THE INCIDENCE OF NEUTRALIZING ANTIBODIES FOR HUMAN INFLUENZA VIRUS IN THE SERUM OF HUMAN INDIVIDUALS OF DIFFERENT AGES

By THOMAS FRANCIS, Jr., M.D., and T. P. MAGILL, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, January 14, 1936)

Evidence has been presented which shows that, following experimental infection with the virus of human influenza, animals develop a state of resistance to reinfection, and the sera of such recovered animals manifest a specific power to protect mice against infection with the homologous virus (1–3). Furthermore, the sera of human individuals develop protective properties against human strains of virus following recovery from naturally acquired influenza (3). The antibodies which develop have been found to persist for 6 to 8 months at least.

Since the presence of antibodies against the human influenza virus in the serum appears to be an expression of a previous infection with the virus, it is important to determine the frequency with which these antibodies occur in the serum of human individuals in the population at large. To this end, serum was obtained from 136 human beings in New York, Philadelphia, Princeton, and Baltimore, and the sera were tested for their capacity to protect mice against a strain of human influenza virus (P. R. 8). So far as possible, histories concerning previous attacks of influenza were also obtained from the same individuals.

The present report deals with the results of mouse protection tests made with these sera against a strain of human influenza virus (P. R. 8). The individuals from whom the sera were obtained ranged from newborn infants to adults in the eighth decade of life.

Methods and Materials

Sera.—The samples of serum used in these tests were derived from clotted venous blood. The majority of the children's sera were obtained from children

in the wards of the Hospital for the Ruptured and Crippled, New York City.[1] In practically all instances the children were hospitalized because of orthopedic disabilities.

Most of the specimens of serum of elderly individuals were obtained from healthy persons at the Baltimore City Hospital, Baltimore.

Serum was obtained from 18 individuals of various ages in Philadelphia.[2]

A few specimens were obtained by Dr. Shope at Princeton.

The majority of the other samples were obtained from staff members or attendants at The Rockefeller Institute.

Virus.—The strain of virus employed in these tests was recovered from an epidemic of influenza in Puerto Rico (4), and has been maintained in white mice through serial passages by the inoculation of suspensions of infected mouse lungs into the nasal passages of normal mice.

Mouse Protection Tests.—The capacity of serum to protect white mice from the action of the virus was tested in the following manner.

Mice infected with virus were killed when moribund, and their lungs removed with aseptic precautions. Weighed amounts of the infected mouse lung were ground with alundum and suspended in physiological salt solution to form a 10 per cent suspension. The suspension was centrifugalized for 10 to 15 minutes at 1500 R.P.M. The supernatant liquid was removed and 0.3 cc. of the fluid was mixed with 0.3 cc. of the serum to be tested. The serum-virus mixture was incubated at 37°C. for 30 minutes. Each of 4 Swiss mice was then inoculated intranasally, while anesthetized with ether, with 0.03 cc. of the mixture.

The mice were observed for 6 days after inoculation. All animals which died during this period were autopsied, and their lungs examined. On the 6th day, all survivors were killed and their lungs were examined for gross pulmonary lesions. The severity of the pulmonary lesions in each mouse was graded by the amount of pulmonary tissue involved as viewed by the naked eye. The various degrees of involvement varied from ±, in which only small pinpoint areas of congestion were seen, to ++++, in which all five lobes of the mouse lung were completely involved. The result of the test with a given serum was determined by making an approximate average of the extent of the lesions in the 4 mice inoculated with each serum-virus mixture. For example, if the lesions in 4 mice were +, +, +, ++, respectively, the final result would be considered +. If the lesions were ++, +++, +, ++, respectively, the result would be classified as ++. If +, +, 0, 0, the result would be considered incomplete protection and graded ±; but for practical purposes these latter results may be considered to indicate complete protection. Mice presenting average lesions of +++ or greater were considered not to have been protected, while in those tests in which an average of +, or ++, lesions appeared, the serum was considered to have afforded partial protection to the mice. In the absence of pulmonary lesions, the result was accounted complete protection.

---

[1] Through the courtesy of Dr. Stefanson.

[2] Through the kindness of Dr. Joseph E. Stokes, Jr., and Dr. Stuart Mudd.

With each series of tests, control tests were made, using one sample of human serum which did not protect mice, and another which afforded complete protection to mice. In all instances the results with the control sera were negative and positive, respectively.

While certain variations in the extent of the pulmonary lesions obtained with the same serum in different tests were observed, they were usually of a minor nature, and were never of sufficient degree to require a complete reclassification of the serum. With the strong concentration of virus used, a 10 to 20 per cent variation in the concentration of virus in different tests might occur without seriously altering the results. Another source of variation which was impossible to control was the size of the mice. So far as possible, mice 4 to 6 weeks of age were used. However, considerable differences in the size of animals occurred, and it has been recognized that large mice are more resistant to infection than small ones of 12 to 15 gm.

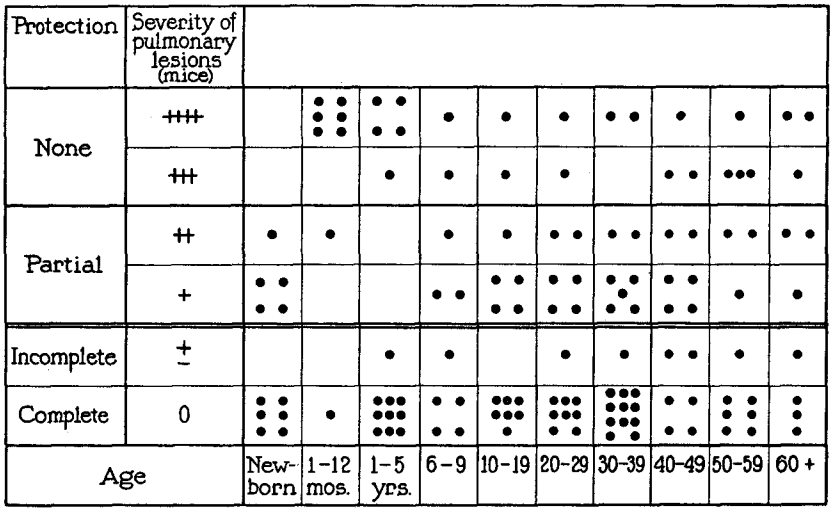

CHART 1. The results of mouse protection tests made with human serum classified according to age of donor. Human influenza virus (P.R. 8).

RESULTS

The results are presented in Chart 1, in which each black circle represents the test made with the serum of a single individual, and the results are distributed according to the age of the donor from whom the serum was obtained. The number of individuals in the different age groups varies, with a relative preponderance of children below 5 years of age, and a disproportionately small number between the ages of

10 and 19. While the entire group is small from a statistical point of view, the trend of the results is sufficiently regular to appear significant.

The sera have been classified, as affording complete protection, partial protection, or no protection. Complete protection was effected by 49 per cent of all sera, partial protection by 29 per cent, and 21 per cent were considered to be non-protective. Distinct differences were noted in the percentage of protective sera at different ages. These figures are presented in Table I and Chart 2.

TABLE I

The Protective Action of Sera of Individuals of Different Ages against Human Influenza Virus

Age of donor	No. of sera	Sera affording					
		Complete protection		Partial protection		No protection	
		No.	*per cent*	*No.*	*per cent*	*No.*	*per cent*
Newborn	11	6	54.5	5	45.5	0	0.0
1 mo.–1 yr.	8	1	12.5	1	12.5	6	75.0
1–5 yrs.	15	10	66.6	0	0.0	5	33.3
6–9 "	10	5	50.0	3	30.0	2	20.0
10–19 "	12	7	58.3	3	25.0	2	16.6
20–29 "	17	9	52.9	6	35.3	2	11.8
30–39 "	23	12	52.1	9	39.1	2	8.8
40–49 "	16	6	37.5	7	43.7	3	18.8
50–59 "	14	7	50.0	3	21.4	4	28.5
60+ "	10	4	40.0	3	30.0	2	20.0
Total.....	136	67	49.2	40	29.4	29	21.3

The frequency of completely protective sera in newborn infants is approximately the same as in middle aged individuals. After the 1st month of life, a sharp drop in the percentage of positive sera occurs and persists through the 1st year of life. Between the 1st and 5th years of life, however, the percentage of positive results reaches its height, and 66 per cent of the sera in this age group possess the capacity to protect mice completely against the virus. From that time until the 40th year of life, complete protection was nearly or quite as frequent; thereafter the incidence of protective sera declined somewhat. The trend of the findings is more even if the age groups are combined so as to include greater numbers in each group (Chart 2).

The results have also been classified according to whether or not the individuals from whom serum was obtained had experienced an attack of influenza (Table II). Tests with the sera of children below the age of 12 years were not included because of the frequent lack of satisfactory histories. In spite of the inaccuracies which occur, a definite

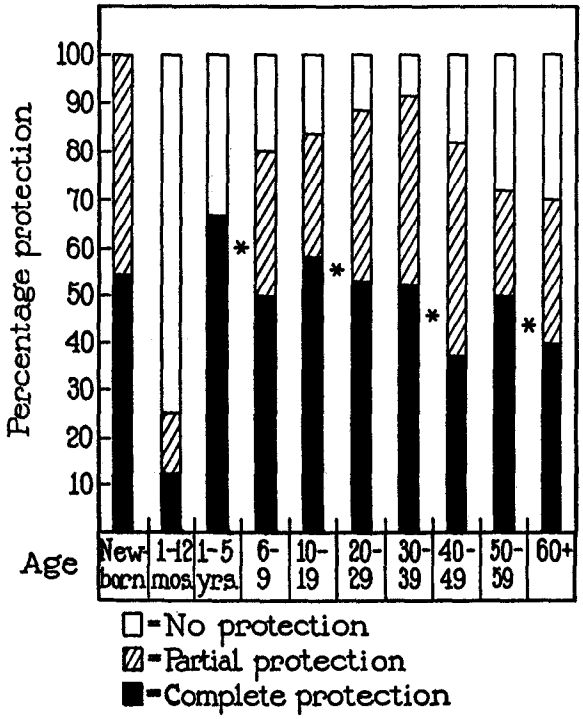

CHART 2. The percentage of human sera exerting protective action against human influenza virus (P.R. 8).

* Indicates per cent of completely protective sera when the two adjacent age groups are combined.

statement regarding previous influenza was obtained from 78 individuals. In this group there appears to be some relation between a history of influenza and a strongly protective serum, since 55.5 per cent of the individuals who were said to have had influenza, and only 39.3 per cent of those without histories of influenza, possessed sufficient circulating antibody to furnish complete protection to mice.

The sera of the individuals giving positive histories of influenza may again be grouped according to the time at which the illness was experienced (Table III). The results show that the sera of individuals alleged to have had influenza in 1918 were equally divided among the protective and non-protective groups, a result not largely different from the average of the entire series which was studied. The individ-

TABLE II

The Relation of Results of Serum-Virus Protection Tests to History of Influenza

History of influenza	No. of sera	Protecting	Not protecting
		per cent	*per cent*
Yes	45	55.5 (25)	44.5 (20)
No	33	39.3 (13)	60.7 (20)
Total.....	78	48.7	51.3

Numbers in parentheses indicate the number of samples included.

TABLE III

The Relation of the Time of Illness to Protective Property of the Serum

Time of illness	No. of sera	Protective	Non-protective
1918–23	18	9	9
1933–35	12	10	2
1918 and 1930–35	6	3	3
Indefinite	11	3	8
1934–35 (Convalescents, Puerto Rico and Alaska)	19	17	2

uals who did not clearly remember the time of illness were in the older age groups, and may be considered not to have suffered from influenza recently. On the other hand the incidence of completely protective sera was considerably greater among the 12 individuals in this series who had suffered from influenza in the past few years, the majority of whom were observed by one of us. In addition to the 12 samples included in this series, taken from the population along the eastern

seaboard, it has been possible to test the convalescent serum of 4 subjects in Puerto Rico from whom the original virus material was obtained, and the sera of 15 individuals convalescent from the influenza epidemic which occurred in Alaska in May, 1935.[3] Complete protection resulted with 27, or 87 per cent, of these 31 sera, an incidence of positive sera not otherwise encountered. These results indicate clearly that the occurrence of recent influenza in a group of individuals is followed by a high proportion of strongly protective sera.

In addition to the sera which contained sufficient antibody to protect mice completely against the human influenza virus, 29 per cent were found to afford partial protection; the latter may, therefore, be considered protective sera of lower titer than those of the former group. With this granted, the sera of all new-born infants are found to contain protective antibodies. After the 1st year of life the incidence of antibodies increases to its maximum between the 20th and 40th years, when 90 per cent of the sera confer complete or partial protection on mice. After the 40th year a gradual but progressive decline in the percentage of protective sera occurs.

Quantitative Aspects of the Protective Tests.—Certain sera which were tested by the present method were also tested by Andrewes, Laidlaw, and Smith (5). These sera were the ones mentioned above, which were obtained from Alaskan convalescents, and in addition there were 6 specimens of dried serum from Philadelphia.[4] The British workers attempt to measure the antibody content of a serum more accurately by testing the protective action of progressive dilutions of the serum against a filtrate of virus suspension. The results with the unknown serum are then interpreted by direct comparison with corresponding dilutions of a standard hyperimmune horse serum. The serum is given a rating on this basis. Thus, S indicates that the test serum is as potent as the standard; S/5 or S/25 indicates that the unknown serum is 1/5 or 1/25 as strong as the standard.

A comparison of the results obtained when the same sera were tested by the method used in the present study and by the method of the

[3] The Alaskan sera were obtained by Drs. H. Pettit and D. S. Pepper, of the University of Pennsylvania, immediately following the epidemic, and were kindly made available to us by Dr. Mudd.

The serum of the Puerto Rican subjects was obtained by Dr. W. C. Earle, of the International Health Division of the Rockefeller Foundation, to whom we should like to express our appreciation.

[4] Kindly sent to us by Dr. Mudd and Dr. Stokes.

TABLE IV

Comparison of Results Obtained with Dried Serum

Sample No.	Severity of pulmonary lesions in Mouse No.				Result	Andrewes' results (5)
	1	2	3	4		
C-91	++++	++++*	++++*	+	NP	S/25
C-74	++	+	+	+	PP	S/25
C-109	0	0	+	±	IP	S/25
C-111	0	0	0	0	CP	S
C-82	0	0	0	0	CP	S
C-121	+++	+++	++++	++++	NP	S/25

NP = no protection.
PP = partial protection.
IP = incomplete protection.
CP = complete protection.
0 = no gross pulmonary lesions.
± to ++++ = progressive degrees of pulmonary involvement.
S = test serum equal in potency to standard serum.
S/5 = " " " " " " " " " diluted 1/5.
S/25 = " " " " " " " " " " 1/25.
* = mouse died.

TABLE V

Comparison of Results Obtained in Tests Made with Alaskan Sera

Serum No.	Influenza convalescent	Severity of pulmonary lesions in Mouse No.				Result	Andrewes' classification (5)
		1	2	3	4		
1	22 yrs., 6 wks. ago	0	0	0	+	CP	S—S/5
2	60 " severe, 3 wks. ago	0	0	±	0	CP	S
3	40 " mild, 2 " "	0	0	0	0	CP	S/25
4	16 " " 1 wk. ago	0	0	0	0	CP	S
5	70 " severe, 2 wks. ago	0	0	0	0	CP	S—S/5
6	63 " " 2 " "	+	±	++	++	PP	S/5—S/25
7	70 " " 2 " "	0	0	0	0	CP	S
8	44 " " 1 wk. ago	+	0	0	0	CP	Less than S/25
10	17 " mild, 2 wks. ago	0	0	0	0	CP	S
11	21 " severe, 2 " "	0	0	0	0	CP	S/5
13	42 " mild, 2 " "	0	±	0	0	CP	S/5—S/25
14	18 " " 2 " "	0	0	0	0	CP	S/5
15	19 " " 2 " "	0	0	±	0	CP	S/5
16	25 " severe, 3 " "	+	+	++	+	PP	Less than S/25
17	19 " mild, 1 wk. ago	0	0	0	0	CP	S—S/5

English workers shows that the correlation is quite good (Tables IV and V). All the sera which the latter workers have assayed S or S/5 have been found by our method to be completely protective. Among the sera which are graded as S/25 by the titration method, the agreement is not so consistent; certain of them have, in our hands, been completely protective while others protect partially or very little. Nevertheless, the results indicate that both methods afforded information indicative of the antibody content of the sera studied. The severity of lung lesions in the mouse which result from the intranasal inoculation of a serum-virus mixture is inversely proportional to the antibody content of the serum.

DISCUSSION

The present report presents the results of tests designed for the demonstration of protective antibodies against a strain of human influenza virus in the serum of 136 individuals of all ages. In addition to the fact that statistically their numbers are small, interpretation of the results is limited by several factors: (*a*) that the sera were collected in three large cities along the Atlantic seaboard; (*b*) that the age distribution of the individuals from whom the sera were collected was not the same in all three cities; (*c*) that the history of influenza is unreliable; and (*d*) that the previous data regarding persistence of immunity following influenza are very inconclusive. Nevertheless, the similarity to the results recently reported by Andrewes, Laidlaw, and Smith (5) with sera of human individuals of different ages in England suggests that the results are significant. Although their tests were made with a different strain (W. S.) of human influenza virus, the percentages of completely protective sera at different age periods found in the present study do not differ greatly from theirs.

For purposes of discussion it may be assumed that, apart from newborn infants, the presence of specific protective antibodies in the serum of an individual is the result of past infection with the virus of human influenza. Evidence for the validity of this assumption has previously been presented, which shows that recovery from influenza in the human individual has been accompanied by the development of circulating antiviral bodies, whereas, in the course of the ordinary common cold or of pneumococcus pneumonia, the development of antibodies

against the human influenza virus has not been observed (3). Moreover, the distribution of the virus infection is widespread, as evidenced by the fact that in the past 2 years strains of virus have been recovered from epidemics of human influenza in England (1, 7), Puerto Rico, the United States (2), and Alaska (7). In view of these facts it is reasonable to utilize the results of the protection tests in an attempt to interpret the epidemiology of human influenza.

Considering only those sera which contained sufficient antibody to protect mice completely against human influenza virus, it was found that, excluding the 1st year of life, influenza attacks individuals of all ages. 59 per cent of those between 1 and 20 years of age, 52.5 per cent of those between 20 and 40 years, and 42.5 per cent of those over 40 years of age possessed a high titer of protective antibodies. The occurrence of antibodies at all ages agrees with the customary experience that in outbreaks of influenza individuals of all ages are attacked. The high incidence of antibodies in children and young adults constitutes further evidence that the human influenza virus is prevalent at the present time.

The interpretation of the results of protection tests with sera which afford partial protection to mice is at the present time impaired by lack of knowledge. Except when based upon history—and this is unreliable—observations regarding the persistence of anti-influenzal antibodies for more than 1 year have not been made. Furthermore, the presence of protective antibodies in the blood of adults does not indicate whether they were acquired as the result of a recent infection or whether they have persisted for years following an attack of influenza. That the partially protective action of a serum does not belong in the category of natural antibodies is shown by the fact that this capacity is not destroyed by heating the serum at 60°C. However, 2 cases of influenza have been observed in which the serum of the individual taken at the height of the disease exerted partial protection against the virus, but after recovery the serum completely neutralized the virus. These observations suggest that other individuals whose sera do not protect completely against the virus of human influenza may be susceptible to infection with the virus. Moreover, of 31 sera obtained from individuals who had suffered from influenza in 1934–35, 27, or 87 per cent, possessed sufficient antibody to neutralize the virus completely.

It seems quite probable, therefore, that the individuals from whom the partially protective sera were obtained are those who have suffered from influenza in the past, but whose circulating antibodies have decreased with the passage of time. This interpretation is supported by an observation of Andrewes, Laidlaw, and Smith (5), in which they noted a marked decline in the antibody titer of the serum of an individual within 2 years following the attack.

If the partially protective sera are included with the fully protective sera, the trend of the findings is modified. Under these conditions all newborn infants are found to possess antibodies, most probably of maternal origin. These are lost to a great extent in the 1st year of life, and are superseded by antibodies presumably developed in response to infection. Between the 1st and 5th years the serum either protects completely or not at all, suggesting recent infection. Thereafter, the incidence mounts to the 20 to 40 age period, when 90 per cent of the sera contain measurable amounts of protective antibodies. This peak may be attributed to the increased percentage of partially protective sera during these years. Beyond the 40th year the percentage of negative sera increases. Whether the decline in the percentage of positive results among older persons indicates that these individuals have wholly escaped infection, or whether it indicates a further step in the loss of antibodies previously acquired, is problematic. If, however, as the statistics of the 1918 epidemic indicate (8), the case rate was highest in children and young adults, the high incidence of antibodies in the present age groups between 20 and 40 years of age might be considered to be a result of infection experienced in 1918. The decreased percentage of protective sera in the older age groups corresponds with the decreased incidence of infection in adults in 1918. Regardless of the relation of the present study to the epidemic of 1918–19, the results suggest that the human influenza virus is related etiologically to the disease which has prevailed in the isolated epidemics of recent years.

Shope (9) tested 124 of the sera used in the present study against the virus of swine influenza. Certain distinct differences appear in the distribution of antibodies against swine influenza virus when compared with those against human influenza virus. The chief differences observed are, that against swine influenza virus little or no protective

action is observed with sera of children under 10 years of age, whereas practically all adult sera protect completely against the swine virus. The period of highest incidence of protective antibodies against swine influenza virus is the same (from 20 to 40 years) as that in which the combined percentage of complete and partial protection against human influenza virus is greatest.

The serum of 40 of 79 individuals over the age of 12 years exerted complete protection against both human and swine strains of influenza virus. On the other hand, certain sera protected completely against human (P. R. 8) virus, but not against the swine virus, and *vice versa*. It is seen, therefore, that in addition to the differences in the protective action noted in the serum of children when tested against the human and swine influenza viruses, specific differences are to be observed in the protective action of the serum of adult individuals tested against the human and swine viruses.

Studies (10) with the sera of animals convalescent from experimental infection with human or swine influenza virus, or animals hyperimmunized by repeated inoculations of virus, have shown that the immune response to the primary infection is specific for the virus employed. After repeated reinfections, however, the serum may develop a broader zone of activity, and may confer some degree of passive immunity to mice against the heterologous virus as well. This effect is most noticeable in animals repeatedly infected with the human influenza virus, and it suggests that the swine influenza virus possesses antigenic components which are also present in the human influenza virus as secondary antigens. It seems possible that a similar set of conditions may obtain in the human population. The serum of children 1 to 5 years old exerts a specific effect against the human influenza virus, owing presumably to a single attack. The serum of older children and adults who may have had repeated exposures to the human influenza virus might, on the other hand, have developed the broader immune zone which would then afford a certain degree of cross-protection against the virus of swine influenza. Conversely, if human individuals had been attacked by the virus of swine influenza, partial protection might be exhibited by the serum against human influenza virus.

The recent studies by Paul and Trask (11), with different strains of

poliomyelitis virus deserve to be considered in this general relation. It was found that in neutralization tests performed with the serum of normal children or of children convalescent from poliomyelitis against the older passage strain of poliomyelitis, no difference could be detected between the effects of normal and convalescent serum. The results appeared to bear a closer relationship to age than to actual illness. When the same sera were tested against a more recently isolated strain, it was found that convalescent children's serum protected against this strain, while normal serum did not. The age differences in serum protection against the older strain of poliomyelitis virus are very similar to those noted by Shope (9) with the serum of human individuals tested against swine influenza virus. The high incidence of protective antibodies in the serum of recent poliomyelitis convalescents against the recently recovered strain of poliomyelitis is also similar to that obtained with the serum of recent influenza convalescents against the human influenza virus.

No attempt at a final interpretation of the epidemiological significance of the results of the present study can be made. Sufficient evidence regarding the persistence of immunity to human influenza has not yet been accumulated to justify detailed inferences concerning the prevalence of influenza in the past. The proportion of positive sera in young children certainly indicates a prevalence of infection due to the human influenza virus at the present time. This indication is strengthened by the recovery of strains of the virus during recent epidemics of human influenza, as well as by the fact that the serum of recently recovered individuals, in the great majority of instances, possesses a high titer of antibodies against strains of human influenza virus.

SUMMARY

The results of mouse protection tests with 136 human sera and a strain of human influenza virus are described. After the 1st year of life, the sera of approximately half the individuals tested contained sufficient antibody to furnish complete protection to mice. A much higher percentage of the sera obtained from individuals recently convalescent from influenza exerted a completely protective effect. On the other hand, certain sera protected only partially under the conditions of the tests.

The results have been compared with those obtained by Shope in tests done with the same sera against swine influenza virus. The possible epidemiological significance of the results is discussed.

BIBLIOGRAPHY

1. Andrewes, C. H., Laidlaw, P. P., and Smith, W., *Lancet*, 1934, **2**, 859.
2. Francis, T., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 1172.
3. Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1935, **62**, 505.
4. Francis, T., Jr., *Science*, 1934, **80**, 457.
5. Andrewes, C. H., Laidlaw, P. P., and Smith, W., *Brit. J. Exp. Path.*, 1935, **16**, 566.
6. Laidlaw, P. P., *Lancet*, 1935, **1**, 1118.
7. Francis, T., Jr., unpublished data.
8. Jordan, E. O., Epidemic influenza, Chicago, The American Medical Association, 1927.
9. Shope, R. E., *J. Exp. Med.*, 1936, **63**, 669.
10. Francis, T., Jr., and Shope, R. E., *J. Exp. Med.*, 1936, **63**, 645.
11. Paul, J. R., and Trask, J. D., *J. Exp. Med.*, 1935, **61**, 447.