

## ANTIBODY RESPONSE OF HUMAN BEINGS FOLLOWING VACCINATION WITH INFLUENZA VIRUSES

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A number of investigators (1-7) have reported the results of studies on the antibody response of human beings following the parenteral administration of various preparations containing influenza virus. With one exception (6) these earlier investigations were carried out on relatively small groups of individuals since the difficulty of examining large numbers of sera by means of the neutralization test in mice was often a limiting factor. Most workers used a single variety of vaccine, although Horsfall, Lennette, and Rickard (5) investigated a number of different preparations in small groups of volunteers. Despite the efforts which have been expended in the study of influenza virus vaccines in human beings, there is at present relatively little known concerning the factors which influence the specific antigenicity of the various kinds of vaccine which can be prepared with this agent.

While extensive work has been done on the antibody response of experimental animals vaccinated with various preparations of influenza virus, the results obtained in such studies are not necessarily applicable to man. Most human beings, unlike most experimental animals, have had at least one contact with influenza virus, and these previous infective experiences are probably one of the factors which condition their responses to vaccination with the virus in an unpredictable manner. Since man is the subject whom it is desired ultimately to protect against attacks of influenza, it seems apparent that direct testing in human beings facilitates the evaluation of the effectiveness of various kinds of vaccines.

Previous workers (1-7) have found that there was marked individual variation in the antibody response of human beings to any one variety of influenza virus vaccine. Because of the individual differences in response it seemed necessary, in order to obtain significant data for the comparison of the specific antigenicity of different virus preparations in man, to use large numbers of individuals in the study of each vaccine.

With the demonstration that influenza viruses agglutinate chicken red blood corpuscles, and that this capacity is specifically inhibited by immune serum (8), a new and relatively simple *in vitro* test for the determination of influenza antibodies became available (9). As has been shown, the titers of influenza

antibodies in normal human sera, as determined by this method, very closely paralleled the titers obtained by means of the virus neutralization test in mice. This parallelism also obtained with sera from human beings who had been vaccinated recently with influenza virus. Furthermore the test was sensitive to relatively small changes in antibody concentration, and the end points obtained had a high degree of reproducibility. Because this method could be applied relatively easily to large numbers of sera it became possible to undertake a large-scale study of the antibody response to vaccination with influenza viruses in human beings.

The experiments in the present paper were designed to explore several questions related to human vaccination with influenza viruses: (a) How much variation in individual antibody response occurs in a large group which has been given a single type of vaccine, and how does the individual response vary with the prevaccination antibody level? (b) What are the relative merits as specific antigens of whole chick embryo vaccines prepared with and without the X strain of distemper virus? (c) How does the antibody response vary with dosage of influenza virus? (d) How does active virus compare with inactive virus as an antigen? (e) What is the effect of increasing the inert protein content of the vaccine? (f) How rapidly do individuals lose the additional antibodies produced following vaccination? (g) How closely does the antibody response to influenza A virus parallel that to influenza B virus under various conditions?

Allantoic fluid from infected chick embryos has been shown to be an excellent source of influenza viruses (10-12) and has been used as the source of material for most of these experiments. In addition to a high virus titer, allantoic fluid contains relatively little non-virus protein. Furthermore the virus present in such fluids can be concentrated readily by several methods, permitting the administration of much larger amounts of virus than heretofore.

#### *Methods*

Inasmuch as these studies were designed to compare the specific antigenicity of vaccines modified in one factor only, it was essential that the basic source material be the same for all preparations. For this purpose two large pools of infected allantoic fluid were prepared, one containing the PR8 strain of influenza A virus (13) and the other the Lee strain of influenza B virus (14). Furthermore to insure uniformity the various vaccines were prepared at the same time with the exception of 53, 54, and 64, which were handled separately as will be explained in the text. Usually, equal amounts of the two allantoic fluid pools which had been treated similarly were combined to make the final preparation. Chick embryo passage derivatives of the mouse-virulent PR8 and Lee strains were the only viruses used throughout this work, except in vaccine 64 which contained the W.S. strain (15) in addition and vaccine 54 which was prepared with a mixture of the PR8 strain and the X strain of distemper virus (16).

*Preparation of Allantoic Fluid Pools Used for Vaccines.*—To prepare the basic allantoic fluid pools 11-day old chick embryos were used. The embryos were inoculated with 0.1 cc. of infected allantoic fluid (diluted to  $10^{-8}$  in saline) through a small puncture in the shell over the chorioallantoic membrane. No attempt was made to drop this membrane. After 2 days incubation at  $37^{\circ}\text{C}$ . blood-free allantoic fluid was removed under sterile conditions as described in a previous report (9). A portion of the fluid from each egg was cultured in blood broth, and while the results of culture were being determined, the remainder of the fluids from individual eggs was stored frozen at  $-72^{\circ}\text{C}$ . All contaminated fluids were discarded and the sterile fluids were pooled. The combined sterile fluids were clarified by low-speed centrifugation, and the sediments were discarded. The pools were then stored at  $-10^{\circ}\text{C}$ . until the various vaccines were prepared. Each pool was titered in mice, after it had been diluted serially in steps of  $10^{-0.5}$ , and the 50 per cent mortality end point was determined. About 3,500 cc. of PR8 and 2,000 cc. of Lee fluids were prepared.

The individual vaccines (except 53, 54, and 64) were all made from these pools on the same day. The technique used for each preparation will be described in the text. All vaccines except 61 and 64 were frozen and dried (17) and were rehydrated with distilled water just before use.

*Method of Vaccination.*—In all cases the vaccinated subjects were patients in state hospitals. Their ages varied from 15 to 60 years, and they were predominantly males. In most instances 150 individuals were inoculated with each vaccine. A number of different vaccines were given on each ward in order to rule out possible variation in response from one group to another. A blood specimen was taken from each individual just before vaccination and another 2 weeks following vaccination. The sera were separated and stored at  $4^{\circ}\text{C}$ . until they were tested for antibody titer. Some of the subjects were bled also at 6 weeks, 9 weeks, and 5 months following vaccination.

*Determination of Antibody Titer in Serum.*—All of the human sera, both pre- and postvaccination specimens, were tested for their capacity to inhibit the agglutination of chicken red cells by the PR8 strain and the Lee strain of influenza virus. The virus suspensions used for these tests were prepared in the same manner as the allantoic fluid pools used for vaccination. Large quantities of each virus were prepared at one time. Each pool of test virus was distributed in small amounts (2 to 25 cc.) in lusteroid tubes, which were then stored at  $-72^{\circ}\text{C}$ . until used. Two such pools of influenza A virus and two of influenza B virus were necessary to complete the serum titrations.

The chicken red cells used for these tests were obtained from a local slaughterhouse. After the cervical vessels of the chickens were severed, the blood was collected in a flask containing a liter of 2 per cent sodium citrate solution. Two liters of chicken blood were collected at one time. The cells were washed three times in saline and after the final washing were centrifuged at 900 R.P.M. for 11 minutes. The packed cells were stored at  $4^{\circ}\text{C}$ . and were diluted just before use with 49 volumes of saline. Red cells were not used more than 1 week after being obtained.

The sera to be tested were inactivated by heating at  $56^{\circ}\text{C}$ . for 30 minutes. Serial dilutions were then made in saline, using twofold steps. To  $\frac{1}{2}$  cc. of each serum dilution was added  $\frac{1}{2}$  cc. of virus dilution, and to this mixture was added 1 cc. of a 2 per cent suspension of chicken red cells. The red cells were added with an automatic

pipetting machine which delivered the cells with such force that adequate mixing took place at once. The mixtures were allowed to stand at room temperature for  $1\frac{1}{4}$  hours before readings were made. This was found to give slightly sharper end points than readings made at 1 hour.

The degree of red cell agglutination was read by comparing the densities of the supernatant fluids in the various serum dilutions with the densities of standard red cell suspensions. The red cell concentrations of these standards were 1.0, 0.87, 0.75, 0.67, 0.50, 0.37, and 0.25 per cent, and they were made up in each instance from the same suspension that was used for testing the sera. The end point of a titration was considered to be the tube which had a density falling between that of the 0.67 per cent and the 0.50 per cent standard. Frequently, however, the transition from complete inhibition to maximum agglutination was such that there was no tube with a density falling within this range. The end point then was considered to be halfway between the tube of density higher than 0.67 per cent and the adjacent tube of density lower than 0.50 per cent. Serum titers were expressed as the reciprocal of the dilution of serum in the end point tube.

The same method of determining end points was used to standardize the virus preparations against which the sera were tested. For this measurement twofold dilutions of virus suspension were made in saline. To 1 cc. of each dilution was added 1 cc. of 2 per cent chicken red cells, and the end points were read after the mixtures had stood for  $1\frac{1}{4}$  hours. Four times the concentration of virus capable of producing the end point described above was used for testing the human sera. This usually resulted in the use of a final concentration of 1:64 for the PR8 strain and 1:32 for the Lee strain. This amount of virus generally produced a residual red cell density of 0.25 per cent or less in the supernatant of the virus control tube.

Since slight day-to-day fluctuations were known to occur in the degree of agglutination obtained with a given dilution of virus suspension, the following controls were set up to determine the amount of this variation. An agglutination titration of each virus was set up with every series of antibody determinations. A more important control, however, was the titration of standard immune ferret sera. With each series of determinations three duplicate titrations of standard sera against each virus were set up at the beginning, middle, and end of the test. Usually there was little or no difference between the results obtained with these standards, both in the same series and from one series to the next. When the controls indicated that the level of the test was markedly altered, the experimental results were corrected to a degree indicated by the deviation of the controls.

A further method for eliminating errors due to unpredictable fluctuations was to distribute the sera obtained from individuals given a single vaccine over many series of determinations so that each series usually contained some sera from individuals given each of the vaccines. In this way the antibody responses produced by all the vaccines were submitted to an equal amount of variation in testing. The prevaccination and postvaccination sera from each individual were always tested in the same run. Each run consisted of approximately 250 titrations, or 125 pairs of pre- and postvaccination sera.

In addition to the sera from the vaccinated individuals, acute and convalescent phase specimens from 232 cases of influenza A were tested at the same time and in

the same way. This was done in order to establish by the same technique the levels of antibody obtained following infection with influenza virus. In general, the acute sera were taken within the first 4 days of the illness and the convalescent sera about 2 weeks after the onset. All of these sera came from institutional epidemics in Alabama in 1941, and had been tested previously by the complement fixation or neutralization test and showed at least a fourfold rise in antibody titer by one technique or by both.

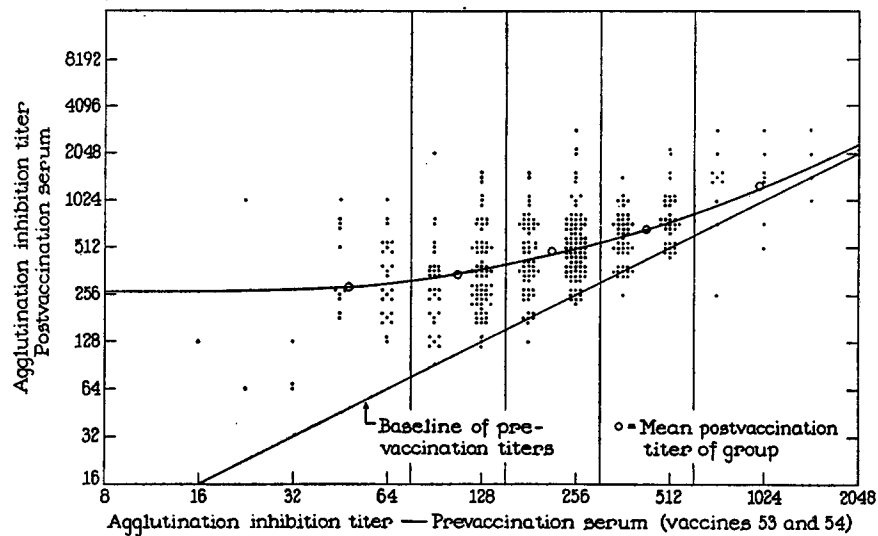


FIG. 1. Antibody response against the PR8 strain at 2 weeks of 400 human beings who were given formalinized chick embryo influenza vaccines 53 and 54.

#### EXPERIMENTAL

*Antibody Response of the Individual to Vaccination.*—Before comparing the average antibody responses of groups of human subjects to various vaccines, it was necessary to determine first the individual variations in response to one type of preparation.

Fig. 1 is a spot chart of the antibody response of 400 individuals 2 weeks after the subcutaneous injection of formalinized preparations of chick embryo containing influenza A virus (vaccines 53 and 54). The details of the preparation of these vaccines will be given below. Each dot on the chart represents the prevaccination and the postvaccination antibody levels of one individual against the PR8 strain of virus. The scale for both the pre- and the postvaccination levels is logarithmic. The entire group of subjects was divided into subgroups on the basis of their prevaccination titers, and the geometric mean of the postvaccination levels of each subgroup was determined. These mean values are represented by open circles in Fig. 1.

Since all the vaccines which were tested gave this same type of distribution in antibody response, certain generalities concerning the results shown in Fig. 1 may be of significance in the problem of vaccination against influenza in general. The most obvious finding was the enormous variation in antibody response which occurred even among those individuals who possessed similar prevaccination antibody titers. A considerable number failed to show a sufficient increase in antibody to be detected by this test, and the proportion of non-reactors was significantly greater among those individuals with high prevaccination levels than among those with low levels.

The curved line in Fig. 1 represents the level that would have been obtained if an equal amount of antibody had been added to each prevaccination specimen. Since this curve closely fits the geometric means of the postvaccination levels found experimentally, it follows that the average actual antibody production as opposed to increase in titer in each of these subgroups was nearly the same, regardless of the prevaccination antibody level.

From a consideration of the foregoing results it was decided to compare different vaccines in terms of the geometric mean antibody level obtained 2 weeks following vaccination. A significant comparison between mean levels can be made only if the prevaccination titers are nearly the same in the groups under consideration. This method is in contrast to that used by Horsfall, Lennette, and Rickard (5) who selected individuals of low antibody titer and based their conclusions mainly on the incremental increases (times rise) obtained in the low antibody group. As this study was based on unselected groups, the incremental rises observed were lower than those found by Horsfall, Lennette, and Rickard. In addition, the method of testing which has been used in this study gives results which show smaller differences between the antibody titers of sera than are shown either by serum neutralization end points in mice or by the calculation of neutralizing capacities. This in no way invalidates the relationship between the two tests previously demonstrated (9) but merely means that the range of titers by *in vitro* measurements are expressed on a compressed scale.

In this study the geometric mean has been used for comparison of different vaccines because of the ease of statistical handling. If influenza antibody titers are plotted on a geometric scale, they give fairly symmetrical probability curves (Fig. 2), and the geometric mean of the titers is at or near the peak of the curve. If the same titers are plotted on an arithmetic scale, however, the arithmetic mean is not at the peak of the curve, and the value of this mean is greatly affected by the presence or absence of a few sera of very high titer. The use of the arithmetic mean would involve the employment of extremely large groups in order to obtain significant comparisons. It should be emphasized that even when a large number of subjects are used, the variation in individual human response is so great that only rather large differences in the geometric mean levels have any statistical significance.

*The Effect of the X Strain of Distemper Virus on the Antigenicity of Influenza Vaccines.*—Horsfall, Lennette, and Rickard (5) reported a series of vaccination studies in human beings using a number of different virus preparations. From their results they concluded that a preparation of formalinized chick embryo virus prepared by inoculating embryos with a mixture of the PR8 strain of influenza A virus and the X strain of distemper virus had greater antigenicity

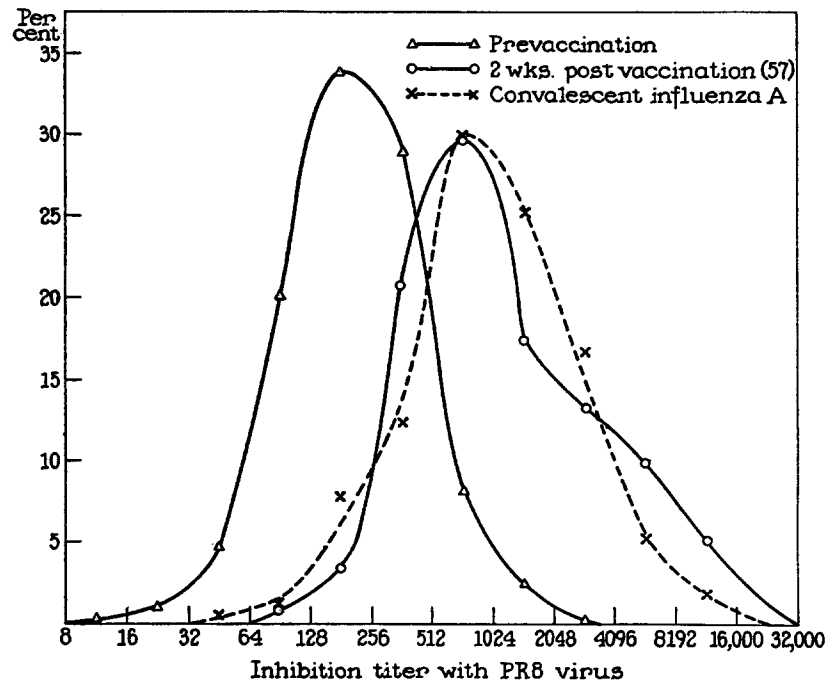


FIG. 2. Curves showing the distribution of antibody titers against influenza A among a normal population, a recently vaccinated population, and a group of convalescent cases of influenza A.

than a similar preparation made by inoculating the PR8 strain alone. However, during the past year, further observations in this laboratory have not borne out these conclusions. Since it is now known that great individual variation occurs in the antibody response to vaccination, a review of the evidence on which the comparison between these vaccines was made suggests that the results may have been due to errors introduced by random sampling. It was decided, therefore, to repeat these experiments on a larger scale.

*Vaccine 53.*—Eleven-day old white Leghorn embryos were inoculated with 0.05 cc. of a  $10^{-3}$  dilution of allantoic fluid containing the PR8 strain plus 0.05 cc. of saline.

The inoculations were made directly into the embryo. After 48 hours incubation at 37°C., the embryos were removed, homogenized in a Waring mixer, and diluted to 20 per cent concentration with saline. The sediment was removed by centrifugation and discarded. Formalin was added to a concentration of 1:4,000 formaldehyde. After standing for 48 hours at 4°C. the suspension was frozen and dried. The active embryo suspension gave a 50 per cent mortality titer of  $10^{-5.8}$  in mice. The finished vaccine was not infectious for mice when 0.05 cc. of a 20 per cent suspension was given intranasally. The vaccine was rehydrated to its former volume with distilled water just before use, and each subject was given 1 cc. of this 20 per cent suspension subcutaneously. This was the equivalent of 632,000 fifty per cent mouse mortality doses of active virus.

*Vaccine 54.*—The preparation was identical with that of vaccine 53 except for the inoculum used for the embryos. The inoculum contained 0.05 cc. of a  $10^{-3}$  dilution of allantoic fluid containing the PR8 strain and 0.05 cc. of a  $10^{-3}$  dilution of ground ferret spleen. The spleen was obtained from a ferret that had been infected with the X strain of distemper virus. Ferrets, when given 1 cc. of a  $10^{-4}$  dilution of this spleen suspension, came down with typical distemper. The 50 per cent mouse mortality titer of this vaccine was  $10^{-5.1}$ , and like vaccine 53, the finished preparation was non-infectious for mice. Individuals who were given 1 cc. of this vaccine subcutaneously received the equivalent of 500,000 fifty per cent mouse mortality doses.

While the technique of preparing these vaccines was not identical with that of Horsfall, Lennette, and Rickard, the differences in technique are not considered important. Furthermore the augmentation of antibody level produced by vaccines 53 and 54 was of the same order of magnitude as that observed by Horsfall, Lennette, Rickard, and Hirst (6) in a later and more extended series of vaccinations with the complex material.

The antibody response to the PR8 strain produced by these two vaccines is shown in Table I. It will be seen that both the mean prevaccination levels and the mean postvaccination levels of the two groups were nearly the same. It is probable, therefore, that the addition of the X strain of distemper virus did not significantly enhance the antigenicity of influenza A virus.

Although these two vaccines did not contain influenza B virus, it was decided to test all the sera against the Lee strain of this virus. The results, which are also shown in Table I, emphasize the complete lack of antigenic relationship between these strains of influenza A and influenza B viruses. They also show that the injection of chick embryo material, in the form of a vaccine, did not give rise to antibodies which seriously interfered with the specificity of the *in vitro* test, even though a chick embryo source was used for the test virus.

*Effect of the Amount of Virus Injected upon the Antibody Response.*—Since subcutaneously administered virus is not known to multiply in the human organism, the amount of virus given should have a pronounced effect upon the magnitude of the antibody response obtained, at least over a considerable range of virus concentrations. The following experiment was an attempt to



determine how much the response varied when different amounts of virus were given.

*Vaccine 56.*—This vaccine was prepared by mixing equal volumes of the PR8 and Lee allantoic fluid pools. The mixture was frozen and dried and rehydrated just before use. Each individual received subcutaneously 0.1 cc. of the mixed fluids diluted to a volume of 1 cc. with saline. This was the equivalent of 36,200 fifty per cent mortality doses of the PR8 strain and 1,000 fifty per cent mortality doses of the Lee strain per person. The viruses were active when injected.

*Vaccine 55.*—This preparation was made in the same way as vaccine 56, but each individual received subcutaneously 1 cc. of the mixed fluids, or 362,000 fifty per cent

TABLE I  
*Effect of the X Strain of Distemper Virus on the Antigenicity of a Vaccine Containing Influenza A Virus*

Vaccine No.	Preparation of vaccine	Influenza A virus (PR8 strain)			Influenza B virus (Lee strain)			No. vaccinated
		No. 50 per cent mouse mortality doses per person*	Mean prevaccination antibody titer	Mean 2-wk. post-vaccination antibody titer	No. 50 per cent mouse mortality doses per person	Mean prevaccination antibody titer	Mean 2-wk. post-vaccination antibody titer	
53	Formalinized whole chick embryo, inoculated with PR8 only	632,000	205	477	0	79	84	196
54	Formalinized whole chick embryo, inoculated with PR8 and X strain of distemper virus	500,000	205	486	0	77	84	201

\* Before inactivation.

mortality doses of the PR8 strain and 10,000 fifty per cent lethal doses of the Lee strain.

*Vaccine 57.*—A large amount of PR8 allantoic fluid was centrifuged at 11,000 R.P.M. for 2 hours, and the sediment was resuspended in a small amount of the supernatant fluid. Over 95 per cent of the virus was recovered in the sediment. A somewhat smaller amount of Lee allantoic fluid was likewise centrifuged and the sediment resuspended in a small amount of supernatant. These resuspended sediments were combined, frozen, and dried. The mixture was resuspended in saline at the time of administration and given subcutaneously in a volume of 1 cc. Each individual was given that quantity of virus originally present in 5 cc. of PR8 fluid and 2 cc. of Lee fluid, or 3,620,000 fifty per cent mortality doses of the PR8 strain and 40,000 fifty per cent mortality doses of the Lee strain. This represents ten times the amount of influenza A virus and four times the amount of influenza B virus in vaccine 55. As with vaccines 55 and 56, the viruses were injected in the active state.

*Vaccine 64.*—Although this preparation was made and given to human beings prior to any other vaccine reported in this paper, the sera were tested simultaneously with those from other vaccine groups. The vaccine was prepared in essentially the same manner as vaccine 57. Infected allantoic fluid was centrifuged at high speed and the sediments were pooled. The vaccine was not dried before use but was kept at  $-72^{\circ}\text{C}$ . until administered. Allantoic fluid containing the W.S. strain was included, as well as preparations of the PR8 and Lee strains. The amounts of virus given were calculated from mouse titrations on the concentrated suspensions. Each subject received subcutaneously 190,000,000 fifty per cent mortality doses of the W.S. strain, 24,000,000 doses of the PR8 strain, and 1,000,000 doses of the Lee strain. These quantities were obtained by concentrating 24 cc. of PR8 fluid, 10 cc. of W.S., and 5 cc. of Lee allantoic fluid.

TABLE II  
*Variation in the Antibody Response When Different Amounts of Influenza Viruses Were Used in Vaccines*

Vaccine No.	Influenza A virus (PR8 strain)			Influenza B virus (Lee strain)			No. vaccinated
	No. 50 per cent mouse mortality doses per person	Mean prevaccination antibody titer	Mean 2-wk. postvaccination antibody titer	No. 50 per cent mouse mortality doses per person	Mean prevaccination antibody titer	Mean 2-wk. postvaccination antibody titer	
56	36,200	183	369	1,000	85	183	145
55	362,000	168	461	10,000	87	276	146
57	3,620,000	163	935	40,000	84	443	120
64	PR8 = 24,000,000 W.S. = 190,000,000	103	1,100	1,000,000	57	444	43
*	Influenza A	104	865		90	84	232

\* Cases of clinical infection included for comparison.

*Influenza A Cases.*—In this experiment were included the 232 cases of influenza A previously mentioned. Each case had previously been diagnosed as influenza A on the basis of a fourfold or greater rise in antibody titer, as determined by complement fixation or neutralization tests.

The results of antibody titrations on sera obtained from these groups are shown in Table II. With vaccines 55, 56, and 57 the antibody response definitely increased with each tenfold increase in the amount of virus given. This is true of the response to both the PR8 and the Lee strains. However, the degree of change of antibody titer is of a considerably smaller magnitude than the differences in the amount of virus given.

With vaccine 57, the levels of antibody obtained were of the same order of magnitude as those which occurred following actual infection. A graphic comparison of these two groups is given in Fig. 2. It will be observed that not only

were the mean antibody levels similar but the distribution of titers was very nearly the same. It should be pointed out, however, that the levels in the convalescent group were measured with a heterologous strain of virus, while the sera from group 57 were titered with a homologous strain of virus. This might tend to cause the infected group to show lower titers than otherwise. On the other hand, the cases of influenza A were arbitrarily selected on the basis of a fourfold antibody rise. If the lesser antibody responses among the clinical cases had been included in this group, the mean final titer would have been 655. It seems unlikely that it will be possible to obtain, by vaccination, antibody levels which are greatly higher than those following actual infection by influenza viruses.

While vaccine 64 was given to a relatively small group, and the material used was not strictly comparable to that used in other experiments, the results are included as another example of the response obtained with a very large amount of virus. In terms of lethal doses of both the PR8 and the Lee strains injected, vaccine 64 was considerably superior to vaccine 57. In spite of this, the antibody response was of almost the same order of magnitude with the two preparations. This strongly suggests that further increases in the amount of virus injected at one time probably would not give a greatly improved antibody response. The large number of lethal doses of W.S. virus given may be misleading and was probably not an indication of the relative amount of antigen given.

*Relative Antigenicity of Active Versus Inactive Virus.*—In the following experiment a comparison was made between the antigenicity of active influenza viruses (the PR8 and Lee strains) and preparations of the same viruses which had been inactivated by heat or formalin. Since freezing and drying also inactivate influenza virus to some extent, a preparation was included in this experiment in which the vaccine was kept frozen at  $-72^{\circ}\text{C}$ . but was not dried.

All of the vaccines in this group were made from the allantoic fluid pools. Each individual received in one subcutaneous injection  $\frac{1}{2}$  cc. of the PR8 pool and  $\frac{1}{2}$  cc. of the Lee pool. The 50 per cent mortality end point of the PR8 pool was  $10^{-4.4}$  and of the Lee pool was  $10^{-3.0}$ . Therefore, each individual received the equivalent of 362,000 fifty per cent mortality doses of influenza A virus and 10,000 fifty per cent mortality doses of influenza B virus.

*Vaccine 55.*—This preparation has been described previously. The virus was frozen and dried without inactivation. Titration of the finished vaccine, however, showed a tenfold drop in mortality titer due to drying.

*Vaccine 59.*—After equal quantities of the PR8 and Lee pools were mixed, formalin was added to a final concentration of 0.2 per cent formaldehyde. The mixture stood at  $4^{\circ}\text{C}$ . overnight and was frozen and dried the following day. The formalinized mixture gave no evidence of infectivity when administered intranasally in full concentration to mice. Each subject received subcutaneously 1 cc. of the pooled fluids.

*Vaccine 60.*—Equal amounts of the PR8 and Lee pools were combined, and the

mixture was heated at 56°C. in a water bath for 15 minutes. The heated preparation gave no evidence of infectivity when administered to mice intranasally in full concentration. The vaccine was frozen and dried immediately after heating. Each subject received subcutaneously 1 cc. of the mixture.

*Vaccine 61.*—The preparation was the same as for vaccine 55 except that it was stored at -72°C. until administered and was not dried. There was no significant loss of infectivity for mice between the time of preparation and the time when it was given to human beings.

TABLE III  
*Effect of Inactivation of Influenza Viruses on Antigenicity in Human Beings*

Vaccine No.	Preparation of vaccine	Influenza A virus (PR8 strain)			Influenza B virus (Lee strain)			No. vaccinated
		No. 50 per cent mouse mortality doses per person	Mean prevaccination antibody titer	Mean 2-wk. postvaccination antibody titer	No. 50 per cent mouse mortality doses per person	Mean prevaccination antibody titer	Mean 2-wk. postvaccination antibody titer	
55	Active virus in allantoic fluid, frozen and dried	362,000	168	461	10,000	87	276	146
59	Virus in allantoic fluid inactivated with formalin	362,000*	183	429	10,000*	87	293	143
60	Virus in allantoic fluid inactivated by heating	362,000*	163	515	10,000*	90	278	144
61	Active virus in allantoic fluid, kept frozen, not dried	362,000	136	479	10,000	86	328	146

\* Before inactivation.

The results of this experiment are recorded in Table III. Against both influenza A virus and influenza B virus the mean postvaccination antibody titers showed only negligible variation among the vaccines used. Such variation as occurred was not statistically significant. The same results were obtained with vaccine 55 where 90 per cent of the virus activity was lost through drying, yet the effect was not significantly different from vaccine 61, which had an unaltered mouse virulence at the time of administration. In view of the fact that the previous experiment showed a significant drop in antibody response when the injected antigen was reduced 90 per cent (vaccines 55 and 56), it seems evident that the virus which is inactivated during desiccation is not significantly altered as an antigen.

*Effect of Adding Infected Chick Embryos to Allantoic Fluid Vaccines.*—In order to determine the effect of increasing the protein content of a vaccine, it

was decided to test preparations in which infected chick embryos were added to infected allantoic fluid. Since the chick embryos used for this purpose had the same virus titer as the fluids to which they were added, the effect would seem to be limited to increasing the proportion of non-virus material in an allantoic fluid vaccine.

*Vaccine 62.*—For this experiment chick embryos were used from the same eggs from which the allantoic fluid pools were prepared. Embryos infected with the PR8 strain were prepared separately from those containing the Lee strain. The chick embryos were ground in a Waring mixer, after the addition of enough of the allantoic fluid pool to make a 20 per cent chick embryo suspension. The sediment was removed by low-speed centrifugation and equal quantities of the PR8 and Lee suspensions were mixed, frozen, and dried. The vaccine was rehydrated just before use, and each subject received 1 cc. subcutaneously of the 20 per cent chick embryo suspension. The 50 per cent mouse mortality titers of both the PR8 and the Lee suspensions were the same as the respective allantoic fluid pools. Each individual received 362,000 fifty per cent mouse mortality doses of the PR8 strain and 10,000 fifty per cent mouse mortality doses of the Lee strain.

*Vaccine 63.*—This was prepared in an identical manner with vaccine 62 except that after mixing the two virus preparations formalin was added to a final concentration of 0.2 per cent formaldehyde, and the mixture stood at 4°C. overnight before freezing and drying. The formalinized mixture was not infective for mice when given intranasally in full concentration.

The results of vaccination with these preparations are shown in Table IV. For purposes of comparison the results obtained with vaccine 55 prepared from active allantoic fluid alone are included. A comparison of the mean post-vaccination antibody titers shows that the addition of whole chick embryo definitely enhanced the antigenicity of both influenza A and B viruses, when the viruses were in the active state. However, when the preparation was formalinized the antibody response was reduced to levels similar to those obtained with active allantoic fluid (vaccine 55) and with formalinized chick embryo alone (vaccines 53 and 54).

Since the mouse mortality titers of the viruses in vaccines 55 and 62 were similar, it is necessary to assume either that the added chick embryo suspension itself has some stimulatory effect or, as seems more likely, that the mouse titration does not accurately measure all of the virus in the chick embryo suspensions. It seems possible that such suspensions contain considerable quantities of non-infective but nevertheless antigenic virus. Why this additional antigenicity should disappear after formalinization is not clear.

Since no preparation was tested to which uninfected chick embryo tissue had been added, the precise effect of inert proteins themselves in influenza virus vaccines was not determined. It is fairly clear, however, from a comparison of the results obtained with vaccines 53, 54, 55, 62, and 63 that the

relatively low concentration of inert protein in allantoic fluid has little, if any, beneficial effect upon the antigenicity of the virus present therein.

*Rate of Disappearance of Antibody Following Vaccination.*—The groups that received vaccines 55 and 57 were bled 2 months following their vaccination. The smaller group that received vaccine 64 was bled 6 weeks and 5 months following vaccination. These sera were tested with the same lots of virus<sup>5</sup> used

TABLE IV  
*Effect of Added Ground Chick Embryo Tissue on the Antigenicity of Influenza Virus*

Vaccine No.	Preparation of vaccine	Influenza A virus (PR8 strain)			Influenza B virus (Lee strain)			No. vaccinated
		No. 50 per cent mouse mortality doses per person	Mean pre-vaccination antibody titer	Mean 2-wk. post-vaccination antibody titer	No. 50 per cent mouse mortality doses per person	Mean pre-vaccination antibody titer	Mean 2-wk. post-vaccination antibody titer	
62	20 per cent infected chick embryo diluted with infected allantoic fluid. Active virus	362,000	165	658	10,000	97	396	144
63	Same as vaccine 62 except preparation was formalinized	362,000*	166	477	10,000*	90	253	146
55	Active virus in allantoic fluid alone	362,000	168	461	10,000	87	276	146

\* Before inactivation.

TABLE V  
*Change in Mean Antibody Levels at Different Lengths of Time after Vaccination*

Vaccine No.	Antibody titer against influenza A virus (PR8 strain)					Antibody titer against influenza B virus (Lee strain)				
	Pre-vaccination	2-wk.	6-wk.	9-wk.	22-wk.	Pre-vaccination	2-wk.	6-wk.	9-wk.	22-wk.
55	168	461		315		87	276		213	
57	163	935		537		83	443		300	
64	103	1100	540		345	57	444	188		136

for the other titrations, and the 2-week postvaccination sera were retested at the same time. The mean antibody levels are shown in Table V. The pre-vaccination and the 2-week postvaccination titers are included for comparison.

There was a rapid and considerable drop in antibody levels after the peak at the 2-week period. While the loss in antibody was greater with vaccine 57 than with vaccine 55 at 9 weeks, the former group still had a higher titer at that time. Even at 22 weeks the group that had received vaccine 64 possessed anti-

body levels considerably above their initial level. When individual responses were examined, it was seen that the incremental drop in titer in all individuals was fairly uniform, *i.e.*, those who had responded poorly subsequently lost about the same percentage of their added antibody as did those who had responded well.

#### DISCUSSION

These experiments in large groups of individuals indicate that quantitative rather than qualitative factors influence the specific antigenicity of influenza virus vaccines as tested in human beings. The chief and most significant differences in antibody response were encountered when different amounts of virus were injected. Inactivation of influenza virus, whether the result of formalinization, heating, or drying, caused but little alteration in the antigenicity of the preparations. Furthermore the addition of inert chick embryo protein, containing inactive virus, to a given vaccine did not significantly change its specific antigenicity. However, when infected chick embryo tissue was added to infected allantoic fluid and the virus in the mixture was not inactivated, a significantly more marked antibody response was obtained than with the allantoic fluid alone. It seems possible that this unexpected result may have been due to the presence in the embryo tissue of a greater quantity of virus than was indicated by the titration of its infectiousness for mice.

With both influenza A virus and influenza B virus evidence was obtained that the average antibody response of human beings is directly related, though not strictly proportional, to the amount of virus given. Although higher antibody levels resulted from the administration of relatively great quantities of virus in concentrated preparations (vaccine 57) than were encountered after the injection of unconcentrated vaccines, the data suggest that there are definite limits to antibody response since a further increase in the quantities of virus (vaccine 64) did not increase the response appreciably.

The present uncertain state of knowledge concerning the significance of circulating antibodies in resistance to infection by influenza viruses makes it hazardous to attempt any prediction as to the possible increased resistance which might follow the use of any of the vaccines tested in this study. It has been shown that antibody levels tend to be lower in acute phase sera obtained from cases of influenza than they are in the general population (18, 19). Furthermore it has been found that some reduction in the incidence of influenza A occurred in groups given a formalinized vaccine (6, 7, 20) which had increased the level of circulating antibodies. While these observations show that there is some relationship between specific circulating antibodies and resistance to clinical infection, there is also considerable evidence (18, 19) which indicates that individuals possessing the highest normal levels of circulating antibody are still susceptible to influenza.

None of the vaccines used in this study was capable of increasing the antibody level of all the individuals in a group to titers higher than those possessed by some of the members of the group before vaccination. Consequently the antibody level distribution curves before and after vaccination in each group overlapped by an appreciable extent (Fig. 2). Since the increased antibody titers which followed vaccination rapidly decreased with time, there is reason to think that whatever degree of increased resistance might result from the administration of a particular influenza vaccine would also become progressively less effective with the passage of time.

It should be reemphasized that the only satisfactory test of the possible efficacy of vaccines against influenza is extensive field trial under carefully controlled conditions. It seems probable that the better the antibody response following vaccination the more likely is the development of increased resistance. From the results of these studies it appears that concentrated virus suspensions produce greater antibody responses than do other kinds of vaccines.

#### SUMMARY

Eleven different preparations of influenza virus were used to vaccinate large groups of human beings. The antibody response to these vaccines was measured by means of the *in vitro* agglutination inhibition test, and the geometric mean titers of sera taken 2 weeks after vaccination were compared. From these comparisons the following conclusions were drawn:

1. There was a wide individual variation in the antibody response of human beings to the same preparation of influenza virus administered subcutaneously. The amount of antibody produced by a group with a low prevaccination antibody level was very nearly the same as the amount produced by groups that had higher initial levels.
2. The use of the X strain of distemper virus in the preparation of an influenza vaccine did not enhance the antigenicity of the influenza virus present.
3. Within certain limits the mean antibody response of human beings increased as the amount of virus injected was increased. When large amounts of influenza A virus were given, the antibody response was of the same order of magnitude as that which occurred following actual infection by this virus.
4. When the vaccine was prepared from allantoic fluid, there was no significant difference in the antibody response of human beings given active virus, formalin-inactivated virus, heat-inactivated virus, or virus inactivated by the drying process.
5. Ground infected chick embryos, when diluted with infected allantoic fluid, gave a greater antibody response than allantoic fluid alone (when the virus remained active). The antigenicity of such a preparation was diminished when the virus was inactivated by formalin.
6. Antibody levels 6 and 9 weeks after vaccination showed a marked drop



from the 2-week postvaccination levels. In a small group the antibody levels at 5 months were still further reduced. Those individuals who possessed the higher titers tended to lose their antibodies faster than did those at a lower level.

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