THE DIAGNOSIS OF VIRUS INFECTIONS

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With the advent of specific chemotherapy, precise diagnosis is important in the efficient management of virus infections. The sulphonamides were known to be of use against the viruses of lymphogranuloma venereum (Findlay, 1940), inclusion conjunctivitis (Thygeson, 1941; 1942), and trachoma (Loe, 1938); penicillin was shown to have some effect against the virus of psittacosis (Bedson and May, 1945; Heilman and Herrell, 1944). The development of the so-called newer antibiotics—*aureomycin*, *terramycin* and *chloramphenicol*—which are effective against a wider range of virus and rickettsial infections, suggests that other as yet undiscovered substances may eventually control all diseases due to viruses and rickettsiae.

The diagnosis of some viral infections can be made at the bedside; others require close co-operation between the clinician and laboratory virologist. Each plays an important role in the management of the case, and so in the subsequent welfare of the community. The clinician, from the history, epidemiological data and clinical findings, may narrow the differential diagnosis or indeed reach the correct diagnosis. The laboratory worker may confirm the diagnosis by isolating the aetiological agent, by showing a specific immunological response, or by demonstrating characteristic pathological changes in the patient's tissues.

To improve this co-operation the clinician must know the laboratory worker's requirements in any given case; he must know which are the most appropriate specimens, the optimum stage of the disease for their collection, and the value of paired acute-phase and convalescent sera for demonstrating rising antibody titres.

The Physician's Role

(a) History.

(b) Clinical findings.

(a) *History*. Indications of importance in diagnosis are: the prevalence of a certain disease in the community, the seasonal incidence of certain viral infections, or a history of contact with rabid dogs, infected syringe needles (hepatitis), sheep and horses (encephalitis), mice (lymphocytic choriomeningitis) or arthropods (rickettsiae). Viral and rickettsial diseases are

usually spread by direct contact, air-borne droplets, or arthropod vectors. Unlike bacterial infections, water-borne and food-borne epidemics, apart from infective hepatitis and possibly poliomyelitis, are relatively uncommon.

(b) *Clinical Findings*. The exanthemata are characterized by a skin eruption which appears in the prodromal or acute phase of the disease. Measles, German measles, smallpox and chickenpox, which are of viral origin, are infectious diseases of this type. Herpes simplex and herpes zoster are other examples in which eruptive skin lesions are usually characteristic.

If all viral infections had such outstanding characteristics, laboratory help would be unnecessary in diagnosis. Such is not the case, for instance, in the diagnosis of the viral pneumonias which are aetiologically distinct but clinically indistinguishable. Virus pneumonia can be caused by the viruses of influenza A and B, psittacosis, lymphocytic choriomeningitis and the rickettsia of Q fever, as well as the unidentified virus (or viruses) of the so-called 'primary atypical pneumonia.' In such an example the diagnosis can only be made with the help of a laboratory. Similarly, a better understanding of the various encephalitides, which are clinically indistinguishable (as are the benign aseptic meningitides), can only be obtained by recourse to the laboratory.

It is often assumed that the white blood cell count is a reliable index for differentiating viral from bacterial infections. Bacterial infections are usually accompanied by a polymorphonuclear leucocytosis, whereas viral infections are associated with a normal white cell count or perhaps a relative lymphocytosis. Occasionally, however, the white cell count is misleading. Encephalitis, lymphocytic choriomeningitis, and primary atypical pneumonia may be accompanied by a polymorphonuclear leucocytosis, whereas mild or fulminating bacterial infections may show a normal white cell count. Furthermore, associated viral and bacterial infections which occur commonly in the respiratory group make this rule-of-thumb even more misleading. Today, probably the commonest source of confusion with viral pneumonia is the inadequately treated bacterial pneumonia, in which the offending pneumococcus has been masked by small amounts of antibiotic.

The varying clinical manifestations of the Cox-sackie virus are worth stressing because they enter into the differential diagnosis of benign aseptic meningitis, non-paralytic poliomyelitis, acute pleurisy, coronary thrombosis, the acute abdomen and vesicular tonsillitis. Coxsackie is a village on the banks of the Hudson River in upper New York State. During an outbreak of poliomyelitis there in 1947, Dalldorf and Sickles isolated a virus, distinct from that of poliomyelitis. In the last three years, much interest has been focused on this virus, and the present knowledge of its relation to Bornholm disease is due to close co-operation between clinician and virologist. Its precise role as a causative agent in disease is difficult to assess. Dalldorf has rightly said that we are in the anomalous position of having discovered the cause before the disease. Such infection should be borne in mind in the diagnosis of the clinical pictures mentioned above.

The Laboratory Role

This resolves itself into (1) isolation of the virus from the patient, (2) serological tests to show immunological responses on the part of the patient and (3) the demonstration of characteristic inclusion bodies in the patient's tissues.

(1) *Isolation of the Virus.* This is often difficult, time-consuming or impossible. Such work, which is undertaken in special virological units, is not possible in routine hospital laboratories. The clinician, however, should know the diseases in which isolation of the virus can be undertaken conveniently, what specimens are needed and how they should be packed.

The viruses of influenza A and B, mumps and herpes simplex are recoverable early in the disease from throat washings, saliva and vesicle fluid, respectively.

The early identification of the virus responsible for an influenzal outbreak is important, for epidemics may be transmitted by international air travel. Throat washings should be obtained as early as possible in the disease, using a broth or saline gargle. The patient should cough before gargling, so that infective bronchial material is included in the specimen. Penicillin (500 units/ml.) is added to suppress bacterial growth. The amnion of fertile hens' eggs is inoculated with this throat washing. If there is a delay, or for transport, the specimen should be frozen in a thermos flask packed with carbon dioxide snow. A result may be obtained in 2-4 days. The infected, amniotic fluid agglutinates chick and human erythrocytes and this haemagglutination can be inhibited by appropriate Influenza A or B specific antisera (Hirst, 1942).

A clinical diagnosis of mumps usually renders

examination of saliva for virus unnecessary. The virus is recoverable from the spinal fluid in mumps meningo-encephalitis; this has been done in patients without parotitis on the second day of illness (Henle and McDougall, 1947). It is found that some cases of benign aseptic meningitis are due to the mumps virus (Kane and Enders, 1945). The best results are obtained by inoculating fertile hens' eggs as soon as possible after obtaining the fluid, but if necessary it can be frozen and kept until the results of other investigations are available. Diagnosis may be made within four to seven days, using the haemagglutination phenomenon mentioned above and the appropriate immune serum.

The herpes simplex virus may affect the skin (herpes simplex, Kaposi's eczema herpeticum), muco-cutaneous junctions (herpes labialis, proctitis and vulvo-vaginitis), mucous membranes (gingivo-stomatitis), eye (conjunctivitis) and central nervous system (meningo-encephalitis). Identification of the virus may be of clinical importance in such cases. It has been isolated from mouth lesions (Dodd, *et al.*, 1939), brain (Smith, *et al.*, 1941), spinal fluid (Armstrong, 1943), skin lesions (Ruchman, *et al.*, 1947) and blood (Scott, *et al.*, 1949). Specimens may be used directly or stored in phosphate-buffered saline pH 7.2 with $\frac{1}{2}$ per cent. gelatine, treated with penicillin 500 units/ml. Storage during transfer should be in a carbon dioxide snow container as with other viruses. Isolation of the virus is made by inoculation of the chorio-allantoic membrane of the fertile hen's egg, inoculation of the scarified cornea of rabbits or intracerebral inoculation in rabbits. A result may be obtained in two to seven days.

Isolation of the viruses of poliomyelitis, infective hepatitis and the Coxsackie virus can be undertaken from specimens of faeces, but results are not available sufficiently quickly to be of diagnostic value in an individual case. They are, at present, of epidemiological and research interest.

A complement-fixation test for smallpox, using vesicle fluid as antigen and potent anti-vaccinal serum as antibody, may help to prove the diagnosis early in this disease. Material from several lesions should be submitted, and a result can be anticipated within 24 hours (Craigie and Wishart, 1936; Downie, 1946).

(2) *Serological tests* to show an immunological response on the part of the patient are easier to perform, quicker, and give satisfactory results in many virus diseases. These tests are usually employed in confirming the clinical diagnosis. They depend upon the demonstration of rising titres of specific antibodies in convalescent, as compared with acute-phase sera, but they suffer from the disadvantage that it is necessary to wait

for convalescent serum. Thus the diagnosis is often retrospective, although high-acute-phase serum titres may be a helpful provisional guide.

Serological methods employed are:

- (a) The *in vitro* complement-fixation test, using virus antigen.
- (b) The *in vivo* animal neutralization test.
- (c) The *in vitro* haemagglutination-inhibition test.
- (d) Non-specific tests.

(a) *The in vitro complement-fixation test.* Potent virus antigen is titrated against serial dilutions of the patient's serum; the highest dilution of serum in which complement is fixed is the end-point. A fourfold or greater increase in titre in the convalescent, as compared with the acute-phase serum, constitutes a significant antibody response. A typical result (after Horsfall, 1950) shows:

Serum	1:10	1:20	1:40	1:80	1:160	1:320	Titre
Acute	+	o	o	o	o	o	1:10
Convalescent	+	+	+	o	o	o	1:40

+ = complement fixation
o = no complement fixation

This experiment shows a two-tube, or fourfold, increase, which is significant. Such tests have the advantage that they can be performed in any laboratory where Wassermann tests are undertaken, without recourse to animal work. They are helpful in typhus (epidemic, murine and scrub); the encephalitides (distinguishing St. Louis, Japanese B and equine types); psittacosis, lymphogranuloma venereum, Q fever, lymphocytic choriomeningitis, mumps, influenza A and B, and Coxsackie infections.

(b) *The in vivo Animal Neutralization Test.* Animal neutralization tests depend upon the comparative powers of the patient's acute-phase and convalescent sera to neutralize the appropriate viral disease in a number of test animals. Several groups of animals are infected with the standard virus, and each group is inoculated with varying dilutions of the patient's serum. The survival rate in the varying groups is an index of the amount of antibody in the serum. This method is employed only when absolutely necessary, because of the cost and complexity of the experiment. It is impractical for routine hospital use, but remains an important research tool. For the diagnosis of some viral diseases it is essential, because the simpler *in vitro* complement-fixation test has not been developed.

Typical result (after Horsfall, 1950):

Serum	1:10	1:20	1:40	1:80	1:160	Titre
Acute	S	D	D	D	D	1:10
Convalescent	S	S	S	S	D	1:80

S = animals surviving
D = animals died

There is a significant increase in titre, indicating a rise in antibody in convalescent compared with acute-phase serum.

This method is employed with the viruses of herpes simplex and yellow fever.

(c) *The in vitro Haemagglutination-Inhibition Test* is easy to perform, but is useful only in influenza and mumps. It depends upon the ability of the patient's serum, containing antibody, to inhibit haemagglutination by influenza and mumps virus. The patient's serum, in varying serial dilutions, is used and the highest dilution which inhibits the agglutination is taken as an end-point. There should be at least a fourfold increase in titre in the convalescent as compared with acute-phase serum. Typical result (after Horsfall, 1950):

Serum	1:10	1:20	1:40	1:80	1:160	1:320	Titre
Acute	o	+	+	+	+	+	1:10
Convalescent	o	o	o	o	+	+	1:80

o = haemagglutination inhibited
+ = haemagglutination

The acute-phase serum has prevented haemagglutination at 1:10, whereas the convalescent serum inhibited agglutination at 1:80. This indicates a significant increase in titre of antibody.

With all these serological tests, it is important to remember to collect blood as early in the disease as possible, and again two or three weeks later. About 6 ml. whole blood is sufficient. This is spun in the centrifuge, and the serum stored in a refrigerator at 4° C. until used.

(d) *Non-Specific Tests.* There are three diseases in which non-specific tests are useful: infectious mononucleosis, primary atypical pneumonia and typhus.

Infectious Mononucleosis. Apart from a characteristic blood count in infectious mononucleosis, the majority of cases develop heterophile antibodies which agglutinate sheep red blood cells (Paul and Bunnell, 1932; Bunnell, 1933). The test is positive in about 70 per cent. of cases by the second week of the disease, but may be positive in the first few days. Five ml. blood is collected and centrifuged to obtain the serum. Normal blood may contain sheep red cell agglutinins, which are also present in serum sickness in significant titres, and in people who have received horse serum injections. In order to avoid errors, it is well to remember the differential adsorption of these agglutinins by guinea-pig kidney and beef red blood cells (Stuart *et al.*, 1936).

Adsorbed by	Infectious Mononucleosis	Serum Sickness	Normal Serum
Guinea pig kidney	—	+	+
Beef R.B.C.	+	+	—

These heterophile antibodies are also present in the spinal fluid of cases of infectious mononucleosis (Silberstein *et al.*, 1948).

Primary Atypical Pneumonia. During World War 2 several groups of workers independently observed the occurrence of cold haemagglutinins in primary atypical pneumonia (Peterson *et al.*, 1943; Turner, 1943; Turner *et al.*, 1943; Shone and Passmore, 1943). In the absence of any specific tests, this phenomenon is at present of some diagnostic help, especially when it fits in with a suggestive clinical picture. The patient's serum agglutinates human (and various animal) red blood cells at 0 to 5° C., and agglutination is absent at 37° C. unless the titre is very high.

Cold haemagglutinins appear during the second week of the pneumonia, and decline during the first or second month after the onset. Titres should be performed at weekly intervals because a rising titre is more significant than a stationary level. Repeated examinations may show the presence of cold haemagglutinins which would otherwise remain undetected. Kneeland (1951) has observed that they may be extremely transient, as in one case where they were absent two days before and two days after a significant titre of 1:512.

There seems to be a rough correlation between the titre of haemagglutination and the severity of the illness; high titres being associated with a longer duration of fever (Finland *et al.*, 1945; Laurell, 1948), and a more extensive pulmonary involvement (Jordan *et al.*, 1951).

It is important that blood be kept warm in an incubator until centrifuged, otherwise some of the agglutinin will be lost with the patient's red cells. Five ml. blood is sufficient for the estimation. Serial dilutions of serum are titrated against a fresh 0.5 per cent. suspension of human Group O red cells. Since significant titre varies with individual laboratories, it should be ascertained whether the result is expressed as the original dilution of the serum or as the final dilution after the red cell suspension has been added.

Agglutination may be detected on the rim of the syringe when blood is taken from the patient; or it may be suspected because of difficulty in performing a blood count. This clue led to their detection by Turner in the epidemic in southern England in 1943.

About 44 per cent. of cases of primary atypical pneumonia show the phenomenon of 'streptococcus M.G. agglutination' (Horsfall, 1947). This is a further non-specific test of some diagnostic value. The serum of such cases agglutinates this non-haemolytic streptococcus. Serum from 5 ml. of acute-phase and a further 5 ml. of convalescent blood is sent to the laboratory. A four-

fold increase in titre is significant. This test resembles the Weil-Felix reaction, in that both depend upon the non-specific agglutination of a bacterium by the patient's serum.

Typhus. The Weil-Felix reaction is a simple and useful test which helps to confirm the diagnosis of epidemic, murine and scrub typhus. Serial dilutions of the patient's serum are mixed with constant amounts of *B. proteus* of the OX 19, OX 2 and OX-K strains. The result is expressed as the highest dilution which causes aggregation of these test suspensions:

	OX 19	OX 2	OX-K
Epidemic typhus	++	+	o
Murine typhus	++	+	o
Scrub typhus	o	o	++

+ = agglutination
O = no agglutination
(modified from Plotz, 1946)

The table summarizes the results obtainable in the different varieties of typhus. It is seen that epidemic and murine typhus cannot be differentiated by this test. The test is of great value in epidemic and murine typhus because agglutinins for OX 19 develop as early as the fifth to eighth day. Results for scrub typhus become positive in the second week, and help to confirm the clinical diagnosis, in an endemic area, of primary eschar and a rash appearing on the fifth day of fever. The test is negative in Q fever.

(3) **Inclusion bodies.** Specific anatomical changes in the patient's tissues are characteristic of certain virus infections. These intracytoplasmic or intranuclear inclusion bodies are found in chickenpox, smallpox, herpes zoster, herpes simplex, psittacosis and lymphogranuloma venereum, inclusion conjunctivitis and trachoma, rabies and yellow fever. At one time these bodies were of great diagnostic importance, but they have now to a certain extent been superseded by serological tests.

However, an examination of scrapings from the base of vesicles in cases of smallpox, chickenpox, herpes zoster and herpes simplex, and from the conjunctiva in trachoma and inclusion conjunctivitis, may help to establish an early diagnosis. Smears in cases of chickenpox, herpes zoster and herpes simplex show identical features, which are characteristic but quite distinct from other vesicular diseases of the skin. They show greatly enlarged giant multinucleated epithelial cells, in which the normal chromatin granules and nucleoli are absent. The nuclei (stained with Giemsa stain) contain reddish-violet stringy amorphous material, and green melanin granules are seen in a blue-staining cytoplasm (Blank *et al.*, 1951). These characteristics are not seen in smallpox. Instead, smears from cases of smallpox, when

stained by Paschen's method, show enormous numbers of elementary bodies (van Rooyen and Illingworth, 1944).

The cytoplasmic inclusion bodies of trachoma and inclusion conjunctivitis are morphologically identical, but in trachoma they are more numerous on the upper tarsal conjunctiva, whereas the reverse holds for inclusion conjunctivitis (Braley, 1940).

Autopsy may reveal inclusion bodies in the liver in yellow fever, and Negri bodies in the hippocampus in rabies.

the control, is an index of a positive reaction. A positive response means that the patient is, or has been at some time in his life, infected with the viruses of the psittacosis-lymphogranuloma group; it is no more specific than this, and should therefore be evaluated in the light of the clinical picture and serological results.

Skin tests have been used in mumps (Enders *et al.*, 1945, 1946), but are unsatisfactory. They are being used in herpes simplex (Rose and Molloy, 1947) and infective hepatitis (Henle *et al.*, 1950), but their value is at present uncertain.

INCLUSION BODIES

Disease	Intra-nuclear	Intra-cytoplasmic	Tissue in which found	Staining characters
Chickenpox	+	Occasionally	Skin lesions	Acidophilic
Smallpox		+	Skin lesions	Acidophilic
Herpes zoster	+	Occasionally	Skin lesions	Acidophilic
Herpes simplex	+		Skin lesions, nerve cells	Acidophilic
Rabies		+	Nerve cells	Acidophilic
Yellow fever		+	Liver cells	Acidophilic
Psittacosis		+	Alveolar exudate, hilar lymph glands, Kupffer cells, spleen	Basophilic Castaneda +
Lymphogranuloma venereum		+	Buboes	Matrix shows positive glycogen stain
Inclusion conjunctivitis		+	Conjunctiva of lower tarsus	
Trachoma		+	Conjunctiva of upper tarsus	

Skin Tests

Intradermal tests with virus antigen have been used in the diagnosis of individual cases, as well as in community surveys for epidemiological data. The difficulty attendant upon this method has been in preparing antigens free from impurity, which are sufficiently potent to give a specific reaction. The most popularly used is the Frei test. Frei prepared his original antigen from bubo pus in cases of lymphogranuloma venereum (Frei, 1925). It was diluted in saline and inactivated by heat. Today the most potent antigen is prepared from lymphogranuloma virus grown in the yolk-sac of fertile hens' eggs. The final preparation is called *lygramum* (Grace *et al.*, 1940): 0.1 ml. is injected intradermally into the skin of one forearm, and a similar amount of uninfected yolk-sac suspension as control into the other. Readings are made at two and again at four days. A raised inflammatory papule, at least 7 mm. in diameter, compared with a negative response in

Conclusion

Certain points of practical value in the diagnosis of virus infections should be stressed:

(1) Specimens for virus isolation should not be sent through the post. They should be placed immediately on ice or in a thermos flask containing carbon dioxide snow, and should be taken as soon as possible to the appropriate laboratory.

(2) Virus isolation is not a routine procedure and should only be considered in suitable cases. Serological methods form the backbone of virus diagnostic procedures. Acute-phase serum should be taken as early as possible in the disease, and a second specimen two or three weeks later. Such specimens can be despatched by post.

(3) A fourfold or greater increase in titre of antibodies is significant. Such a finding should be correlated with the clinical picture in an assessment of the case.

(4) Blood for cold agglutinins should be centrifuged whilst warm, so that the patient's red cells do not precipitate any of the serum cold agglutinin.

Disease	Virus grown in	Virus recoverable from	Serological tests	Inclusion bodies in	'Incubation period' (days)
Mumps	Fertile hens' eggs	Blood, saliva, C.S.F.	(a) Haemagglutination inhibition test (b) Complement-fixation test (C.F.T.)	—	18
Chickenpox (varicella)	—	—	—	Vesicles	12-16
Smallpox (variola)	Fertile eggs	Vesicles, pustules	C.F.T. using vesicle fluid	Vesicles	12
German measles (rubella)	Monkeys	Blood, nasopharynx	—	—	16-18
Measles	Monkey, man	Nasopharynx, blood	—	—	11-14
Herpes simplex	Fertile eggs	Vesicles, saliva, C.S.F.	Animal neutralization, using acute and convalescent sera	Vesicles	3
Herpes zoster	—	—	—	Vesicles	7-14
Influenza	Fertile eggs Mice Ferrets	Nasopharynx	(a) Haemagglutination inhibition (b) C.F.T.	—	1-2
Common cold	Chimpanzee* Man* Fertile eggs**	Nasopharynx	—	—	Up to 14
'Primary atypical pneumonia'	—	—	(a) Cold haemagglutination (b) Strep. M.G.	—	Up to 21
Q fever	Fertile eggs	Nasopharynx, blood	C.F.T.	—	19
Psittacosis (ornithosis)	Fertile eggs	Nasopharynx, blood	C.F.T.	Lungs, spleen lymph glands	4-15
Lymphogranuloma venereum	Fertile eggs	Buboes	C.F.T.	Gland or granuloma	3-21
Hepatitis (a) Infective	Man† Fertile eggs††	Blood, faeces	—	—	Up to 40
(b) Serum	Man only	Blood	—	—	Up to 180
Infectious mononucleosis	—	—	(a) Blood picture (b) Paul-Bunnell	—	5-10

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Disease	Virus grown in	Virus recoverable from	Serological tests	Inclusion bodies in	'Incubation period' (days)
Lymphocytic choriomeningitis	Mice Guinea-pigs	Blood C.S.F.	C.F.T.	—	1½-3 days experimental infection, not known in natural
Poliomyelitis	Monkeys	Faeces, nasopharynx	—	—	4-35
Epidemic myalgia (Bornholm dis.)	(a) suckling mice (b) adult mice on cortisone	Nasopharynx, faeces	C.F.T.	—	Probably about 5
Encephalitis St. Louis; Western and Eastern equine; loup- ing ill)	Mice Fertile eggs	Blood, brain, C.S.F., spinal cord	(a) C.F.T. (b) Animal neutralization tests	—	4-21
Rabies	Mice, rabbits dogs	Saliva, brain, spinal cord	—	Negri bodies in hippocampus	10 days to 7 months
Typhus	Guinea pigs, Fertile eggs	Blood	(a) Weil-Felix (b) C.F.T. (c) Agglutination	—	8-18
Yellow fever	Mice, monkeys	Blood	Animal neutralization	Liver	3-6
Trachoma and inclusion conjunctivitis	Man, monkey	Conjunctiva	C.F.T. using psittacosis - L.G.V. antigens may be positive because of antigenic similarity	Scrapings from tarsal conjunctiva	4-12
Dengue	Man, mice, fertile eggs	Blood	Mouse neutralization tests	—	5-9
Sandfly fever	Man	Blood	—	—	2½-6

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