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Infection of Pigeons by Airborne Venezuelan Equine Encephalitis Virus

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INTRODUCTION

The experiments described were designed to provide information on the infectivity of Venezuelan equine encephalitis (VEE) virus for birds by the respiratory route. Laboratory studies by Chamberlain (2) showed that wild birds, including pigeons, could be infected with VEE virus by mosquito bite or by subcutaneous (sc) injection of virus. Overt signs of disease were absent in avian hosts, but viremia was produced for periods of 1 or 2 days, followed by the appearance of specific serum-neutralizing (SN) antibodies. The respiratory route of infection with VEE virus has been suggested previously. In noting that virus occurs in the nasopharyngeal washings of infected humans, Olitsky and Casals (10) recognized a potential for epidemics without insect vectors. Perhaps the most striking evidence of invasiveness of VEE virus by the respiratory route was provided by Slepushkin (11), who reported on infections in a large group of laboratory personnel after exposure to aerosols produced by breakage of a vial of virus. The susceptibility of birds to infections by the respiratory route had not been investigated. However, the possibility that the respiratory route was involved with arboviruses in nature was suggested by Holden (6) in studies with pheasants and eastern equine encephalitis (EEE) virus. Other cases of contact infection among birds by EEE and western equine encephalitis viruses were reviewed by Bourke (1).

COMPARATIVE SUSCEPTIBILITY OF FOWL TO AEROSOLS OF VEE VIRUS

The selection of an avian host for the subsequent studies of response to static aerosols of VEE virus was preceded by screening a number of species of fowl. Birds were exposed for 1 min to aerosols of the Trinidad strain of VEE virus. The particle size distribution of the clouds was characterized by a mass median diameter of 1.5 to 2.5 μ and a slope of 3.5 probits per log diameter. Those values indicated that about 60% of the cloud mass was in particles between 1 and 3 μ in diameter. After exposure, the birds were isolated by dosage group in gas-tight cabinets and bled daily for viremia determinations. Serum neutralization tests were conducted on host sera collected before exposure and 21 days after exposure.

Included in the host range were leghorn chickens that had previously been shown to respond to an intravenous dose of <10 mouse intracerebral LD_{50} ($MICLD_{50}$) units. However, these birds, like ring-necked pheasants, hybrid chickens, and Peking ducks, were resistant to doses of 2,500 to 10,000 $MICLD_{50}$ inhaled. By contrast, White Carneau pigeons (3) proved susceptible. A viremic and serological response was obtained in 60 to 80% of the birds tested at an inhaled dose as low as 374 $MICLD_{50}$ (9). The marked cut-off in response below this level and high percentage infected with higher doses are illustrated in Table 1, with data taken from a number of experiments. All aerosol exposures were for 1 min only.

In general, viremic levels ranged as high as 10^6 $MICLD_{50}$ per ml of blood with no obvious dependence upon dose, once a minimal infective dose was given. The duration of viremia averaged 3 days, beginning on the 1st or 2nd day after exposure. VEE virus infections in pigeons, as in other birds (2), did not result in apparent illness or in histopathology. Such histopathological evidence of disease as did appear was usually ascribed by the pathologists to other causes, including agents of pneumonia, trichomoniasis, and bacterial meningitis, but even these were rare. During the course of these studies, no hypersensitivity was detected in previously exposed birds. Tests were

TABLE 1. *Response of White Carneau pigeons to respiratory doses of VEE virus presented in 1 min*[a]

Dose ($MICLD_{50}$ inhaled)	No. of birds viremic (positive/total)	Serological response (no. of birds showing positive SN indices/ no. of birds tested)
3,770	4/5	4/5
2,291	6/9	8/9
1,960	5/8	4/6
1,349	5/8	5/7
589	5/6	3/3
374	7/8	6/7
135	0/8	0/6
76[b]	0/8	0/8
51[b]	0/5	0/5
Controls	0/24	0/24

[a] Aerosol conditions: 80% relative humidity at 80 F (26.7 C).

[b] Doses estimated by extrapolation from cloud concentrations at earlier cloud ages.

conducted for delayed skin reactions after injection of VEE virus antigen into the margin of the eye.

Almost without exception, the detection of viremia in pigeons over a 2-day period was followed by a significant rise in the titer of SN antibodies (>1.0 log increase in SN index). These same animals were resistant to respiratory challenge 3 weeks after the original exposure. Table 2 illustrates a typical set of responses to an initial and to a challenge dose of VEE virus. Although not shown, control studies in which nonresponders were later challenged indicated susceptibility indistinguishable from that of normal birds.

COMPARISON OF RESPONSES AFTER INHALATION AND INJECTION OF VEE VIRUS

The objective of one series of experiments was to compare the responses of pigeons to respiratory and subcutaneous doses of VEE virus (8). The respiratory dose was 1,349 $MICLD_{50}$ inhaled; the subcutaneous dose was 506 $MICLD_{50}$. The results of the experiment are summarized in Table 3. Viremic responses are presented as a function of day after dosage. Birds receiving virus by the respiratory route were not tested beyond day 4 because of previous data indicating that viremias normally terminated prior to that time. No data were available to indicate the duration of viremia after dosage by the sc route. Therefore, blood samples were collected and assessed for VEE virus on each of 10 successive days after injection.

Among eight pigeons receiving 1,349 $MICLD_{50}$ by the respiratory route, approximately 75% exhibited viremias that first became evident in blood samples collected on the 1st or 2nd day after exposure. In the group injected with 506 $MICLD_{50}$ sc, viremias occurred in all eight birds, and virus was uniformly found in the blood on the 1st day after injection. However, the more rapid response of the injected birds was not obtained with a smaller dose. At a dose of about 5 $MICLD_{50}$, by the sc route, only 50% were found to be viremic, and this condition first occurred from 1 to 2 days after injection. Thus, as the minimal infective dose was approached by each route, the characteristics of viremias were indistinguishable.

The similarity of the serological responses after infection by each route is illustrated in Fig. 1. Apparently, once an infection was established, the rate and extent of appearance of SN antibodies was independent of the route by which the virus entered.

An additional criterion for the comparison of responses by each route of infection was the detection of virus in the cloaca and in the oral cavity. These tests were considered to be qualitative only because of the frequent occurrence of low titers that could not be reliably confirmed. However, with both the sc and respiratory groups, it was possible during the period of viremia to isolate and confirm the presence of VEE virus in the oral cavity, but not in the cloaca. Subsequent attempts to isolate virus on days 42, 43, 80, and 81 after infection was initiated were unsuccessful. These results add to the concept of a subclinical, but immunizing, type of infection.

BIRD-TO-BIRD TRANSMISSION OF VIRUS

It appeared logical to investigate cross-infections between birds, because both the data on respiratory susceptibility and the demonstration of virus in the oral cavity suggested the possibility of contact infections.

A device was fabricated to provide passage of air from viremic birds to normal animals (8). Two boxes of about 3-ft^3 capacity were interconnected by a 3-inch duct through which air flowed at about 12 liters per min. To insure that arthropods would not pass from infected birds in one box to normal birds in a second, a 60-mesh screen was placed in the duct.

Six birds were infected by head exposure to static aerosols of VEE virus. These animals were placed in one box, and six normal birds were placed in the second. The birds remained in the enclosures for 3 weeks, except for short periods during the first 10 days when blood samples were collected daily. One of six normal birds developed specific viremia on days 9 and 10 of the 10-day test period. Considering that viremia and oral

TABLE 2. *Responses of White Carneau pigeons to VEE virus administered by the respiratory route and subsequently challenged with virus by the same route*[a]

Dose ($MICLD_{50}$) with 95% confidence limits	Bird no.	Viremia (log $MICLD_{50}$ per ml of blood)				SN[b]		Challenge[c] dose ($MICLD_{50}$) with 95% confidence limits	Viremia (log $MICLD_{50}$) per ml of blood)				SN[b] post-exposure
		1 day	2 days	3 days	4 days	Pre-exposure	Post-exposure		1 day	2 days	3 days	4 days	
3,715 inhaled in 1 min (2,344–5,888)	35	>3.5	>3.5	3.5	<1.5	0.1	2.0	3,379 inhaled in 1 min (2,361–4,781)	<1.5	<1.5	<1.5	<1.5	2.5
	28	<1.5	>3.5	>3.5	<1.5	0.6	2.0		<1.5	<1.5	<1.5	<1.5	1.8
	63	<1.5	>3.5	>3.5	<1.5	0.4	2.9		<1.5	<1.5	<1.5	<1.5	2.5
	40	<1.5	2.7	2.8	<1.5	−0.6	2.2		<1.5	<1.5	<1.5	<1.5	2.9
None (controls)	45	<1.5	<1.5	<1.5	<1.5	−0.2	0.4		3.3	3.1	2.3	<1.5	2.9
	38	<1.5	<1.5	<1.5	<1.5	1.0	0.7		2.9	2.9	2.5	<1.5	2.3
	34	<1.5	<1.5	<1.5	<1.5	−0.5	0.8		1.8	3.2	2.6	<1.5	2.2
	50	<1.5	<1.5	<1.5	<1.5	0.4	0.1		<1.5	3.5	2.5	<1.5	1.9
	62	<1.5	<1.5	<1.5	<1.5	0.5	0.1		<1.5	<1.5	2.4	3.0	1.1

[a] Aerosol conditions: 80% relative humidity at 80 F.
[b] Log units of virus neutralized.
[c] Birds were challenged 21 days after the original exposure.

TABLE 3. *Response of pigeons to respiratory and subcutaneous doses of VEE virus*

Day post-infection	Respiratory group[a]		Injected group[b]	
	Viremic (no. positive/total)	Confirmed oral swab isolate (no. positive/total)	Viremic (no. positive/total)	Confirmed oral swab isolate (no. positive/total)
1	3/8		8/8	
2	5/8		6/8	
3	5/8	1/8	6/8	
4	2/8		2/8	
5	No data		1/7	1/7
7–10	No data		0/7	

[a] The respiratory group inhaled 1,349 $MICLD_{50}$ in 1 min.

[b] The injected group received 506 $MICLD_{50}$ subcutaneously.

virus were apparent among exposed birds, the potential of cross-infection among pigeons appeared to be low. It might be noted that additional opportunities to detect cross-infections were afforded by placing normal animals in holding cabinets with viremic hosts. All such tests were negative.

EFFECT OF EXPOSURE TIME ON RESPONSE TO INFECTION

One possible explanation for the lack of cross-infections was an effect of exposure time, i.e., that dose-response data from a 1 - min exposure could not be extrapolated for the interpretation of effects when the same doses were given over extended periods of time. One might assume in this case that the passage of virus through the duct mechanism would not be in numbers equal to the minimal infective dose per minute that was noted in controlled aerosol trials. If, then, one were to postulate that infection does not occur unless a specific *rate* of exposure is achieved, regardless of the total dose presented, the lack of cross-infections could be explained. A system was developed (8) to permit exposure of a group of pigeons to aerosols at a dose rate less than the minimal infective dose per minute. With prolonged exposure, however, a total dose far in excess of the minimal infective dose could be inhaled. The scheme employed is illustrated in Fig. 2. After dissemination of virus, the aerosol chamber was mechanically purged for a period of 20 min. The remaining aerosol was then assessed, and subsequently was allowed to undergo biological decay until the concentration approached the minimal level for estimation of viral content. At this time, the aerosol was again sampled for estimation of viral concentration. Further cloud aging occurred to the extent necessary to yield desired doses. The level of infective virus during exposures was estimated by extrapolation from the line established by the two assays. Justification for the procedure was given by earlier work which indicated that biological decay was linear, and respiratory infectivity of VEE virus, for guinea pigs, was consistent over the cloud ages of interest to this study.

The results of five experiments in which birds were exposed to aerosols for extended periods of time are presented in Table 4. Periods of exposure, total inhaled doses, and doses in the first minute of exposure were varied. In four of five experiments, birds were given total doses over periods of 25 to 180 min that far exceeded the infective doses discussed previously for 1-min exposures. In only one experiment did pigeons generally respond with viremia and production of SN antibodies. In that test, 6,037 $MICLD_{50}$ were inhaled over 60 min, but, more important, the dose in the 1st min of exposure was 304 $MICLD_{50}$ inhaled. This value was within the minimal infective dose range established in 1-min exposure trials. Note that in the test where the 1st min dose was 124 $MICLD_{50}$ inhaled, about three times the minimal infective dose was accumulated in the first 5 min of exposure. By the end of 60 min in that trial, 10 to 20

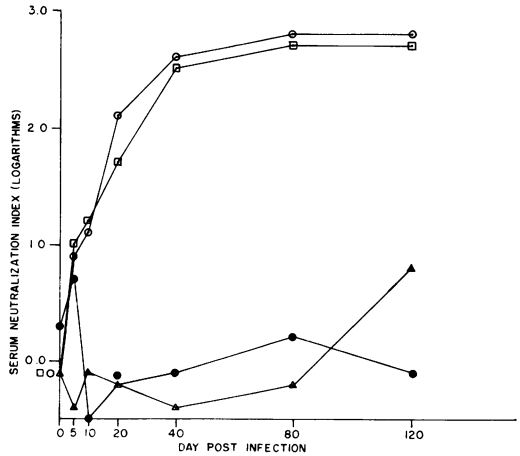

FIG. 1. *Results of neutralization tests against Venezuelan equine encephalitis virus by sera collected from White Carneau pigeons at various periods after injection. Symbols:* ○ = *birds developed viremia after respiratory dosage;* △ = *birds did not develop viremia after respiratory dosage;* □ = *birds developed viremia after dosage by the subcutaneous route;* ● = *birds served as environmental controls and were not viremic. The open square and circle to the left of the vertical axis should be read as being superimposed on the triangle on that axis.*

times the minimal infective dose had been accumulated, and yet viremias and SN antibodies did not occur. It thus appeared from these data that infection was dependent upon rate of exposure and not total dose.

It was of interest to test pigeons for effects of extended exposure in terms of response to subsequent challenge. Accordingly, the dose-response curve was re-estimated by exposure of birds that had not shown viremia or neutralizing antibody formation after exposure to a total dose of 2,934 $MICLD_{50}$ inhaled over a 60-min period. The results indicated that the previous experience with virus had no detectable influence on the subsequent disease response to infective doses. Birds were viremic after an inhaled dose of 589 $MICLD_{50}$ and, as expected, failed to respond to 19 $MICLD_{50}$.

EFFECT OF ANTIMICROBIAL DRUGS ON SUSCEPTIBILITY

It is of interest, both epidemiologically and academically, to detect mechanisms that alter the normal dose-response relationship of virus and host. More specifically, it was of interest to detect factors which altered the rate process indicated above. Such a mechanism was suspected when a group of birds responded with viremia and formation of neutralizing antibodies to what appeared to be an abnormally low dose. This group had been given a supplement of Cosa-terramycin (oxytetracycline with glucosamine and vitamins; Chas. Pfizer and Co., Inc., New York, N.Y.) and HepZide (nithiazide; Merck and Co., Inc., Rahway, N.J.) in the feed for 2 weeks prior to the test. To test for a possible relationship between the drugs and susceptibility to VEE virus, 15 birds were held for 2 weeks without supplement, 10 birds received Cosa-terramycin in the drinking water at a dosage of 400 mg per gal of water, and 5 birds received Cosa-terramycin plus HepZide at a dosage of 800 mg per gal for 2 weeks. All birds were then exposed to a total inhaled dose of 2,934 $MICLD_{50}$ units over a 60-min period. The highest dose per min was 22 $MICLD_{50}$ units inhaled, or about one-tenth the usual infective dose. Of the untreated birds, 13% developed viremia and neutralizing antibodies, 40% of the Cosa-terramycin-treated birds responded, and 60% of the birds receiving Cosa-terramycin plus HepZide responded. The results of a second test in which oxytetracycline alone was included in the drinking water (200 mg per gal) of birds for 2 weeks prior to exposure to VEE virus aerosols gave different results. There was no viremic response among eight birds to total doses of 668 $MICLD_{50}$ inhaled over 60 min with 1st-min doses of 27 $MICLD_{50}$ inhaled. In this case, susceptibility was not increased by antibiotic treatment. It is possible that the effects noted here are complex and will require extensive investigation for a full definition.

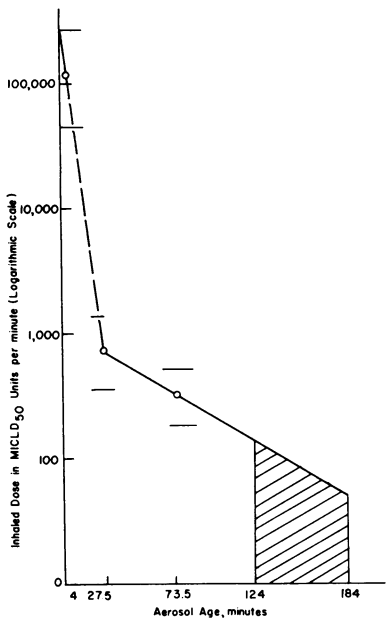

FIG. 2. *Aerosol concentration of Venezuelan equine encephalitis virus as a function of age. Concentrations are in terms of doses per minute for White Carneau pigeons expressed as $MICLD_{50}$ units. The open circles indicate aerosol concentrations estimated by sampling. The solid line indicates the periods of natural cloud total decay; the dotted line indicates a period of mechanical purging. The hatched area illustrates the dosage inhaled by pigeons during a 60-min exposure. Bands above and below the open circles indicate 95% confidence limits.*

TABLE 4. *Responses of White Carneau pigeons to respiratory doses of VEE virus presented over extended periods*[a]

Total dose ($MICLD_{50}$) inhaled)	Maximal dose per min	No. viremic (no. positive/ total)	Serological response (positive/ total)
1.7 in 60 min	Minute	0/5	0/4
1,236 in 180 min	51	1/5	0/4
2,934 in 60 min	22	2/15	2/15
4,544 in 60 min	124	0/8	0/6
6,037 in 25 min	304	5/7	5/7

[a] Aerosol conditions: 80% relative humidity at 80 F.

CONCLUSIONS AND DISCUSSION

The principal findings of these studies may be summarized as follows.

(i) VEE virus can infect avian hosts through the lower respiratory tract, although marked species differences occur. The minimal infective dose for White Carneau pigeons was between 135 and 374 $MICLD_{50}$ units inhaled in not more than a 1-min period. Infection was characterized by viremia over a 2- to 3-day period, virus in the oral cavity during the viremic period, and production of neutralizing and protective antibodies. Far higher concentrations of virus were usually tolerated without viremic or serological response if inhaled at a rate less than 374 $MICLD_{50}$ per min. The full potential of this resistance mechanism in terms of duration of effectiveness is not known. This phenomenon, however, is possibly involved in natural resistance to cross-infections between birds. Further, the possible implication of the rate process for successful aerogenic immunization should not be overlooked.

(ii) Treatment of pigeons with Cosa-terramycin and HepZide, or Cosa-terramycin alone for 2 weeks prior to exposure to viral aerosols altered a normal resistance mechanism associated with respiratory challenge. With such treatment, birds became susceptible to a dosage rate 1 log lower than that normally seen.

(iii) Subcutaneous injection of 5 $MICLD_{50}$ of virus into pigeons resulted in infections which could not be distinguished from those which followed respiratory exposure to 374 $MICLD_{50}$ inhaled. Comparisons were based on level and duration of viremia, level and duration of neutralizing antibodies, and occurrence of virus in the oral cavity. In view of the similarity of responses, it is reasonable to assume that the sites of infection were the same regardless of route. Thus, the difference in minimal infective doses was not a function of requirements of the infection sites, but, rather, a function of factors which inhibit arrival at such sites.

A fraction of the difference was due to incomplete retention in the respiratory system. On the basis of data presented by Hatch and Gross (5) for mammals, the particle size range employed in these studies would yield retention of 25 to 50% of inhaled particles in the lower respiratory system or about 100 to 200 $MICLD_{50}$. The precise amount of inhaled dose which was retained, however, is not known. Hatch and Gross point out a number of factors that affect retention of aerosol particles, including tidal volume, breathing frequency, particle size, and species of host. These variables have been controlled to the maximal extent in our aerosol studies to permit valid, though relative, estimates of treatment relationships. The *precise* effects of each factor on retention in pigeons are not known, however, and thus comparisons of responses by route have limitations.

A component of the remaining difference in effective doses by the two routes would appear to be nonspecific resistance associated with the respiratory system. Resistance of the pigeon against VEE virus by the respiratory route was considered from the standpoint of virus-induced and host-induced mechanisms. In the case of the former, autointerference was a distinct possibility because of the test procedures. Where graded doses, as in dose-response studies or in extended exposure time trials, are achieved by cloud aging, the aerosols will contain increasing proportions of dead virus. Thus, effects of decreasing dosages which might be ascribed to the host could be due to interference, because the percentage of inactive virus increases with decreasing amounts of active virus. This was not a problem, however, as shown by a study in which birds were given a large dose of inactive virus in aerosol form. Following this procedure, 1-min exposures were made to viral aerosols at two concentrations for dose-response estimation. The birds responded to 513 but not to 19 $MICLD_{50}$ inhaled, suggesting that inactive virus was ineffective in preventing infection.

The host-associated mechanisms of nonspecific resistance are not known, but might include phagocytosis, antiviral substances present in the lower respiratory tract (4), or physical removal by the proteinaceous fluid film of the alveolar membrane and the mucous blanket which begins in the respiratory bronchioles (5). Whatever the system, it must be compatible with the rapid rate of viral inhibition indicated in these experiments.

The mechanism associated with the reduction of the nonspecific resistance rate by drugs is also not clear. One may postulate direct antagonism of the drugs towards a protective substance or mechanism, or a withdrawal of a substance or mechanism due to the presence of effective drugs. One interesting possibility is that the antibiotic may eliminate gram-negative endotoxin producers of the intestinal tract. According to Ho (7) and Stinebring and Youngner (12), endotoxins induce the formation of interferon or cause the release of preformed interferon in rabbits and mice, and thus may indirectly affect general resistance of the host to viruses. A similar and additive effect could be attributed to nithiazide in those birds in which trichomonads existed and possibly stimulated nonspecific viral inhibitors. In brief, the drugs employed may have eliminated organisms or their products which induced or released active interferon. This proposal is purely speculative, however, and must be examined experimentally.

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Discussion

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Dr. Miller has presented some intriguing observations on the response of white Carneau pigeons to airborne Venezuelan equine encephalitis (VEE) virus.

Infection by subcutaneous and by respiratory inoculation was benign. Not surprising are the relatively brief viremias, prompt antibody responses, and resistance to reinfection.

Despite demonstration of virus in the oral cavity of infected birds, airborne bird-to-bird transmission was demonstrated to be a rare occurrence, perhaps best explained by the size of the minimal airborne infecting dose and the poor aerosol-generating capacity of the bird itself.

The response of the pigeons to graded acute doses of airborne VEE is quite unlike that of mammals exposed to the virulent virus. It does resemble, in some respects, the responses of mice and of monkeys to airborne attenuated VEE (1). In rhesus monkeys, an abrupt threshold of infection is manifest at approximately 1,000 guinea pig intraperitoneal 50% infectious doses ($GPIPID_{50}$), with no infections occurring below this point, and consistent infection above this level. This, to a degree, is comparable to the abrupt cutoff in the pigeons at a level of approximately 374 $MICLD_{50}$. On the other hand, the continuing partial response in groups of birds at doses ranging up to 10 times this dose is remarkably similar to the partial response of mice over a 2-log range of exposure to the attenuated virus.

To me, the most intriguing observations reported are the resistance of the pigeons to infections when exposed to large doses of virus presented at rates less than one ID_{50} per minute. No parallel in mammals is known to the author. Indeed, in mice exposed to virulent VEE at the rate of 20 $MICLD_{50}$ per minute (2), the respiratory LD_{50} was 27 $MICLD_{50}$ presented, a value in consonance with those obtained in short exposure times.

Additional data obtained by Miller, but not presented in his paper, substantiate the validity of extrapolation of the linear decay of VEE in the system used. It does not appear reasonable to challenge the validity of the dose estimation in these studies.

A slightly different type of experiment might eliminate some factors inherent in the studies described. The role of decaying, as against dead, virus might be eliminated from consideration if the doses were presented at the same rates with a