EXPERIMENTAL INFECTION WITH INFLUENZA A VIRUS IN MICE

THE INCREASE IN INTRAPULMONARY VIRUS AFTER INOCULATION AND THE INFLUENCE OF VARIOUS FACTORS THEREON

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There are numerous reports in the literature on the infectivity and virulence of human influenza A virus[1] and the histopathological response to infection in several species of animals, particularly mice and ferrets. There is, however, lack of satisfactory information on the rapidity and extent of increase of the virus relative to the dosage of the inoculum and the lung lesions and mortality produced.

Smorodintseff and Ostrovskaya (3) were the first to point out that following intranasal inoculation of mice with lethal doses, the maximum concentration of the virus in the lungs was reached in 24 to 48 hours, although at this interval no macroscopic lesions were manifest. The amount of virus in the inoculum was not always precisely given nor did these authors determine the increase of the virus following inoculation of sublethal doses.

It has been established that with a "fixed" strain the virus content of a suspension may be rather accurately measured by its lethal effect upon mice inoculated intranasally and that through serial dilution a point can be reached where lung lesions are produced but the mice survive (4).

This paper embodies the results of studies on the titration of the virus in the lungs and the macroscopic lung lesions and mortality in mice at varying intervals following the administration of graded doses of the virus, and the effect of intranasal instillation of fluid following infection with a sublethal dose.

Material and Methods

Virus.—The virus strain PR8 was used throughout these experiments. This strain which has been through 333 mouse passages was chosen because its virulence to mice is well fixed and it is possible to prepare dilutions that will give predictable results in respect to mortality and production of lung lesions.

[1] Name suggested by Horsfall, Lennette, Rickard, Andrewes, Smith, and Stuart-Harris (1) for the virus discovered by Smith, Andrewes, and Laidlaw (2).

Infected mouse lungs were ground in a porcelain mortar with a small quantity of alundum sand. A 20 per cent suspension by weight of the ground mouse lungs was made in broth containing 20 per cent normal horse serum and the whole centrifuged for 15 minutes at 2500 R.P.M. The supernate was withdrawn and stored in a low temperature cabinet at −76°C. (5). This stock suspension had been titrated each week for a number of months and was found to have an average 50 per cent mortality titer of $10^{-6.5}$. Dilutions between 10^{-7} and 10^{-8} usually produced lesions but the mice survived. This virus stock was employed for infecting all mice. The required dilutions were made immediately before use in broth containing 10 per cent normal horse serum.

Titration of Virus Content of Mouse Lungs.—Groups of four mice were sacrificed at varying intervals following inoculation and the lungs removed aseptically. After inspection for lesions, the lungs were mixed, weighed, and ground in a mortar with alundum sand. A primary 10 per cent suspension of the lung pulp was made and centrifuged for 10 minutes at 2500 R.P.M. Serial tenfold dilutions were prepared from the supernate. 10 per cent normal horse serum in broth was used as diluent.

Groups of four mice were inoculated intranasally while under light ether anesthesia with each dilution tested. Each mouse received 0.05 cc. The mice were kept under observation, and all dying were autopsied for verification of the characteristic pulmonary consolidation of viral infection. At the conclusion of 10 days, all surviving mice were autopsied and the lung lesions noted.

The titration end-point was based on 50 per cent mortality calculated according to the formula of Reed and Muench (6).

EXPERIMENTAL

Groups of mice were inoculated intranasally with varying amounts of virus ranging from less than 1/10 to more than 300,000 M.L.D.[2] At intervals following inoculation, four mice from each group were sacrificed and the lungs titrated for virus content. The results are shown in Table I and Fig. 1. The base line in the figure represents 1 M.L.D., and the first series of points to the left shows the log of the number of M.L.D. inoculated. The succeeding points on each lineal diagram indicate the log of the observed content of M.L.D. per mouse lung at varying intervals following inoculation.

The macroscopic lung lesions in the mice sacrificed for titration are recorded in Table II. Previous titrations of the virus suspension used for inoculation had amply demonstrated that 31 M.L.D. or more were consistently fatal, and that 0.06 M.L.D. though usually producing some pulmonary lesions failed to cause death.

It will be observed that: (*a*) When the dose of virus administered was large, the maximum titer was reached within 24 hours. (*b*) Although the proportional increase following smaller doses was greater during the first 24 hours, the maximum titer was not reached until 48 hours. (*c*) In each instance the maximum titer was attained before the appearance of gross lesions in the lungs. (*d*) When a lethal dose was administered, the M.L.D. content of the lungs approached log +7 and remained in this neighborhood until death ensued. But with the sublethal inoculum the M.L.D.

[2] M.L.D. refers to minimum lethal dose when inoculated intranasally.

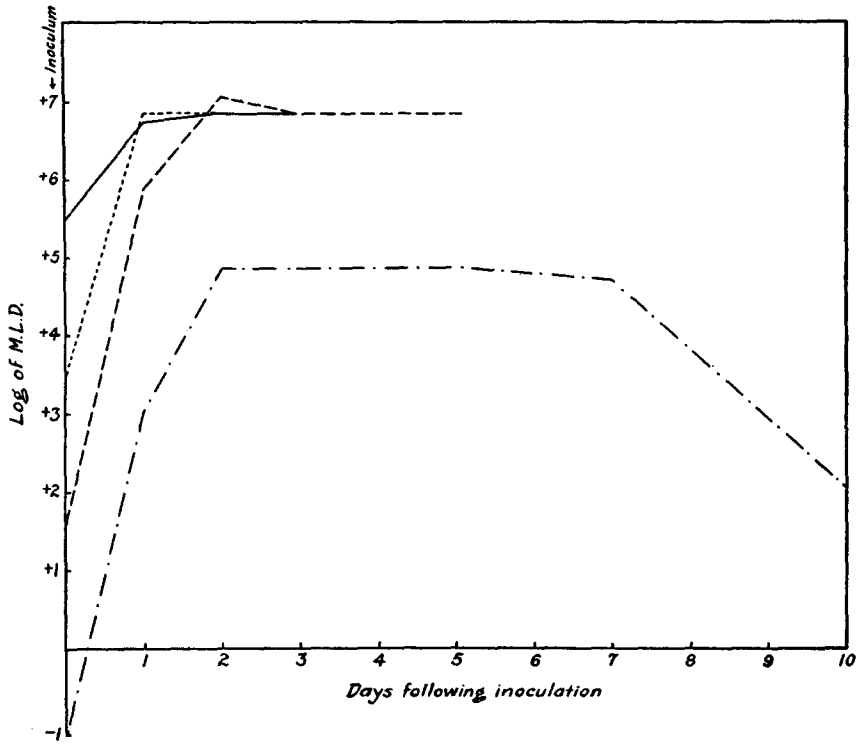


FIG. 1. Lung content of virus at varying intervals following intranasal inoculation of graded doses. Plotted logarithmically according to M.L.D.

TABLE I

Lung Content of Virus at Varying Intervals Following Intranasal Inoculation of Graded Doses. Expressed in Log and Actual Number (Antilog) of M.L.D.

	Minimal lethal doses							
	Log	Number	Log	Number	Log	Number	Log	Number
Inoculum	−1.2	0.06	+1.5	31	+3.5	3,100	+5.5	310,000
Virus content of lung after 1 day	+3.04	1,100	+5.88	760,000	+6.88	7,600,000	+6.71	5,130,000
Virus content of lung after 2 days	+4.88	76,000	+7.04	11,000,000	+6.88	7,600,000	+6.88	7,600,000
Virus content of lung after 3 days	+4.88	76,000	+6.88	7,600,000	+6.88	7,600,000	+6.38	2,410,000
Virus content of lung after 4 days	+4.88	76,000						
Virus content of lung after 5 days	+4.88	76,000	+6.88	7,600,000				
Virus content of lung after 7 days	+4.71	51,400						
Virus content of lung after 10 days	+2.12	130						

content did not exceed log +4.9. (*e*) However, within 48 hours following the sublethal dose, the virus had increased more than 1,000,000 times. At this interval the lungs of one of these mice contained sufficient virus to

infect fatally 76,000 normal mice, yet the mouse whose lungs harbored this enormous amount of virus in some way managed to master the infection and survive. Nor did the virus further increase after it reached a maximum at 48 hours following inoculation.

Repeated experiments have shown that the amount of virus in the lungs of mice receiving a sublethal dose was always less than in mice which had

TABLE II

*Lung Lesions in Mice Inoculated Intranasally with Graded Doses of Virus and Sacrificed at Varying Intervals for Titration of Virus Content of Lungs**

M.L.D. inoculated		Days after inoculation									
Log	Number	1	2	3	4	5	6	7	8	9	10
+5.5	310,000	+†	±	+++							
		−	−	+++							
		−	−	++							
				D++++							
+3.5	3,100	−	−	+++	D++++	D++++					
		−	−	+++	D++++	D++++					
		−	−	++							
		−	−	++							
+1.5	31	−	−	±		+++		D++++			D+++
		−	−	±		++		D++++			
		−	−	−		++		D++++			
		−	−	−		+					
−1.2	0.06	−	−	−	−	−		++			++
		−	−	−	−	−		+			++
		−	−	−	−	−		±			++
		−	−	−	−	−		−			−

D indicates death and the number of pluses the degree of lesion.

* Only the lungs of living mice were used for titration.

† Atypical.

received a fatal inoculum. The crucial titer appears to be around 10^{-6}; that is, mice which have been given a fatal dose and are destined to die will, at 48 hours following inoculation, have a lung titer above 10^{-6}, while in mice which received a sublethal dose and consequently would survive, the titer was usually below this level.

Having found that the lungs of sublethally infected mice contained several hundred M.L.D. within 24 hours and many thousand within 48 hours following inoculation, we decided to see what effect the instillation of a few

additional M.L.D. would have. With this object in view the following experiment was performed.[3]

A series of mice was inoculated intranasally with 0.06 M.L.D. and on the 1st, 2nd, 3rd, 4th, 5th, 7th, and 10th days following the sublethal inoculation, ten mice of the series received intranasally 31 M.L.D. of virus. At the same periods an equal number of the

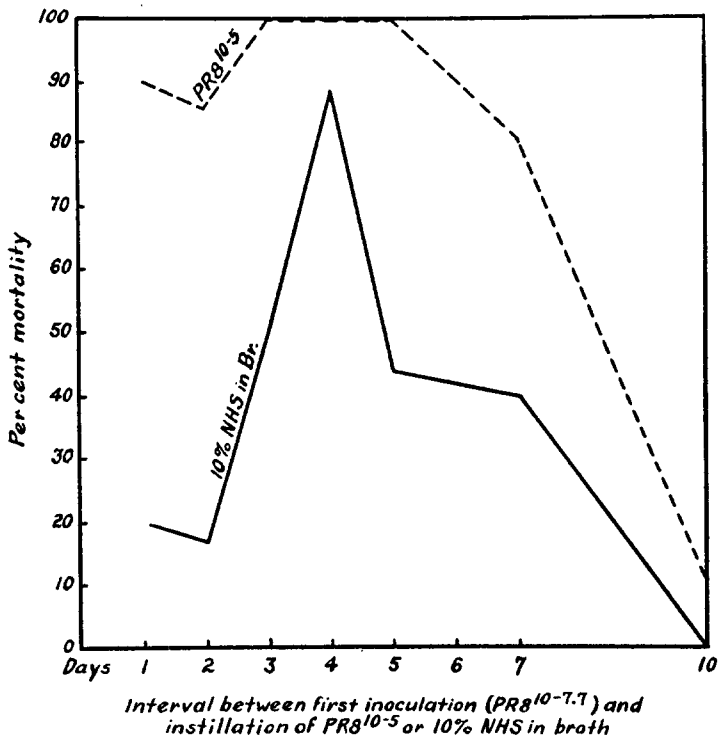


FIG. 2. Effect of intranasal instillation of 31 M.L.D. ($PR8^{10-5}$) of virus and 10 per cent normal horse serum in broth at varying intervals after infection with a sublethal dose of virus, 0.06 M.L.D. ($PR8^{10-7.7}$). All mice survived which received only 0.06 M.L.D. ($PR8^{10-7.7}$).

series received 10 per cent normal horse serum in broth, which was the diluent used for the virus. These were intended as controls upon inoculation of the virus. In addition

[3] In this and the succeeding experiments the quantity of 0.05 cc. per mouse was used for intranasal instillation. The mice were held for observation for at least 10 days following the last instillation, and all dying during this period were autopsied for verification of lung consolidation. Also, all surviving mice were autopsied and the lung lesions recorded. Mice which failed to show typical lung consolidation at time of death are not included in the protocols of the experiments.

ten mice received no intranasal instillation beyond the original inoculum of 0.06 M.L.D. As anticipated, none of the latter ten succumbed; but as will be seen in Fig. 2, which illustrates the percentage mortality following the secondary instillations, most of the mice died that received the relatively small addition of virus during the first 5 days following the original inoculation. There was a decline in the mortality among the group reinoculated on the 7th day, and by the 10th day following the original sublethal inoculum immunity to 31 M.L.D. of virus was almost complete.

In interpreting these results it should be borne in mind that the mice subinoculated between the 2nd and 5th days already had in their lungs between 50 and 100,000 M.L.D. from which they would have recovered, yet the administration of 31 M.L.D. caused them to die.

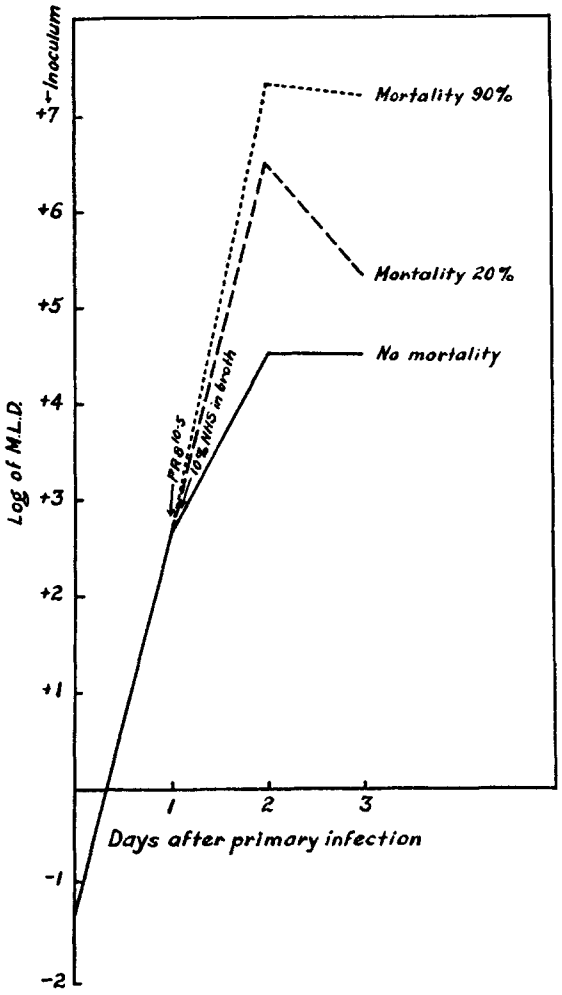
It was still more surprising to find that many of the mice which received an instillation of sterile 10 per cent normal horse serum in broth also died. While the mortality was low among the groups receiving the instillation on the 1st and 2nd days, it rose to nearly 90 per cent among those receiving the instillation on the 4th day following sublethal infection. Thereafter the effect, as judged by mortality, diminished until by the 10th day the mice were completely resistant.

The lungs of the mice which died following either the instillation of 31 M.L.D. of virus or 10 per cent normal horse serum in broth presented the characteristic consolidation of virus infection. Moreover, deaths did not begin to occur until 3 or 4 days following the secondary instillations, which is about the interval required for a heavy dose of virus to cause death.

An additional number of mice was included in this experiment and sacrificed at intervals for titration of virus content of the lungs. The results are shown in Fig. 3. It will be seen that following the instillation of either 31 M.L.D. of virus or 10 per cent normal horse serum there was within 24 hours a sharp increase in virus titer above that observed in the sublethally infected mice which received no subsequent treatment. The apparent severe drop in titer on the 2nd day after administration of 10 per cent normal horse serum may be related to the element of chance involved in choice of mice sacrificed for the titration. Only two of the ten mice retained in this group subsequently died. It is probable, therefore, that the amount of virus in a suspension prepared from the pooled lungs of four mice would be influenced by whether there happened to be a mouse among the four which would later have died. Indeed one of the striking features of this phenomenon is that the mice either die or the course of the disease is apparently unaffected. The results are probably influenced by both the stage of the infection and the success in flooding the lungs with the subsequent instillate.

A number of experiments were made using various bland fluids for instillation. The results of one of these experiments are recorded in Fig. 4, in which each square on the histograms represents a mouse.

All of the mice as in the previous experiment first received an intranasal inoculation of 0.06 M.L.D. of virus. 4 days thereafter groups of ten of these mice were given intra-



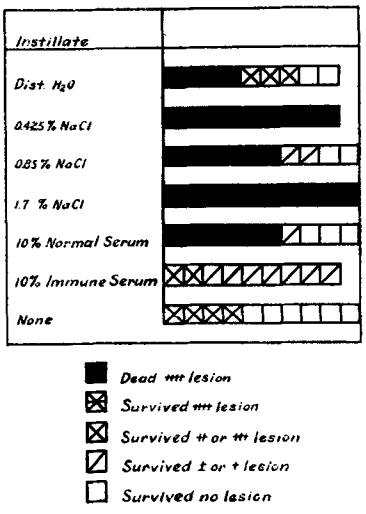


FIG. 3 FIG. 4

FIG. 3. Effect on virus titer in lungs of intranasal instillation of 31 M.L.D. ($PR8^{10-5}$) of virus and 10 per cent normal horse serum in broth 24 hours after infection with a sublethal dose of virus (0.06 M.L.D.).

FIG. 4. Effect of intranasal instillation of various fluids on 4th day after infection with a sublethal dose of virus (0.06 M.L.D.).

nasally sterile distilled water, 0.425, 0.85, and 1.7 per cent NaCl solution, 10 per cent normal goat serum, and 10 per cent serum from a goat immunized against influenza virus,[4] respectively. A group of ten mice was also held as controls. This group received no subsequent instillation but was placed under light ether anesthesia on the 4th day, in order to control the effect of the anesthesia which was used for all intranasal instillations.

It will be observed that varying proportions of the mice died with typical lesions in the groups receiving distilled water, graded concentra-

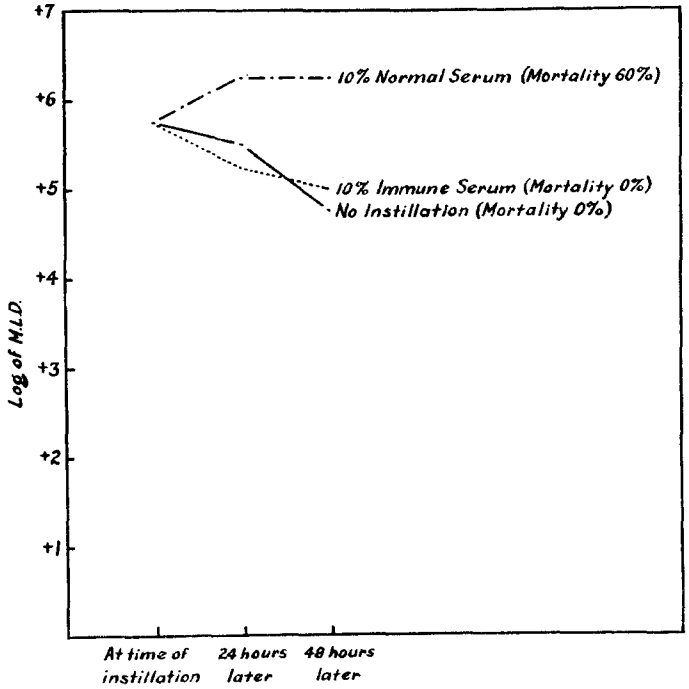


FIG. 5. Effect on virus titer in lungs of intranasal instillation of 10 per cent normal and 10 per cent immune serum on 4th day after infection with a sublethal dose of virus.

tions of NaCl, and 10 per cent normal goat serum, but all in the control group, as well as all which received 10 per cent immune serum, survived.

It is doubtful that the differences in mortality among the groups receiving distilled water, NaCl solutions, and 10 per cent normal serum are significant, for, as has been pointed out, there appears to be a certain element of chance involved in the results of the secondary instillations. It seems clear, however, from this and other experiments, that immune serum

[4] This goat serum was prepared by the intratracheal inoculation of a mouse lung suspension of active virus (7) and had a "standard titer" (8) of 1:512.

blocks or offsets the usually fatal effect of fluids such as distilled water, saline, and normal serum solutions.

Again, additional mice were included in the groups receiving normal serum and immune serum and in the control group for titration of the virus content of the lungs. The results of the titrations are illustrated in Fig. 5. The height of the titer at the time the instillations were given was higher than was commonly encountered in mice receiving this quantity of virus. However, as will be noted in Fig. 4, none of the control group held for observation succumbed from the infection. Following the instillation of normal serum the titer rose and remained above what is considered to be the crucial level. On the other hand, the titer of the group receiving immune serum declined in parallel with the control group.

Histopathology.—In order to follow the histopathological processes, at daily intervals sections were prepared of the lungs of mice receiving 0.06 M.L.D. of virus, of the lungs of the same series of mice after the intranasal instillation of 0.85 per cent NaCl solution on the 4th day following the sublethal infection, and for purposes of comparison, of the lungs of mice which had received graded lethal doses of virus.

The pathological changes following infection with a fatal dose were found to be similar to those described by Straub (9, 10), Nelson and Oliphant (11), de Balogh (12), and others. The outstanding features were a swelling of the bronchial mucosa followed by degeneration and desquamation of the cells leading frequently to a plugging of the bronchioles which was superseded by dilation and then collapse of the alveoli with edema, congestion, and not uncommonly extravasation of blood in the alveolar spaces. Peribronchial infiltration of mononuclear cells was found in varying degrees. Death was apparently due to collapse of the alveoli and the resulting cessation of lung function. In the lungs of mice sublethally infected the process was much retarded and limited in extent. Although after the 1st or 2nd day, there was some indication of thickening of the bronchial mucosa, the cells were apparently intact, and the nuclei retained their normal staining qualities. By the 5th to the 7th day there was observed patchy desquamation which was followed by a very marked hyperplasia with the conversion of the columnar to stratified epithelium. At this time parts of the lung were emphysematous and other portions beginning to collapse, but the collapsed pneumonic areas never became extensive and resolution soon followed. Here and there the peribronchial infiltration was quite marked.

However, within 24 to 48 hours after the instillation of the saline solution the picture was markedly altered and the lungs began to present an appearance quite analogous to that observed in the mice which had received many lethal doses of the virus. By the 4th day the mice began to die with lungs completely consolidated by alveolar collapse, edema, and congestion. Straub (10) remarks in his more recent paper, which came to attention after this work was in progress, that the intranasal administration of Tyrode's solution 1 to 2 days following inoculation of a "weak" virus enhanced the pathological changes.

DISCUSSION

Several questions emerge from these experiments which provoke discussion. Why is it that a mouse can have within 24 hours following infection several hundred, and within 48 hours many thousand, fatal doses of virus in its lungs and yet survive? Why is it that a small quantity (0.05 cc.) of fluid instilled into the lungs of a mouse infected with a sublethal dose of virus will cause a rapid increase of the virus and usually death within 3 to 8 days? Also, how is it that immune serum which ordinarily has little effect after the infection is once implanted prevents this deleterious action? The following postulates which embody ideas expressed by others (13) would offer an explanation for these phenomena.

When a virus particle becomes associated with a cell, it proceeds to multiply to the supporting capacity of the infected cell. It would also seem that for some reason it is easier for the virus to enter or to become attached to the cell than to escape it; that is, the cell acts in the manner of a trap, as well as a haven, for the virus. The injury inflicted by the virus is primarily confined to the attacked cell, and the death of the host occurs only when a sufficient proportion of the cells of a vital organ have been affected to prevent the essential functioning of that organ. In the case of the influenza virus it is probably the damage to the mucosa of the bronchioles which produces occlusion and the resulting fatal pneumonia.

The channel of dissemination of the influenza virus is largely through the bronchial tree rather than through the blood or lymphatic system. This conception seems justified by the fact that the virus is confined almost exclusively to the lungs and the difficulty of producing fatal pneumonia by any other than through the intranasal route of inoculation. More than 100,000 intranasal M.L.D. may be given intraperitoneally and at least 20,000 M.L.D. given intravenously without the production of a fatal pneumonia.

In the course of infection immunity of neutralizing antibodies against the influenza virus develops rather rapidly. The action of immune serum is mainly upon virus which is outside of or has escaped from the cell. It will, therefore, prevent the virus from passing from one cell to another although it has a very limited effect, if any, upon virus protected by the cell.

This would explain how it is possible for a mouse receiving a sublethal inoculum to develop rapidly a large quantity of virus and harbor it for some days and still recover. It would seem that the mouse does not die because an insufficient number of cells have been infected by the initial inoculum, and by the time the virus begins to escape in any considerable

quantity from the infected cells it is prevented from entering additional cells by the presence of neutralizing antibodies which in the interval have begun to appear. Thus, while an enormous stock of virus has been produced and is retained, possibly within the infected cells, a balance is established between the liberation of the virus and the production of antibodies. In consequence the infection is prevented from spreading to the required number of cells to produce a fatal pneumonia. This would also explain the differential between the virus titer of the lungs of mice receiving a lethal and sublethal dose, the virus titer being proportional to the number of cells primarily infected.

It is readily conceivable that the introduction of some unnatural influence which would tend to favor the escape of the virus from the infected cells or its spread through the bronchial tree would upset what is probably a rather delicate balance and result in the invasion of cells that would have otherwise escaped. This is probably what happens when fluid is instilled into the nose of a sublethally infected mouse. That the effect is attributable to the dissemination of the virus rather than to direct injury of the pulmonary cells *per se* is supported by: (*a*) the rise in the virus titer, (*b*) the occurrence of deaths only after an interval of 3 to 8 days, (*c*) the histopathological picture which resembles that of lungs of mice having received a lethal inoculum of the virus, and, lastly, (*d*) the fact that the effect is not produced if a dilution of immune serum instead of normal serum is used for the instillation. The physical properties of 10 per cent immune serum are no different from those of a like solution of normal serum and presumably would cause the same insult to the lung tissue. It is believed, therefore, that the reason for the failure of the immune serum to bring about death is that it neutralizes the virus as the latter escapes and thus prevents the invasion of additional cells.

Although it is dangerous to venture far in the realm of speculation, one cannot avoid the thought that there may be an analogy between the deaths induced in sublethally infected mice by the intranasal instillation of fluid and the influenzal pneumonias of human beings. It is conceded that in nearly all of the fatal influenzal pneumonias seen in man there is a supervening bacterial infection, and the secondary bacterial invasion is generally believed to be the immediate cause of death. On the other hand, is it justifiable to conclude that the influenza virus only paves the way for the bacteria and that the latter are entirely responsible for the final issue? May it not be that, in keeping with the hypothesis above propounded, the inflammation induced by the bacteria causes, like the intranasal instillation of fluid in mice, an excessive release of the virus and that the virus

then spreads and invades new cells? According to this conception, the sequence of events would be as follows: primary and in all probability sublethal virus infection with a breaking down of the barrier to bacterial invasion, the bacteria in turn causing an excessive release of the virus from the cells, and finally both the virus and the bacteria contributing to the terminal pneumonia. Such a procession would explain the rather singular pathology of influenzal pneumonias which presents a certain uniformity in appearance irrespective of the type of the secondary invader.

Instillation of fluid after infection (sterile salt solution, broth, or 10 per cent serum) may prove of assistance in adapting influenza virus strains to mice. The procedure has not yet been adequately tried, but thus far it has been found that infection could be more readily established with two strains relatively avirulent for mice if the mice were given intranasal instillation of broth on the 2nd day following inoculation.

SUMMARY

Following intranasal inoculation of influenza A virus (strain PR8) there is a rapid increase of the virus in the lungs which with large doses reaches a maximum within 24 hours. With smaller doses, although the proportional increase is greater, the maximum concentration is not reached until 48 hours following inoculation. If a lethal dose is administered, the ultimate concentration of the virus in the lungs is the same, irrespective of the size of the dose.

If a sublethal dose is given, the titer of the virus in the lungs does not achieve the titer reached in mice receiving a lethal dose. Within 48 hours following inoculation of a sublethal dose the lungs of a mouse may contain at least 76,000 M.L.D., yet the mouse survives.

The intranasal instillation of sterile fluid (distilled water, varying concentrations of NaCl, broth, or 10 per cent normal serum) into a mouse sublethally infected produces a sharp rise in the virus content of the lung usually followed by death within 3 to 8 days. If, however, the instillate consists of 10 per cent immune serum, there is no rise in the virus titer, and no apparent harm results from the instillation.

The implications of these phenomena are discussed and an hypothesis presented to explain their occurrence.

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