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IMMUNITY IN VIRUS INFECTIONS[1]

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FOR many years it has been recognized that one attack of certain diseases, now classified as virus maladies, induces an enduring immunity. Because of this fact and since many workers believed that viruses are radically different from other types of infectious agents, there arose in some quarters the idea that immunological and serological phenomena in the virus field differ from those in other fields of infection. This was an erroneous idea because the basic principles underlying serological and immunological phenomena in all fields of biology are identical. These principles, however, may evidence themselves in different manners in the various fields, and the techniques of studying the phenomena associated with them in different fields frequently vary because of the nature and mode of action of the infecting agents. It will be impossible to discuss at this time all the different phenomena and peculiarities associated with immunity in the numerous virus diseases. Therefore, a few general remarks will be made, following which immunological and serological phenomena associated with vaccinia will be discussed in detail.

Immunity is resistance to infection or injury and is demonstrable only in a living host. Such resistance

[1] Delivered on September 26, 1941, as part of a symposium on immunity at the Fiftieth Anniversary Celebration of the University of Chicago.

may be natural or acquired. The acquired form may be associated with certain serological phenomena, for example, complement fixation, agglutination, precipitation and neutralization. It must be remembered, however, that these phenomena, while interesting, may not be directly responsible for the immune state of a host.

One of the most striking immunological phenomena in the virus field is the complete and enduring immunity that follows many of the diseases in this category. It is so striking that there was a tendency at one time to make generalizations to the effect that all virus diseases are followed by a lasting immunity. Now we know that this is not true. While smallpox, chicken pox, measles and yellow fever are followed by a protracted immunity, other virus diseases, *e.g.*, influenza and common colds, produce protection of short duration. An awareness of the difference in the duration of immunity following different virus diseases immediately raises the question as to why some of them induce a lasting immunity and others do not. Many workers in this field believe that a life-long immunity to a second attack of a virus disease is due to a persistence of the virus in the recovered host. Inasmuch as circulating antibodies against yellow fever virus have been found in a recovered host for as long as fifty years after an attack of the disease during which time no further contact with the active agent has occurred, the statement just made about persistence of virus is particularly applicable to yellow fever. Such a persistence does not mean that an immune individual is capable of spreading disease, because it is most likely that the virus is stored in some remote part of the body within living cells where it can not come in contact with circulating antibodies and from which point it can not, for one reason or another, reach the outside world.

A statement to the effect that an infectious agent can persist for so long a time in a host in the presence of neutralizing antibodies seems to shock some workers. There is nothing strange about the situation if one realizes that viruses multiply and carry on all their activities within living cells. Furthermore, while situated intracellularly the active agents can not be attacked by antibodies. Consequently, if a virus and its host cells multiply at approximately the same rate and if the host cells are not destroyed by the virus, the two can live together happily year after year without the infecting agent ever being subjected to the action of humoral antibodies. There is ample experimental evidence to show that such a state of affairs can and does exist under certain conditions.

Thus, following certain virus diseases the prolonged or enduring immunity is probably due to a long-term sojourn of the virus in the host or its persistence throughout the host's life. From such a statement one might infer that the reason for the absence of an enduring immunity following attacks of certain virus diseases is due to the fact that the inciting agents of these maladies are not capable of permanently establishing themselves in an infected host or that they do not persist long enough in a host to bring about a profound immunity. Why the virus can not permanently establish itself in some diseases is not known. Perhaps the type of tissues involved is responsible. For example, it is possible that in the case of the common cold and influenza the viruses attack superficial tissues, establish themselves in such tissues and would lead to an enduring immunity if these tissues were not themselves temporary. In other words, the superficial cells lining the respiratory tract are being thrown off at regular intervals to be replaced by new cells from deeper layers and do not provide a permanent abode for viruses.

This explanation for the persistence and lack of persistence of immunity to certain virus diseases may not cover the field completely, and doubtless some workers will object to it. On the other hand, there are good reasons for believing that the conditions described play a part in the picture. In any event, it is well known that a plant once infected with a virus, although it may later assume a fairly normal appearance, still carries the virus and is refractory to reinfection. Moreover, Kunkel has shown that one virus disease of plants can be cured by treatment at a temperature which inactivates the virus without injury to the plants and that the cured plants return to a state of susceptibility to infection.

What has been said about the persistence of immunity following attacks of virus diseases has a bearing on the production of immunity by means of vaccines. At the beginning of the virus era it was not unusual to hear that a protection could not be induced except by the use of vaccines containing active viruses. While it is true that immunity against many virus diseases has not been induced except by the use of active agents, it has been possible in the case of others, *e.g.*, equine encephalomyelitis, rabies and influenza, to produce an appreciable amount of protection by means of inactive agents, provided sufficient quantities of them are used. If enduring immunity is due to persistence of virus in a host, then one would not expect inactive viruses to produce a prolonged immunity because of their inability to establish themselves and continue to multiply. This appears to be the case, because whenever inactive viruses are used it is recommended that vaccinations be repeated at stated intervals.

The fact that viruses multiply within living susceptible cells and can not be attacked by antibodies while

within such cells has a bearing on serotherapy of virus diseases. It is generally believed that once a virus has invaded a host and has manifested itself by signs and symptoms of illness, it is too late to modify materially the course of the disease by the therapeutic administration of immune serum. This belief has arisen as the result of many negative reports regarding the value of immune serum in the treatment of virus maladies. If the belief is justified, and it undoubtedly is in the case of many virus diseases, one has to seek for an adequate explanation of such findings further than the fact that viruses multiply in cells and can not be attacked by antibodies while so situated. In order to account completely for the negative findings regarding serum therapy, it is necessary to assume that all the cells that are going to be infected in a particular host by a virus have been infected by the time signs and symptoms of illness become evident. This assumption is substantiated by a considerable amount of experimental work. Fite and Webster have shown that after instillation of louping ill virus in the noses of mice the active agent is present in the brain four days before the animals evidence signs and symptoms of illness. Galloway and Perdrau found that after instillation of louping ill virus in the noses of monkeys the active agent was well distributed throughout the central nervous system several days before the animals showed signs of sickness. Hurst instilled equine encephalomyelitis virus into the noses of monkeys, sacrificed them at different times after inoculation, tested various parts of their central nervous systems for the presence of virus, and correlated his findings with clinical observations made on the monkeys before they were killed. According to him, all parts of the central nervous system except the cord contained virus within thirty hours after onset of fever, and several hours later, at the time of onset of nervous symptoms, even the lumbar cord was infectious. Webster and Clow dropped the virus of the St. Louis type of encephalitis into the noses of mice, sacrificed some at different times in order to test for the presence of virus in various parts of the brain and cord, killed others to determine the time of appearance and progression of lesions, and allowed others to sicken and die in order to determine the time of onset of clinical signs and symptoms. The data obtained in this manner clearly showed that virus was present in tissues twenty-four to forty-eight hours before the appearance of lesions detectable by means of the microscope and that all parts of the brain and cord contained large amounts of virus before the animals became ill. Faber and Gebhardt conducted similar experiments with monkeys that had been infected by means of intranasal instillation of poliomyelitis virus. Their findings indicated that by the fifth, sixth or seventh day after inoculation, at which time only an occasional rise of temperature or tremor and hyperesthesia were present, and before paralysis had occurred, virus was distributed throughout the central nervous system.

Following the general remarks about immunity in virus diseases, it seems advisable to discuss in detail certain immunological and serological phenomena associated with vaccinia. It is generally accepted that a successful vaccination against smallpox in which active vaccine virus is used results in a satisfactory immunity which endures for a varying period of time, *i.e.*, from several years to a lifetime. Many attempts have been made to obtain satisfactory immunity both in lower animals and in human beings by means of inactive vaccine virus. While a low-grade protection can be obtained in this manner in lower animals, provided large amounts of inactivated vaccine virus are used, no definite evidence as yet has been brought to indicate that any of the inactivated vaccines employed are of real value in the protection of human beings against smallpox.

In some virus diseases, the only humoral antibody that has been demonstrated is one which neutralizes the inciting agents, as is the case in poliomyelitis. In other virus diseases, for example, in Shope's papilloma of rabbits, complement-fixing and neutralizing antibodies have been demonstrated, and apparently they are identical, reacting to a single antigen, the virus, in different ways under different conditions. In still other virus diseases, for example, in infectious myxomatosis of rabbits and vaccinia, there appears to be a multiplicity of antigens and antibodies. In fact, the serological studies of vaccinia and infectious myxomatosis of rabbits have gradually made it obvious that many of the phenomena observed in other types of infectious diseases are also observed in the virus field. Moreover, methods used for investigating these phenomena in the virus field are in many instances identical with or similar to those employed in other biological fields.

Sternberg in 1892 demonstrated that the serum of an animal recovered from vaccinia possesses the property of neutralizing the activity of the virus. In 1899 Béclère, Chambon and Ménard confirmed and extended Sternberg's observations. In addition to this, they showed that sera of human beings and monkeys convalescent from variola possess neutralizing antibodies for vaccine virus. The first definite demonstration that flocculation occurs in a mixture of vaccinia-immune serum and vaccine virus was made in 1904 by Freyer. In 1906 Jobling demonstrated that complement is fixed in the presence of a mixture of vaccine virus and serum from calves convalescent from vac-

cinia. Paschen reported in 1913 that elementary bodies of vaccinia are specifically agglutinated by vaccinia-immune serum.

In 1925 Gordon in a Medical Research Council Report presented the results of his extensive study of the antibodies found in vaccinia- and variola-immune sera. He again took up the question of complement fixation and definitely showed that the viruses of vaccinia and variola specifically fix complement in the presence of homologous antisera. He also showed that vaccine virus fixes complement in the presence of variola-immune serum and *vice versa*—phenomena indicating the antigenic similarity or identity of the viruses. This work has been fully confirmed by Parker and Muckenfuss and by Craigie and Wishart. Furthermore, Gordon found that a flocculation occurs in mixtures of vaccinia-immune serum and vaccine virus, and showed that the reaction is specific in that vaccinia-immune serum flocculates only in the presence of vaccine virus or smallpox virus. He spoke of the reaction as an agglutination; but as will be pointed out presently this was a mistake, because the flocculation was undoubtedly due to two phenomena, *viz.*, a precipitation of soluble substances and an agglutination of elementary bodies. Indeed, some of the tabulated results of his experiments should have indicated to him that a soluble substance separable from the virus was playing a part in the complement-fixation reactions as well as in the flocculation tests.

In 1929 Burgess, Craigie and Tulloch described in a Medical Research Council Report the results of their work on the vaccinia-variola flocculation test in which they were able to confirm most of Gordon's observations. In 1931 another report by Craigie and Tulloch on the same matter extended their previous observations. Ledingham, in 1932, using specially prepared elementary bodies of vaccinia, showed by means of the hanging drop method a specific agglutination of the bodies in the presence of vaccinia-immune serum. In 1932 Tulloch reported that in ordinary preparations of vaccine virus there is present a soluble fraction, separable from the virus itself, which gives a precipitin reaction when mixed with immune serum. Shortly after this report, Craigie confirmed Tulloch's observation and stated that the flocculation test described by Gordon with crude virus suspensions involved both a precipitin and an agglutination reaction, the precipitin reaction being caused by a soluble substance separable from the virus, while the agglutination reaction represented an aggregation of elementary bodies. In 1934 Craigie and Wishart reported that the soluble fraction is made up of two components; one, designated as L, is labile and no longer active after being heated at 56° C; the other, called S, is stable at 95° C. Craigie and Wishart later stated that L and S are probably not separate entities but form an LS complex. Inasmuch as the stable substance gives a Molisch reaction, Craigie, Smith and Ch'en at one time believed it to be a polysaccharide derived from the virus. Parker and I have confirmed most of Craigie's observations, but do not agree with him that the stable substance (S) is a polysaccharide; it is a protein with particular properties. Moreover, Parker later showed that under certain conditions L and S are separable and are not always united in an LS complex. Recently, in 1938, Craigie and Wishart stated that they now believe the LS complex to be capable upon storage of breaking up into its separate components L and S.

At this stage of our knowledge there appeared to be in vaccinia-immune serum antibodies capable of doing the following things: neutralize virus; agglutinate elementary bodies; fix complement; and precipitate specific substances which had been separated from the virus. The agglutination of elementary bodies may take place because of the reaction between the soluble substances, L and S, adsorbed on their surfaces, and the L and S antibodies. On the other hand, Craigie believes that there is in addition to the L and S substances adsorbed on elementary bodies, another agglutinogen, X, which gives rise to X agglutinins. The antibodies that fix complement and produce the precipitin and agglutination reactions are likely to be the same, merely exhibiting their activity in different ways when they cause agglutination, complement fixation and precipitation. There is reason to believe that neutralizing antibodies are different from the others, because, as Parker has shown, an animal immunized with purified S is not immune to vaccine virus nor does its serum neutralize more than a minimal amount of virus in spite of the fact that it causes the other reactions that S antibodies should. Moreover, it is easy by adsorption to remove from vaccinia-immune serum L and S antibodies and with some difficulty the agglutinins also without diminishing the neutralizing titer of the serum.

Several years ago Dr. Smadel and I undertook further investigations of the LS complex spoken of by Craigie. In addition to this, we also endeavored to obtain more information regarding the existence of unrecognized antigens in vaccine virus. The first thing we did was to extract washed elementary bodies of vaccinia with N/20 NaOH at 56° C. for a short time and then quickly neutralize the material. This extraction yielded a nucleoprotein which gave a precipitin reaction with unadsorbed vaccinia-immune serum. Such a serum from which all the L and S antibodies had been removed by adsorption still possessed its full ability to precipitate in the presence of the nucleoprotein antigen extracted from the ele-

mentary bodies. Inasmuch as Smadel, Dubos and Lavin have shown that S is not a nucleoprotein and in view of the results of adsorption just described, it is obvious that S and the nucleoprotein extracted from elementary bodies are distinct antigenic entities.

We next investigated the relation of the nucleoprotein antigen to Craigie's X agglutinogen and an antigen which produces neutralizing antibodies. According to Craigie, the X agglutinins are the antibodies left in a vaccinia-immune serum following adsorption with L and S. Such a serum adsorbed with the nucleoprotein antigen removed some of Craigie's X agglutinins but not all of them. Consequently, we believe that Craigie's X agglutinins are produced as a reaction in part to the nucleoprotein extractable from elementary bodies and in part to some other antigen or antigens of the virus. In other words, Craigie's X agglutinogen is not a single substance. Serum adsorbed to remove all L and S and all nucleoprotein antibodies still possesses full neutralizing value. Thus, it is obvious that the extractable nucleoprotein antigen is not responsible for the production of neutralizing antibodies.

By the proper means L or S antiserum can be obtained. When either of these sera is mixed with a fresh filtrate from the skin of a rabbit infected with vaccine virus, both L and S are removed. Because of this fact Craigie stated that the two substances L and S probably do not occur separately but as an LS complex. On one occasion, however, Parker obtained a dermal filtrate in which he thought that L and S occurred separately in that one could be removed completely without the entire removal of the other component. Craigie also was able on one occasion to confirm this finding of Parker. Nevertheless, the original findings of Craigie are the ones usually encountered. Inasmuch as questions about the identity of L and S were still open, Dr. Smadel and I for several years have been pursuing the matter.

Fresh virus-free filtrate from vaccinia-infected skin of rabbits contains both L and S. When heated at 56° C., L no longer is precipitated in the presence of its antiserum; however, it inhibits L antibodies so that when unheated L is added to the mixture no precipitate occurs. When heated dermal filtrate is treated with dilute alkali and heat for a short time and then neutralized, it neither inhibits L antibodies nor does it precipitate in the presence of S antibodies; but a filtrate treated in such a manner inhibits S antibodies. If the filtrate is degraded further, it no longer inhibits S antibodies. Moreover, purified S, obtained according to the method of Parker and Rivers, was found to inhibit L antibodies while still capable of precipitating with S antibodies. After the purified S was treated with dilute alkali and heat and then neutralized, it no longer inhibited L antibodies, did not precipitate in the presence of S antibodies, but did inhibit S antibodies. From these findings it was concluded that LS is probably a single substance with two reactive components, L and S, which are degraded with different degrees of ease. At that time we were aware of the fact that definite proof of this idea of the nature of L and S was lacking and that such a conception was directly opposed to the occasional findings of Parker and Craigie in which L and S apparently were separated from each other by means of adsorption.

Believing that some of the newer techniques might be of assistance in purifying the LS complex and acquiring further information regarding its nature, Shedlovsky and Smadel attempted by means of the Tiselius apparatus to obtain LS in pure form from a filtrate of dermal pulp. They soon found that it would not be possible to obtain pure LS by this means alone. Then they used a combination of methods, fractionation by means of precipitation and by electrophoresis. When the dermal filtrate was brought to a pH of 4.5, a precipitate formed which was partially soluble at pH 6.5. If this procedure was carried out properly, it was found that the material which went back in solution at pH 6.5 possessed the nature of a globulin and gave evidence of being a single substance both in the ultracentrifuge and in the Tiselius apparatus. Moreover, this substance reacted equally well with L and S antibodies. When heated at 56° C. it inhibited L antibodies but still precipitated with S antibodies. When treated with dilute alkali and heat and then neutralized, it did not inhibit L antibodies, did not precipitate with S antibodies, but did inhibit S antibodies. As a result of this work it seems obvious that L and S are components of a single substance.

The data just presented regarding L and S are believed to be correct and their meaning is obvious, but they do not contain an explanation of the occasional findings of Parker and Craigie in which L and S were apparently different substances. These men are excellent investigators and it is not reasonable to believe that their observations were false. Therefore, some explanation for their occasional findings was sought. A hypothesis was formed to the effect that L and S is one substance with two serologically reactive components and that under usual conditions the activity of L is more easily suppressed than that of S. On the other hand, it was believed that it might be possible for events to occur the other way around under exceptional conditions, as for instance in the presence of special enzymes. Consequently, Smadel and Hoagland tested the effect of certain crystalline enzymes on the activity of purified LS. Very quickly it was shown that crystalline chymotrypsin acting on pure LS destroys the activity of S, leaving that of L

unimpaired. With these findings all the parts of the puzzle, L and S of vaccinia, seemed to fall in place, and now more than ever it seems reasonable to conclude that they are nothing more than different components of a single protein molecule.

Little or nothing is known about the antigen that gives rise to antibodies that neutralize the virus of vaccinia. Indeed, most workers have been unable to remove these antibodies from immune serum by means of adsorption with purified elementary bodies. On the other hand, Salaman believes that there is a union between elementary bodies of vaccinia and neutralizing antibodies and that if sufficient amounts of elementary bodies are used the neutralizing substances can be adsorbed from immune serum. An assessment of information regarding the antigen that incites the production of neutralizing antibodies and the manner in which such antibodies act reveals that much remains to be learned concerning this the most important of all subjects connected with immunity to vaccinia.

From my remarks regarding viruses in general and vaccine virus in particular, it should be evident that there is nothing peculiar about immunity in virus diseases. Principles that hold in other fields operate also in the virus domain. Furthermore, it should be obvious that generalizations about immunity in virus maladies can be made with no more assurance than about resistance to other types of infection. Immunological and serological phenomena in each virus disease present special problems that have to be met not through generalizations but by specific experiments.

NORMS OF GROWTH

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IN the *Proceedings* of the National Academy of Sciences (Vol. 21, pp. 633–4, 1935) I gave average heights and weights of 275 school girls for consecutive ages 7 to 16 years inclusive with the correlations of the heights and of the weights in the different years based on the measurements obtained in Dr. W. F. Dearborn's growth study. As many persons knew that the study involved many more than 275 girls, some have wondered why I took only the 275 for whom records were available for each and every one of the ten years from 7 to 16.

The answer, unless I am mistaken, is to be had by considering the aims of a growth study. If we desire to establish stable norms of height and weight or of other measurements at different ages we should take, of course, large samples because the standard error σ of an average is that of the distribution divided by the square root of the number n in the sample. Thus at 13 the average height was 153.40 cm with a standard deviation of 7.42, which means $153.40 \pm .45$ for the average if based on only 275 girls, whereas if we had measurements of four times as many the standard deviation of the mean would be only .22. However, for such norms one need not trouble with the continuity involved in growth studies; one could make a cross-sectional survey involving a large number of persons at each of the different ages.

Growth, however, is a continuous process and the amount of growth between two given ages is measured by a difference or increment in the measures. If we have l girls of one age and m different girls of another age as in cross-sectional studies with means Z and W, respectively, for some measurement and with standard deviations σ_z and σ_w, the sampling error of the difference $W - Z$ would be

$$\sigma^2_{W-Z} = \frac{\sigma^2_w}{m} + \frac{\sigma^2_z}{l};$$

but if we had n girls of both ages, as in growth studies, and the averages were X and Y, the sampling error of the difference could be obtained directly from the differences $y - x$ or indirectly from the correlation coefficient r between corresponding values of x and y as

$$\sigma^2_{Y-X} = \frac{\sigma^2_x + \sigma^2_y - 2r\sigma_x\sigma_y}{n}.$$

In cases in which r is high this value may be much smaller than the former when the number of persons involved is about the same, or, to put it differently, the second value may be statistically as good from a relatively small number of individuals, as the first is from a much larger number.

For example, the lowest correlation of the heights in successive years was found to be $r = .96$. If we assume σ_x and σ_y nearly enough equal so that they may be put equal, and equal to σ_z, σ_w for corresponding ages, without serious error, and if we take $l = m = n$ the first formula gives $2\sigma^2_x/n$ and the second gives $2\sigma^2_x(1-r)/n$ or only .04 as much; to put it inversely, we should have to have $l = m = 25n$ to obtain from the first formula a sampling error as small as that obtainable from the second, or we should need nearly 6,400 girls taken at each of the years to give as good an estimate of average growth as we got from 275 taken at both years—provided we trust our statistical formula.[1]

[1] This proviso may seem odd. We have, however, to remember that statistical formulas are mathematical theorems proved on certain assumptions which may not hold