IMMUNOLOGICAL STUDIES WITH THE VIRUS OF INFLUENZA*

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On the basis of clinical observations, a widespread opinion prevails that immunity to influenza is of brief duration. This has usually been explained as due to one of two circumstances: (1) An attack of influenza is not followed by the formation of specific antibodies in the serum of the patient, or, if so, they persist for only a short time. (2) Many strains of the infectious agent are commonly in circulation, and the human individual may be attacked repeatedly by strains of different serological specificity. The lack of knowledge of the nature of the causative agent, and the failure to transmit the disease consistently to laboratory animals, have made it impossible heretofore to submit the various hypotheses to experimental tests.

Recent studies (1–3), however, appear to have established the fact that the primary causative agent of human influenza is a filterable virus. In this laboratory the virus has been recovered from the nasopharynx of patients suffering from influenza in Puerto Rico and Philadelphia. These strains, (designated P.R.8 and Phila.) have been repeatedly transferred both in ferrets and mice. In both species of animals an experimental disease characterized by involvement of the respiratory tract, especially of the lungs, has been produced. Serum of ferrets recovered from infection with either of these strains of virus was found to neutralize the infectivity of both strains for mice. Furthermore, the serum of a horse immunized by Andrewes, Laidlaw and Smith (2) against a strain of virus (W.S.) recovered in England, was also found to neutralize both the P.R.8 and Phila. strains of virus. The serum of swine and ferrets which neutralized the virus

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of swine influenza (Shope (4)) failed, however, to neutralize the recently isolated human strains of virus. These results (5) show that the strains of virus recovered from human cases of influenza are immunologically identical, while the virus of swine influenza differs from them serologically. Nevertheless, certain evidence to be presented in the present paper indicates that an immunological relationship exists between the strains of human and swine influenza virus. Similar observations have been reported by the English investigators (6) who have, in addition, recovered several immunologically identical strains of human virus from different outbreaks of influenza in England.

The present report deals with additional studies of the immunity reactions of animals to the P.R.8 and Phila. strains of virus, and with certain results obtained with human sera which indicate the etiological relationship of the virus to the human disease.

Materials and Methods

Strains of Virus.—The strains of virus used in the present study were obtained from sputum or pharyngeal washings of patients suffering from influenza in Puerto Rico (P.R.8) and Philadelphia. In ferrets the disease produced by the P.R.8 strain is somewhat more severe than that produced by the Phila. strain. The pulmonary lesions in ferrets infected with the P.R.8 strain are usually more extensive, and the respiratory symptoms are more marked. The characteristics of the experimental disease produced by these two strains in mice are identical.

Mice.—Albino mice of the Swiss strain were used throughout. Young mice 4–6 weeks of age are most satisfactory. In older mice irregular survivals are more frequent, and death of the infected animals is somewhat delayed.

Neutralization Tests.—The technique of the virus neutralization tests has previously been described (5). The serum to be tested is mixed with a 10 per cent suspension of infected mouse lung in saline, and, after incubation, 0.03 cc. of the mixture is inoculated into the nasal passages of 4 or 5 anesthetized mice. If the mice die, their lungs are removed and the extent of the pulmonary consolidation is noted. All surviving mice are killed on the 6th day after infection, and the extent of pulmonary involvement recorded. The absence of pulmonary lesions in mice receiving virus and serum is taken to indicate the fact that the serum possesses specific neutralizing antibodies.

Isolation.—When ferrets are received from the breeders they are placed in quarantine for a period of 2–4 weeks and observed for any signs of illness. During the winter certain of the animals were found to carry hemolytic streptococci or small Gram-negative bacilli in the respiratory tract, and among these animals

purulent rhinitis sometimes occurred. Such animals were segregated from other stock.

After this period of observation, the ferrets are transferred to another quarantine room, from which they are transported to the isolation units when needed for experimental purposes. At no time are they exposed to experimentally infected animals or to persons who have contact with infected animals.

Active Immunity in Ferrets

In a previous publication (5) it was shown that the serum of ferrets which have recovered from infection with either the P.R.8 or Phila. strain of influenza virus is capable of passively protecting white mice against both strains of virus.

In addition to possessing neutralizing antibodies in their blood, ferrets recovering from infection with one strain have been found to be actively immune to reinfection with either the homologous or heterologous strain. The duration of this active immunity is somewhat variable. In most instances, tests made 4 months after previous infection have shown the animals to be resistant to reinfection. In certain instances, however, reinoculation has been followed by a single sharp rise of temperature in the first 24 hours, without subsequent evidence of infection. Reactions of this type have been considered to be either non-specific or of the nature of accelerated immune reactions.

These results appear to indicate clearly that the active immunity which follows infection with one strain is fully effective against reinfection with the other strain. The fact that a comparatively firm active immunity may persist for months in previously infected ferrets is in complete agreement with the observations of the British workers (1, 6). Infrequently, however, reinfection has occurred in animals receiving a second intranasal inoculation within a period of 2 months. In these cases the only evidence of reinfection was the persistence of fever for 2–3 days after the second inoculation.

It was of interest, therefore, to note that one shipment of animals from a known susceptible stock was found to be resistant to experimental infection. Another group of animals from the same source was completely susceptible. A third group, obtained from this breeder, on arrival at the laboratory exhibited nasal discharge. Certain individual animals of this latter group, when used for experi-

mental purposes and inoculated intranasally with active influenza virus, developed no febrile or constitutional reaction, and attempts to recover virus from the nasal mucous membranes or lungs of such animals were unsuccessful. In other instances a moderate fever without other symptoms persisted for 3–4 days after inoculation, but the inoculation of suspensions of the lungs and turbinates of these animals into normal susceptible ferrets failed to produce the experimental disease. It was assumed, therefore, that these ferrets had become immune, although they had not been subjected to experimental infection.

To test this assumption, serum was obtained from 5 ferrets of the third group and of several other ferrets which had been kept in the same room for a period of months. The serum of 2 of the 5 ferrets of this group was found to contain antibodies which neutralized the P.R.8 virus in mouse tests.

6 months after they had been received from the dealer, the 2 ferrets whose serum contained neutralizing antibodies, together with a third animal of the same group which did not possess neutralizing antibodies, were inoculated simultaneously with the Phila. virus. The control animal developed fever in 24 hours, lost its appetite and exhibited catarrhal rhinitis and mild respiratory distress. When sacrificed on the 4th day after the onset of fever, involvement of practically all of both lower lobes of the lung was found. The 2 ferrets, presumably immune, developed fever on the 2nd day after inoculation, but remained well thereafter. When sacrificed on the 4th day after the development of fever, the lungs of both animals were normal in appearance. The turbinates were somewhat mucoid.

From one of these latter animals a 20 per cent suspension of ground lung and turbinate tissue was made and inoculated intranasally into a normal ferret. Likewise, the lung and turbinates of the control animal were ground to form a 10 per cent suspension and administered to another normal ferret. The animal which received the latter material became sick, and when sacrificed on the 4th day pulmonary consolidation was found. The ferret which received the passage material from the animal possessing neutralizing antibodies had a mild febrile reaction on the 2nd day, but appeared perfectly well. When sacrificed on the 4th day, no pulmonary involvement was observed, although the nasal mucous membranes were swollen and contained some mucopurulent material from which hemolytic streptococci were recovered.

The fact that the two ferrets which possessed circulating neutralizing antibodies exhibited only a brief febrile reaction and failed to develop lung lesions, in contrast to the normal control animal of the same group, is of interest. Furthermore, passage from one of these 2 animals failed to elicit more than a minimal febrile reaction in a

normal ferret, whereas the animal which received material from the control ferret developed distinct pulmonary lesions. This indicates that following the experimental infection of the ferrets whose serum contained neutralizing antibodies the virus was either completely neutralized or persisted in only small amounts, since heavy suspensions of the tissues of these animals failed to induce a typical infection when injected into a normal ferret.

The fact that immune ferrets may be encountered among stock animals not infected experimentally must be taken into account in all studies relating to immunity. Whether the neutralizing antibodies present in the serum of these animals develop solely in response to contact infection of human origin, is a problem the solution of which must await further study. In view of these observations, it is extremely important that strict isolation be carried out in the care of animals used for experimental purposes.

Immunization of Rabbits

During the course of experiments with Rift Valley fever virus, it was found that rabbits which were inoculated intraperitoneally with the virus presented no evidence of infection. These animals, however, subsequently developed in the circulating blood neutralizing antibodies for the virus of Rift Valley fever (7).

It seemed important, therefore, to determine whether rabbits inoculated intraperitoneally with the influenza virus subsequently developed demonstrable neutralizing antibodies in their serum.

Two adult male Chinchilla rabbits were used. Serum was obtained for control purposes before inoculation. Both animals were then given, intraperitoneally, 5.0 cc. of a 10 per cent emulsion of lung and turbinates of a ferret infected with the P.R.8 strain of influenza virus. No fever or other evidence of infection occurred. 28 days later a similar inoculation was made by the same route. The serum obtained 14 days after the second injection was found to contain antibodies which neutralized both the P.R.8 and Phila. strains of virus in mice, while the serum before inoculation was ineffective. This immune serum was tested by Shope against the virus of swine influenza and found to neutralize that virus in mouse tests. Similar results have been obtained in rabbits inoculated with the Phila.

strain of virus. This evidence, like that obtained in the case of Rift Valley fever, indicates that rabbits, although apparently insusceptible to infection, are nevertheless capable of giving rise to the formation of specific antibodies in response to the antigenic action of the virus.

Immunization of Mice

The virulence of influenza virus for mice has been progressively enhanced by repeated passage through these animals. The virulence of the strains used in the present studies is such that 0.03 cc. of a 1:1000 dilution of infected mouse lung inoculated intranasally produces a fatal infection, and a similar amount of a 1:10,000 dilution produces pulmonary lesions from which the mice may recover. In contrast to the virulence by the intranasal route, it has been repeatedly observed that as much as 0.2 cc. of a 10 per cent suspension subcutaneously, or 0.5 cc. intraperitoneally, produces no evidence of infection. Similarly, suspensions of infected ferret lung or Berkefeld filtrates of lungs and turbinates which contain active virus, as shown by intranasal infection, produce no evidence of infection in mice when injected in large amounts by the subcutaneous or the intraperitoneal route. It was found, however, that animals so treated become actively resistant to intranasal infection with the virus.

Immunization of Mice with Influenza Virus (Strain P.R.8).—Each of 20 normal young mice was given, subcutaneously, 0.2 cc. of a 10 per cent broth emulsion of the lungs of mice infected with P.R.8 strain of human influenza virus. As controls, 20 normal mice were each given 0.2 cc. of a 10 per cent suspension of normal mouse lung by the same route. After 14 days the virus-vaccinated mice each received, intraperitoneally, 0.2 cc. of a 10 per cent suspension of the lungs of mice infected with P.R.8 virus. At the same time, the control mice each received the same amount, intraperitoneally, of a 10 per cent suspension of normal mouse lung. 1 week after the second injection, all mice, slightly anesthetized with ether, were inoculated intranasally with 0.05 cc. of a 10 per cent suspension of P.R.8 mouse virus. By the 6th day after inoculation all but one of the control mice vaccinated with normal mouse lung were dead, and at autopsy all exhibited consolidation of both lungs. The virus-vaccinated mice were all well and presented no evidence of infection. 6 of these mice were sacrificed and their lungs examined. No pulmonary lesions were detected. The remaining 14 mice which had been found actively immune to intranasal infection with the strain of virus used for vaccination were then retested to determine their resistance to a strain other than that used for immunization. To this end they were inoculated intra-

nasally with 0.05 cc. of a 10 per cent suspension of the Phila. strain of virus. All the vaccinated mice were found to be immune to the second strain of virus.

Similar results have been obtained in mice vaccinated against the Phila. strain of virus and subsequently retested with the P.R.8 strain of mouse virus. Furthermore, the serum of mice so treated has been found to contain antibodies which neutralize the infectivity of both strains of virus. In a similar manner mice have been vaccinated with virus-containing material derived from infected ferrets. Both unfiltered suspensions of virus material and Berkefeld filtrates of these suspensions have been used. The resultant active immunity appears to be quite as effective as that obtained in mice vaccinated with mouse passage virus. The living virus, therefore, functions as an immunizing agent irrespective of the species of animal from which the infectious material is derived. Smith, Andrewes and Laidlaw (8), in a recent article, have described their results regarding the immunization of mice by the subcutaneous inoculation of mouse virus.

Different intervals between the vaccinating injections have been tried, and the interval between the last vaccinating dose and the intranasal infection has been varied. The best results have been obtained, so far, by giving 3 consecutive subcutaneous injections at 7–10 day intervals, or with a single subcutaneous dose followed 2 weeks later by an intraperitoneal injection. Single subcutaneous or intraperitoneal doses of living virus have not, up to the present, resulted in effective immunity. Nevertheless, mice recovering from the experimental disease induced by intranasal infection have been found actively resistant to reinfection.

Certain groups of these immunized mice have been tested by Shope and found to be actively resistant to infection with swine influenza virus as well. The significance of these results will be considered in a later publication.

Neutralization Tests with Serum of Patients Suffering from Respiratory Infections

Serum was obtained from 3 patients admitted to the Hospital of The Rockefeller Institute suffering from influenza. The serum of these individuals during the acute and convalescent stages of the

disease was tested for the presence of neutralizing antibodies against both the P.R.8 and Phila. strains of influenza virus. In comparison with normal ferret or normal horse serum, most human sera have an inhibitory effect upon the activity of the virus. Nevertheless, the serum of these individuals taken during the acute stage of influenza failed to prevent the development of pulmonary lesions in mice inoculated with serum-virus mixtures, while the convalescent sera uniformly protected the animals. Furthermore, the antibodies develop-

TABLE I

Neutralization Tests in Mice with Serum of Influenza Patients

Serum		Influenza virus (P.R.8 strain)			
		Severity of pulmonary lesions in mice			
		No. 1	No. 2	No. 3	No. 4
H.F.	Acute	+++++	++++	+++	+++
	Convalescent	0	0	0	0
	6 mos. later	0	0	0	0
B.P.	Acute	++	++	+++	++
	Convalescent	0	0	0	±
	6 mos. later	0	0	0	±
M.B.	Acute	++	+	++	+++
	Convalescent	0	0	0	0
	6 mos. later	0	0	0	0

0 = no pulmonary lesion.

± to +++++ = progressive degrees of pulmonary involvement.

ing in early convalescence were found to persist for 6–8 months at least.

Similar tests were made with the serum of patients acutely ill with, and convalescent from, pneumococcus pneumonia. In general, the effect of convalescent pneumonia serum upon the influenza virus appeared to be no different from that of the serum taken at the height of the illness. Neutralizing antibodies for the influenza virus did not develop in response to the pneumococcus infection. If neutralizing antibodies were present at the onset of the illness, they were likewise present in the convalescent serum. In the one instance in which

neutralizing antibodies did develop in convalescence from pneumonia, it seemed quite likely from the clinical history that influenza may have been associated with the onset of the pneumonia.

TABLE II

Neutralization Tests in Mice with Serum of Pneumonia Patients

Serum		Influenza virus (P.R.8 strain)			
		Severity of pulmonary lesions in mice			
		No. 1	No. 2	No. 3	No. 4
M.C.	Acute	0	0	0	0
	Convalescent	0	0	0	0
J.C.	Acute	+++	++	++	0
	Convalescent	+++	++	+	0
A.W.	Acute	++	±	+	±
	Convalescent	++	+	++	0
R.O.	Acute	0	+	0	++
	Convalescent	0	+	0	++
C.P.	Acute	+	0	+++	+
	Convalescent	++	+	+	0
C.M.	Acute	0	0	+	0
	Convalescent	0	0	0	+
H.C.	Acute	+++	+++	++	+++
	Convalescent	+++	+++	++	++++
B.W.	Acute	+	++	++	+
	Convalescent	0	0	0	0
Control influenza H.F.	Acute	+++	++	++	+++
	Convalescent	0	0	0	0

0 = no pulmonary lesions.

± to ++++ = progressive degrees of pulmonary involvement.

Further studies were made with the serum of 4 individuals taken before, during and after the course of a common cold. In the 4 cases studied, the effect of the individual's serum on the influenza virus was entirely uninfluenced by the common cold.

These results indicate that the development of antibodies which neutralize the influenza virus is a specific response to the infectious agent, and that the virus is causally related to the human disease—influenza.

TABLE III

Neutralization Tests in Mice with Serum of Human Individuals before, during and after a Common Cold

Serum		Influenza virus (P.R.8 strain)			
		Severity of pulmonary lesions in mice			
		No. 1	No. 2	No. 3	No. 4
R.L.	Before cold	++	+	++	++
	During "	+	+	++	+
	After "	++	+	++	+
T.A.	Before cold	+	+	++	++
	During "	+	++	+	+
	After "	±	++	±	+
F.H.	Before cold	0	±	0	0
	During "	0	0	0	0
	After "	0	+	0	0
T.F.	Before cold	+++++	+++	+++	+
	During "	+++++	+++++	+++++	++++
	After "	++	++++	++	++

0 = no pulmonary lesions.

± to +++++ = progressive degree of pulmonary involvement.

DISCUSSION

The evidence presented in this report demonstrates that a state of immunity as measured by circulating antibodies and active resistance follows recovery from infection with the virus of influenza. That the mere presence of neutralizing antibodies in the circulating blood may not necessarily assure a complete refractory state to reinfection is recognized. Ferrets which have developed neutralizing antibodies following experimental or presumably direct infection exhibit little or no reaction to reinfection. In certain instances, however, reinoculation may elicit a brief febrile reaction without other evidence of in-

fection. Attempts to recover virus from these animals have in general been unsuccessful. The results in experimental animals indicate that although the immunity acquired as a result of infection may not be sufficiently absolute to prevent febrile reactions on reinfection, the virus is quickly neutralized and from these animals is not so readily recoverable as it is from normal animals infected for the first time. If a similar set of circumstances prevails in the natural disease in man, the experimental results suggest a possible explanation for the lack of uniform success in attempts to recover virus from all patients with influenza.

Virus neutralization tests with serum of influenza patients taken during the acute stage of the disease, during early convalescence and at later periods, have shown that the serum of the individual at the height of the disease fails to neutralize the influenza virus, whereas serum taken from the same patient during convalescence does contain specific antibodies. These antibodies are not evanescent, but persist for several months at least, as evidenced by the neutralizing capacity of serum obtained from patients 6–8 months after recovery from influenza.

Similar studies with the serum of patients ill with, and recovering from, pneumococcus pneumonia, have shown that in general specific antibodies neutralizing the influenza virus do not develop in response to pneumococcus infection. Studies of the antibody content of the serum of human individuals before, during and after a common cold were made. The results indicate that this type of respiratory infection does not stimulate the formation of antibodies against the virus of influenza. It appears, therefore, that the neutralizing action of the serum of human individuals is a specific response to infection with the influenza virus.

That the virus of swine influenza is not serologically identical with the strains of virus recently isolated from human cases of influenza seems definitely established (5). Nevertheless, that the strains of human influenza virus and of swine influenza virus are related is shown by the active cross-immunity in ferrets (6), and in mice immunized with the P. R. 8 or Phila. virus, as well as by passive neutralization of swine influenza virus by the serum of rabbits immunized with P. R. 8 virus. Further consideration of this problem will form the basis of a

subsequent report. It has been suggested, moreover, that the virus of swine influenza is the etiological agent which gave rise to the 1918 pandemic of influenza in man (6). If this is subsequently shown to be true, the problem of immunity to influenza will of necessity involve consideration of the possible existence of multiple strains of virus of related but not wholly identical antigenic structure.

SUMMARY

Following infection with the virus of influenza, both ferrets and mice develop a state of active immunity to reinfection. The serum of these animals contains neutralizing antibodies, as evidenced by the capacity of the serum to confer passive protection to mice against infection with the P.R.8 and Phila. strains of the virus of human influenza.

Rabbits which are apparently insusceptible to infection with the virus of influenza produce specific antibodies in response to repeated injection of virus-containing material. The serum of immunized rabbits affords passive protection to mice against mouse-virulent virus.

Although the subcutaneous or intraperitoneal injection of the living virus does not produce infection in mice, animals so treated acquire active immunity against subsequent infection by the intranasal route.

Neutralization tests with the serum of patients before and after recovery from influenza, pneumonia and the common cold indicate that neutralizing antibodies arise as a specific response to infection with the virus of influenza.

The immunological identity of strains of influenza virus recovered from human sources has been established, and the possible existence of strains of related, but not identical, antigenic structure is discussed.

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