A COMPLEX VACCINE AGAINST INFLUENZA A VIRUS

Quantitative Analysis of the Antibody Response Produced in Man

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Numerous studies have been made to determine the effectiveness in man of vaccines containing influenza A virus (1). Andrewes and Smith (2) showed that the subcutaneous administration of formolized filtrates of mouse lungs infected with the virus caused an increase in neutralizing antibodies in human beings. Francis and Magill (3, 4) found that active tissue culture virus given either subcutaneously or intradermally stimulated the production of neutralizing antibodies in a group of volunteers selected because of their low level of antibodies. Stokes, Chenoweth, Waltz, Gladen, and Shaw (5) and Stokes, McGuinness, Langnor, and Shaw (6) vaccinated large groups of human beings intramuscularly with either active tissue culture or active mouse lung virus and followed these and larger groups of unvaccinated persons through epidemics of respiratory disease which they considered to be influenza A (1). These authors found that the attack rate for "febrile respiratory disease" was lower in vaccinated than in unvaccinated groups. Smith, Andrewes, and Stuart-Harris (7) and Taylor and Dreguss (8) studied the antibody response in man which followed the subcutaneous injection of formolized mouse lung virus. These authors also followed large groups of vaccinated volunteers and unvaccinated control groups through acute respiratory disease epidemics which they showed to be influenza A. They obtained no evidence that vaccination had produced immunity to the clinical disease. There is as yet no conclusive evidence that any one of the various vaccines which have been studied regularly produces active immunity to influenza A in man.

It has been shown previously (9) that a formolized complex vaccine containing both influenza A virus and the X strain (10) of canine distemper virus was very effective in immunizing ferrets against influenza A virus. A single subcutaneous injection of this vaccine in normal ferrets stimulated the production of antibodies in a concentration entirely comparable with that which followed actual infection by influenza A virus. It was also shown (9) that ferrets which had been given the complex vaccine were solidly immune to infection by antigenically different strains of influenza A virus for at least 3 months. Preliminary observations on small numbers of human volunteers who received the complex ferret vaccine were also

reported (9). It seemed possible that if the complex vaccine resulted in the production of a concentration of antibodies in man comparable with that observed in ferrets, it might prove more effective as a prophylactic agent against influenza A than other types of vaccines thus far studied. Consequently, an investigation of the specific antigenicity of various single and complex influenza A vaccines was undertaken. It is the purpose of this paper to report the results of quantitative serological studies on groups of human volunteers who were given these vaccines.

Methods

VIRUSES.—

Influenza A Virus.—The following three derivatives of the PR8 strain (11) maintained in three distinct biological environments during the past 6 years in this laboratory were used.

PR8-f has been kept continuously in ferrets, having been carried through 91 consecutive passages in this species.

PR8-m, after a number of passages in ferrets, was adapted to mice and since has been carried through 332 mouse lung passages.

PR8-tc, after 70 mouse lung passages, was introduced into chick embryo-Tyrode-tissue culture (12) and has been carried through 533 continuous passages in this medium.

For the purposes of this investigation, PR8-f and PR8-m were each inoculated on the chorio-allantoic membranes of developing chick embryos (13), and both were carried independently through 9 consecutive serial passages. 9 to 10 day old embryos were used. 0.1 cc. of virus suspension was inoculated onto the membrane in an artificial air sac. The embryos were incubated for an additional 4 days after inoculation. The infected embryos and the chorio-allantoic membranes were then harvested, pooled, and ground to a 10 per cent suspension in 0.85 per cent NaCl. This suspension was used for passage.

The W.S. strain (14) was a mouse-adapted strain which had been maintained subsequently by serial passage in ferrets.

Canine Distemper Virus.—Two derivatives of the X strain (9) of canine distemper virus, which was encountered accidentally in ferrets inoculated with strain 188 (15) of influenza A virus, were used.

X-f, ferret passage series. This strain had been passed repeatedly in ferrets and was known to produce typical signs of distemper after either subcutaneous or intranasal injection (10). Moreover, it had been shown to alter markedly the usual course of influenza A virus infection in ferrets (10).

X-ch, chick embryo passage series. For the purposes of this study, attempts were made to propagate the X strain in developing chick embryos. Plummer (16) has reported previously the successful passage of canine distemper virus on the chorio-allantoic membrane. After a number of unsuccessful attempts, it was found that the X strain could be maintained in an active state for at least 5 passages on the membrane. 9 day old white Leghorn embryos were used. 0.1 cc. of an unfiltered 10 per cent suspension in 0.85 per cent NaCl of infected membrane was dropped onto the chorio-allantoic membrane in an artificial air sac. The embryos were then incubated an additional 6

days. Ferrets were inoculated intranasally with suspensions prepared from membranes of the 1st, 4th, and 5th passages, and all developed typical signs of severe distemper 8 to 11 days after inoculation.

The Y strain (10) of canine distemper virus was obtained from the spleen of an infected dog. This strain was also capable of producing typical signs of distemper after inoculation in ferrets. It had been shown previously (10), however, that the Y strain in no way changed the normal course of influenza A virus infection in this species.

Preparation of Experimental Vaccines.—

Vaccine No. 1. Tissue Culture.—Influenza virus *PR8-tc* was introduced into flasks containing 5 cc. of minced chick embryo-Tyrode-tissue culture medium. The flasks were incubated for 48 hours at 37°C. The liquid contents were then centrifuged for 30 minutes at 8000 R.P.M. at 4°C. The undiluted supernate was kept in a low temperature storage cabinet (17) at −76°C. and was not thawed until immediately before it was used for vaccination. This vaccine corresponded quite closely to that used by Francis and Magill (4).

Vaccine No. 2. Mouse Lung.—Influenza virus *PR8-m* was inoculated intranasally into a large number of lightly anesthetized mice. The infected mouse lungs were harvested aseptically 4 days later. The lungs were cultured individually and stored in the low temperature cabinet for 48 hours. They were then thawed, and those that were bacteriologically sterile were pooled and ground to a 10 per cent suspension in 0.85 per cent NaCl. This suspension was centrifuged for 30 minutes at 8000 R.P.M. at 4°C. Sufficient formalin was added to make a final concentration of formaldehyde of 1:5000. The suspension was then kept at 4°C. for 7 days, after which the excess formaldehyde was neutralized by the addition of sufficient ammonia water to make a final concentration of 1:8000 ammonia. The inactivated vaccine was stored at −76°C. and was not thawed until immediately before use. This vaccine corresponded rather closely to those used by Smith, Andrewes, and Stuart-Harris (7) and by Taylor and Dreguss (8).

Vaccine No. 3. Ferret Lung and Spleen.—Influenza virus *W.S. strain* was inoculated intranasally in an anesthetized normal ferret. 3 days later the ferret was killed, and the lung and spleen were removed aseptically. Approximately 90 per cent of the lung showed typical consolidation. Equal amounts by weight of the lung and spleen were ground to a 20 per cent suspension in 0.85 per cent NaCl. The suspension was centrifuged for 20 minutes at 2000 R.P.M. The supernate was diluted to 10 per cent, and sufficient formalin was added to give a final concentration of formaldehyde of 1:4000. This suspension was stored at 4°C. for 10 days, after which the excess formaldehyde was neutralized by the addition of sufficient ammonia water to make a final concentration of 1:8000 ammonia. The inactivated vaccine was stored at −76°C. and was not thawed until immediately before it was used.

Vaccine No. 4. Complex Ferret Lung and Spleen.—A normal ferret was inoculated intranasally with a mixture containing equal parts of a 10^{-2} dilution of influenza virus W.S. strain and a 10^{-1} dilution of canine distemper virus *X-f*. Following a typical course of both influenza and distemper, the ferret was killed 11 days after inoculation. The lung and spleen were removed aseptically, and equal amounts of these organs were ground to a 20 per cent suspension in 0.85 per cent NaCl. The suspension was centrifuged for 20 minutes at 2000 R.P.M. The supernate was diluted to 10 per cent, and sufficient formalin was added to make a final concentration of formaldehyde of 1:4000. This suspension was stored at 4°C. for 10 days after which the excess formaldehyde

was neutralized by the addition of sufficient ammonia water to make a final concentration of ammonia of 1:8000. The inactivated vaccine was stored at −76°C. and was not thawed until immediately prior to administration.

Vaccine No. 5. Complex Ferret Lung and Spleen.—A normal ferret was inoculated intranasally with a mixture containing equal parts of a 10^{-2} dilution of influenza virus W.S. strain and a 10^{-1} dilution of dog spleen containing canine distemper virus, Y strain. The ferret was killed 11 days after inoculation, and the lung and spleen were removed aseptically. Equal amounts of these organs were ground to a 20 per cent suspension in 0.85 per cent NaCl. The remainder of the procedure followed in the preparation of this vaccine was identical in every respect with that used for vaccine No. 4. The inactivated vaccine was stored at −76°C. and was not thawed until just before use.

Vaccine No. 6. Chick Embryo.—The 9th chick embryo passage of influenza virus *PR8-f* was inoculated onto the membrane of 9 day old chick embryos. The embryos were incubated for an additional 10 days and were then harvested aseptically. The heads and legs were removed, and a 20 per cent suspension of the embryos and the chorio-allantoic membranes was made in 0.85 per cent NaCl. The suspension was centrifuged for 10 minutes at 1500 R.P.M. The supernate was stored in the low temperature cabinet and was not thawed until immediately before use.

Vaccine No. 7. Complex Chick Embryo.—The 9th chick embryo passage of influenza virus *PR8-f* diluted 10^{-2} and the 2nd chick embryo passage of canine distemper virus *X-ch* diluted 10^{-1} were mixed and inoculated onto the membrane of 9 day old chick embryos. After an additional incubation of 10 days the infected embryos were harvested aseptically. The heads and legs were removed. The embryos and the chorio-allantoic membranes were ground to a 10 per cent suspension in 0.85 per cent NaCl. Sufficient formalin was added to make a final concentration of formaldehyde of 1:4000. This suspension was stored at 4°C. for 5 days when the excess formaldehyde was neutralized by the addition of sufficient ammonia water to make a final concentration of ammonia of 1:8000. The inactivated vaccine was stored at −76°C. and was not thawed until immediately before use.

Vaccine No. 8. Chick Embryo.—The 8th chick embryo passage of influenza virus *PR8-m* was inoculated onto the membrane of 12 day old chick embryos. The embryos were incubated for an additional 4 days and then harvested aseptically. The heads and legs were removed. A 10 per cent suspension of the embryos and the chorio-allantoic membranes was made in 0.85 per cent NaCl. This suspension was centrifuged for 10 minutes at 1500 R.P.M. The supernate was stored at −76°C. until immediately before it was used.

Vaccine No. 9. Complex Chick Embryo.—The 9th chick embryo passage of influenza virus *PR8-m* in a dilution of 10^{-2} and the 2nd chick embryo passage of canine distemper virus *X-ch* diluted 10^{-1} were mixed and inoculated onto the membranes of 10 to 12 day old chick embryos. After 6 days' additional incubation the embryos were harvested aseptically. The heads and legs were removed and the embryos and membranes ground to a 20 per cent suspension in 0.85 per cent NaCl. Sufficient formalin was added to make a final concentration of formaldehyde of 1:4000. This suspension was stored at 4°C. for 3 days, after which the excess formaldehyde was neutralized by the addition of sufficient ammonia water to make a final concentration of ammonia of 1:8000. The suspension was then centrifuged for 10 minutes at 1500 R.P.M. The supernate was

distributed in pyrex ampoules, frozen rapidly in a mixture of alcohol and solid CO_2, and desiccated in the frozen state by the method of Bauer and Pickels (18). The desiccated vaccine was stored at 4°C. It was rehydrated 15 days after drying by the addition of sterile distilled water and was used immediately.

Serum.—

Serum was obtained from each volunteer immediately before vaccination and at various intervals thereafter. In many instances weekly serum specimens were taken during the 1st month after vaccination. In all cases serum was obtained between 2 and 3 weeks following vaccination. Sera were stored at 4°C. In almost every instance prevaccination and postvaccination sera from the same individual were run in the same neutralization test.

Neutralization Test.—

The exact technique used in the neutralization tests has been described previously (19). Serum was inactivated at 56°C. for 30 minutes, and serial fourfold dilutions were made in 0.85 per cent NaCl. Equal quantities of the various serum dilutions and a constant amount of the PR8 strain of influenza A virus were mixed. In some tests, depending upon the titer of the serum, $10^{3.5}$ fifty per cent mortality doses of virus were used, while in others $10^{2.5}$ doses were added. Four mice lightly anesthetized with ether were inoculated intranasally with each serum-virus mixture and were observed for 11 days. Deaths were recorded daily as they occurred. All mice which survived the observation period were autopsied, and the extent of pulmonary consolidation was recorded by the grading method previously described (19). Dilutions of single immune ferret serum of known titer against the PR8 strain were included in each test.

Virus Titrations.—

The virus titration technique corresponded exactly to that previously described (19). In the case of the neutralization tests, groups of six mice were inoculated with each decimal dilution of virus. To determine the virus content of the various vaccines, however, only four mice were inoculated with each decimal dilution of the initial suspension. The test mice were observed for a period identical with that indicated above, and the survivors were autopsied in a similar manner.

Calculation of End Points.—

Both the serum dilution end points and the virus titration end points were calculated by the 50 per cent end point method of Reed and Muench (20). In the neutralization tests the 50 per cent mortality end point was used as previously described (19). In the case of titrations of the virus content of the vaccines, both the 50 per cent mortality and 50 per cent mouse pneumonia end points were determined. This latter end point corresponded to that dilution of virus which was just capable of producing definite pneumonia in 50 per cent of mice. Definite influenza A virus pneumonia was considered to be present when one-fourth or more of the mouse lung showed consolidation. This dilution was considered equal to one 50 per cent *mouse pneumonia dose* of virus, and it will be termed hereafter M.P.D.

Calculation of Neutralizing Capacity of Serum.—

The neutralizing capacity of a serum was calculated from its neutralizing titer and the quantity of virus used in the neutralization test in the manner described previously (21). The neutralizing capacity of a serum is the maximum number of 50 per cent mortality doses of influenza A virus which can be neutralized by 0.05 cc. of the serum. With the great majority of human sera this number is large, and consequently it is con-

venient to express the neutralizing capacity logarithmically. The advantages of using neutralizing capacities instead of serum titers have been discussed already (21). Throughout the remainder of this paper *neutralizing capacity* will frequently be referred to merely as N.C.

EXPERIMENTAL

For the purposes of testing the antigenicity of various vaccines, volunteers were chosen at random. No effort was made to select individuals on the basis that their serum had a particular neutralizing capacity. A preliminary serum specimen was obtained from each volunteer immediately before vaccination, and additional serum was taken at varying intervals after vaccination. The neutralizing capacities of these sera against the PR8 strain of influenza A virus were determined as described above. In a previous report it has been demonstrated (22) that the standard neutralizing antibody titers of the sera of a large number of individuals studied for a period of 2 years, although they remained relatively constant for each individual during this period, showed marked differences from one individual to another. However, it was found that approximately 97 per cent of normal individuals possessed sera which fell into one or the other of the first three standard neutralizing titer groups. These titer groups were: (*a*) less than 1:4, (*b*) 1:4 to 1:15, and (*c*) 1:16 to 1:64. To facilitate a correlation between what has previously been published (15, 21) concerning standard neutralizing antibody titers and the interpretation of the effectiveness of various types of vaccines containing influenza A virus, neutralizing capacity ranges were chosen which corresponded quite closely to the three titer groups mentioned. Volunteers were arranged in order of increasing neutralizing capacity of their prevaccination serum, and those in each neutralizing capacity range were considered as a group. In the various tables which are to be presented the neutralizing capacity range log 2.50 to 4.35 would include only those persons whose prevaccination sera possessed a so called standard titer of <1:4. The neutralizing capacity range log 4.36 to 5.22 would include only those individuals whose prevaccination sera had a standard neutralizing titer of 1:4 to <1:16, while the neutralizing capacity range log 5.23 to 6.09 would contain only those volunteers whose prevaccination sera had a standard neutralizing titer of 1:16 to <1:64.

Results of Human Vaccination.—

Vaccine No. 1.—Each of the ten human volunteers was given a single subcutaneous injection of 2 cc. of tissue culture vaccine No. 1. This quantity of vaccine contained $10^{5.8}$ fifty per cent M.P.D. of fully active influenza A virus. Serum was obtained from each individual immediately before vaccination, as well as 1 and 2 weeks thereafter. The neutralizing

capacities of these sera were determined against the PR8 strain of influenza A virus in the manner described above. The results of these tests are shown in Table I. It will be seen that almost no increase in neutralizing capacity (N.C.) occurred during the 1st week after vaccination and that

TABLE I

Vaccine No. 1. Neutralizing Capacities of Sera of Human Beings before and after Administration of Tissue Culture Vaccine Containing Active Influenza A Virus

Volunteer*	Neutralizing capacity of serum log				Increase in N.C. log	
	Range	Prevaccination	Postvaccination		Postvaccination	
			1 wk.	2 wks.	1 wk.	2 wks.
1	2.50	3.93	4.94	4.94	1.01	1.01
8	to	3.93	3.93	4.36	0.00	0.43
	4.35					
Mean		3.93	4.43	4.65	0.50	0.72
					3×	5×
4		4.36	4.36	4.94	0.00	0.58
2		4.62	4.94	4.94	0.32	0.32
9	4.36	4.62	4.80	5.51	0.18	0.89
6	to	4.80	4.80	5.67	0.00	0.87
10	5.22	4.80	4.80	4.80	0.00	0.00
3		4.94	4.94	5.23	0.00	0.29
5		4.94	4.94	5.67	0.00	0.73
Mean		4.72	4.79	5.25	0.07	0.53
					0×	3×
7	5.23	5.67	5.67	5.67	0.00	0.00
	to					
	6.09					
Mean		5.67	5.67	5.67	0.00	0.00
					0×	0×

* One subcutaneous injection containing $10^{5.8}$ fifty per cent M.P.D. of influenza A virus was given each individual.

even in the low N.C. range a mean increase of but fivefold occurred in 2 weeks. In the mid N.C. range the mean increase 2 weeks after vaccination was but threefold.

Vaccine No. 2.—Each of ten human volunteers was given a single subcutaneous injection of 2 cc. of mouse lung vaccine No. 2. This vaccine had been inactivated by the addition of formaldehyde, as described above. The quantity injected represented $10^{9.4}$ fifty per cent M.P.D. of influenza A virus before inactivation. Serum was obtained from each individual imme-

diately before vaccination, as well as 1 and 2 weeks thereafter. Serum was also obtained from some volunteers 17 weeks after vaccination. The neutralizing capacities of these sera against the PR8 strain of influenza A virus were determined as described above. The results are shown in Table II. It will be noted that a definite increase in mean neutralizing

TABLE II

Vaccine No. 2. Neutralizing Capacities of Sera of Human Beings before and after Administration of Mouse Lung Vaccine Containing Inactivated Influenza A Virus

Volunteer*	Neutralizing capacity of serum log					Increase in N.C. log		
	Range	Prevaccination	Postvaccination			Postvaccination		
			1 wk.	2 wks.	17 wks.	1 wk.	2 wks.	17 wks.
1	2.50	3.93	4.94	5.51	4.94	1.01	1.58	1.01
4	to	3.93	4.80	4.80	4.94	0.87	0.87	1.01
7	4.35	3.93	3.93	3.93	3.93	0.00	0.00	0.00
Mean.....		3.93	4.55	4.75	4.60	0.62	0.82	0.67
						4×	7×	5×
2	4.36	4.62	5.51	6.53	5.46	0.89	1.91	0.84
3	to	4.80	5.67	6.53	5.67	0.87	1.73	0.87
10	5.22	4.80	6.53	6.53	6.53	1.73	1.73	1.73
5		4.94	5.23	5.80	5.80	0.29	0.86	0.86
Mean.....		4.81	5.75	6.37	5.86	0.94	1.56	1.07
						9×	36×	12×
6	5.23	5.67	5.67	5.67	—	0.00	0.00	—
8	to	5.67	5.80	5.67	—	0.13	0.00	—
9	6.09	5.67	5.67	6.53	—	0.00	0.86	—
Mean.....		5.67	5.71	5.95		0.04	0.28	
						0×	2×	

* One subcutaneous injection which contained, before inactivation, $10^{9.4}$ fifty per cent M.P.D. of influenza A virus was given each individual.

capacities had occurred in both the low and mid N.C. ranges during the 1st week. This increase was considerably more definite at the end of 2 weeks, at which time the mean increase in N.C. in the low range was sevenfold, while in the mid N.C. range it was 36 times. In the high N.C. range, however, the mean increase during the same period was but twofold. 17 weeks after vaccination the mean increase in N.C. in the low range was fivefold, whereas in the mid N.C. range it had dropped to 12 times.

Vaccine No. 3.—Each of ten human volunteers was given a single injection of 2 cc. of ferret lung and spleen vaccine No. 3 subcutaneously. This

vaccine had been inactivated by the addition of formaldehyde in the manner described above. The quantity injected contained, before inactivation, $10^{5.3}$ fifty per cent M.P.D. of influenza A virus. Serum was obtained from each individual immediately before vaccination, as well as 1 and 2 weeks

TABLE III

Vaccine No. 3. Neutralizing Capacities of Sera of Human Beings before and after Administration of Ferret Lung and Spleen Vaccine Containing Inactivated Influenza A Virus

Volunteer*	Neutralizing capacity of serum log				Increase in N.C. log	
	Range	Prevaccination	Postvaccination		Postvaccination	
			1 wk.	2 wks.	1 wk.	2 wks.
1	2.50 to 4.35	3.93	4.36	4.94	0.43	1.01
Mean		3.93	4.36	4.94	0.43 3×	1.01 10×
9	4.36 to 5.22	4.36	4.62	4.80	0.26	0.46
4		4.62	4.80	5.23	0.18	0.61
5		4.62	4.36	5.67	−0.26	1.05
8		4.62	4.80	5.80	0.18	1.18
2		4.80	4.80	5.67	0.00	0.87
3		4.94	4.80	5.80	−0.14	0.86
6		4.94	5.67	5.80	0.73	0.86
10		4.94	5.23	5.67	0.29	0.73
Mean		4.73	4.93	5.55	0.20 0×	0.82 7×
7	5.23 to 6.09	5.23	5.51	5.23	0.28	0.00
Mean		5.23	5.51	5.23	0.28 2×	0.00 0×

* One subcutaneous injection which contained, before inactivation, $10^{5.3}$ fifty per cent M.P.D. of influenza A virus was given each individual.

thereafter. The neutralizing capacities of these sera were determined against the PR8 strain of influenza A virus. The results are shown in Table III. It is evident that little or no increase in mean neutralizing capacity had occurred during the 1st week. At the end of the 2nd week, however, the single individual in the low N.C. range showed an increase of tenfold, whereas those in the mid N.C. range had a mean increase of sevenfold.

Vaccine No. 4.—Each of nine human volunteers was given a single injection of 2 cc. of complex ferret lung and spleen vaccine No. 4 subcutaneously. This vaccine had been inactivated by the addition of formaldehyde in the manner described above. Before inactivation the quantity injected con-

TABLE IV

Vaccine No. 4. Neutralizing Capacities of Sera of Human Beings before and after Administration of Complex Ferret Lung and Spleen Vaccine Containing Both Inactivated Influenza A Virus and Canine Distemper Virus

Volunteer*	Neutralizing capacity of serum log					Increase in N.C. log		
	Range	Prevaccination	Postvaccination			Postvaccination		
			2 wks.	4 wks.	12 wks.	2 wks.	4 wks.	12 wks.
10	2.50 to 4.35	3.93	4.80	—	—	0.87	—	—
Mean		3.93	4.80	—	—	0.87 7×	— —	— —
9		4.62	5.67	—	—	1.05	—	—
7		4.62	5.67	5.51	5.12	1.05	0.89	0.50
1	4.36	4.80	5.51	5.23	5.67	0.71	0.43	0.87
3	to	4.80	5.23	—	6.28	0.43	—	1.48
4	5.22	4.80	5.67	5.67	5.67	0.87	0.87	0.87
6		4.94	5.67	5.89	—	0.73	0.95	—
2		5.05	5.51	5.89	5.67	0.46	0.84	0.62
Mean		4.80	5.56	5.64	5.68	0.76 6×	0.79 6×	0.87 7×
5	5.23 to 6.09	5.23	5.51	5.67	5.23	0.28	0.44	0.00
Mean		5.23	5.51	5.67	5.23	0.28 2×	0.44 3×	0.00 0×

* One subcutaneous injection which contained, before inactivation, $10^{6.1}$ fifty per cent M.P.D. of influenza A virus was given each individual.

tained $10^{6.1}$ fifty per cent M.P.D. of influenza A virus. Serum was obtained immediately before vaccination from each individual, and 2, 4, and 12 weeks after vaccination from as many volunteers as possible. The capacities of these sera to neutralize the PR8 strain were determined, and the results are shown in Table IV. 2 weeks after vaccination the single individual in the low N.C. range showed an increase of sevenfold, whereas those in the mid N.C. range had a mean increase of sixfold. It is obvious that these

increases in specific neutralizing antibodies were almost identical in magnitude to those presented in Table III. However, it is also evident from the results shown in Table IV that during the first 3 months following vaccination there was no significant reduction in the increased antibody levels. It will be recalled that ferret lung and spleen vaccine No. 3 contained only

TABLE V

Vaccine No. 5. Neutralizing Capacities of Sera of Human Beings before and after Administration of Complex Ferret Lung and Spleen Vaccine Prepared with Y Strain of Canine Distemper Virus

Volunteer*	Neutralizing capacity of serum log				Increase in N.C. log	
	Range	Prevaccination	Postvaccination		Postvaccination	
			2 wks.	4 wks.	2 wks.	4 wks.
9	2.50	3.93	3.93	3.93	0.00	0.00
10	to	3.93	3.93	3.93	0.00	0.00
	4.35					
Mean		3.93	3.93	3.93	0.00	0.00
					0×	0×
1		4.62	4.80	4.94	0.18	0.32
5		4.62	4.36	4.62	−0.26	0.00
7	4.36	4.62	3.93	3.93	−0.69	−0.69
3	to	4.80	4.80	4.80	0.00	0.00
2	5.22	4.80	4.80	4.62	0.00	−0.18
8		4.80	4.94	4.94	0.14	0.14
6		4.80	4.80	—	0.00	—
Mean		4.72	4.64	4.64	−0.08	−0.07
					0×	0×
10	5.23	5.67	5.67	5.51	0.00	−0.16
	to					
	6.09					
Mean		5.67	5.67	5.51	0.00	−0.16
					0×	0×

* One subcutaneous injection of this vaccine was given each individual.

inactivated influenza A virus, whereas complex ferret lung and spleen vaccine No. 4 contained both inactivated influenza A virus and the X strain of canine distemper virus.

Vaccine No. 5.—Each of ten human volunteers was given a single subcutaneous injection of 2 cc. of complex ferret lung and spleen vaccine No. 5 subcutaneously. This vaccine was prepared with the Y strain of canine distemper virus. Even before inactivation with formaldehyde this vaccine

contained no demonstrable active influenza A virus, as was to be expected from the results of experiments previously reported (10). Serum was obtained immediately before vaccination from each individual and from all but one volunteer 2 and 4 weeks after vaccination. The capacities of these sera to neutralize the PR8 strain were determined, and the results are presented in Table V. No increase in mean neutralizing capacity occurred in any of the three N.C. ranges at either 2 or 4 weeks following the administration of this vaccine. In fact, the neutralizing capacities of the three sera from each of the individuals were found to be almost identical.

Vaccine No. 6.—Each of five human volunteers was given a single subcutaneous injection of 2 cc. of chick embryo vaccine No. 6. This vaccine contained fully active influenza A virus which had not been adapted to mice. The titer of the virus as determined in this species was, therefore, meaningless. Serum was obtained immediately before, as well as 2, 4, and 21 weeks after vaccination from as many individuals as possible. The capacities of these sera to neutralize the PR8 strain of influenza A virus were determined. The results are presented in Table VI. The single individual in the low N.C. range showed an increase of sevenfold, both at 2 and 4 weeks after vaccination. The three individuals in the mid N.C. range showed no significant increase at 2 weeks, a twofold increase at 4 weeks, and a threefold increase at 21 weeks.

Vaccine No. 7.—Each of five human volunteers was given a single subcutaneous injection of 1 cc. of complex chick embryo vaccine No. 7. This vaccine had been inactivated by the addition of formaldehyde. The influenza A virus used had not been adapted to mice. Even before inactivation, therefore, its titer in this species was of no significance. Serum was obtained immediately before vaccination, as well as 1, 2, 4, and 21 weeks thereafter from as many individuals as possible. The neutralizing capacities of these sera against the PR8 strain of influenza A virus were determined. The results are presented in Table VII. 1 week after vaccination the single individual in the low N.C. range showed an increase of sevenfold, whereas those in the mid N.C. range showed an increase of twofold. At 2 weeks after vaccination the increases in these ranges were found to be 55 times and 10 times, respectively. 4 weeks after vaccination they were 74 times and 10 times, respectively, while at 21 weeks they were 10 times and 7 times, respectively. It is quite evident that the results obtained with this vaccine were very different from those which are presented in Table VI. It will be recalled that chick embryo vaccine No. 6 contained only influenza A virus which was fully active when injected, whereas complex chick embryo vaccine No. 7 was prepared from embryos inoculated with both

TABLE VI

Vaccine No. 6. Neutralizing Capacities of Sera of Human Beings before and after Administration of Chick Embryo Vaccine Containing Active Influenza A Virus

Volunteer*	Neutralizing capacity of serum log					Increase in N.C. log		
	Range	Prevaccination	Postvaccination			Postvaccination		
			2 wks.	4 wks.	21 wks.	2 wks.	4 wks.	21 wks.
4	2.50 to 4.35	3.93	4.80	4.80	—	0.87	0.87	—
Mean		3.93	4.80	4.80	—	0.87 7×	0.87 7×	—
2	4.36	4.80	5.23	5.67	5.81	0.43	0.87	1.01
5	to	4.80	4.94	4.94	5.05	0.14	0.14	0.25
3	5.22	4.94	4.94	4.80	5.05	0.00	−0.14	0.09
Mean		4.84	5.03	5.13	5.31	0.19 0×	0.29 2×	0.45 3×
1	5.23 to 6.09	5.23	6.10	5.67	5.81	0.87	0.44	0.58
Mean		5.23	6.10	5.67	5.81	0.87 7×	0.44 3×	0.58 4×

* One subcutaneous injection was given each individual. Since this influenza A virus had not been adapted to mice, the exact titer could not be determined in this species.

TABLE VII

Vaccine No. 7. Neutralizing Capacities of Sera of Human Beings before and after Administration of Complex Chick Embryo Vaccine

Volunteer*	Neutralizing capacity of serum log						Increase in N.C. log			
	Range	Prevaccination	Postvaccination				Postvaccination			
			1 wk.	2 wks.	4 wks.	21 wks.	1 wk.	2 wks.	4 wks.	21 wks.
3	2.50 to 4.35	3.93	4.80	5.67	5.80	4.94	0.87	1.74	1.87	1.01
Mean		3.93	4.80	5.67	5.80	4.94	0.87 7×	1.74 55×	1.87 74×	1.01 10×
4	4.36	4.36	4.36	4.80	4.80	5.05	0.00	0.46	0.46	0.69
1	to	4.80	5.51	5.67	5.67	—	0.71	0.87	0.87	—
2	5.22	4.80	5.23	6.53	6.53	—	0.43	1.73	1.73	—
5		4.80	4.80	5.67	5.67	5.81	0.00	0.87	0.87	1.01
Mean		4.69	4.97	5.67	5.67	5.43	0.28 2×	0.98 10×	0.98 10×	0.85 7×

* One subcutaneous injection was given each individual. Since this influenza A virus had not been adapted to mice, the exact titer could not be determined in this species.

influenza A virus and the X strain of canine distemper virus. Moreover, the latter vaccine was inactivated by formaldehyde and might have been

TABLE VIII

Vaccine No. 8. Neutralizing Capacities of Sera of Human Beings before and after Administration of Chick Embryo Vaccine Containing Influenza A Virus

Volunteer	Neutralizing capacity of serum log				Increase in N.C. log	
	Range	Prevaccination	Postvaccination		Postvaccination	
			2 wks.	3 wks.	2 wks.	3 wks.
5*	2.50 to 4.35	3.50	5.05	4.80	1.55	1.30
8*		3.50	4.18	4.36	0.68	0.86
7*		3.93	4.62	4.18	0.69	0.25
5**		3.93	—	3.93	—	0.00
8**		3.93	—	3.93	—	0.00
10**		3.93	—	4.94	—	1.01
3**		3.93	—	4.94	—	1.01
9**		3.93	—	4.62	—	0.69
3*		4.18	5.05	5.05	0.87	0.87
Mean		3.86	4.72	4.53	0.94 9×	0.67 5×
4*	4.36 to 5.22	4.80	5.67	5.23	0.87	0.43
6*		4.80	5.67	4.80	0.87	0.00
9*		4.80	5.67	4.80	0.87	0.00
4**		4.80	—	5.67	—	0.87
Mean		4.80	5.67	5.12	0.87 7×	0.32 2×
7**	5.23 to 6.09	5.51	—	5.67	—	0.16
2**		5.51	—	5.51	—	0.00
1*		5.67	5.67	5.67	0.00	0.00
2*		5.67	6.53	5.74	0.86	0.07
10*		5.67	5.67	5.67	0.00	0.00
1**		5.80	—	6.37	—	0.57
Mean		5.64	5.95	5.77	0.28 2×	0.13 0×

* One subcutaneous injection containing $10^{6.3}$ fifty per cent M.P.D. was given.

** Vaccine given intramuscularly.

expected to produce a less vigorous rather than a considerably more definite specific neutralizing antibody response. It seems of considerable interest that the increased neutralizing capacities which followed the administration of this vaccine declined but little during the first 5 months.

Vaccine No. 8.—Each of nineteen human volunteers was given a single

injection of 1 cc. of chick embryo vaccine No. 8. Ten volunteers received the vaccine subcutaneously while nine were given intramuscular injections. This quantity of vaccine contained $10^{6.3}$ fifty per cent M.P.D. of influenza A virus which was fully active when administered. Serum was obtained from each individual immediately before vaccination and 3 weeks afterwards. From those persons who received the vaccine subcutaneously serum was also obtained 2 weeks after vaccination. The capacities of these sera to neutralize the PR8 strain were determined, and the results are shown in Table VIII. It will be noted that the four individuals in the low N.C. range had a mean increase of ninefold at 2 weeks, whereas at 3 weeks the total group in this range showed a mean increase in N.C. of fivefold. The individuals in the mid N.C. range showed an increase at 2 and 3 weeks of sevenfold and twofold, respectively.

Vaccine No. 9.—Each of twenty-four human volunteers was given a single injection of 1 cc. of complex chick embryo vaccine No. 9 subcutaneously. This vaccine was inactivated by the addition of formaldehyde and had been desiccated in the frozen state. Before inactivation the quantity of vaccine injected contained $10^{6.9}$ fifty per cent M.P.D. of influenza A virus. Serum was obtained from each individual immediately before vaccination and from as many as possible 2 and 4 weeks thereafter. The capacities of these sera to neutralize the PR8 strain were determined, and the results are presented in Table IX. It will be seen that those individuals in the low N.C. range whose sera were studied 2 weeks after vaccination showed a mean increase of 79 times, whereas those whose sera were obtained 4 weeks after vaccination had a mean increase of 31 times. The seven volunteers in the low N.C. range from each of whom serum was obtained 2 and 4 weeks after vaccination showed a mean increase in N.C. at these intervals of 68 times and 58 times, respectively. Those individuals in the mid N.C. range whose sera were examined 2 weeks after vaccination showed a mean increase of 15 times, while the larger number whose sera were obtained at 4 weeks had a mean increase of 9 times. From five volunteers in the mid N.C. range serum was obtained both at 2 and 4 weeks after vaccination. In this group the mean increase in N.C. at these periods was 12 times and 9 times, respectively.

It is apparent that these results are very different from those presented in Table VIII and that the increases in neutralizing capacity which followed the administration of the complex vaccine were considerably greater than those which followed the administration of a comparable vaccine containing influenza A virus alone. It will be recalled that chick embryo vaccine No. 8 contained only fully active influenza A virus, while complex chick

TABLE IX

Vaccine No. 9. Neutralizing Capacities of Sera of Human Beings before and after Administration of Complex Chick Embryo Vaccine

Volunteer*	Neutralizing capacity of serum log				Increase in N.C. log	
	Range	Prevaccination	Postvaccination		Postvaccination	
			2 wks.	4 wks.	2 wks.	4 wks.
11		2.50	5.10	4.96	2.60	2.46
1		3.36	6.40	6.11	3.04	2.75
10		3.50	6.68	—	3.18	—
24		3.50	—	4.13	—	0.63
7	2.50	4.05	5.10	—	1.05	—
9	to	4.05	5.32	5.53	1.27	1.48
4	4.35	4.23	4.96	5.20	0.73	0.97
8		4.23	6.11	5.53	1.88	1.30
15		4.23	5.10	5.10	0.87	0.87
16		4.23	6.68	6.68	2.45	2.45
23		4.23	—	4.67	—	0.44
Mean		3.83	5.72	5.32	1.90 79×	1.48 31×
14		4.37	5.96	5.10	1.59	0.73
19		4.37	—	6.11	—	1.74
6		4.67	4.96	5.11	0.29	0.44
17		4.67	5.10	5.96	0.43	1.29
20	4.36	4.80	—	5.96	—	1.16
21	to	4.90	—	5.96	—	1.06
25	5.22	4.90	—	5.96	—	1.06
12		4.96	6.40	6.83	1.44	1.87
22		5.10	—	4.96	—	−0.14
3		5.10	6.68	5.53	1.58	0.43
13		5.10	6.83	—	1.73	—
Mean		4.81	5.96	5.66	1.18 15×	0.96 9×
2	5.23	5.53	5.82	—	0.29	—
18	to	5.96	6.40	6.40	0.44	0.44
	6.09					
Mean		5.74	6.00	6.40	0.36 2×	0.44 3×

* One subcutaneous injection containing, before inactivation, $10^{6.9}$ fifty per cent M.P.D. of influenza A virus was given each individual.

embryo vaccine No. 9 was prepared from embryos inoculated with both influenza A virus and the X strain of canine distemper virus. Moreover, the latter vaccine was inactivated by the addition of formaldehyde. It was

to be expected that the active simple vaccine might produce a greater specific antibody response than the inactive complex vaccine, whereas quite the reverse was found to be the case.

A summary analysis of the mean increases in neutralizing capacities which occurred 2 weeks after the administration of each of the nine vaccines studied is presented in Table X. The mean antibody increases observed in each of the three N.C. ranges, as well as that which was observed irrespective of range, are shown. It is apparent that the greatest increase in neutralizing capacity followed the injection of complex chick embryo vaccine No. 9 and that the next most marked increase occurred after the injection of

TABLE X

Mean Increase in Neutralizing Capacities of Human Sera 2 Weeks after Administration of Various Vaccines Containing Influenza A Virus

Vaccine			Mean neutralizing capacity increase				
			Log N.C. range[1]			All ranges	
No.	Source	Type	2.50 to 4.35	4.36 to 5.22	5.23 to 6.09	Log	Arith.
1	t.c.	Influenza alone	0.72	0.53	0.00	0.52	3.2×
2	m.l.	" "	0.82	1.56	0.28	0.95	9.0×
3	f.l.s.	" "	1.01	0.82	0.00	0.76	5.7×
4	f.l.s.	Complex X	0.87	0.76	0.28	0.72	5.2×
5	f.l.s.	Complex Y	0.00	−0.08	0.00	−0.06	0.0×
6	ch.em.	Influenza alone	0.87	0.19	0.87	0.46	2.9×
7	ch.em.	Complex X	1.74	0.98	—	1.13	13.5×
8	ch.em.	Influenza alone	0.94	0.87	0.28	0.77	5.9×
9	ch.em.	Complex X	1.90	1.18	0.36	1.47	29.2×

complex chick embryo vaccine No. 7. It should again be noted that vaccine No. 5, which was prepared with the Y strain of canine distemper virus, produced no increase whatever in antibody levels.

DISCUSSION

Since normal experimental animals do not possess circulating antibodies against influenza A virus, evidence concerning the effectiveness of vaccines containing this virus obtained in these species may not be directly applicable to man. The fact that the great majority of normal human beings possess considerable quantities of specific neutralizing antibodies against influenza A virus makes it difficult to assess the efficacy of a vaccine containing this virus in man. Inasmuch as certain normal individuals may possess very different amounts of neutralizing antibodies, in some cases as much as

10,000 times more than in others, it is essential that individual antibody levels be determined exactly if accurate data are to be obtained concerning the production of antibodies which results from the administration of a given vaccine.

During the course of the present studies it became apparent that the quantitative increase in circulating antibodies which followed the subcutaneous injection of a particular vaccine was dependent to a considerable extent upon the initial quantity of antibodies possessed by a given individual. In general, the lower the prevaccination antibody level, the greater was the postvaccination production of antibodies. Because of this observation it has seemed desirable to group individuals with certain initial antibody levels so that a reasonable comparison could be made between the antibody responses produced in comparable groups by different vaccines. However, the degree of difference in specific antigenicity which was possessed by the complex chick embryo vaccines as opposed to the others tested was sufficiently great to be reflected even in the results obtained in the whole groups irrespective of their initial antibody levels.

Under the conditions of these experiments it was found that the parenteral administration of fully active tissue culture virus or chick embryo virus in man was followed by a definite, though not very marked, production of specific neutralizing antibodies. The increases in antibody levels which were observed after the injection of active tissue culture virus were probably somewhat less than those previously reported by Francis and Magill (4). However, it should be pointed out that these workers gave multiple injections of the virus, whereas in the present study only one injection was made. It seems worthy of note that no particularly striking differences in antigenicity for man were encountered with either the active tissue culture or the active chick embryo viruses even though three distinct passage series of the original PR8 strain were used. These three series for more than 4 years have been maintained in very different biological environments; *viz.*, tissue culture, ferret lung, and mouse lung.

The inactivated mouse lung virus in man was found to stimulate the production of a considerably greater quantity of neutralizing antibodies than did the inactivated ferret lung virus. It may be that this difference in antigenicity is related to similar differences observed in mice with related vaccines by Andrewes and Smith (23). However, it seems more reasonable in the present circumstances to explain the different antigenicity of these preparations on the basis of their very obviously different virus content. The mouse titration results indicated that the mouse lung vaccine contained over 10,000 times more virus than the ferret lung and spleen vaccine. In fact, the mouse lung vaccine contained some 300 times more virus than any

other vaccine studied. The increases in antibody levels which were observed after the injection of inactivated mouse lung virus appear to be of approximately the same order of magnitude as those which were obtained previously with similar preparations by Smith, Andrewes, and Stuart-Harris (7) and by Taylor and Dreguss (8). However, it should be noted that a considerable decrease in these raised antibody levels had occurred after 4 months.

The complex ferret lung and spleen vaccine containing inactivated influenza A virus and the X strain of canine distemper virus was found to be no more antigenic in man than the comparable ferret vaccine which contained only influenza A virus. This vaccine was prepared in a manner as nearly as possible identical with the complex ferret vaccine previously described (9). Moreover, it was tested subcutaneously in ferrets and was found to stimulate the production of antibodies in 10 days in this species equal to a mean neutralizing capacity of log 4.94. Furthermore, it resulted in the production in ferrets of definite immunity to the intranasal inoculation of 1000 infectious doses of either the PR8 or the W.S. strain.

The complex ferret lung and spleen vaccine prepared with the Y strain of canine distemper virus failed to stimulate the production of any additional antibodies against influenza A virus. It was stated previously (9) that this strain, recently isolated from the spleen of an infected dog, was ineffective when used in the preparation of a complex vaccine. It was shown also (10) that the X strain, although possessing no demonstrable antigenic differences from the Y strain, markedly altered the usual course of influenza virus infection in the ferret, whereas the Y strain did not. It seems reasonable to suggest that the X strain possesses certain peculiarities which may not be common to other strains of canine distemper virus.

The complex chick embryo vaccines, although containing inactivated virus, caused a definitely greater production of neutralizing antibodies than did comparable vaccines prepared from embryos infected only with influenza A virus, even though the latter vaccines contained active virus. In fact, vaccine No. 9 stimulated the formation of more specific antibodies than any other vaccine tested. In individuals with comparable prevaccination antibody levels vaccine No. 7 appears to have been equally as effective an antigen as vaccine No. 9, although the number of individuals tested with the former vaccine is probably too small to permit of accurate comparison. It may again be worth noting that different passage series of the PR8 strain, *i.e.*, ferret lung and mouse lung viruses which had both been established in the chick embryo, were used in the production of these two complex vaccines.

Following the injection of either complex ferret lung and spleen or com-

plex chick embryo vaccines prepared with the X strain of canine distemper virus, the increased antibody levels against influenza A virus remained relatively unaltered for a period of at least 12 weeks and 21 weeks, respectively. It was shown in a previous paper (24) that after an attack of influenza A striking reductions in the initially increased antibody levels occurred during similar periods. Despite the fact that the mean increases in neutralizing antibodies which occurred in the first 2 weeks after the onset of influenza A were more marked than those which followed the injection of complex vaccines, the residual antibody levels in comparable neutralizing capacity groups, approximately 12 and 21 weeks after an attack of influenza A or the administration of a complex vaccine, appear to have been of the same order of magnitude. If, as seems not unlikely, neutralizing antibody levels actually reflect relative immunity to infection by influenza A virus (22), it seems possible that the administration of the complex chick embryo vaccine may have resulted in the production of a degree of immunity at least as satisfactory and as persistent as that which followed the disease itself. Field tests under natural conditions in large groups of vaccinated and unvaccinated individuals exposed to epidemics of proven influenza A will obviously be necessary before the possible prophylactic effectiveness of the complex chick embryo vaccine can be definitely determined.

The actual biological phenomena which cause the complex chick embryo vaccine to be more effective in stimulating the production of specific neutralizing antibodies against influenza A virus than a similar vaccine prepared with influenza A virus alone remain obscure. It has previously been shown (10) that no demonstrable antigenic relationship existed between influenza A virus and the X strain of canine distemper virus. Nonetheless, from the results presented herein it seems evident that the inoculation of chick embryos with a mixture of both influenza A virus and this particular strain of distemper virus leads to the production of preparations more antigenic for man than would have been anticipated on the basis of the concentration of influenza A virus in them as determined by titrations in mice. Whether this result is due to as yet unmeasured quantitative or qualitative modifications in either virus or in both is not clearly evident, and to enumerate the various theoretical possibilities seems pointless.

CONCLUSION

A quantitative study of the antigenicity of various vaccines containing influenza A virus has been made in human beings. A complex vaccine prepared from chick embryos inoculated with both influenza A virus and

the X strain of canine distemper virus was found to be more effective than other vaccines in stimulating the production of neutralizing antibodies against the former virus. The increased antibody levels which resulted from the administration of this vaccine remained almost unaltered for at least 5 months.

BIBLIOGRAPHY

1. Horsfall, F. L., Jr., Lennette, E. H., Rickard, E. R., Andrewes, C. H., Smith, W., and Stuart-Harris, C. H., *Lancet*, 1940, **2**, 413.
2. Andrewes, C. H., and Smith, W., *Brit. J. Exp. Path.*, 1937, **18**, 43.
3. Francis, T., Jr., and Magill, T. P., *Proc. Soc. Exp. Biol. and Med.*, 1936, **33**, 604.
4. Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1937, **65**, 251.
5. Stokes, J., Chenoweth, A. D., Waltz, A. D., Gladen, R. G., and Shaw, D. R., *J. Clin. Inv.*, 1937, **16**, 237.
6. Stokes, J., McGuinness, A. C., Langnor, P. H., and Shaw, D. R., *Am. J. Med. Sc.*, 1937, **194**, 757.
7. Smith, W., Andrewes, C. H., and Stuart-Harris, C. H., *Great Britain Med. Research Council, Special Rep. Series, No. 228*, 1938.
8. Taylor, R. M., and Dreguss, M., *Am. J. Hyg.*, 1940, **31**, 31.
9. Horsfall, F. L., Jr., and Lennette, E. H., *Science*, 1940, **91**, 492.
10. Horsfall, F. L., Jr., and Lennette, E. H., *J. Exp. Med.*, 1940, **72**, 247.
11. Francis, T., Jr., *Science*, 1934, **80**, 2081.
12. Francis, T., Jr., and Magill, T. P., *Science*, 1935, **82**, 353.
13. Burnet, F. M., *Great Britain Med. Research Council, Special Rep. Series, No. 220*, 1936.
14. Smith, W., Andrewes, C. H., and Laidlaw, P. P., *Lancet*, 1933, **1**, 66.
15. Horsfall, F. L., Jr., Hahn, R. G., and Rickard, E. R., *J. Clin. Inv.*, 1940, **19**, 379.
16. Plummer, P. J. G., *Canad. J. Comp. Med.*, 1939, **3**, 96.
17. Horsfall, F. L., Jr., *J. Bact.*, 1940, **40**, 559.
18. Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, 1940, **71**, 83.
19. Horsfall, F. L., Jr., *J. Exp. Med.*, 1939, **70**, 209.
20. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.
21. Horsfall, F. L., Jr., and Lennette, E. H., *J. Exp. Med.*, 1941, **73**, 327.
22. Rickard, E. R., Lennette, E. H., and Horsfall, F. L., Jr., *Pub. Health Rep., U. S. P. H. S.*, 1940, **55**, 2146.
23. Andrewes, C. H., and Smith, W., *Brit. J. Exp. Path.*, 1939, **20**, 305.
24. Horsfall, F. L., Jr., *Am. J. Pub. Health*, 1940, **30**, 1302.