

CROSS-REACTION STUDIES WITHIN THE PICORNAVIRUS GROUP
BY IMMUNOFLUORESCENCE AND ELECTRON MICROSCOPY

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GENERAL INTRODUCTION

The fluorescent antibody (FA) technique has a great potential for diagnostic use both for the identification of viruses in tissue cultures and, in certain cases, for direct identification of viral agents in clinical and postmortem specimens. It is routinely used for the diagnosis of respiratory syncytial virus infection by staining the cells from nasal washings (Gardner & McQuillin 1968) and a similar method is used for influenza viruses (Tateno et al. 1965, Tatenoebisawa et al. 1966).

The FA staining is also used for the diagnosis of rabies from clinical specimens. This method is found to be as efficient as the mouse inoculation test and is routinely used for examining brain smears from rabid animals (Beauregard et al. 1965).

In most virus groups, the number of member viruses is large and antigenically related. The degree of antigenic relationship varies from group to group. When antigenic relationships are present between the members of a given virus group, the identification and typing of these viruses by FA staining would require a knowledge of these cross-reactions.

The picornavirus group consists of a large number of viruses and the identification and typing of these viruses are time consuming and require the use of a large number of pooled antisera. The work reported by Hatch et al. (1961) and Hatch (1963) suggested that it might be possible to use the direct method of FA staining for the identification of picornaviruses, but unfortunately it has the same disadvantage of requiring a large number of conjugated antisera. On the other hand the observations of Forsgren (1966) indicated that there may be a broad antigenic relationship among the picornaviruses since he found antigenic relationship between echovirus type 6 antigen and poliovirus type 1, 2 and echovirus type 9 antisera by immunodiffusion. This indicates that it might be possible to detect similar cross-reaction by FA staining. Indirect method of FA staining is more sensitive than the direct method.

If similar cross-reactions could be detected by FA staining they could greatly facilitate the diagnostic use of the technique since a small number of sera might be capable of detecting all members of the group.

The detection of viruses by electron microscopy from clinical specimens is used with increasing frequency (Doane et al. 1969 & Joncas et al. 1969), but in the case of picornaviruses the limiting factor is their similar morphology. Immuno-electron microscopy could provide a useful additional tool both for their detection and rapid

identification. This method was first used to examine tobacco mosaic virus by Anderson & Stanley (1941). It has the potential of identifying a virus by electron microscopy within a few hours. In the case of related virus groups the problem of cross-reactions might however appear. Ball & Brakke (1969) reported that the immunoelectron microscopic test for tobacco mosaic virus and southern bean mosaic virus and their respective antisera was more sensitive than microprecipitin, ring interface and density gradient centrifugation tests.

The rapid diagnosis of viral infections is becoming more important because of the availability of a few antiviral drugs, as these agents tend to be highly specific against one virus or a group of viruses.

The experimental approach was therefore aimed to:

1. Study the cross-reactions between some of the members of the picornavirus group by indirect FA staining.
2. Study the complexes formed by some of the members of the picornavirus group and their homologous and heterologous antisera under the electron microscope.

LITERATURE REVIEW

A. CROSS-REACTIONS

1. Cross-reactions within picornavirus groups

Polioviruses: The polioviruses are classified into three types by the highly specific neutralization reaction, but the occurrence of cross-reactions has in fact been observed using several serological methods. Barnett and Baron (1961) observed the formation of heterologous antibodies in 3 groups of rabbits separately inoculated with poliovirus type 1, 2 and 3 respectively. The antisera were tested by an immun-activation-plaque-neutralization test. The antisera from 6/6 rabbits inoculated with poliovirus type 1 neutralized 90-100% of the plaques produced by poliovirus type 2, and antisera from 5/6 rabbits neutralized 80-100% of the plaques produced by poliovirus type 3 on post-inoculation day 7. The antisera from 2/6 rabbits inoculated with poliovirus type 2 neutralized 100% of the plaques, whereas the antisera from the remaining rabbits only neutralized 50-70% of the plaques produced by poliovirus type 1 on post-inoculation day 7. The plaques produced by poliovirus type 3 were not neutralized by the antisera on this

day, but on post-inoculation day 14, the antisera from 4/6 rabbits neutralized 60-90% of the plaques and the antisera from 2/6 rabbits neutralized 40-50% of the plaques produced by poliovirus type 3. The antisera from rabbits inoculated with poliovirus type 3 were tested on post-inoculation day 46. The antisera from 4/6 rabbits neutralized 80-100% of the plaques produced by poliovirus type 1, but the neutralization pattern for the plaques produced by poliovirus type 2 was: 3/6 neutralized 75-90% and 2/6 neutralized 40-60% of the plaques. In all the three cases the homologous plaque neutralization was 100%. The cross-reactions were not detected in many of the serum samples by neutralization test against 100 TCID₅₀/0.1 ml of the virus tested. It indicates that the detection of cross-reactions also depends on the sensitivity of the test used and they may be demonstrated even by the highly specific neutralization test under appropriate conditions.

The stimulation of heterologous antibody responses in children who have been given monovalent attenuated poliovirus vaccine has been reported (Ashkenazi and Melnick 1962). Vonka et al. (1967) studied the results of feeding poliovirus type 3 (USOL-D bac) attenuated vaccine to children with pre-existing type 1, 2 and 3 antibodies. They observed a 1.75, 2.6 and 24.3 fold

rise in antibodies titers for the three polioviruses respectively.

Hammon and Ludwig (1957) found that the prevalence of poliovirus type 2 antibodies prevented paralytic poliomyelitis due to poliovirus type 1. Plotkin et al. (1962-63) reported that when poliovirus type 1 antigen was used to immunize rabbits, not only the homologous antibodies appeared but also heterologous precipitating and neutralizing antibodies against poliovirus type 2 and 3 were detected. Melnick (1955) detected antigenic crossing between poliovirus types by complement fixation (CF) test. The homologous titers of 41 strains of poliotype 1 virus was 1:60 - 1:240 by CF test and 54% of them reacted with poliovirus type 2 antiserum by CF test, the titers varied from 1:15 - 1:120. Two of poliovirus type 1 strains reacted with poliovirus type 3 antiserum. Discussing his observations Melnick reported that heterotypic responses are much more pronounced by CF test than by neutralization test. Hummeler and Hamparian (1958) demonstrated "type" and "group" specific antigens in native (N) and heated (H) poliovirus respectively. A chimpanzee serum reacted with the "H" antigen of the three polioviruses. The chimpanzee was fed attenuated poliovirus type 1 and the pre-inoculation serum was negative for antibodies against poliovirus type 1.

The absorption of chimpanzee antiserum with "N" antigen of the three polioviruses, did not alter the titer of antibodies against "H" antigen from type 1 and 2 polioviruses. Results with poliovirus type 3 "H" antigen were inconclusive. An individual with no CF antibodies against "N" and "H" antigens of polioviruses, developed CF antibodies against "N" and "H" antigens of all three polioviruses after a febrile illness. The "H" antibodies could be removed from the serum by absorption with "H" antigen from any one of the three polioviruses, without altering the titer of "N" antibodies. This indicates that a heat-stable antigenic component is shared by all the three polioviruses. Scharff et al (1964) reported the presence of three types of antigens in polioviruses; (1) the infectious virus which is called "D" antigen, (2) the empty capsid which is called "C" antigen, and (3) a soluble or "S" antigen which is a degradation product. "D" antigen could be converted by heating into "C" antigen.

Coxsackieviruses: Cross-reactions are found not only among polioviruses but to the same extent in the coxsackievirus group. Beeman and Huebner (1952) found that persons infected with some of the group A coxsackieviruses developed type specific neutralizing antibodies against the infecting viruses, but they also developed CF antibodies against viruses not

associated with their infection. A similar result of antigenic sharing was demonstrated by immunodiffusion in sera from patients infected with coxsackievirus type B1, B3, B4, B5 and A9. These sera formed two lines of precipitation with homologous antigen, one line was considered "type specific" whereas the second line was considered "group specific". It was further observed that the "group" precipitation line formed by coxsackievirus type B1, B3, B4, B5, B6 and A9 were immunologically identical as judged by reactions of identity (Schmidt and Lennette 1962).

The "type" and "group" antigens could also be demonstrated by separating them by cesium chloride density gradient centrifugation. The separation of coxsackievirus type B1, B2, B3, B4, B5, B6 and A9 antigens was undertaken. Seven fractions were collected from the gradient. The fractions 1, 2, 3, and 5 had no immunological activity. Infectivity was associated mainly with fraction 4 which also reacted specifically and produced a single line of precipitation with anti-serum while fractions 6 and 7 produced "group" precipitation. This pattern was common for all the viruses tested with the exception of coxsackievirus type B2 which did not produce any precipitating antigen. The unfractionated antigen produced two lines of precipitation with homologous human sera and one line

with heterologous antisera. The "group specific" line was closer to the antigen well while the "type specific" line was closer to the serum well. The "group" line produced by homologous serum fused with the line produced by heterologous antiserum, indicating immunological identity. By CF test it was found that fractions 1, 2 and 3 had no CF activities whereas fractions 4, 6 and 7 contained relatively large amounts of CF antigen. Fraction 4 fixed complement with monkey sera only. Fractions 6 and 7 fixed complement with human sera and both fractions possessed "group" CF activities and fixed complement with heterologous antisera as well as with homologous antiserum. The "group" precipitating antigen in fractions 6 and 7 would therefore seem to be identical to "group" CF antigen of these fractions. It was also observed that the "type specific" antigen of fraction 4 could be converted to "group" antigen by heating at 56°C for 30 minutes. These results strongly suggest that coxsackieviruses have a common "group" antigen (Schmidt et al 1963). Schmidt et al (1965) studied the immunological response of rhesus monkeys experimentally infected with coxsackieviruses types B1, B2, B3, B4, B5 and A9 by oral route. Infection was produced by coxsackievirus type B3, B4, B5 and A9 only. The CF antibody response to initial infection was type specific, but successive

infections with 2nd, 3rd and 4th viral types recalled antibodies to previously infecting viral types and also elicited CF antibodies to heterologous viruses with which the animals had no previous experience. In most instances a second infection was sufficient to produce a broad heterotypic CF antibody response. The precipitating antibody responses were similar to that of CF antibody responses and both "group" and "type" antibodies were produced. Fractionation studies on serum samples were performed to locate the "group" and "type" antibodies. Human and monkey sera were fractionated by density gradient centrifugation. The monkeys were experimentally infected with individual coxsackievirus types (B and A9) or with their combinations. The test on different fractions showed that the "type specific" homotypic neutralizing and precipitating antibodies were present in the 19S fraction whereas the same types of antibodies for heterologous viruses were present in the 7S fraction. The "type specific" CF antibodies were present in the 7S fraction but the heterotypic CF antibodies were present in 19S fraction (Schmidt et al 1968).

Echoviruses: They are also related to each other, although they are not as thoroughly investigated as the polio and coxsackieviruses. A large group of sera from patients infected with echovirus types 4, 6 and 9

showed a four fold or greater increase in CF antibodies for echovirus type 12 and 19 (Schmidt et al 1962). Wenner (1962-63) reported that following infection with echovirus type 4, heterotypic antibody rises were observed against echovirus type 6 and 9 by CF test. Styk and Schmidt (1968) tested echovirus type 9 antigen against homologous antiserum and echovirus type 2, 11, 14, and 30 antisera by immunodiffusion. The homologous reaction produced 1-3 faint lines and one major line of precipitate, but the heterologous antisera produced only one line of precipitate. The heterologous precipitation line was considered to be a virus-antibody reaction because the host antigen did not form a precipitate with these antisera. The treatment of echovirus type 9 antigen with guanidine improved the reaction and the heterologous precipitation line became similar to the homologous precipitation line. All these findings support the point of view that echoviruses do share an antigenic relationship.

2. Cross-reactions between picornavirus groups

Cross-reactivity is also detectable between the major groups of picornaviruses. The presence of cross-reactivity was very well illustrated by the studies conducted by Halonen et al (1959). They studied the presence of homologous and heterologous CF antibodies

in naturally or experimentally infected individuals, with certain of the echo, coxsackie and polioviruses. In their study, echovirus type 2, 4, 5, 6, 7, 8, 11, 14, 16 and 20 and coxsackievirus type B1, B3, B4 and B5 were linked to each other by significant heterologous CF antibody responses. The studies conducted by Lennette et al (1961) also support the view that there is antigenic relationship among picornaviruses. They reported that a high proportion (18%) of patients with echovirus type 9 infection and with no evidence of dual infection or of vaccination against polioviruses showed a four fold or greater rise to CF antibody titer for polioviruses. Wenner (1962-63) also reported that infection with echovirus type 1, 3, 4, 6, 9, 12 and 16 had been followed by CF antibody rises to polioviruses. Patients with coxsackievirus type B3 infection showed an increase in CF antibodies against echovirus type 6. The testing of WHO reference sera by neutralization test also revealed some cross-reactions within the picornavirus group. In general, the cross-reacting titer was low (Melnick and Hampil 1965). The immunodiffusion study by Forsgren (1966) also revealed cross-reactions between polio and echoviruses. He examined native echovirus type 6 antigen against homologous antiserum and against poliovirus type 1, 2 and echovirus type 9 antisera (Human). The antigen

produced two lines with homologous antisera but also produced one line with heterologous antisera which fused with one of the lines of the homologous antiserum. Schmidt et al (1967) conducted an elaborate experiment to study the heterologous responses in rhesus monkeys infected by oral route with coxsackievirus type B1, B3, B4, B5, B6, A9 and echovirus type 4. The sera from these monkeys were tested against echo and coxsackieviruses by CF, neutralization, immunodiffusion and haemagglutination inhibition (HI) tests. The successive infections with coxsackieviruses resulted in heterotypic CF antibody rises to echovirus type 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 14, 16, 17, 18, 19, 20, 31, 24, 25, 29 and 30. Heterologous neutralizing antibodies were detected in four cases only. Precipitating antibodies were detected against echovirus type 1, 2, 4, 6, 8, 12, 13, 14, 19, 20, 21, 24, 25 and 29. HI antibodies were seen only for echovirus type 3, 6, 7, 19, 21 and 29, with titers varying from 1:32 - 1:256. One monkey infected with echovirus type 4 was fed with coxsackievirus type B5 and heterotypic CF antibodies against all of the group B coxsackieviruses and A9 developed. Precipitating antibodies were found only against coxsackievirus type B1, B4 and B6.

The results of all these studies clearly indicate that the picornaviruses are related to each other between the groups as well as within the groups.

3. Cross-reactions associated with other virus groups

Cross-reactions are not limited to the picornaviruses but they can also be found in many other virus groups. A brief survey of the cross-reactions within: Pox-, Adeno-, Herpes-, Arbo- and Myxoviruses is presented here. Discussion is mostly limited to human viruses.

(a) Poxviruses: Poxviruses are divided into five subgroups. Serological cross-reactions have been demonstrated within each subgroup by CF, HI, precipitation, neutralization and FA techniques. These tests failed to show antigenic relationship between the subgroups. The vaccinia subgroup includes: vaccinia, cowpox, rabbitpox, ectromelia, monkeypox, variola, alastrim and raccoonpox viruses (Wilner 1969). These viruses are very closely related to each other serologically. Gispen (1955) observed that there is no essential difference between the pattern of vaccinia, rabbitpox, smallpox, and alastrim virus antigens when diffused against standard antivaccinia antiserum. The study of cross-reactions by neutralization test was undertaken by Downie and McCarthy (1950). They observed a broad cross-reaction between cowpox, vaccinia,

ectromelia and variola viruses. The summary of their results are given in Table 1.

TABLE 1

THE RESULTS OF CROSS-NEUTRALIZATION TEST WITHIN
POXVIRUS GROUP (DOWNIE AND MCCARTHY, 1950)

Antigens tested	Neutralizing titers of the antisera			
	Cowpox	Vaccinia	Ectromelia	Variola
Cowpox	1:660	1:890	1:1020	1:2750
Vaccinia	1:49	1:3760	1:660	1:3350
Ectromelia	1:63	1:470	1:560	1:2060
Variola	1:9	1:460	1:102	1:4470

McCarthy and Hebert (1960) tested the antigenic relation of cowpox, ectromelia, vaccinia and variola viruses by HI test. The homologous and heterologous titers were close to each other. Behbehani et al (1969) reported that the monkeypox virus and vaccinia virus do cross-react with each other, but they do not cross-react with yabavirus.

(b) Adenoviruses: Human adenoviruses consist of 31 serotypes. All members of the group except the avian types share a common CF antigen associated with "Hexons" (Wilner 1969). Rowe and Hartley (1962-63) observed that antisera against human adenoviruses cross-react with canine, murine, simian and bovine

adenoviruses. Sochier et al (1965) also reported that the CF antigen is common to all types of adenoviruses and the CF titer varies from 1:64-1:128 in general. The neutralization and HI tests are specific.

(c) Herpes viruses: A detailed review of the antigenic and biological properties of Herpes virus hominis is given by Nahmias and Dowdle (1968). H. hominis is divided into two antigenic types, type 1 and type 2. There is a one way cross by neutralization test between type 1 and 2. B. virus (H. Simiae) anti-serum cross-reacts with H. hominis type 1 but H. hominis anti-serum generally does not neutralize B. virus. Pseudorabies (H. suis) virus shows partial antigenic relationship with H. hominis. Equine abortion virus type 1 reacts with hyper-immune rabbit antiserum against H. hominis type 1 in CF test. Wilner (1969) reported that CF test showed some antigenic sharing among pseudorabies, equine abortion virus and bovine rhinotracheitis viruses. The existence of minor antigenic relationship between H-Simplex and varicella viruses was also reported by CF test.

(d) Arboviruses: Casals (1961) injected mayarovirus (group A) into guinea pigs which developed high titered homologous HI antibodies and also heterologous HI antibodies acting against other viruses of the group.

Following reinoculation with sindbis virus (group A) the immune response of the guinea pigs showed a marked overlap by HI test. The guinea pigs inoculated with group B and C viruses reacted in a similar manner as those inoculated with group A viruses showing a broad cross-reactivity within their groups by HI test. The homologous and heterologous HI titers are given in Tables 2, 3 and 4 respectively for A, B and C group arboviruses. These results clearly show that the arboviruses are related to each other within the group and that the inoculation of a heterologous virus of the same group results in a broadening of the cross-reactions.

McLean (1968) reported that members of arbovirus group show antigenic sharing by HI test, to a lesser extent by CF test, while they are antigenically distinct by neutralization.

(e) Myxoviruses: Influenza virus group has three serotypes A, B and C. They are distinguished from each other by their type-specific internal nucleoprotein CF antigen. All strains of a particular type, regardless of their original host species, share a common nucleoprotein antigen detectable by complement fixation. This means that influenza virus type A of human, swine, avian and equine origin are antigenically related. Pereira et al (1965) reported that avian

TABLE 2RESULTS OF GROUP A ARBOVIRUSES CROSS-HI TESTS
(CASALS, 1961)

Antigens (8 units)	Response in guinea pigs by HI	
	Mayaro virus + Sindbis virus	
Mayaro	2560	5120
Chikungunya	80	320
Semliki	160	1280
AMM 2021	320	1280
AMM 2350	20	640
Sindbis	0	640
WEE	20	1280
Aura	0	320
Middelburg	0	320
EEE	0	320
VEE	0	160

influenza A viruses share a soluble nucleoprotein antigen common to all influenza A viruses. The CF titers of avian influenza A viruses against antisoluble influenza A antisera varied from 1:240 - 1:320. Groth (1969) studied the antigenic relationship among human subtypes of influenza virus type A by haemagglutination inhibition, and found that all the strains were related although the heterologous titers varied with different strains.

TABLE 3RESULTS OF GROUP B ARBOVIRUSES CROSS-HI TESTS
(CASALS, 1961)

Antigens (8 units)	Response in guinea pigs by HI	
	Japanese B encephalitis virus + SAH 336 virus	
Japanese B encephalitis	640	5120
SAH 336	80	5120
Russian SSE	40	1280
Powassan	20	640
Yellow fever	80	2560
Zika	320	10240
Dengue 1	40	1280
Dengue 2	80	2560
Dengue 3	80	2560
Spondowen1	20	640
Bussuquara	320	10240

The paramyxoviruses appear to be serologically distinct from the influenza viruses. Chanock et al (1960) observed that parainfluenza type 1 and 3 are related antigenically but not the other viruses of the parainfluenza group. Similar findings were reported by Chanock et al in 1963.

The pseudomyxoviruses, measles, Canine distemper and rinderpest, are antigenically related but are distinct from the myxoviruses and paramyxoviruses.

TABLE 4RESULTS OF GROUP C ARBOVIRUSES CROSS-HI TESTS
(CASALS, 1961)

Antigens (4 units)	Response in guinea pigs by HI	
	Caraparu virus + Oriboca virus	
Caraparu	640	5120
Oriboca	20	2560
Apeu	320	2860
Marituba	40	640
Murutucu	40	640

Cross-reactions between measles and rinderpest viruses were reported by Plowright (1962-63), while Imagawa (1968) reported that distemper virus is neutralized by measles antiserum although the neutralization of measles virus with distemper antiserum was less efficient. The neutralization of distemper virus by rinderpest antiserum is reported but the level of antibodies in distemper antiserum for rinderpest virus is low. Measles antiserum neutralizes rinderpest virus and rinderpest antiserum neutralizes measles virus.

Conclusions: One can draw a conclusion from all these results that the detection of the heterologous reactions depends on the type of test employed. When an antiserum is tested by a particular system, depending on its sensitivity and specificity one could detect a

"type-specific" or a "group-specific" reaction. As in the case of arboviruses if an antiserum against a member of the group A virus is tested against a heterologous virus of the same group by neutralization test, the result will show no antigenic crossing, but the same antigen and antiserum will show some crossing by CF test and a broad antigenic overlap by HI test (McLean 1968).

In the case of polioviruses Barnett and Baron (1961) detected a broad cross-reaction among polioviruses by immunoactivation-plaque-neutralization test, while the neutralization test with 100 TCID₅₀/0.1 ml of the virus showed very limited crossing.

At present serological typing systems are geared to precise identification of virus isolates but the high degree of specificity which makes this possible and which is so valuable in epidemiological studies becomes a positive disadvantage in the diagnostic field because of the multiplicity of antisera required to make an identification. For diagnostic purposes group identification is usually sufficient for immediate diagnosis and treatment.

Even for the purposes of chemotherapy, group identification will normally prove to be adequate since most of the anti-viral drugs act by inhibiting some step in the replication cycle, which is similar for

different members of the same virus group.

In the picornavirus group, therefore, development of test procedures to give broad antigenic coverage would provide useful diagnostic tools.

B. IMMUNOFLUORESCENCE

Immunofluorescence is a technique which employs fluorescein-labelled immune serum globulin to locate the corresponding antigen using fluorescence microscopy. It has been used for the localization and identification of viruses, bacteria, fungi, serum antibodies and parasites, and already plays an important role in the diagnostic laboratory.

In this section of the literature review, attempts will be made to analyze the recent findings in the field of immunofluorescence related to viruses.

(a) Technique: The origin of the FA technique could be traced to the successful conjugation of a fluorochrome dye to antibody molecules by Coons et al in 1941. Microbiologists became interested when Coons et al (1942) used fluorescein isocyanate-labelled pneumococcal antibodies to localize pneumococci in tissues of infected mice and this was called the "direct method". Further modification was made by Weller and Coons (1954). First they treated the antigen with unlabelled homologous antiserum and then with the conjugate which was directed against the globulin of the species in

which specific antiserum was prepared. This is called the "indirect method". Recently Goldwasser and Shepard (1958) introduced the "complement staining method" as a further modification. In this case the antigen, antibody and complement complexes are stained with conjugated anti-guinea-pig globulin.

There are certain advantages and disadvantages associated with these three staining methods. The "indirect method" is simpler and it requires only one conjugate for each animal species contributing the unlabelled homologous antisera, whereas the "direct method" requires separate conjugate for each antigen to be located or identified. The "complement staining" is even simpler than the "indirect" method as it requires only one conjugate. Both the "indirect" and "complement staining" methods are more sensitive than the "direct" method and require less antibody in the intermediate layer (Borek 1961). These two methods could also be used for titrating antibodies.

Conjugation: The dye most commonly used for this purpose is fluorescein isothiocyanate (FITC). McDevitt and Coons (1964) reported that purification of ammonium sulfate-precipitated gamma-globulin by column chromatography provides better material for conjugation and the conjugate gives specific and brighter fluorescence. Gordon et al (1962) observed that gel filtration through a Sephadex (G-25) column is the best way to purify a conjugate.

Non-specific staining (NSS): This is one of the major problems associated with FA staining. The NSS could be defined as the uptake by tissue cells and matrix of fluorescent components of a conjugate. Neither autofluorescence nor heterologous staining could be properly designated as NSS (Pittman et al 1967). The NSS could be due to a variety of causes, some of them are now known but attempts at their elimination still remain largely empirical.

The NSS could be due to:

- 1) Presence of unbound dye in the conjugate: This is no longer a major problem since unbound dye can be removed completely by gel filtration with Sephadex (G-25), although some dissociation does occur after storage. Dissociated free dye can be removed by absorbing the conjugate before use with a small amount of Sephadex (G-25).
- 2) Conjugated serum protein: Nairn (1969) reported that conjugation increases the net negative charge at pH 7.0, the conjugate then behaves as an acid dye and binds to tissue proteins. The molecules having these properties can be selectively removed by absorbing the conjugate with tissue powders. Goldstein et al (1961) reported that the addition of too many fluorescein groups per molecule of protein leads to a marked lowering of isoelectric point and thus results in NSS. They recommended gradient elution chromatography on diethylaminoethyl cellulose column to remove these molecules. This type

of NSS could also be removed by dilution.

- 3) High fluorescein protein ratio (F/P): It was observed by Herbert et al (1967) that NSS increased directly with increase in F/P ratio. It is considered that the increase in F/P ratio leads to protein denaturation and NSS. They also considered that impure fluorescein also contributes to NSS. These factors could be easily corrected by using smaller amount of FITC as recommended by McDevitt and Coons (1964).

- 4) Protein interaction and NSS due to natural antibodies: It is reported that serologically inactive components of antisera could give rise to NSS. Luis (1958) reported that fluorescein and rhodamine labelled normal serum globulin, albumin and egg albumin when used showed NSS. Antibodies against cellular components could be present in an antiserum which could result in NSS. The presence of immunologically inactive component could be removed by proper purification of the gamma-globulin and the antibodies against cellular components could be removed by absorbing the antiserum with uninfected homologous cells.

- (b) Diagnostic application for the detection of viral antigens and antibodies

Picornaviruses: Kalter et al (1959) used direct FA staining to identify polioviruses from stool specimens inoculated in monkey kidney tissue culture. The cells showing 2+

cytopathic changes were trypsinized and centrifuged. A smear was prepared from the sediment and fixed in acetone. The staining was done with conjugate prepared from antisera against poliovirus type 1, 2 and 3. By this method they were able to type 30/33 poliovirus-containing stool specimens. Similar findings were reported by Hatch et al (1961). They were also able to type 34/38 poliovirus-containing stool specimens by direct FA staining. Occasionally they observed cross-reactions between poliovirus type 1 and 3. Kanamitsu et al (1967) observed that it was possible to localize poliovirus antigens by direct FA staining in different tissues of monkeys experimentally infected with poliovirus type 1. Fluorescence was detectable in the pharyngeal cells before viraemia. After viraemia fluorescence could be seen in a number of cells from different tissues; mononuclear cells of the terminal ileum, cells of the lamina propria of the large intestine and cells from spleen, liver and lymph-nodes. In paralytic cases the fluorescence was observed in the spinal cord, the highest concentration of viral antigen was in the nerve cells of the anterior horn in all the segments of spinal cord. Riggs and Brown (1962) were able to differentiate between passive and active poliovirus antibodies by using indirect FA staining. Paired sera from cases recovered from polio infection and from vaccinated children showed a marked increase in fluorescent antibody reactions between acute and convalescent sera and between

pre- and post-vaccination sera whereas most of the sera from infants containing high titers of passive antibodies failed to show fluorescence.

The identification of coxsackie and echoviruses by direct FA staining was reported by Shaw et al (1961). They were able to identify and type correctly known coxsackievirus type B1, B2, B3, B4, B5, A9 and echovirus type 1, 2, 6 and 9 in tissue culture by using conjugated pools of antisera. No cross-reactions were observed. Hatch (1963) was also able to identify coxsackie and echoviruses from stool specimens by direct FA staining. From these stool specimens an enterovirus was isolated and identified by neutralization test. The results of the two tests were comparable. Crossing was observed between echovirus type 4 conjugate and echovirus type 9. One should note that only a single staining was performed. Burch et al (1969) examined renal tissues from autopsies by direct FA staining to detect coxsackievirus type B antigens. Out of 104 cases examined 11 showed positive fluorescence for coxsackievirus type B antigens in the kidney. They suggested that FA staining could be useful in detecting viral antigens in renal tissues. Hatch (1969) studied the direct FA staining reactions of conjugated antisera prepared against heated and unheated poliovirus type 1 antigen. The conjugated sera against heated virus gave higher FA staining titers than the conjugated sera against native infectious virus. The CF antibodies

which react with heated antigen, was responsible for FA staining. By using the conjugated sera against heated virus, he was able to identify 21 poliovirus type 1 isolates. Hummeler and Hamparian (1958) observed that "group specific" antigen was associated with heated polioviruses and it was common for all the three polioviruses.

Myxoviruses: Liu (1956) was first to report his results of detecting influenza virus antigens in cells from nasal washings of patients by direct FA staining. These specimens were also tested by egg inoculation. There were 17 confirmed cases of influenza, but only 12 were positive by FA staining. Hinuma et al (1962) found that the complement staining was more sensitive than the indirect method of FA staining for the identification of certain myxoviruses. Carski et al (1962) studied the detection of respiratory viruses by indirect FA staining from clinical specimens. They identified 4 positive cases of influenza virus type B infection by this method as well as by isolation. Hers (1963) results showed that the influenza virus antigen could be demonstrated by indirect FA staining in cells from nasal washings. He examined epithelial cells from sputum and nasal exudates of patients suffering from influenza A or B infection by this method. All positive cases showed bright fluorescence either in the nucleus or in the cytoplasm or in both. He considered that the indirect FA staining could also be used for demonstrating specific antibodies in serum

by using a known antigen. Baratawidjaja et al (1962) employed indirect FA staining and egg inoculation to identify influenza viruses from throat washings of patients suffering from respiratory infections. The results obtained by both systems were comparable. The advantage of the FA staining was that the result could be reported within 4-5 hours after the arrival of the specimen. There were cross-reactions between strains within the type. Tatenoebisawa et al (1965) examined nasal smears from serologically confirmed and from clinical cases of influenza A2 infections by direct FA staining. The fluorescence was chiefly in ciliated and unciliated epithelial cells. Tatenoebisawa et al (1966) made rapid diagnosis of influenza virus type B infections by direct FA staining of the nasal smears. By this method 77% (33/43) of confirmed cases of influenza B infection showed positive fluorescence. In their view the diagnosis of influenza B infection could be made within 2 hours after the specimen is received by FA staining. Lyarska et al (1966) examined the epithelial cells from respiratory tract of cases naturally or experimentally infected with influenza viruses by direct FA staining and autoradiography. The impression smears were made from the nasal mucosa of patients in acute phase of infection and from volunteers given live influenza vaccine intranasally. Also smears from trachea and lungs were prepared from mice inoculated with PR8 virus intranasally. Horse gamma-globulin against influenza virus type A, A1, A2 and B

were labelled with ^{131}I . The radioactivity was detected by radio-autography. Their results showed traces of radioactivity in smears from trachea and lungs of mice. The FA staining showed fluorescent aggregate of cells. The smears from volunteers showed virus antigens in 40/48 cases by auto-radiography, whereas all the smears were positive by FA staining. Out of 35 influenza patients 23 showed viral antigen by auto-radiography and 24 by FA staining. The comparison of the two methods showed that the FA staining was superior to radioactive labelled gamma-globulin for detecting viral antigen.

Liu et al (1961) reported that when parainfluenza infected cells were stained by direct FA technique, they showed bright fluorescence in the cytoplasm but no cross-reactions were observed. They also stained cells from nasal epithelium, trachea, bronchi and lungs of intranasally infected hamsters all of which showed bright fluorescence. Their results indicate that immunofluorescence could be successfully used for the identification of parainfluenza viruses by staining the cells from clinical specimens.

Fedova and Zelenkova (1969) studied the multiplication of parainfluenza viruses in monkey kidney tissue culture by indirect FA staining. They observed that the FA staining was much more sensitive than haemagglutination and haemadsorption tests in detecting the virus in tissue culture. They recommended that FA staining could be used for rapid

diagnosis of respiratory infections. Fedova et al (1969) used indirect FA staining for the laboratory diagnosis of respiratory infection in children. They were able to diagnose 17 cases of parainfluenza virus type 1, 7 cases of influenza virus type B and 1 case of mixed infection out of 54 specimens. In most cases the fluorescence was type-specific, but partial cross-fluorescence was found between parainfluenza type 1 virus and type 3 antiserum. The cross-reaction was also observed between SV5 antiserum and mumps virus. The results indicate that the indirect FA staining could be a useful technique for the identification of respiratory viruses from clinical specimens.

Lesso and Vaskebova (1965) demonstrated mumps virus in chorio-allantoic membrane of eggs inoculated with clinical specimens from parotitic meningitis by direct FA staining. They were able to identify mumps virus in 4/8 cases which were also confirmed by serological test. Sattar et al (1967) used conjugated goat milk antibodies against mumps virus for the identification of the virus in tissue culture. They observed bright and typical fluorescence in the cytoplasm. The detection of mumps antibodies by indirect FA staining was reported by Cerini and Cabasso (1968). They studied the appearance of mumps antibodies in sera, from children inoculated with attenuated mumps vaccine. Mumps virus grown in chick cells was used for testing the antisera at a dilution of 1:5. The result showed that 26/27

postvaccination sera were positive for mumps antibodies. The infected cells showed a granular fluorescence in the cytoplasm. Cohen et al (1955) used indirect FA staining to identify measles virus from clinical specimens inoculated in tissue culture. They identified measles virus from three patients.

Arboviruses: The FA staining is generally used to study the viral antigens in experimentally infected tissue culture or in mice inoculated with the virus. Metzger et al (1961) demonstrated by direct FA staining that the Venezuelan equine encephalomyelitis (VEE) virus antigen develops in the cytoplasm of the infected cells. The whole cytoplasm was filled with viral antigen in 72 hours after infection as demonstrated by bright fluorescence. Michael (1966) used indirect FA staining to localize VEE virus adsorbed on goose erythrocytes and a bright fluorescence of viral aggregates was observed. Erlandson et al (1967) studied the growth of semliki forest (SF) virus in HEP-2 cells by direct method. A granular fluorescence in the cytoplasm was demonstrated 8 hours after infection. Dzhivanian and Zasukhina (1967) reported that the apathogenic and pathogenic strains of western equine encephalitis (WEE) virus could be differentiated by FA staining of tissues from mice inoculated with these viruses. Mice inoculated with the apathogenic strain showed fluorescence in the neuron cells which almost disappeared in 32 hours. In the case of pathogenic virus the specific

fluorescence was present in the neuron cells up to the end of the incubation period. The number of fluorescent cells increased during the period of clinical disease. The cells from spleen, liver and intestine were also fluorescent. Feigin et al (1967) infected 40 volunteers with live attenuated VEE virus vaccine. Blood samples were taken every 12 hours to detect the presence of the viral antigen in leukocytes by direct FA staining. The leukocytes from 80% of the vaccinated men showed specific fluorescence within 2-6 days post vaccination.

Albrecht (1965) used indirect FA staining to detect group B arboviruses (tick-borne encephalitis (TBE), Japanese B encephalitis (JBE, West Nile (WN), St. Louis encephalitis (SLE), dengue and yellow fever (YF) viruses) inoculated in CEC cultures. The TBE, WN, and SLE viruses showed bright fluorescence in the cytoplasm 3 days after inoculation. The dengue and YF viruses were unable to multiply readily in the CEC culture. Atchison et al (1966) reported that it is possible to identify dengue viruses from infected mouse brains by complement staining. There was cross-reaction between dengue type 1 viral antigen and antisera against other dengue viruses, which extended to the other group B members. The results are given in table 5.

Karpovich et al (1968) identified 18 TBE virus strains from 175 tick suspensions by direct FA staining after inoculation in CEC cultures.

TABLE 5

RESULTS OF THE COMPLEMENT STAINING OF GROUP B ARBOVIRUSES
(ATCHISON ET AL, 1966)

Viruses	Antisera against				
	Dengue 1	Dengue 3	JBE	MVE	EEE(A)
Dengue 1	4+	4+	±	3+	-
Dengue 3	4+	4+	-	1+	-
Japanese B encephalitis	3+	4+	4+	±	-
Murray Valley encephalitis	4+	2+	-	4+	-
Eastern equine encephalitis	-	-	-	-	4+

Emmons and Lennette (1966) reported the rapid diagnosis of Colorado tick fever by FA staining of smears prepared from 18 patients' blood clots and also from infected mice brains, which was confirmed by isolation of the virus. Arbovirus antigens were present in the cytoplasm of the infected cells. These findings indicate that immunofluorescence could be used for the diagnosis of arboviruses; in some cases directly from the clinical specimens, in others following inoculation in cell cultures or mice.

Respiratory syncytial virus: In recent years immunofluorescence has proved to be a useful tool for the identification of respiratory syncytial (RS) virus from clinical specimens. Kisch et al (1962) demonstrated the development of RS virus

antigen in HEP-2 cells by indirect FA staining. Granular specific fluorescence was detected 10-12 hours after infection in the cytoplasm indicating that viral antigen was formed in this location. Fluorescent syncytia were detected 24 hours after infection. Similar findings were reported by Dreizin et al (1967) about RS virus growing in human fetal lung culture. Schieble et al (1965) used direct FA staining to identify RS virus from clinical specimens which were inoculated in human fetal lung culture. They correctly identified that 71/75 specimens contained RS virus. The 4 RS virus negative specimens yielded: 1 adenovirus type 2, 1 type 5, 1 mumps and 1 SV40 viruses. The identification of RS virus from clinical specimens by FA staining was therefore 100% accurate. Gray et al (1968) stained throat-swab smears from children with respiratory infections by direct FA technique and they obtained positive results in 80% of the cases. However, when FA results were compared with serological (CFT) and isolation results the positive identification by FA staining was reduced to 70%. Still FA staining has the advantage of giving a positive result in 70% of cases within 1-2 hours after the specimens were received, whereas the positive results by CFT and isolation were 68% and 48% respectively. Gardner and McQuillin (1968) used indirect staining to identify respiratory syncytial (RSV) virus from clinical specimens, either directly or after inoculation in tissue culture. When the

cells showed CPE, they were scraped off from the tubes and transferred to slides for staining and some of the cells were also used for neutralization test. For direct staining of the specimens, the cells from nasopharyngeal secretions were washed and a smear was prepared on a slide. The direct staining gave positive diagnosis of RS virus infection in over 90% of cases on the day of admission. When the infected cell smears were stained and examined the results were: 53% of positive cases diagnosed on second day, 71% on fourth day and 82% on the seventh day after inoculation.

Rabies virus: A quick diagnosis of rabies virus is very essential due to the risk involved with the infection and also because the immunization should be started as soon as possible in positive cases. Immunofluorescence has proved very effective and reliable for the diagnosis of rabies infection. Goldwasser et al (1959) examined 157 salivary glands from suspected animals by FA staining. Virus isolation was also tried from these specimens. Rabies virus was isolated from 49 of these specimens and 48 of them were also positive for rabies virus by direct FA staining. This shows that FA staining is as reliable as isolation technique. Results of the direct FA staining for the diagnosis of rabies had been compared with the results obtained by mouse inoculation (McQueen et al 1960). Seventy positive specimens were diagnosed by both the systems, which indicate that the FA staining is as sensitive as mouse inoculation test.

Beauregard et al (1965) obtained positive diagnosis for rabies from 175 brain specimens by different tests. Out of these, 58 were diagnosed by histological staining and the rest by mouse inoculation test which coincides with the number of positive cases diagnosed by FA staining. The possibilities of detecting rabies virus during the incubation period by indirect FA staining were investigated by Serokowa et al (1967) in experimentally infected mice. The mice were sacrificed at different time intervals and the brain smears were stained and examined for rabies antigen. Rabies virus was demonstrated in the brain during the incubation period by FA staining. Krawczynski et al (1967) examined sera taken at different intervals from mice experimentally infected with rabies virus by indirect FA staining to detect the development of antibodies during the incubation period. The neutralization test was also used for comparison. The appearance of antibodies in the serum was first detected by neutralization test, about 120 hours after inoculation whereas by FA staining not before 144 hours. Thomas et al (1963) tested 588 sera from vaccinated persons by indirect FA staining and neutralization test for rabies antibodies. Results showed that 280 sera were positive by both the tests used. In addition to these samples, 78 other sera samples were only positive by FA staining which indicates that FA staining is more sensitive than the neutralization test for the detection of antibodies against rabies.

Smears from mouse brains infected with street virus were used as antigen for FA staining.

Rubella virus: The interference test is used for the detection of rubella virus in tissue cultures, but does not provide a definitive diagnosis. In recent years encouraging results are being obtained by FA staining for the identification of rubella antigens and antibodies. Woods et al (1966) reported that the indirect FA staining is specific and sensitive for the identification of rubella virus isolates in tissue cultures. They demonstrated fluorescent foci in heart and skeletal muscle of an infant with rubella syndrome. Schmidt et al (1966) supported the findings of Woods et al that the indirect method is very sensitive for the identification of rubella virus in infected tissue cultures. They examined tissue culture cells from a total of 269 isolation attempts by FA staining. The results showed that 63 of them contained rubella virus. Recently Estella (1967) has reported that hyperimmune rubella sera from roosters gave brighter fluorescence than the rabbit sera when tested by direct FA staining. Throat washings from 120 suspected cases of rubella were inoculated in green monkey kidney cultures and examined after FA staining with rooster anti-rubella conjugate. The results of FA staining showed that 101/120 throat-washings contained rubella virus, whereas 109 were positive by interference test. Estella also used indirect FA staining to detect antibodies

against rubella virus in the sera of the same patients from whom throat-washings were obtained. The antibodies against rubella virus were present in 109/120 serum samples tested. Rubella virus infected cell cultures were used as antigen for the detection of antibodies in the patient's sera. Brown et al (1964) used indirect FA staining not only for the detection, but also for the titration of antisera suspected of containing antibodies against rubella virus. They tested sera from 11 patients against cell cultures chronically infected with rubella virus. The sera were positive for rubella antibodies and the titer varied from 1:8 - 1:32. The sera from two nonexposed persons were negative for rubella antibodies. Lennette et al (1967) used a modified indirect FA staining to detect rubella antibodies in sera from 35 patients. These sera were also tested by neutralization and CF tests. Throat washings were also obtained for virus isolation. Rubella virus was isolated from 13 patients and the sera from these 13 patients showed a 4 fold rise in antibody titers by FA staining. Whereas neutralization and CF tests showed a 4 fold rise only in 10/13 and 12/13 cases respectively. The antibody titers demonstrated by FA staining were slightly higher than those obtained by neutralization and CF tests. The sera from 5 isolation negative patients showed a 4 fold rise in antibody titer by FA staining but not by other tests. The sera from the rest of the patients did not show a 4 fold rise in antibody titers

by any of the tests used.

Cohen et al (1968) undertook studies, by indirect FA staining to detect whether antibody against rubella virus was present in IgG or in IgM immunoglobulins at different stages of infection. IgM antibody titers of 1:16 or more were detected in sera from 9/13 cases of recent infections. The sera from 14 infants with congenital anomalies also showed IgM antibody titers of more than 1:16. These results indicate that in recent infections with rubella virus the antibodies are predominantly present in IgM fraction which could be detected by FA staining for early diagnosis of recent infection. BHK21 cells infected with rubella virus were used as antigen. Conjugates were prepared from anti-human IgG and anti-human IgM antisera.

Schmidt and Lennette (1970) were able to determine the immunological responses of 21 individuals vaccinated with rubella vaccine by testing the sera by indirect FA staining against known rubella antigen. Seventeen of the 21 vaccines showed a 4 fold or greater increase in antibodies by FA staining and the titers varied from 1:16 - 1:128. They also tested the sera by CF test and the results showed a 4 fold increase only in 12/21 vaccinees. The report indicates that FA staining is more sensitive than the CF test.

Herpes viruses: Immunofluorescence is used successfully for the diagnosis of external herpes infections by direct

staining of clinical specimens. Biegeleisen et al (1959) identified herpes simplex virus from 8/15 clinical specimens by direct FA staining. The results were confirmed by egg inoculation test. Kaufman (1960) examined smears from corneal scrapings of herpetic keratitis patients after direct FA staining. They identified herpes simplex virus from 6/17 cases by immunofluorescence as well as by isolation. In one case identification was possible only by FA staining. Pettit et al (1964) used direct FA staining for the diagnosis of herpetic keratitis from experimentally infected rabbits as well as from natural human cases. The smears from the epithelial scrapings were prepared, fixed and then stained. Bright fluorescence was present in the nucleus and cytoplasm of the epithelial cells from both rabbits and human cases. Fifteen of the 21 human cases were diagnosed by FA staining, while only 12 cases were diagnosed by isolation. This indicates that immunofluorescence is even more sensitive than the isolation technique. Uchida and Kimura (1965) examined the corneal cells from rabbits experimentally infected with herpes simplex virus by direct FA staining. The results showed that the virus antigen was localized in the cytoplasm and nuclei of the conjunctival epithelial cells.

Griffin (1963) reported that FA staining is reliable for the diagnosis of herpes skin infections. A positive diagnosis was made in 54/56 cases by FA staining as well as

by virus isolation.

Lesso and Szanto (1969) studied the multiplication of herpes simplex virus in Hela cells by indirect FA staining. The viral antigen was first observed in the nuclei about 5 hours after infection; by 12/16 hours the viral antigen was localized in the vicinity of the nuclear membrane and the paranuclear area of the cytoplasm. They also studied the persistent herpetic infection in Hela cells. They found that the persistently infected cell culture contains small amount of intracellular virus.

Nahmias et al (1969) used direct FA staining to differentiate between herpes simplex virus type 1 and 2. The 1:8 dilution of the conjugated anti-type 1 serum gave 3+ to 4+ staining with type 1 infected cell culture, whereas type 2 infected cell cultures was negative. The differentiation was not so clear with 1:32 dilution of conjugated anti-type 2 serum, which showed a 1+ staining of type 2 infected cell culture and no staining with type 1 infected cell culture. But any dilution lower than those mentioned above gave a positive staining of the cells infected with either of the two types.

Benda et al (1966) demonstrated B virus (herpes simiae) in infected mouse brain by direct FA staining. The staining of the impression smears from infected mouse brain proved to be very simple and reliable for diagnosis. Benda (1966) showed cross-reactions between B virus and herpes

simplex virus by indirect FA staining. B virus infected cells showed medium fluorescence when stained with herpes simplex antiserum. Similar results were obtained when B virus antiserum was used for staining herpes simplex infected cells.

Poxviruses: Avakyan et al (1961) made a comparative study between immunofluorescence and other conventional methods for the diagnosis of smallpox infections. The detection of variola virus by FA staining was rapid and more sensitive than other tests. Murray (1963) prepared smears from vesicular lesions of 5 patients and examined them after staining with indirect method with vaccinia antiserum. All of them showed bright fluorescence indicating the presence of variola antigen. Smears from 5 chickenpox cases were negative when stained with vaccinia antisera. He could not distinguish between variola and vaccinia viruses by FA staining. Kirsch and Kissling (1963) reported that FA staining is very useful for the diagnosis of smallpox infections by using the smears from vesicular materials.

Gurvich and Roibel (1965) used direct FA staining to differentiate variola, alastrim, vaccinia, cowpox, varicella herpes, and herpes zoster virus in tissue cultures. The vaccinia and herpes zoster antisera were conjugated. The conjugated vaccinia antiserum gave a strong specific fluorescence when used for staining cells infected with variola, alastrim, vaccinia and cowpox viruses, but cells

infected with herpes simplex, herpes zoster and varicella virus were negative. The conjugated herpes zoster anti-serum showed a bright fluorescence when cells infected with herpes zoster, varicella virus and herpes simplex were stained. The cells infected with poxviruses did not show fluorescence when stained with herpes zoster conjugate. The site of localization of the antigen was different in these two groups of viruses. The poxviruses are characterized by cytoplasmic fluorescence whereas herpes viruses showed a nuclear as well as cytoplasmic fluorescence. Carter (1965) reported the detection, titration and differentiation of variola and vaccinia viruses by direct FA staining in tissue cultures. Kratchko et al (1964) also reported that direct FA staining is reliable and sensitive for the diagnosis of vaccinia virus in tissue cultures. Sokolov and Parfanovich (1964) studied the development of vaccinia virus in tissue culture by direct FA staining. They observed that the specific fluorescence was detected in the cytoplasm, first at 8 hours after infection, which gradually increased and by 18-22 hours most of the cells showed fluorescence of the whole cytoplasm. Benda et al (1968) demonstrated antigens in materials taken from vaccinia lesions in rabbits and from smallpox infiltrates in monkeys by direct staining.

Meszaros et al (1969) identified vaccinia virus from infected chorioallantoic membrane by direct FA

staining.

Adenoviruses: Immunofluorescence is mostly used for the study of adenoviruses in tissue cultures. Boyers et al (1959) reported about the development and localization of adenovirus type 4 antigens in Hela cells. The specific fluorescence was detected in the nuclei about 14-24 hours after infection. The whole nucleus became covered with fluorescent material within 30-38 hours after infection. The findings were correlated with the growth curve of the infectious virus. Similar findings were reported for adenovirus types 5 and 7.

Pape and Wallace (1964) reported that cells from tumors produced by adenovirus type 12 in hamsters showed fluorescence in the cytoplasm only. The cultured cells from these tumors showed fluorescent flecks in the cytoplasm and nucleus when hamster serum was used for staining. In vitro transformed cells showed fluorescent flecks in the nucleus only. The cells infected with adenovirus types 1, 2, 5, 7 and 18 also showed fluorescence when stained with antisera from hamsters bearing adenovirus type 12 tumors.

Shimojo et al (1967) differentiated between "T" antigen and "virion" antigen of adenovirus type 12 in hamster embryonic kidney (HaEk) cells and hamster's tumor cells by direct FA staining. The HaEk cells infected with adenovirus type 12, showed fluorescence 6 hours after infection in the nucleus and soon it was present in the cytoplasm

with anti-"T" conjugate. The "C" antigen was detected in the nucleus with anti-"C" conjugate 15-20 hours after infection. The "A" antigen was also detected in the nucleus with anti-"A" conjugate about 20 hours after infection. In tumor cells "T" antigen was detected but "C" and "A" antigens were not present.

Other viruses: There have been reports of successful application of immunofluorescence for the studies of other viruses. Joncas (1964) reported that cytoplasmic granular fluorescence was detected 10 hours after infection with reovirus type 1 in green monkey kidney cells. The cytoplasm was filled with fluorescent materials, 40 hours after infection.

Coyne et al (1970) examined smears of liver cells, (biopsy specimens) taken from 61 patients with infectious hepatitis, serum hepatitis or other diseases which were stained by direct method to detect Australia antigen (a particulate substance associated with hepatitis). All 26 patients who had Australian antigen (1) in their serum, showed specific fluorescence in their liver cells. The fluorescence was either as a discrete particle in the nucleus or in the form of diffuse fluorescence of the entire nucleus.

Hinuma et al (1967) used indirect FA staining to detect viral antigen in Burkitt lymphoma cell line (P₃HR-1) with selected human sera. P₃HR-1 cell line initially contained 1 to 5% fluorescent cells, which increased to 15-40%

after 4 months of propagation. When the cells were incubated at 35° or 32°C for 9 to 15 days without refeeding more than 50% of the cells became fluorescent. Thirteen different cultures which contained up to 75% fluorescent cells were thin-sectioned and examined under the electron microscope. The percentage of cells showing herpes-like particles varied from <3 to 78%. There was generally a good correlation between number of fluorescent cells and the number of cells containing virus-like particles.

(c) Applications for the quantitation of virus infectivity

Immunofluorescence has been successfully used for the quantitative estimation of viruses in tissue culture. It is specially useful for those viruses which do not produce cytopathic changes in tissue cultures. In most cases it also takes less time than the conventional methods.

Hobbins and Smith (1968) developed a fluorescent plaque assay for rubella virus in rabbit kidney (RK13) cells. Fluorescent plaques were counted 72 hours after inoculation. The number of fluorescent plaques counted showed a linear relationship with the virus concentration. The efficiency of absorption was 90%. The sensitivity of the fluorescent plaque assay was equal to that of the interference titration method.

Schieble et al (1967) used fluorescent cell counting method for the assay of respiratory syncytial virus in

human fetal diploid cells. The efficiency of adsorption was 85% in 2 hours. The coverslip cultures were stained 20 hours after virus inoculation. A linear relationship between the virus concentration and the number of fluorescent cells counted was established. The method was more sensitive and less variable than TCID50 endpoint titration method. McClain et al (1967) compared immunofluorescent cell count and plaque methods for the infectivity titration of reoviruses. Both procedures were found equally sensitive and reliable.

Rapp et al (1959) determined the infectious units of measles virus, by counting the number of fluorescent foci in infected coverslip culture of H.Ep-2 cells under methyl cellulose overlay. The cells were stained by indirect method, 5 days after incubation. The results showed a linear relationship between fluorescent focus count and the concentration of virus used.

Sattar and Westwood (1967b) developed a rapid and sensitive immunofluorescent method for the assay of herpes simplex virus in green monkey kidney cells. The fluorescent foci could be counted within 20-22 hours after virus inoculation. A direct relationship was detected between virus concentration and the number of fluorescent foci counted. The technique was reported to be more sensitive than the conventional plaque assay.

Sattar and Westwood (1968) reported that influenza

virus (A/PR8) could be assayed by counting the fluorescent cells within 12-13 hours after inoculation in green monkey kidney cells. A direct relationship between virus concentration and the number of fluorescent cells counted was established. The fluorescent cell count assay was compared with quantitative haemadsorption technique and was found to be more sensitive.

Hahon and Cooke (1967) used fluorescent cell counting method for the titration of VEE virus in McCoy cells. An efficiency of absorption of 97% was achieved by centrifuging the virus on cell monolayer. The overlay medium contained antiserum to prevent secondary spread of the virus. The cells could be enumerated as early as 12 hours after inoculation. The relationship between virus concentration and infecting units was linear. The fluorescent cell counting method was equivalent in sensitivity but more precise and rapid than mice inoculation test. Carter (1969) developed a fluorescent focus assay method for semliki forest virus in Hela cells. Hahon (1966) found that a quantitative assay of infective yellow fever virus could be achieved by fluorescent cell counting within 24 hours after inoculation in McCoy cells under antiserum overlay. An efficiency of adsorption of 95% was achieved by centrifugation. The cells were stained 24 hours after inoculation. The relationship between virus concentration and cell infecting units was linear. The sensitivity of the assay was

comparable to mouse inoculation test. Hahon and Hankins (1970) assayed Chikungunya virus by counting fluorescent foci in BHK21/C13 cells. The efficiency of adsorption was enhanced by centrifugation of the virus on the cell monolayer. The foci could be counted as early as 16 hours after inoculation. A linear function was demonstrated between focus count and relative virus concentration.

Spendlove and Lennette (1962) reported the development of fluorescent plaque assay method for vaccinia virus. The plaques were developed on infected coverslip cultures under agar overlay. The cells were stained after 24 hours of incubation and fluorescent plaques were counted. The results obtained by fluorescent plaque assay were compared with the results of conventional plaque assay method and found to be more sensitive. Hahon (1965) used fluorescent cell counting technique for the assay of variola virus in McCoy cells. The efficiency of adsorption was improved by centrifugation of the virus on the cell monolayer. The optimal period for enumerating fluorescent cells was 16-20 hours after inoculation.

(d) Other applications: In recent years FA staining has been used for the titration of anti-viral drugs and interferon in tissue cultures. FA staining has the advantage of allowing a study of the local effect of these compounds in individual cells, and also by using fluorescent cell

counting technique the antiviral activity could be quantified.

Kashiwazaki and Ishida (1965) used FA staining to determine the inhibitory effect of N-isobutylbiguanide hydrochloride on sendai virus grown in L-cells. In the presence of a nontoxic concentration of the compound the total number of fluorescent cells was reduced to one half of those present in control 24 hours after infection. At 72 hours after infection the number of fluorescent cells was the same in treated as well as in control groups except that the virus in the treated group could not reach to maturation stage. The results show that the effect of the compound tested is on the inhibition of the late maturation mechanism of sendai virus synthesis.

Vigario et al (1968) studied the multiplication of sheep pox virus in sheep embryo cell culture exposed to 5-fluorodeoxyuridine (FUdR) by autoradiography and immunofluorescence. No progeny virus was detected by infectivity titration when the cells were exposed to a concentration of 10^{-5} M of FUdR at the time of infection. The autoradiographic preparations of the treated cells showed cytoplasmic labelling of viral DNA in 4-9% of the cells during the first 24 hours of infection. The FA staining demonstrated abnormal fluorescent aggregates and coccoids in infected cells exposed to FUdR. In conclusion the results show that viral antigens are formed in FUdR treated cells but the synthesis

of infectious virus was prevented.

Oxford and Schild (1968) used immunofluorescent cell counting technique to investigate the virostatic effect of aminoadamantane, ammonium acetate and a number of aliphatic amines on the development of influenza A and B virus antigens in BHK-21 cells. The coverslip cultures were incubated at 37°C in maintenance medium containing the appropriate amount of the compounds 1 hour before virus infection. Cells were then infected with influenza virus and incubated for 24 hours at 37°C in a CO₂ incubator. In virus control cultures amines were omitted. At the end of the incubation period the cells were stained by indirect FA technique. The number of fluorescent cells were counted from both treated and untreated cells. The two counts were compared to give relative inhibition of the virus.

Influenza A₂/Scotland/49/57 showed 88% inhibition with 25 ug/ml of aminoadamantane in the medium. When the cells were serially subcultured, 10 times in the presence of 25 ug/ml of aminoadamantane and then infected, the percentage of inhibition went up to 99.4%. Influenza A/NWS virus was slightly inhibited but influenza B/England/939/59 was not inhibited. The degree of inhibition by ammonium acetate was similar for the influenza viruses as with aminoadamantane. Two amines tested showed a significant inhibition of influenza B/England/939/59 virus.

Kozikowski and Hahon (1969) used fluorescent cell counting technique for the quantitative assay of interferon. The effect of interferon was tested against, vaccinia, yellow fever, psittacosis agent and VEE viruses in McCoy cells. The interferon was produced in the same cell line with irradiated vaccinia virus. The dilutions of interferon were added to coverslip cultures and incubated for 24 hours at 35°C. Virus and cell controls were included. At the end of the incubation period the cells were washed and challenged with 7×10^3 cell infective units of respective virus. They were incubated at 35°C for 18-24 hours, then stained and fluorescent cells were counted. The reciprocal of the interferon dilution that reduced the number of fluorescent cells to 50% of the control served as measure of interferon activity. The yellowfever virus was the most sensitive virus to the action of interferon out of the 4 viruses tested.

Chaudhary and Westwood (1970) used immunofluorescence to detect cross-reactions within picornavirus group.

Conclusions: This review presents a broad picture of the usefulness of the immunofluorescence technique in the field of virology.

It is obvious that FA staining could be used for many different purposes. The direct staining of clinical specimens has proved satisfactory for the diagnosis of

influenza and respiratory syncytial infections. Rabies virus has been accurately diagnosed by FA staining.

In the case of herpes and poxviruses an accurate diagnosis could be achieved by direct staining of the clinical specimens or after inoculation in tissue cultures.

The rubella virus requires a lengthy interference neutralization test for identification, but the FA staining has simplified the whole process of identification.

Titration and detection of antibodies in rubella, mumps and rabies infections could also be achieved by FA staining. The quantitative estimation of virus infectivity in tissue cultures has been reported by FA staining for most of the viruses.

In recent years FA staining has been used to detect the antiviral activities of drugs and interferon in tissue cultures. By using fluorescent cell counting technique the antiviral activities could be quantified.

Immunofluorescence has added substantially to our knowledge and has solved many of the problems in the field of virology.

C. IMMUNE ELECTRON MICROSCOPY

The immune electron microscopy technique was specially developed for visualizing the interaction between antibody and antigen molecules:

(a) Methods: Almeida and Waterson (1969) outlined the

general procedures for this technique. They reported that the frequently encountered difficulty lies in obtaining a clean virus preparation for use, and that it is a sensitive method to detect immune reactions with as little as 10^6 virus particles. The specimen could be prepared by mixing a concentrated virus antigen (0.1-0.2 ml) with an equal volume of antiserum and then incubating it at 37°C for 1 hour and at 4°C overnight. At the end of the incubation period the mixture was centrifuged and the sediment was washed twice with PBS. The pellet from the final wash was suspended in 0.1 ml of distilled water and a drop of the suspension was mixed with a drop of phosphotungstic acid pH6.0. A drop of this mixture is then placed on a 400-mesh carbon-formvar-coated grid, excess fluid was withdrawn with filter paper and then examined under electron microscope. The speed at which the antigen-antibody aggregate should be centrifuged, depends on the size of the virus. In some cases the IgG and IgM fractions of the serum was used. Norrby et al (1969) used purified IgG antibodies to study adenovirus type 3 antigens. They incubated the virus and IgG antibodies for 1 hour and then directly used the mixture for negative staining without any centrifugation. Brown and Smale (1970) studied the antigenic sites on foot-and-mouth disease virus by using IgG and IgM fractions.

(b) Structure of antigen-antibody complexes: Anderson and Stanley (1941) were the first to observe tobacco mosaic

virus and its antibody complex under electron microscope. The antibody seemed to have joined the antigen molecules to give an outward appearance of "lattice" formation. Easty and Mercer (1958) reported that the complexes formed by ferritin and its antibody showed a halo of antibodies around the ferritin antigen. They were not able to observe the details of the antibody molecules. Lafferty and Oertel (1961) did kinetic analysis of virus neutralization by using influenza virus and its antibodies. They thought that irreversible neutralization depends on the stabilization of the attachment of antibody molecules to virus particles. The stabilization depends on divalent antibodies as monovalent antibodies could not form a stable combination with virus particles. The first stage in neutralization would be the attachment of antibody molecules to the virus particles through one combining site and then stabilization would be brought about by the attachment of the second combining site of the divalent antibody to the same virus particle thus the antibody molecule will form a loop on the surface of the virus. This is what they observed in electron microscope. There was no loop formation with monovalent antibodies.

Almeida et al (1963) examined complexes formed by verruca vulgaris virus and polyoma virus with their respective antibodies under the electron microscope, and they observed three main types of arrangements; at the

lowest relative concentration of antibody about two molecules of antibody could be seen on any one virus particle; as the concentration of antibody was increased, the number of linked virus particles also increased; when the relative concentration of antibody was further increased a dense aura of antibody was found around virus particles. Such particles were not linked to each other by antibodies.

Feinstein and Rowe (1965) studied the complexes formed between ferritin and its antibody. When there was an excess of antigen there was no cross-linking, but when antigen-antibody were in equivalence ratio, the antigen and antibody molecules were cross-linked. When pepsin treated antibodies were used the strands were similar but less complex. When monovalent antibodies were used for complexing there was no cross-linking but 100 $\overset{\circ}{\text{\AA}}$ long rod-shaped antibody could be seen attached to the antigen. These results showed that for cross-linking one needs a divalent antibody molecule. Valentine and Green (1967) examined antibody-hapten (dinitrophenyl octamethylenediamine) complexes under electron microscope. Their findings were similar to those reported by Feinstein and Rowe.

Hoglund (1967a) reported the interaction of T2-phage and its IgG-and-IgM-antibodies. The IgG-antibody treated phages showed a head to head attachment. The antibodies formed bridges of about 150 $\overset{\circ}{\text{\AA}}$ and they often appeared in dense tangle surrounding the head membrane.

The IgM-antibodies were much less effective in precipitating the T2-phages, however small virus-antibody complexes were seen. The IgM-antibodies were attached to the outer edge of the phage head and also in few cases to the distal end of the tail. The results also showed that the IgG-antibodies are more specific than the IgM-antibodies.

Svehag (1968) thought that the studies of virus-antibody complexes by electron microscopy might be a useful complement to kinetic and hydrodynamic studies of the neutralization process.

(c) Identification of antigens and antibodies: Hummeler et al (1962) reported the identification of poliovirus of different antigenicity by immune electron microscopy. They examined the complexes formed between poliovirus type 1 antigen and immunoglobulin prepared against native (N) and heated (H) virus after negative staining. The results showed that the anti-"H" globulin mostly agglutinated empty particles, whereas anti-"N" globulin agglutinated full particles. The reaction with antiserum containing antibodies against both "N" and "H" antigens showed aggregates containing both full and empty virus particles. These results showed the usefulness of immune electron microscopy for the identification of viral antigens. Beale and Mason (1968) were also able to differentiate the two antigenic types of polioviruses by immune electron microscopy. The antigen-antibody complexes were extracted from precipitation

lines from agar and they were examined after negative staining. Their findings were similar as those reported by Hummeler et al (1962).

Lafferty and Oertelis (1963) mixed influenza virus type A and B separately with influenza A antiserum. The two preparations were examined under electron microscope after negative staining. The result showed that the influenza A and B viruses were not related antigenically as no aggregate was formed between influenza A antiserum and B virus whereas the homologous reaction showed large aggregates of virus and antibody molecules.

The presence of antibodies could be demonstrated in sera by immune electron microscopy with a known virus. Almeida and Goffe (1965) obtained sera from 19 human cases of warts. Each serum sample was mixed separately with warts virus and after proper incubation, they were negatively stained and examined under the electron microscope for the presence of clumping and attachment of antibody molecules to virus particles. The sera from 10 cases were positive as they showed clumping of wart virus. Ball and Brakke (1969) used electron microscopy and density gradient centrifugation to detect serological reactions of tobacco mosaic virus and southern bean mosaic virus with their respective antisera; clumps of virus were seen under the electron microscope and the test was reported to be more sensitive than other serological tests for these two viruses tested.

(d) The morphology of antibody molecules

IgG antibody: has a molecular weight of 150,000 and a sedimentation coefficient of 7S. Hall et al (1959) reported that the electron microscopic examination of rabbit IgG after shadow casting showed a length of $250\overset{\circ}{\text{\AA}}$, but further details were not revealed. Almeida et al (1967) examined negatively stained aggregates of 7S antibody and foot-and-mouth disease virus. Electronmicrographs showed that 7S antibody was of a filamentous form, measuring $27 \times 270\overset{\circ}{\text{\AA}}$.

Green (1969) reviewed the electron microscopic structure of immunoglobulin. He observed that different workers reported a different dimension for IgG immunoglobulin. The most likely dimension was suggested to be $35\text{--}40 \times 250\text{--}270\overset{\circ}{\text{\AA}}$. Some workers reported the shape of IgG to be "Y" shaped but electron microscopic observation did not provide a strong evidence in their support. While the simple linear model yielded dimensions of $35 \times 250\overset{\circ}{\text{\AA}}$ the "Y" model was in the range of $35 \times 150\overset{\circ}{\text{\AA}}$. The controversy on linear shape or "Y" shape of IgG is still not resolved.

IgM antibody: has a molecular weight of 900,000 and a sedimentation coefficient of 19S. Svehag and Bloth (1967) studied the configuration and attachment of antibody to poliovirus antigen under electron microscope. They observed that IgM antibody was of a filamentous form. Almeida and Waterson (1969) in their review paper reported that the IgM antibody appeared as "staple-shaped" structure with a

clear angular outline under the electron microscope. The "staple" form was also confirmed by Feinstein and Rowe (1965). They used bacterial flagella as antigen. The IgM molecules can also appear in a "linear" form with an average dimension of $350 \times 50\text{\AA}$.

Green (1969) reported that each IgM molecule contains 7S subunits linked together by single disulphide bonds. Uncombined IgM appeared as a multiarmed structure spread out on the carbon film. It is reported that IgM molecule is a "cyclic pentamer" held together by sulphide bonds. The dimension of the arms are $35 \times 125\text{\AA}$.

Monovalent antibody: IgG antibody can be degraded in two identical fragments by digestion with papain. The two fragments have antigen binding capacity and they are called "Fab". When they attach to antigens, they appear as rigid radially oriented rods about $70\text{-}90\text{\AA}$ long. They could not bind two antigens together (Almeida et al 1965).

These results indicate that the negative staining enables both IgG and IgM antibodies to be visualized and distinguished from each other, but a final appraisal of the exact anatomy of these molecules has not been achieved.

(e) Localization of specific antigens

In electron microscopy the counterpart of fluorescein-conjugated antibody is the ferritin-conjugated antibody for the localization of antigens. However ferritin

marker introduces problem of specificity and it is technically not easy to handle. Since the antibody molecules without a marker could be visualized; the negative staining of the antigen and antibody complex should be the technique of choice to localize an antigen. The work reported by Watson and Wildy (1963) illustrated the usefulness of immune electron microscopy for the localization of host specific antigen in the herpes virus envelope. Herpes virus in the presence of homologous anti-host cell antibodies showed a marked clumping of the enveloped particles, but there was no clumping of enveloped particles with antiherpes antiserum, but it did agglutinate naked particles. These results demonstrated the presence of host cell material in the envelope of herpes virus. Similarity of the envelope to host cell material was also demonstrated by the attachment of influenza virus particles to the envelope.

Berry and Almeida (1968) also demonstrated the presence of host cell material in the envelope of avian infectious bronchitis virus, grown in eggs, by immune electron microscopy. Antisera prepared in fowl and rabbit were used for complexing and for neutralization test. The result showed that the neutralization titers of unheated antisera from both sources were 3 logs higher than heated antisera and activity of heated sera could be restored by adding complement. The electron microscopic examination of the heated and unheated fowl antiserum and virus aggregates

showed that the antibody molecules were attached solely to the spikes. The reaction between heated rabbit antiserum and virus showed that the antibodies were not only attached to the spikes but also to the envelope. In addition, the unheated rabbit serum produced a number of holes, about 100 Å across, in the envelope. The holes were produced due to the action of complement.

Heated rabbit anti-chick-embryo antiserum showed clumping of virus particles, but the antibody molecules were attached to the envelope only not to the spikes, while the unheated chick-embryo antiserum produced holes in the envelope.

The findings that the fowl antiserum did not produce holes in the envelope, could be explained in the light that the fowl was unable to recognize the virus envelope as being antigenically different from their own tissues, so no antibodies were produced against the envelope, whereas a heterologous species (rabbits) recognized the envelope as foreign and reacted by producing antibodies against it. This argument is also supported by the fact that the antiserum prepared in rabbits against normal chick-embryo tissues was also capable of reacting with envelope and produced holes. Thus it proves that the envelope contains host material which could be easily detected by immune electron microscopy.

Norrby et al (1969) examined the relationship between soluble antigen and the virion of adenovirus type 3 by immune electron microscopy. The results showed that the virions incubated with homotypic hexons antiserum had antibody coating but not with heterotypic hexons antisera. These anti-hexons antisera did show heterotypic CF antibodies (1:640). The intact virions were able to fix complement only with homotypic hexons antibodies, but disrupted particles showed complement fixation and clumping with heterotypic antibodies. They suggested that the hexons might have a specific orientation, so that only their type specific part is available at the surface of the virion, but the group specific CF antigen is exposed when the virions are disrupted.

The type specific antifiber antibodies showed a small meshwork of molecules between vertices of aggregated virions. The anti-penton serum absorbed with fiber antigens did not reveal any attachment of antibodies.

The results showed that the "group specific" and "type specific" antigen associated with hexons could be differentiated by this technique along with type specific fiber antigens.

Brown and Smale (1970) demonstrated three specific antigenic sites on the surface of foot-and-mouth disease virus by this technique. The results showed that the virus particles formed complexes both with IgG and IgM antibodies.

The IgG-virus complex showed that the entire virus surface was covered with antibody molecules and the virus outline was obscured. Whereas the IgM-virus complex showed a clear outline of the virus particles and the antibody molecules were attached at distinctly spaced sites. These observations suggested that the virus particles have more than one combining site.

When the IgG was absorbed with excess of 12S proteins (subunits prepared by heating the virus at 56°C for 1 hour) then their complex with virus showed a similar pattern of attachment as those observed with IgM antibodies. It showed that IgG preparation contained antibodies for two antigenic sites, one of which was absorbed out by 12S subunits. Evidence for the third antigenic site was presented by examining the complex formed between trypsin treated virus particles and IgG antibodies whereby two antigenic sites were demonstrated on the treated virus and serum absorbed with trypsin-treated virus still reacted with untreated virus, demonstrating a possible third combining site. The trypsin treated virus particles did not react with IgM antibodies indicating that the IgM is monospecific in nature and reacts only with site removed by trypsin.

Conclusions: The applications of this technique have been known for about 8 years but during this time only a small number of people have attempted to use it. This would seem to be mainly because of the unawareness of the potential of

this technique. The method is a straightforward one, giving direct visual evidence of the immune reactions to be studied, with an amount of virus in the region of 10^6 particles, which would be too small to give a visible reaction with the agar gel immuno-diffusion technique or with most of the standard immunological techniques.

In view of the growing importance of the electron microscope as a direct diagnostic tool, its simultaneous development as a means of detecting serological reactions suggests a wide field of applicability in the diagnostic as well as in the research field. Since the electron microscopic identification of a virus as belonging to one of the major groups can be so rapidly carried out, the further steps of precise serological identification by the same means would appear to be a logical extension of this powerful technique.

PART I

CROSS-REACTION STUDIES WITHIN THE PICORNAVIRUS GROUP BY IMMUNOFLUORESCENCE

INTRODUCTION

The identification and typing of viruses by FA staining which is still to be developed, would require a detailed study of cross-reactions within different groups of viruses. In fact, cross-reactions are reported by different serological methods for most of the virus groups.

The studies concerning the cross-reactions by immunofluorescence are limited. Hatch et al (1961) reported the identification of polioviruses from stool specimens by direct staining and observed cross-reactions between poliovirus type 1 and 3. Hatch (1963) was able to identify coxsackie and echoviruses by direct FA staining and crossing was observed between echovirus type 4 conjugate and echovirus type 9. The direct method has certain limitations for groups having a large number of viruses. It would require a large battery of conjugated antisera. The indirect method has certain advantages; it requires only one conjugate and is more sensitive than the direct method. If broad cross-reactions could be detected in the picornavirus group as in the case of poxviruses (one could identify variola, vaccinia,

alastrim and cowpox viruses by using vaccinia antiserum conjugate; Gurvich and Roihel, 1965) then the identification of picornaviruses as a group could be greatly simplified.

The conjugate plays an important role in achieving a specific staining. McDevitt and Coons (1964) considered that a ratio of more than 6-8 μ gs of fluorescein per mg of protein will produce nonspecific staining due to the binding of too many fluorescein groups to each protein molecule.

The experimental approach was therefore aimed to study the cross-reactions between some of the members of the picornavirus group by indirect FA staining.

MATERIALS AND METHODS

VIRUSES

Poliovirus type 1, 2 and 3 (Sabin strain), echo-virus type 3, 9, 11 and 25, and coxsackievirus type A9 and B3, B4 and B5 were selected for experiments. The type specificity of these viruses was tested by the Canadian Communicable Disease Centre, Ottawa or by typing antisera kindly supplied by them. The virus pools were prepared and titrated in BS-C-1 cells or in green monkey kidney cells.

The titer was computed by the "Kärber" method.

PREPARATION OF ANTIVIRAL ANTISERUM

Rabbits were immunized with viruses grown in BS-C-1 cells. Rabbits were given four intravenous injections (2 ml) at weekly intervals. Rabbits were bled 10 days after the last injection and the serum was tested by neutralization test for the presence of antibodies. Rabbits whose serum showed a titer of 1:500 or above were bled by cardiac puncture and serum was separated by centrifugation and stored at -20°C. Rabbits having titers of less than 1:500 were further immunized until they had a titer of 1:500 or above. All the antiserum pools were titrated by neutralization test against 100TCID₅₀/0.1 ml of homologous and heterologous viruses. Details of the virus pools used for immunization are given in Table 6. The pre-immunization antisera were negative.

TABLE 6VIRUS ANTIGENS USED FOR THE IMMUNIZATION OF RABBITS

Virus Antigens	Cell culture used for antigen production	Virus antigen titers *per ml.
Poliovirus type 1	BS-C-1	7.25
Poliovirus type 2	"	7.75
Poliovirus type 3	"	8.25
Echovirus type 3	"	6.50
Echovirus type 9	"	7.00
Echovirus type 11	"	7.50

* The titers were computed by the ["]Kärber method.

CELLS AND MEDIUM

1. BS-C-1 cells were used for the production of viral pools in the first series of experiments. The cells were continuously maintained in this laboratory in 32 ounce bottles. Medium-199 was used for growth with 10% fetal calf serum (FCS). The cells were trypsinized every week with 0.2% Versene-trypsin solution and seeded with fresh medium in culture bottles.
2. Primary African green monkey kidney cells were purchased from Grand Island Biological Company (GIBCO) New York, U.S.A., and were grown in 32 ounce bottles with medium-199 containing 10% FCS. When a complete monolayer was formed the cells were trypsinized and were used for

seeding plastic slide chambers or culture tubes. The cell cultures in the chambers were used for inoculating virus for fluorescent antibody studies, whereas the tube cultures were used for virus or antiserum titration. In the later series of experiments virus pools were also prepared in GMK cells. The medium-199 and FCS were also purchased from GIBCO.

PREPARATION OF CONJUGATE

The procedures were similar as those reported by McDevitt and Coons (1964). The antiviral antiserum was prepared in rabbits and therefore in order to apply the indirect method of FA staining guineapig anti-rabbit gammaglobulin immunoglobulin was prepared and conjugated as follows:

(i) Gammaglobulin was prepared from normal rabbit serum by precipitation at 33% saturation of ammonium sulphate which produced a nearly pure gammaglobulin (Cohen et al 1940). The precipitate was dialysed against 0.15 M sodium chloride to remove the ammonium sulphate. The gammaglobulin was further purified by fractionation through a Sephadex G-200 (Pharmacia Fine Chemicals) Column (2.5 x 100 cm). The column was loaded with 5 ml of gammaglobulin and eluted with 0.01 M phosphate buffered saline pH 7.3. The fractions were collected in 2 ml volume on a Gilson fractionator. The optical density (O.D.) of the different fractions were taken

at 280 mμ in a B.D. Spectrophotometer (Beckman). The O.D. values were plotted and the fractions of the two peaks were pooled separately and concentrated. They were then tested for purity by agar gel immunoelectrophoresis against goat anti-rabbit serum (Hoechst Pharmaceutical) on a microscope slide. The electrophoresis chamber was filled with 0.05M Veronal buffer pH 8.2 and the slides were placed in the chamber and a constant current of 42 volts was applied to the slides. At the end of electrophoresis, the slides were washed and dried and then stained with 0.1% thiazine red and decolourized with 1% acetic acid. They were then photographed.

(11) Anti-rabbit-gammaglobulin immunoglobulin was produced in guinea pigs. The latter were injected in the foot-pads once every week for four weeks with rabbit gammaglobulin emulsified with Freund's complete adjuvant (Difco). Guinea pigs were bled by cardiac puncture 10-14 days after the last injection and the serum was tested by precipitation technique against rabbit gammaglobulin to detect antibodies. Guinea pigs showing a titer of 1:320 or higher were bled by cardiac puncture and the serum was separated and pooled. The pooled serum was titrated again.

The immunoglobulin was prepared from guinea pig antiserum by the same procedure reported for the preparation of rabbit gammaglobulin.

The immunoglobulin protein concentration was estimated by measuring the O.D. value at 280 mμ and then interpolating on a standard graph.

(iii) The guinea pig anti-rabbit-gammaglobulin immunoglobulin was conjugated with crystalline fluorescein isothiocyanate (FITC) fraction I (Mann Research). The protein concentration of the immunoglobulin was adjusted to 20 mg/ml with 0.5 M carbonate-bicarbonate buffer. The immunoglobulin was chilled in an ice bath and the FITC solution was added slowly to give a final concentration of 6 μg of the fluorescein per mg of protein, while the immunoglobulin was stirred gently with a magnetic stirrer. The protein-FITC mixture was then transferred to 4°C and stirred overnight and then kept frozen at -20°C till purified.

(iv) The conjugate was purified by passing through a column (2.5 x 45 cm) packed with Sephadex G-25. The column was loaded with 3 ml of the conjugate and eluted with 0.01 M phosphate buffered saline pH 7.3. The purified fraction of the conjugate eluted first from the column.

The purified conjugate was concentrated and absorbed with washed GMK cells three times to reduce nonspecific staining.

The conjugate was also absorbed with a small amount of Sephadex G-25 before use to remove any dissociated free FITC.

Near the end of the experiments antirabbit and anti-human conjugates obtained from Microbiological Associates were also used after proper absorption and dilution (1:5).

CULTIVATION OF CELLS IN PLASTIC SLIDE CHAMBERS

The plastic slide chamber (Sattar and Westwood 1967) consists of four parts: a plastic square frame (17 x 17 x 10 mm), a silicone rubber gasket, two stainless steel clips and a microscope slide. The assembled chambers were sterilized by exposing them to ultraviolet light for 20-30 minutes. The used chambers were cleaned by boiling in soapy water, followed by rinsing in tap and deionized water.

Each chamber was seeded with $16-18 \times 10^4$ second passage GMK cells in 1 ml of medium-199 containing 10% FCS. Three chambers were placed in a square petriplate (100 x 15 mm Falcon plastics) and incubated in an atmosphere of 5% CO₂ at 36°C (Chaudhary and Westwood 1969). Chambers were examined periodically under the microscope and used for virus inoculation when a complete monolayer was formed. Generally a monolayer was formed within 20-24 hours after seeding.

MICROSCOPY AND PHOTOGRAPHY

The stained slides were examined under a Reichert binolux microscope (HBO 200 lamp) with exciter filter E₃ (BG12/6 mm) and barrier filter Sp3 (GG 9/1 mm + OG 1/1.5 mm).

A light field condenser was used. A Reichert photo-automatic camera (35 mm) was attached to the microscope for photography. The black and white pictures were taken on high speed Kodak tri-X film (ASA400) and the coloured slides were taken on a high speed Anscochrome 500 (ASA500) film. An exposure time of 2 minutes was given for the slides.

EXPERIMENTAL ARRANGEMENTS

The cells in the plastic chambers were examined under the microscope and those showing a complete monolayer were selected for virus inoculation. Prior to inoculation the medium from the chambers was discarded and the monolayers were washed with phosphate buffered saline (PBS) pH = 7.3, after which 1 ml of fresh medium-199 containing 2% FCS was added. A virus concentration of 10^4 /TCID₅₀/0.1 ml was used. The cell culture in each chamber was inoculated with 0.1 ml of the virus preparation. After inoculation the chambers were placed back in the square petri plates and incubated in an atmosphere of 5% CO₂ at 36°C. The inoculated cultures were examined at intervals under the microscope and used for fluorescent staining when 50-60% of the cells showed cytopathic changes. The plastic chambers were removed from the slides and the culture washed in PBS. They were then fixed in acetone for 10 minutes at room temperature. Fixation at 4°C and -20°C was also tested. The fixed slides were stored at -20°C. In-

fectured cultures on slides were thus prepared for poliovirus type 1, 2 and 3, echovirus type 3, 9, 11 and 25, coxsackievirus type A9, B3, B4 and B5, and vaccinia virus. The indirect method of fluorescent antibody staining was used throughout the experiments. The cell sheet was layered with 3 drops of rabbit antiserum (1:2) and incubated at 37°C in a moist chamber for 30 minutes. At the end of the incubation period the slides were washed in two changes of PBS (5 minutes in each), air dried, and 3 drops of conjugate (1:3) were applied and processed as above. The conjugate (Microbiological Associates) was diluted 1:5 before use. The cover-slips (22 x 22 mm) were mounted on the stained cell sheet by using a nonfluorescent mountant (Hartman-Leddon).

The controls included in the experiments were:

- (i) Staining of virus infected cells with normal rabbit serum
- (ii) Staining of virus infected cells with conjugate alone
- (iii) Staining of noninfected cells with antiviral antisera.

The arrangement for staining was done in such a way that cross-reactions could be studied within the group as well as between the groups of viruses. In most cases three slides were stained with each antiserum, but the controls were stained in duplicate.

The cells infected with each virus were in turn stained with antiserum against most of the other viruses used in this experiment; e.g. GMK cells infected with polio type 1 virus were stained with homologous antiserum and with antisera against poliovirus type 2 and 3 and echovirus type 3, 9 and 11. The other viruses mentioned above were treated in the same way. Coxsackievirus type A9, B3, B4 and B5 and echovirus type 25 infected cells were stained with their respective antisera as well as with antisera against polio and echoviruses. The antisera against these viruses (coxsackievirus type A9, B3, B4, B5 and echovirus type 25) were not tested against polio and echovirus infected cells.

Cross-reactions within each group and also between the groups were tested.

To determine the specificity of the cross-reactions, cells infected with vaccinia virus were stained with homologous, poliovirus type 1 and echovirus type 9 antisera. This would reveal nonspecific staining properties of the antisera.

The specificity was also tested by absorbing the antisera against echovirus type 3 with packed GMK cells, and then using the absorbed antiserum for staining cells infected with homologous virus and with echovirus type 9.

In order to determine whether the cross-reactions observed could be eliminated by dilution of the test anti-

serum, an experiment was performed in which the reactions of poliovirus type 1 and type 3 and echovirus type 3 antisera were tested in doubling dilutions up to 1:32 against cells infected with homologous and heterologous viruses.

Anti- BS-C-1 serum was produced in two rabbits. Four intravenous injections (2 ml) were given at weekly intervals. The rabbits were bled 10 days after the last injection and the serum was tested for fluorescence by staining cells infected with coxsackie type A9 virus.

Antiserum from a child infected with echo type 9 virus was kindly supplied by Virus Diagnostic Laboratory, Civic Hospital, Ottawa. The neutralizing titers of the antiserum against echo type 9, 11, coxsackie type A9 and polio type 1 viruses were; 1:640, <1:4, <1:4 and 1:4 respectively. This antiserum was used to stain cells infected with each of the viruses.

RESULTS

VIRUS TITRATION: The results of the titration of polio and echoviruses except echovirus type 25 are given in Table 6. The titers of echovirus type 25, coxsackievirus type A9, B3, B4 and B5 are given in Table 7.

TITRATION OF SPECIFIC VIRAL ANTISERA: The antisera against poliovirus type 1, 2 and 3 and echovirus type 3, 9 and 11 were titrated against 100 TCID₅₀/0.1 ml of both homologous and heterologous viruses. The titers obtained for the antisera are given in Table 8. It can be seen that a high degree of specificity was shown by the antisera in neutralization tests with the possible exception of poliovirus type 3 antiserum which gave some degree of cross-reaction with type 1 and type 2 poliovirus and to a lesser extent with type 3 echovirus.

The homologous titers of the rabbit antisera obtained from Microbiological Associates were specified as: coxsackievirus type A9 1:1250, B3 1:2800, B4 1:1850, B5 1:2800 and echovirus type 25 1:1250 respectively.

PREPARATION OF CONJUGATE: Normal rabbit gammaglobulin showed the presence of YG, YA and YM globulins as indicated by immunoelectrophoresis (figure 1).

The pooled guineapig anti-rabbit gammaglobulin serum showed a precipitation titer of 1:640 against rabbit gammaglobulin.

Immunoglobulin was separated from guineapig anti-serum and conjugated with FITC. The conjugate following purification and absorption, gave little nonspecific staining when used in a 1:3 dilution.

The conjugate obtained from Microbiological Associates also gave satisfactory results after absorption and dilution (1:5).

IMMUNOFLUORESCENT CROSS-REACTIONS

Cross-reactions within the groups: The GMK cells infected with poliovirus type 1, 2 and 3 showed almost similar fluorescence when stained with either homologous or heterologous antisera. In some cases heterologous reactions appeared to be slightly less intense than the homologous reactions. The results of the three different controls showed an acceptable reaction. The infected cells stained with normal rabbit serum showed some nonspecific staining which was within an acceptable limit. The infected cells stained with conjugate alone and uninfected cells stained with virus antiserum were negative for fluorescence. The results may therefore be regarded as virus-specific which clearly indicate the presence of cross-reactions within the poliovirus group detectable by indirect FA staining. The reactions of poliovirus type 3 antiserum with poliovirus type 1, 2 and 3 infected cells and controls are shown in figures 2-7. The results of staining with one antiserum

only are presented in the above figures in order to show the actual staining differences between different viruses and not the differences between individual antisera.

The results of the staining with other antisera were similar to those shown and a visual estimation of the intensity of fluorescence is given in Table 9.

The staining of cells infected with echovirus types 3, 9, 11 and 25 using homologous and heterologous antisera showed the presence of cross-reactions among these viruses. The antisera against echovirus type 25 was not used for staining the other three echoviruses. In most cases heterologous reactions appeared to be less intense than the homologous reactions. This could be due to differences in individual antisera.

The infected cells, stained with normal rabbit serum showed some degree of nonspecific staining, but the infected cells stained with conjugate alone and the uninfected cells stained with viral antisera showed no fluorescence.

With some variations the results were similar to those obtained with polioviruses. A visual estimation of the intensity of fluorescence is presented in Table 9. The illustrations of the staining reactions with echovirus type 9 antiserum are presented in figures 8-11. The three controls shown in figures 5, 6 and 7 are common for all the figures as well as for the results reported in the tables.

The cross-reactions were also present within the coxsackievirus group. The coxsackievirus type A9, B3, B4 and B5 were stained with homologous and heterologous antiserum: the coxsackievirus type B4 antiserum was not used for staining A9 and B5 infected cells due to some technical difficulties. The staining reactions of coxsackievirus type B5 antiserum for cells infected with A9, B3, B4 and B5 viruses are shown in figures 12-15.

The results of the visual estimation of fluorescence are given in Table 10.

Cross-reactions between the groups: The studies were completed by testing the degree of cross-reaction between polio, echo and coxsackieviruses. When antisera against echoviruses were used for staining cells infected with polioviruses, the results showed that the intensity of fluorescence varied with different combinations. Poliovirus type 1 and 3 infected cells showed medium fluorescence with echo antisera whereas poliovirus type 2 infected cells showed a medium bright fluorescence. The results are presented in Table 11.

In the same way antisera against polioviruses were tested against cells infected with echoviruses. The intensity of fluorescence varied from medium to bright fluorescence with different antisera used. The visual estimation of the intensity of fluorescence is given in Table 11. Some of the results of staining echovirus infected cells

with poliovirus type 3 antiserum are shown in figures 16-18. The staining of coxsackievirus infected cells with antisera against polio- and echoviruses also showed the presence of cross-reactions between these viruses. In general, the intensity of fluorescence was medium but some cells also showed a medium dull fluorescence. Medium bright fluorescence was only encountered in a few cases. This might be due to the fact that the individual antisera reacted differently or because of less close antigenic relationship between coxsackieviruses and the polio and echovirus antisera.

The results of staining the coxsackievirus infected cells with poliovirus type 3 antiserum are shown in figures 19-22. The results of the visual estimation are given in Table 12.

Effect of staining vaccinia virus infected cells: When vaccinia virus infected cells were stained with poliovirus type 1 and echovirus type 9 antisera, they showed no fluorescence, however there was some nonspecific staining similar to that shown by normal rabbit serum. The vaccinia virus infected cells stained with homologous antiserum showed bright fluorescence. The results are shown in figures 23-26.

Effect of antiserum absorption: That the cross-reactions were not due to anti-cellular antibodies but due to

virus-specific antisera is shown by the absence of fluorescence from the normal cell controls and from cells infected with vaccinia virus. However, to make doubly sure one of the anti-viral sera showing good cross-reaction (anti echovirus type 3) was extensively absorbed with GMK cells and retested. No reduction in the intensity of either the homologous or heterologous reactions was observed following absorption, indicating that anti-cellular antibodies were not involved.

Effect of antiserum dilution: When poliovirus type 1 antiserum was used for staining poliovirus type 1 and 3 infected cells in doubling dilutions up to 1:32, the results showed that both homologous and heterologous reactions were almost identical in this series up to 1:8 dilutions. Cells infected with heterologous virus were not stained by a 1:32 dilution of the antiserum whereas the cells infected with homologous virus showed medium dull fluorescence.

In the same way poliovirus type 3 antiserum was tested in doubling dilutions against cells infected with homologous, polio type 1 and echo type 3 viruses. The results showed that the homologous and poliovirus type 1 infected cells gave similar fluorescence up to 1:16 dilutions. Echovirus type 3 infected cells showed fluorescence only up to a 1:4 dilution of the test antiserum.

The results of staining with doubling dilutions of echovirus type 3 antiserum of cells infected with homologous

and polio type 3 viruses showed that there was fluorescence with homologous antiserum up to a 1:16 dilution whereas poliovirus type 3 infected cells showed fluorescence only up to a 1:8 dilution of the antiserum. The results of the visual estimation of the intensity of fluorescence for all three antisera are given in Table 13.

Effects of varying the temperature of fixation: The fixation at room temperature, and at 4°C gave similar fluorescence but fixation at -20°C gave a slightly less bright fluorescence.

Effect of anticellular serum: The infected cells (coxsackievirus type A9) stained with BS-C-1 antiserum, showed a medium dull fluorescence.

Effect of staining with human antiserum: When human echovirus type 9 antiserum from a naturally infected person was used for staining cells infected with homologous, polio type 1, echo type 11 and coxsackie type A9 viruses; the results showed a medium bright fluorescence for homologous staining and a medium fluorescence for heterologous staining. The absence of nonspecific staining was characteristic. A human serum control which had a neutralizing antibody titer of 1:2 against poliovirus type 1 showed some fluorescence. All other controls were satisfactory.

TABLE 7
THE TITERS OF THE VIRUS PREPARATIONS

Viruses	Cell culture used for cultivation	Titers*/ml.
Echovirus type 25	GMK	10^6
Coxsackievirus type A9	GMK	10^8
Coxsackievirus type B3	GMK	$10^{5.75}$
Coxsackievirus type B4	GMK	$10^{7.5}$
Coxsackievirus type B5	GMK	10^6

*The titers were computed by Karber method.

TABLE 8

NEUTRALIZATION TITERS OF ANTISERA: HOMOLOGOUS AND
HETEROLOGOUS REACTIONS

Viruses tested	Titers of antisera against*					
	Polio-virus Type 1	Polio-virus Type 2	Polio-virus Type 3	Echo-virus Type 3	Echo-virus Type 9	Echo-virus Type 11
Polio-virus Type 1	1:1400	-	1:16	-	-	-
Polio-virus Type 2	-	1:1300	1:64	-	-	-
Polio-virus Type 3	-	-	1:800	-	-	-
Echo-virus Type 3	-	-	1:8	1:500	-	1:4
Echo-virus Type 9	1:4	-	1:4	1:4	1:1000	1:8
Echo-virus Type 11	-	-	1:4	1:8	1:4	1:800

* All the titres are tested against 100 TCID₅₀/0.1 ml of the viruses listed.

- Less than 1:4.

TABLE 9RESULTS OF THE CROSS-REACTIONS WITHIN
POLIOVIRUS AND ECHOVIRUS GROUPS

Cells infected with	Antiserum used for staining	Results
Poliovirus Type 1	Homologous	++++
	Poliovirus type 2	+++
	Poliovirus type 3	+++
Poliovirus Type 2	Homologous	++++
	Poliovirus type 1	+++
	Poliovirus type 3	++++
Poliovirus Type 3	Homologous	++++
	Poliovirus type 1	++++
	Poliovirus type 2	++++
Echovirus Type 3	Homologous	++++
	Echovirus type 9	+++
	Echovirus type 11	+++
Echovirus Type 9	Homologous	++++
	Echovirus type 3	++++
	Echovirus type 11	++++
Echovirus Type 11	Homologous	++++
	Echovirus type 3	+++
	Echovirus type 9	+++
Echovirus Type 25	Homologous	+++
	Echovirus type 3	++
	Echovirus type 9	++
	Echovirus type 11	++

+++++ Bright fluorescence
 +++ Medium bright fluorescence
 ++ Medium fluorescence
 + Medium dull fluorescence
 - No fluorescence
 All controls were satisfactory.

TABLE 10RESULTS OF THE CROSS-REACTIONS
WITHIN COXSACKIEVIRUS GROUP

Cells infected with	Antiserum used for staining	Results
Coxsackievirus type A9	Homologous	++++
	Coxsackievirus type B3	+++
	Coxsackievirus type B5	++
Coxsackievirus type B3	Homologous	++++
	Coxsackievirus type A9	++
	Coxsackievirus type B4	++
	Coxsackievirus type B5	+++
Coxsackievirus type B4	Homologous	+++
	Coxsackievirus type A9	++
	Coxsackievirus type B3	++
	Coxsackievirus type B5	++
Coxsackievirus type B5	Homologous	+++
	Coxsackievirus type A9	+++
	Coxsackievirus type B3	+++

+++++ Bright fluorescence
 +++ Medium bright fluorescence
 ++ Medium fluorescence
 + Medium dull fluorescence
 - No fluorescence

All controls were satisfactory.

TABLE 11RESULTS OF THE CROSS-REACTIONS BETWEEN
POLIOVIRUS AND ECHOVIRUS GROUPS

Cells infected with	Antiserum used for staining	Results
Poliovirus type 1	Echovirus type 3	++
	Echovirus type 9	++
	Echovirus type 11	++
Poliovirus type 2	Echovirus type 3	+++
	Echovirus type 9	+++
	Echovirus type 11	++++
Poliovirus type 3	Echovirus type 3	++
	Echovirus type 9	++
	Echovirus type 11	++
Echovirus type 3	Poliovirus type 1	+++
	Poliovirus type 2	++++
	Poliovirus type 3	++++
Echovirus type 9	Poliovirus type 1	++++
	Poliovirus type 2	+++
	Poliovirus type 3	++++
Echovirus type 11	Poliovirus type 1	+++
	Poliovirus type 2	+++
	Poliovirus type 3	++++
Echovirus type 25	Poliovirus type 1	++
	Poliovirus type 2	++
	Poliovirus type 3	++

++++ Bright fluorescence
 +++ Medium bright fluorescence
 ++ Medium fluorescence
 + Medium dull fluorescence
 - No fluorescence

All controls were satisfactory.

TABLE 12

RESULTS OF THE CROSS-REACTIONS BETWEEN
COXSACKIEVIRUSES AND POLIO AND ECHO-
VIRUSES ANTISERA

Cells infected with	Antiserum used for staining	Results
Coxsackievirus type A9	Poliovirus type 1	++
	Poliovirus type 2	++
	Poliovirus type 3	+++
	Echovirus type 3	++
	Echovirus type 9	++
	Echovirus type 11	+++
Coxsackievirus type B3	Poliovirus type 1	++
	Poliovirus type 2	+
	Poliovirus type 3	++
	Echovirus type 3	+
	Echovirus type 9	++
	Echovirus type 11	++
Coxsackievirus type B4	Poliovirus type 1	++
	Poliovirus type 2	+
	Poliovirus type 3	+++
	Echovirus type 3	++
	Echovirus type 9	+
	Echovirus type 11	++
Coxsackievirus type B5	Poliovirus type 1	++
	Poliovirus type 2	+
	Poliovirus type 3	++
	Echovirus type 3	+++
	Echovirus type 9	++
	Echovirus type 11	++

++++ Bright fluorescence
 +++ Medium bright fluorescence
 ++ Medium fluorescence
 + Medium dull fluorescence
 - No fluorescence

All controls were satisfactory.

TABLE 13

THE EFFECTS OF ANTISERUM DILUTIONS ON
HOMOLOGOUS AND HETEROLOGOUS STAINING

Antiserum used for staining	Dilution	Poliovirus type 1 inf. cells	Poliovirus type 3 inf. cells	Echovirus type 3 inf. cells
Poliovirus type 1	1:8	++	++	NT
	1:16	++	+	NT
	1:32	+	-	NT
Poliovirus type 3	1:4	NT	+++	++
	1:8	++	++	-
	1:16	+	+	-
	1:32	-	NT	NT
Echovirus type 3	1:4	NT	++	+++
	1:8	NT	+	++
	1:16	NT	-	+

++++ Bright fluorescence
 +++ Medium bright fluorescence
 ++ Medium fluorescence
 + Medium dull fluorescence
 - No fluorescence
 All controls were satisfactory.
 NT Not tested.

PLATE 1

Figure 1: The result of immunoelectrophoretic analysis of
Sephadex purified rabbit gammaglobulin.

Centraltrough (d) - Goat antirabbit serum
antiserum

Peripheral wells (b) - Sephadex purified rabbit
gammaglobulin

PLATE 1

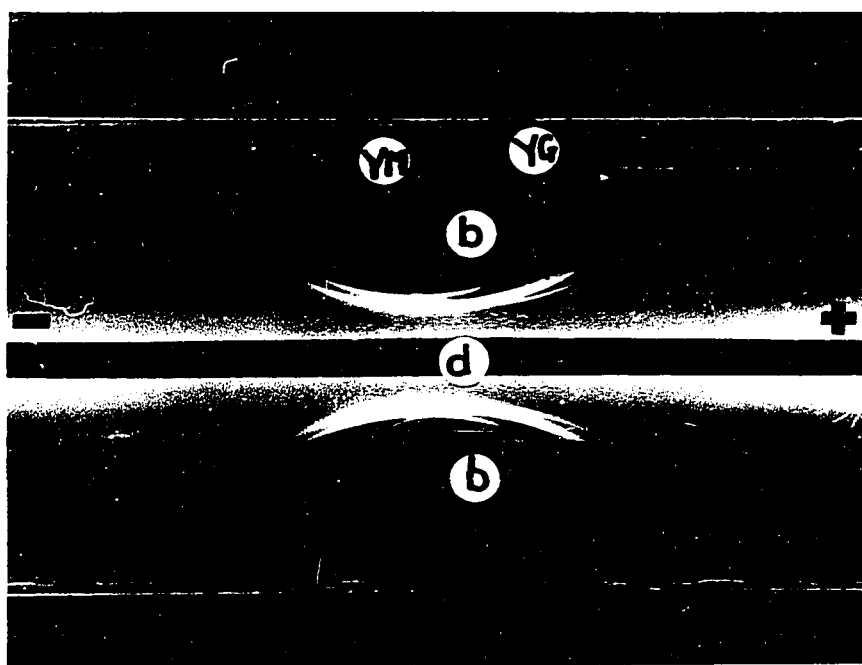


PLATE 2

- Figure 2: Poliovirus type 1 infected GMK cells (1250 X) stained with poliovirus type 3 antiserum by indirect FA technique.
- Figure 3: Poliovirus type 2 infected GMK cells (1250 X) stained with poliovirus type 3 antiserum by indirect FA technique.
- Figure 4: Poliovirus type 3 infected GMK cells (1250 X) stained with homologous antiserum by indirect FA technique.
- Figure 5: Poliovirus type 3 infected GMK cells (1250 X) stained with normal rabbit serum by indirect FA technique.
- Figure 6: Poliovirus type 3 infected GMK cells (1250 X) stained with conjugate alone.
- Figure 7: Uninfected GMK cells (1250 X) stained with poliovirus type 3 antiserum by indirect FA technique.

PLATE 2

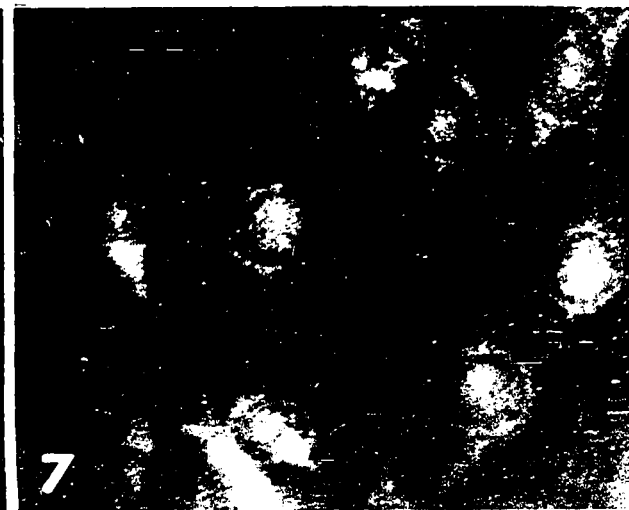
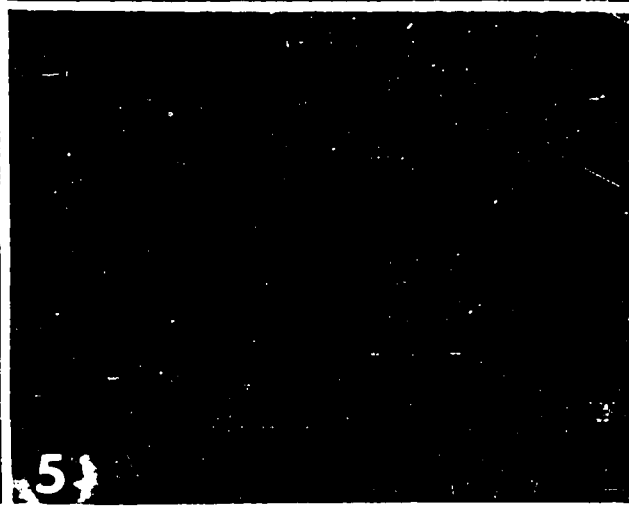
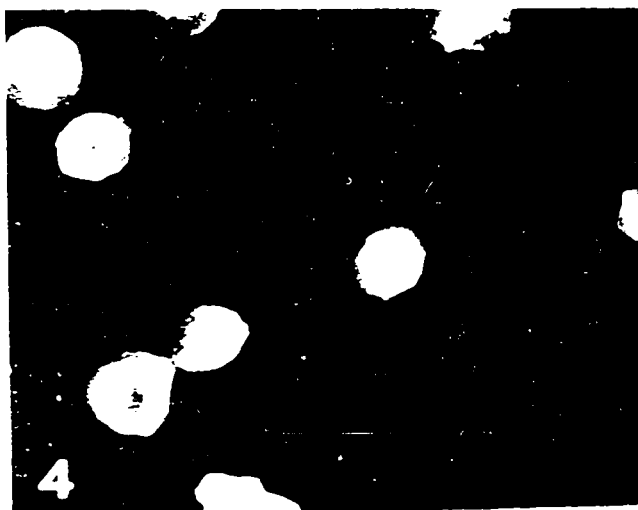
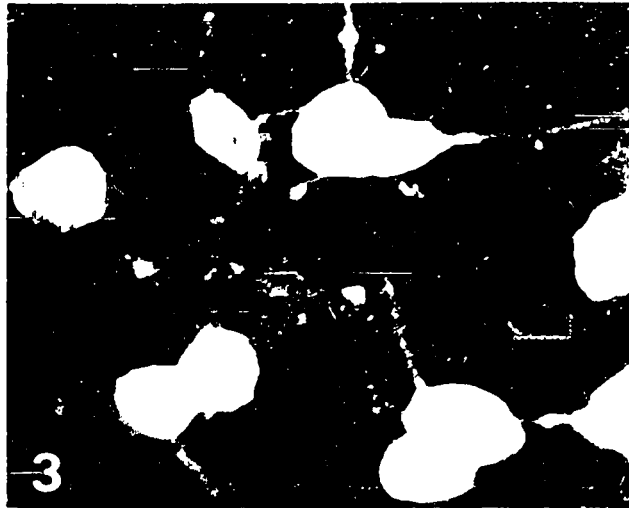
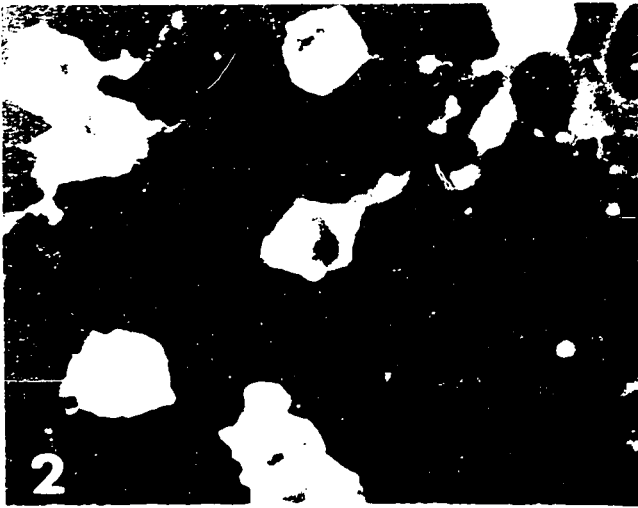


PLATE 3

- Figure 8: Echovirus type 3 infected GMK cells (1250 X) stained with echovirus type 9 antiserum by indirect FA technique.
- Figure 9: Echovirus type 9 infected GMK cells (1250 X) stained with homologous antiserum by indirect FA technique.
- Figure 10: Echovirus type 11 infected GMK cells (1250 X) stained with echovirus type 9 antiserum by indirect FA technique.
- Figure 11: Echovirus type 25 infected GMK cells (1250 X) stained with echovirus type 9 antiserum by indirect FA technique.
- Figure 12: Coxsackievirus type A9 infected GMK cells (1250 X) stained with coxsackievirus type B5 antiserum by indirect FA technique.
- Figure 13: Coxsackievirus type B3 infected GMK cells (1250 X) stained with coxsackievirus type B5 antiserum by indirect FA technique.

PLATE 3

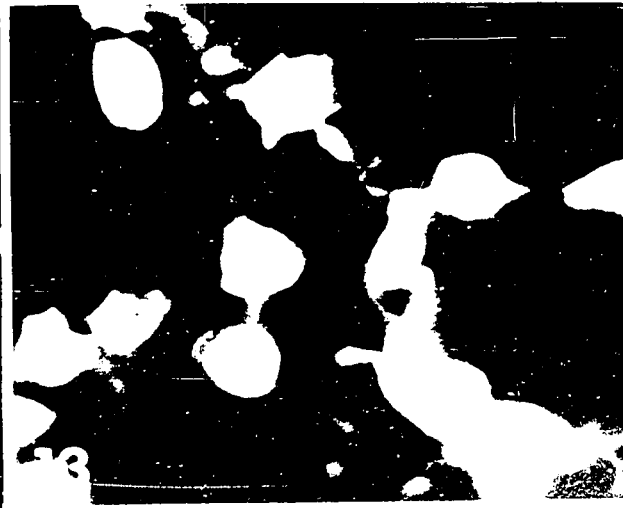
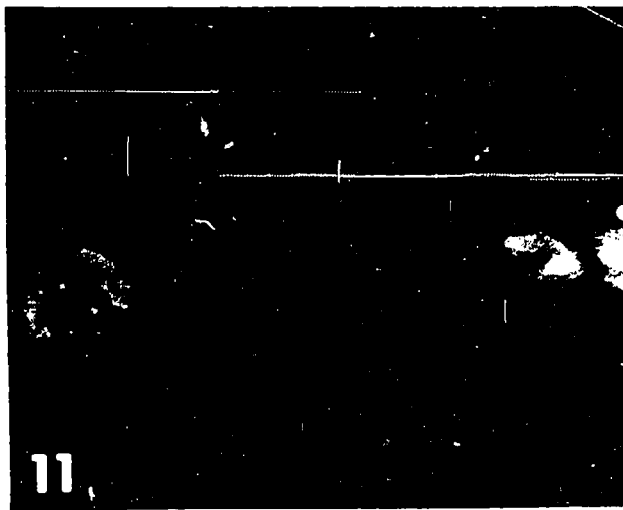
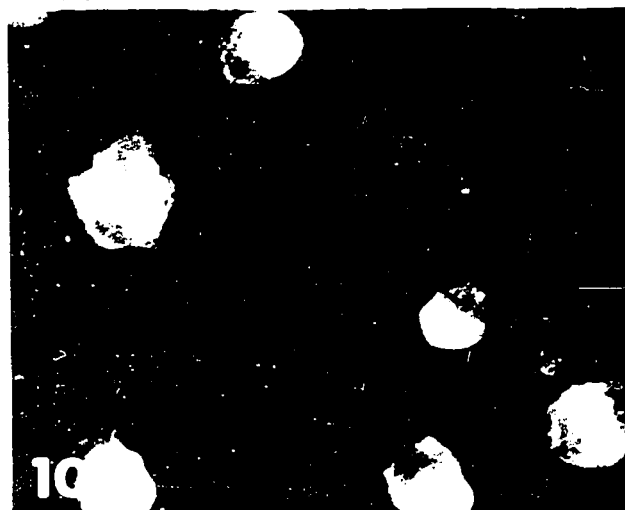
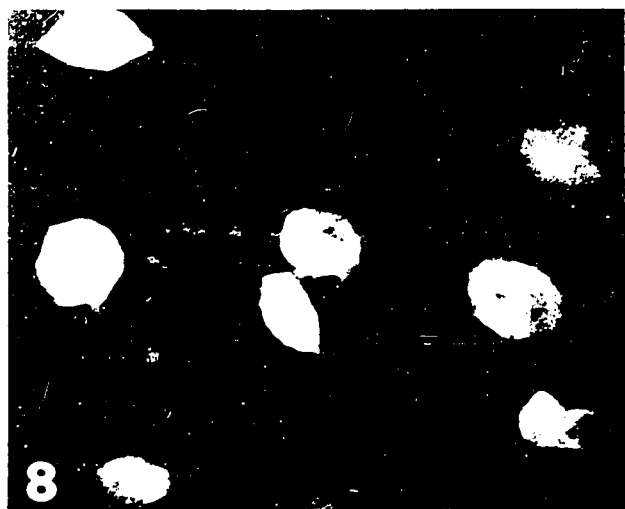


PLATE 4

- Figure 14: Coxsackievirus type B4 infected GMK cells
(1250 X) stained with coxsackievirus type B5
antiserum by indirect FA technique.
- Figure 15: Coxsackievirus type B5 infected GMK cells
(1250 X) stained with homologous antiserum by
indirect FA technique.
- Figure 16: Echovirus type 3 infected GMK cells (1250 X)
stained with poliovirus type 3 antiserum by
indirect FA technique.
- Figure 17: Echovirus type 9 infected GMK cells (1250 X)
stained with poliovirus type 3 antiserum by
indirect FA technique.
- Figure 18: Echovirus type 11 infected GMK cells (1250 X)
stained with poliovirus type 3 antiserum by
indirect FA technique.

PLATE 4

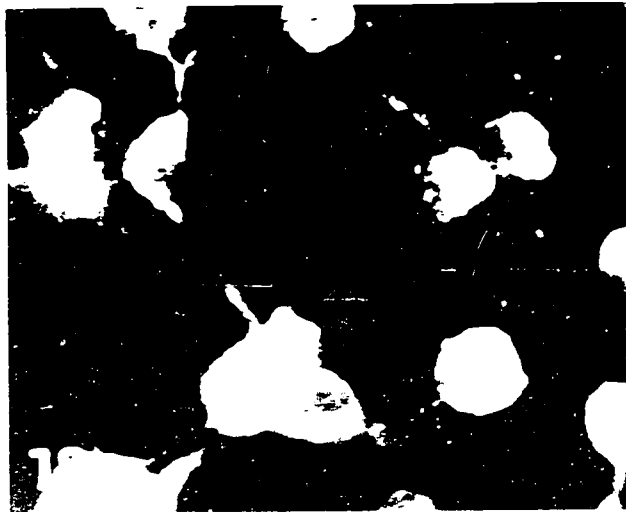
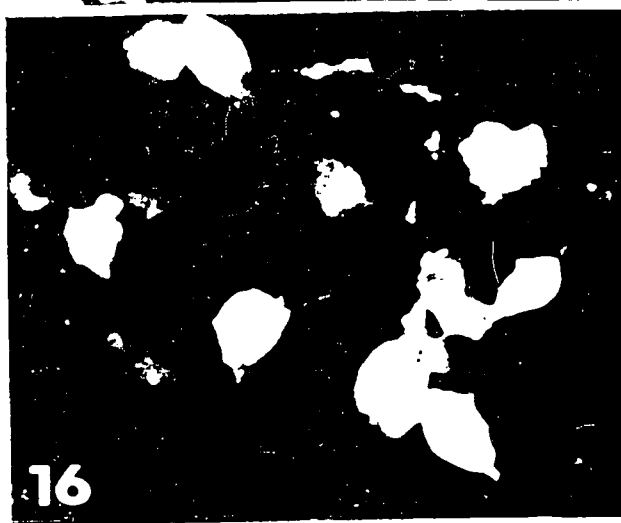
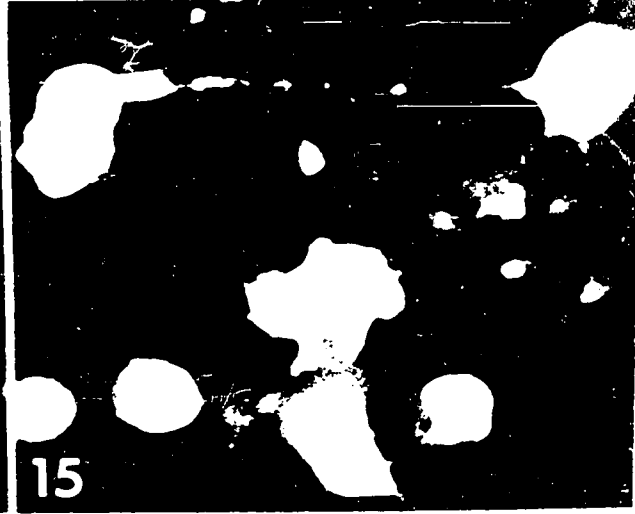
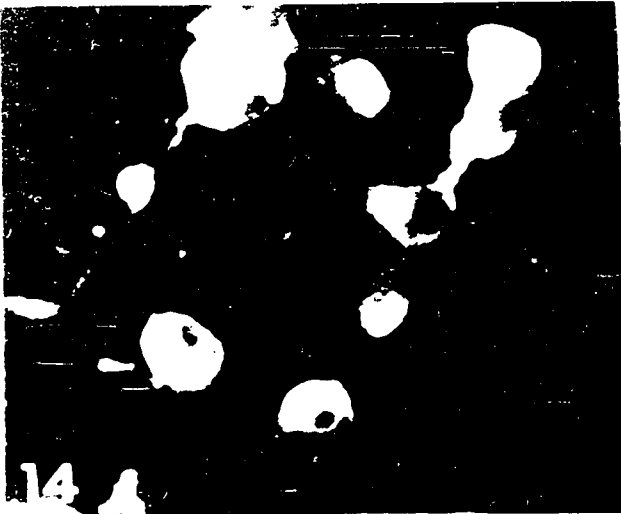


PLATE 5

Figure 19: Cocksackievirus type A9 infected GMK cells
(1250 X) stained with poliovirus type 3 anti-
serum by indirect FA technique.

Figure 20: Cocksackievirus type B3 infected GMK cells
(1250 X) stained with poliovirus type 3 anti-
serum by indirect FA technique.

Figure 21: Cocksackievirus type B4 infected GMK cells
(1250 X) stained with poliovirus type 3 anti-
serum by indirect FA technique.

Figure 22: Cocksackievirus type B5 infected GMK cells
(1250 X) stained with poliovirus type 3 anti-
serum by indirect FA technique.

PLATE 5

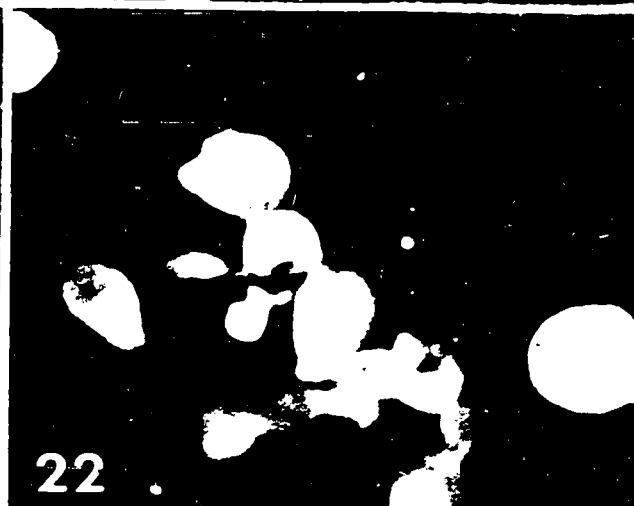
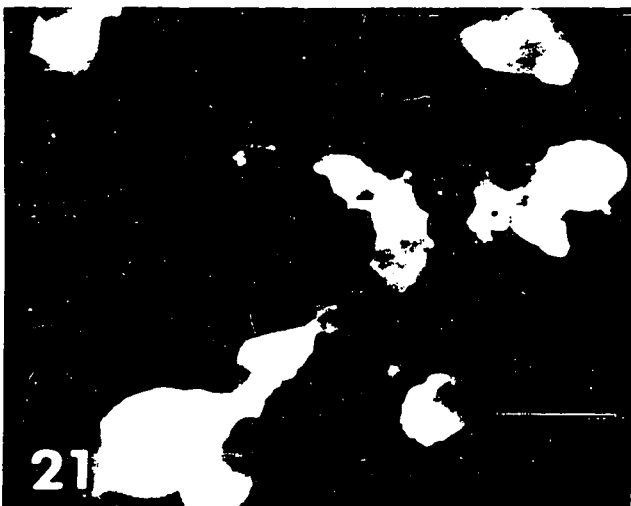
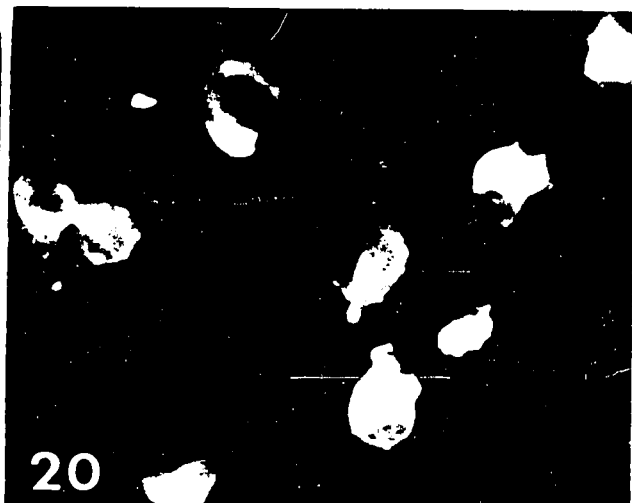
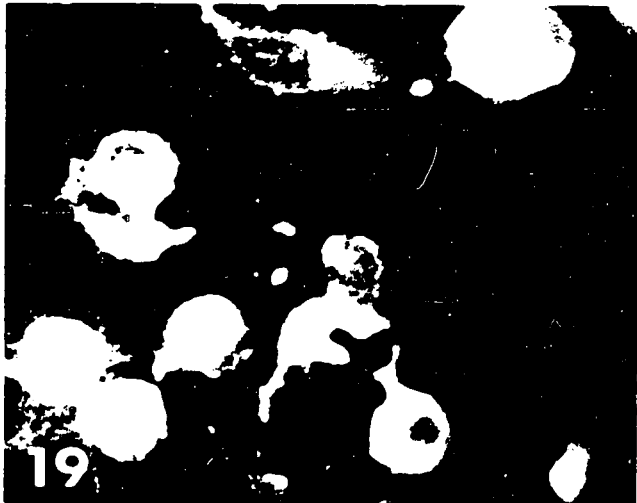
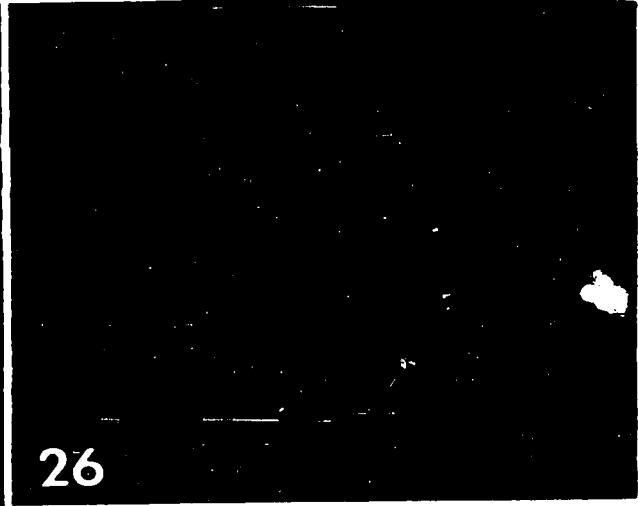
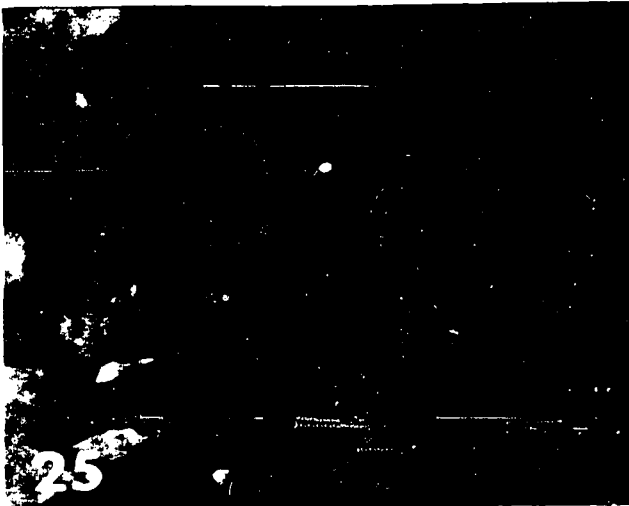
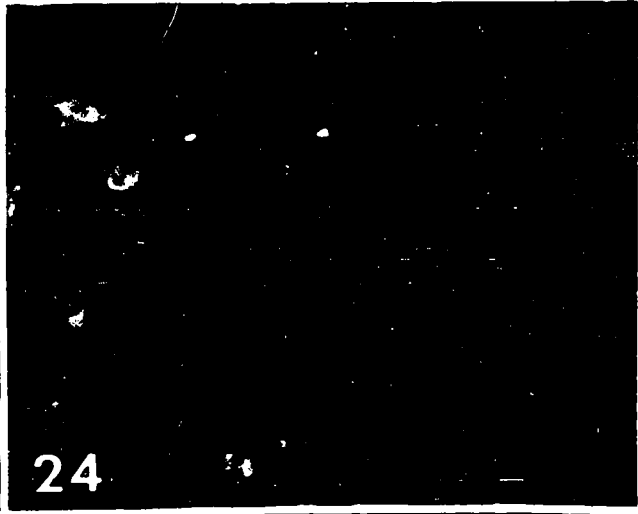
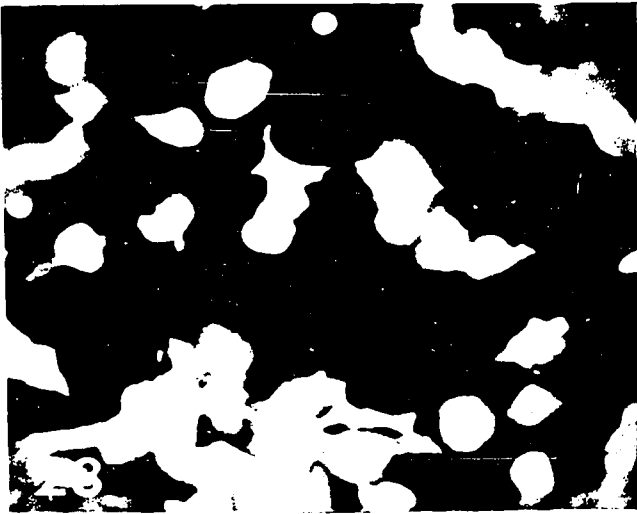


PLATE 6

- Figure 23: Vaccinia virus infected GMK cells (600 X)
stained with homologous antiserum by indirect
FA technique.
- Figure 24: Vaccinia virus infected GMK cells (600 X)
stained with poliovirus type 1 antiserum by
indirect FA technique.
- Figure 25: Vaccinia virus infected GMK cells (600 X)
stained with echovirus type 9 antiserum by
indirect FA technique.
- Figure 26: Vaccinia virus infected GMK cells (600 X)
stained with normal rabbit serum by indirect
FA technique.

PLATE 6



DISCUSSION

The major contribution of the present work is the demonstration of previously unsuspected broad cross-reactions within the picornavirus group (human enteroviruses) by indirect FA staining. This is an observation which is not only of considerable theoretical interest but may be of far reaching practical importance in the diagnostic field.

The validity of the strong cross-reactions observed was tested by staining uninfected and vaccinia virus infected GMK cells with enterovirus antisera. No evidence of non-specific staining or of cross-reactions was obtained with these systems. A human echovirus type 9 antiserum was used to stain cells infected with homologous and heterologous viruses. Fluorescence was observed in cells infected with all the test viruses. This indicates that the heterologous fluorescence was due to antigenic sharing.

The presence of such a broad cross-reaction which could enable group identification of picornaviruses was not demonstrated by any serological technique before the present studies were reported (Chaudhary and Westwood 1970). The cross-reactions obtained by indirect FA staining could either be due to; (i) specific shared antigen, (ii) broad reactivity of related antigens, (iii) infection-associated-group antigen or due to (iv) sensitivity of the test system used.

The evidence in support of these hypotheses is present in the literature. Specific antigenic sharing was revealed by the neutralization test in some of the enterovirus reference antisera but it was not broad enough to cover the whole group (Melnick and Hampil 1965). The stimulation of a heterologous antibody response in children who have been given monovalent attenuated poliovaccine indicates the presence of a broadly reacting antigen within polioviruses (Ashkenazi and Melnick 1962 and Vonka et al 1967). Antigenic sharing was also demonstrated within various picornaviruses by immunodiffusion (Schmidt and Lennette 1962, Schmidt et al 1965, Forsgren 1966 and Styk and Schmidt 1968), and by complement fixation test (Halonen et al 1959, Lennette et al 1961, Schmidt et al 1962, Schmidt et al 1963 and Schmidt et al 1967). The presence of a group reactive infection-associated antigen in cells infected with foot-and-mouth disease virus was reported by McVicar and Sutmoller (1970).

The sensitivity of the test used could also influence the results. Hajak (1969) showed that by using a 50% virus plaque neutralization test it was possible to detect about 50,000 times smaller amount of antibody than by CF test and about 2,000 times smaller than by passive haemagglutination test. Kern et al (1969) reported that cross-reactions between bovine adenovirus type 1 (non-oncogenic) and type 3 (oncogenic) were demonstrated by indirect FA

staining, but not by direct FA staining.

The diagnostic problem associated with picornaviruses is the relatively large size of the group and the availability of only one system of identification that is the neutralization test which is highly type specific. In consequence the present system requires a large battery of antisera to identify an isolate and this makes identification too time consuming and expensive for the routine laboratory. The group identification of picornaviruses by indirect FA staining would provide a simple and rapid means of establishing a clinical diagnosis, leaving the neutralization test for the reference laboratory and for the purpose of epidemiological study.

Such a system is of proven value for the adenovirus group where group identification is dependent on the group antigen detected by complement fixation while reference work demanding type identification may be carried out by using the neutralization test.

The present studies will make a similar situation possible for picornaviruses by using indirect FA staining. In this case a single or a small combination of antisera directed against a few selected members of the group could be used for group staining. In future this technique could also be tried for other viruses which do not have a group identification system.

The overall results show that the indirect FA stain-

ing could be successfully used for group identification of picornaviruses.

SUMMARY

A rapid and sensitive method has been developed to determine the degree of cross-reaction within the picornavirus group by indirect fluorescent antibody staining in green monkey kidney cells. The viruses included in the study were: poliovirus type 1, 2 and 3, echovirus type 3, 9, 11 and 25, and coxsackievirus type A9, B3, B4 and B5. It has been found that cross-reactions are indeed extensive amongst the viruses tested although heterologous reactions were somewhat weaker than homologous reactions. The significance of the cross-reaction observed was tested by staining GMK cells infected with vaccinia virus with poliovirus type 1 and echovirus type 9 antisera, uninfected monolayers with all the viral antisera under test, infected cells with normal rabbit serum and infected cells with conjugate alone.

The present results indicate that it could be possible to use a single antiserum directed against any one member to serve as a group specific antiserum covering the whole group of enteric viruses.

PART II

CROSS-REACTION STUDIES WITHIN THE PICORNAVIRUS GROUP BY ELECTRON MICROSCOPY

INTRODUCTION

The first virus to be seen in the electron microscope was tobacco mosaic virus. It is therefore a matter of interest that the first electron microscope study of virus-antibody interaction was also carried out using this virus (Anderson and Stanley 1941). Morphological differences were demonstrated between particles treated with specific antiserum and those treated with normal serum. The development of negative staining (Brenner and Horne 1959) has broadened the field of immune electron microscopy and it is possible now to recognize and distinguish the 7S and 19S antibodies (Green 1969). Lafferty and Oertel (1963) were able to show distinct changes in influenza type A virus after reaction with homologous antiserum. The reaction was serologically specific as influenza virus type A antiserum only reacted with type A virus but not with type B virus. It indicates that electron microscopy could also be used for antigenic studies.

A serological test based on the visualization of antigen and antibody should be more sensitive than one

based on indirect evidence. Ball and Brakke (1969) used immune electron microscopy for the titration of antisera against tobacco mosaic and southern bean mosaic viruses. They reported that the method was relatively more sensitive than other serological tests. It is therefore reasonable to expect that the electron microscopy technique could also be used as a sensitive tool for serotyping of virus.

Almeida and Waterson (1969) commented that immune electron microscopy could be both a simple and accurate method for checking the specificity of an antiserum.

The present work was aimed to study the serological relationships between members of the picornavirus group by immune electron microscopy.

MATERIALS AND METHODSVIRUSES

Poliovirus type 1 and 3 (Sabin strain), echovirus type 9, coxsackievirus type A9 and adenovirus type 7 were selected for this study. The type specificity of these viruses except adenovirus type 7 was tested by the Canadian Communicable Disease Centre, Ottawa or by typing antisera kindly supplied by them.

All viruses were grown in green monkey kidney (GMK) cells except adenovirus type 7 which was grown in human fetal lung culture.

Picornaviruses were purified and concentrated by differential centrifugation in an ultra-centrifuge ("Spinco" model L-2, Beckman).

The virus infected tissue culture fluid was clarified by low speed centrifugation at 3000 rpm for 10 minutes, then centrifuged at 42,000 rpm (106,000g) for 3 hours at 10°C in sterile polyallomer tubes using a type "50" rotor. At the end of centrifugation the supernatant fluid was discarded and the pellets were resuspended in sterile deionized water and made up to 9 ml, which was again centrifuged in the same rotor at 10,000 rpm (6000g) for 30 minutes; the supernatant was removed and centrifuged for 3 hours at 42,000 rpm. At the end of the centrifugation the supernatant fluid was discarded and the pellet was resuspended in 0.5 ml of

sterile deionized water when the starting fluid was 100 ml. The concentrated virus preparation was then given a final low speed centrifugation and stored in a labelled vial at -70°C .

In this way, poliovirus type 1 and 3, echovirus type 9 and coxsackievirus type A9 were purified and concentrated. Adenovirus type 7 was used as tissue culture fluid after low speed centrifugation.

The concentrated virus preparations were titrated in GMK cells using the "Kärber" method.

PREPARATION OF ANTISERA

Poliovirus type 3 and echovirus type 9 antisera were selected for study. The antisera were prepared in rabbits in this laboratory and the details of the preparation are given in part I materials and methods. The titers of the antisera are given in Table 8. Serum from an unimmunized rabbit was included as control. The antisera were used without any treatment or fractionation.

PREPARATION OF ANTIGEN-ANTIBODY COMPLEXES

Almeida and Waterson (1969) outlined the procedure for the preparation of complexes, which included centrifugation of the virus-antiserum mixture following incubation at 37°C and 4°C for 1 and 14 hours respectively.

In the present experiments the same procedures were followed except that the centrifugation was not carried out since this was found to cause clumping of control virus suspensions.

In this series of experiments the virus preparation (0.05 ml) was mixed with an equal volume of antiserum and incubated for 1 hour at 37°C and for 24-48 hours at 4°C. At this point the mixture was ready for the preparation of grids. In this way complexes were prepared for each virus and antiserum; for example, polio type 1 virus was mixed with poliovirus type 3 and echovirus type 9 antisera, and also with normal rabbit serum and PBS in separate vials and then incubated as mentioned above. In turn poliovirus type 3, echovirus type 9 and coxsackievirus type A9 were each treated separately in the same way as poliovirus type 1.

For homologous reactions antisera were diluted 1:2-1:10 with PBS, but for heterologous reactions they were generally used undiluted or in 1:2 dilution. For heterologous reactions viruses were diluted 1:2-1:10 with PBS depending on the original virus concentration and each virus preparation was used as a native (N) or a heat inactivated (H) antigen (10 minutes at 56°C).

The controls included in the experiment were:

- (i) Virus mixed with normal rabbit serum.
- (ii) Virus mixed with PBS.
- (iii) Adenovirus type 7 mixed with poliovirus type 3 and

echovirus type 9 antisera.

PREPARATION OF GRIDS AND NEGATIVE STAINING

The methods reported by Doane et al (1969) were followed with slight modification. One drop of the virus-antiserum mixture was added to a mass of deionized water consisting of 30-60 drops on a parafilm. The volume of water was increased in order to reduce the concentration of serum present in the preparation to an acceptable level. When a few drops of water were used the preparation showed blotches of stained serum on the grid.

A formvar-carbon-coated copper grid (400 mesh) was touched quickly to the surface of the drop and a drop of 2% sodium phosphotungstate (PTA) at pH.7 was then placed on the grid to act as negative stain, the excess fluid was removed with filter paper and the grid was allowed to dry in air. It was then placed in a small plastic petri dish on a filter paper properly numbered and left there till examined under electron microscope. In the case of adenovirus type 7 where the concentration of virus was low, a drop of mixture was placed on a formvar-carbon-coated grid and then washed 3 times with a drop of PTA, allowed to air dry and placed in a labelled plastic petri dish.

ELECTRON MICROSCOPY AND PHOTOGRAPHY

Grids were examined under a Philips EM-300 electron microscope at magnifications varying from 22,000 to 120,000 with a double condensor illumination at accelerating voltage of 60 or 80 kv. The objective aperture of 20 or 25u diameter was used throughout the experiments.

Some of the grids were also examined under Hitachi HU-110-1 electron microscope at the accelerating voltage of 75 kv. The objective aperture of 30 or 50 u was used. The magnification used was 30,000 or 60,000. For photography in Philips microscope, Kodak fine grain positive 35 mm films were used. The photographs in Hitachi were taken on Kodak electron image plates.

RESULTS

VIRUS TITRATION: The viruses were concentrated by ultracentrifugation. The titers of the individual virus preparations of poliovirus type 1 and 3, echovirus type 9 and coxsackievirus type A9 are given in Table 14. They were titrated by the "Kärber" method in GMK cells.

REACTION OF VIRUS WITH ANTIBODIES: The criterion used to detect the presence of an antigen-antibody reaction was the clumping of virus and attachment of antibody-like strands to the virus particles. In addition, the virus in these aggregates tended to have its outline obscured resulting in a "fuzzy" appearance. A clump of virus particles, shown in figure 35A was produced by centrifugation in the absence of specific antibody and is included for comparison. The outline of the virus particles in the centrifuged clumps are clear and defined whereas antibody treated particles are fuzzy.

The homologous reactions between polio type 3 virus (N) and its antiserum showed that several antibody molecules were attached to the virus particles, which formed a large clump. Many viruses were linked together with antibody molecules which formed bridges between adjacent particles. Full and empty particles of both types were present within the clumps. Figure 28 shows the appearance of polio type 3 virus reacted with specific antiserum.

The reaction between polio type 1 virus (N) and type 3 antiserum also showed clumping of the virus particles and attachment of antibody molecules on the surface of virus particles. Some virus particles showed halos around them. The only differences between homologous and heterologous reactions were that more antibody molecules were attached to the virus particles and the clumps were denser in the former case. The results showed that polio type 1 virus and type 3 antiserum do cross-react with each other, and the reaction could be easily detected by the electron microscope. The result is shown in figure 27. Coxsackievirus type A9 reacted with poliovirus type 3 antiserum and showed clumping, the antiserum reacting both with heated (H) and native (N) antigen. In the case of coxsackie- A9 viruses the clumps were smaller and only a few antibody molecules were attached to virus particles. Most of the clumped virus particles were empty and they had a blurred outline. The result is shown in figure 29.

Adenovirus type 7 was treated with poliovirus type 3 antiserum to check the specificity of the antiserum. The results showed that there was no clumping of adenotype 7 virus and the viruses were seen as single particles (figure 30).

The reactions were also controlled by treating the viruses with normal rabbit serum and PBS. There was no clumping of virus particles and they were evenly

distributed. The results are shown in figures 31 and 32 respectively. The virus particles were so evenly distributed that it was difficult to get more than 2-3 virus particles in each field even at a low magnification. This is the reason why these two controls are shown at a low magnification. No clumping of echo type 9 viruses with poliovirus type 3 antiserum could be detected.

When these viruses were treated with echovirus type 9 antiserum almost similar results as those with poliovirus type 3 antiserum were obtained.

The complex formed between echovirus type 9 and its homologous antiserum showed a blurred appearance and the outline of the virus particles was obscured because they were coated with antibodies. The reaction shows the presence of an excess of antibodies. Almeida et al (1963) reported similar findings. The result is shown in figure 35.

Echovirus type 9 antiserum also reacted with polio type 1, type 3 and coxsackie type 9 viruses. Antiserum reacted with polio type 1 (N) virus but it only reacted with heated (H) polio type 3 and coxsackie type A9 viruses. The clumps formed by coxsackievirus type A9 were small. The clumps formed by polio type 1 and type 3 viruses with echovirus type 9 antiserum were of medium size, the antibody molecules could be seen attached to the virus particles, however the attachment was not as clear as in the case of homologous reactions. The results of the reactions between

echovirus type 9 antiserum and polio type 1, type 3 and coxsackie type A9 viruses are shown in figures 33, 34, and 36 respectively.

Adenovirus type 7 treated with echovirus type 9 antiserum showed no clumping and were distributed as single particles and the picture was similar to those shown in figure 30. The other two controls were similar to those shown in figures 31 and 32.

A visual estimation of the size of the clumps with different viruses and the two antisera are given in Table 15.

TABLE 14

TITERS OF VIRUSES USED FOR THE ELECTRON MICROSCOPY

Viruses	Cells used for cultivation	Titers of the* purified virus per ml.
Poliovirus type 1	GMK	$10^{11.0}$
Poliovirus type 3	GMK	$10^{10.25}$
Echovirus type 9	GMK	$10^{10.0}$
Coxsackievirus type A9	GMK	$10^{10.0}$

* Computed by "Karber" method.

TABLE 15

THE RESULTS OF IMMUNO-ELECTRON MICROSCOPY

Viruses	Poliovirus type 3 Antiserum	Echovirus type 9 Antiserum	Normal Rabbit Serum	PBS
Polio- virus type 1	++(N)	++(N)	-	-
Polio- virus type 3	+++ (N)	++(H)	-	-
Echo- virus type 9	-(N&H)	+++ (N)	-	-
Coxsackie- virus type A9	++(N&H)	+(H)	-	-
Adeno- virus type 7	-	-	-	-

+++ Large clumps of viruses
 ++ Medium clumps of viruses
 + Small clumps of viruses
 - No clumps
 (N) Native virus
 (H) Heat inactivated virus

PLATE 7

Figure 27: Interaction between poliovirus type 1 native antigen and poliovirus type 3 antiserum (230,000x).

Figure 28: Interaction between poliovirus type 3 native antigen and homologous antiserum (230,000x).

Figure 29: Interaction between coxsackievirus type A9 heated antigen and poliovirus type 3 antiserum (230,000x).

Figure 30: Interaction between adenovirus type 7 and poliovirus type 3 antiserum (170,000x).

Figure 31: Interaction between poliovirus type 1 antigen and normal rabbit serum (100,000x).

Figure 32: Interaction between poliovirus type 1 antigen and PBS (160,000x).

PLATE 7

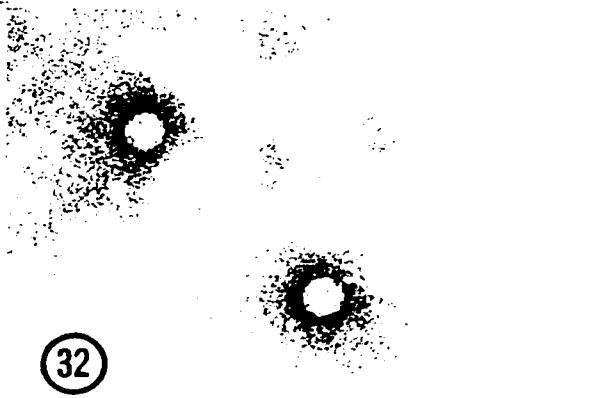
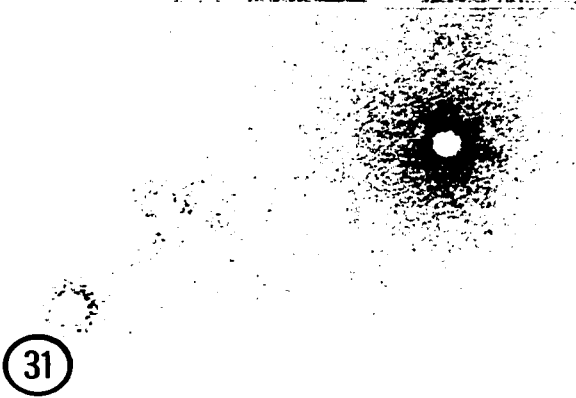
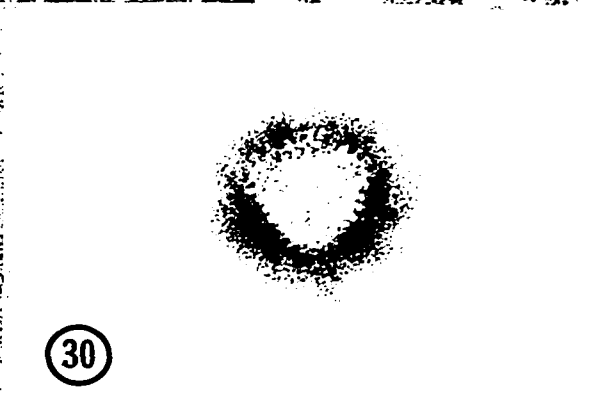
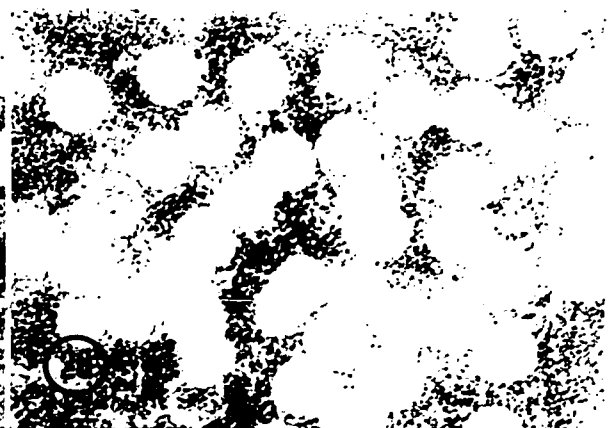
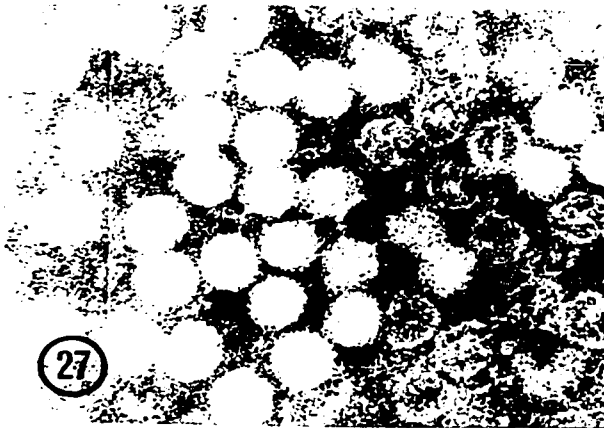
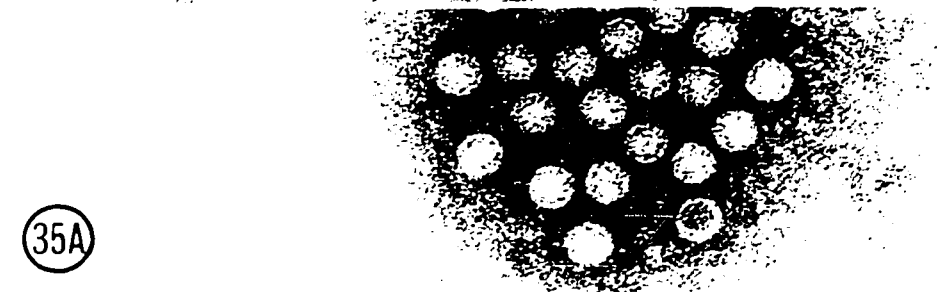
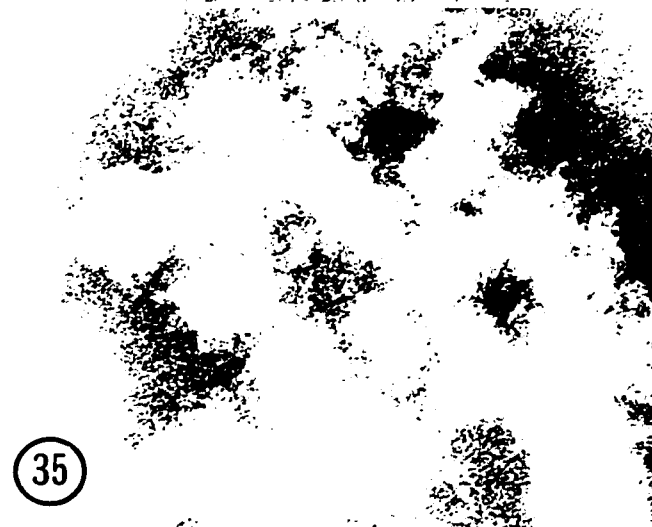
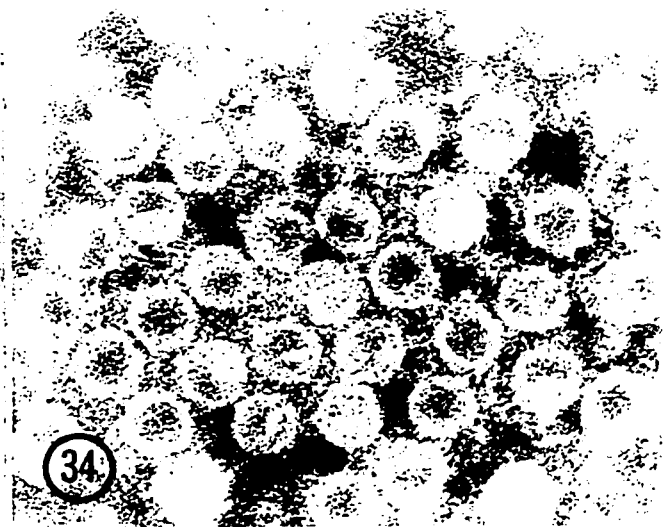


PLATE 8

- Figure 33: Interaction between poliovirus type 1 native antigen and echovirus type 9 antiserum (250,000x).
- Figure 34: Interaction between poliovirus type 3 heated antigen and echovirus type 9 antiserum (250,000x).
- Figure 35: Interaction between echovirus type 9 native antigen and homologous antiserum (250,000x).
- Figure 35A: Clump of echovirus type 9 produced by centrifugation (170,000x).
- Figure 36: Interaction between coxsackievirus type A9 heated antigen and echovirus type 9 antiserum (250,000x).
-

PLATE 8



DISCUSSION

Visual evidence of cross-reactions within the picornavirus group was obtained by immune electron microscopy. The selection of viruses, poliovirus type 1, type 3, echovirus type 9 and coxsackievirus type A9 was made at random. Only two antisera, poliovirus type 3 and echovirus type 9 were selected for the studies in order to avoid the individual variations of the antisera. The results obtained with each antiserum should show their antigenic sharing with the three viruses under test.

The electronmicrographs showed that the homologous reactions were quite obvious and large clumps of virus particles were formed. Antibody molecules were attached to virus particles and in some cases formed a bridge between them. In the case of the homologous reaction with echo type 9 virus, the particles were completely blurred and coated with antibody molecules.

Poliovirus type 3 antiserum showed cross-reactions with polio type 1 and coxsackie type A9 viruses. Echovirus type 9 antiserum showed cross-reactions with polio type 1, type 3 and coxsackie type A9 viruses. In all these reactions medium size clumps were seen except in the case of coxsackie type A9 virus and echovirus type 9 antiserum, where the clumps were small.

The homologous reactions showed a large number of antibodies attached to the virus particles and the clumps were larger than in the case of heterologous reactions. These results were similar to those reported by Lafferty and Oertel (1963) and Almeida and Waterson (1969).

No cross-reactions were observed between echo type 9 virus and poliovirus type 3 antiserum. The lack of reaction might be due to an excess of antigen. Almeida et al (1963) reported that at low concentration of antibody no aggregate could form, even in the case of homologous reactions. This might well explain the reason why no clumping was seen in this case.

Neutralization test results showed that echovirus type 9 antiserum had less than 1:4 titer against polio type 1 and type 3 viruses, but they still did show cross-reactions by electron microscopy. Echovirus type 9 antiserum reacted only with heated polio type 3 and coxsackie type A9 viruses. This could be well explained by the fact that the "group" specific antigen is associated with heated virus. Hummeler and Hamparian (1958) demonstrated "type" and "group" specific antigens in native and heated polioviruses respectively (CF).

The significance of the results was tested by examining adenotype 7 virus treated with poliovirus type 3 and echovirus type 9 antisera. The results showed that there was no clumping or attachment of antibody molecules to

adenotype 7 virus, indicating that the clumping of the picornaviruses was due to the presence of antibodies directed against them.

The nonspecific clumping of the virus preparations was tested by treating them with normal rabbit serum and PBS. The results of both these experiments showed that there was no nonspecific clumping of the viruses tested.

The study of antibody molecules was not undertaken. The results show that cross-reactions between the viruses and antisera tested could be visualized by immune electron microscopy.

In the field of clinical virology there is a possibility that the electron microscope technique of negative staining will become standard method of diagnosis (Doane et al 1969). It is not hard to imagine that an extension of this approach employing immune electron microscopy will allow particles not only to be visualized but also to be serotyped (Almeida and Waterson 1969). The present studies will supplement this imagination.

Visualization of cross-reactions by electron microscope was undertaken on a limited scale to verify the broad cross-reactions obtained by indirect FA staining.

SUMMARY

A sensitive method has been developed to determine the antigenic relationship within the picornavirus group by immune electron microscopy.

Negatively stained mixtures of antigens (poliovirus type 1 and 3, echovirus type 9 and coxsackievirus type A9) and antibodies (poliovirus type 3 and echovirus type 9 antisera) were examined under the electron microscope.

The results showed clumping of homologous and heterologous viruses with both antisera, except in the case of echovirus type 9 antigen and poliovirus type 3 antiserum. Clumping and attachment of antibody molecules to virus particles were considered as evidence of cross-reaction. The controls showed no clumping or attachment of antibody molecules.

The significance of clumping was tested by treating adenovirus type 7 with the two test antisera, which showed no clumping or attachment of antibody molecules to the adenovirus particles.

The results showed that cross-reaction does exist between the viruses and antisera tested.

GENERAL CONCLUSIONS

The present investigation was aimed to provide a foundation for the application of immunofluorescence for the group identification of picornaviruses. The results showed that a high degree of cross-reaction exists within the picornaviruses examined by indirect FA staining.

It seems likely that such cross-reactions could also be found among other members of the group. In the presence of such an extensive cross-reaction it should be possible to identify picornaviruses as a group by using either a single antiserum directed against any one member or by use of a pool of a small number of sera. This will greatly reduce the time and effort required for individual typing by neutralization tests.

Visual evidence of cross-reaction was also demonstrated by immune electron microscopy. The results obtained by electron microscopy corroborated the findings by immunofluorescence.

The electron microscope technique of negative staining will increasingly be used as a method of virus diagnosis and an extension of this approach employing immune electron microscopy will allow particles not only to be visualized but also to be serotyped.

APPENDIX

PLAQUE ASSAY OF POLIOVIRUS IN PLASTIC CHAMBERS

INTRODUCTION

A more precise assay of viral infectivity is afforded by plaquing in cell culture. This technique was first adopted for animal viruses by Dulbecco and Vogt in 1954 and ever since it has been widely used. Spendlove and Lennette (1962) reported the development of an immunofluorescent plaque method for the assay of vaccinia virus on cover slip culture with agar overlay.

The cover slip cultures are very fragile and difficult to handle. Sattar and Westwood (1967a) developed a plastic slide chamber by which cells are grown on a microscope slide. It overcomes all the handicaps of a cover slip culture.

These chambers were successfully used for fluorescent focus assay of Herpes simplex virus by Sattar and Westwood (1967b). It was found very suitable for cell cultivation, virus assay, and staining.

The present studies were undertaken to determine the suitability of these chambers for the development of plaques with poliovirus type 1 under agar overlay and to compare the results obtained by the chamber method to

those obtained with the conventional method of plaquing in bottles. This method can also be used for fluorescent plaque assay of poliovirus.

MATERIALS AND METHODS

The plastic slide chamber consists of four parts: a plastic square frame (17x17x10mm), a silicone rubber gasket, two stainless steel clips and a microscope slide. An assembled chamber is represented in figure 37. All the components of the chamber were cleaned by boiling in soap water for 20 minutes. They were then rinsed in tap water for 30 minutes and in three changes of deionized water.

The assembled chambers were sterilized by exposing them to ultraviolet light for 20-30 minutes. The virus contaminated chambers were also processed in the same way. Each chamber was seeded with $16-18 \times 10^4$ second passage green monkey kidney (GMK-2) cells in 1 ml of medium-199 containing 10% fetal calf-serum (FCS). Three chambers were placed in a square petri plate (100x15 mm Falcon plastics which is used for staphylococcal phage typing) and incubated in an atmosphere of 5% CO_2 at 36°C . One ounce bottles were also seeded at the same time with 64×10^4 GMK-2 cells in 4 ml of medium-199 containing 10% FCS, and were incubated with the chambers. A complete monolayer was formed within 18-24 hours after seeding in both chambers and bottles.

Poliovirus type 1 (LSC-ab) was used for the production of plaques. The dilution of the virus was made in phosphate buffered saline (PBS) pH = 7.3 to 10^{-5} which was further diluted to $10^{-5.301}$ and $10^{-5.602}$. Before virus inoculation, the monolayers in both the chambers and bottles were washed with PBS. Each virus dilution was inoculated into 6 chambers and 5 bottles. The inoculum volume was 0.03 ml for the chambers and 0.09 ml for the bottles. Different inoculum volumes (0.02-0.3 ml) were tried before selecting the one which is used in this experiment. The controls were inoculated with PBS. All the inoculated chambers and bottles were incubated at 36°C for 2 hours in a CO_2 incubator for adsorption. At the end of the incubation period the inoculum from the chambers and bottles was removed and the cell monolayer was washed with PBS to remove the unadsorbed virus. The inoculum collected from the chambers at the end of the incubation period was further inoculated into chambers containing fresh monolayers to determine the efficiency of adsorption. After the monolayer was washed, 1 ml of agar overlay medium was added to each chamber. The overlay medium consisted of 0.6% agar (Nobel Bacto) in medium-199 with 1% FCS and 25 mM MgCl_2 . The bottles were overlayed with 6 ml of the same medium (0.8% agar). The concentration of agar was increased in order to incubate the bottles in an inverted position.

When the agar overlay has solidified, the bottles and chambers were incubated in a CO₂ incubator. At the end of the incubation period (30-34 hours) they were taken out from the incubator and the agar overlay was removed from both chambers and bottles. The monolayer was washed with PBS and fixed in 10% formalin for 10 minutes. They were then stained with methylene blue.

The number of plaques was counted and tabulated. A Student t-test was used to compare the results obtained in chambers and in bottles at the point where they were inoculated with the lowest dilution of the virus. A graph was plotted to examine the relation between the virus dilutions and the number of plaques.

RESULTS

The agar overlay was removed from the chambers without disturbing the cell monolayer. The plaques developed as early as 30 hours after incubation in both systems; but they were more clear and defined after an incubation period of 32-34 hours. The results are presented in figures 38 and 39.

The inoculum volume of 0.03 ml in the chambers gave a linear relation when the average numbers of plaques were plotted against the virus dilutions (figure 40). The plaques were counted in both chambers and bottles and the average number of plaques obtained in chambers was

multiplied by three in order to correct for volume difference. The results are given in Table 16. The computed t value is -0.91 with a critical region where $t > 2.132$ or where $t < -2.132$ with 4 degrees of freedom. The t value is outside the critical region which indicates that there is no significant difference between the results obtained by the two systems at that point. The calculated efficiency of adsorption was between 80-85%.

DISCUSSION

The results of poliovirus titration obtained by slide chambers are comparable to those obtained from the bottles. Clear and countable plaques developed within 30-34 hours which is a significant improvement. Schmidt (1964) reported that polio virus generally takes 48-96 hours to develop plaques by conventional method of plaquing. The reduction in time could be attributed to the use of $MgCl_2$ in the overlay medium. Melnick et al (1964) reported that incorporation of $MgCl_2$ enhances the production of plaques by enteroviruses. Wallis and Melnick (1968) reported about the mechanism of enhancement of virus plaques by cationic polymers. The advantages of the plastic chamber technique include: simplicity, economy of materials, flexibility and the convenience of keeping a permanent record of the results. Other applications of this technique could be plaque inhibition, plaque size reduction, immunofluorescent plaque assay and cytological studies.

SUMMARY

A method for the plaque assay of poliovirus type 1 using plastic chambers on glass microscope slides with an agar overlay has been developed. This microtechnique offers a reliable system for plaque assays which is directly comparable with other systems. The maximum efficiency of adsorption was 80-85% and clear and countable plaques appeared within 30-34 hours. The advantages of this system include: simplicity, economy of materials, and flexibility. Other applications of the technique include plaque inhibition, plaque size reduction, immunofluorescent plaque-assays, and cytological studies.

TABLE 16COMPARATIVE ASSAY OF POLIOVIRUS TYPE-1 BY
PLASTIC CHAMBER AND BOTTLE PLAQUE METHOD

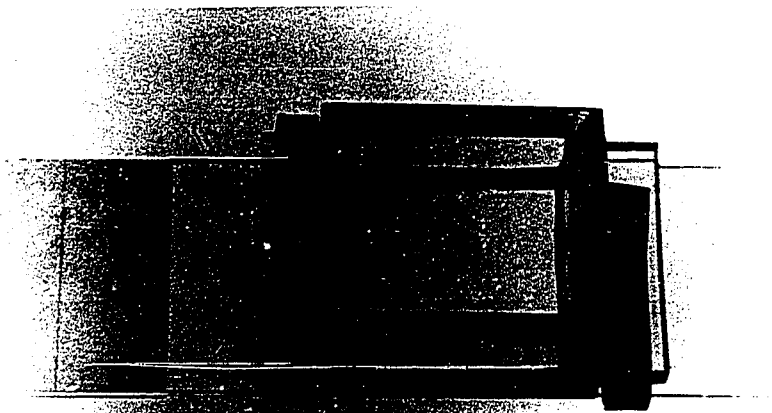
Method	Dilution	Number of Plaques							Average Count/0.09 ml.
Chamber ^a	10 ⁻⁵	30	30	24	42	36	30		31.98
	10 ^{-5.301}	30	18	9	27	12	21		19.5
	10 ^{-5.602}	15	12	9	12	12	15		12.3
Bottle ^b	10 ⁻⁵	36	48	33	31	37	-		37.0
	10 ^{-5.301}	23	21	26	28	26	-		24.8
	10 ^{-5.602}	16	12	15	12	16	-		14.2

^a Inoculum vol. = 0.03 ml. (No. plaques x 3)^b Inoculum vol. = 0.09 ml.

PLATE 9

Figure 37: An assembled plastic slide chamber.

PLATE 9



37

PLATE 10

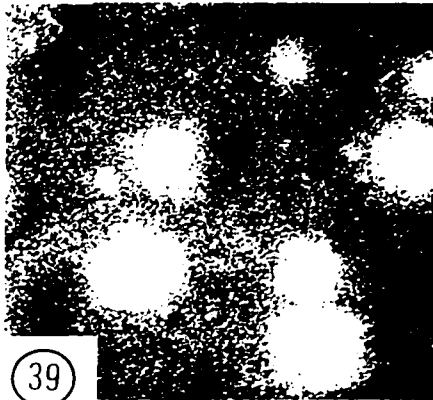
Figure 38: Poliovirus type-1 plaques (left) and control (right) in GMK-2 cells in 1 oz. bottles under agar overlay after an incubation period of 34 hours (Magnification 1.5 times)

Figure 39: Poliovirus type-1 plaques (left) and control (right) in GMK-2 cells in slide chambers under agar overlay after an incubation period of 34 hours (Magnification 3.3 times).

PLATE 10



38



39

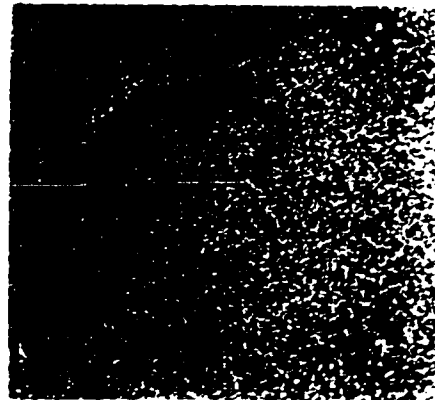
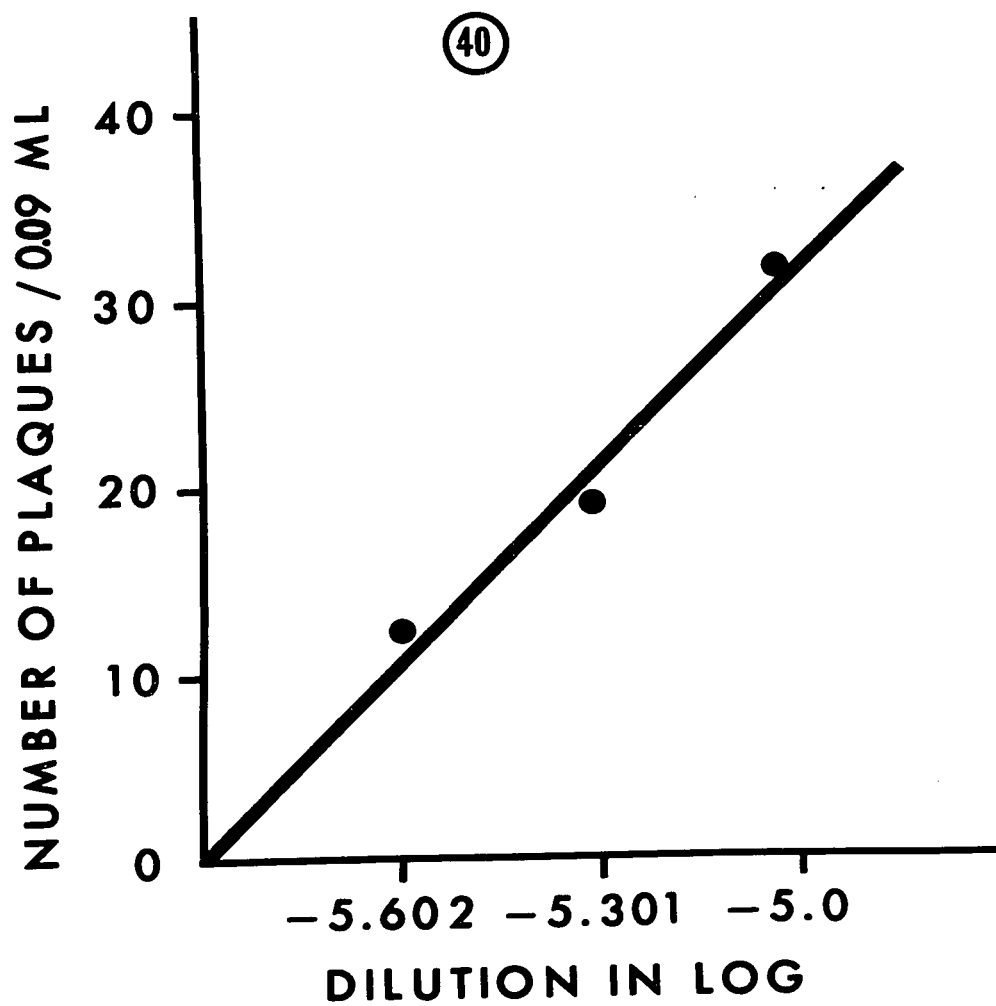


PLATE 11

Figure 40: Relation between poliovirus type-1 dilutions and the number of plaques obtained from the chambers in GMK cells.

PLATE 11



CARBONATE-BICARBONATE BUFFER0.5M; pH 9.0NaHCO₃ 3.7 gm.Na₂CO₃ 0.6 gm.

Distilled water ... 100 ml.

Used immediately after preparation to avoid
absorption of CO₂ from the atmosphere.

VERSENE-TRYPSIN SOLUTION

Trypsin (1:250) 0.2 gm.

Versene (EDTA) 0.05 gm.

Calcium and Magnesium free PBS pH 7.2 100 ml.

pH was adjusted to 7.2 and sterilized by
filtration through millipore filter.

PHOSPHATE BUFFERED SALINE WITHOUT
CALCIUM AND MAGNESIUM

NaCl 8.0 gm.

KCl 0.2 gm.

Na₂HPO₄ 1.15 gm.KH₂PO₄ 0.2 gm.

Deionized water 1000 ml.

pH was adjusted to 7.2 and sterilized by
autoclaving.

PHOSPHATE BUFFERED SALINE FOR SEPHADEXCOLUMN 0.01M., pH 7.4

- A. Na_2HPO_4 1.419 gm.
 Distilled water 1000 ml.
- B. NaH_2PO_4 1.379 gm.
 Distilled water 1000 ml.

Solution B was added gradually to Solution A until the pH reached 7.4. Then 9.0 gm. of NaCl was added in 1000 ml. and pH checked again.

VERONAL BUFFER0.2M pH 8.2

- A. Barbital 2.763 gm.
 Distilled water 1000 ml. (0.015M)
- B. Sodium barbital 103.0 gm.
 Distilled water 1000 ml. (0.5M)
- C. NaCl 29.23 gm.
 Distilled water 1000 ml. (0.5M)

A, B and C solutions were
mixed as follows:

A 218 ml.
B 25 ml.
C 375 ml.
Distilled water 182 ml.

Checked the pH and adjusted to 8.2 and
the solution was diluted 1:4 to give
0.05 μ before use.

AGAR GEL FOR IMMUNOELECTROPHORESIS

Agar (oxoid Ionagar) 1 gm.
Vernol buffer 0.025 μ 100 ml.

Dissolved and sterilized by autoclaving
and 2 ml. was used for each slide.

PACKING OF SEPHADEX COLUMN

The Sephadex G-200 was allowed to soak in water for
3 days and G-25 only for 24 hours. The fine particles from
the Sephadex were scanted out and the air bubbles were re-
moved by negative pressure. The column was fixed on a
stand and filled with distilled water and a glass funnel
was fixed on the top of the column with rubber cork.

Sephadex was poured into the funnel and allowed to settle down in the column with closed outlet. When a layer of Sephadex about 3" high was formed, the outlet was opened and the water was allowed to run out drop by drop. When the column was filled, the Sephadex was washed 4 times with PBS. Now the column was ready for use.

SODIUM-PHOSPHOTUNGSTIC ACID

Phosphotungstic acid 2 gm.

Deionized water 100 ml.

pH was adjusted to 7.0 with 2%
sodium hydroxide solution.

SATURATED AMMONIUM SULFATE SOLUTION

$(\text{NH}_4)_2\text{SO}_4$ 53.1 gm.

Distilled water 71.7 ml.

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