QUANTITATIVE RELATIONSHIPS BETWEEN THE IMMUNIZING DOSE OF EPIDEMIC INFLUENZA VIRUS AND THE RESULTANT IMMUNITY

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(Received for publication, October 4, 1938)

In the problem of subcutaneous immunization of human individuals with the virus of epidemic influenza the question of what constitutes an effective dose of virus is of great importance. Francis and Magill (1) and Stokes *et al.* (2) have shown that the administration of one or more subcutaneous doses of active virus to human individuals elicits a rise in circulating antibodies, but conclusive evidence as to the degree of active immunity engendered by this procedure has not been obtained. In mice it has been well recognized that intraperitoneal vaccination with active virus results in a firm immunity to intranasal infection. To a lesser extent the same effect is obtained by subcutaneous vaccination, and Andrewes and Smith (3) have reported that high concentrations of virus induce a better degree of immunity than low ones. As a result of subcutaneous vaccination ferrets develop antibodies but not complete immunity to intranasal infection (4), while intraperitoneal vaccination is again more efficacious than the subcutaneous (5). Little attention has been paid, however, to the strength of virus used in vaccination and to the severity of the intranasal test for immunity.

The object of the present studies was to ascertain the relationship between the concentration of influenza virus used in vaccination and the extent of the immunity so induced. The details of experiments designed for this purpose comprise the substance of this article.

Immunity of Mice to Intranasal Infection after Intraperitoneal Vaccination with Graded Doses of Virus

Experiment 1.—The PR8 mouse passage strain of epidemic influenza virus (6) was used. A suspension of infected mouse lung was made by grinding the lungs

in meat infusion broth. Using the original weight of the material as a unit, serial tenfold dilutions ranging from 1:10 to 1:1 million were made.

Seven groups of 50 mice each were selected. The mice of one group were given intraperitoneally 0.5 cc. amounts of the 1:10 dilution of virus; mice of the next group were given 0.5 cc. amounts of the 1:100 dilution of virus, etc. At weekly intervals the mice were reinoculated with virus in the same concentration for a total of three doses. One group of mice was kept as untreated controls. A certain number of animals died during the course of immunization, death being due apparently to intercurrent bacterial infection. 1 week after the last intraperitoneal inoculation, each mouse, including the controls, was given intranasally 0.05

TABLE I

Relation of Immunizing Dosage (Intraperitoneal) to Resistance to Intranasal Infection with 1,000 M.L.D. *(Mice)*

Immunizing dose of virus	Number of mice	Deaths		Pulmonary lesions in survivors	Titration of virus used for infection
		No.	Per cent		
10^{-1}	39	0	0	0 0 0 0 0 0 0 0 0 0	
10^{-2}	49	0	0	0 0 0 0 0 0 0 0 0 0	
10^{-3}	50	0	0	0 ± 0 ± 0 0 0 ± 0 0	
10^{-4}	50	1	2	+ 0 ± 0 + ++ 0 ++ 0 ±	5, 7, 7, 8, 9, 10
10^{-5}	50	28	56	++ + + ++ ++	8, 9, ++++, ++, ++++, ++++
10^{-6}	48	47	98	—	9, 10, ++++, ±, +++, ++
Unvaccinated controls	49	45	92	—	

± to ++++ = increasing degrees of pulmonary involvement.
0 = no pulmonary involvement.
Numerals in titration results indicate day of death of individual mice.

cc. of a 1 per cent suspension of virus (approximately 1,000 lethal doses). It should be noted that mouse passage virus in a dilution of 10^{-5} produces fatal infection in the great majority of mice. The animals were observed for 10 days, and on the 11th day representative groups of survivors were sacrificed and the presence of pulmonary lesions was determined. The results are presented in Table I.

A definite relationship between the strength of virus used for immunization and the resultant degree of immunity is seen. Mice vaccinated with virus in 1:10 and 1:100 dilutions were completely resistant to the intranasal infection, as evidenced by the survival of

all the mice and the absence of pulmonary lesions. Mice vaccinated with the 1:1,000 concentration of virus exhibited less complete immunity in that, while all mice survived, occasional pulmonary lesions of minimal severity were seen. One of the mice immunized with the 1:10,000 concentration died and pulmonary lesions were more frequent and more extensive in the survivors. Approximately half of the mice immunized with virus diluted to 1:100,000 died and the survivors uniformly showed pulmonary lesions of moderate extent. When given by the intranasal route virus in a concentration of 1:1 million proved capable of producing extensive pulmonary lesions and some deaths, but when given intraperitoneally it failed to elicit any immunity to the test dose, since mice so treated died as uniformly as the controls.

Thus complete immunity to 1,000 lethal doses was observed only in those mice vaccinated intraperitoneally at each injection with the equivalent of 1,000 lethal intranasal doses or more. Fatal infections developed in mice vaccinated with amounts of virus equivalent to less than 100 lethal doses. The regular gradation in immunity paralleling the gradation in immunizing dose is extremely clear and indicates that statements regarding immunity must under these conditions be accompanied by quantitative data.

The fact that complete immunity to intranasal infection with 1,000 lethal doses was acquired only by mice vaccinated with virus of similar strength, or more, suggested that a given amount of virus injected intraperitoneally induces a degree of immunity effective against no more than the equivalent strength of virus injected intranasally. The following experiment was devised to study this possibility.

Experiment 2.—The Melbourne strain of epidemic influenza virus (7) in its 128th to 131st transfer in chick embryo-Tyrode's tissue culture medium was used. The virulence of the virus was such that 0.05 cc. of a 1:10,000 dilution of the supernatant fluid given intranasally caused a fatal infection; a 1:100,000 dilution invariably caused well marked pulmonary lesions, with occasional deaths; and inconstant, mild pulmonary lesions followed injection with the virus in a dilution of 1:1 million. Groups of 50 mice each were inoculated intraperitoneally with 0.5 cc. amounts of the undiluted culture supernatant and with similar amounts of the supernatant diluted 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}, respectively. Two injections were given, 1 week apart. A group of 50 untreated control mice was kept.

TABLE II

Relation between the Immunizing Dose of Virus and the Resultant Immunity (Mice)

Intranasal test dose of virus	Immunizing dose of virus																									
	Undiluted					10^{-1}					10^{-2}					10^{-3}					10^{-4}					Unvaccinated controls				
Undiluted	++	+	+	±	0	4	5	5	7	10	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	0	0	0	0	0	+++	++	0	0	0	4	4	4	+	±	3	4	4	6	0	5	5	5	5	5	3	3	3	5	6
10^{-1}	0	0	0	0	0	+	+	+	0	0	4	4	5	5	++	3	4	4	4	4	3	3	4	4	4	5	5	5	5	5
	0	0	0	0	0	0	0	0	0	0	+	+	±	0	0	6	+	±	0	0	4	4	4	4	4	5	5	5	5	5
10^{-2}	0	0	0	0	0	++	++	+	+	+	0	0	0	0	0	4	5	6	6	6	4	4	4	4	4	4	4	4	4	5
	0	0	0	0	±	+	0	0	0	0	0	0	0	0	0	6	7	8	+++	0	4	5	5	6	6	5	5	5	5	6
10^{-3}	0	0	0	0	0	±	±	0	0	0	++	+	+	0	0	6	7	+++	++	++	5	6	6	6	6	4	5	5	6	6
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	++	±		0	0	6	7	7	++	++	6	6	6	6	+++
10^{-4}	0	0	0	0	0	+	0	0	0	0	6	10	+	0	0	7	9	+	+	+	6	7	8	8	8	7	7	8	9	10
	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	++	+	±	0		10	++++	+++	++	+	10	++++	++++	++++	+
10^{-5}																										8	+++	++		
																										+++	+++	++		

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

0 = no pulmonary lesions.

Numerals indicate day of death of individual mice.

A week after the second intraperitoneal injection ten mice of each group were infected intranasally with 0.05 cc. of full strength culture virus; other groups of ten each received 0.05 cc. of culture diluted 1:10, 1:100, 1:1,000, and 1:10,000, respectively. Thus, mice vaccinated with a given concentration of virus were tested for immunity with graded amounts containing from 1 to 10,000 lethal doses of the same strain of virus. Unvaccinated control mice were similarly infected. The mice were observed for 10 days and deaths recorded. All survivors were sacrificed and the degree of pulmonary involvement was noted. It should be observed that, in the experiment presented, minute lesions were seen in the lungs of many of the surviving mice. This may be due to the fact that only two vaccinating injections were given, or more probably is related to the use of chick embryo culture material.

The results are presented in Table II.

There is a striking relationship between the strength of virus used for vaccination and the dose of virus against which the mice were protected. Mice vaccinated with full strength virus survived infection with full strength virus. Half the mice vaccinated with culture virus diluted 1:10 died when tested against full strength virus but were immune to the 1:10 and weaker dilutions of virus. Similarly, mice vaccinated with the 1:100 dilution of virus were resistant to infection with a 1:100 or higher dilution of virus, but exhibited only partial immunity to the 1:10 concentration and little or no immunity to the undiluted material. The same sequence of events was seen in mice vaccinated with the 1:1,000 dilution; they exhibited an increased resistance to virus of the same strength as that used for vaccination but not against the higher concentrations. Vaccination with a dilution of 1:10,000 induced no apparent resistance since the mice so treated showed no appreciable difference from the controls in their reaction to infection even with virus of the same strength as that used in their vaccination.

When measured by survival and death the results strongly suggest that, under the conditions of this experiment, intraperitoneal vaccination with a given dose of virus induces a degree of immunity sufficient to protect mice against intranasal infection with virus of equivalent strength or less. Below a certain level of virus concentration vaccination does not afford protection even against infection with virus of the same concentration. On the other hand, in the upper range of immunizing doses a firm immunity is established, effective against the strongest concentrations of virus. It appears, therefore,

that there is a threshold of immunity which varies with, and is closely proportionate to, the concentration of virus used in the process of immunization. Suspensions of virus containing less than 10 lethal or 100 infectious doses per 0.05 cc. produce little immunity, but above that level the concentration of virus which mice will resist is approximately the same as the concentration of virus used for vaccination.

The Relation of Vaccinating Dosage to the Development of Antibodies and Resistance to Infection in Ferrets

Vaccination of ferrets with influenza virus administered by the subcutaneous route does not ordinarily result in a complete resistance of the animal to intranasal infection. That immunity is produced is shown, however, by the fact that properly vaccinated ferrets when subsequently infected do not develop the pneumonic lesions typical of experimental influenza in that species of animal (4). Since, however, no studies on the quantitative aspects of the problem in ferrets have been reported, it seemed of interest to determine what relationship, if any, exists between the size of the immunizing dose for that animal and the subsequent response to infection. The following experiments were, therefore, undertaken.

Experiment 3.—The PR8 ferret passage strain of epidemic influenza virus was used. The lung of an infected ferret placed in glycerine 10 days previously served as the source of virus. Virus so obtained almost invariably produces infection when given to normal ferrets intranasally in dilution of 10^{-6} and frequently in lower concentration. Using the weight of lung tissue as the unit, a suspension was made by grinding with alundum, and six tenfold serial dilutions ranging from 10^{-1} to 10^{-6} were made in Locke's solution. Twelve normal ferrets, previously bled from the heart, were selected and 4.0 cc. of the virus dilution was then given subcutaneously, in the flank, to each of two ferrets. The site of the inoculation was then painted with alcohol and iodine and observed for leakage. This painting discouraged the animal from licking the site of injection, a habit infrequent in ferrets even in the absence of such precautions. In this manner groups of two ferrets were vaccinated with the respective dilutions of virus. Those receiving the lowest concentrations of virus were vaccinated first to prevent deterioration of the virus through standing. In order to demonstrate the infectivity of the virus suspension control animals were inoculated intranasally, under ether, with 2.0 cc. of the 10^{-6} dilution.

Temperatures of the vaccinated ferrets were taken daily and the animals were observed for any other signs of infection. None was detected.

One week later and again 2 weeks later the same procedure was carried out, each time using the same glycerinated lung as the source of material.

Two weeks after the third weekly subcutaneous injection all animals, while anesthetized with ether, were given intranasally 2.0 cc. of a 10 per cent suspension of freshly infected ferret lung. Subsequently, their temperatures were taken twice daily, their appetites noted, and records of nasal and respiratory symptoms made by a trained caretaker who was uninformed as to the nature of the study. One or two unvaccinated control animals were infected at the same time. 2 weeks after infection each animal was bled from the heart and the serum removed for antibody titrations. The animals were then sacrificed in order that the lungs might be examined for pneumonic lesions.

Antibody titrations of each serum were made in mice by the method described by Francis and Magill (8) in which serial dilutions of serum are tested against 1,000 lethal doses of mouse lung suspension of the PR8 strain of virus. The mice are observed for 10 days and the dilution of serum which protects 50 per cent of the mice from fatal infection is taken as the end-point.

The results of the clinical, serological, and pathological observations are summarized in Table III. Antibodies were not detected in the serum of animals vaccinated with the 10^{-6} or 10^{-5} concentration of virus, in one of the two vaccinated with the 10^{-4} concentration, but were present in the serum of all those receiving the 10^{-3}, 10^{-2}, or 10^{-1} concentrations. Hence antibodies were detected only in those animals vaccinated with concentrations of virus which were equivalent to 100 or more infectious doses. Complete immunity as measured by absence of fever and clinical symptoms was not produced even in the presence of antibodies. Nevertheless, the ferrets which had received the largest vaccinating doses of virus presented the most modified reactions in the immunity test. Their fever was mild and of brief duration; in the first four animals a single rise of temperature to the upper limit of normal was observed. Nasal symptoms were slight or absent, and no respiratory distress was detected. Animals vaccinated with the 1:100 dilution presented higher fever of longer duration beginning earlier after infection. Furthermore, mild respiratory symptoms were noted. In animals vaccinated with smaller doses the febrile course was more prolonged; both nasal and pulmonary symptoms became progressively more marked so as to approach the type of disease noted in the control animals. When the animals were sacrificed 2 weeks after infection, it was found that those vaccinated with the smaller doses presented characteristic pneumonic lesions in their lungs, whereas those which had received the larger doses had been

TABLE III

Relation between Vaccinating Dose and Resistance to 100,000 Infectious Units in Ferrets

Ferret No.	Vaccinating dose	Antibody titer after vaccination	Response to intranasal infection				Lung lesions 2 wks. later	Antibody titer after infection
			Fever		Signs			
			Highest	Duration	Nasal	Pulmonary		
			°F.	hrs.				
6-94	10^{-1}	100	104.0	12	±	0	0	3,200
6-95	10^{-1}	335	103.6	12	0	0	0	3,200
6-96	10^{-2}	150	104.0	8	+	0	0	1,600
6-97	10^{-2}	162	104.1	8	±	0	0	1,140
6-98	10^{-3}	162	105.1	24	+	+	0	1,140
6-99	10^{-3}	100	105.3	48	±	+	0	3,200
7-00	10^{-4}	0	104.2	48*	+	0	+	280
7-01	10^{-4}	25	105.7	48*	+	±	±	1,200
7-02	10^{-5}	0	106.7	48*	++	++	++	140
7-03	10^{-5}	0	105.9	72	++	+++	+++	100
7-04	10^{-6}	0	105.7	144	+++	+++	+	280
7-05	10^{-6}	0	107.2	216†	++	+++	+++	960
7-06	Control	—	105.0	48*	++	++	Sacrificed 4th day +++	
7-07	Control	—	105.9	Moribund 4th day	+++	++++	++++	

In Tables III and IV.

	Nasal	*Pulmonary*
±	= slight nasal discharge, 1 day.	Respirations irregular.
+	= discharge definite, 1 day.	Respirations increased, 1 to 2 days.
++	= dirty nasal discharge, 2 to 3 days.	Respirations increased, 3 to 6 days.
+++	= dirty nasal discharge, 3 to 5 days.	Respirations labored, 2 to 5 days.
++++	= dirty nasal discharge, 5 or more days.	Respirations labored, 5 to 7 days.

* Subsequent secondary rise of temperature.

† Temperature subnormal, first 3 days.

protected from pulmonary consolidation. The pulmonary lesions were first detected in the ferrets receiving the 10^{-4} vaccinating dose, which was also the critical level for antibody formation.

It appears, therefore, that the concentration of virus in the immunizing dose exerts a distinct influence upon the degree of immunity which is evoked and upon the production of antibodies. Immunizing doses of virus equivalent to 100 intranasal infectious doses or more appear to elicit evidence of immunity, while smaller doses fail to induce antibody formation, to influence the severity of the clinical disease, or to protect ferrets from pulmonary involvement when they are tested for immunity with approximately 100,000 infectious doses of virus.

Experiment 4.—The same procedure as that of Experiment 3 was repeated, except that the vaccinating material was produced from a freshly infected ferret each week in place of stock glycerinated virus. A 1 per cent suspension of virus was used in the immunity test instead of a 10 per cent suspension, as in Experiment 3. Furthermore, the animals were not sacrificed for pathological sections after the immunity test. Control animals were inoculated intranasally with the 10^{-6} preparation of virus each week at the time of vaccination. In one of the three instances virus was not demonstrable at that dilution, hence ferrets vaccinated with the 10^{-6} dilution of virus received only two injections of virus.

Table IV presents the tabulated data.

The results of the experiment were closely similar to those of Experiment 3. Antibodies were not detectable in animals vaccinated with doses containing less than 100 intranasal infectious units (10^{-5}, 10^{-6}). In general the temperature reaction was more marked than in the previous experiment although ferret 10-51 showed almost no clinical evidence of infection and ferret 10-50 had only borderline fever. The animals which developed no demonstrable antibodies exhibited a much more severe type of infection than those in whose serum antibodies had developed. The animals receiving the largest vaccinating doses (10^{-1}, 10^{-2}) had somewhat higher titers of antibodies and less prolonged febrile reactions than those receiving the intermediate sized doses (10^{-3}, 10^{-4}). Additional evidence of response to the virus administered intranasally was obtained, both in these animals and those of Experiment 3, in that all ferrets possessing circulating antibodies developed a further marked increase in antibodies as a result of the immunity test, and the level attained was much higher than that observed in the ferrets which showed no antibodies as a

result of vaccination. This phenomenon is not observed in fully immune animals and is interpreted as evidence of modified susceptibility.

The Relation of Infecting Dose of Virus to Severity of Disease and Immunity

The previous evidence of a marked quantitative relationship between the size of the virus dosage and the resultant immunity was

TABLE IV

Relation between Vaccinating Dose and Resistance to 10,000 Infectious Units in Ferrets

Ferret No.	Vaccinating dose	Antibody titer after vaccination	Response to intranasal infection				Antibody titer after infection
			Fever		Signs		
			Highest	Duration	Nasal	Pulmonary	
			°F.	hrs.			
10-54	10^{-1}	100	104.7	24	0	0	3,000
10-53	10^{-1}	143	104.8	12	±	0	2,250
10-52	10^{-2}	100	105.4	24	+	±	960
10-51	10^{-2}	100	None	—	±	0	1,350
10-50	10^{-3}	20	103.2 103.5	48	++	+	960
10-49	10^{-3}	90	106.2	24*	+	0	1,140
10-48	10^{-4}	25	104.4	72*	±	+	1,140
10-47	10^{-4}	2	105.9	48*	±	+	1,140
10-46	10^{-5}	0	104.6	96‡	++++	++++	565
10-45	10^{-5}	0	104.1	120	+++	0	565
10-44	10^{-6}	0	106.0	72‡	++++	+++	400
10-43	10^{-6}	0	104.6	48*	++	+	400
9-83	Control	0	106.3	72*	+++	++	Not done

‡ Temperature dropped to subnormal level.

based upon intraperitoneal or subcutaneous vaccination of animals. It seemed of interest, therefore, to compare the effects when similar quantitative experiments were performed with different amounts of virus given intranasally so as to produce infection. The characteristics of the resultant disease could be studied clinically and the degree of immunity produced by graded infectious doses of virus could be ascertained.

Experiment 5.—A group of twelve ferrets (Nos. 6-44 to 6-55) was divided into six groups of two each. Serum was obtained from each animal by cardiac puncture. A suspension of the lungs of a ferret infected with the PR8 strain of epidemic influenza virus was prepared, and serial tenfold dilutions of virus, 10^{-1} to 10^{-6}, were made. A pair of ferrets while anesthetized were inoculated intranasally with 2.0 cc. of a given virus dilution. Temperatures were taken twice daily and all symptoms were recorded. 14 days after infection samples of serum were again obtained, and in order to test for immunity all animals were reinoculated intranasally 1 week later with 2.0 cc. of a 10 per cent suspension of ferret passage virus. 14 days after the immunity test serum was again obtained. Titrations of the neutralizing antibody content of each serum were done in mice, with 1,000 lethal doses of the PR8 mouse passage strain of virus as a standard concentration.

A marked difference in the severity of infection resulting from different infectious doses was seen. The larger doses produced a marked, sustained fever, loss of appetite, severe respiratory distress, and profuse nasal discharge. With the smaller doses the incubation period was prolonged and the disease, in general, was much milder. In two ferrets, one receiving the 10^{-5} dilution and one receiving the 10^{-6} dilution, extremely mild infection resulted. In each instance a brief rise of temperature was detected on the 5th day although few clinical signs of infection were observed. When the series of ferrets was tested for immunity on the 21st day after the original inoculation, all were immune as shown by the lack of fever or other evidence of illness. All animals had developed antibodies of similar titer as a result of the primary infection, except the two ferrets mentioned above whose titers were lower. In response to reinoculation only these two animals showed additional rise in antibody titer, indicating that despite the absence of clinical response to infection, they had not reached as complete a state of immunity as the others which seemed completely refractory. The evidence suggests, then, that with the possible exception of two animals exhibiting the slightest degree of infection, the immunity present immediately after infection bears no relation to the size of the infectious dose and that fully immune animals when reinoculated do not respond with additional antibody formation.

It was conceivable, however, that, were the interval between the primary and secondary infections prolonged until some of the immunity was lost, a correlation between the size of the original infecting dose and the degree of immunity which persisted could be had. Accordingly, the experiment was repeated.

Experiment 6.—Ferrets were infected in the same manner as in Experiment 5. Those which received the largest dose succumbed to a severe infection. Both ferrets receiving the smallest dose, 10^{-6}, developed fever on the 7th day after inoculation and exhibited minimal signs of disease. There was a distinct tendency to gradation of symptoms with infectious doses of the intermediate size. 14 and 93 days after the first inoculation the animals were bled. On the 98th day all ferrets were reinoculated intranasally with 2.0 cc. of a 10 per cent virus suspension, two normal controls receiving the same dosage. The course of the reaction to the second dose of virus was again recorded and a third bleeding obtained 10 days after reinoculation. Antibody titrations were performed as previously described

The results of the study in half the series are presented graphically in Chart 1. In each instance the ferret represented is the second animal of the pair. The sequence of events in the group comprising the alternates was similar but somewhat obscured by the fact that three of those animals developed secondary complications late in the disease.

It can be seen that the animals which developed the most severe illness as a result of the original infection were those receiving the larger doses. When tested 3 months later with a large concentration of the same virus, the animals which originally developed the least severe (in No. 6-79 practically subclinical) infection appeared to be most severely affected, while those which had undergone the most marked primary infection appeared to be the most resistant to the second inoculation of virus. The level of antibodies in the interval between tests had tended to decline although in No. 6-79 a pronounced increase was observed. This may be due to the fact that the interval between the onset of the first infection and the time of bleeding was relatively shorter than in the other ferrets. The mate of No. 6-79, however, had a relatively low antibody titer throughout.

The evidence, then, suggests that with the passage of time there may be a relationship between the size of the infecting dose, the severity of the disease, and the relative resistance to infection after the period of firm immunity has passed. In this connection it should be noted that, compared with the previously untreated control animals. the disease resulting from reinoculation of the partially immune ferrets was distinctly modified. Moreover, in response to the second infection the antibody development was strikingly accelerated, reaching in almost every instance the limit of titer, 1:3,200. This type of serological response characterizes the partially immune animal, as

shown by comparison with the titers in the same animals after the first infection. As described in Experiment 5, the fully immune ani-

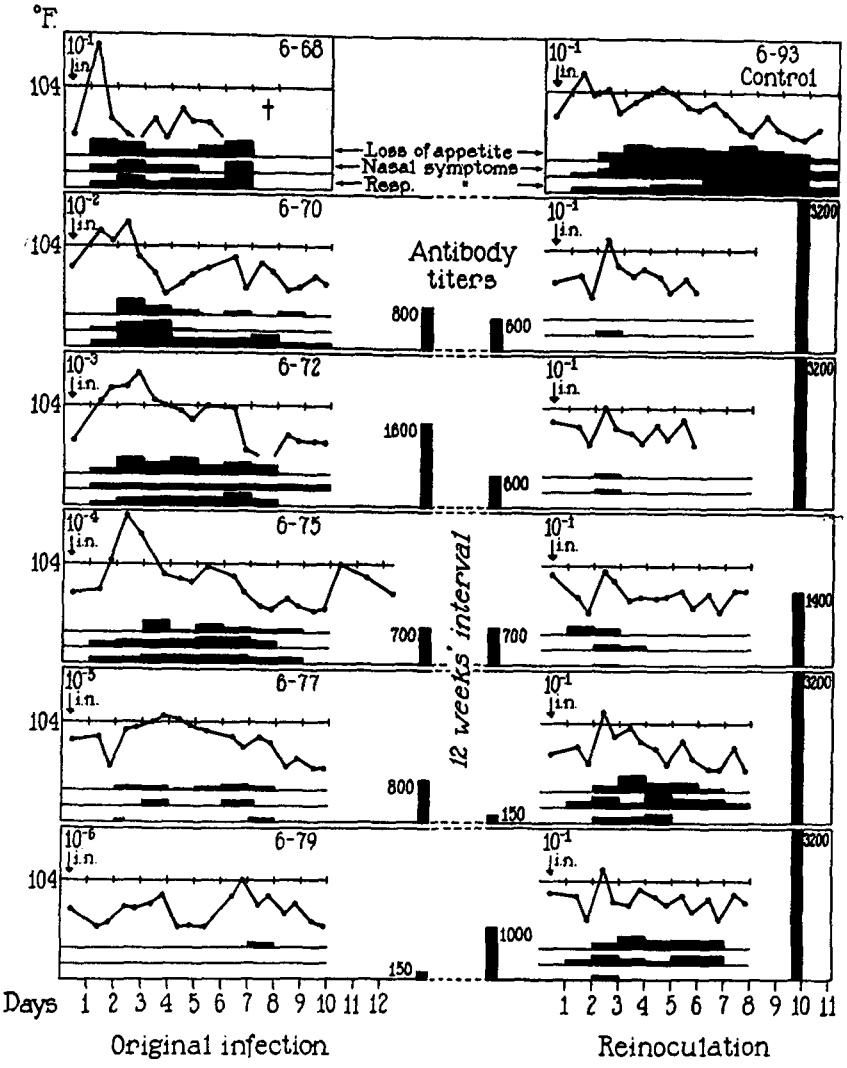

CHART 1. The relation of infectious dose to severity of infection and immunity.

mal ordinarily shows little or no serological response to the second stimulus. In fact, evidence is at hand which suggests that the anti-

body response to second inoculation is inversely proportionate to the degree of immunity.

DISCUSSION

The results of experiments dealing with the immunization of mice with epidemic influenza virus injected by the intraperitoneal route seem clearly to establish a quantitative relationship between the concentration of virus in the immunizing dose and the level of immunity which is reached. Thus it was found that a progressive increase in the virus concentration of the immunizing dose was paralleled by a similarly increasing degree of immunity as measured by infection with 1,000 intranasal lethal doses of the same virus. Complete immunity to this infecting dose was demonstrable only in mice vaccinated with concentrations of virus containing approximately 1,000 intranasal lethal doses, or more, per 0.05 cc. Vaccination with smaller doses resulted in decreasingly effective immunity until the vaccinating range of approximately one lethal dose per injection was reached, when no evidence of immunity was observed.

Subsequently, mice vaccinated with serially decreasing doses of virus were tested with a parallel range of infectious doses. In this instance the correlation between the concentration of virus used for vaccination and the concentration of virus which the animals could overcome is remarkable. In fact, it appears to be a direct proportion. Mice vaccinated with a given concentration of virus are protected against infection with virus in approximately the same concentration or less, but are either not protected or only partially protected against larger concentrations of virus. With the smallest vaccinating doses, approaching the range of one lethal intranasal unit per 0.05 cc., no detectable immunity is produced even against the equivalent concentration of virus. Thus there appears to be an irreducible minimum of approximately ten intranasal lethal doses required for the induction of even the mildest degree of immunity, and there is a definite threshold to the level of immunity which a given concentration of virus can elicit. The threshold of resistance corresponds in general with the concentration of virus used in vaccination.

In ferrets, while complete immunity is not obtained as a result of subcutaneous vaccination with active virus, a distinct resistance develops. When vaccinated ferrets are tested intranasally with

10,000 to 100,000 infectious units, the resultant clinical response is clearly conditioned by the concentration of virus used in vaccination. This is further shown by determination of the antibody production after vaccination. Antibodies are not produced until the concentration of virus in the immunizing dose reaches 100 infectious units, an amount corresponding roughly with the minimal requirements for the development of effective immunity in mice. The animals at the borderline are somewhat more severely affected than those immunized with the higher concentrations, but the state of partial immunity and antibodies appear uniformly together.

The observations reported are somewhat similar to those of Olitsky and Cox (9–11) with the virus of equine encephalomyelitis. These authors noted that approximately 1,000 mouse lethal units of virus were required to induce immunity in guinea pigs by the subcutaneous route, even though fatal infection frequently occurred when larger immunizing doses were used. In mice the equivalent of 1,000 lethal units in formolized vaccine was required to give immunity to 20 to 50 intracerebral lethal doses, and when no immunity developed no demonstrable antibodies were present in the blood. Study of their protocols suggests that a still closer proportionate relationship, somewhat similar to that of Experiment 2 of the present series, actually existed.

The results of the vaccination experiments in which a definite, proportionate relationship between size of the immunizing dose and immunity is found to exist are in sharp contrast to those obtained as a result of infection. Infection is followed by a staunch immunity to reinfection even though the infectious dose is so small as to elicit little more than a subclinical infection. The severity of the disease tends to vary with the infecting dose, but, in general, the immediate immunity is related to infection and not to the amount of virus inoculated. In certain instances, after infection with the smallest doses, reinoculation results in a second rise of antibody titer although the animal is clinically immune. Moreover, when an interval of months elapsed between the first and second tests, the impression was gained that animals receiving originally the largest doses and exhibiting the most severe infections presented the mildest reactions to reinoculation, whereas ferrets which experienced the mildest infection originally

tended to have the most marked reactions to reinoculation. Nevertheless, all animals retained a distinct but modified state of immunity.

It becomes obvious that the action of the virus in inducing immunity following vaccination differs from that in play during infection. Introduction of minute amounts of virus by the intranasal route results in infection, multiplication of the virus, and a staunch, immediate immunity essentially unrelated to the amount of virus introduced. By the intraperitoneal route in mice, or by the subcutaneous route in ferrets, it is necessary to inject virus in a concentration of ten fatal intranasal units for mice or 100 infectious units in ferrets before eliciting any evidence of immunity. Mice receiving less than subfatal doses intranasally become solidly immune and a single infectious unit in the ferret intranasally is followed by complete resistance to reinfection. Since relatively large amounts of virus as measured by infectious capacity are required to induce any degree of immunity by vaccination, and since there is such a relatively sharp limit to the amount of immunity any given effective dose can create, there can be little appreciable multiplication of virus or multiplication in such limited extent as to maintain a strict proportion with the amount of virus injected. This is especially striking since multiple doses of virus were used in vaccination and the length of time over which each experiment was carried was sufficient to permit the development of any effects which virus multiplication might produce.

From the point of view of vaccination of human individuals with active virus, the criticism that the procedure might induce infection seems, on the basis of the present results, not to be sound since, with infection, multiplication of virus ordinarily ensues. Moreover, in ferrets the immunity induced by minimal intranasal infection is far superior to that produced by maximal vaccinating doses. The results also tend to answer in the negative the question previously raised (12) as to whether virus which reaches the lung after intraperitoneal inoculation actually multiplies. It is not necessary for virus in demonstrable amounts to reach the lung from the peritoneum in order that immunity may develop, nor apparently does multiplication of virus occur in the development of immunity following vaccination. The even gradations in immunity militate against the possibility of virus multiplication.

Another observation reported in this communication deserves com-

ment. It has been uniformly found in ferrets vaccinated subcutaneously with influenza virus that with adequate doses a state of partial immunity is produced and circulating neutralizing antibodies can be detected. When subjected to intranasal inoculation with virus these ferrets exhibit definite evidence of infection, but the disease is clearly of a modified type. Subsequently a marked secondary rise of antibodies occurs, greatly exceeding the level reached in animals infected without previous treatment. The same phenomenon is observed when ferrets previously infected are reinoculated intranasally after an interval of months, when sufficient immunity has been lost to permit of clinical reaction to reinfection (Chart 1). This accelerated serological response appears, therefore, to typify the reaction of the partially immune animal to reinfection. In the human individual it has been noted that as a result of infection a certain proportion of affected subjects develop much higher antibody titers than others (8). It may be that in these instances the accentuated serological response characterizes the individual in a state of partial immunity, whereas the mild rises in titer observed in individuals previously possessing no antibody indicate that they may be undergoing their first infection with epidemic influenza virus.

SUMMARY AND CONCLUSIONS

A direct proportion exists between the concentration of epidemic influenza virus used for intraperitoneal immunization of mice and the degree of immunity to intranasal infection which develops. Mice vaccinated with virus of a given strength resist infection with virus of the same concentration but not more. An irreducible minimum exists since mice vaccinated with less than ten intranasal lethal doses do not develop sufficient immunity to overcome intranasal infection with virus of the same strength. The fact that there exists a limiting threshold for the degree of immunity which a certain strength of virus will induce indicates that the virus does not multiply after intraperitoneal inoculations.

In ferrets a state of partial immunity is induced as a result of subcutaneous vaccination with active influenza virus. Vaccination with doses containing 100 or more intranasal infectious units is required for the production of circulating antibodies, protection of the animals

from pulmonary involvement, and modification of the severity of the disease. On the other hand, intranasal inoculation with one infectious unit results in a firm, immediate immunity, although the duration of immunity may bear a relation to the severity of the original infection and consequently to the size of the infecting dose.

Ferrets in a state of partial immunity resulting from subcutaneous vaccination, or from the waning of a firm immunity following infection, respond to intranasal inoculation of influenza virus with an accelerated production of neutralizing antibodies. The antibody titer under these conditions reaches a much higher level than occurs following a primary infection. Fully immune animals, however, show no further antibody response to a second inoculation.

BIBLIOGRAPHY

1. Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1937, **65**, 251.
2. Stokes, J., Jr., Chenoweth, A. D., Waltz, A. D., Gladen, R. G., and Shaw, D., *J. Clin. Inv.*, 1937, **16**, 237.
3. Andrewes, C. H., and Smith, W., *Brit. J. Exp. Path.*, 1937, **18**, 43.
4. Smith, W., Andrewes, C. H., and Laidlaw, P. P., *Brit. J. Exp. Path.*, 1935, **16**, 291.
5. Shope, R. E., *J. Exp. Med.*, 1936, **64**, 47.
6. Francis, T., Jr., *Science*, 1934, **80**, 457.
7. Burnet, F. M., *Med. J. Australia*, 1935, 651.
8. Francis, T., Jr., Magill, T. P., Rickard, E. R., and Beck, M. D., *Am. J. Pub. Health*, 1937, **27**, 1141.
9. Olitsky, P. K., and Cox, H. R., *J. Exp. Med.*, 1936, **63**, 311.
10. Cox, H. R., and Olitsky, P. K., *J. Exp. Med.*, 1936, **63**, 745.
11. Cox, H. R., and Olitsky, P. K., *J. Exp. Med.*, 1936, **64**, 217.
12. Rickard, E. R., and Francis, T., Jr., *J. Exp. Med.*, 1938, **67**, 953.