VI CONGRESSO INTERNAZIONALE DI MICROBIOLOGIA

ROMA 6-12 SETTEMBRE 1953

Segretario Gen.: E. BIOCCA *Presidente: V. PUNTONI*

ATTI

DEL VI CONGRESSO INTERNAZIONALE DI MICROBIOLOGIA

VOLUME III

SEZIONI VIII-X

N. 1-162

SEZIONE VIII - Virus degli animali

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ATTI

DEL

VI CONGRESSO INTERNAZIONALE DI MICROBIOLOGIA

SEZIONE VIII

VIRUS DEGLI ANIMALI
ULTRAVIRUS DES ANIMAUX
ANIMAL VIRUSES

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MORPHOLOGY, PATHOGENESIS AND IMMUNOLOGY

Presiding: A. J. RHODES

1.

AN ELECTRON MICROSCOPY OF VIRUSES IN HOST CELLS (*)

REN KIMURA

(Microbiological Institute, Faculty of Medicine, Kyoto University, Kyoto, Japan)

With a view to studying interaction between viruses and host cells, I tried the technique of tissue culture. The tissue cells infected with viruses were grown in a special kind of culture flask devised by myself. Then the cells were examined with the electron microscope. Several kinds of tissue culture techniques were taken as test for the purpose, and the following two techniques were found most satisfactory:

1. A cover-glass was covered with a membrane made of 3% collodion solution, and tissue fragments were embedded in 2 or 3 small plasma clots on the membrane. The glass was turned over in a special sort of culture flask containing a nutrient medium, and the cultivation was made at 37° C. After two or three days' culture, the tissue fragments were fixed with 3% Ringer-formalin solution for ten to fifteen minutes so that they were attached to the cover-glass. The collodion membrane with the newly grown cells attached was then carefully stripped from the glass surface by dipping it in distilled water. They were, then, put on the specimen holder of the electron microscope, selecting the suitable part of the cultured tissue cells.

2. A special plate of synthesized resin was prepared to support the specimen holder. A collodion membrane could be made simultaneously on the surface of the plate as well as on the top surface of the specimen holder as they came into contact with each other, holding the same level. The tissue fragments were placed on the

(*) This work was done in collaboration with Drs. Noboru Higashi, Yoshikatsu Ozaki and Tsutomu Shimizu.

collodion membrane near the pin hole of the specimen holder, expecting the result that the margins of grown cells might cover the pin hole, and they were thus cultivated in a flask.

In both techniques, as nutrients were used amnion water and the embryonic juice of embryonated chicken egg at the ratio of 2:1, or amnion water and the embryonic juice of embryonated chicken egg mixed with rabbit serum at the ratio of 2:1:1.

In electron micrographs of normal cells, there were found entangled fibers and vacuoles in the central part of the cells, revealing extremely complicated images. On the contrary, the peripheral part of a cell was thin, and it had a simple structure appearing sometimes as if it were almost non-structural. The peripheral part seemed, therefore, to be the most appropriate region for examining such minute intracellular bodies as viruses.

In my first experiment the vaccinia virus of the 6th passage through rabbit testicles was used. The virus was inoculated into the chorio-allantoic cavity of 9-day embryonated chicken egg. A large number of minute white spots were produced in the infected chorio-allantoic membrane, especially along blood vessels. This part was cut into small pieces and cultivated for 48 hours by the first technique already described. Then, specimens for electron microscopy were prepared.

Minute bodies of the size of vaccinia virus could be seen clearly in the extremely thin part, i.e., the reticular part of the cells grown from the original tissue fragment infected. Some of them were scattered and some aggregated in the form of diplococci, sarcinae or staphylococci. It was, however, impossible to ascertain if the protoplasm umderwent any changes by the present technique.

Electron microscopic studies on the ectromelia virus were done with a 48 hours' culture of the chorio-allantoic membrane which had been infected with virus for 48 hours by the allantoic method. The results showed that it was almost impossible to find virus in the cell. But vacuolar degeneration as well as a little degree of transparency of the nucleus was observed, and this could not be seen in normal cells.

Electron microscopy of ectromelia inclusion bodies seemed interesting as revealed in the experiment described in the foregoing. As ectromelia inclusion bodies were formed remarkably in the epithelial cells of infected mouse sole skin, I prepared the frozen section of the infected skin and tried to sodden the tissue cells after putting the section into distilled water and placing it in an ice box for a period of 24-48 hours. The inclusion bodies were found floating in the water.

The suspension with the bodies was centrifuged at 3,000 r.p.m. for an hour, and the sediments were examined with the electron microscope. There were observed a large number of viruses embedded, separately or in clumps, in the stroma of the inclusion bodies as well as the extra-inclusional free viruses which were supposed to have been released out of the stroma.

Now allow me to refer to the results obtained with regard to the interaction of bacterial virus with host cells. The virus used was T_2 phage and the host cells

were strain B of Escherichia coli. The titer of the phage was 1.4×10^{11}. Colon bacilli were incubated for 2½ hours in broth by forced aeration, giving a bacterial concentration of about 10^8/cc. The broth culture was centrifuged, and the bacterial pellets were re-suspended in the buffer solution (M/15 phosphate buffer + 10^{-3} M M_gSO_4 + 0.5% NaCl). T_2 phage was added to the bacterial suspension at the rate of $\frac{[T_2]}{[B]} \fallingdotseq 10$, namely, the multiplicity of infection or so-called multiple infection. This sort of test tube containing both bacteria and phage was placed in a water bath regulated at 37° C, and the aeration was continued. Since the minimal latent period of T_2 was 21 minutes, the infected bacilli were treated after 5 minute-, 10 minute-, 15 minute-, or 18 minute infection, respectively. The method adopted was as follows:

After a fixed period of time, 2 cc were transferred from the test tube into the centrifuge tube, and then centrifuged. The pellet was re-suspended in 0.5% osmic acid, and it was centrifuged with the angle centrifuge. The pellet was washed with distilled water and re-centrifuged. Then, the pellet was dehydrated in situ with an alcohol series ranging from 25% to 100%. The pellets were transferred into a gelatin capsule containing N-butyl methacrylate monomer with a catalyst, 2,4- dichloro-benzoyl peroxide. Next it was polymerized for 7 hours at 45° C., and then the embedded bacterial block was cut with the Minot's ultra-microtome at a thickness of 0.1-0.05μ. Electron micrographs of the sectioned bacteria were taken, removing the monomer in the shadowing apparatus.

The results obtained may be summerized as follows:

Phage particles were unobservable in any section of the bacteria of 5 minute infection, while the protoplasm was seen remarkably expanded in 2 or 3 parts. Occasionally I could see the bacilli showing a forky developing form.

The bacillus bodies of ten minute infection became considerably rough, and there appeared a fibrillar structure. But no phage particle could be seen in the section.

After 15-18 minute infection a large number of newly grown phage particles could be clearly observed in sectioned cells. The phages with a tail appeared on rather rare occasions.

In a sample of 18 minute infection, the size of every phage measured 60 × 80 mμ, and was square in shape. Definitely it had a limiting membrane completely corresponding to free phages. Some of the sections were found to have 30 phage particles or more, while some of the cells of 18 minute infection became considerably fragile.

(1) Kimura, R. et al, 1952: Studies on *Bacillus aneurinolyticus* Kimura et Aoyama. Vitamine (Japanese), 5, 51.

(2) Kimura, R. & T. H. Liao, 1953: A new thiamine decomposing anaerobic bacterium, *Clostridium thiaminolyticum* Kimura et Liao. Proc. Japan Academy, 29, 132.

(3) Studies on the thiamine decomposing bacterium.

(4) Aoyama, S., 1952: Bacteriological researches on a new thiamine decomposing bacillus, *Bacillus aneurinolyticus* Kimura et Aoyama. Acta Schol. Med. Univ. Kioto, 30, 127.
(5) Aoyama, S., 1953: The characteristics of thiaminase of *Bacillus aneurinolyticus* Kimura et Aoyama. ibid. 30, 239.
(6) Liao T. H., 1953: Immunological studies on *Bacillus aneurinolyticus* Kimura et Aoyama. ibid. 30, 270.
(7) Liao T. H., 1953: Observations on *Bacillus aneurinolyticus* Kimura et Aoyama in synthetic media. ibid. 31, 27.
(8) Liao T. H., 1953: On the anaerobic thiamine decomposing bacterium, *Clostridium thiaminolyticum* Kimura et Liao. ibid. 21, 33.
(9) Liao T. H., 1954: Studies on the growth factors and amino acid requirements of a new bacterium, *Clostridium thiaminolyticum* Kimura et Liao. ibid. 32, 52.

DISCUSSIONE

(J. D. Verlinde, Institute for Prevent. Medicine, Leiden, Holland).

Die elektronenoptischen Bilder zeigen nur erwachsene Teilchen des Vacciniavirus. Sind auch Hohlformen beobachtet worden, wie von Gaylord und Melnick beschrieben und auch von uns in Schnitten des Chorioallantois des Hühnereies gesehen worden sind?

(Risposta dell'Autore).

Bei unserer Methode sind die sog. Hohlformen des Vacciniavirus nicht beobachtet worden.

2.

VIRUSES AS ORGANISMS WITH PARTICULAR REFERENCE TO INSECT VIRUSES (*)

G. H. Bergold

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Schlesinger (1) in 1934 suggested that bacteriophages might be a nucleoprotein. Stanley found in 1935 (2) and surprised the II. International Congress for Microbiology in London in 1936 with isolation of a crystalline substance from tobacco leaves which had the specific pathogenic action of TMV. Shortly afterwards Bawden, Pirie, Bernal, and Fankuchen (3) reported that TMV is a ribonucleoprotein. Although this revolutionary idea was a great surprise to all biologists, it was soon more or less generally accepted that all viruses are macromolecules of nucleoprotein. The main reason for this attitude was that in these years chemists and physicists conditioned strongly with their concepts the ideology of virus research. The results were often unsatisfactory, because of the quantitative and qualitative limitations of the methods. This situation has lately been silently recognized by many virologists: the term nucleoprotein molecule is less frequently used and « old fashioned » concepts of biological terminology like « life cycle, morphology, sexuality, mating, vegetative and resting stage » etc. have sneaked in without any comments. The majority of virus workers of today believe even, whether they say it or not, that viruses are organisms. Burnet (4) expressed this opinion as early as 1944.

In the past most definitions of an organism were offered by philosophers rather than by scientists. Furthermore this concept usually is mixed somehow with that of life, which is unnecessary. On the other hand Whitehead (5) and Sinnot (6, 7) are certainly right to stress the importance of organization within an organism. One may argue, however, whether an organism is for principal reasons a product of a « system » plus a « substance » as Sinnot (6) has pointed out, since « forma » and « materia » may fuse into each other in a virus organism of minimal dimensions.

(*) Contribution no. 183, Forest Biology Division, Science Service, Department of Agriculture, Ottawa, Canada.

However one can safely state that an organism consists of organs, that is tools for certain functions. Theoretically the simplest organism must possess at least two such organs and since multiplication is the most important function of an organism, one of the two characteristic organs must be responsible for the reproduction. It may consist of one molecule of nucleic acid. This is based on the discovery of the nucleic acid nature of the transforming factor in pneumoccocci (8), the finding of Hershey and Chase (9), and on the absolute weight of the smallest virus (about 15 mμ in diameter), which amounts to about 2.2×10^{-18}g or a molecular weight of about 1.35 million.

We can only speculate about the nature and particular function of the second or the several other necessary organs. It is generally accepted, however, that a virus does contain protein. As to the function we can only assume that it might serve as protection and/or steric support of the reproductive organ (that is the nucleic acid), or represent the tool for attaching and entering the host cell (9).

Before we try to give a definition of an organism in general and a virus in particular, we should refer to the definition introduced recently by Lwoff (10): « the theoretical virus would be defined as a specific reproducible nucleoprotein or molecule, which was, is, or may become infectious and pathogenic ».

Characteristic of this definition and of the kind of reasoning of many other scientists is the complete neglect of morphological characters such as « tails », membranes and other differentiated organelles, which are believed to be only secondary features. It is true that reproduction is the most important character of a virus but certainly not the only one and the morphochemical differentiation into membranes or tails is just as intrinsic for the continuous preservation of a phage. It is this morphochemical differentiation of organs and their co-ordination for the continuation of the whole which distinguishes qualitatively a virus from a definable nucleoprotein molecule, a gene, or a transforming factor. Furthermore the relative ease with which in some cases the DNA can be separated from the rest of the phage, and the separation of DNA during infection, makes it hard to believe that DNA and protein are combined into a macrostructure.

It seems therefore that Lwoff's definition of a virus as a nucleoprotein is not satisfactory. On the other hand everybody will agree with Lwoff's statement that a virus « was, is, or may become infectious and pathogenic ». Since these are long and well known characteristics of a parasite one could briefly say that viruses are parasites.

Summarizing the above one could arrive at the following definition:

A functional virus is a parasitic organism; that is, a unit consisting of at least a minimum amount of protein and nucleic acid and of such shape, content, and activities that its continuation and multiplication are ensured in a suitable environment.

This definition postulates 4 properties for a virus which are well known characteristics of a parasitic organism (Table 1, 1-4). Additionally 6 more properties can be provided which establish, by analogy to recognized principles in the general field

of microbiology, the organismal nature of viruses. Some of these properties (2-4, 8, 10) were already presented by Andrewes (11).

TABLE I - TYPICAL PROPERTIES OF ORGANISMS SHARED BY DIFFERENT VIRUSES

No.	Property	Viruses			
		Insect	Animal	Bacterial	Plant
1	Morphological differentiation . . .	+	+	+	− ?
2	Fact of multiplication	+	+	+	+
3	Chemical complexity	+	+	+	− ?
4	Parasitism	+	+	+	+
5	Mode of multiplication	+	+ ?	?	?
6	Exponential rate of multiplication .	?	+	+	+
7	Recombination	?	+	+	?
8	Variability	?	+	+	+
9	Relationship	+	+	+	+
10	Gradient	+	+	+	+ ?

Because of the time limitation it is impossible to discuss in detail the 10 properties and to check exhaustively whether or not they do apply to Insect, Animal, Bacterial, and Plant Viruses:

1) Morphological differentiation certainly is characteristic of insect, and probably of all animal and bacterial viruses. Plant viruses, as far as we now know, are morphologically comparatively simple. However, it may well be that the multiplication stages, of which we know nothing, are morphologically differentiated. This question is therefore by no means decided.

2) The fact of multiplication is of course characteristic of all viruses.

3) Insect, animal and bacterial viruses have a complex chemical composition. The plant viruses apparently consist of nothing but protein and nucleic acid. This is again proven only for the somewhat inert stage of plant viruses and the vegetative stages may be of a complex nature (Andrewes, 11).

4) Parasitism, if understood in its widest sense, is characteristic of all viruses.

5) In insect viruses and a few animal viruses we know something about the mode of multiplication. It is too early to compare it with that of other microorganisms but there seem to be certain similarities.

6) Animal, bacterial (Dulbecco and Vogt, 12), and possibly plant viruses too (Bawden, 13) seem to multiply at an exponential rate, which is exclusively characteristic of organisms. Nothing is known about this point in insect viruses.

7) Recombination has been demonstrated in bacterial viruses, and seems to occur in animal, but has not been investigated in insect and plant viruses.

8) Variability is characteristic of animal, bacterial and plant viruses but has not been checked in insect viruses.

9 Between the members of each of the four groups of viruses there exist certain relationships along serological and morphological lines.

10) There is no sharp break either in size or properties between members of insect, animal, bacterial viruses (Andrewes, 11). The plant viruses however fit in the gradient as far as the size is concerned but until we know more about their other properties this point cannot be discussed any further.

As Andrewes (11) has already pointed out, not all of these properties may be characteristic of a given virus and no one alone is proof of organismal nature. But I hope I have shown that as a set these 10 postulates are quite convincing that viruses are not molecules but rather parasitic organisms. I am sure that this concept will better help us to understand viral multiplication than the molecule theory.

(1) Schlesinger, M., Biochem. Z., 1934, *273*, 306.
(2) Stanley, W.M., Science, 1935, *81*, 644.
(3) Bawden, F.C., Pirie, N.W., Bernal, J.D. and Fankuchen, I., Nature, 1936, *138*, 1051.
(4) Burnet, F.M., Virus as organism. Harvard University Press, Cambridge, U.S.A., 1944.
(5) Whitehead, A.N., Science in the modern world, Lowell Lectures 1925, Mentor, 28, New American Library.
(6) Sinnott, E.W., Amer. Naturalist, 1946, *80*, 497.
(7) Sinnott, E.W., Amer. J. Orthopsych., 1952, *22*, 457.
(8) Avery, D.T., MacLeod, C.M. and McCarty, M., J. Exp. Med., 1944, *79*, 137.
(9) Hershey, A.D. and Chase, M., J. Gen. Phys., 1952, *36*, 39.
(10) Lwoff, A., In The Nature of Virus Multiplication, Cambridge University Press 1953, 2, 149.
(11) Andrewes, C.H., Proc. Roy. Soc. B., 1952, *139*, 313.
(12) Dulbecco, R. and Vogt, M., Symposium Interaction of Viruses, VI. Int. Cong. Microbiol., 1953, 73.
(13) Bawden, F.C., Discussion remark to Dulbecco and Vogt 1953 (12).

3.

VIRUS MULTIPLICATION AND THE PROBLEM OF AGE RESISTANCE

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When a suspension of neurotropic virus is injected into the host by a peripheral route, young animals are almost always more susceptible than old ones. The present work was undertaken in an attempt to throw light on this very general phenomenon.

Four strains of virus were used for the work, the GDVII strain of mouse encephalomyelitis, encephalomyocarditis (EMC) virus, and two strains of Coxsackie virus, the Texas strain belonging to Dalldorf's Group A, and the Connecticut-5 strain from Group B. Each of these four was titrated in both the brain and the muscle of mice of different ages by the injection of serial dilutions of a standard virus suspension. In this way age resistance curves were built up.

In addition, a small dose of virus was injected into the muscle or brain of mice of different ages, and the virus content of the injected tissue titrated after a two-day interval. This gives an approximate estimate of the amount of multiplication relative to the initial inoculum which a small amount of virus will undergo.

Five day old mice were highly susceptible to all four viruses given by either route, and abundant virus multiplication was demonstrated in both muscle and brain, ranging from several hundred times in the case of GDVII virus in muscle, to several million times for the Coxsackie viruses. Thirty day old mice, though still just as susceptible to EMC and GDVII viruses given into the brain, were partially resistant to both these viruses given intramuscularly, and wholly resistant to the Coxsackie viruses given by either route. In these older mice, virus multiplication relative to the initial inoculum could only be demonstrated for GDVII and EMC viruses in the brain.

In short, wherever some degree of resistance was encountered, no virus multiplication could be demonstrated, and where there was no resistance, multiplication was obtained in every case.

The question now arises: what is the basis for this correlation, and what light does it throw on the mechanism of age resistance? An explanation in terms of the increased antibody-producing power of older animals is considered, and reasons given for its rejection. An alternative theory is discussed, in which our failure to demonstrate virus multiplication in older animals following the injection of small amounts of virus is interpreted as being due to an actual decrease in the ability of older tissues to support virus multiplication. This theory entails the intervention of specific

antibody to account for the age resistance data, and would lead to a tendency for the slope of incubation period plotted against dosage to be flatter in older animals than in younger ones, because of the postulated slower rate of virus multiplication. An analysis of our results shows no such tendency.

The hypothesis which seems most satisfactorily to account for our results is to assume that the permeability of cells to virus decreases with age — that is, that the probability of a given virus particle entering a cell is very much higher in young than in old mice. Unless it enters a cell, the virus can neither multiply nor invade the central nervous system; so that this single assumption wold account both for the increased resistance to intramuscular inoculation which develops with age, and for our failure to detect any virus multiplication when a very small amount of virus was injected into older mice.

Certain consequences would follow if this hypothesis were true. In older mice, a considerable dose of virus would be needed for any to get into the cells at all. If a large amount were given and a small fraction did enter the cells and multiply, the virus content of the tissue might still never exceed the level of the large initial dose, and so the method described above would be inadequate to detect the multiplication which had occurred. But if the virus content of the tissue was titrated at a number of intervals after injection, it might be possibe to detect virus multiplication even in older mice.

This proved to be so. In two experiments of this type with EMC virus and one with GDVII, a large dose of virus was injected into six months old mice, and batches of muscles titrated at intervals. In each case, after a sharp initial fall, the virus content showed an appreciable rise, though never exceeding the initial dose by a sufficient amount to enable one to say that multiplication relative to the initial inoculum had occurred.

Again, if a decline in cell permeability is a factor in age resistance, then the intramuscular administration of substances known to increase cell permeability might be expected also to increase susceptibility to virus inoculation in older mice. The substance chosen to test was physostigmine, which has been shown to increase the permeability of several types of cell. Injection of physostigmine after GDVII virus had been inoculated into the same muscle, increased the susceptibility of thirty day old mice to the virus approximately tenfold. The experiment was repeated three times with the same result, giving an overall probability of less than one in a hundred of obtaining such a difference through chance alone.

Finally, an attempt was made to answer the following question: is the greater degree of virus multiplication relative to the initial inoculum which we had demonstrated in young animals due to the fact that the muscle is growing rapidly during youth, or is it due to some other factor of the physiology of the young? When the sciatic nerve is crushed, the calf muscle atrophies, and then when the nerve fibres have grown down, the reinnervated muscle increases very rapidly in size. Virus was