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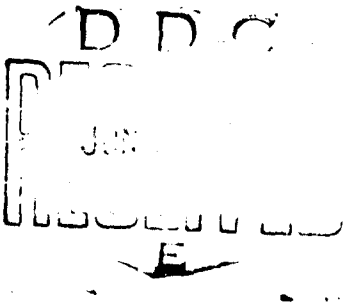

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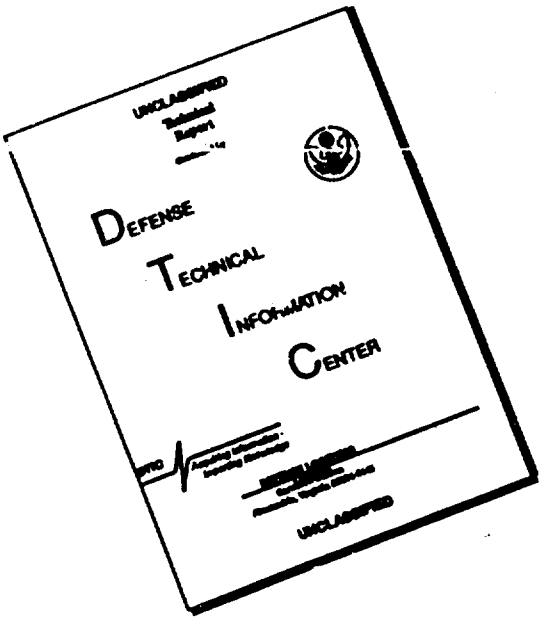

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RECENT ADVANCES IN SERODIAGNOSIS OF VIRUS DISEASE

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RINSHO BYORI
(Journal of Clinical Pathology)
Vol. 13, pp 21-26, 1964

Introduction

Recent advances of virus studies showed remarkable results such as the discovery of new phenomenon, development of new technique and concept, and discovery and classification of new type of virus. Of course, the virus studies, searching for the main body of micro creature with all the characteristics of life, did not advance alone. Thus improvement of electron microscope enabled one to study the shape and mechanism of virus multiplication, and the technology of tissue culture opened the door for developing the method of separating and refining process. And the improvement of refining process would show several shapes of biological species. The method of serodiagnosis reported in this paper contributes to the knowledge of virus multiplication method such as tissue culture process, virus refining process and the development of immunology.

There are two types of serodiagnosis of virus diseases.

(A) Detection of antigen

Employing the disease material to be examined as an antigen, serum reaction is conducted on already known highly immune serum. If the result is positive, the disease material is diagnosed to be virus antigen.

(B) Detection of anti-body

The anti-body of human serum is detected serologically using the known virus antigen. This method is the most common type. In the

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following, it is reported according to the following classification.

I. Detection of antigen

The advantage of this method is to detect the source of disease, and compares fundamentally with the virus separation method. Ordinarily the source of disease exists in a large amount during the initial period. Thus, the detection of these would contribute to the purpose of an early diagnosis.

1. Fluorescent antibody technique; immunofluorescent staining

The fluorescent antibody technique completed by Coons[1] is the method of staining the antibody laveled with fluorescein isothiocyanate instead of using ordinary dye solution. Therefore, the characteristics of antigen-antibody reaction are utilized in staining and antigen itself is stained by this method.

The early diagnosis of clinical material and successful practical use are as shown in table 1. Selection in the type of staining are classified as follows. (figure 1)

The detailed technique is described in the references.[2,3,4] Here, we outline briefly the staining process.

2. Experiment on supplementary body

Diagnosis[5,6] of pox conducting the supplementary body combination reaction of blood during the acute period (until 5th day of disease), blister pus and outer part of blister as the antigens with rabbit anti vaccinia serum:

When men are bitten by a dog with a possible rabies and the supplementary body combination reaction is conducted on standard molmot(transliteration) serum of the dog and animal brain or salivary gland as an antigen, a highly accurate diagnosis can be done a few hours after the administration of detecting material.

II. Measurement of antibody

This method is generally called as a serum reaction and classified into (1) neutralization test, (2) red blood-corpuscle clot control test, (3) supplementary body combination reaction, (4) descending reaction and (5) clotting reaction. In addition, there are a cold red blood-corpuscle clot reaction of different type pneumonia and a serum reaction by non-characteristic antigen such as Paul-Bannel reaction of contageous single nucleus cell disease. In this report, the recent advances of item (1) to (5) above are described.

Table 1

Main Virus Antigen and Practical Use of Experimental Diagnosis According to Fluorescent Antibody Technique

(1) ウイルス	(12) 核酸	(13) 抗原 (14) 核 (15) 細胞質	(16) 実用化	(17) ウイルス	(28) 核酸	(29) 抗原 (30) 核 (31) 細胞質	(32) 実用化
(2) ワタナビア	DNA	− +	●	(18) レ か	RNA	− +	
(3) エクトロメリア	◆	− +		(19) インフルエンザ	◆	+ +	●
(4) アデノ	◆	+ (+)	●	(19) MS	◆	− +	●
(5) イヌ肝炎	◆?	+ −		(20) ソトリペスト	◆	+ +	
(6) ヘルペス	◆	+? +	●	(21) ウス肉腫	◆	− +	
(7) パピローマ	◆	+ −		(22) 犬 病	◆	− +	●
(8) ポリオーマ	◆	+ (+)		(23) 日本脳炎	◆	− +	●
SV40	◆	+ (+)		(24) 熱 病	◆	− +	
(9) オウム病	DNA+RNA	− +		(25) リ ヤ	◆	− +	
(10) ニューカッスル病	RNA	− +		(26) クサッキー	◆	− +	
(11) ムンプス	◆	− +	●	(27) コ −	◆	− +	

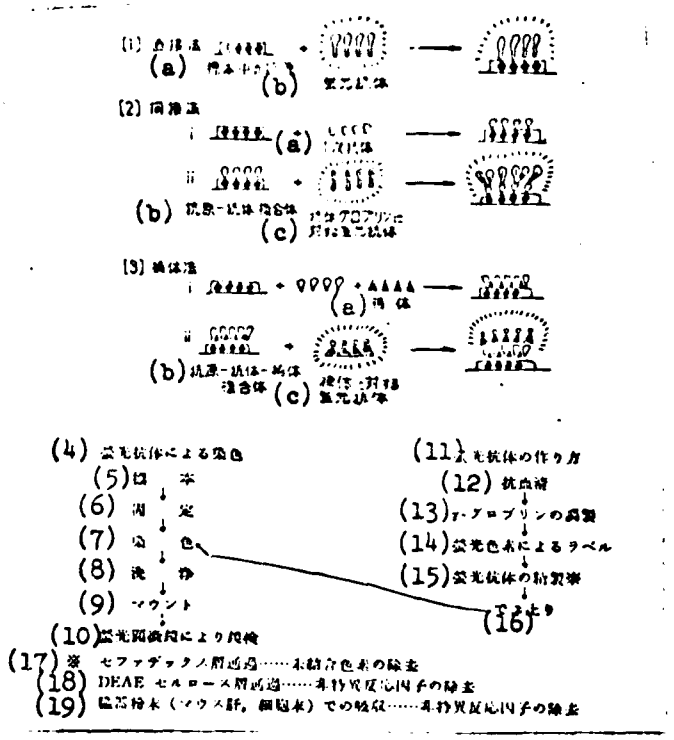
(33)) は従属的な出現を示す。? は統一的な見解の得られていないもの。

(34) ● は一部において実用化に成功しているもの。

1. Virus
2. Wakuchinia (transliteration)
3. Ekutoromeria (")
4. Adeno (")
5. Hepatitis of dog
6. Herubes (transliteration)
7. Papiroma (")
8. Porioma (")
9. Parrot disease
10. New castle disease
11. Mumpus (transliteration)
12. Nucleicacid
13. Virus antigen
14. Nucleus
15. Cell
16. Practical use
17. Virus
18. Measles
19. Influenza
20. Chicken pest
21. Mouse sarcoma
22. Rabies
23. Japanese meningitis
24. Yellow fever
25. Polio
26. Coxsackie
27. Echo
28. Nucleic acid
29. Virus antigen
30. Nucleus
31. Cell
32. Practical use
33. ( ) indicates subordinate appearance. ? indicates that opinions are not uniform.
34. ● indicates the partial success of practical use.

Figure 1

Model of Antibody Staining by Fluorescent Technique



1. Direct method
   a. Antigen in the samples
   b. Fluorescent antibody
2. Indirect method
   a. First antibody
   b. Antigen-antibody
   c. Fluorescent antibody for globulin antibody
3. Supplementary body method
   a. Supplementary body
   b. Antigen-antibody-supplementary body
   c. Fluorescent antibody for supplementary body
4. Staining by fluorescent antibody technique
5. Sample
6. Fixed
7. Staining
8. Washing
9. Mount

(Key for Figure 1 continued)

10. Detection by fluorescent microscope
11. Formation of fluorescent antibody
12. Antiserum
13. Manufacture of γ -glovulin
14. Lavel by fluorescent color element
15. Refinement of fluorescent antibody
16. Completion
17. Passage through sephadex layer -- Removal of uncombined color element
18. Passage through DEAE cellulose layer -- Removal of non-characteristic reaction factor
19. Absorption by powder of intestinal apparatus (mouse liver, cell) -- Removal of noncharacteristic reaction factor.

1. Neutralization test

A mouse egg incubation has been used for this experiment, but by the introduction of cell culture method, the technique of this method has rapidly advanced. Measuring 50% infectious value (TCD_{50}) against cell susceptible to virus, a constant amount of virus liquid, i.e. 100 TCD_{50}/ml, and an equal amount of serum diluted with culture liquid in stepwise (1:4, 1:8, 1:16 ...) are mixed. After this mixture is sufficiently reacted by leaving it for an hour at 37°C or overnight at 4°C, 0.2 ml of virus and serum reaction mixture is inoculated into the tissue culture tube (0.8 ml). For each dilution of serum, at least more than four tubes are continuously observed for about a week (depending on virus, the reaction and judgement times are different) and the change in the appearance of tissue is examined. Taking the serum concentration of 50% infection control as a terminal point, antibody value is expressed by dilution number. As a comparison of virus, the virus solution diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} is added to equal amount of tissue culture and 0.2 ml is inoculated. It was confirmed that the amount of virus used in neutralization test was 100 TCD_{50}/ml. And for improvement of accuracy, a constant amount of virus is introduced by the plaque formation technique. The simplified plaque formation of virus is (Japanese) gelatine cover slip method[7]. Making the heavy layer of gelatin solution used for plaque count, the solution is gelatinized into 1 mm height on plane box. A circular plate of diameter 14 mm is prepared with sterilized cruni hat. This is called the gelatin cover slip. After the reaction, the content of test tube is taken out by platinum ear which was burnt and immersed in salt solution, and placed on the gelatin cover slip. The single layer tissue culture is covered on the surface plane. Usually for tissue cultured on syare(transliteration) (90 mm diameter), eight sheets are placed. Immediately afterwards, 7 ml of gelatin solution for heavy layer is flowed and solidified. The rest is same as plaque formation method. At serum concentration of neutralization

point, either a sparse plaque or no plaque is formed. In this method, the neutralization test of a serum sample can be done with a sheet of syare(transliteration).

In order to eliminate the troublesome microscopic observation of cytopathic effect on each test tube in neutralization test, the methods called metabolic inhibition test (MIT) and color change test are developed by Melnick J.L. (Bull. World Health Org. 14, 129 (1956)). When cells conduct an active metabolism, acid is accumulated in the culture fluid and lowers pH of the solution. Phenol-phthalein indicator in culture solution shows yellow color. If metabolism is inhibited by virus infection, the culture fluid remains as an alkali solution and the color does not change. Utilizing this change, the neutralization test is conducted from the fixed quantity of virus. Plastic pannel is generally used for this test but the test tubes can be also used. The reaction system consists of 0.1 ml serum, 0.2 ml virus (100 TCC_{50}), 0.2 ml cell fluid (2×10^4 cells) and 0.6 ml of drakeol no. 35. It seems to be a rather simple method but not widely adopted method at the present time.

2. Red blood-corpuscle clot control test

The phenomenon of influenza virus discovered by Hirst in 1941 is widely spread. Research on red blood-corpuscle clot mechanism has advanced and a difference in opinions also exists. Virus for red blood-corpuscle clot reaction are the following types.

i) Mixer virus group influenza A. A_2. B. C., Para influenza, Mumpus.

ii) Pox virus, parrot disease, mouse rimpa(transliteration) granulation virus group.

iii) Albovirus group. Japanese meningitis, Yellow fever.

iv) Adeno, Echo(transliteration), measle virus.

v) Other riftopare(transliteration) fever, mouse meningitis, mouse pneumonia virus.

Among these, i) and iii) are most frequently used in actual clinical diagnosis. Antibody measurement by this method is simple but inhibitor for virus clot exists in serum and is removed by treating with RDE (receptor destroying enzyme), trypsin, KIO_4, kaolin and acetone. Research on the removal of this material was successful to a certain degree and its virus can be said to be i) and iii). The status on influenza inhibitor is as shown in table 2. Antigens of influenza, Japanese meningitis, mumpus used in red blood-corpuscle clot control test and RDE can be purchased now.

Table 2

Noncharacteristic Inhibitor of Influenza Virus HA

α inhibitor (Francis)	β inhibitor (Chu)	γ inhibitor (Belyavin)
(1) 耐熱性	(6) 耐熱性	耐熱性 (11)
(2) 活性ウイルスよりも加熱ウイルスのHAを測定し易い。	(7) 活性、加熱不活化両ウイルスに作用する。	
(3) RDE で破壊される。 trypsin で破壊される。 $NaIO_4$, KIO_4 で破壊される。	(8) RDE, KIO_4, $NaIO_4$ で破壊されない。 trypsin で破壊される。	RDE で破壊されない。 KIO_4, $NaIO_4$ で破壊される。 (12)
(4) 活性ウイルスで破壊される。	(9) 活性ウイルスと結合するが破壊されない。	A_2 ウイルスの感染性を中和。 (13)
(5) 主成分は N-acetyl neuramine acid で、この物質は赤血球 receptor 成分でもある。従ってウイルス材料に対し赤血球と競合する。	(10) α-globulin 中に含まれ、糖体ではない。	γ-globulin 中に含まれる。 (14)

1. Thermal stability
2. Heated virus HA is easier to control than active virus
3. Destroyed by RDE. Destroyed by trypsin. Destroyed by $NaIO_4$, and KIO_4
4. Destroyed by active virus
5. Main component is N-acetyl neuramic acid and also a red blood-corpuscle receptor. Therefore this material competes with red blood-corpuscle for virus ferment.
6. Thermal stability
7. Works on both active and heated inactive virus
8. Not destroyed by RDE, KIO_4 and $NaIO_4$. Destroyed by trypsin
9. Combine with active virus but is not destroyed
10. Included in L-globulin but is not a glucoside
11. Thermal stability
12. Not destroyed by RDE. Destroyed by KIO_4 and $NaIO_4$
13. Neutralize the infectious ability of A_2 virus
14. Included in γ-globulin.

There is a method of adsorbing antigen (virus) to red blood-corpuscle after treating virus (Herpes, polio) which is not reactive in red blood-corpuscle clot with tannic acid. The method of measuring anti-body value in clot reaction by tannic acid receptive red blood-corpuscle is developed by Scott[5]. The red blood-corpuscle clot reaction of echo coxsackie and adenovirus is useful in determining the present status and type of virus as shown in table 3 and 4.

3. Supplementary body combination reaction

The measurement of antibody for supplementary body combination is very important from the standpoint of clinical diagnosis among serological

dia- ...thods of virus diseases. Also, if the standard anti serum ...n be administered, the supplementary body combination re... ...s disease would be one of many techniques whose serum reactio... ...yphilis (Wasserman reaction) can be carried out at the central laboratory of hospital.

Table 3

Reaction Temperature of Entro Virus HA

4°C	37°C	4°C と 37°C (1)
ECHO 3	Coxsackie B1	Coxsackie B3
11	〃 B5	ECHO 7※
13	ECHO 29	〃 12※
19	〃 6	Reo 1, 2, ヒト O 型 (2)
21		3 ウシ (3)
Coxsackie A-21 (Coe)		

(4) ※ ECHO-7, 12 はサル血球も凝集する。

1. 4°C and 37°C
2. Human O type
3. Cow
4. ECHO 7 and 12 monkey blood-corpuscle

Table 4

Classification by Red Blood-Corpuscle Clot of Adeno Virus
(by Ginsberg, H.S. - 1962)

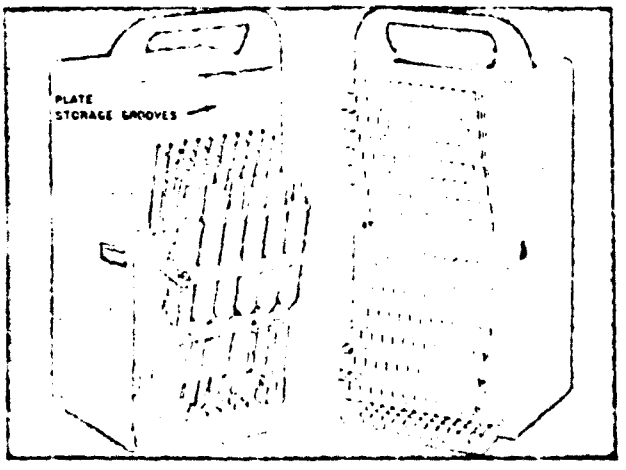
群 (1)	型 (2)	使用血球 (3) サル (4)	ラット (5)
1	3, 7, 11, 14, 16, 20, 21, 25, 28	+	0
2	8–10, 13, 15, 17, 19, 22–24, 26, 27 (6)	+※ ※は12 0	+
3	1, 2, 4, 5, 6	0	不完全 (7)

(8) + : HA…+ 0 : HA…—
※ : 9, 13, 15 も例外 +
HA を示さない型 : 12, 18

1. Group
2. Type
3. Blood-corpuscle
4. Monkey
5. Rat
6. + or -
7. Incomplete
8. Type 9, 13, 15 + type which doesn't indicate HA: 12, 18.

At present, with the cooperation of National Preventive Health Research Institute and Bacteria Manufacturing Association, the antigen and antiserum such as Japanese meningitis influenza, polio, mumps, adeno and herubes (transliteration) reached the stage of commercial marketing. The recent tendency is to move micro-method of high accuracy since the diagnosis has to be made on infant patient of virus disease with insufficient amount of serum and many tests on antigen are required. Since 1945, the U.S. Army Medical School method of total amount 1.5 ml is replaced by Kolmer micro amount method of 0.6 ml total amount (serum 0.1 ml, antigen 0.1 ml, supplementary body 0.2 ml, blood system 0.2 ml). Cassals (1958) used a depression tray (depth about 1 cm, 96 hollows with 2 cm inner diameter and thin half transparent plastic plate 8 x 12 (24.5 x 33.5 cm)), and a drop of serum (0.02 ml), a drop of antigen (0.02 ml) and two drops of supplementary body (0.04 ml) are left at 4°C overnight. Two drops of blood system (0.04 ml) are added to these, thus making up total 12 drops or 0.12 ml. Thus, this method was successful with 1/5 amount of Kolmer method. This technique became popular.

The details of the technique is referred to new edition of books[2] in this field. Here, we describe briefly the microtiter method reported by Sever[9]. This method uses a hard thick plastic plate and stainless steel loop in place of depression tray and the dilution of 0.025 ml can be done directly in the hollow part. Loop dilution eliminates the operation of master dilution by pipet in a separate test tube and transfer to the tray. The inspection of loop is conducted by the spread of water drop (diameter) on the filter paper. The syringe needle, 16 guage, 1.5 cm length is attached to the end of 10 ml pipet after cutting the needle tip horizontally. When the content is dropped vertically, one drop is 0.025 ml. (see photograph)



4. Immune Adherence Hemagglutination Test (IA)

This is the new method discovered by Nelson[10], and the phenomenon that combination of antigen, antibody and, supplementary body adheres strongly to human red blood-corpuscle is designated as immune adherence. Since human blood-corpuscle has the clot reaction, this method is sensitive blood-corpuscle reaction which can be conducted simply by various antigen-antibody reaction. Nishioka[11] has reported on fazi(transliteration), and Ito[12] has investigated animal virus. Thus, the first step in practical use has begun. However, many problems remain to be investigated and new sensitive immunological reaction can be utilized in animal virus area.

Diluted solution for reaction includes 0.00015 M $CaCl_2$, 0.000 M $MgCl_2$, 0.1% gelatin, 4% sucrose, 0.005 M veronal buffer and 0.09 M NaCl as the final concentrations. Blood-corpuscle for reaction is 2×10^8/ml (about 2%) in human O type. Determination of concentration is done by the measurement of optical density at 541 mμ using Beckman DU spectrophtometer on ten times diluted solution. The solution is made by comparing with the standard. Reaction system consists of 0.2 ml antigen, 0.2 ml antiserum, 0.5 ml mixture of supplementary body and human blood-corpuscle (using supplementary body diluted about 200 times, blood-corpuscle 2×10^8/ml of 0.1 ml solution is included). The reaction mixture is placed in 37°C water bath for 15 minutes with stirring and then left at 37°C. The reaction mixture is returned to room temperature and the results are observed. About 50% of clot point is judged to be reaction positive.

Conclusion

Finally, serological diagnostic methods are summarized. If antibody (patient's serum) is not taken under the best condition and the related items are not recorded accurately the test becomes a vain effort no matter how advanced technique is employed for antigen. For the same patient, serology should be conducted during the acute period (3 days after illness) and twice during the recovery period (2-3 weeks of illness) and minimum 3 ml of serum is desirable.

Except 2-3 virus diseases, therapy on causes of disease is not done at the present time. Therefore, serological diagnosis by antibody value rise is a mere review. But it is necessary to understand thoroughly the meaning of laboratory diagnosis and educate clinical physicians and public. Diagnostic fluid for virus diseases is begun to be marketed, but they are a very small part of viruses. According to an increasing demand of society, the test system should be improved in the future. Also the improvement on technique and method requires an effort and the planned publication of revised health textbook is natural from the standpoint of virology. The books on the experimental technique in virology were very few but since 1964, many books appeared on this subject. These seem

to be indicative of increasing number of persons studying virology directly or indirectly. Finally, meeting for virus laboratory diagnostic method including the serological diagnostic method is held every year. (Clinical virus conversazione) In 1965, this meeting is planned at Sendaishi under the auspices of Professor Nakooh Ishida (7 and 8th of August).

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