EFFECT OF THE LESION DUE TO INFLUENZA VIRUS ON THE RESISTANCE OF MICE TO INHALED PNEUMOCOCCI*‡

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Plate 4

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Increased susceptibility to bacterial pneumonia is well known following influenza in human beings and experimental animals. Previous work from this laboratory (2) demonstrated that mice with sublethal influenza viral infections are more susceptible than normal mice to pneumococci or hemolytic streptococci administered by inhalation of fine droplets. The object of the present investigation was to determine the mechanisms by which resistance is lowered under these particular experimental conditions. Controlled experiments in mice with known infectious agents were thought to be capable of elucidating principles concerning resistance which might be applicable to various types of human respiratory infection. The results indicate that inception of superimposed pneumococcal pneumonia is not due merely to the presence of virus in the lung but is dependent upon visible lesions in the lung caused by viral action.

Methods and Materials

Techniques were the same as those previously described (2) except for modifications and additions to be noted.

Administration and Titration of Virus.—In the preliminary experiments recorded in Table I, a sublethal dose (10^{-7}) of the PR8 strain of influenza virus was used. Test of this sublethal dose was carried out in Experiments 3 and 4 in Table I of the previous paper (2). In all the other experiments, the Weiss strain of influenza virus was employed in lethal doses (dilutions 10^{-3} or 10^{-5}). Diluents were tryptose phosphate broth (Difco), beef infusion broth, or 0.1 M phosphate buffer (3).

Injection into the left bronchus under anesthesia with chloral hydrate was done as before and further experience with this method has confirmed its reliability as judged by the constancy of viral lesions in the left lobe of the lung. Also, the operative fatality has been reduced by use of mice 4 weeks of age or older and by heating the mice with an electric lamp after inoculation in order to terminate anesthesia more promptly (4, 5).

Titrations to determine the amount of virus in the left lobe of the lung were done by intratracheal inoculation of tenfold dilutions of the ground pulmonary tissue. The technique of intratracheal inoculation was the same as intrabronchial except that the inoculum was de-

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livered while the cannula was in the trachea. The purpose of this method was to obtain viral infection of the whole lung so that results of titration would be clear cut.

For histologic study of early viral lesions, the virus was administered also by inhalation of fine droplets using the same technique as for pneumococci. For this purpose, a dilution of 10^{-3} in buffer solution was employed and the mice were exposed to viral droplets for 2 to 3 hours.

Bacteria.—The A5 strain of Type I pneumococci was used throughout. In the preliminary experiments (Table I) the organisms were suspended in saline. In all the other experiments, pneumococci were grown for 4 to 8 hours in serum infusion roth before passage through the atomizer. Normal rabbit serum was added to the broth in concentrations of 5 to 10 per cent and in later experiments 0.05 to 0.2 per cent glucose was used.

Inhalation of Fine Droplets.—The fluid culture of bacteria was passed through a metal atomizer[1] under a pressure of 500 mm. of mercury. A fine fog was emitted that was barely visible against a dark background and did not condense to form visible droplets when allowed to impinge upon a surface.

The fog was directed by rubber tubing from the atomizer into a small bottle serving as a trap for larger droplets and then into a chamber made of galvanized iron 40 × 30 × 86 cm. in size. The mice were contained in wire cages that were passed into the chamber through a tightly fitting door and the stream of droplets was allowed to pass through the chamber for 20 minutes before the mice were placed inside. In the first experiments (Table I) the mice were allowed to inhale bacteria for 3 hours. In all the other experiments, inhalation lasted for 1 hour.

The outlet of the chamber was directed through two coils of brass tubing connected in series. The coils were heated constantly with large Bunsen burners and blood agar plates held over the final outlet showed that pneumococci were not penetrating the coils to contaminate the room. Ultraviolet light from a lamp was directed over the door of the chamber while open in order to minimize bacterial contamination of the room. Operators wore flannel masks.

Mice.—In each experiment, mice from a single shipment were used so that ages did not vary by more than a few days and sizes were essentially uniform. When mice were observed for survival after inhalation of bacteria, those with wounds from the bites of cage mates were eliminated in order to avoid confusion of results by contamination of wounds with droplets containing pneumococci.

In order to test for the presence of spontaneous pneumotropic viruses in mice from the source used, 19 passages (some in series) were carried out with pooled suspensions of ground pulmonary tissue. In some instances, small macroscopic pulmonary lesions were found but further passage of such lungs failed to reveal a transmissible agent.

Enumeration of Bacteria in the Lungs.—In the previous experiments, recovery of pneumococci from the lung was done by transferring a loopful of minced tissue to the surface of a blood agar plate. For the present purpose, a quantitative and more sensitive method was used, namely, grinding in a mortar and preparing poured blood agar plates.

In order to find out whether grinding with sand would have a significant effect on the viability of bacteria in the lung, the following experiment was done: The left lobes of two mice were removed aseptically, placed in each of two sterile mortars, and covered with sterile sand. An undiluted broth culture of pneumococci was added to one mortar before grinding with a pestle and the same amount of the same culture was added to the other mortar after grinding. The number of viable bacteria in each suspension was determined by making serial tenfold dilutions and poured blood agar plates of the dilutions 10^{-6} and 10^{-7}. Essentially no difference was noted in the number of colonies counted on the plates of the two series.

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[1] De Vilbiss No. 180.

Accordingly, the following procedure was adopted: The mouse was killed with ether after which the left lobe of the lung was removed aseptically, ground with sand in a mortar, and the ground pulmonary tissue was suspended in 5 ml. of serum infusion broth or tryptose phosphate broth. After thorough mixing, 1 ml. of this suspension was used to prepare a poured blood agar plate with tryptose phosphate agar and defibrinated rabbit blood. Colony counts were done after incubation overnight.

In some instances, too many colonies developed to be counted with accuracy and such plates have been indicated in the tables (TNC). For purposes of calculation, plates with such numerous colonies were counted as 400 in order to make a conservative evaluation of the data. Occasional colonies of contaminating organisms were disregarded but plates were not included if contaminants were numerous enough to cause any difficulty in counting colonies of pneumococci.

In the first experiments, lungs could be removed immediately after the mice were killed because only a few animals were tested at a time. Later, when approximately 20 mice were used in the test group and a similar number for controls, the mice were all killed with ether at the same time and kept in the refrigerator during the time needed for the manipulations. One worker carried out aseptic removal of the lungs while another ground them and prepared the poured plates. With this arrangement, 20 mice could be processed in 1 hour. In Tables II, III, and IV, the order of tabulation of plate counts corresponds with the order in which the lungs were removed and it may be seen that no significant number of pneumococci were lost by storage of killed mice for 1 hour in the refrigerator. In those experiments requiring that both groups of mice be killed at the same time, animals from test and control groups were processed alternately and the duration of manipulations was 2 hours. Likewise, the results shown in Tables III and IV do not show a decrease in colony count due to delay necessitated by manipulations.

Observation of Macroscopic Viral Lesions.—Removal of the left lobe of the lung within 6 hours after inhalation of pneumococci gave an opportunity to observe the lung for the presence or absence of macroscopic lesions due to influenza viral infection. In viral infections of 5 days' duration, the left lobe was completely consolidated in nearly every instance. In these experiments, any left lobes showing less than about 90 per cent consolidation were discarded.

Blood Cultures.—Determination of the presence and number of pneumococci in the blood of mice was carried out by aseptic reflection of a flap of axillary skin under deep anesthesia with chloral hydrate followed by severance of the axillary artery and collection of a pool of blood in a pocket formed by the flap of skin (6). The blood was removed from this pocket by a 1 ml. pipette and mixed with 1 ml. of 4 per cent sterile sodium citrate in a slanted Petri dish. When less than 0.75 ml. of blood was obtained from a mouse, enough defibrinated rabbit blood was added to give this amount so as to ensure sufficient blood for growth of pneumococci in the poured plate. Incubation and colony counts of these poured plates were carried out as in the others.

Variability of Data and Validity of Results.—Comparison of different experiments in Tables II, III, and IV will disclose that there was considerable variation on different days in the number of pneumococci present in the lung immediately after inhalation. Because of this variation, conclusions have been drawn only from comparison of test and control series of animals in each experiment. In this way, the effect of day-to-day variation was avoided inasmuch as all the mice of a given experiment were allowed to inhale bacterial droplets at the same time.

Variation has been encountered also in counts of the same experiment but the magnitude of differences between test animals and controls has been so great as to render its significance obvious by inspection. Nevertheless, the data have been subjected to standard statistical calculations (7) and the probability of the differences being due to chance has been found to be extremely small.

Further, it may be mentioned that the errors inherent in plate-counting techniques for the enumeration of bacteria have been realized and efforts made to reduce these to a minimum (8).

EXPERIMENTAL PROCEDURES AND RESULTS

Survival of Mice after Implantation of Pneumococci on Viral Infections of Varying Duration.—It is well known (9, 10) that the titer of influenza virus increases greatly within the first 2 days after inoculation of sublethal doses, persists at high levels for about 1 week, and then decreases gradually. On the other hand, gross lesions are absent before 2 days but are full-blown at 5 to 7 days. Accordingly experiments were designed to determine the effect of viral infections of varying duration on susceptibility to superimposed pneumococci in order to evalute the relative importance of the virus itself and the lesion produced by the virus.

TABLE I

Fatal Pneumococcal Infection after Implantation of Pneumococci on Sublethal Viral Infections of Different Ages

Experiment No.	Duration of viral infection	Results																							
	days																								
1	2	4*	6	8	8	8	8	S‡	S	S	S	S	S	S	S	S	S	S							
	7	2	2	2	2	2	2	3	3	3	4	4	4	4	4	5	S	S							
2	1	3	3	3	3	4	5	5	5	7	8	8	9	9	S	S	S	S	S	S	S	S	S		
	14	3	5	5	5	6	S	S	S	S	S	S	S	S	S	S	S								
3	1	2	2	3	3	3	4	5	6	6	12	S§	S	S	S	S	S	S							
	5	2	2	2	2	3	3	3	3	3	3	3	3	3	3	4									
	14	2	2	3	3	3	3	3	3	4	4	5	S	S	S										

* Each numeral represents a single mouse and indicates the number of days elapsing between inhalation of pneumococci and death from pneumococcal infection.

‡ Each S represents a single mouse and indicates survival for 15 to 17 days after inhalation of pneumococci at the end of which time the animal was killed and autopsy disclosed consolidation in all or part of the left lobe of the lung and culture of this lobe failed to reveal pneumococci.

§ Culture showed pneumococci.

Sublethal doses of virus were given to groups of mice on different days planned so that animals with viral infections of different duration could be permitted to inhale the same culture of pneumococci in the chamber at the same time. The mice were observed for survival. The results are presented in Table I and show the highest incidence of fatal pneumococcal infection in mice with viral infections of 5 to 7 days duration. However, the differences in fatality were not striking and many mice dying of pneumococcal infection after viral infections of only 1 to 2 days duration showed longer incubation periods than the others. In view of these longer incubation periods, the possibility arose that maturation of the viral lesion during the time that mice were

observed for survival could have enabled persisting pneumococci to take advantage of susceptibility accompanying development of the viral lesion.

Bacterial Content of the Lung after Inhalation of Pneumococci.—Because of the probability that viral lesions developed during the long period of observation for survival, an immediate criterion of resistance to pneumococcal infection was needed. Preliminary experiments with normal mice showed that a striking diminution of the number of pneumococci in the lung occurred within a few hours after inhalation and raised the question as to whether viral infection or viral lesions would have an effect on this eliminatory mechanism.

TABLE II

Inhaled Pneumococci in Lungs of Normal Mice

Experiment No.	Time after inhalation	Plate counts																				Mean	Statistical evaluation*
	hrs.																						
1	0	1207	481	836	669	535	764	941	322	405	456	812	1184	478	622	487	755	662	922	425		682	11.4
	6	4	14	3	12	5	2	1	0	25	4	2	9	1	0	2	2	15	1	9		6	
2	0	189	171	211	134	238	145	431	143	107	163	173	133	117	133	154	103	189	301	194	141	178	10.2
	3	0	2	1	0	0	1	6	9	1	10	3	7	0	0	2	0	10	13	1	5	4	
3	0	275	229	278	152	234	228	245	254	426	229	280	309	476	275	73	163	312	158	37		244	9.6
	3	10	43	2	11	13	20	2	9	27	3	2	4	13	29	2	6	3	27	36	5	13	

* The difference of the means divided by the standard error of the difference.

Therefore, bacterial counts were made of lungs immediately after inhalation, and at 3 and 6 hours after inhalation. Normal mice and mice with viral infections of 24 hours and 5 days duration were tested in this manner. Pilot experiments were done first in order to determine the nature of the results to be expected and then controlled experiments were done to establish the findings on a firm basis. The results of the controlled experiments are shown in Tables II, III, and IV.

The experiments of Table II showed that a decrease in the bacterial content of the left lobe of the normal lung took place not only within 6 hours after inhalation but even in 3 hours. Although the decrease was striking, the bacteria did not disappear completely.

In Table III, Experiment 1 demonstrated that lungs with viral infections of 5 days duration and tested 6 hours after inhalation contained many more bacteria than the normal. Experiment 2 revealed that significantly fewer pneumococci were taken into the lung with a 5 day viral lesion than into the normal lung. Although the bacterial content of the normal lung decreased in 3 hours, Experiment 3 showed no such decrease after 3 hours in lungs with 5 day viral infection. Experiments 4 and 5 showed an increase in numbers of pneumococci 6 hours after inhalation in lungs with 5 day viral lesions.

Experiment 1 of Table IV compared lungs with 5 day and 1 day viral infections respectively 6 hours after inhalation of pneumococci and revealed many more bacteria in the 5 day viral

TABLE III

Inhaled Pneumococci in Lungs with Well Developed Viral Lesions

Experiment No.	Duration of viral infection	Time after inhalation	Plate counts																								Mean	Statistical evaluation*
	days	*hrs.*																										
1	0	6	0	0	0	4	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0						0	3.1
	5	6	TNC‡	112	169	3	98	18	269	17	1	28	17	35	1	TNC	4	2									86	
2	0	0	281	275	200	131	151	436	482	248	494	253	236	177	160	186	386	230	206	366	207	405	293	256			275	10.8
	5	0	25	17	4	21	0	3	1	3	1	56	1	46	9	20	10	12	65	3	98	30	30	2	1		20	
3	5	0	83	35	27	3	35	117	45	17	182	87	6	1	113	64	40	68	12								55	0.7
	5	3	43	715	55	17	40	TNC	12	15	52	12	4	57	2	71	6	95	18	54	34	6					80	
4	5	0	0	6	0	5	14	8	5	4	3	2	4	8	1	5	1	8	16	15							6	4.6
	5	6	45	56	23	10	TNC	8	339	519	315	576	59	451	99	489	38	35	563	87	141	366	6	511	13		220	
5	5	0	6	1	11	5	4	12	2	3	5	2	5	0	1	2	2										4	3.6
	5	6	583	186	12	261	319	940	13	1092	108	61	61	5	65	430	40	403	0	93	12	37	352	93			235	

* As in Table II.

‡ Too numerous to count.

TABLE IV

Inhaled Pneumococci in Lungs with Early Viral Infections

Experiment No.	Duration of viral infection	Time after inhalation	Plate counts																												Mean	Statistical evaluation*
	days	*hrs.*																														
1	5	6	134	62	1	464	74	0	43	282	0	0	12	260	17	230	270	0	81	220	43	110	0	31							106	3.4
	1	6	0	0	7	7	0	3	55	58	0	13	30	4	0	2	2	45	0	12	1	1	5								12	
2‡	1	0	17	21	77	28	34	43	21	6	22	50	42	30	23	18	27	55	47	26	59	45	8								33	7.7
	1	3	2	0	0	9	1	4	1	2	0	1	1	0	2	1	2	1	5	0	7	1									2	
3§‡	1	0	8	30	30	21	20	27	37	27	43	43	53	13	36	61	31	33	18	12	77	55	40								34	7.8
	1	3	1	2	11	3	7	2	1	9	3	16	0	0	3	3	4	12	0	16	3	18									6	
4‡	1	0	29	42	278	78	201	65	36	107	118	111	139	205	94	28	52	121	168	82	95	78									106	3.6
	1	3	426	34	59	108	21	24	51	140	0	1	217	14	5	12	0	0	60	131											72	
5§‡	0	0	102	35	56	94	58	125	48	56	42	111	31	93	74	76	93	44	101	168	46	38	76	47	44	88					73	2.4
	1	0	27	85	67	19	50	112	57	13	52	56	28	145	34	29	26	9	73	54	72	52	10	42	9						49	

* As in Table II.

‡ Virus diluted in 0.1 M phosphate buffer.

§ The culture of pneumococci was concentrated by centrifuging and resuspending the sediment in half the original volume.

TABLE V

Bacterial Content of Blood and Lungs of Mice with Pneumonia Due to Influenza Virus and Pneumococci

Experiment No.		Mouse No.																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	Plate counts (lung)	17	0	0	4	519	15	0	1118	0	7	384	5	8	33	1524	589	TNC*	638	217	171	0
	Plate counts (blood)	0	0	0	0	4	0	0	1	0	0	0	0	0	0	10	0	5	13	0	0	0
	Amounts of blood, *ml.*‡	0.3	0.7	0.4	0.7	0.65	0.85	0.4	0.75	0.6	0.65	0.6	0.65	0.3	0.75	0.6	0.75	0.8	0.7	0.6	0.65	0.6
2	Plate counts (lung)	104	302	0	69	719	912	196	0	2	699	545	792	331	875	0	358	2378	617	0		
	Plate counts (blood)	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
	Amounts of blood, *ml.*‡	0.35	0.5	0.4	0.4	0.1	0.8	0.65	0.3	0.85	0.35	0.55	0.55	0.6	0.45	0.5	0.55	0.3	0.65	0.5		

* Too numerous to count.

‡ Estimates.

lesion. Experiments 2, 3, and 4 demonstrated that the bacterial content of the lung with a 24 hour viral infection decreased within 3 hours after inhalation of pneumococci in the same manner as in the normal lung. Experiment 5 indicated that the number of bacteria gaining entrance to the normal lung was slightly greater than that of the lung with a 24 hour viral infection.

Inspection of the left lobe of each mouse was done at the time it was removed for determination of its bacterial content. Every lung with 5 day viral infection had complete or nearly complete consolidation since the few with lesser degrees of macroscopic change were discarded. Normal lungs or those with 1 day viral infections showed no gross lesions.

The results in Tables II, III, and IV demonstrate that pneumococci disappeared rapidly from the normal lung after inhalation but increased in the lung with a 5 day viral lesion. Also, the larger numbers in the 5 day viral lesion were rendered more striking by the fact that fewer organisms gained entrance to the viral consolidated lung than to the normal lung. The viral infection of 24 hours duration was not manifested by a macroscopic lesion and showed rapid diminution in numbers of pneumococci as did the normal lung.

Bacterial Content of the Blood of Mice with Combined Viral and Bacterial Infection of the Lung.—In the foregoing experiments, increase in the number of pneumococci in the 5 day viral lung 6 hours after inhalation suggested that the bacteria were growing in the viral lesion. However, the possibility remained that pneumococci penetrated to other parts of the body immediately after inhalation where they multiplied and caused bacteremia. On this hypothesis the bacteria recovered from the lung would be chiefly those contained in the pulmonary blood vessels. In order to decide this point, mice with 5 day viral consolidation were allowed to inhale pneumococci and 6 hours later determinations of the pneumococcal content of blood and the left lung were made in each mouse. The findings are recorded in Table V and it is apparent that practically no pneumococci were present in the blood under these circumstances and indicated that growth of bacteria took place in the viral lesion of the lung.

Viral Content of the Lung 24 Hours after Inoculation.—Although evidence existed already (9, 10) that influenza virus multiplies rapidly in the mouse lung within 24 hours after inoculation, confirmation of this fact under the particular circumstances of our experiments seemed desirable. Accordingly, mice were given a lethal dose of influenza virus intrabronchially; the left lobes of some were removed immediately, pooled, and titrated for their viral content by intratracheal inoculation of serial tenfold dilutions into mice. The left lobes of the remaining mice were removed 24 hours later, pooled, and titrated in a similar manner. The results presented in Table VI show that the virus multiplied approximately 10,000-fold in the first 24 hours after inoculation.

Early Pulmonary Lesions.—The foregoing experiments demonstrated that extensive multiplication of virus takes place within 24 hours after inoculation and yet the lung continues to have the power of rapidly reducing its content of pneumococci. Therefore, it was necessary to make a careful histologic study

to determine whether any lesions due to viral action could be detected 24 hours after inoculation. The result of this study has shown no lesion due to viral infection occurring at this time. However, some lesions were seen that were found to be due to the diluent used as a vehicle for the virus.

Macroscopically, the lungs of mice appeared normal 24 hours after intrabronchial inoculation of virus. However, in sections, many lungs showed mild degrees of acute inflammation with polymorphonuclear exudate on some portions of the bronchial[2] epithelium or in a few alveoli. There was also slight edema and infiltration of interstitial tissues. In several instances, the bronchial epithelium appeared denuded as if from erosion by the cannula.

TABLE VI

Viral Content of Mouse Lungs 24 Hours after Inoculation

Experiment No.	Time after inocuiion	No. of left lobes of mice pooled and ground	Dilutions of ground pulmonary tissue						
			10^{-1}	10^{-2}	1^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	Immediate	5	DDDD* DDD3‡	DDD3 33342	D322 110	1000 0000	D000 0000		
	24 hrs.	4			DDDD DD	DDDD DDDD	DDDD DDDD	DDDD DDD4	3222 110
2	Immediate	4		2221 10	0000 000	0000 0	0000 000		
	24 hrs.	3			DDDD DD	DDDD	DDDD D	D443 32	3221 000

* Each D represents one dead mouse.

‡ Each figure represents a single mouse and gives an estimation of the amount of consolidated pulmonary tissue found at autopsy 11 days after inoculation. 0–0; 1–1/4; 2–1/2; 3–3/4; 4–4/4.

At this same early interval after intrabronchial inoculation many of the cells of the bronchial epithelium showed a change consisting mainly in swelling and a light or vacuolated appearance of the cytoplasm particularly those portions adjacent to the nucleus. For convenience, such cells have been designated "halo cells." These cells were easily distinguished from the normal even under low power of the microscope; under oil immersion, fine granules were noted in the cytoplasm or a fine reticulated appearance similar to that seen in foam cells. The nuclei maintained their vesicular character and did not show pyknosis although frequently appearing shrunken and more deeply stained than normal. Halo cells and normally appearing cells are shown in Figs. 1 and 2.

Sections of lungs 24 hours after intrabronchial inoculation with the diluent alone (usually 0.1 M phosphate buffer) showed acute inflammatory lesions that could not be distinguished

[2] For the purposes of the present study, no distinction has been made between bronchi and bronchioles in the mouse.

from those found 24 hours after similar inoculation with virus. Also, halo cells were reproduced readily by intrabronchial inoculation not only of broth or buffer solution without virus but also by normal mouse serum or distilled water. These cells were not present immediately after inoculation but were evident 18 to 24 hours later and were nearly gone by 48 hours. This effect on the cells was reversible since necrosis did not occur 48 or 72 hours after inoculation of diluent alone.

In order to distinguish definitely between changes due to the diluent and true viral lesions, observations have been made on the latter for comparison. As mentioned above, halo cells occurring after intrabronchial administration of diluent without virus were practically gone 48 hours after inoculation and the cells appeared normal thereafter. In addition, it has been found that inhalation of fine droplets of diluent without virus did not result in halo cells or any other pulmonary lesion although inhalation of such droplets containing virus resulted in definite viral lesions. Therefore, by observing bronchial epithelium 48 to 72 hours after intrabronchial inoculation of virus and also in mice receiving virus by inhalation of fine droplets, it was possible to distinguish true viral necrosis for comparison with the transient halo cells due to the diluent alone. In this manner, it was found that the earliest manifestations of true viral necrosis detected by the methods used consisted of pyknosis or karyorrhexis of the nucleus. The nuclear membrane was lost and the finely divided chromatin of the nucleus coalesced to form large deeply staining masses. Cells undergoing viral necrosis did not have cytoplasmic changes characteristic of halo cells and were definitely distinguished from them. Lesions of epithelial cells due to viral infection are illustrated in Fig. 3.

DISCUSSION

The finding of a rapid decrease in pneumococcal content of the normal mouse lung shortly after inhalation[3] has been found to be useful in studying the effect of viral infection on resistance to bacterial pneumonia. In addition, this striking change in the pneumococcal content of the normal lung indicates the existence of a quickly acting mechanism for elimination of bacteria from the lung that must be a potent factor in natural pulmonary resistance to bacterial infection. It is known that mice may inhale pneumococci deeply into the lung and yet survive (2, 11–14). The mechanism of this survival is unknown but it appears likely that the marked decrease of pneumococci that takes place within a few hours after inhalation may reflect an important means by which the mouse resists ultimate general infection.

Previous studies and opinions concerning mechanisms by which influenza

[3] Stillman has reported (12) that pneumococci were not found in the normal mouse lung 3 hours after inhalation while in the present experiments a few of these organisms were present at 3 and usually 6 hours later. This discrepancy may be due to the fact that Stillman employed a qualitative and probably less sensitive method for detection of pneumococci in the lung.

viral infection lowers resistance to secondary bacterial infection may be summarized as follows:[4]—

In the case of swine influenza, Shope (15, 16) has suggested that the virus creates a portal of entry for *H. influenzae suis* and has an effect on pulmonary tissue rendering it a favorable medium for bacterial growth.

In experiments done before the discovery of human influenza virus, McCordock and Muckenfuss (17) studied pulmonary infections of rabbits due to the combined action of vaccine virus and bacteria. They thought that this viral infection in rabbits and possibly also a viral infection in human influenza changed the lung into a favorable culture medium for bacteria and that the bacteria were allowed to spread more widely because of dilated lymphatics.

Russian workers (14) have presented evidence that infection of mice by influenza virus lowers resistance to subsequent inhalation of droplets of pneumococci, hemolytic streptococci, or influenza bacilli. They found that this effect was accentuated by increasing the dose of virus and by increasing the time interval between inoculation of virus and inhalation of bacteria. A viral effect was thought to occur before the appearance of viral lesions but it is not clear that allowance was made for the development of the viral lesion during the time of observation of mice for bacterial infection. Administration of bacteria subcutaneously, intravenously, or intraperitoneally also showed the effect of the viral infection of mice in lowering resistance. *In vitro* studies did not show that the virus had any direct effect on bacterial growth.

Because destruction of the bronchial epithelium occurs both in human influenza and in experimental infections with influenza virus, Burnet and Clark (18) reasoned that interference with ciliary action of the mucosa results in retention of bacteria which grow in the inflammatory viral lesion. The importance of ciliary action in this connection has been discussed by O'Hara (19).

Robertson (20) has postulated that human influenza affects resistance to bacterial pneumonia not only by damaging the lung but also in a manner similar to upper respiratory illnesses. It is thought that upper respiratory secretions passing the barrier of the epiglottis may initiate the pneumonic process in the same way that pneumococci suspended in mucin cause pneumonia when injected into the lungs of experimental animals (21, 22). Knowledge concerning minor respiratory infection in the pathogenesis of pneumonia has been reviewed by Heffron (23).

Francis and de Torregrosa (24) have shown that influenza bacilli superimposed upon influenza viral infection of mice are more apt to cause fatal infection if inoculated after the viral infection has become established. Also, influenza bacilli may increase in virulence by passage in mice infected with the virus.

By intranasal inoculation of monkeys with influenza virus and hemolytic streptococci, Ohio investigators (25–27) have presented evidence indicating that the virus alone induces an inapparent infection resulting in a granulocytopenia and impairment of the phagocytic power of the polymorphonuclear leucocytes as judged by

[4] Without consideration of the important problem as to whether bacterial pneumonia affects viral infection of the lung. Experiments bearing on this problem have already been reported in the preceding paper (2).

lowering of the opsonocytophagic index. Impairment of the phagocytic mechanism by viral infection renders monkeys more susceptible to subsequent inoculation with hemolytic streptococci. Furthermore, viral infection was found to interfere with output of antistreptococcal antibodies. These authors have suggested also that damage to epithelial barriers may be an additional factor in the effect of viral infection on subsequent bacterial infection.

Carlisle and Hudson (28–30) found mice most susceptible to superimposed hemolytic streptococcal infection if intranasal inoculation of these bacteria was done 4, 8, or 12 days after infection with the virus. These authors consider the viral effect a local one in the lung since reduction in bacterial content of the lung was not as rapid in viral mice as in controls and because no viral effect on bacterial infection was discerned if the bacteria were given intraperitoneally or intravenously.

Recently, Volkert, Pierce, Horsfall, and Dubos (31) have shown that intraperitoneal inoculation of tubercle bacilli into mice results in more severe pulmonary lesions of tuberculosis if the mice have a preceding or concomitant infection with influenza virus or pneumonia virus of mice (PVM). Furthermore, small doses of these viruses result in such mild infection that viral lesions are minimal or absent, yet the potentiation of pulmonary tuberculous infection is still demonstrable. The authors suggest that in some manner infection of the lung with these viruses makes conditions more favorable for bacterial growth and for the development of tuberculous lesions.

The experiments reported in the present study demonstrate that the power of the normal lung to reduce rapidly its content of inhaled pneumococci is not lost simply through the presence and multiplication of influenza virus in the pulmonary tissue. On the other hand, lungs with fully developed lesions of viral pneumonia are capable of supporting the growth of inhaled pneumococci.

It will be apparent from the foregoing survey of the literature that several authors (15–18, 31) have suggested that the action of influenza virus is to change the lung in such a way that it becomes a favorable culture medium for bacteria. Our experiments offer evidence that this change actually takes place with the experimental conditions under study.

The findings also show that the action of the virus is through its damage to the tissue of the host and confirm the generally held concept that respiratory viruses potentiate bacterial infection by interference with mechanisms of defence. In accordance with this viewpoint, it seems probable also that the rapid eliminatory mechanism of the normal lung referred to above is destroyed by the viral lesion. However, there is a possibility that bacterial growth in the viral lesion has merely overshadowed this immediate reducing mechanism.

The concept that lowering of resistance to inhaled pneumococci is due to the lesion resulting from viral action suggests an explanation for several observations of others. Experiments indicating that resistance is lowered to the greatest extent after 2 or more days following the administration of virus (14, 24, 28, 29) may well be explained by the fact that gross and microscopic changes due to viral action take that much time to develop. Likewise, the observation

that severe viral infection due to large inocula (14, 31, 32) or to particularly virulent strains (33, 34) causes a greater susceptibility to secondary bacterial infection may be due to the fact that such severe infections cause more definite anatomical lesions in the lung.

A report has been made (30) indicating a pulmonary localization of viral action in lowering resistance of the mouse to secondary bacterial infection. Our experiments furnish additional evidence of this pulmonary action of the virus by showing the association of the viral lesion with lowered resistance to pneumococci and by the finding of few or no pneumococci in the blood of mice with pneumococcal infection superimposed on viral pneumonia.

There is difficulty in reconciling the finding of potentiation of pulmonary tuberculosis in mice by viral infection so mild as to produce few or no lesions (31). However, it may by pointed out that microscopic viral lesions apparently were not sought in these mild viral infections and that small lesions were associated with lesser degrees of potentiation of tuberculous infection. In contrast, absence of influenza viral lesions in our experiments was confirmed by histologic study and was associated with severe infection with many lethal doses of virus. Nevertheless, it seems probable that different mechanisms are being studied in these two experimental situations. Although both types of experiment involve bacterial infection of the mouse lung in conjunction with pneumotropic viral infection, the kinds of bacteria, routes of inoculation, and times of observation differ markedly.

The association of deranged natural resistance to pneumococci with visible viral alterations in pulmonary tissue in our experiments does not imply that all changes due to respiratory viruses resulting in lowered resistance to bacterial infection in man or other animals must always be visible. For example, the results with tubercle bacilli and pneumotropic viruses cited above may have been due to physiologic effects not reflected in viral pneumonia as suggested by the authors. Nevertheless, the association of visible lesions with decreased resistance may be of value in the further investigation of these mechanisms in the mouse since histologic study of the viral lesion may be expected to yield clews as to what particular structures and antibacterial mechanisms may be involved in the lowered resistance.

Finally, it is necessary to assess the significance of the lesions observed 24 hours after intrabronchial inoculation of viral suspensions. Descriptions in the literature of influenza viral lesions in mice after intranasal inoculation have indicated that some changes occur as early as 24 hours after inoculation (35–39). Alterations in bronchial epithelium somewhat similar to halo cells have been noted also (35, 36, 38). In addition, control inoculations of diluent alone (35, 39), suspensions of normal pulmonary tissues (37, 39), or virus suspension mixed with antiserum (37, 39) have produced mild lesions. The evidence given in the present report makes it possible to distinguish between lesions due to

diluents used for suspending the virus and changes due to viral action itself since the microscopic changes due to diluents alone could be compared with lesions due to viral infection alone. Histologic study of lungs 24 hours after inoculation utilizing these criteria has indicated no lesion due to viral action but showed that lesions observed were due to the diluent.

Examination of the data suggests that these non-viral lesions may have interfered to a slight extent with the number of bacteria gaining entrance to the lung (Experiment 5, Table IV) and also may have lessened to some degree the resistance of the normal lung (Experiment 4, Table IV). Whether or not these mild lesions due to the diluents had any influence at all on the results, it is apparent that the bacterial content of the lung diminished rapidly in spite of them.

It is important to distinguish the halo cells in our experiments from the lightly staining hypertrophic cells described as an early manifestation of a number of other viral infections (40). In the present case, halo cells were shown to be transient effects of the diluents and could be distinguished from influenza viral necrosis appearing later. The reproduction of halo cells not only with the usual diluents but also with distilled water suggests that this phenomenon may be due to an osmotic effect (41, 42). This concept would help to explain the absence of this change when the fluids were administered by inhalation of fine droplets since it is probable that evaporation of the aqueous portion of the droplet takes place before contact with the bronchial epithelium. In addition, cells resembling halo cells have been produced in the renal tubules of rats by treatment with desoxycorticosterone and sodium chloride (43). In this circumstance, an osmotic effect may also be concerned.

SUMMARY

1. The normal lung of the mouse possesses the power of reducing markedly its content of Type I pneumococci within 3 hours after inhalation of the organisms in the form of fine droplets.

2. Lungs with fully developed influenza viral pneumonia not only fail to reduce the pulmonary content of pneumococci administered in this manner but, on the contrary, support their growth.

3. After intrabronchial inoculation into mice, influenza virus multiplies rapidly in the lung within 24 hours.

4. Criteria have been established for distinction between true viral lesions of the lung and changes due to the inoculation of diluents as vehicles for the virus.

5. 24 hours after inoculation of virus, there are no macroscopic lesions in the lung and the microscopic changes are due to the diluent.

6. Presence and multiplication of the virus in the lung 24 hours after inoculation have no apparent effect on the power of the lung to reduce rapidly its content of inhaled pneumococci.

7. The effect of the virus in lowering resistance to secondary bacterial infection appears to be due to the presence of the lesion produced by the virus.

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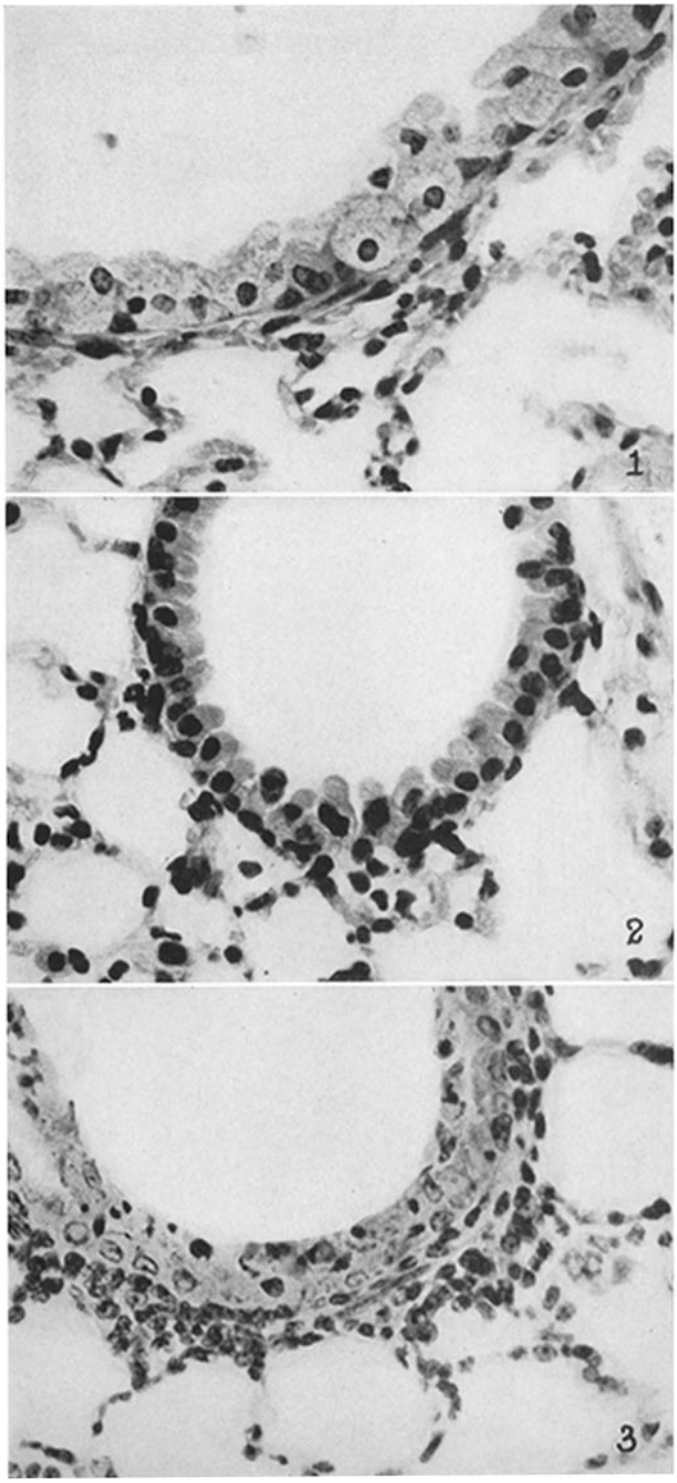
EXPLANATION OF PLATE 4

Sections were stained with hematoxylin and eosin and photographed by Mr. Cramer Lewis.

FIG. 1. Halo cells of bronchial epithelium 18 hours after intrabronchial inoculation of tryptose phosphate broth without virus. × 400.

FIG. 2. Normally appearing bronchial epithelium 6 hours after intrabronchial inoculation of a lethal dose of virus in broth. × 400.

FIG. 3. True viral necrosis of bronchial epithelium 48 hours after intrabronchial inoculation of a lethal dose of virus in broth. × 400.



(Harford et al.: Viral lesion and pneumococci)