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Immunity in Human Subjects Artificially Infected with Influenza Virus, Type B*

THOMAS FRANCIS, JR., M.D., F.A.P.H.A., HAROLD E. PEARSON, M.D., JONAS E. SALK, M.D.,† AND PHILIP N. BROWN, M.D.

Department of Epidemiology and the Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor, Mich., and the Ypsilanti State Hospital, Ypsilanti, Mich.

EPIDEMIOLOGICAL evidence indicates that immunity to influenza is acquired by the human subject, presumably as the result of infection, but its duration has not been clearly established. During the winter of 1942–1943 an effort was made to gain further information on this point by studying the reaction of a group of individuals to induced infection with influenza virus, Type B, and determining the response of the same subjects to the same virus four months later. The present report comprises the evidence obtained.

MATERIAL AND METHODS

Virus—The Lee strain [1] of influenza virus, Type B, was used throughout. The extra-embryonic fluid from infected hen's eggs, 48 hours after inoculation, was harvested and concentrated in physiological salt solution by the procedure of Francis and Salk.[2] For the first exposure, concentrate was used from the 20th and 21st egg passages after 8 passages in ferrets and 137 in mice. The material had been stored in the refrigerator at 4° C. for a month prior to use. In the second test similar material from the 34th and 35th egg passages was used after 7 to 16 days' storage at 4° C.

The titer of virus in the two lots of concentrate was determined by agglutination of chicken erythrocytes and by

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† Fellow in Medical Sciences of the National Research Council.

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the intranasal infection of mice. Hemagglutinin titers were determined by a modification of the method described by Hirst.[3] Five-tenth ml. volumes of twofold dilutions in physiological salt solution of the fluid to be tested were mixed with equal volume of 0.25 per cent suspension of washed fowl erythrocytes. The test was allowed to stand at room temperature for 1½ hours, when all the cells had settled, and then read by viewing the pattern formed at the bottom of the tubes. The end point was considered to be the highest dilution of virus which completely agglutinated all the cells. The titer is expressed in terms of final dilution of virus, after addition of cell suspension.

The titers of the two lots of material are as follows:

		Mouse Infectivity		
	R C A	10^{-4}	10^{-5}	10^{-6}
Lot 1	20,000	7,7,9	9, ++++, +++	+ ± ±
Lot 2	12,800	6,6, ++++	++++, ++, ++	+ + ±

Numerals indicate day of death after inoculation
± to ++++ indicate different degrees of pulmonary consolidation at autopsy on 10th day

Method of Infection—The nebulizers used contain glass baffles against which the material to be sprayed is blown. The result is almost a dry mist which condenses on a cold surface with fine, uniform coating. The instruments were attached by rubber connections to a tank of compressed air and 10 lb. pressure was applied. Under these conditions the different nebulizers delivered 0.1 to 0.17 ml. per minute. Glass nasal adapters were attached to the dispensing end of each nebulizer and were inserted into the nostril. The spray was inspired by the subject through one nostril for 2½ minutes and then for the same time through the other nostril. In the process of spraying, the patient exhaled a large proportion of the material which settled as a fine cloud. The adapter was washed with antiseptic solution before use by another subject. The spraying was done in a small treatment room off the corridor leading to a large day room. After spraying, each subject was permitted to return to the large room where he mingled with the controls. Although the latter did not enter the treatment room, there was opportunity for atmospheric interchange between the spray room and the day room where both treated and control individuals congregated.

Subjects — The individuals participating in the clinical trial were residents of one ward of the Ypsilanti State Hospital, Ypsilanti, Mich. They were ambulatory males, many of them in the older age groups, who were confined to the ward except for a daily walk outdoors or occasional tonsorial expeditions. Since no preliminary information as to their serological levels was obtained, no selection on this basis occurred. There had been no evidence of heightened incidence of respiratory disease in the previous 8 months. All were carefully examined for signs of acute respiratory illness before being included in the group.

Clinical Observations—At the time of the first spray, records of rectal temperature were begun the day after inoculation and continued until the temperature reached normal. In many instances satisfactory stories of the individual's symptoms could not be obtained but attendants familiar with the customary behavior of the subjects were able to recognize significant modifications in this respect.

Previous to the second test, temperatures were taken *per os* for 2 days; thereafter rectal temperatures were

taken. In addition, from 9 men receiving virus a second time and from 9 receiving virus for the first time, leukocyte counts were obtained on the day before and the 2 days succeeding the spray.

Laboratory Studies — Venous blood was collected from each individual immediately before and 14 to 16 days after spraying. The sera were examined at one time for the titer of antibodies to the Lee strain of virus, using a modification of the agglutinin-inhibition test of Hirst.[4] The modified test * involves the use of a 0.25 per cent suspension of fowl erythrocytes in which the serum dilutions are made directly and the result is read by the sedimentation pattern obtained after complete settling at room temperature. To 0.5 ml. dilutions of the serum in the 0.25 per cent red cell suspension is added 0.5 ml. of virus dilution corresponding to twice the agglutinin titer, thus giving a mixture which contains one unit of hemagglutinin. The end point chosen is the last tube showing complete inhibition of agglutination and is expressed in terms of the final serum dilution, after the addition of virus. The lowest serum dilution tested in the routine test was 1:32, since significant changes in titer are usually well above this level. However, in a few instances lower dilutions were tested.

Throat washings were obtained 48 hours after inhalation from half the individuals sprayed in the first test but, owing to an accident, were rendered unsatisfactory for conclusive study. No virus was detected in samples examined for the presence of red cell agglutinins or tested by ferret inoculation. In the second experiment garglings were collected 24 hours after the spray from 19 receiving a second inoculation, from 10 receiving their first exposure and from 2 untreated controls. The samples were kept on dry ice until tested 10 days later. Each specimen was injected into the nostrils of 5 anesthetized mice which were reinoculated with sterile broth by the same route 4 days later. All animals were sacrificed on the 11th day and their lungs examined for virus lesions. A few samples were also examined for their capacity to agglutinate chicken's erythrocytes. The results are discussed in the text.

Dissemination of Virus—At the time of the second spraying experiment, 10 mice in a box of wire mesh were placed in the room where the inhalations were administered. Another set of 10 was placed on the floor in the ward where the men congregated after spraying. They were kept in these locations for 4 hours and then transported to the laboratory. Two days later, half the mice of each group were inoculated intranasally with sterile broth, and all were observed for signs of infection with influenza virus.

EXPERIMENTAL INFECTION OF HUMAN SUBJECTS WITH INFLUENZA VIRUS, TYPE B

Clinical Evidence—On November 28, 1942, 60 individuals inhabited the ward. Thirty were designated to serve as untreated controls; the remaining 30 were divided into three groups of 10. The first group was sprayed with virus which had been concentrated and then diluted to the strength of the original material; the second, with virus concentrated twofold; the third group received virus which had been concentrated tenfold. In the next 24 hours, 27 of the 30 individuals receiving the sprayed virus exhibited fevers of 100° F. or greater. In 20 instances the temperature was 101° F. or greater. The data presented in Table 1 indicate that the severity of febrile reaction was somewhat related to the amount of virus received. Thus, 6 of 10 receiving the smallest dose had temperatures of

* Details of the procedure are soon to be published.

TABLE 1

Febrile Response to Spray with Different Doses of Influenza Virus, Type B

Group	Dose	Maximal Temperature (per rectum)			
		<100° F.	100°–100.9° F.	101°–101.9° F.	102°+ F.
1	Unconc.	1	5	1	3
2	2 x Conc.	1	2	3	4
3	10 x Conc.	1	0	2	7
	Total	3	7	6	14

less than 101° F. whereas, 9 of the 10 given the largest concentration of virus had fever of 101° F. or greater. The temperatures of those receiving the intermediate dose were more widely distributed.

From 14 subjects in the series there were either no complaints or information was not obtainable. Among the remaining 16 the following symptoms were noted:

Chills or chilliness	14
Loss of appetite	9
Nausea	5
Malaise	4
Headache	2
Nausea and vomiting	2
Body aches	1

Seven of the patients took to bed. It should be observed that coryza, sore throat, and productive cough were essentially absent.

Physical examination revealed little. No evidence of pulmonary involvement was noted.

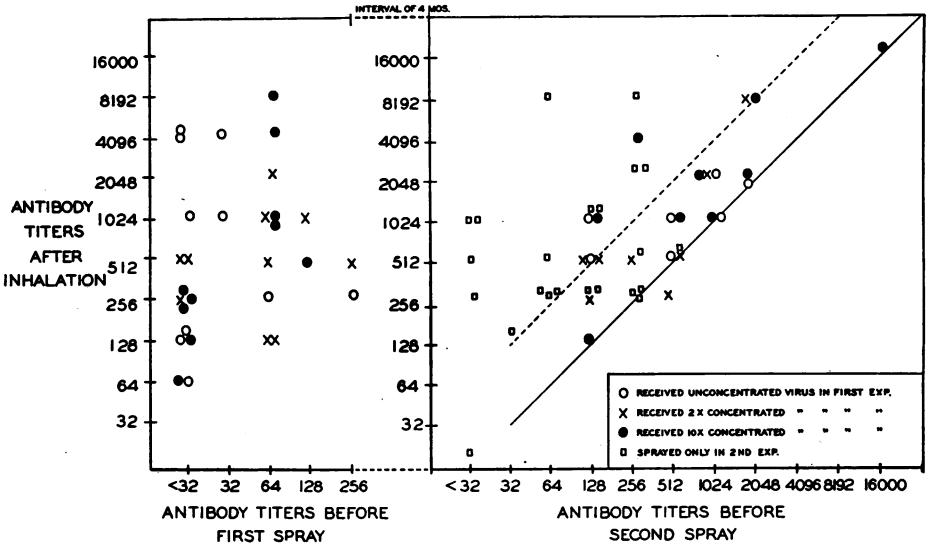
The period of illness was brief. In all but 6 instances the temperature reached normal on the 2nd day; in 5 cases on the 3rd day and in 1 case on the 4th day. With the subsidence of fever, symptoms disappeared and the patients rapidly returned to their customary states of activity and appetite. In general, the symptoms seemed to be somewhat proportionate to the febrile reaction but 2 of the individuals who had no febrile reaction were among those with most definite complaints. Patients with various degrees of illness, apart from fever, were divided in all groups though a tendency to more pronounced symptomatology was observed in those with the larger doses of virus.

Antibody Titers—The serological responses measured by the inhibition of erythrocytic agglutination are shown in the tables and charts. In 26 of the 30 individuals a fourfold or greater rise in titer of antibodies was noted; two of the four failures occurred in the only 2 subjects with initial titers as high as 256. At the beginning of the experiment titers greater than 64 occurred in only 4 instances, while 2 weeks later only 2 individuals had titers as low as 64 (Chart 1). There was no discernible relation between the height of fever or the dosage of virus and antibody response. Those with normal temperatures and those with the milder degrees of fever after the weakest dose of virus responded equally as well as those who had the highest temperatures after the largest doses of virus. The mean titers before and after spraying were 48 and 1,516, respectively, in those receiving the unconcentrated virus; 46 and 1,581 in those receiving virus concentrated tenfold; 75 and 659 in those receiving the twice concentrated material. In 11 individuals with temperatures of 101° or less, the mean titer was 54 before spraying and 1,059 afterward, while the mean titers in 19 individuals with temperatures of 101.8 or greater were 56 and 1,336, before and after spraying, respectively.

The distribution of titers at the onset was scarcely diffuse enough to gain a satisfactory impression of relation be-

CHART 1

ANTIBODY TITERS BEFORE AND AFTER INHALATION OF TYPE B INFLUENZA VIRUS



tween antibody levels and clinical response. Moreover, three different concentrations of virus were employed. Nevertheless, all ranges of reaction were observed at any one initial level of antibodies (Chart 2). For example, of the 14 individuals with the lowest measured titer of less than 32, the recorded maximal temperatures varied from normal to 103.6°. Within the limited scale available the antibody level at the onset of the experiment appeared to have little relation to the extent of the fever which ensued.

Contacts—Among the 30 individuals selected as untreated controls, temperatures were not recorded but no recognizable signs of illness were noted. Nevertheless, serological studies revealed that 11 of them had developed in the period of 14 days an increase to at least four times the original antibody titer so as to indicate that infection had occurred. Three others had twofold rises in titer (Table 2).

TABLE 2

Positive Serological Responses among Contact Controls—First Series

Subjects	*Antibody Titer—Before*	*Antibody Titer—After*
C2 ac	64	>1,024
C5 jf	64	256
C6 bg	64	512
C4 tl	16	64
C10 pm	16	64
C12 cr	64	1,024
C15 rs	<32	128
C29 wt	<16	64
C22 ow	32	2,048
C24 sw	128	2,048
C25 jw	16	64
Dr. hpl	64	1,024

One physician who had watched the spraying procedure experienced generalized aching, pharyngeal irritation, nausea, and a temperature of 99.4° the day following exposure. He subsequently developed a high antibody titer.

The observations clearly indicate that the virus became distributed in the environment so that individuals occupying the quarters in which the spraying was done and mingling with subjects purposely given the spray, underwent in-

fection which was not detected by ordinary observation. Here again infection was not limited to contacts with the least antibody titers but was distributed over the range present in the control group.

SUMMARY

As a result of intranasal spray with influenza virus, Type B, a mild illness ensued. The disease was characterized by an incubation period of 10 to 24 hours; the onset was most commonly accompanied by chilly sensations, aching, loss of appetite, and nausea. The absence of respiratory symptoms or signs was notable. In one-fourth of the subjects the illness was sufficiently severe to put the individual to bed. Twenty-seven of 30 subjects had fever of 100° or greater in the first 24 hours; two-thirds had temperatures of 101° or higher. In the majority, signs and symptoms of illness had disappeared by the 2nd day after inoculation. The height of fever appeared to be more closely related to the amount of virus received than to any single level of antibodies present. All but 4 subjects had at least fourfold rises in antibody titer following the inhalation irrespective of the amount of virus received or the severity of the febrile reaction. Of 30 untreated controls, 11 developed significant serological changes indicating infection incurred while residing in the same quarters.

REINFECTION

In the latter part of March, 1943, 65 subjects on the same ward were available for study. Three groups were formed. The first consisted of 24 of the 30 subjects who had received virus in November. The second group comprised 10 individuals who had served as untreated controls in the first experiment and 13 new arrivals on the ward. A third group made up of 2 individuals previously sprayed, 9 of the earlier controls, and 7 new arrivals, represented untreated controls. Temperatures were taken by mouth on the 2 days preceding the spray and subsequently by the rectal route. Efforts to gain information of clinical symptoms and signs were intensified. On March 29, 1943, each of the 47 individuals in the first two groups received an intranasal spray of the Lee strain of Type B influenza virus, concentrated tenfold, for 5 minutes.

Clinical Response — Symptoms and fever first occurred within 12 hours of the exposure and in 24 hours the effect was at its height. Essentially the same symptoms were noted by those given virus the second time and by controls receiving their first inoculation. They were most prominent again in individuals with highest fevers.

	Resprayed	*Sprayed Controls*
Chilly	8	7
In bed	4	8
Headache	4	7
Malaise	3	2
Anorexia	2	3
Cough	2	2
Bodily aches	1	3
Nausea	1	2
Dizzy	0	3
Sore Throat	0	3
Runny Nose	0	1
Vomiting	0	1

Thirteen of the reinoculated and 14 of the sprayed controls had definite symptoms; 4 and 8, respectively, were in bed. In most instances the illness subsided 24 to 36 hours after inhalation, but in a few cases temperatures remained elevated until the 3rd day.

Among the 24 individuals tested for immunity by reinoculation of virus, 21 developed temperatures of 100° or higher, as did all 23 of those sprayed for the first time. However, 19 (82.6 per cent) of the latter developed fever of 101° or greater while only 9 (37.5 per cent) of the reinoculated group reached that level (Table 3, Chart 2). The rapidity with which fever developed

CHART 2

RELATION OF ANTIBODY TITER AND FEBRILE RESPONSE TO TYPE B INFLUENZA VIRUS

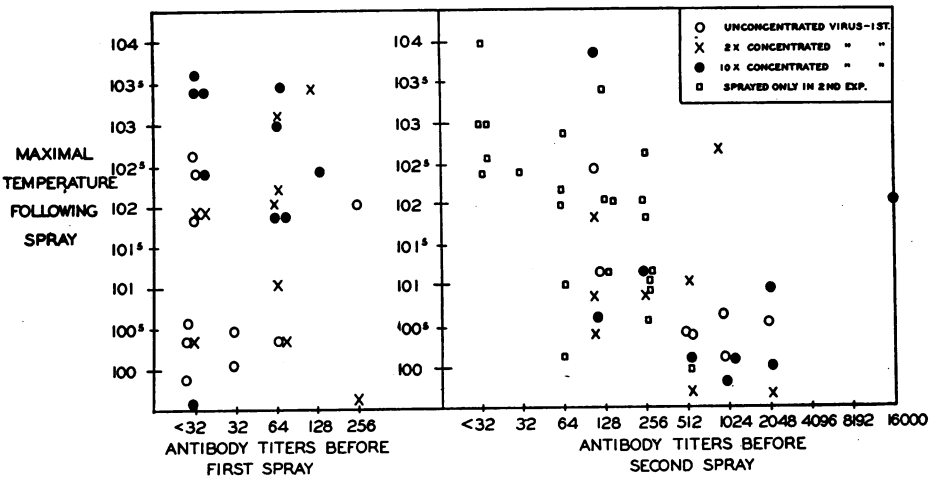


TABLE 3

Febrile Responses of Subjects Resprayed with Influenza Virus, Type B, and Controls

		Maximal Temperature (per rectum)			
Group	*No.*	*<100° F.*	*100°–100.9° F.*	*101°–101.9° F.*	*102°+ F.*
Resprayed	24	3	12	5	4
Sprayed Controls	23	0	4	5	14
Contact Controls	18	15	2	1	0

and declined was essentially the same in both groups.

No sharp change in the leukocyte count occurred. The mean count of individuals before spraying was 12,300 and on the 1st and 2nd days after spraying was 11,300 and 8,900, respectively. No difference was observed in the trends exhibited by those receiving a first or second inhalation of the virus.

It becomes quite evident that 4 months after infection with influenza virus, Type B, inhalation of the same virus induced in the same group of individuals illness clinically similar to that which followed the first exposure. The majority of those retested had lower temperatures and symptoms were generally less pronounced than in control subjects receiving their first inhalation of virus.

Serological Response — Prior to the original spraying of the group the titers of antibody to influenza virus, Type B, were preponderantly low. In consequence of that experience, much higher levels were reached and, to a great extent, maintained through the interval of 3½ months between the termination of the first study period and the beginning of the second. Moderate shifts occurred, however. In 8 instances the titers decreased within a range which might be anticipated with the lapse of time. Increases which were noted in 12 instances are probably attributable to continued accessions of antibody beyond the time at which the second sample was obtained rather than to deterioration of the earlier samples of serum during the period of storage. This was evidenced by comparing results of tests conducted in December with the first two specimens of serum

TABLE 4

Changes in Antibody Titer in Interval Between Studies

	Decreased			Increased		
Unchanged	2 x	4 x	8 x	2 x	4 x	8 x
6	3	4	1	4	5	3

and those in April which involved all four specimens.

Except for one value of 16,000 the titers of the group immediately before reinoculation ranged evenly from 128 to 2,048 with a mean of 848 and a median of 512. The titers of the con-

TABLE 5

Serological Responses to First and Second Inoculations of Influenza

Antibody Titer Before Inhalation	Sprayed—First Series				Resprayed				Sprayed Controls			
		Increase in Titer				Increase in Titer				Increase in Titer		
	No.	0	2 x	4 x +	No.	0	2 x	4 x +	No.	0	2 x	4 x +
64 or less	21	1	1	19	..	..	..	..	11	1	0	10
128–256	3	1	1	1	9	1	2	6	11	3	3	5
512–1,024	0	..	..	..	10	5	5	0	1	1	0	0
2,048+	0	..	..	..	5	3	0	2	..	..	..	..
Total	24	2	2	20	24	9	7	8	23	5	3	15

TABLE 6

Summary of Clinical and Serological Responses to First and Second Inoculations of Type B Influenza Virus

Subject	Original Dose	Severity of Symptoms		Maximal Temperature			Antibody Titers	
		1st	2nd	1st	2nd	Diff. 1–2	1st	2nd
9 th	U	+++	0	99.8	100.2	+0.4	<32/4,096	1,024/2,048
11 gb	2 x	+++	+++	103.2	102.6	−0.6	64/1,024	1,024/2,048
22 ak	10 x	+++	0	103.6	100.2	−3.4	<32/128	512/1,024
26 jp	10 x	+++	++	103.4	101.2	−2.2	<32/64	256/4,096
29 cs	10 x	+++	+++	103.4	103.8	+0.4	<32/256	128/1,024
14 lb	2 x	++	++	101.8	100.4	−1.4	<32/512	128/512
23 al	10 x	++	0	101.8	100.2	−1.6	64/4,096	1,024/2,048
25 wo	10 x	++	++	102.8	101.0	−1.8	<32/256	2,048/2,048
30 ws	10 x	++	+	98.6	100.6	+1.0	32/256	128/128
12 db	2 x	+	++	101.0	100.8	−0.2	64/128	128/512
16 ld	2 x	+	+	100.4	100.8	+0.4	<32/512	256/512
17 cd	2 x	+	0	98.6	99.6	.....	256/512	512/512
8 jg	U	+	+++	100.4	102.4	+2.0	<32/128	128/1,024
21 fj	10 x	+	+	103.4	100.0	−3.4	64/8,192	2,048/8,192
4 ec	U	0	0	100.4	100.6	+0.2	32/1,024	2,048/2,048
6 eb	U	0	0	100.0	100.4	+0.4	32/4,096	512/1,024
7 hf	U	0	0	100.4	100.6	+0.2	64/256	1,024/1,024
13 jb	2 x	0	+++	102.0	101.8	−0.2	64/64	128/256
15 tc	2 x	0	0	100.4	101.0	+0.6	64/512	512/256
1 rb	U	NH	+	102.0	100.4	−1.6	256/256	512/512
2 rb	U	NH	+++	102.6	101.2	−1.4	<32/64	128/512
19 jh	2 x	NH	NH	101.8	99.6	−2.2	<32/256	2,048/8,192
27 js	10 x	NH	NH	102.4	102.0	−0.2	128/512	16,000/16,000
28 vs	10 x	NH	NH	101.8	99.8	−2.0	64/1,024	1,024/1,024
3 wb	U	NH	....	100.6	.....	.....	<32/128	.....
5 tc	U	+	....	102.4	.....	.....	<32/1,024	.....
10 wh	U	0	....	101.8	.....	.....	<32/4,096	.....
18 bf	2 x	+++	....	103.4	.....	.....	128/1,024	.....
20 hh	2 x	+++	....	102.2	.....	.....	64/2,048	.....
24 cn	10 x	0	....	103.0	.....	.....	64/1,024	.....

Symptoms: 0 to +++ = increasing severity of constitutional reaction.
NH = no information from patient.

Antibody titers: Numerator = titer before inoculation.
Denominator = titer after inoculation.

U = inhalation of virus—unconcentrated.
2 x = inhalation of virus—concentrated twofold.
10 x = Inhalation of virus—concentrated tenfold.

trols who were to receive the spraying for the first time had a mean of 141 and a median of 128. The responses of the two groups are seen in Chart 1 and are tabulated (Tables 5, 6, 7).

TABLE 7

Responses Among Controls Sprayed in Immunity Test

Subject	Maximal Temperature	Symptoms	Antibody Response
C8 ck	104.0	+++	<32/1,024
C36 eg	103.4	+++	128/1,024
C32 wb	103.0	+++	<32/1.024
C42 wn	103.0	0	16/16
C19 ct	102.8	+	64/256
C41 hm	102.6	+++	<32/256
C23 jw	102.6	0	256/8,192
C43 ns	102.4	+++	<32/512
C37 jh	102.4	0	32/128
C33 jc	102.2	0	64/8,192
C31 db	102.0	+++	64/256
C38 lh	102.0	+++	256/256
C14 js	102.0	+++	128/256
C40 jm	102.0	+	128/256
C6 bg	101.8	+	256/2,048
C11 sn	101.2	++	128/1,024
C5 jf	101.2	0	256/256
C18 pt	101.0	++	64/256
C15 js	101.0	0	256/512
C9 dMc	100.8	++	256/256
C35 ce	100.6	0	256/2,048
C34 ed	100.2	0	64/512
C39 jk	100.0	0	512/512

Symptoms: 0 to +++ = increasing severity of constitutional reaction

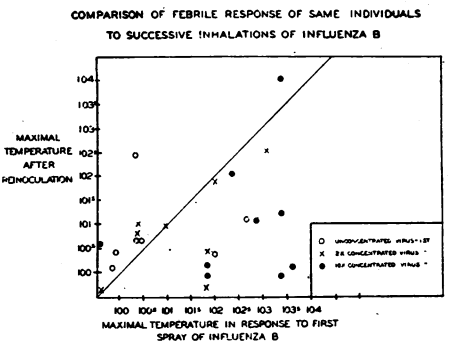
Antibody titers: Numerator = titer before inoculation. Denominator = titer after inoculation.

Following the inhalation of virus, 8 of the experienced and 15 of the new subjects developed significant increases in titer. The increment of increase, however, was much greater in the controls whose mean titer rose from 141 to 1,260 (median 512) while the rise in resprayed individuals was from a mean titer of 848 to one of 1,776 (median 1,024). Significant serological responses in both groups were most common in those with the lower antibody levels and least frequent in subjects with high titers.

COMPARISON OF RESPONSE OF SAME INDIVIDUALS TO FIRST AND SECOND EXPOSURES

Fever—Of the 24 individuals who received the two inhalations of virus, 21 developed fever of 100° or more each time; only 1 remained below that point in both tests. In 10 instances the maximal temperature following the second exposure was within 0.5° F. of the height reached in the primary attack; the majority of these individuals were those who, following the smaller amounts of virus, had the milder fevers originally (Chart 4). One of them, however, with fever of 103.4° in the primary episode responded with 103.8° to the second inoculation (Chart 5). On the other hand, of the 14 cases who exhibited fever higher than 101° on the first exposure, 10 showed decidedly lower temperatures, with a mean decline of 1.5° F., in the second test (Chart 6). In only 3 instances was the second fever significantly higher than the first (Chart 3, Table 6).

CHART 3



Two interpretations are suggested by the results. The fact that primarily those with the highest original fevers exhibited reduced temperatures following reinoculation (Chart 4) might be considered to indicate that they alone had profited from the earlier experience and that the reactive state of the remaining individuals had been essentially unaltered. Since, however, most of the subjects with fever of about the same extent in both tests were those who received the smaller doses of virus the first time, it may be inferred that the milder response to the first inhalation of virus had been just as beneficial in

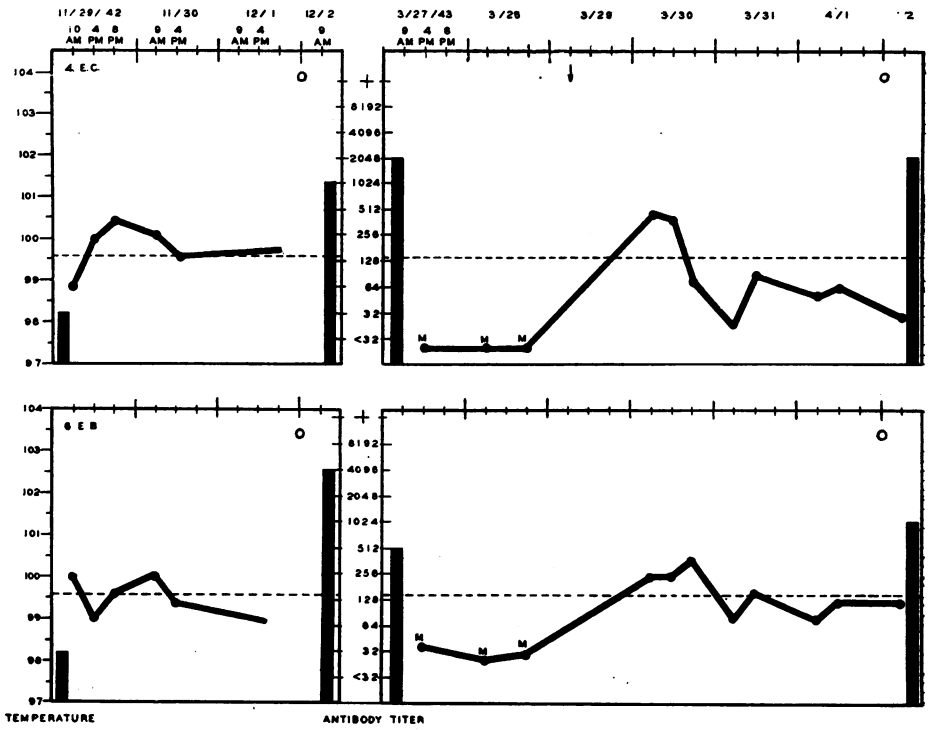


CHART 4—Subjects 4 and 6 received unconcentrated virus in November. Temperature was low, no symptoms were noted but sharp rises in antibody occurred. Four months later virus concentrated tenfold was administered. The second clinical response was similar to the first but no further increase in antibodies took place.

The left half of each chart represents the original febrile response beginning the day after inoculation; the right half the response to reinoculation. The vertical columns indicate antibody titers immediately before and 2 weeks after inoculation. Symbols in upper right corners indicate severity of symptoms. (See Table 6). *M* signifies oral temperatures.

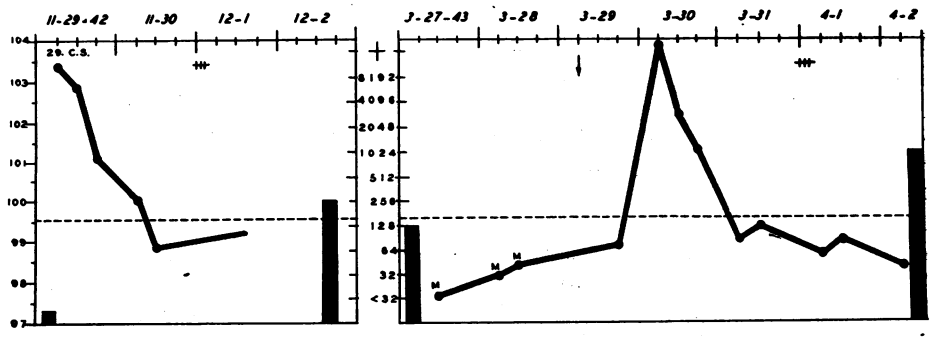


CHART 5—Illness was among the most marked of the group after each exposure to virus concentrated tenfold. Significant antibody increases occurred both times (Subject 29).

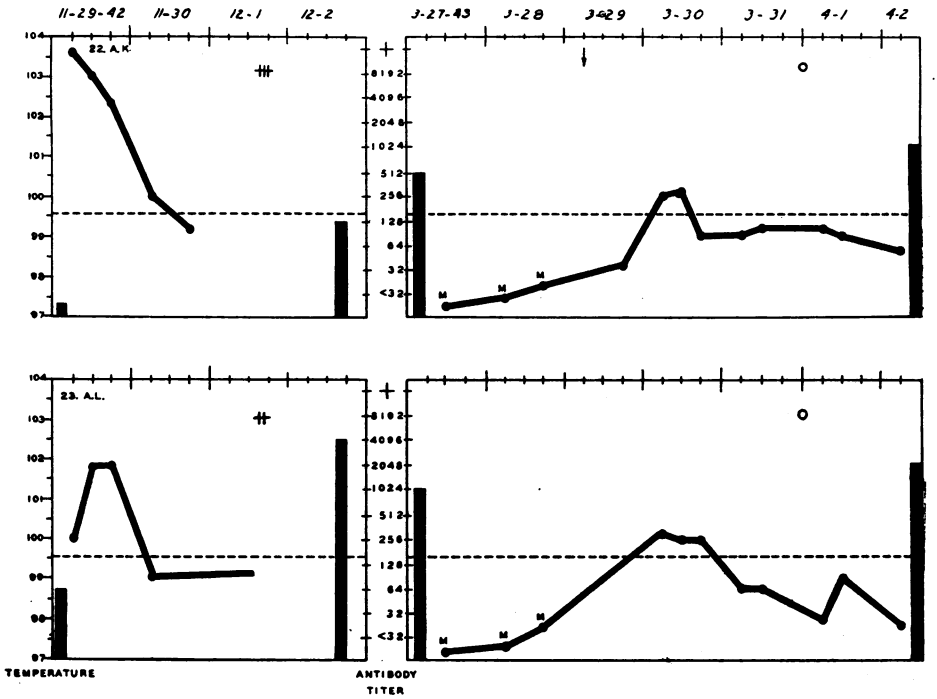


CHART 6—The charts of subjects 22 and 23, who had sharp febrile reactions, pronounced symptoms, and significant antibody responses to the first inhalation of virus on November 28, 1942. Second inoculation on March 29, 1943, was followed by markedly reduced temperatures and symptomatology.

limiting the second response of these individuals as was the more severe reaction in reducing the height of fever in the other group. Had this not been the case higher temperatures, equivalent to those of the controls, might have occurred (Chart 3).

Symptoms—Although the symptomatic evidence is of limited value, definite complaints were obtained from 14 of the group after the first spray and from 13 of them following the second. A comparison of the complaints of the same individuals in the two experiences revealed the following:

No symptoms either test	4
Decreased severity in second test	6
Increased severity in second test	5
Same severity both tests	6
No information either test	3

In both periods symptoms tended to be most severe among those with the highest fevers. In the individuals exhibiting the more marked symptoms after the second exposure, however, only 1 showed higher fever than in the original test. Diminished febrile reactions to the second spray, therefore, were not consistently accompanied by a reduction in symptoms, nor was the reaction to reinoculation clearly referable to the severity of initial response. The more marked symptoms in the second test followed just as commonly after a severe as after a mild primary response (Table 6).

Serology—The individuals subjected to reinoculation had considerably higher titers of antibody at that time than were observed at the time of their original inoculation. In general, they were less responsive, serologically, to

the second inhalation than to the first. The mean titers before and after the first exposure were 65 and 1,180; before and after the second, 848 and 1,776. Whereas, originally 20 of the 24 had antibody rises to four times or more the initial titer, and 2, twofold; after the second spray 7 showed twofold increases and only 8 had a fourfold or greater increase (Tables 5 and 6, Chart 1). Although these 8 individuals had exhibited significant responses to the first inoculation, the medians of their titers preceding and following the first infection were low, 16 and 96, respectively. At the time of the immunity test, 5 of them had titers of 128; the lowest level encountered in the retested series. It is well to emphasize that they belonged to the group with most definite symptoms and fever in each test. Despite the sharp rise in antibodies from the original level these subjects were again clearly susceptible to reinoculation with serological and clinical responses to second inoculation in many respects parallel to the first.

Briefly, inhalation of Type B influenza virus 4 months after exposure to the same virus induced again febrile response in the majority of the group. In general, temperatures were either lower or about the same level as those exhibited in the first attack. When symptoms could be obtained there was no relation demonstrable between their severity in the first instance and the response to the second attack (Chart 7).

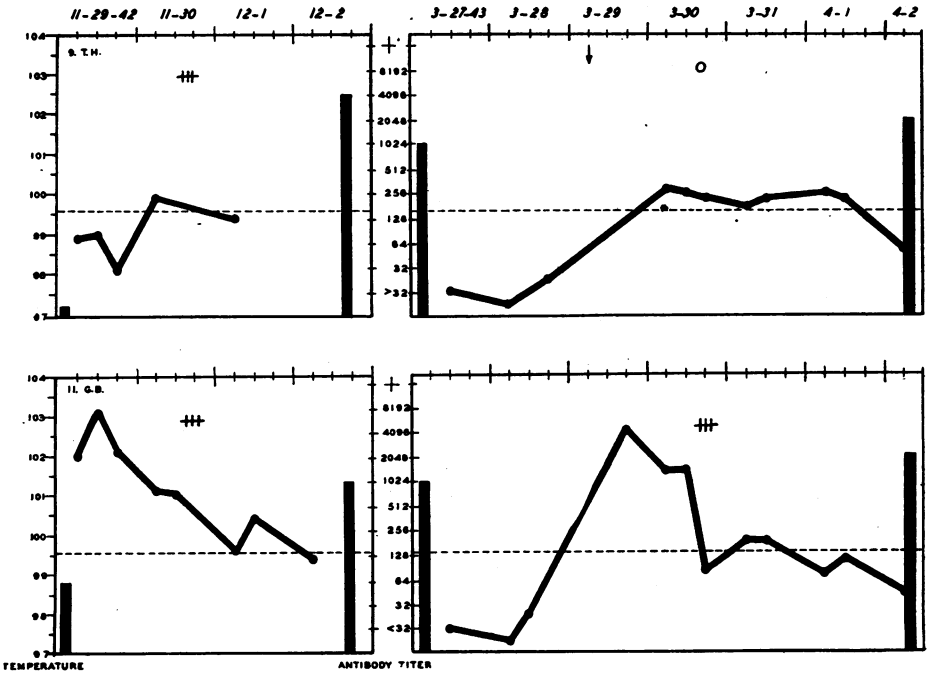


CHART 7—*Subject 9.* Illustrates the presence of pronounced symptoms and sharp antibody response with minimal febrile reaction following inhalation of unconcentrated virus. Test for immunity with concentrated virus elicited no symptoms, slight fever and no further rise in antibody.

Subject 11. Despite well marked fever, symptoms and antibody response to the first inoculation, the severity of the second clinical reaction was undiminished. The antibody titer was not significantly affected.

Although antibody titers were decidedly higher at the time of reëxposure than originally, one-third of the subjects, comprising largely those with the most severe second illness, responded with significant serological reactions again. The major effect of previous infection appeared, therefore, to be a reduction in severity of the second attack.

PERSISTENCE OF VIRUS

No virus was recovered from the throat washings obtained from 29 sprayed subjects 24 hours after inhalation of the virus. Each specimen was tested in mice with the appearance of pulmonary lesions taken to indicate infection. With five specimens attempts were made to adsorb virus from the washings with chicken erythrocytes and concentrate it by elution. No significant agglutination of red cells by the eluate was noted. The failure to find virus under these conditions was somewhat surprising in view of the short period elapsing after the spray.

On the other hand, that virus was in the air was demonstrated by the infection of mice exposed during the spraying procedure. The 5 mice which were given a stimulating intranasal inoculation of broth following exposure in the spray room, died of influenza virus infection; 2 of the remaining 5 died and the other had extensive pulmonary lesions when autopsied on the 10th day. All the mice exposed in the large day room occupied by the patients survived for the 10 day period of observation but had extensive pulmonary lesions. Influenza virus, Type B, was identified. These results show that the virus was distributed in the atmosphere by the spray itself and probably for a limited period, at least, by the infected subjects. It is of interest, however, that in this second series only 2 of the 18 contact controls gave serological evidence of having been infected through the environment in contrast to 11 instances in the first contact group.

RELATION OF CLINICAL RESPONSE TO SEROLOGICAL STATUS

In the first experiment, the use of three different strengths of virus apparently accounted for certain variations in clinical response. Partly because of these conditions, no intimate relation between symptoms or fever and the initial or subsequent antibody titer was evident. For the second spraying, virus concentrated tenfold was used throughout and the influence of other factors can be considered on the basis of uniform dosage.

In Chart 2 and Table 8 it is seen that when compared with themselves in the first test or with the controls for the second test, individuals undergoing their second experience had in general the higher antibody titers and lower temperatures. Moreover, the controls with lowest antibody titers presented a preponderance of high temperatures. In the intermediate range of titers (128–256) both controls and reinoculated subjects were found. The temperatures of individuals in this antibody zone were more widely distributed than those in the higher or lower antibody levels. But because of the limited numbers and since practically all the titers above 256 belong to individuals previously inoculated while the low titers of 64 or less are limited to those who had not undergone recent infection, no strict conclusion can be drawn. Nevertheless, a comparison of the responses of the resprayed and the controls of the second series does suggest a trend of decreasing severity of fever as antibody levels rise. Only 4 of the 24 reëxposed subjects had temperatures of 102° or more, while 14 of the 23 controls reached that level. Totalling all groups it is seen that temperatures of 101° or more occurred in 22 of the 32 (68.7 per cent) with titers of 64 or less, in 16 of 23 (69.5 per cent)

TABLE 8

Relation of Febrile Response to Influenza B and Antibody Titer

Antibody Titer Prior to Inoculation	Maximal Temperature: Sprayed—1st				Resprayed				Sprayed Controls—2nd				Totals			
	<100	100–100.9	101–101.9	102+	<100	100–100.9	101–101.9	102+	<100	100–100.9	101–101.9	102+	<100	100–100.9	101–101.9	102+
<32	2	2	2	5	..	..	..	..	..	..	0	5	2	2	2	10
32–64	..	5	2	3	..	..	..	..	..	1	1	4	0	6	3	7
128–256	1	..	0	2	..	4	3	2	..	2	4	5	1	6	7	9
512–1,024	..	..	..	..	2	6	1	1	..	1	..	..	2	7	1	1
2,048+	..	..	..	..	1	2	1	1	..	..	..	..	1	2	1	1
Totals	3	7	4	10	3	12	5	4	0	4	5	14	6	23	14	27

with titers of 128 and 256, in 4 of 16 (25 per cent) with titers of 512 or more.

The tendency for symptoms to be proportionate to fever was noted in both periods of observation of the original group and in the sprayed controls. At the time of the respraying, 9 of the 13 reinoculated subjects who presented positive signs or symptoms resided in the antibody levels of 128 or 256, the lowest of the group. This suggests again an association between antibody titers and severity of disease.

The 9 reinoculated individuals (2, 8, 11, 12, 13, 14, 25, 26, 29) with most definite symptoms (++ or +++) included 7 of the 9 with fever of 101° or greater, 7 of the 9 with lowest antibody titers (128 or 256), and among this 7 were 6 of the 8 who showed a fourfold or greater rise in antibodies following the second inoculation. In these instances, involving individuals with the lowest titers of the group, fever, symptoms, and antibody response combined to give the most complete evidence of infection (Chart 5). Of the 8 reinoculated subjects without complaints only 1 had fever as high as 101°, their antibody titers were all 512 or greater, and none showed a significant increase in antibodies. The suggestion arises that under the conditions of the present experiment significant rises in antibody are most common in those with the most definite clinical signs of disease. On the other hand, the absence of a fourfold increase in antibodies cannot be interpreted as freedom from infection when fever occurs in the presence of high antibody titers since they are less readily affected by additional antigenic stimuli (Chart 7, subject 11).

Among the sprayed controls a broad relation was also found between height of fever and the extent of antibody response. Of 10 with temperatures greater than 102°, all but 1 showed a fourfold or higher rise in antibody and 6 had more than an eightfold increase; of 13 with 102° or less only 6 had an increase of fourfold or more and none had more than eightfold increase. It should be recalled, however, that no such correlation was found in the original group receiving the different doses of virus.

In summary, the relation between the height of circulating antibodies and clinical response to infection is not clearly demonstrable in the limited data available from the present study. There exists a tendency, most evident in the previously infected, for high antibody titers to be associated with the lower temperatures. Among the controls sprayed in the second experiment there was also a tendency toward an inverse relation between the severity of reaction and the level of circulating antibody. On the other hand, all degrees of reaction were found at any given antibody level.

The results among the reinoculated individuals and their controls at the time suggest that the most prominent serological responses to infection occur in subjects with the most marked clinical reaction, although this was not noted among the individuals sprayed with different doses of virus in the first experiment. The fact remains that despite a rather uniform increase in antibody to levels beyond those encountered in the group originally, reinoculation 4 months later caused clinical evidence of infection in a high proportion of the 24 subjects a second time and in 8 individuals a second significant increase in antibody titer took place.

SPECIFICITY OF FEBRILE REACTIONS

Since the great majority of subjects in all three sprayed series, irrespective of dose or experience, had fever of 100° or more, it is apparent that if the lower fevers represent disease, infection 4 months earlier did not prevent reinfection. Although the serological responses and the presence of symptoms in afebrile subjects indicate that the mild fevers were associated with infection, the possibility that certain of the lower temperatures in the reinoculated group might be nonspecific remained. In order to study the question a series of individuals were sprayed with virus inactivated by ultra-violet irradiation.

Concentrated virus of the same lot as that used in the second test was irradiated with ultra-violet light for sufficient time to inactivate the virus as measured by loss of infectivity for mice and chick embryos. Eight subjects not previously studied and 4 who had been sprayed with active virus 4 months earlier were then given inhalations of the inactive virus for 5 minutes. Temperatures were taken before and after spraying and serological tests were made with serum obtained before and 2 weeks after the inhalation. One of the previously untreated subjects developed chilly sensations and a temperature of 102° the following day but an antibody titer of 32 remained unchanged. It seems likely that this represented an incidental illness. One of the 4 individuals sprayed earlier with active virus had a temperature of 100.4° on the 3rd day after spraying. The tardiness of this fever in comparison with the rapidity with which fever follows active virus indicates that it was not of the same nature. No other reactions were observed. None of the individuals showed a fourfold rise of antibodies as a result of spraying with inactivated virus.

The evidence clearly indicates that the elevated temperatures observed in the studies with active virus are not related to nonspecific factors associated with the spraying and that allergic reactions to egg fluid or the virus played no rôle in the symptomatic or febrile responses to the inhalation of active virus. Subsequent studies have added ample confirmation of this conclusion.

DISCUSSION

The inhalation of nebulized influenza virus, Type B, by human subjects produced an illness closely resembling that seen in the milder outbreaks of the natural disease. The course of the disease was brief and the severity varied among individuals, depending to some extent upon the concentration of virus administered. It is noteworthy that in most instances the incubation period was 12 to 24 hours, that temperatures greater than 102° F. were common, and that respiratory signs and symptoms were conspicuously infrequent while constitutional symptoms were predominant. No complications ensued.

Four months after the induction of clinical influenza, reëxposure of the same group of individuals to the same virus was again followed by clinical disease evidenced in fever, symptoms, and

serological reactions. Nine of them had temperatures of 101° or greater and 8 exhibited a second significant rise in antibodies. That a state of increased resistance had persisted over this period was seen, however, by the reduced severity of the second response in comparison with the primary reactions of the same subjects or controls. In estimating the degree of resistance, the significance of the febrile reactions among the reinoculated individuals must be appraised. The milder febrile reactions in the original group were largely limited to those receiving the smaller concentrations of virus. In the second instance, where one preparation of virus was used throughout, they were essentially exhibited by the reinoculated subjects in contrast to the inoculated controls. The infrequent and irrelevant fevers in untreated controls and in those sprayed with inactive virus strongly suggest that the temperatures of 100° to 100.9° in the reinoculated subjects represent, in the main, reactions to the inhalation of active virus rather than allergic or nonspecific responses to the sprayed material. This is further supported by the presence, as in subjects 12, 14, and 21, of symptoms and further increases in antibody titer comparable to those of individuals with higher fevers. Hence, it must be concluded that few of the previously infected subjects were wholly refractory to reinfection. On the other hand, the fact that 15 of the 24 reinoculated had temperatures below 101° while 19 of the 23 controls had temperatures of 101° or more indicates that the earlier infection had conferred a significant degree of resistance upon a majority of the individuals involved. Had the infection been milder the effect would probably have been enhanced, but this would require the employment of much larger numbers of individuals than seems feasible at the present time.

Information as to the influence of circulating antibodies upon the response to infection is not readily drawn from the present data. Combining the results from all groups it was found that 69 per cent of those with titers below 512, and 16 per cent of those with titers of 512 or more developed temperatures of 101° or higher. However, the low titers of antibody were preponderant among those receiving their first inoculation while those who had been previously infected possessed uniformly the higher titers. The recently experienced subjects with high titers had, in general, lesser febrile reactions than the inexperienced with low titers. On the other hand, the persistence of an increased titer of circulating antibodies from the first infection did not necessarily prevent the development of clinical disease a second time. Thus, although resistance and circulating antibodies were enhanced by infection, the evidence does not permit any conclusion as to a causal relationship. As has been shown elsewhere, an increase in circulating antibodies following infection [5] or vaccination [6] results in an increased neutralizing capacity of the nasal secretions. In these terms, circulating antibodies serve more as an index of the activity of the local protective mechanisms than representing the actual agency of resistance. This concept would appear clearly applicable under the conditions of the present study.

It may be objected that the procedures adopted constitute too severe a test of the immune state and that the respiratory tract is much more intensely exposed to the virus than is the case in natural infection. This implies that under natural conditions a minute amount of virus enters the upper respiratory tract during a brief period of exposure. On the other hand, recent studies of air-borne infection call attention to the fact that, in closed spaces, contamination of the air by infected individuals constitutes an opportunity

for prolonged exposure and permeation of the respiratory tract with much larger amounts of virus than mere contact would afford. The serological evidence among 11 of 30 contact controls in the first experiment demonstrates that the virus employed in the present investigation also produced infection after entering the respiratory tract by the more physiological method of normal respiration in an infected atmosphere. Although approximately 0.5 ml. of the virus was delivered to each individual the actual amount retained is not known because much of it was promptly exhaled. Moreover, the differences in response among the groups receiving different concentrations of virus suggests that the amount was not greatly in excess of that needed for adequate clinical reaction. The frequency of infection was clearly heightened by the manner of observation, detecting brief and mild responses which under conditions of normal activity would not have been noted. The incidence of clinical disease in an epidemic is related to the care with which a population is observed and the infectiousness of the prevalent strain. The fact that the incidence of influenza, Type B, has been low in recent epidemics may well reflect a need for relatively large doses of the current strains in order to produce the disease naturally as well as experimentally. For the purposes of this investigation it has been advantageous to employ amounts of virus sufficient to compensate for natural defects in the strain and yet to permit a demonstration of the differences in response of individual subjects. Under other conditions the same procedures have clearly demonstrated firm immunity in groups under investigation.

Bull and Burnet [7] have recently reported observations concerning the immune response of human subjects to small intranasal doses of an attenuated form of the same strain of influenza virus, Type B, as was used in these studies. It is of interest to point out certain differences in effect and interpretation. Their subjects were selected because of low antibody titers, and the presence of immunity was, to a large extent, determined by failure to show a rise in antibodies to the second inoculation. The demonstration of virus in nasal secretions was also involved. The symptoms were extremely mild and mostly nasal; fever is not recorded. Certain of the rises in antibody to which they ascribe importance, seem of doubtful significance; an increase in antibody after reinoculation may not occur even with sharp clinical response. The predominance of nasal symptomatology in their cases as evidence of response is in contrast to its relative infrequency in the clinical disease. The absence of controls for the immunity test and for the nasal symptoms furnishes little basis for evaluating the results. Certainly, there is no evidence in our studies that subjects with highest antibodies respond with most marked symptoms; quite the contrary. Moreover, if frank infection fails to give uniform staunch resistance over a period of 4 months, it seems unlikely that single exposure to a much milder single stimulus by the same route will have a greater effect. Bull and Burnet have measured essentially the serological reactions to a mild application of attenuated virus and it seems doubtful that further inference of its effect upon resistance to clinical infection can be drawn at present.

From the studies here reported the suggestion is derived that if procedures devised for prophylaxis against influenza are to be effective they should be repeated at intervals shorter than 4 months. The evidence shows that the degree of resistance persisting 4 months after infection is less influenced by the severity of the original infection than by the responsiveness and con-

tinued activity of the individual's immune mechanisms, suggesting that selection may be made of those most in need of the additional stimuli.

SUMMARY

Inhalation of finely dispersed Type B influenza virus by human subjects resulted in a high incidence of clinical infection resembling a mild form of the natural disease.

Four months later 24 of the same subjects received a second inhalation of the same virus. Fever, symptoms and serological responses were noted a second time. The illness was milder in the majority of previously infected individuals than in controls inoculated at the same time. Few were refractory to reinoculation.

The diminished response to reinfection was most evident in those who had the highest fevers in the first test. Nevertheless, the evidence indicates that mild primary infections were as beneficial as the more severe ones.

Although there was a trend toward association between high antibody titers and low temperatures, no uniform correlation between the antibody titer of an individual and his response to inoculation was found.

REFERENCES

1. Francis, T., Jr. A New Type of Virus from Epidemic Influenza. *Science*, 92:405–408 (Nov. 1), 1940.

2. Francis, T., Jr., and Salk, J. E. A Simplified Procedure for the Concentration and Purification of Influenza Virus. *Science*, 96:499–500 (Nov. 27), 1942.

3. Hirst, G. K. The Agglutination of Red Cells by Allantoic Fluid of Chick Embryos Infected with Influenza Virus. *Science*, 94:22–23 (July 4), 1941.

4. Hirst, G. K. The Quantitative Determination of Influenza Virus and Antibodies by Means of Red Cell Agglutination. *J. Exper. Med.*, 75:49–64 (Jan.), 1942.

5. Francis, T., Jr., and Brightman, I. J. Virus-Inactivating Capacity of Nasal Sécretions in the Acute and Convalescent States of Influenza. *Proc. Soc. Exper. Biol. & Med.*, 48:116–117 (Oct.), 1941.

6. Francis, T., Jr., Pearson, H. E., Sullivan, E. R., and Brown, P. N. The Effect of Subcutaneous Vaccination with Influenza Virus upon the Virus-Inactivating Capacity of Nasal Secretions. *Am. J. Hyg.*, 37:294–300 (May), 1943.

7. Bull, D. R., and Burnet, F. M. Experimental Immunization of Volunteers against Influenza Virus B. *M. J. Australia*, 1:389–394 (May 1), 1943.