produced by cowpox virus at this time are 2–3 mm diam and red throughout due to hemorrhage in the underlying mesoderm. Varicella virus produces no change on the chorioallantois and the only other virus from clinical material likely to give rise to difficulty is that of herpes simplex. However, this virus usually produces tiny lesions, smaller than those of variola, although occasional strains may produce appearances similar to those of variola. Where there is doubt, this may be resolved by preparing histologic sections of the lesions, or testing an extract of heavily infected membranes for hemagglutinin or for precipitating and CF antigen with immune sera (see below).

2. Isolation of variola virus in tissue culture—Variola virus will grow and produce CPE in a variety of cells derived from human and animal tissues (*32*). It grows more rapidly in cultures of human cells; cultures of human embryo skin muscle, HeLa and KB cells, human amnion or monkey kidney give satisfactory results. In cultures of chick embryo cells, the virus grows more slowly and CPE may not be detectable for several days.

a. Procedure—The method of obtaining monolayer cultures of HeLa and other cells is described in Chapter 3. Leighton tubes with monolayers growing on cover slips are particularly suitable for the study of stained preparations and for examination by FA technic. Material for inoculation is prepared as for inoculation of chick embryos, although it is preferable to use Hanks' BSS with broth added to make 10% as suspending and diluting medium. If serum is to be used in the maintenance medium after inoculation of virus—as for example for HeLa cells grown in Medium #199 or in Eagle's basal medium with 20% calf serum added—the serum must be tested beforehand to ensure that it contains no virus-inhibiting antibody. Cell cultures are washed with Hanks' BSS before adding to each tube 0.1 ml of the inoculum to be tested for virus. The tubes are then incubated at 36–37 C for 1 to 3 hr before adding 0.9 ml of medium to each tube; they are then reincubated. At least 3 tubes are inoculated with each dilution prepared from the specimens submitted. The tubes are examined for CPE with low-power binocular microscope twice daily. Uninoculated cultures are included as controls with every test.

b. Interpretation—*Variola virus:* The time required for CPE to appear varies with the amount of virus in the inoculum. With clinical specimens changes may appear in 24 hr but may be delayed for 3 or more days. In HeLa cells, or in other cell lines derived originally from human malignant tissue and in some continuous cell lines derived from normal tissues e.g., human embryo lung, variola produces small discrete hyperplastic foci in cell monolayers (*36*). Such hyperplastic foci appear in 1 to 3 days. Later, as a result of cell necrosis, these foci are replaced by small clear plaques surrounded by rounded ballooned cells and sometimes by giant cells. These hyperplastic foci are not formed in primary cultures from normal tissue and are not produced by vaccinia or cowpox viruses. In human amnion and monkey kidney cell cultures small plaques are produced in 3 or 4 days and CPE spreads only slowly through the cell sheet. Human fetal diploid cells are particularly suitable for growth of variola virus (*25*). With a large inoculum CPE may be seen in these cultures in 24 hr in the form of focal areas of cell degeneration; cells become rounded and multinucleated cells appear in the areas affected. The hyperplastic foci and small plaques may

be readily seen by naked eye after staining the cell sheet with strong carbol fuchsin for 20–60 sec. In infected cover slip preparations cytoplasmic inclusions may be demonstrated by appropriate staining methods in 24 hr or less, and by FA staining foci of fluorescent cells may be found in 24 hr (*6*).

Vaccinia virus: CPE often appears in 24 hr and clear plaques 2–3 mm in size surrounded by rounded up cells are present in 40–48 hr. Soon afterwards secondary foci appear and in 3 or 4 days the whole cell sheet may be involved. CPE appears more rapidly and spreads more quickly than in cultures infected with variola virus.

Herpes simplex virus: CPE produced by this virus appears early and rapidly involves the whole cell sheet.

Varicella virus: Does not produce CPE before 4–6 days on primary isolation. Then foci of rounded up cells appear and soon multinucleated giant cells are to be found. Varicella virus produces CPE in cultures of human and monkey cells but not as a rule in cells derived from other animal species. Both herpes simplex and varicella viruses induce the formation of intranuclear inclusions in infected cells and these are readily demonstrable in fixed and stained cover slip preparations.

3. Identification of virus grown in embryonated eggs or in tissue culture

a. Technics available—The following tests may be used in the identification of virus isolated on CAM: (a) macroscopic appearance of the lesions as described above, (b) histologic appearance of the lesions in paraffin section of CAM—vaccinia and variola form cytoplasmic inclusions whereas herpes simplex lesions show intranuclear but not cytoplasmic inclusions, (c) 20% saline extract of heavily infected membranes will hemagglutinate, (d) will form a precipitate in agar gel, (e) fix complement with an antivaccinial rabbit serum. These tests will serve to distinguish variola from herpes simplex but, with the exception of (a), will not distinguish vaccinia from variola virus. Variola produces much smaller lesions than vaccinia on CAM, but if lesions are confluent so that individual pocks are not readily visible, a suspension of the membranes should be inoculated in several 10-fold dilutions into 2 sets of chick embryos, one to be incubated at 36 C and the other at 39–40 C. If the virus is variola, lesions will appear only in the eggs incubated at 36 C, whereas, if the virus is vaccinia, lesions will appear at both temperatures. The virus of variola minor is readily distinguished from variola major by a difference in ceiling temperature of the 2 viruses (*16*). Variola major virus produces lesions on CAM at 38–38.5 C as well as at 36 C, whereas variola minor virus does not produce lesions at 38 C or over.

For virus grown in tissue cultures the hemadsorption (HAd) test will serve to distinguish a virus of the variola-vaccinia group from one of the herpes group (see below). If cover slips have been used in the tissue culture tubes for the isolation of virus, the cytologic appearances in stained preparations will confirm the differentiation. FA examination of the cell sheet will confirm the presence of a virus of the vaccinia-variola group. To distinguish vaccinia from variola, inoculation of the tissue culture suspensions into eggs, incubated at 36–37 C and 39–40 C, will be decisive.

b. Methods of choice

1) *Isolation on CAM*—The size and appearance of the lesions on the chorioallantois is usually sufficient to distinguish variola from vaccinia and from herpes simplex. If doubt should remain, the following procedure is recommended: From heavily infected membranes a 20% suspension is made by shaking the membranes in a stoppered sterile glass bottle containing glass beads. The suspension is spun at 1500 rpm for 10 min. The supernatant fluid is tested in doubling dilutions from 1:5 upwards against a 0.5% of susceptible fowl RBC for hemagglutinin (see D, 3, b below). Viruses of the variola-vaccinia group produce hemagglutinin, whereas members of the herpes virus group do not. A known vaccinial hemagglutinin should be tested as control. The same supernatant fluid is tested for variola-vaccinia antigen in agar gel precipitation test and in a CF test against antivaccinial serum, with appropriate controls. If the appearance of the pocks does not seem to identify the virus as variola but rather as vaccinia, the ceiling temperature test on subculture to additional embryonated eggs (see above) should resolve the issue.

2) *Isolation in tissue culture*—In human fetal diploid cells and in monkey kidney cultures, the lesions appear more slowly than in cultures infected with vaccinia virus. Definite lesions may not be seen until after 48 hr; the foci enlarge more slowly, and spread of CPE through the cell sheet may take several days more. In HeLa cells the appearance of hyperplastic foci is characteristic of variola virus. Confirmation may be obtained by the HAd test and FA staining.

Chick RBC found suitable by prior tests on vaccinia infected cultures are used for HAd test. Of a 0.5% suspension of washed RBC, 0.2 ml is added to the cultures of the new isolate and to uninfected cultures. Tubes are held at room temperature for 15 min, then gently shaken and examined with a dissecting microscope. Hemadsorption is shown by the adhesion of RBC around infected cells, or groups of cells, in the monolayer. This test will often be positive in variola and vaccinia cultures in 24–48

hr even before manifest CPE is seen. Herpes simplex virus shows no hemadsorption.

Finally, if isolation has been made on primary tissue cultures and doubt exists whether the virus is variola or vaccinia, a suspension of the infected cultured cells and fluid is subcultured in embryonated eggs and in further tissue cultures incubated at 36 C and 39–40 C. If the virus produces lesions at both temperatures it is vaccinia, if only at the lower temperature it is variola.

D. Serologic Diagnosis

Antibody as measured by various technics is often undetectable before the 5th or 6th day of illness. In the average case of smallpox, the skin eruption has developed by this time and diagnosis in doubtful cases can be readily established by the tests described above for the detection of virus or virus antigen in the skin lesions. However, in cases in which the diagnoses have been missed, or in suspected cases of *variola sine eruptione* when no material from skin lesions is available, the examination for serum antibody may be the only laboratory test available to confirm the nature of the infection.

Serum antibody may be estimated by various methods including: (a) precipitation in agar gel, (b) complement fixation, (c) hemagglutination-inhibition, (d) neutralization tests. Various methods have been introduced to determine the neutralizing activity of serum, for example the reduction of pock counts on the chorioallantois, the reduction of plaque counts on tissue culture monolayers, the reduction of fluorescent foci in monolayers treated with fluorescent antibody (*6*), and the reduction in hemadsorption centers on monolayers to which a suspension of suitable fowl RBC has been added. All these methods of determining neutralizing antibody titers involve the incubation of mixtures of serum dilutions with a constant amount of virus suspension prior to inoculation of the mixtures on the chorioallantois or in tissue culture monolayers. Only the first two neutralization technics are described below. These methods are simpler and more sensitive than Nt tests in the skin of rabbits.

1. Precipitation tests in agar gel

The technic to be used here is the same as the one for detection of antigen described above, except that undiluted sera are tested against a known precipitating antigen. This antigen may be prepared from vaccinia-infected rabbit skin or from vaccinia-infected chorioallantoic membranes. The preparation of soluble antigen from vaccinial infected rabbit skin is described in section III. Antigen is prepared from vaccinia-

infected CAM as follows: 12 day old chick embryos are inoculated on the chorioallantois with a dose of virus calculated to contain 1000 PFU. Three days later the eggs are opened, the infected CAM is excised and placed in a glass bottle containing sterile glass beads. For each membrane 1.0 ml of physiologic saline solution is added. The contents of the bottle are shaken vigorously for 5 min and the suspension it then spun at 5000 rpm in an angle centrifuge for 10–15 min. The clear supernatant fluid is used as antigen.

In tests for antibody by precipitation technic a positive serum—either smallpox convalescent-phase serum or rabbit antivaccinial serum—is always included. A positive result is shown by the appearance, usually within a few hours, of a white line in the agar between the cups containing the patient's serum and the antigen. This line should link up with the precipitation line between the neighboring known positive serum and the antigen.

a. Interpretation—This simple test is not quantitative but is useful as it will give positive results with sera from most cases of smallpox after 8 days of illness. Sera from toxic or malignant cases will usually be negative. In absence of a history of smallpox, sera from unvaccinated healthy persons never, and from recently vaccinated or revaccinated individuals rarely give positive results.

2. Complement fixation

a. Antigen—The antigens prepared from infected rabbit skin or chorioallantois are used as in the precipitation test. The optimal concentration for testing of patient's serum is determined by a block titration of antigen dilutions against immune serum dilutions as shown in Table 2. In this example, the constant antigen dilution used in testing sera for antibody was 1:100.

b. Procedure—All sera are inactivated by heating at 58 C for 15 min prior to testing. Doubling serum dilutions from 1:5 upwards are then mixed with 2 doses of complement and constant antigen and held at 4 C overnight for the test next morning. The usual controls are set up, including test of serum dilutions with control antigen (extract of normal rabbit skin or CAM), serum dilutions without antigen, and specific antigen control without serum. A positive serum of known titer is included with each test.

c. Interpretation—After 7 days of illness most of the sera from smallpox patients will give positive results and by the 10th or 11th day the titer may be as high as 1:640 or 1:1280. When smallpox occurs in previously vaccinated individuals the test may be positive 5 days after onset of illness. After vaccination or revaccination the titer of CF antibody is usually low, rarely more than 1:40 and may be negative at 1:5. As CF antibody elicited by vaccination is rarely detectable 6–12 months later, CF titer of 1:20 or higher in a

person who has not been vaccinated within the previous year provides presumptive evidence of smallpox infection. A 4-fold rise in titer between an early serum taken before the 6th day of illness and a sample taken 1 to 3 weeks later would be even stronger evidence in support of the diagnosis.

3. Hemagglutination-inhibition test

a. Preparation of hemagglutinin—Vaccinia infected CAM, as in the preparation of precipitating antigen, are ground with glass powder and then suspended in 0.85% saline solution containing 1.0% inactivated normal rabbit serum, using 2 ml of fluid per membrane. The suspension is spun at 2000 rpm for 15 min. The supernatant fluid contains the hemagglutinin which usually has a titer of 1:128 or 1:256; it may be stored for months in the frozen state at —20 C. Hemagglutinin may also be prepared from vaccinia infected monkey kidney monolayers. The medium is removed from confluent monolayers and the cell sheets are washed with Hanks' BSS. Vaccinia virus, about 1000 TCD_{50} grown in monkey kidney, is used to infect the cell sheets, and a medium consisting of equal parts of Medium #199 and Eagle's basal medium without serum is added. The cultures are incubated until the cell sheets show widespread CPE (3 to 4 days). The cells are scraped off the glass into the medium and the fluid is oscillated for 5 min. The fluid is spun at low speed, 1500 rpm for 10 min, and the supernatant fluid is used as hemagglutinin. Virus passed repeatedly in monkey kidney tissue cultures usually produces hemagglutinin with a titer of 1:128.

b. Chicken red cells—The red cells from only certain hens are suitable; this can be determined by testing with a 1:10,000 dilution of cardiolipin microflocculation antigen. Blood is collected from several chickens in Alsever's solution and washed in 3 changes of 0.85% saline solution. Washed packed RBC are used to make 0.5% suspensions in saline solution. The cell suspensions are tested by adding 0.25 ml amounts of doubling dilutions of hemagglutinin to 0.25 ml of RBC suspension and making the total volume in each tube up to 0.75 ml with saline solution. The tubes are held at room temperature for 1 hr before reading the results. Hemagglutinin is indicated by the settling of the agglutinated RBC over the bottom of the tube. In tubes showing no hemagglutination, the RBC settle as a compact button in the center of the bottom of the tube. Chickens with susceptible cells are kept for further tests.

c. Procedure—Sera to be tested for HI are inactivated as for CF tests and then absorbed by adding to 0.2 ml of serum, 0.6 ml of saline solution and 0.2 ml of 20% suspension of washed chicken RBC. This mixture is kept at 4 C for 1 hr and spun at 1500 rpm for 10 min. The clear supernatant fluid, representing a 1:5 dilution of the serum, is used for the test.

The hemagglutinin is diluted to contain 4 hemagglutinin doses in 0.25 ml, as shown by the previous titration. Of the absorbed sera under test, 0.25 ml amounts of doubling dilutions are placed in a row of tubes. To each tube 0.25 ml of hemagglutinin is added. In addition, 0.25 ml

of hemagglutinin undiluted, and diluted 1:2, 1:3, 1:4, and 1:6 are placed in 5 additional tubes and 0.25 ml of saline solution is added to each to check the potency of the hemagglutinin. The rack of tubes is placed in a waterbath at 37 C for 1 hr; 0.25 ml of chicken RBC suspension is added to each tube and the rack is held at room temperature for 1 hr before reading the results. The titer of antihemagglutinin in the serum is indicated by the highest dilution of the serum inhibiting the agglutination of the chicken RBC. A positive control serum of known HI titer is included with each test.

d. Interpretation—This test is relatively simple. Postvaccination sera may show HI titers up to 1:80 or 1:160 but these antibodies usually disappear after 1 or 2 years. In sera of smallpox patients HI antibodies appear about the 4th or 5th day of illness and in the convalescent-phase sera the titer may rise to 1:1000 or more. A 4-fold rise in titer between sera taken early (1st to 4th day) and sera taken later (after 7th or 8th day) provides presumptive evidence of infection with variola or vaccinia virus.

4. Neutralization test—This test may be made on CAM, or in tissue cultures. In either case a virus suspension of known infectivity must be used.

a. For tests on CAM—A partially purified suspension of virus is preferable. Variola virus producing small pocks on CAM gives excellent results, but vaccinia is safer to handle and gives comparable titers. The vaccinia virus suspension is prepared from infected rabbit skin (see section III) or from CAM. Infected membranes are vigorously shaken in a bottle containing glass beads in sterile buffered distilled water. The suspension is spun at 1500 rpm to deposit tissue and cell debris. The supernatant fluid is spun at 10,000 rpm in a Spinco or suitable angle-head centrifuge to deposit the virus. This may be partially purified by resuspending in buffered distilled water and again centrifuged at high speed once or twice. The final suspension of virus is oscillated to disperse the virus. If sterile glycerol is added to make 40%, this suspension may be stored at −20 to −30 C without appreciable loss in titer. The suspension is titrated by inoculating 0.1 ml of 10-fold dilutions, prepared in saline solution containing 10% nutrient broth, on CAM of 12 day old chick embryos. Four or 5 eggs are inoculated with each dilution; after 48 hr incubation, the eggs are opened and the membranes are excised. The lesions are counted and the mean count for each group of 4 eggs is determined. The dilution calculated to give 100 to 150 pocks in 0.1 ml is used for the test.

Procedure: Sera are inactivated at 58 C for 20 min before the test. Doubling dilutions of serum are made in 0.85% saline solution to which 10% nutrient broth was added. To 0.5 ml of each dilution, 0.5 ml of diluted virus suspension is added. The tubes are placed in a waterbath at 37 C for 2 hr; 0.1 ml per egg of the suspension is then inoculated on CAM prepared 30 min to 1 hr before. A positive serum of known titer and a negative serum are tested in the same manner. The opening over

CAM is closed with paraffin wax and the eggs are rolled gently to distribute the inoculum. Five or 6 eggs are inoculated with each serum-virus mixture. The eggs are incubated at 36 C for 48 hr. The eggs are then opened, the infected CAM excised and placed in formol saline solution (2% formalin) for counting of lesions. The counts are averaged for each serum-virus mixture and the results, expressed as a percentage of the mean count for the negative serum-virus mixture, are plotted on graph paper. The dilution of serum which reduces the pock count to 50% of the control is the neutralizing titer of the serum.

b. For neutralization tests in tissue culture—Monkey kidney monolayers in Leighton tubes are convenient. Monkey kidney cell suspension is grown in Leighton tubes in a medium consisting of 50% Medium #199 and 50% Eagle's MEM (containing 10% calf serum). It is preferable to use vaccinia virus which had undergone several passages in monkey kidney tissue cultures. The virus is used in a dilution determined by titration to produce 100-150 plaques from 0.1 ml on monkey kidney monolayer cultures.

Procedure: The sera to be tested, previously inactivated, are diluted 2-fold from 1:5 upwards in maintenance medium. This is similar to the growth medium but with the calf serum omitted. To 0.5 ml quantities of serum dilutions, 0.5 ml of suitably diluted virus (in maintenance medium) is added. The mixtures are kept at 37 C for 2 hours. A known negative serum and a positive serum of known titer are always included. The growth medium is removed from the Leighton tubes and the cell sheets are washed with warm Hanks' BSS; 0.1 ml of each serum-virus mixture is inoculated into each of 3 or 4 tubes. To each tube, 0.9 ml of maintenance medium is added and the tubes are incubated in appropriate racks.

The control tubes inoculated with virus mixed with 1:10 dilution of negative serum are examined under a dissecting microscope after 38–40 hr. If lesions are readily visible, the fluid is removed from all tubes in the test and the cell sheets are stained by application of strong carbol fuchsin solution for 20–60 sec. The stain is then washed off with saline solution and the clear plaques on the stained sheet of cells are counted. The average number of plaques per tube for each virus-serum mixture is determined and the results are plotted on graph paper to determine the 50% reduction of plaque count, as compared with the control tubes, for the serum under test.

c. Interpretation—This tissue culture Nt test gives similar results to those obtained by tests of the same serum-virus mixtures on CAM. For laboratories where tissue cultures are in regular use, this method may be more convenient than tests on CAM. Nt antibody may persist in low titer for years after vaccination and especially after revaccination. A rising titer is, therefore, more significant than a low titer on a single sample of serum. Smallpox convalescent-phase sera often have a neutralizing titer of 1:1000 and may reach 1:10,000. However, the simpler tests described under D, 1, 2 and 3 are often as informative and conclusive as is the test for Nt antibody.

5. **Other tests**—Biopsy of skin lesions may provide rapid confirmation of diagnosis, but this method is not often used. In stained sections prepared from a biopsy, a herpes or varicella lesion will be indicated by the superficial nature of the vesicle, the presence of multinucleated cells and intranuclear inclusions in epithelial cells in and around the vesicle or pustule. The smallpox lesion is more deeply embedded in the skin, it rarely shows giant cells, and cytoplasmic inclusions are present in epithelial cells at the base and sides of the lesion.

E. Summary of Laboratory Diagnostic Tests in Smallpox

1. **In preeruptive illness**—Test white cells from heparinized blood for virus by culture on CAM or by tissue culture of human cells. Test serum for antigen by CF test or by precipitation in agar gel. **These tests are likely to be positive only in very severe toxic cases.**

2. **In early eruptive period, macular and papular stage**—Test smears from lesions for presence of virus by examination with electron microscope, if available, or by stained smears. Isolate virus on CAM or in tissue culture and identify.

3. **Vesicular and pustular stage**—In smears from base of lesions examine for virus by electron or light-microscopy. Test fluid from lesions for virus antigen by CF or by precipitation in agar gel for variola-vaccinia antigen and isolate virus on CAM or in tissue culture.

4. **Crusting stage**—Examine extract of crust for virus particles with electron microscope. Test extract for virus antigen by CF or by precipitation in agar gel. Isolate virus from extract on CAM or in tissue culture.

At all stages of the disease *proof* of smallpox infection in a person with suggestive clinical illness can be firmly established only by the isolation of virus from the patient on CAM or in tissue culture, and by its identification by serologic and other characteristics.

5. **Variola sine eruptione**—After crusts have disappeared or no skin lesions are present, i.e., in missed cases or cases of *Variola sine eruptione:* examine patient's serum for antibody by CF, precipitation in agar gel or by HI test. The interpretation of the result will depend on history of previous smallpox vaccination and on the antibody level found. Where early and late serum samples are available a 4-fold rise in titer will be significant.

In arriving at a final diagnosis, the laboratory findings should be assessed together with history of exposure, history of vaccination and the clinical features of the case.

F. Diagnosis of Vaccinial Infection

The same methods of diagnosis are used as in smallpox. Serologic tests do not distinguish vaccinia from variola. Except in some cases of vaccinia necrosum, virus or viral antigen will not be present in the blood. Vaccinia virus isolated from the patient's lesions on CAM or in tissue cultures will be distinguished from variola virus on the basis of the following:

1) Vaccinia virus produces on CAM much larger lesions and in tissue culture more rapid CPE.

2) Vaccinia virus does not produce hyperplastic foci on HeLa cell tissue cultures, whereas variola does.

3) Vaccinia virus produces pocks on CAM and CPE in tissue cultures at 39–40 C, whereas variola does not.

4) Vaccinia virus produces lesions on the rabbit skin which can readily be passed in series, whereas this is not possible with variola virus.

G. Laboratory Diagnosis of Cowpox Infection in Man

The lesions, usually seen on the hands, forearm or face of farm workers, have the same appearance as vaccinia. The virus resembles vaccinia in morphology, antigenic structure and host range. The same laboratory tests as for smallpox are used in diagnosis. The cowpox virus can be readily distinguished from vaccinia in that: (1) it produces typical hemorrhagic pocks on CAM and hemorrhagic lesions in the skin of rabbits and guinea pigs, and (2) in these lesions and in infected tissue cultures there are present numerous large homogeneous, strongly eosinophilic cytoplasmic inclusions which are not a feature of lesions caused by vaccinia or variola viruses (*13*).

H. Diagnosis of Milker's Nodules Infection

The clinical diagnosis may be suggested by a history of contact with cows suffering from spurious or pseudo-cowpox lesions on the udder, and by the appearance of firm cherry red nodules on the skin of the hands of the patient. Laboratory diagnosis may be attempted by the tests used in the examination of patients with the lesions of Orf (see Chapter 22, III). Microscopic and electron microscopic examination of material from the lesions will give results similar to those seen in Orf. No lesions will be produced with the material in chick embryos, baby mice, or in other laboratory animals. The virus may be isolated in cultures of bovine tissue (*18*) or of primary human amnion (*30*). CPE may not appear in primary culture for 10 to 12 days but on subculture it may appear in 2 to

3 days. The virus shows little serologic cross reaction with the vaccinia-variola group and produces no hemagglutinin. The patient of Friedman-Kien et al (*18*) developed CF antibody to tissue culture fluid antigen 3 weeks after his infection.

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