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STUDIES ON THE PATHOGENESIS OF INFLUENZAL PNEUMONITIS. INTRANASAL VS. INTRAVENOUS INFECTION OF MICE†‡

The respiratory epithelium is the only tissue of the adult mouse that is uniformly susceptible to influenza virus infection. The virus is most effective when administered intranasally[1, 4] although pulmonary lesions can be produced less regularly by direct intrathoracic inoculation.[1] Rickard and Francis[13] were the first to demonstrate virus multiplication and pulmonary consolidation after parenteral injection of large doses of PR8 influenza virus, a finding confirmed by Oakley and Warrack[12] for the WS strain. The Henles[9] noted marked variations in the incidence of pneumonitis after massive intravenous and intraperitoneal injections of mouse-adapted influenza viruses. Although each of these investigators observed that the mortality rate in parenterally infected mice was lower than in those infected by the intranasal route, they did not report any basic differences in pulmonary pathology.

During the course of an investigation on virus distribution in mouse tissues, it was found that intravenous injection of the highly pathogenic WS strain of influenza A virus usually produced a nonfatal pneumonitis. The nature of the infectious process, as reflected in the type of virus multiplication and the character of the pulmonary lesions, was deemed to be sufficiently different from classical intranasal infection to warrant a detailed description.

MATERIALS AND METHODS

Male Swiss mice (Webster strain) weighing 10-14 gm. were used in all experiments. The infectivity studies were carried out with the mouse lung-adapted WS strain of influenza A virus using allantoic fluid preparations of the 100th-107th consecutive chick embryo passages. Fluids were obtained from 12-day-old eggs that had been infected 44 hours previously with 0.1 ml. of a 10^{-8} dilution of seed inoculum.

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Mouse inoculation techniques. Intranasal injections were performed under light ether anesthesia by placing 0.05 ml. of the inoculum on the external nares. In experiments involving large numbers of mice, an attempt was made to induce the same level of anesthesia for each animal. Intravenous injections were made in the ventral or dorsal vein of the tail of unanesthetized mice, the usual volume of the inoculum being 0.1 ml. Mice were discarded if there was any question of their not having received a full intranasal or intravenous dose.

Method for obtaining bronchial washings. This method was used to determine the amount of free virus on the respiratory surface of the tracheo-bronchio-alveolar tree. Mice were killed with illuminating gas, the anterior skin reflected laterally and the lungs collapsed by severing the diaphragm just below the xiphoid process. The anterior thoracic cage was then removed without disturbing the visceral pleura. After exposing the trachea a 19-gauge needle on the end of a 1.0-ml. tuberculin syringe was inserted to a point just above the tracheal bifurcation and 0.5 ml. of sterile broth-saline solution introduced into the lungs. This volume of fluid produced equal expansion of all lobes without leakage from the pleural surface. The wash fluid was allowed to remain in the lungs for approximately two minutes before aspiration; each pair of lungs was washed twice with the same fluid. Approximately 95 per cent of the broth-saline solution could be recovered after this procedure; the aspirate was usually slightly cloudy but rarely blood-tinged even in the presence of considerable pulmonary consolidation. It is not certain, of course, that the returns were purely from the respiratory surface unmixed with interstitial fluid, but Fazekas de St. Groth and Donnelly,[8] using a similar technique, have presented evidence for the efficacy of this method.

Infectivity determinations. Lung tissue was emulsified without abrasive in a sufficient volume of broth-saline solution to make a 10 per cent suspension (wet weight). This material was then further diluted in 10-fold steps and 0.1-ml. aliquots of each dilution injected into the allantoic cavities of 4-6 eggs. After 44 hours' incubation the allantoic fluids were harvested and tested individually for their capacity to agglutinate an equal volume of one per cent chicken erythrocytes. In addition to the egg infectivity studies, many of the lung suspensions were tested for hemagglutinin content. This method was less reliable for estimating virus content due to the presence of nonspecific agglutinins at low dilutions which tended to obscure the classical pattern[14] of specific viral hemagglutination. In addition, the direct hemagglutination method is not sufficiently sensitive to detect small quantities of virus since approximately 10^5-10^6 infective doses are required to produce one hemagglutinating unit.

Egg infectivity and hemagglutinin determinations of bronchial washings were made in the same manner, using undiluted fluid as the 10° reference point. The results obtained with this material were eminently more satisfactory than those with whole lung suspensions. The bronchial washings contained no particulate matter, provided more uniform suspensions, were invariably free of nonspecific hemagglutinins, and frequently gave higher egg infectivity and hemagglutinin titers than the lung suspensions.

Scoring pulmonary consolidation. The method used was essentially that of Ginsberg and Horsfall[7] by which the degree of lung consolidation is estimated by gross inspection on a scale of 0 to 5+. A percentage rating was then given to each group of similarly treated mice and a mean score (MS_{50}) calculated on the basis of dilution of infective material required to produce a 50 per cent consolidation endpoint.

EXPERIMENTAL RESULTS

Comparative effectiveness of intranasal and intravenous routes of inoculation

The variations in response of mice to intranasal and intravenous inoculation were determined by comparing the amount of WS virus required to produce infection, pulmonary consolidation, and death at the 50 per cent level. Twelve mice were injected by each route with serial 10-fold dilutions of a single allantoic fluid preparation of WS virus having a hemagglutinin titer of 1 : 4096 and an egg infective titer of $10^{-9.83}$. Six mice in each group were killed in 4 days and their lungs tested individually for the presence of virus. The incidence of death and the degree of pulmonary consolidation were noted in the remaining animals over a 10-day period.

TABLE 1. THE AMOUNT OF WS VIRUS REQUIRED TO PRODUCE A 50 PER CENT INCIDENCE OF INFECTION, PULMONARY CONSOLIDATION, AND DEATH IN MICE INJECTED INTRANASALLY OR INTRAVENOUSLY

	—Log EID_{50}		*I.V./I.N. ratios (Antilog)*
	I.N.	*I.V.*	
ID_{50}*	0.83	6.0	147,000
MS_{50}†	1.83	9.33	31,700,000
LD_{50}†	2.5	>9.83	.....

* In 4 days.
† In 10 days.

The striking differences in susceptibility by the two routes of inoculation are shown in Table 1. As noted, the virus was approximately 100,000 times more infective when administered intranasally. This refractoriness to intravenously injected virus was even more apparent when gross pulmonary lesions and death were considered. In order to produce equivalent degrees of pulmonary consolidation, 31,000,000 more egg infective doses were required by the intravenous route than by intranasal injection. A 50 per cent lethal endpoint was not achieved with the most concentrated intravenous inoculum. Two of six mice died in 48 hours after receiving $10^{9.83}$ EID_{50} intravenously (undoubtedly due to the toxicity of the preparation[9]), but there were no deaths attributable to late pulmonary consolidation.

The fate of the inoculum

It seemed possible that the marked resistance of mice to virus injected intravenously could be due to failure of a sufficient number of infective particles to reach the lung. Or, alternatively, the post-inoculation virus

content of the lung might be high but cells capable of supporting virus multiplication were relatively inaccessible by this route. These questions were explored by comparing the amount of "free" virus recovered in the bronchi and whole lungs at intervals after inoculation. In this manner some estimate was possible of what proportion of the total virus population of the lung could traverse the "blood-lung barrier" and find its way to susceptible cells.

As in the previous experiment, various concentrations of virus were injected intranasally or intravenously in groups of 18 mice. At arbitrarily chosen times of 10 minutes and 3 hours after inoculation, when the virus present represented original inoculum, 6 mice were sacrificed and infectiv-

TABLE 2. WS VIRUS CONTENT OF LUNGS AND BRONCHIAL WASHINGS AT INTERVALS AFTER INTRANASAL AND INTRAVENOUS INJECTION

Route	*Dose/mouse (Log EID_{50})*	*Infectivity ratios (—Log EID_{50}), Whole lung/Bronchi*		
		10 min.	*3 hr.*	*24 hr.*
I.V.	9.83	6.0/0.75	4.37/2.5	5.5/6.17
	7.83	4.5/1.5	3.0/0	4.33/4.5
	6.83	4.67/0*	1.33/0	4.25/5.5
	5.83	4.25/0	0/0	0.67/0
I.N.	3.83	2.75/2.5	1.23/1.75	7.5/7.0
	1.83	0/0	0/0	4.67/3.5

*0 = $<10^{0.5}$.

ity determinations made of pooled samples of bronchial washings and lung suspensions. The procedure was repeated with the remaining 6 mice in each group 24 hours after inoculation to compare the distribution of actively multiplying virus.

The results are summarized in Table 2. The proportion of the intravenously injected virus recovered from whole lung in 10 minutes varied with the infecting dose, ranging from 2.6 per cent of the most dilute inoculum to 0.01 per cent of the most concentrated. By the 3d hour after intravenous injection a considerable decline in the infective virus content of whole lung was noted at each dose level. Relatively few infective particles were found in bronchial washings at 10 minutes and 3 hours and only in the most heavily exposed mice, representing but a small fraction of the total lung virus. In the animals that received $10^{6.83}$ EID_{60}, no virus could be demonstrated in bronchial washings, but its presence there was attested to

by the appearance of bronchial virus in moderately high concentration at 24 hours. Whereas the post-inoculation samples showed a preponderance of virus located extrabronchially, when multiplication ensued, the bronchial titers exceeded those of whole lung. Undoubtedly, the presence of small amounts of bronchial virus could not be detected due to the virus-binding capacity of the cells. This is most clearly demonstrated by intranasal inoculation which resulted in rapid absorption of virus by respiratory epithelium leaving less than 10 per cent of the inoculum free in the bronchi after 10 minutes. As opposed to intravenous injection, the whole lung/bronchial ratios of intranasally administered virus were about equal.

There were no significant differences in the recovery rates of intravenously injected virus when the volume of the inoculum was increased to as much as two ml.

It would appear, therefore, that the relative innocuousness of intravenously injected virus is due, at least in part, to its inability to reach susceptible cells in adequate concentration to initiate infection.

Virus growth curves

The interrelationship of pathogenicity and multiplication rate of influenza viruses has been stressed by many investigators. Different strains vary in their capacity to produce transmissible pneumonitis after intranasal injection depending upon their multiplication potentials which, in turn, are related to their degree of adaptation.[21] Rapidly proliferating strains cause fatal pneumonitis with inocula of low infectivity, whereas avirulent, unadapted strains grow poorly and do not produce pulmonary consolidation unless massive doses are injected.[19] Multiplication of adapted strains may also be limited with very dilute inocula, in which case the onset of pulmonary consolidation is correspondingly delayed.[7] In the last analysis, probably the most important factor in pathogenicity is the total amount of virus that the respiratory epithelium is exposed to at any one time, regardless of whether this be from newly formed virus or weakly infective inoculated virus. This has been most clearly demonstrated by Ginsberg[6] who found that in vaccinated mice immunity could be overcome and extensive pneumonitis produced with massive intranasal inoculations, in spite of the absence of a detectable cycle of virus replication.

An example of the influence of the infecting dose on the multiplication rate of intranasally injected WS virus is shown in Figure 1. When the inoculum contained $10^{3.83}$ EID_{50}, the maximum lung and bronchial concentrations of infective virus were achieved within 24 hours and all the mice died in 5 to 7 days with complete pulmonary consolidation. On the other

hand, intranasal inoculation of $10^{1.83}$ EID_{50} resulted in a slower increase of virus, generally lower titers late in the course of infection, and an over-all incidence of only 50 per cent lung lesions. Also noteworthy is the fact that the bronchial titers frequently exceeded the total lung titers in the same mice, indicating that a considerable amount of virus was "free" in the bronchial secretions and that the cells were "saturated."

Returning for a moment to Table 1, it can be seen that a more dilute intranasal inoculum ($10^{0.83}$ EID_{50}) retained its infectivity but completely lost its pathogenicity. By the intravenous route, on the other hand, greater

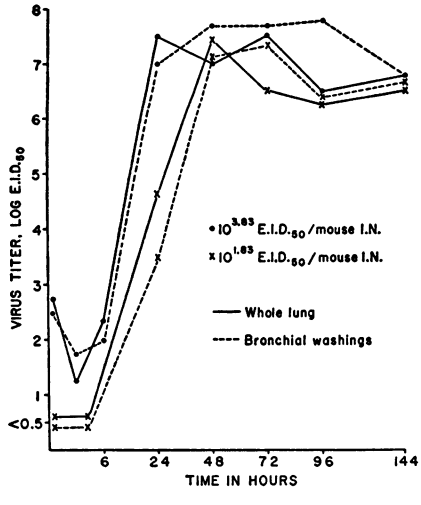

FIG. 1.

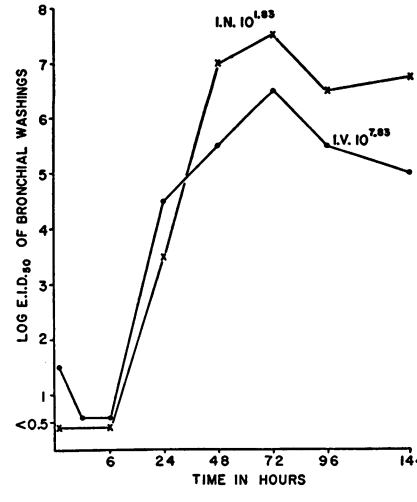

FIG. 2.

FIG. 1. The relation of multiplication potential to dose of intranasally administered WS virus.

FIG. 2. WS virus growth curves after intranasal and intravenous inoculation.

than a 1000-fold increase in the ID_{50} level was required to produce 50 per cent consolidation and an LD_{50} concentration was unattainable. These data suggested, that, regardless of the amount of virus reaching the respiratory epithelium after intravenous injection, it was severely limited in its proliferative potential, both as to rate and extent of multiplication.

Support for this hypothesis was obtained by examining in detail the virus growth curves after intravenous injection of various concentrations of WS virus. As seen in Table 3, there was little difference in production of infective virus over a wide intravenous dose range and, most strikingly, the titers rarely exceeded $10^{-6.5}$ EID_{50} even with the heaviest inoculum. Also, there was no evidence of significant virus multiplication outside of the

respiratory epithelium for, as was the case with intranasal infection, the virus content of the bronchial washings was usually greater than that of whole lung in the same mice. This, incidentally, would appear to be presumptive evidence that bronchial epithelial cells are infected by intravenous as well as intranasal injection.

Bronchial growth curves are presented in Figure 2 to show more clearly the comparative response of mice to a small intranasal and a large intravenous dose of WS virus. It is apparent that the mechanism of infection is quite different, not only in regard to the invasiveness of virus by the two routes, but also in the essential dissimilarity of virus growth.

TABLE 3. DAILY WHOLE LUNG/BRONCHIAL WS VIRUS TITERS (—LOG EID_{50}) AFTER INTRAVENOUS INJECTION

Log I.V. dose	*Days after injection*					
	1	*2*	*3*	*4*	*5*	*7*
9.83	5.5/6.17	5.5/6.5	6.0/6.29	6.25/7.0	5.5/6.4	4.75/4.5
7.83	4.33/4.5	5.5/5.5	5.78/6.5	4.75/5.5	.....	4.33/5.5
6.83	4.25/5.5	4.33/4.25	4.5/5.5	5.5/5.33		
5.83	0.67/0*	0/0	4.33/5.25	3.76/4.33		
4.83	0/0	0/0	0/0	0/0		

* $0 = <10^{0.5}$.

Hemagglutinin-infectivity ratios

One possibility that required consideration was that intravenous infection results in an entirely different mode of virus replication involving the production of noninfectious particles, the so-called "incomplete virus" of von Magnus.[11] Such a cycle of influenza virus multiplication has been demonstrated in insusceptible tissues like mouse brain after direct inoculation of very large doses of the PR8 strain.[15] Since mouse lung also exhibited a relatively poor response to massive parenteral doses of virus, further studies were carried out to determine whether noninfectious hemagglutinins appeared at any stage after intravenous infection.

An allantoic fluid preparation of WS virus was used that contained approximately $10^{5.5}$ EID_{50} for each unit of hemagglutinin, a ratio which is in the usually accepted range for highly infective influenza virus. This material was injected undiluted into 24 mice by the intravenous route and individual assays made of the hemagglutinin and egg infective titers of both the bronchial washings and whole lung suspensions of 4 mice at daily intervals thereafter. Hemagglutination titrations of all specimens were carried

out in citrate-saline solution after treatment with receptor-destroying enzyme in order to inactivate virus inhibitors.

As shown in Table 4, the expected ratios of hemagglutinating to infective virus were obtained throughout the 6-day experimental period. It appears reasonable to assume, therefore, that only fully infective particles are being produced in response to intravenous infection. Similar ratios were also obtained after intranasal injection. Furthermore, these experiments provide additional evidence that the differences in the virus growth curves after intravenous infection are not due to significant extrabronchial multiplication of either infective or "incomplete" virus.

TABLE 4. HEMAGGLUTININ/INFECTIVITY RATIOS AFTER INTRAVENOUS INJECTION OF UNDILUTED WS VIRUS

	Day				
	1	*2*	*3*	*4*	*6*
Whole lung	$0/10^{3.5}$	$0/10^{4.2}$	$2/10^{6.38}$	$0/10^{5.5}$	$3.2/10^{5.3}$
Bronchi	$0/10^{3.0}$	$0/10^{4.75}$	$2/10^{5.7}$	$6.4/10^{5.88}$	$8/10^{6.0}$

The values are the reciprocal geometric mean.
HA/EID_{50} titers of 4 mice. $0 = <2$.

Although the hemagglutinin titer may provide a more accurate means for following the course of well-established infections, it is quite apparent that this method is insufficiently sensitive to detect the presence of low grade infections. Nevertheless, most of the detailed studies of influenza virus infection of mouse lung that have been done in the recent past have used viral hemagglutinins as the index of infection. For example, in the exhaustive series of experiments reported by Fazekas de St. Groth and Donnelly[8] there was no hemagglutinin detectable in the lung after large intraperitoneal or subcutaneous injections of the Melbourne A strain, leading these authors to conclude that "active virus given by extrarespiratory routes is as incapable of causing infection as inactivated vaccines given by any route."

Serial intravenous passage

In their studies on influenza virus toxicity the Henles[9] found considerable variation in the frequency with which late pulmonary consolidation could be produced after parenteral injection of mouse-adapted strains. Since it was conceivable that these strain differences might have been related to degree of adaptation, an attempt was made to augment the intravenous pathogenicity of WS virus by serial passage in the hope that a more

virulent form would arise. Presumably, this might occur by mutation or selection of a variant with greater invasive and proliferative potential by the intravenous route of inoculation. The series was initiated by injecting 10 mice intravenously with 0.1 ml. of undiluted WS allantoic fluid. After 4 days 5 of the animals were sacrificed and their bronchial washings collected, titered, and pooled. This material was then immediately injected intravenously into 10 new mice and the cycle repeated thereafter at 4-day intervals. The incidence of death and the degree of pulmonary consolidation over a 10-day period were recorded in the remaining 5 mice of each group.

No deaths occurred and only minimal pulmonary consolidation was noted during the first 9 passages. Deaths were occasionally encountered in subsequent passages, but the mortality rate did not increase consistently. The degree of pulmonary consolidation was also more marked in the later passages but was almost always sporadic and rarely exceeded an over-all score of 50 per cent, occasionally reverting to less than 10 per cent for no apparent reason. Successive transfer did not result in increasing infectivity and hemagglutinin titers of the bronchial washings nor was there any definite correlation between virus content of the lungs and extent of pneumonitis. The experiment was terminated at the 28th consecutive intravenous passage at which time the virus was somewhat more pathogenic but exhibited no alteration in its multiplication potential in mouse lung by either the intravenous or intranasal route.

The Taylor phenomenon

As previously mentioned, an important limiting factor in the intranasal pathogenicity of any virus strain is the dose of the inoculum. Small doses of virus undergo a reproductive cycle characterized by a slower rate of increase and invariably lower titers than do lethal inocula. However, at 48 hours after infection the lungs of surviving mice may contain more than 1,000,000 infective virus particles. The most succinct expression of this paradox is contained in a paper by Taylor:[20] ". . . 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." Somewhat by accident Taylor[20] discovered that this phenomenon is related to mechanical bronchogenic dissemination and the capacity of the host to localize the sublethal infection. Simple intranasal instillation of sterile water, saline solution, broth, or 10 per cent normal serum four days after a sublethal inoculation "produced a sharp rise in the virus content of the

lungs followed by death in 3 to 8 days." Pathological lesions in these mice were identical to those seen in animals receiving a lethal intranasal injection. Similar observations had also been made by Straub.[18]

The Taylor phenomenon afforded an additional means of comparing sublethal intranasal and intravenous pulmonary infections. A group of 20 mice was injected intranasally with $10^{1.83}$ EID_{50} of WS virus and an equal number with $10^{7.83}$ EID_{50} intravenously. These doses of virus by themselves did not cause death and produced roughly equivalent bronchial titers. Four days after injection each of these 40 mice was given 0.05 ml. of sterile broth-saline solution intranasally. All 20 of the mice originally infected by the intranasal route died with complete pulmonary consolidation between 2 and 8 days after the second inoculation. On the other hand, only 5 of the 20 intravenously infected mice succumbed from 3 to 7 days after intranasal instillation of broth-saline solution. It would appear, therefore, that intravenously administered virus is in a less advantageous position to be disseminated by the bronchogenic route.

Interfering action of intravenously administered virus

Rickard and Francis[18] have presented evidence of a rapidly developing resistance to intranasal PR8 virus infection in mice previously inoculated intraperitoneally with live homologous virus. This protective effect was maximal when the interval to challenge was two days, during a time when circulating antibody could not be detected. Oakley and Warrack[12] were unable to duplicate these results with either PR8 or WS virus but entertained the possibility that their preliminary intraperitoneal injections were too small to confer resistance.

The results of the following experiment support the findings of Rickard and Francis.[18] Four groups of 20 mice were injected with normal allantoic fluid (NAF) or sublethal doses of WS virus, as follows: Group I—NAF 0.05 ml. intranasally; Group II—NAF 0.1 ml intravenously; Group III—$10^{1.83}$ EID_{50} of WS virus intranasally; and Group IV—$10^{7.83}$ EID_{50} of WS virus intravenously. Two days after these preliminary injections all 80 animals were challenged with 50 LD_{50} of WS virus by the intranasal route. As shown in Table 5 all but 1 of the 60 mice in Groups I, II, and III died with complete pulmonary consolidation. On the other hand, 60 per cent of the mice that had received a preliminary intravenous virus inoculation survived the challenge injection, in spite of the fact that extensive pulmonary consolidation was noted at the end of the 10-day experimental period.

It would appear, therefore, that the interfering action of intravenously injected virus represents an additional distinguishing feature from classical intranasal infection.

Pathology

Credit for the earliest and most concise description of the morphology and pathogenesis of murine influenza virus pneumonitis belongs to Straub.[17, 18] He states that following intranasal infection "the first localisation of this process (gross lesion) is often found near the hilum of one or more lobes; later on entire lobes or almost the whole of both lungs except the edges may be affected." The gross morphological changes were not considered to be peculiar to influenza virus infection but many of the microscopic lesions appeared to be quite specific reactions to this organism. His most pertinent observation was that experimental influenza infection in the mouse is primarily a disease of bronchial and bronchiolar epithelium.

TABLE 5. RESISTANCE TO INTRANASAL CHALLENGE OF LD_{50} OF WS VIRUS TWO DAYS AFTER PRELIMINARY INJECTION

	Interfering virus			
Group	*Route*	*Dose (EID_{50})*	*Mortality*	*% Lung lesions*
I	I.N.	0 (NAF)	20/20	100
II	I.V.	0 (NAF)	19/20	99
III	I.N.	$10^{1.88}$	20/20	100
IV	I.V.	$10^{7.88}$	8/20	80

Sequentially, there is destruction of epithelial cells which desquamate in 2 or 3 days followed by replacement with a fibrinoid material poor in inflammatory cells. Even the terminal bronchioles, which in the mouse are lined with cuboidal epithelium to their junction with alveolar sacs, are involved in this process but the alveolar cells themselves are spared. Parenthetically, a true hyaline membrane has not been noted.[10] Associated with these changes are peribronchial edema, collapse of alveolar segments with compensatory emphysema in uninvolved areas, interstitial edema and hemorrhage, and varying degrees of infiltration with polymorphonuclear leukocytes. The final gross appearance of plum-colored consolidation is due, therefore, to atelectasis, the extent of the lesions being dependent upon the degree of bronchiolar involvement. Death ensues only when most of the pulmonary parenchyma is involved, varying with the virus dose from 3 to as long as 10 days after intranasal injection. Somewhat more detailed descriptions of certain aspects of the pathogenicity of this disease, particularly the early cytoplasmic and ciliary changes in epithelial cells, are contained in the reports of Loosli[10] and Harford and Hamlin.[8]

Of the greatest interest, perhaps, is Straub's observation that the reparative process may begin as early as the 5th day even in fatal infections. Proliferation of new bronchial and bronchiolar epithelium is seen at this time which is low cuboidal or squamous in appearance, closely resembling the lesions described by Winternitz *et al.*[22] in pandemic human influenza. Mice surviving the infection may show an extraordinary picture several weeks after inoculation. The involved portions of the lung contain nests of squamous epithelial cells with many mitotic figures, not only in the bronchioles but also proliferating downward to fill the alveoli. However, this metaplastic squamous epithelium does not invade interalveolar spaces, and the alveolar septa themselves appear to be well preserved.

In the present studies the pathological changes in the lungs following intranasal injection of WS virus were essentially similar to those described above. *Macroscopically,* plum-colored consolidation was noted in the perihilar areas in 2 to 4 days, depending on the virus concentration of the inoculum. Mice killed thereafter usually showed more extensive lesions spreading toward the periphery but even the lungs of those mice dead of infection usually exhibited a narrow rim of uninvolved lung tissue. The early *microscopic* lesions in intranasally injected mice were those of bronchitis with progressive desquamation of epithelium, accompanied by peribronchial edema and more extensive peribronchitis and intraluminal exudate than described by Straub[17] and Loosli.[10] This process, particularly with small intranasal doses of virus, began in the large bronchi (Fig. 3) and gradually spread to involve the bronchioles. Replacement of columnar epithelium by squamous or low cuboidal cells was noted as early as the 4th day in the large bronchi (Fig. 4) and 1 to 3 days later in the bronchioles.

In marked contrast, the *gross* pathological findings in intravenously infected mice were characterized by peripheral, frequently wedge-shaped pulmonary lesions with hilar sparing. These focal areas of plum-colored consolidation could be found as early as 3 days after infection and were most extensive by the 4th or 5th day. The lesions usually remained localized and ordinarily did not progress to involve the entire lung as was the case with intranasal infection. Most frequently affected were the right middle lobe, the apical segment of the left lung, and the subcardiac lobe of the right lung, in that order. The earliest *microscopic* lesions in intravenously infected mice occurred in the small and medium-sized blood vessels of the lungs, both arteries and veins. Extensive perivascular infiltration with polymorphonuclear and mononuclear leukocytes was noted 2 to 3 days after injection, remaining stationary with little progression or resolution for at least several weeks (Fig. 5). Only rarely were inflammatory cells seen invading blood vells walls and there was no destruction or proliferation of

vascular endothelium. Thromboses were uncommon. Destruction of respiratory epithelium was noted by the 3d or 4th day after intravenous infection, usually focal in distribution and limited to the terminal bronchioles. Unlike intranasal infection the larger bronchioles and bronchi were never involved and the degree of peribronchiolar inflammation was minimal. Consolidation, hemorrhage, edema, and collapse of alveoli occurred in patchy segments, generally at the periphery of the lung and not unusually involving the visceral pleura. Straw-colored pleural effusions were occasionally seen with intravenous but not intranasal infections.

Squamous metaplasia of the bronchiolar epithelium appeared on the 4th or 5th day after intravenous infection, becoming increasingly more prominent thereafter, and was far out of proportion to the rather minimal degree of bronchiolar destruction that preceded it. The lungs of mice killed two weeks after intravenous infection presented a striking picture (Fig. 6). The periarteritis had not subsided and squamous epithelial cells with numerous mitotic figures filled nests of alveoli. By the 23d day the extent of proliferative epithelialization took on an appearance suggestive of bronchogenic carcinoma of the squamous cell type, but closer examination revealed that the interalveolar spaces were not invaded and the basic architecture of the lung was preserved. There was no desmoplastic reaction in the epithelial cell masses and the degree of interstitial fibrosis was minimal. Very little resolution of these lesions was noted as long as three months after infection.

On the basis of the superficial resemblance of these epithelial changes to neoplasia[17, 18] an attempt was made to transplant lung tissue of intravenously infected mice to the subcutaneous space of homologous hosts. Many of the homografts survived for several weeks but did not exhibit true epithelial proliferation and could not be retransplanted.

The gross and microscopic pathological changes in the lungs of the intravenous serial passage mice were similar to those described above but were generally more extensive. Infected allantoic fluid diluted beyond 10^{-3} or normal allantoic fluid injected intravenously produced no vascular or bronchiolar lesions.

The lungs of mice that survived a challenge intranasal injection of WS virus given two days after an intravenous interfering dose showed the histological effects of intranasal infection plus the perivasculitis of intravenous infection. Grossly and microscopically the consolidative process ten days after intranasal challenge was confined to the hilar and peribronchial areas, almost as if the centrifugal spread of the lesions had been arrested at the terminal bronchioles.

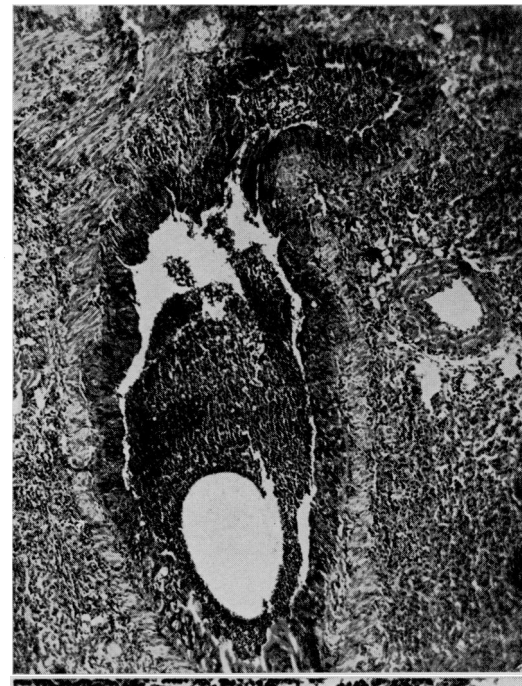

FIG. 3. Intranasal infection. Marked inflammatory response in a major bronchus four days after intranasal inoculation of $10^{1.83}$ EID_{50} of WS virus. Hematoxylin-eosin, x90.

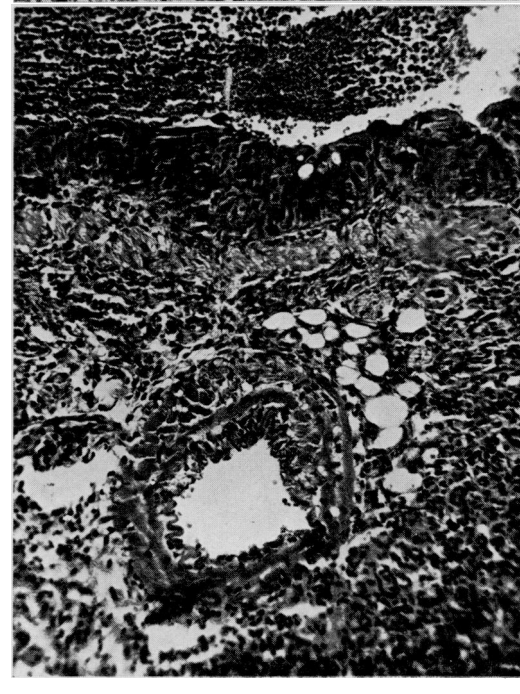

FIG. 4. Intranasal infection. Higher power (x410) view of the wall of the bronchus in Figure 3 showing the thickened cuboidal metaplastic epithelium. Note the lack of perivasculitis of the adjacent arteriole.

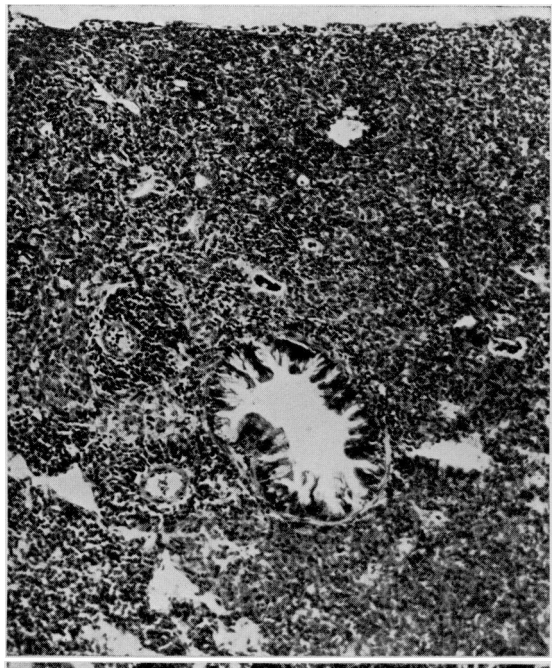

FIG. 5. Intravenous infection. Marked perivasculitis, well-preserved columnar bronchial epithelium and early squamous metaplasia six days after infection. The visceral pleura is also infiltrated with inflammatory cells. Hematoxylin-eosin, x130.

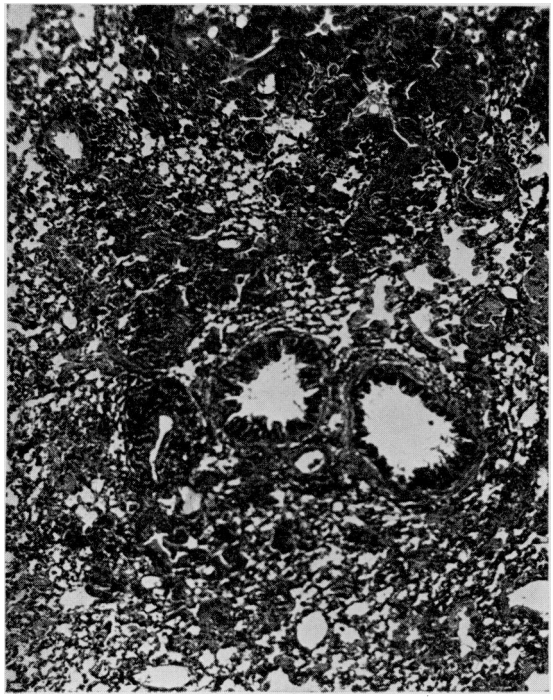

FIG. 6. Intravenous infection. Masses of squamous epithelial cells filling nests of alveoli in the lung of a mouse that survived 14 days. Note the fairly well-preserved bronchial epithelium and residual perivasculitis. Hematoxylin-eosin, x130.

DISCUSSION

Regardless of whether virus is administered intranasally or intravenously, the pathogenesis of influenzal pneumonitis is clearly related to bronchiolitis. However, the rate of virus multiplication and the severity of the disease depend, to a large extent, on the route and virus concentration of the inoculum. Thus, the response to intranasal infection may take three forms depending on the degree of exposure. With large infective doses the bronchi and bronchioles are probably involved simultaneously, the virus multiplying at all sites of primary infection and producing maximum titers within 24 hours. This fulminating infection is accompanied by generalized, uniform destruction of respiratory epithelium and early atelectasis, consolidation, and death. An alternative course may be seen with smaller doses of virus in which the infection is probably confined at first to the upper respiratory tract and major bronchi resulting in a generally slower rate of virus proliferation. Due to involvement initially of fewer cells, maximum titers are not obtained until 48 hours after injection and the occurrence of lung lesions and death is correspondingly delayed. Thirdly, sublethal doses produce relatively few new infective particles, presumably because the infectious process is localized to the major bronchi; the majority of bronchioles are spared, the incidence of atelectatic and pneumonitic lesions is low, and the host, therefore, survives. It is of interest that this capacity to resist a low-grade infection is largely a matter of lessened mechanical dissemination of virus, since simple intranasal instillation of bland fluids can convert the benign course to a fulminating pneumonitis. Notwithstanding these differences in dose response, influenzal pneumonitis produced by intranasal injection is essentially a descending bronchogenic infection.

In contrast, infection by the intravenous route appears to be primarily a focal disease of terminal bronchioles. By and large the virus has no way of ascending the bronchial tree and in only a limited number of animals can disseminated infection be produced by intranasal instillation of sterile broth. Since the infectious process is generally confined to the bronchiolar epithelium, the resulting virus titers are lower than after intranasal infections, rarely attaining the critical levels of a lethal infection. Although severely limited, the multiplication potential of the virus and the degree of pulmonary consolidation depend to some extent on the number of infective particles that reach susceptible cells by the intravenous route which, in turn, is a function of dose of inoculum. Unlike intranasal infection, the range between infective and pathogenetic doses of intravenously injected virus is extremely wide.

One possible explanation of the limited susceptibility of lung tissue to intravenous infection would be that virus replication takes place on the extrarespiratory surface of the capillary-alveolar membrane. An analogy to such a situation can be found in the differential susceptibility of the two sides of the chorio-allantoic membrane of the chick embryo. Fulton and Isaacs[5] clearly demonstrated that the chorionic surface is capable of supporting only a single cycle of influenza virus multiplication and the amount of antigen produced in these cells is dependent on the infecting dose. Significantly, they also found that virus did not penetrate to the susceptible allantoic cells. Of interest also are the findings of Burnet and Fraser[2] that certain strains of virus inoculated intravenously or onto the chorionic surface of the chick embryo chorio-allantois are disseminated to the brain, resulting in a hemorrhagic encephalitis. Previous inoculation of homologous virus by the allantoic route interfered with the development of cerebral hemorrhages, and the embryos survived. These authors suggested that virus multiplication and interference take place in the capillary endothelial cells of the brain. However, the evidence in the present studies appears to be fairly conclusive that multiplication of intravenously administered virus is confined to the bronchiolar epithelial cells of the mouse. Except for the immediate post-inoculation period the virus content of bronchial washings always equalled or exceeded that of whole lung, indicating that little if any infective virus remained in the interstitium or capillaries. In addition, the pathologic lesions were those of bronchiolitis rather than interstitial pneumonia, and perivasculitis without endothelial involvement.

Assuming, therefore, that only the respiratory epithelium of the lung can serve as substrate for influenza virus multiplication, there appear to be three possible routes by which the virus can cross the "barrier" from blood stream to terminal bronchiolar cells. The first is that rupture of submucosal capillaries may spill virus into the bronchial tree. This explanation seems least likely because large volumes of inocula, up to two ml. intravenously, did not enhance pulmonary infectivity, and no extravasation of blood was seen in sections taken immediately after injection. Of greater likelihood is that small blood vessels are permeable to small quantities of virus adherent to their walls. This mode of penetration would be more consistent with the invariable pathological finding of perivasculitis. Having found its way into the interstitial tissues the virus may then invade bronchiolar epithelium by the "back door," that is, the extrarespiratory surface of the cells. An equally tenable explanation, however, would be that virus diffuses across the capillary-alveolar membrane with reflux upward to infect adjacent epithelial cells at their respiratory surface. This last hypothesis would perhaps best

explain the primary infection of terminal bronchioles and the sparing of larger branches.

SUMMARY

The pathogenesis of murine influenzal pneumonitis was studied by comparing the host response to intranasal and intravenous infections with the mouse-adapted WS strain of influenza A virus. Striking differences were found in infectivity, pulmonary consolidation, and death by the two routes of inoculation, as well as post-inoculation distribution of virus in lungs and bronchi, virus multiplication potentials, capacity of the host to localize low-grade infections, and interference phenomenon. The primary pathological findings in either type of infection were early destruction of respiratory epithelium and subsequent metaplastic regeneration. However, the distribution of the lung lesions was quite different. After intranasal infection the major air passages were involved initially, progressing to extensive bronchitis, atalectasis, diffuse pulmonary consolidation, and death; whereas, intravenous infection was essentially a benign, focal disease of terminal bronchioles. It is concluded that the severity of the pneumonitic process is a function of the extent to which the total mass of respiratory epithelium is involved, which is, in turn, related to mechanical bronchogenic dissemination of virus.

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