SECTION ON MICROBIOLOGY

APRIL 21, 1948

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      Frank L. Horsfall, Jr.
      Hospital of Rockefeller Institute for Medical Research

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Studies on the Nature of Red Cell Agglutination by Viruses

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Summary. Some of the facts about the interaction between influenza virus and red cells were reviewed. By testing for the virus adsorbing capacity of red cells, it was found that the virus receptors were very stable to treatment with a number of reagents and to exposure to high temperatures but were inactivated by proteolytic enzymes and by the periodate ion in small concentrations, as well as by influenza virus. These characteristics of the cell receptors were found to be similar to those of the virus inhibitor present in normal serum. Evidence for the destruction of serum inhibitor by proteolytic enzyme, periodate and by influenza virus was given. Preliminary attempts to isolate the inhibitory principle from normal human plasma yielded a fraction in which the inhibitor activity was destroyed by trypsin, concentrated phenol and by heating. These qualities and the small amount of carbohydrate in active preparations make it seem unlikely that the active principle in serum is closely related to the blood group mucins.

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The Significance of Combinations Between Viruses and Host Cells

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Combinations between viruses and host cells occur with great frequency. It is very probable that in the absence of such a combination infection with a virus does not develop. Virus-host cell combinations can be divided, for the purposes of this discussion, into at least three different classes which have various degrees of significance and importance.

These are: 1) contact combinations or virus-cell surface unions, 2) intracellular combinations or virus-cytoplasmic unions, and 3) extracellular combinations or virus-tissue component unions. During the course of infection with a virus these different types of combination are thought to occur in series one after another and in general they appear to occur in the order stated above.

Evidence for the occurrence of combinations between viruses and host cells has been obtained with a number of viruses including some in each of the three chief categories, i.e., so-called bacterial, plant and animal viruses. Because of the apparently constant occurrence of combination, certain workers[1] now doubt that any virus has been obtained in a state entirely free of contaminating host material. Whether or not such a degree of purification has been achieved is not our present concern. Rather it is the purpose of this discussion to examine some of the evidence concerning the occurrence of combinations between viruses and host cells and to discuss the significance of such unions.

It seems fairly obvious that the first step in infection with a virus probably is contact between the virus particle and a susceptible host cell. This results in one type of contact combination or virus-cell surface union. The adsorption of bacteriophage or bacterial viruses by a susceptible micro-organism provides an excellent example of this type of virus-host cell combination.[2] Although it seems essential for such a union to occur before infection with a virus can develop, it does not follow that all virus-cell surface combinations result in infection. As an example, heat-killed bacteria combine with bacterial viruses readily but certainly are not infected by them. Moreover, a number of animal viruses combine with erythrocytes as was shown first with influenza viruses,[3] some even form stable unions,[4] but none causes infection of the red blood cells. The nature of the cell surface component which unites with a virus has been studied in only a few instances. Evidence has been obtained[5] which indicates that certain bacterial viruses combine with polysaccharides at the surface of susceptible micro-organisms. There is some evidence[6] which suggests that certain plant viruses may also combine with complex carbohydrates of the host. In the case of pneumonia virus of mice (PVM) it appears that protein is an essential constituent of the combining component.[7] With influenza virus there is evidence[8, 9] indicating that the combining component of erythrocytes may be a mucoprotein.

After a virus and a susceptible cell have come in contact and surface combination has occurred, there follows a series of mysterious phenomena about which there is more conjecture than information. It is thought that the virus penetrates the cell membrane and undergoes multiplication intracellularly. With some plant and a number of animal viruses there is good evidence for this point of view. In certain instances the virus actually can be visualized within the cytoplasm of susceptible cells and seen to increase in number. Particularly good examples are provided by the so-called pox virus group which are sufficiently large that their elementary bodies can be seen with a good microscope.[10] Although a virus may become dissociated from cell surface components at the moment it penetrates into the cell cytoplasm, there is no reason to think that it remains uncombined with cytoplasmic components while it occupies an intracellular position. If present concepts regarding the mechanism of virus multiplication are valid, it would appear that in order for sufficient energy to become available for the synthesis of additional virus particles within the cell, the initial virus particle should combine with at least one and probably with a series of intracellular enzymes. Therefore, such virus-cytoplasmic unions appear of decisive importance relative to the possibility of virus multiplication, and would seem to control the process which results in infection. In the light of this concept they are of more fundamental significance than any other type of virus-host cell combination. Very little is known of the nature of intracellular combinations and nothing is known as yet of the intracellular enzyme systems which are required for virus multiplication. So far, almost all frontal approaches to these problems have come up against nearly insurmountable technical difficulties. There appears to be no satisfactory means by which a virus-in-action, so to speak, can be studied. Still it

has been possible to obtain evidence with some plant viruses as well as certain animal viruses which suggests that either polysaccharides or proteins, perhaps both classes of substances, combine with viruses inside susceptible cells. It has been shown[10] that digestion with trypsin releases the aggregates of fowl-pox virus from infected tissue. In trachoma there is evidence[11] that the elementary bodies lie in a matrix which is composed largely of carbohydrate and probably is glycogen. Either trypsin or a cellulase obtained from the snail, Helix aspersa, liberates tobacco mosaic virus, tomato bushy stunt virus and potato X virus from infected plant leaves.[6]

After a virus has established contact with a susceptible cell and then has undergone multiplication within the cell, numerous virus particles are released or escape from the cell. They may then infect other susceptible cells and repeat the cycle. When virus particles escape from infected cells they may remain combined with cytoplasmic components or may combine with extracellular components, with substances present in the intercellular fluid or with connective tissue cells which in most instances they do not infect. A number of plant viruses become so firmly combined with the fiber of the leaf after release from infected cells that it is virtually impossible to break the union except by rigorous procedures which largely destroy the viruses.[1] Both mumps[12] and influenza viruses[13] combine with a component present in the allantoic fluid of the chick embryo and it is fairly certain from results recently obtained[14] that such combinations do not dissociate completely. Moreover, it has been shown[15] that influenza virus remains firmly combined with host tissue components even after extensive purification procedures. In the latter instance it is not yet possible to determine whether intracellular, extracellular or both types of combination are responsible for the results obtained. In a few cases there is some indication of the nature of the component which is combined with a virus after it has escaped from an infected cell. With influenza viruses[16] a polysaccharide composed of mannose, galactose and glucosamine units was constantly associated with the purified virus particles and it was thought that host protein was also present. The results of recent experiments[14] suggest that the combining component in allantoic fluid may be a mucoprotein and in this respect, at least, it seems to be analogous to the erythrocyte receptor which appears to be a mucoprotein.[9]

If virus-host cell combinations occur with the frequency suggested by the results of the studies which have been summarized, it is to be expected that they should lead to peculiar findings, difficult to understand if the existence of such combinations is unrecognized. What has been found with one animal virus is pertinent in this connection.

Normal mice harbor in their lungs a virus which can cause fatal pneumonia in its natural host.[17] The agent is termed pneumonia virus of mice (PVM). The name is misleading because PVM is present not only in mice but also in many other species. It appears that among 9 species of mammals, including man, each is subject to latent infection with the virus[18] and it can cause fatal pneumonia in at least 3 animal species, i.e., mice, hamsters and cotton rats. Among avian species it appears that chickens, as well as chick and duck embryos, do not harbor PVM and are not susceptible to infection.[7]

Early investigations[17] showed that the virus is strictly pneumotropic; causes infection of the lungs when given intranasally, but not when given by other routes; stimulates the development of active immunity and is neutralized *in vivo* by immune serum. Complement fixation, however, did not occur in the presence of immune serum and no other *in vitro* test gave positive results with the agent either in the presence or absence of immune serum. Early evidence[17] indicated that the virus was of medium size with dimensions of the order of 100 to 150 millimicrons.

With studies limited by *in vivo* techniques progress was slow and neither the precision nor the quantity of data obtainable was great. This situation changed when it was discovered[19] that PVM causes agglutination of mouse red blood cells. From comprehensive investigations on the hemagglutination phenomenon with the virus there have emerged some unexpected results.[7, 20, 21, 22]

When fluid and red blood cells are expressed from the cut surface of mouse or

hamster lungs infected with the virus, agglutination of the erythrocytes occurs. Suspensions of such infected lungs do not cause hemagglutination. After heating such suspensions at 70 or 80° C., agglutination of red cells is demonstrable. The addition of material from normal lungs to heated suspensions causes hemagglutination to disappear again but, upon further heating, the property reappears. This cycle of masking and unmasking the capacity to cause hemagglutination can be repeated many times. The virus, although rendered non-infectious, remains otherwise unaltered during the process. These findings led to the concept that stable combination between the virus and a heat labile component of lung tissue was responsible for the results.[4, 20]

To obtain maximum hemagglutination titers high temperatures are required and the titer is related to both the temperature and time of heating.[4, 20] Alkali can also be used to unmask hemagglutination with PVM.[22] To obtain maximum titers a high pH is needed. The virus becomes non-infectious when heated or when mixed with alkali. Consequently, the procedures which unmask hemagglutination also cause inactivation. Until recently it was not possible to cause dissociation of the combination between virus and lung tissue component without destroying infectivity. This has now been accomplished;[23] when the electrolyte concentration is sufficiently reduced, free infectious virus dissociates from combination with host tissue. By appropriate variation of the concentration of electrolytes, either combination or dissociation can be caused at will. Of more importance the cycle of combination and dissociation can be repeated many times without causing demonstrable alteration in the combining capacity of either the virus or the host cells.

There is adequate evidence that hemagglutination is caused by free virus particles themselves and not by some other substance in infected lung tissues.[20] Free virus can be obtained from infected lungs without subjecting the tissues to grinding.[21, 23] Such preparations contain infectious PVM in relatively high titer and give, without further treatment, corresponding hemagglutination titers. On the addition of mouse or hamster erythrocytes agglutination occurs and the virus is carried down with the red cells. Erythrocytes from other mammalian species or from chickens are not agglutinated and the virus is not adsorbed by such red cells.[4, 20] When particles from the lungs of normal mammals are added to free virus, agglutination does not occur, but the virus sediments along with the lung tissue particles.[7, 21] Similar particles from tissues other than the lungs of susceptible animals or from chick embryos do not increase the sedimentation rate of the virus.

It is evident that the virus unites with erythrocytes which are agglutinable but does not combine with those which are inagglutinable. Moreover, it also unites with mammalian lung tissue particles but does not combine with particles from other tissues of the same animals or with avian tissues. It is important to emphasize that PVM infects the lungs alone.[17] The combination between PVM and erythrocytes or between the virus and lung tissue particles is stable and does not dissociate at physiological electrolyte concentration. When combined with lung tissue particles, the virus is not capable of uniting with red cells or reacting with specific antibody *in vitro* even though it can be neutralized by specific antibody *in vivo*, probably only after the complex has been split.

In intact infected lung tissue much of the virus appears not to be combined; it is infectious and also capable of combining with suitable erythrocytes. When it is uncombined, the virus is capable of reacting with specific antibody *in vitro* and positive reactions are obtained in both complement fixation[20] and hemagglutination-inhibition tests.[19, 20] Free or uncombined PVM is several times smaller than combined virus and is of relatively uniform particle size. Present evidence indicates that the free virus is actually a relatively small agent with dimensions of the order of 40 millimicrons.[21]

In attempts to determine the physical, chemical or immunological properties of a virus, or to estimate the size of the particles, the results will be influenced by the state of the agent. With spherical particles of similar density with dimensions of the order of free PVM (i.e., 40 millimicrons) as compared to combined PVM (i.e., 140 millimicrons), the difference in particle

weight or volume is more than 42 times. Moreover, at least 95 per cent of the particles of combined PVM consist of host constituents distinct and separable from the virus. It is improbable that stable combination with tissue particles is a phenomenon peculiar to PVM alone. As has been indicated, there are reasons for thinking that the phenomenon is not unique and that other viruses behave in a similar manner. Because of this the available data concerning their physical, chemical and immunological properties may require re-evaluation.

The capacity of mammalian lung tissue to combine with PVM appears to be dependent upon a tissue component, not present in other organs, which interacts with the virus.[7, 22] Only specific antibody possesses greater affinity for the virus than this tissue component.[7] Non-specific adsorption can hardly be invoked as an explanation for combination since suspensions of mammalian tissues other than lung, or of avian tissues, do not bind PVM. Excepting only the red blood cells of mice and hamsters the combining component is present solely in mammalian lungs and is demonstrable in the intact lung.[7] Since crystalline trypsin, but not other enzymes, destroys the combining capacity of the tissue component, as also do heat and alkali, it is probable that protein is an essential constituent.[7]

The available evidence strongly suggests that the combining component in mammalian lungs plays a decisive role in the initiation of infection with PVM.[22] The only mammalian organ which can be infected with the virus is that organ which contains a component capable of combining with the virus. Different animal species are susceptible to infection with PVM in different degree.[18] These differences in susceptibility are directly correlated with the quantity of combining component in the lungs of the several species.[7] It is probable that the first step in the initiation of infection with PVM is combination between free virus and the lung tissue component at the surface of susceptible cells. If this is the case, it may seem paradoxical that combined virus is as infectious as free virus. However, evidence has been obtained that a substance, probably a proteolytic enzyme, present in the intact lung can split the combination and release free virus which then can combine with and infect susceptible cells.[7] It appears now that an essential preliminary step in establishing infection with combined virus is splitting of the virus-tissue component complex in order that free virus, so released, may recombine with the component at the surface of susceptible cells in the lung.[7, 22]

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