is an upper respiratory disease. Experimentally produced upper respiratory illness with this virus has been achieved by nasopharyngeal inoculation (3), by deposition of virus on selected sites in the upper respiratory tract, and by inhalation of large particle aerosols (4), which are primarily deposited in the upper respiratory tract.

In a series of experiments, Buckland and his associates circumvented the problem of precise location of deposition of airborne particulates by direct application of coxsackievirus A-21 to specific locations in the upper respiratory tract. Their findings showed the nasal mucosa to be exquisitely susceptible to infection, whereas the oropharynx and nasopharynx were refractory to doses several orders of magnitude greater. In subsequent studies, volunteers were infected with doses comparable to those directly instilled when presented in relatively large airborne particles, virtually all of which might be expected to be deposited on the nasal mucosa. These authors concluded that only particles retained in the upper respiratory tract are of significance in transmission of naturally occurring disease.

In attributing production of upper respiratory disease to the small particles generated with the Collison atomizer, Gerone and his associates have not rigorously excluded the contribution of that portion of the particles larger than 2 μ, which might be expected to be retained in the upper respiratory tract. From analysis of the particle size spectrum of the aerosol, approximately one-fifteenth the dose presented might be so retained (5). This may well be a significant quantity of virus, of itself capable of initiating infection.

Further experimentation, either by use of aerosols whose upper respiratory retention is negligible, or by bypassing the upper respiratory tract via an artificial airway, are needed if this matter is to be definitively resolved.

Most disappointing to this reviewer is the lack of information presented upon the airborne stability of coxsackievirus A-21 under varying conditions of relative humidity and temperature. The observations of Buckland and his associates indicate a biological decay rate of 50% per min for virus in small particles and roughly 25% per min in the larger particles, if decay is linear. Such values are compatible with droplet infection. Far greater airborne stability is required for significant airborne transmission under ordinary conditions. Valuable information could be obtained by sequential examination of static aerosols with slit samplers or impingers.

In summary, the authors have described an aerosol used to induce infection in man. This discussant believes that further, more critical examination is required to definitively establish the significance of deep respiratory deposition of small particles in production of upper respiratory disease, and hence the appropriateness of the model for the study of naturally acquired infection. It is hoped that further studies will clarify this. Similarly, improvements in high-volume sampling, combined with knowlege of airborne stability of this virus, will permit more critical evaluation of the role of airborne dissemination in coxsackievirus A-21 upper respiratory disease.

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Author's Comments on the Discussion

P. J. GERONE, R. B. COUCH, R. G. DOUGLAS, AND V. KNIGHT

In Col. Gochenour's discussion of our paper, several points were made with which we are in complete agreement. Other issues were raised, however, regarding which we would like to clarify the position or the conclusions that have been reached.

In regard to the large volume air sampler (LVS), the discussant has outlined its limitations as being (i) excessive evaporative loss of collecting fluid, (ii) inability to estimate particle size distribution, and (iii) its failure to "meaningfully quantitate airborne virus." The loss of fluid by evaporation in the LVS has not been a serious problem. When known concentrations of coxsackievirus A-21 were added to the collecting medium and circulated through the LVS for periods three times longer than the sampling periods described in the paper, no virus loss occurred. On the contrary, when low-concentration aerosols were sampled, the reduced volume of fluid that had to be tested served as an advantage.

The LVS was not designed to measure particle sizes of the aerosols it samples; furthermore, no other equipment is available which can analyze particle size and, yet, handle these large volumes of air.

Under the conditions in which the LVS was tested, it was found to be a quantitative sampler. The data in the last figure of the paper were replotted (Fig. 1a) to show the relationship between virus concentration recovered and virus concentration in the room air. It is readily apparent not only that a relationship does exist, but that there is a direct proportionality between the amount of airborne virus in the room and the quantities recovered in the LVS.

The studies comparing the LVS and all glass impinger (AGI; Table 5) raised a question in the

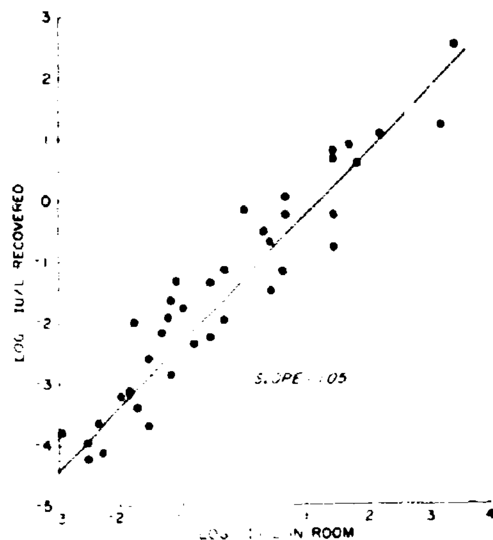

Fig. 1a. *Relationship of coxsackievirus A-21 concentrations in room air and quantities recovered by the large volume air sampler*

Table 1a. *Coxsackievirus A-21 aerosols used in volunteer inoculations*

Run no.	Dilution of inoculum pool	Suspension concn (log_{10} $TCID_{50}$ per liter)	Aerosol concn (log_{10} $TCID_{50}$ per liter)	
			Predicted	Actual
1	$10^{-4.0}$	8.0	1.74	2.08
2	$10^{-4.5}$	7.5	1.30	0 35
3	$10^{-4.5}$	7.5	1.30	1.24
4	$10^{-4.5}$	7.5	1.30	1.42
5	$10^{-4.5}$	7.5	1.30	1.42
6	$10^{-4.6}$	7.4	1.22	1.11

discussant's mind regarding the variability seen in virus recoveries and the consistency demonstrated in the fluorescein recoveries. It should be stated that two variables were present in the virus determinations which were not present in the fluorescein assays. These are: (i) the biological inactivation of the virus and (ii) the sensitivity of the cell cultures used in the assay procedure. The four virus recovery values with the LVS in actual $TCID_{50}$ ranged from 4.6 to 5.1 log_{10}, and are consistent with the 0.25 log_{10} standard deviation of the assay procedure. This standard deviation, however, cannot be applied to the virus values obtained with the AGI, because those end points were calculated by the Fischer-Yates dilution technique. Despite the limitations imposed by this assay procedure, the mean recovery in the LVS and AGI were remarkably similar.

The disc[illegible]t also questioned the predictability of do[illegible] administered to volunteers with the experimental aerosols. We agree with his enumeration of the factors which may influence predictability. Undue emphasis, however, was placed on a few points which strayed from the line shown in Fig. 1 of the manuscript. The maximal deviation between the predicted and actual determination was 1.1 log_{10}, and only 3 of the 27 points plotted on the graph (1 in 9 determinations) were more than 0.5 log_{10} from the predicted values. These results have been interpreted by the authors as representing good predictability for aerosol inoculations with *this virus*. This can be further supported by actual figures, shown in Table 1a, taken from the subsequent volunteer experiments. Five of the six predicted values were within 0.35 log_{10} from the actual determinations and four of these were within 0.15 log_{10} of the anticipated concentration.

The two main points that were established by the studies of sneezes and coughs were: (i) these expiratory events produce large numbers of small aerosol particles capable of remaining airborne for long periods of time, and (ii) sufficient quanti-

ties of coxsackievirus A-21 are present in these particles to induce infection in susceptible subjects. Additionally, it should be noted that most of the particles produced by sneezes and coughs are in a size range comparable to those generated by the Collison atomizer; however, because of the presence of small numbers of large particles in sneezes and coughs, the volume distributions of the natural and experimental aerosols are different. The distribution of virus according to number or volume of particles in natural aerosols has not been determined. The occurrence of airborne virus in cough specimens was found to be statistically related to the quantities of virus in the nasal and oral secretions (1).

The discussant's observation that the larger particles in the experimental aerosol may have initiated upper respiratory infection and illness in the volunteers may be valid, and was recognized by us in a previous report (2).

On the basis of the discussant's comments regarding types of clinical illness produced with coxsackievirus A-21 infections, additional clarification of our findings is necessary. The predominant clinical response produced by this virus is upper respiratory illness, regardless of whether he infection occurs in natural circumstances or follows experimental inoculation by nasal instillation, large-particle aerosols, or *small-particle* aerosols. With one strain (49889 HEK_1), however, lower respiratory illness was the predominant response and occurred only after small-particle aerosol inoculation. There appears to be no doubt that the upper respiratory passages are extremely susceptible to infection with this virus, and we agree that deposition at this site may be responsible for the consistent finding of upper respiratory illness in natural and experimentally induced disease. Finally, as stated in the previous paper (1), the question of how this virus is transmitted in nature has, at the present time, not been answered in this laboratory or elsewhere.

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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-ft³ 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 expocure 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

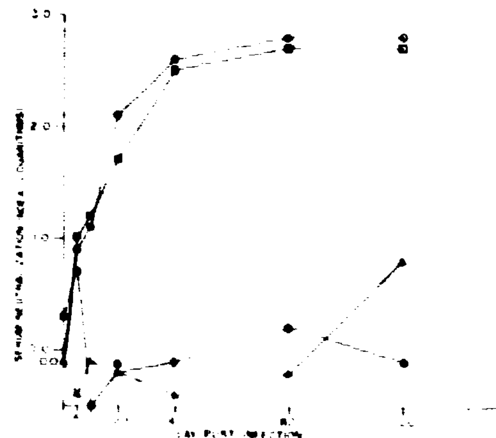

FIG. 1. *Results of neutralization tests against Venezuelan equine encephalitis virus by sera collected from White Carneau pigeons at various periods after injection. Symbols: ○ = birds developed viremia after respiratory dosage; △ = birds did not develop viremia after respiratory dosage; □ = birds developed 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 1-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.

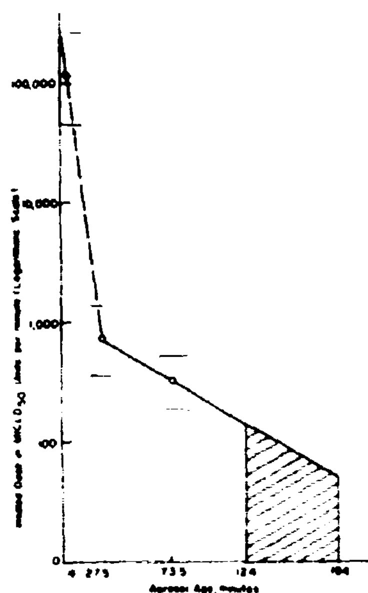

FIG. 2. *Aerosol concentration of Venezuelan equine encephalitis virus as a function of age. Concentrations are in terms of doses per minute for White Carneau pigeons expressed as $MICLD_{50}$ units. The open circles indicate aerosol concentrations estimated by sampling. The solid line indicates the 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
4,344 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.

ACKNOWLEDGMENT

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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

dynamic cloud by use of a modified Henderson apparatus, as was done in the mouse experiment cited. Further, such a system would permit larger volumes of air to be sampled and, hence, direct estimation of the dose presented rather than extrapolation.

It is desirable to extend these studies of the significance of dose rate to other hosts and to other airborne infections wherein the minimal acute respiratory dose is a value larger than 10 organisms presented. Miller has added yet another variable, dose rate, to be entered into the complex equation describing host agent interaction in airborne infection.

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Physiological Responses of Airborne Bacteria to Shifts in Relative Humidity

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Most information currently available on the behavior of airborne cells has been collected by investigators studying aerosols held in static environments. Wells and Riley (15), for example, showed that survival of bacteria was markedly influenced by humidity and temperature, and that the effects varied between bacterial species. Death of airborne bacteria has been observed to increase with a rise in humidity (2, 17), but contrary findings have also been reported (6, 16). Maximal death rates were found between 50 and 60% relative humidity (RH), and death has been reported to occur at more than one rate (5). There are more recent reports of multiple-stage death rates (12, 13, 14).

Few reports have been published, however, describing possible experimental techniques for subjecting microorganisms in air to shifts in RH, although it is well understood that such shifts do occur in natural airborne environments. Brown (1) regulated moisture in static chambers with salt solutions and sprayed water to produce intermediate changes. Hemmes (9) reported similar experiments with shifts in RH produced by spraying water into the aerosol chamber. One may also effectively produce limited rehydration of airborne particles by permitting the incoming air of the atomizer to be at a higher humidity level than that of the final humidity condition (12). The principle of the adiabatic expansion of a gas has been used successfully by Druett (*personal communication*), who found that a rapid decrease in viability occurred if the expansion raised the humidity sufficiently to cause moisture condensation onto the particles. No effect was observed at low relative humidities. Other unreported experiments have apparently been performed, as discussed by Wolfe (cited in 8), wherein pressure changes were produced within an aerosol chamber. A change in temperature would effect a change in RH, although the task of ascribing noted biological effects to humidity alone would be difficult.

Our purpose in studying the effects of sudden shifts in RH on airborne bacteria already equilibrated to one humidity condition was twofold: first, we were interested in applying laboratory findings to natural environments where temperature and humidity are constantly changing; and second, we were interested in possible death mechanisms—noting effects of shifts on subsequent biological behavior might furnish us with additional clues to such mechanisms. It is the purpose of this paper to report our findings and to discuss some of the implications of our results.

DUAL AEROSOL TRANSPORT APPARATUS

We achieved an abrupt shift of humidity in an air stream by diluting it with a second air stream at a different humidity; air temperature was held constant at 21 C. Only a brief description of the equipment and methodology will be made, since details have been previously reported (8). A 45-ft (13.7-meter) duct, 6 inches (15.2 cm) in diameter, was inserted 2 ft (61 cm) into another 45-ft duct, 8 inches (20.3 cm) in diameter. Each duct was equipped with numerous sampling ports. The point of juncture, where mixing of two air streams occurred, was called the confluence point. Linear air flow through both ducts was equal, the transit time per duct was about 5.7 min; total aerosol time was about 11.3 min. The calculated dilution of the primary air stream at the confluence point was 0.56, or approximately 50%. Humidity could either be increased or decreased by the dilution effect. Figures 1 and 2 illustrate the apparatus.

Bacteria were sprayed into the primary air stream. Concentration of particulate matter was

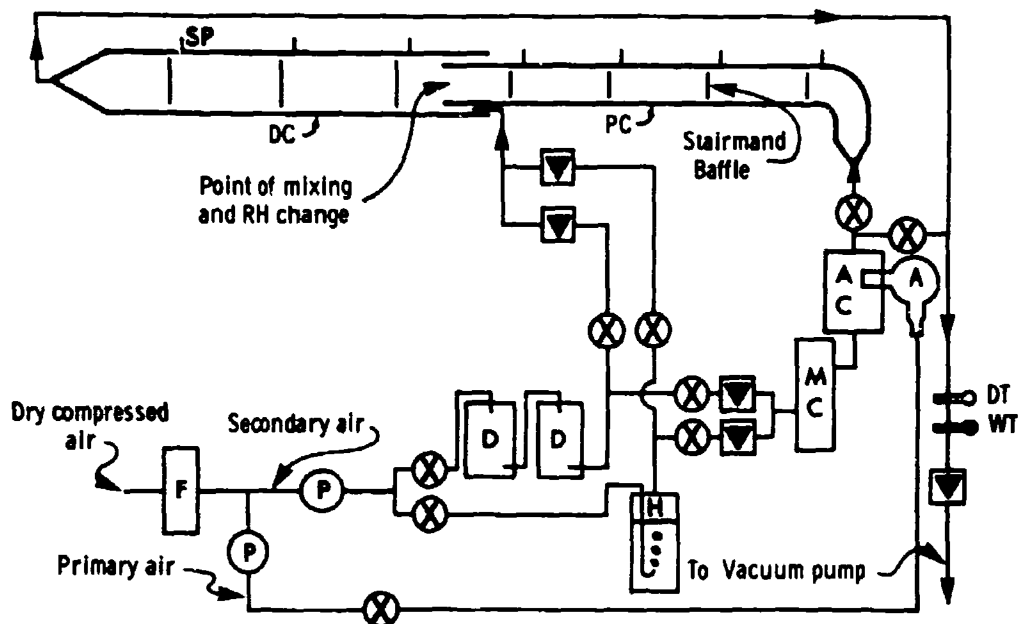

FIG. 1. *Schematic diagram of the dynamic aerosol transport apparatus. PC, primary aerosol chamber; DC, diluted aerosol chamber; A, reflux-type Wells' atomizer; AC, atomization chamber; MC, mixing chamber for conditioning of air; D, Lectrodryer; P, pressure regulators; F, filter; SP, sampling ports; H, humidifying chamber; DT, dry-bulb thermometer; WT, wet-bulb thermometer;* ⊗, *valve;* ▼, *rotometer.*

FIG. 2. *Lateral view of the dynamic aerosol transport apparatus and ancillary equipment. PC, primary aerosol chamber; DC, diluted aerosol chamber; SP, sampling port; CP, control panel; LA, light scatter apparatus.*

measured by forward-angle light scatter, and biological assay was made on samples collected either by slit samplers or impingers. Unless noted otherwise, we cooled 21.5-hr cultures to 4 C and then sprayed them from a refluxing atomizer, wherein the temperature usually increased to 15 C.

Figure 3 shows a hypothetical example, typical of observed results, for the purpose of defining parameters. The physical decay always followed first-order kinetics. The measured concentration of particles in the primary air stream immediately before dilution, compared with that in the secondary stream immediately after dilution (i.e., the apparent dilution ratio, ADR), was usually slightly higher than 0.56; sections A and B of Table 1 list some observed mean ADR values. Analysis of variance of three sets, 20 runs for each condition of shift-up, shift-down, or no shift, and disregarding other variables, indicated a 95% confidence interval of ±0.01 for all sets; differences between these three sets exceeded the 0.1% level of significance. These data were interpreted as indicating that the particles either increased or decreased in volume as a function of shift in RH. For example, if particles decrease in size, they scatter less light; therefore, the apparent dilution seems to increase and the ADR becomes smaller than without a shift in RH, and vice versa.

Since physical decay in the duct system was small and consistent, we refer to the sum of physical and biological loss as biological decay. The latter was always greater than physical decay and, in the primary air stream, usually followed first-order kinetics; "tailing" sometimes occurred after a humidity shift, but for comparative purposes we assumed first-order kinetics in all instances. Usually the biological loss, or biological dilution ratio (BDR), as a result of dilution at the confluence point, corresponded to the ADR (Fig. 3); important exceptions are noted below. Biological loss observed under the final conditions, as compared with loss under the initial condition, was defined as the dynamic-humidity-death (DHD) ratio (Fig. 3). If no change occurred, the theoretical ratio was 1.00; less than this number indicated enhanced death, and a number larger than 1.00 indicated that death process had decreased as a result of the shift. The mean DHD of 21 aerosols subjected to no shift in RH was 1.04 with a 95% confidence interval of ±0.05. We assume from this that DHD ratios greater than 1.10 or less than 0.90 are significant.

Serratia marcescens grown in, and sprayed from, dilute Trypticase Soy Broth (BBL) evinced an increased death rate (sorbed death, 11) when the RH was shifted from low to high values, but this effect was decreased if cells were sprayed from a temperature-controlled, nonrefluxing (TCNR) atomizer at 4 C (section A, Table 1).

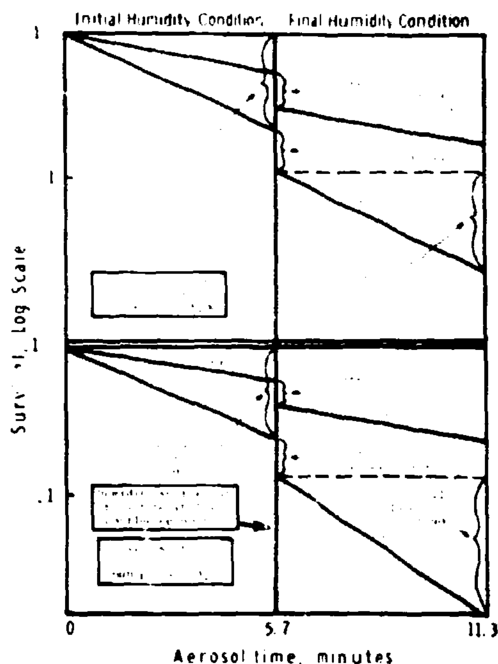

FIG. 3. *Theoretical behavior of bacteria aerosolized in the Dual Aerosol Transport Apparatus. There are two distinct intervals of both biological and physical loss, and there is loss caused by dilution. The theoretical dilution ratio, based on the geometry of the system, is 0.56. The apparent dilution ratio (ADR), measured by light-scatter, was approximately 0.59. A shift up in humidity increased the ADR, whereas a shift down decreased it. If no biological loss occurred at the confluence point, then the biological dilution ratio (BDR) was approximately equal to the ADR. We have frequently observed the BDR to be as much as 10 times the ADR. The initial biological loss divided by the final biological loss has been defined as the dynamic-humidity-death (DHD) ratio. In the upper example, with no change in humidity, the DHD ratio is approximately 1.00. In the lower, hypothetical example, where the change in humidity is unspecified, the DHD ratio shown is 0.77, indicating that the change was detrimental to survival. We have occasionally observed DHD ratios greater than 1.00.*

No sorbed death was noted (section B, Table 1) when *S. marcescens* was grown and sprayed in a chemically defined medium (3). Initially, there appeared to be a "toxic" effect of dilute Trypticase Soy Broth, because the DHD ratio was low (section C, Table 1) when cells were grown in chemically defined medium and resuspended in dilute Trypticase Soy Broth medium. It is of interest to note, however, that sorbed death was eliminated at humidity values above

TABLE 1. *Summary of results obtained when airborne bacteria were subjected to shifts in relative humidity*

Pertinent test conditions[a]	Per cent relative humidity conditions		ADR or BDR[b]	DHD ratio[b]
	Initial	Final		
Serratia marcescens				
(A) Grown and sprayed in dilute Trypticase Soy Broth (DTS)	24	51	ADR 0.61	0.71
	50	50	ADR 0.59	0.97
	24	47[c]		0.89
	90	57	ADR 0.48	1.10
(B) Grown and sprayed in chemically defined medium (CDM)	27	37	ADR 0.60	1.10
	51	51	ADR 0.57	1.08
	93	72	ADR 0.52	1.10
(C) Grown in CDM, resuspended and sprayed in DTS	22	54		0.62
	59	72		1.00
	24	55[c]		1.00
Atomizer fluid at 21 C	25[d]	53		—[e]
Atomizer at 21 C with 1 mg/ml of chloramphenicol	25[d]	53		0.91
(D) Grown in DTS, resuspended and sprayed in CDM	25	52		0.90
(E) Grown in CDM and stored at 4 C for noted times, then resuspended in DTS at 4 C for 30 min and sprayed			Initial loss[b]	
(0 hr)	22[d]	52	71%	0.75
(1 hr)	22[d]	52	50%	0.86
(5½ hr)	22[d]	52	46%	1.20
(7 hr)	22[d]	52	21%	0.53
***Pasteurella pestis* A1122**				
(F) Grown in Heart Infusion Broth	28	46	BDR 0.28	0.44
	28	28	BDR 0.59	1.00
	39	26	BDR 0.70	3.40
	87	61	BDR 0.16	1.00

[a] Refluxing atomizer; fluid chilled to 4 C before spraying, unless otherwise noted.
[b] See Fig. 3.
[c] Sprayed with modified, nonrefluxing atomizer with temperature control at 4 C.
[d] Single experiment; all other data are mean value of three or more aerosols.
[e] No survivors after shift in humidity.

59% RH, and when cells were sprayed from the TCNR atomizer at 4 C, the DHD ratio was 1.00. If cells were grown in dilute Trypticase Soy Broth and sprayed from chemically defined medium (section D, Table 1), the DHD ratio was higher than above; hence, dilute Trypticase Soy Broth was not toxic. Further evidence for nontoxicity is shown in section C, Table 1; cells grown in chemically defined medium and sprayed from dilute Trypticase Soy Broth at room temperature were so sensitive to sorbed death that no viable cells were recovered from the second duct, but the addition of 1 mg/ml of chloramphenicol to a similar suspension practically eliminated the detrimental effects of sorbed water.

The latter effect might be considered a protective one, but a similar result was obtained when cells were grown in chemically defined medium and stored at 4 C for various times before being added to dilute Trypticase Soy Broth to be sprayed (section E, Table 1). Storage in the cold for 5.5 hr changed the DHD ratio from 0.75 to 1.20; additional storage caused the DHD ratio to decrease to 0.53. This decrease was more apparent than real, however, because of the marked change in initial loss that occurred as a result of the cold storage period (see Table 1); the final biological decay, as a result of the 7-hr storage period, was less than that of the final decay of cells stored for 5.5 hr and equivalent to the final

decay of cells grown and sprayed in chemically defined medium.

Pasteurella pestis A1122 was grown and sprayed in Heart Infusion Broth (Difco). Although this species exhibited sorbed death similar to *S. marcescens*, there were three distinct differences between the species (section F, Table 1): (i) there was a change in death rate as a result of a shift-down in RH, a phenomenon we never observed with *S. marcescens*; (ii) the DHD ratio as a result of this change was 3.40, i.e., the death rate decreased markedly rather than increasing, (iii) instantaneous death (i.e., the rate was too rapid to measure) often occurred after humidity shifts (note last BDR, section F, Table 1). Also, preliminary evidence indicated instances of dissonance (3), or dilution shock, and a dependence on both constituents in, and temperature of, the sampling medium. For example, in one experiment in which plates, before being incubated, were chilled for 2 hr after the sample from an aerosol was inoculated, a twofold increase in colony numbers was found over the number on plates incubated immediately. In the same experiment, the addition of 1% whole blood to the medium caused a fourfold increase in colony numbers. Whole blood did not increase the number of colonies produced by unstressed populations. The actual extent of these increases varied with aerosol age.

Applied Interpretations

These findings show that changes in RH do influence subsequent survival of airborne bacteria. The evidence indicates that this effect might be applied to air-sterilization processes. For example, air conditioning equipment might be cycled to lower the air contamination of public places, such as hospitals, schools, institutions, etc. (5). The study has not indicated specific or generally applicable RH changes, or rates of changes, that might be most lethal, nor is there direct evidence that survival after a shift in RH is different than it would have been in the second condition without a change. There is, however, presumptive evidence for this, in that we never observed *S. marcescens* cells to die as rapidly at 53% RH as they did when shifted from 25 to 53% RH (section C, Table 1). The spray temperature in this instance was more equivalent to natural conditions (21 C) than in other experiments (4 to 15 C). Further, little more than 10% of airborne *P. pestis* cells survived a shift-down in humidity from 87 to 61% RH, although the death rates before and after the shift were observed to be identical (section F, Table 1).

The difficulty of finding interpretations meaningful to natural situations lies in the obvious dependence of airborne behavior on the history of environment of the culture before aerosolization. Goodlow and Leonard (7) previously pointed out the importance of such conditions. Rigid standardization in an effort to attain replicability does not aid the interpretation, because we usually do not know the cultural history of bacteria found in nature.

Theoretical Interpretations

There are additional difficulties influencing our attempts to interpret these data from a theoretical viewpoint. We can justifiably point out some previously suspected mechanisms that either are not applicable or act only indirectly. Dehydration alone does not kill cells, otherwise freeze-dried cells would not survive as they do (4). Moreover, Hess (10), in a most important contribution, showed that little or no loss of viability occurred at any RH tested if cells were held airborne in oxygen-free chambers. Our data show that rehydration can cause death; interpreted broadly, these facts imply that part of the observed death may be caused by the act of sampling. In some instances, additional colonies arose when sampling plates were cooled before they were incubated or when nutrients not required by unstressed cells were added. Since cells ruptured by osmotic shock are unlikely to repair such damage, osmotic shock cannot be solely responsible for death. In fact, we may reasonably suggest that no currently used assay is accurate for cells injured by aerosolization.

From these data we theorize that airborne cells may be metabolically active. Substances such as chloramphenicol, or conditions such as low temperature that decrease metabolic functions, tend to increase survival capacity. Dehydration undoubtedly decreases metabolic functions, but probably in a manner that leads to an imbalanced but slowly readjustable condition. Cells sampled before readjustment die as a result of further imbalance unless provided with a situation where additional slow change, or repair, can take place. Cells normally exist in a variety of "states" because of the division cycle and differences in microenvironments. Therefore, individual cellular responsiveness to aerosolization ought to vary, and this is apparently what happened in our studies.

The evidence indicates that no single structural injury (e.g., hydrogen bond breakage, deoxyribonucleic acid denaturation) can account for all death observed and that measured behavior (colony formation) is highly dependent on functional activities of the cell

SUMMARY

In summary, the aerobiologist places a biological system, the bacteria, in a hostile and ill-defined environment, the atmosphere, for the purpose of studying air-bacterium interactions. Measurement of this interaction is in terms of survival. Survival has been shown to depend not only on physicochemical reactions of the somatic, structural components of the cell, but also on those functional, physiological, dynamic properties of all living systems, termed adaptability or responsiveness. The problem, whether one is assaying infectivity or is searching for clues pertinent to death mechanisms, is to separate the two effects.

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Discussion

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In the experiments which Dr. Hatch has described, the immediate effect of an abrupt change in relative humidity on an airborne microorganism was expressed as a deviation from the expected reduction in aerosol concentration due to the dilution by the additional air introduced at the confluence point. The biological dilution ratio, based on samples, was compared with the apparent dilution ratio based upon light scatter measurements. The biological loss observed during the 5.7-min aerosol transit time in the second half of the apparatus was compared with the equivalent loss observed in the first half, and was expressed as the dynamic humidity death ratio.

Regarding the immediate effects of an abrupt change in relative humidity on airborne microorganisms, one might suggest that not only are the effects dependent upon the direction and magnitude of the change but, perhaps, also upon the

rate of change of relative humidity. If, in the apparatus described, one assumes that the aerosol from the first tube mixes perfectly with the additional air introduced at the confluence point and that temperature is constant throughout, one wonders what time is required to achieve uniform relative humidity in the mixed aerosol beyond the confluence point. With adequate mixing, the equilibration time is probably rather short and dependent upon the diffusion rate of water vapor. One could perhaps assume that the small airborne particles containing microorganisms come to equilibrium with their micro-environment at a rate greater than that at which the environment is changing. Undoubtedly the equilibration rate of the airborne microorganisms with their environment would be influenced by the nature of the material in the particle deposited by evaporation of the suspending fluid from which the microorganisms were originally atomized. Other factors such as strain of a given species and the age of a culture and its metabolic state, as influenced by temperature or chemical composition of the suspending fluid, also have been shown to affect the behavior of airborne microorganisms subjected to an additional stress such as a change in relative humidity.

A differing biological loss observed during the initial and final 5.7-min aerosol transit periods was identified by Dr. Hatch as the dynamic humidity death ratio and was based upon the assumption that first order kinetics were followed during the initial and final aerosol transit periods. Assuming that a simple exponential decay does occur, one could as readily express the biological loss as a decay rate, which could perhaps be useful in predicting biological loss for time periods other than those obtained in this apparatus. In addition, by computing decay rates, one could separate the physical and total loss, as measured by light scatter and sampling, respectively, to obtain a true biological decay rate. In using light scatter measurements to indicate particulate concentration of an aerosol, one must be aware of the fact that the light scattered from a sample of the aerosol is not restricted to particles carrying microorganisms.

The employment of a mixed aerosol containing the test organism and a tracer such as *Bacillus subtilis* spores is suggested, since, from the test organism-tracer ratio, one can obtain viability data independent of sampler efficiency and the extent of aerosol dilution. To eliminate the influence of a possible biological loss of the tracer, one could employ radioactively tagged microorganisms as a nonviable tracer.

Such tracer techniques would also be of assistance in elucidating the "tailing" or deviations from an exponential decay rate which have sometimes been observed after a change in relative humidity.

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Effect of Nitrogen Dioxide on Resistance to Respiratory Infection

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INTRODUCTION

In studies of the effect of atmospheric pollutants on health, the basic aspects that must be considered are: the direct damage due to acute and chronic exposure, the role of a pre-existing disease on susceptibility to acute and chronic exposures, and the effects of acute and chronic exposures on resistance to secondary stresses such as respiratory infection.

Air pollutants exert their effect by contact between the pollutant and the body, normally at the surface of skin and exposed membranes. The extent of damage is related to the pollutant's physicochemical properties, its concentration, and the duration of exposure.

For example, among the physicochemical properties, solubility is important. The part of the respiratory system upon which a pollutant may act depends on solubility. A gas of low solubility, such as nitrogen dioxide, penetrates into the lower respiratory tract and exerts its effect in this portion of the respiratory system.

The severity of the tissue response is usually the product of the concentration of the pollutant and the duration of the exposure. Although very low concentrations can sometimes be inhaled for long periods of time without causing any observable effects, inhalation of the same total amount of the gas over a short period of time or as a single breath can result in severe tissue damage and toxic response (14).

The effects of gaseous air pollutants on the membraneous surfaces of the respiratory system are of special interest from the standpoint of resistance to respiratory infection. An irritant gas reaching the epithelium of the trachea or the bronchi can paralyze cilia, alter mucus flow, affect phagocytic activity, and in severe exposures destroy the surface layers of the epithelial lining. These functions constitute the major defense mechanisms and play an important role in respiratory infections.

Nitrogen dioxide is one of the most abundant atmospheric contaminants in many communities. It is emitted in large quantities in the exhausts of automotive engines and is a by-product of natural gas combustion (26). In recent years, it has been increasingly recognized that exposure to oxides of nitrogen (nitrogen dioxide and nitric oxide) can occur in a wide variety of situations. Dangerous accumulations of nitric oxide and nitrogen dioxide can occur, for example, in agricultural silos (17), in enclosed mineshafts after detonation of explosives (3), and in industrial processes requiring the handling of nitric acid (7). A time interval of a few hours after acute exposure usually elapses before symptoms develop (20). After this interval, acute pulmonary edema, cyanosis, severe dyspnea, and bronchopneumonia characteristically develop. When not immediately fatal, the acute episode may be followed by the development of bronchiolitis obliterans, which may cause death during the next few weeks (17) or may lead to persistent abnormalities in airflow.

In the past, the effect of air pollutants on resistance to infection has been studied from two viewpoints, namely, epidemiology and animal experimentation. The discussion in this paper will be limited primarily to the effect of acute and

chronic exposures to the air pollutant nitrogen dioxide on resistance to infection produced by respiratory challenge with airborne *Klebsiella pneumoniae*.

EFFECT OF ACUTE EXPOSURE

The methods used for acute exposure of experimental animals to nitrogen dioxide and for respiratory challenge with aerosols of *K. pneumoniae* have been described in detail in previous publications (21, 22).

Briefly, *K. pneumoniae* type A, strain A-D, was used. It was isolated on Blood Agar Base (Difco) from the heart of an intraperitoneally injected mouse. Stock cultures were prepared on Blood Agar Base in Roux flasks. After 24 hr at 37 C, the growth was harvested in a minimal amount of sterile distilled water and frozen in glass vials containing 2 ml each. For aerosolization, the stock culture was regrown on Blood Agar Base, harvested, and diluted to 10^6 organisms per milliliter in sterile water.

The aerosol chamber was a 200-liter plastic container which was inserted into a microbiological safety hood. A modified University of Chicago Toxicity Laboratory atomizer was used to produce the aerosol. The liquid culture was fed from a 50-ml syringe, the plunger of which was activated by a revolving threaded rod propelled by a 1-rev/min synchronous electric motor. The atomizer delivered 0.4 ml of culture mixed in 32.5 liters per min of air into the chamber. The chamber air was maintained at 73 ± 2 C and 80 ± 5% relative humidity (RH).

Animals were exposed for 10 min to the bacterial aerosol, in particle size of 1 to 5 μ. After the exposure, aerosol production was stopped, and the animals were air-washed for 15 min.

The source of nitrogen dioxide was a gas cylinder containing 10,000 ppm of nitrogen dioxide in air. The flow of the gas was measured on passage from the cylinder to a mixing chamber where it was further diluted with filtered air. For acute exposures, the nitrogen dioxide-air mixture was introduced into a 3.5-ft^3 glass aquarium. For chronic exposure, a walk-in type chamber was used.

Two basic experimental procedures were employed with the use of mice in groups of 10 and hamsters in groups of 6. To determine the effect of pre-exposure to nitrogen dioxide on resistance, experimental animals were exposed to the gas for a 2-hr period before the challenge with the infectious aerosol. To study the effect of nitrogen dioxide on the course of the infection, animals challenged with *K. pneumoniae* were exposed for 2 hr to the gas. The animals were observed for 14 days after aerosol challenge, during which time mortality and survival time data were recorded. Autopsies were performed on all animals at the time of death, and randomly selected lung tissues were subjected to histopathological examination. Blood-agar plates were streaked with heart blood to confirm *K. pneumoniae* as the cause of death. Animals surviving the 14-day observation period were sacrificed and examined in the same way.

In all experiments, control groups of animals were exposed either to nitrogen dioxide or to the infectious agent, simultaneously with the experimental animals. Accordingly, results could be compared on the basis of individual test exposures or could be pooled for statistical analysis. The mortality and the survival data were analyzed statistically by the *t* test. Significance is reported at $P < 0.05$. There were no deaths in any of the animals exposed to nitrogen dioxide only. The mortality in the animals challenged with *K. pneumoniae* was only approximately 40%.

TABLE 1. *Mortality of Swiss albino mice exposed for 2 hr to nitrogen dioxide 1 hr before infectious challenge*

NO_2	Mortality (deaths/total): Infected controls	Exptl group	Change	*P*
ppm			%	
1.5	71/150	86/150	21.1	
2.5	135/400	158/400	16.8	
3.5	44/100	98/100	122.7	<0.05
5	112/250	234/250	108.9	<0.05
10	19/40	39/40	105.3	<0.05
15	13/40	35/40	169.2	<0.05
25	40/100	46/50	130.0	<0.05

Swiss Albino Mice

The effect of a 2-hr exposure to nitrogen dioxide on resistance of Swiss albino mice to infection has been reported in detail in previous publications (8, 9) and will be discussed here only briefly. Table 1 summarizes the data on the effect of nitrogen dioxide in concentrations ranging from 1.5 to 25 ppm. The time interval between the termination of the nitrogen dioxide exposure and the infectious challenge was 1 hr or less. Mice not exposed to the gas but challenged with *K. pneumoniae* aerosol simultaneously with the experimental mice served as controls.

Based on the results shown in Table 1, a threshold value was determined at which the exposure to nitrogen dioxide reduces the resistance of Swiss albino mice to respiratory infection. The threshold is approximately 3 ppm.

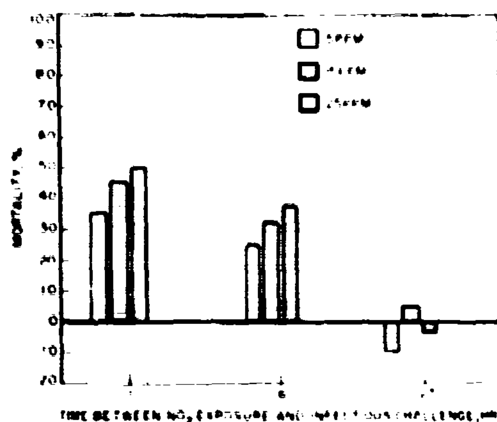

FIG. 1. *Mortality rates corrected for control mortality in mice exposed for 2 hr to various concentrations of NO_2 prior to challenge with infectious agent*

The acute exposure appeared to produce an all-or-none response. There was no effect at 2.5 ppm, a complete effect at 3.5 ppm, and a minimal effect at higher concentrations. The mean survival time of the infected controls, calculated on the basis of a maximal 14-day survival, was 11.1 days. The survival time was not affected by exposure to nitrogen dioxide concentrations of up to 2.5 ppm, but it was reduced to 5.5 days at concentrations ranging from 3.5 to 25 ppm.

No deaths occurred in the mice exposed to nitrogen dioxide only, irrespective of the concentrations used. In mice exposed to 5 ppm or more, the lungs were congested to various degrees, and the veins and capillaries of the lungs were dilated. Concentrations of less than 5 ppm produced little, if any, damage.

For the histopathological examinations, the mice were sacrificed within 1 hr after the termination of exposure to nitrogen dioxide. On the few occasions when the sacrifice was delayed for 24 or 48 hr, pathological findings were reduced or absent. Thus, it was of interest to determine whether the effect of acute exposure to nitrogen dioxide on resistance to infection is transitory. To study this parameter, the time interval between the termination of the exposure to nitrogen dioxide and the infectious challenge was extended from 1 hr to 6 and 27 hr. The data in Fig. 1 show that the decrease in resistance was not permanent and disappeared within 27 hr after the termination of the nitrogen dioxide exposure. The persistence of the effect was not influenced by the concentrations of gas used within the 5 to 25 ppm range. It can be assumed, therefore, that nitrogen dioxide produces a temporary damage to

TABLE 2. *Mortality of Swiss albino mice exposed for 2 hr to nitrogen dioxide 1 to 24 hr after infectious challenge*

NO_2	Time between challenge and exposure	Mortality		Survival time
		Deaths/total	Percent	
ppm	*hr*			*days*
0	0	27/60	45.0	10.9
2.5	1	20/40	50.0	9
2.5	6	33/60	55.0	9.8
2.5	24	32/60	53.3	10.2
0	0	15/30	50.0	10.6
25	1	30/30	100.0*	4.7*
25	6	28/30	93.3*	6.0*
25	24	26/30	86.6*	7.6*

* Significant at $P < 0.05$

the defense mechanisms; this damage disappears within 24 hr.

Concentrations of 2.5 or 25 ppm were used to study the effect of a 2-hr exposure to nitrogen dioxide on mortality of previously infected mice. The mortality increased from 50% in the control mice to 100% in the mice exposed to 25 ppm of nitrogen dioxide. At 2.5 ppm, there was no significant difference in mortality between control and exposed mice. Delaying the exposure to nitrogen dioxide for 6 or 24 hr after the infectious challenge did not significantly alter the mortality increase in mice exposed to 25 ppm (Table 2).

Inbred Mice

Increased immunity due to genetically conditioned natural resistance may manifest itself as resistance to invasion by bacteria or as increased ability to produce bacterial antibodies. In studies of acute toxicity of oxides of nitrogen, Gray and co-workers (13) observed appreciable variations in the response of rats obtained from different sources. An exposure difference of 40 ppm was required to produce an LD_{50} in groups of rats from two different sources. In studies of chronic exposures, Wagner et al. (28) found no effect that could be attributed to the nitrogen dioxide exposures, and therefore strain difference among HLA, $C_{57}BL/6$, and CAF_1/Jax mice were not observed.

The effect of a 2-hr exposure to 5 ppm of nitrogen dioxide on resistance to respiratory infection was determined in BDF_1, BALB/c, $C_{57}BL/c$, and LAF_1 mice. Groups of mice from each inbred strain were exposed simultaneously with Swiss albino Webster strain mice to nitrogen dioxide, and either before or after this exposure were challenged with airborne *K. pneumoniae*.

TABLE 3. *Mortality of various mouse strains exposed to nitrogen dioxide and infectious challenge*

Mouse strain	NO_2 before challenge: Mortality (deaths/total), Infected controls	NO_2 before challenge: Mortality (deaths/total), Exp'd group	NO_2 before challenge: Change	NO_2 after challenge: Mortality (deaths/total), Infected controls	NO_2 after challenge: Mortality (deaths/total), Exp'd group	NO_2 after challenge: Change
			%			%
SA	164/300	262/300	59.6[a]	247/620	372/620	50.8[a]
BDF_1	31/120[b]	40/120	29.1	66/240[b]	105/240	58.9[a]
BALB/c	48/100	72/100	50.0[a]	43/100	62/100	44.2[a]
$C_{57}BL/c$	24/70[b]	36/70	49.9[a]	29/130[b]	34/130	17.4
LAF_1	40/100	50/100	25.0	52/160	91/160	74.8[a]

[a] Significant ($P < 0.05$) change due to NO_2 exposure.
[b] Significant ($P < 0.05$) change from Swiss albino mice.

The interval between these two treatments was 1 hr or less.

Table 3 summarizes the results obtained on the basis of a minimum of eight replicate exposures. The data can be considered from two standpoints. One relates to strain differences in resistance to the infection per se; the other, to effects of exposure to nitrogen dioxide on resistance.

BDF_1 and $C_{57}BL/c$ mice were more resistant to the infection than the other two strains, as measured by mortality. The mean survival times of the two more resistant strains were 12.2 and 12.0 days, respectively. Compared with the 10.9 mean survival time of the Swiss albino mice, this increase was statistically significant ($P < 0.05$). The other two inbred strains showed mortalities and mean survival times similar to those of the Swiss albino mice.

Exposure to nitrogen dioxide followed by infectious challenge significantly increased mortality in the Swiss albino, BALB/c, and $C_{57}BL/c$ mice. The increases due to the exposure were 59.6, 50.0, and 49.9%, respectively. The mortality of BDF_1 and LAF_1 mice also increased to 29.1 and 25.0%, respectively, but the differences were not significant. The LAF_1 data, however, must be considered with caution. In this group of experiments, only a small increase in mortality was observed upon exposure to nitrogen dioxide of the Swiss albino mice challenged at the same time as the LAF_1 mice.

Exposure to nitrogen dioxide prior to infectious challenge increased mortality in all five strains. The increase was significant in all but the $C_{57}BL/c$ strain.

The data suggest that mouse strain differences are of importance in resistance to infection produced by *K. pneumoniae*. The damage produced by nitrogen dioxide, on the other hand, is not closely related to strain differences. In all instances, mice exposed to nitrogen dioxide either before or after the infectious challenge showed increased mortality. The 5 ppm of nitrogen dioxide did not produce any significant damage to the respiratory system, as determined by histopathological examination of the lungs.

TABLE 4. *Mortality of hamsters exposed for 2 hr to nitrogen dioxide 1 hr before infectious challenge*

NO_2	Mortality (deaths/total): Infected controls	Mortality (deaths/total): Exp'd group	Mortality (deaths/total): Change	P
ppm			%	
5	18/96	22/96	21.8	
15	11/126	19/126	73.5	
25	15/72	10/72	−33.2	
35	9/90	40/90	389.0	<0.001
40	6/72	28/72	368.7	<0.005
50	11/138	44/138	298.7	<0.001
65	12/96	61/96	408.0	<0.001

Hamsters

Golden hamsters have a high natural resistance to *K. pneumoniae* infection initiated by the respiratory route. Inhaled respiratory doses as high as 30,000 organisms produced only 12% mortality in our studies; of 690 hamsters challenged with the infectious agent, 82 died. The same challenge dose repeatedly produced 100% mortality in Swiss albino mice used as controls.

A 2-hr exposure to high levels of nitrogen dioxide terminated 1 hr prior to infectious challenge significantly altered the resistance of hamsters. As shown in Table 4, concentrations ranging from 5 to 25 ppm caused some increase in mortality, but it was not significant. Concentra

tions ranging from 35 to 65 ppm increased mortality significantly; the mortality of the control group was 9.6%, but this increased to 44.7% in the exposed group.

Exposure to nitrogen dioxide apparently is a significant factor in hamsters' resistance to respiratory infection by *K. pneumoniae*. The 10-fold increase in nitrogen dioxide required to produce this effect in hamsters, as compared with mice, cannot be explained at present. It can be related only partially to the differences in body weights and respiratory volumes of these two species. However, the all-or-none response and the absence of a graded dose response are similar in the two species.

Squirrel Monkeys

Increased mortality was observed in preliminary studies with squirrel monkeys exposed for 2 hr to approximately 40 ppm of nitrogen dioxide followed by respiratory challenge. Three groups of monkeys were included in the experiments: one challenged with airborne *K. pneumoniae* only, one exposed to nitrogen dioxide only, and one exposed to nitrogen dioxide and within 1 hr challenged with the infectious agent. Deaths occurred only in the last group; of the five monkeys exposed to both stresses, three died.

EFFECT OF RETENTION OF BACTERIA IN LUNGS

The response of the respiratory system to infectious agents involves the activation of such gross defense mechanisms as cough, alterations in the respiratory functions, phagocytosis, mucus flow, and alterations in ciliary activity.

Under normal conditions, inhaled bacteria are

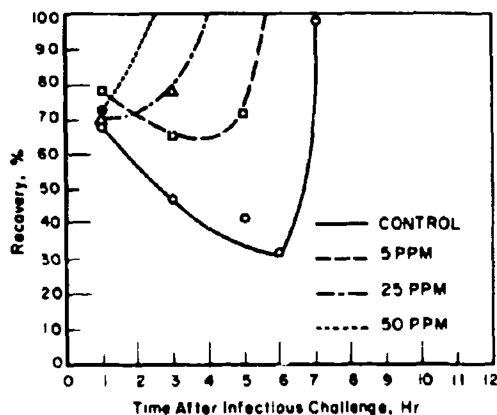

FIG. 2. *Recovery of Klebsiella pneumoniae from lungs of mice exposed to NO_2*

deposited upon mucus, which, through ciliary action, is constantly moved from the deeper part of the lung toward the larynx. Thus, ciliary movement combined with mucus secretion normally prevents an accumulation of particles in the tracheobronchial tree.

This defense mechanism against invasion by bacteria can be altered. Drying, for example, markedly impairs the mobility and the effectiveness of ciliary actions. Irritant gases, such as ozone, sulfur dioxide, ammonia, and nitrogen dioxide, have been reported to interfere with ciliary movement (2, 6, 15). Thus, one parameter that can be utilized to determine the toxicity or the effect of irritant gases is their action on the ciliated epithelium of the respiratory tract.

The role of phagocytosis as a clearance mechanism of inhaled dust particles is well recognized. Defense against bacterial infection in the lung is similar to defense against dusts. In both cases, alveolar macrophages play a key role in the clearance (16). Green and Kass (11) impaired pulmonary clearance mechanisms in mice by a variety of stresses: hypoxia, cold, corticosteroid injection, and ethyl alcohol intoxication. The inhibition of clearance depended on the type and the extent of the treatment and on the bacterial species being cleared.

To study the effect of nitrogen dioxide on clearance of bacteria by the lower respiratory tract, *K. pneumoniae* was used as the infectious agent. Swiss albino mice and hamsters were exposed for 2 hr to nitrogen dioxide in concentrations ranging from 5 to 50 ppm. Within 1 hr after the exposure, they were challenged with the infectious aerosol. Groups of animals were sacrificed immediately after the infectious challenge. The lungs were removed aseptically from each animal, homogenized in sterile saline, and cultured quantitatively. The initial counts were assumed to be 100% recovery. Control animals as well as animals exposed to the nitrogen dioxide were sacrificed at 1, 3, 5, 6, 7, and 8 hr after the combined treatment. The mean number of bacteria present in the lungs of each group of animals was plotted against the time elapsed after the infectious challenge.

Figure 2 shows the data obtained in mice. Recoveries of *K. pneumoniae* from the lungs of mice exceeding 100% are not shown in the figures. However, they were used in construction of the recovery curves. The mean recovery of bacteria from the lungs of control mice challenged only with the infectious aerosol showed a similar pattern in three replicate tests. The bacterial population was markedly reduced (a range of 65 to 90% was observed) during the first 5 to 6 hr after

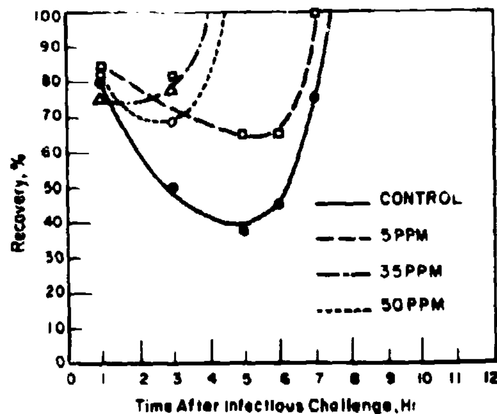

FIG. 3. *Recovery of Klebsiella pneumoniae from lungs of hamsters exposed to NO_2.*

challenge. Thereafter, the population increased and reached the initial concentration after 6 to 8 hr.

In mice exposed to 5 ppm of nitrogen dioxide, the 100% concentration was reached within 5.5 hr. In mice exposed to 25 ppm, the bacterial population decreased during the 1st hr and increased thereafter; the 100% concentration was reached within 3.3 hr. In mice exposed to 50 ppm, the 100% concentration was reached in 2.3 hr. A 4- to 6-log increase in concentration of *K. pneumoniae* occurred in mice 24 hr after the infectious challenge, irrespective of the previous treatment.

Figure 3 shows that similar results were obtained in hamsters. In control hamsters not exposed to nitrogen dioxide, gradual reduction of bacteria occurred during the first 5 hr, and the initial concentration point was reached after 7.3 hr. In hamsters exposed to 5 ppm, this 100% point was observed after 6.4 hr, and in those exposed to 35 ppm, after 2.9 hr.

In the experiments, mice or hamsters exposed to nitrogen dioxide 1 hr before infection were challenged with the infectious aerosol simultaneously with control animals not exposed to the gas. Both groups of animals were thus exposed to the same quantity of *K. pneumoniae*. However, as shown in Table 5, the initial recoveries of *K. pneumoniae* from lungs varied widely. In all instances, fewer organisms were recovered from the animals exposed to nitrogen dioxide. In hamsters, the decrease in organisms appears to be related to the concentration of nitrogen dioxide. At 5 ppm, the recovery was 75% of that in the controls; at 35 ppm, 58%; and at 50 ppm, 44%. However, experiments were not conducted to determine the statistical significance of this relation. In mice, the recovery was approximately 71% of that in controls, irrespective of the nitrogen dioxide concentration.

While the increased mortality in animals exposed to nitrogen dioxide can in part be explained by damage to the ciliary activity and the phagocytic activity, the lower recovery of inhaled bacteria from the lungs of animals exposed to nitrogen dioxide cannot be ascribed to this type of damage. Also, because of the absence of appreciable pulmonary edema in animals exposed to nitrogen dioxide, a dilution effect can be discounted. Three theoretical explanations are possible. One is that the nitrogen dioxide remaining in the lungs inactivates the bacteria in situ. This supposition is questionable, because larger amounts of nitrogen dioxide are usually required to produce any effects on bacteria. Another explanation is that a protective film forms in the respiratory system as a result of inhalation of the gas; this film would make the recovery of bacteria from the lung tissue more difficult. The third possibility is that the respiratory functions are modified by inhalation of nitrogen dioxide.

Our studies in squirrel monkeys showed that exposure to nitrogen dioxide increased the respiratory rate and decreased the tidal volume. Although the animal was breathing more frequently, the breathing was shallow. Thus, it is possible that the bacteria do not penetrate into the alveoli in the same quantities as in normal animals.

The tidal volume of a monkey exposed for 2 hr to nitrogen dioxide is shown in Fig. 4. The monkey was placed in a restraining chair, a mask was fastened to its face, and it was exposed to filtered air for 30 min. Without any interruption, 35 ppm of nitrogen dioxide was introduced into the air, and the respiratory functions were measured with a spirometer and a dual-channel recorder. After the exposure, the tidal volume was approximately 72% of the initial value.

EFFECT ON LACTIC DEHYDROGENASE (LDH) ISOENZYMES

The LDH enzyme system plays a principal role in the glycolytic cycle for the conversion of stored

TABLE 5. *Retention of Klebsiella pneumoniae in lungs of mice and hamsters*

NO_2	No. of organisms/g of lung tissue			
	Control hamsters	Exptl hamsters	Control mice	Exptl mice
ppm				
5	3,948	2,966	3,366	2,457
25	—	—	1,530	1,092
35	7,284	4,253	—	—
50	7,142	3,122	1,289	883

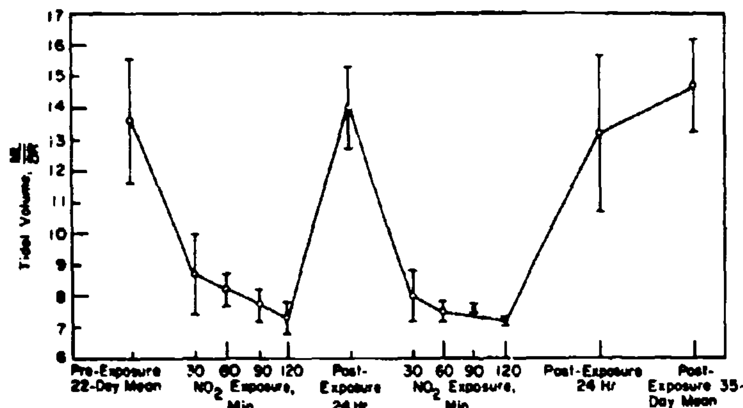

FIG. 4. *Mean tidal volumes of monkey exposed to 35 ppm of NO_2.*

energy. Recently, the enzyme has been separated electrophoretically into five components defined as LDH isoenzymes (29). Diseases, such as cardiac infarction (30), hepatitis (25), and cancer (23), produce abnormal serum and tissue isoenzyme patterns that are indicative of the tissues affected.

Exploratory studies were conducted to determine whether exposure to nitrogen dioxide and infection with *K. pneumoniae* produce an atypical LDH isoenzyme pattern in serum or selected tissues and whether the pattern is indicative of the resulting pathology. The limited number of animals (four) used per point did not permit an exhaustive analysis of the data. However, the differences obtained are large enough to suggest trends and to form the base for additional experimentation. Hamsters were exposed to 5 and 35 ppm of nitrogen dioxide for 2 hr and examined frequently over a 72-hr period.

Heart tissue was removed from each hamster; the LDH enzyme was extracted and resolved into isoenzyme components. Approximately 1 hr after exposure, in either group the enzymatic activity of isoenzymes 1 and 2 was reduced and remained depressed for approximately 1 day. Correspondingly, the isoenzyme activity in bands 4 and 5 increased. This period of altered isoenzyme activity coincides with the period of maximal susceptibility to respiratory infection after exposure to nitrogen dioxide. Hamsters subjected to infectious challenge only did not experience these alterations.

The livers were removed and the LDH isoenzyme was extracted. Although there was a large variability among the hamsters in a group, the overall trend was clear. Hamsters exposed to 5 or 35 ppm showed a decrease of approximately 50% in band 1 and 2 isoenzyme activity 5 hr after exposure.

Exploratory studies were also conducted with squirrel monkeys. One monkey was exposed to 35 ppm for 2 hr and subsequently was infected intravenously with *K. pneumoniae*. The LDH activity in the lung tissue increased 5-fold 24 hr after exposure. The increase in activity was accompanied by a marked and disproportionate increase in the LDH activity in bands 4 and 5.

Two monkeys exposed to 50 ppm of nitrogen dioxide and to *K. pneumoniae* aerosol displayed similar isoenzyme alterations. Upon autopsy, the lung tissue appeared gray, with patches of marked reddish congestion clearly demarcated from the gray areas. Tissue sections from both gray and red areas showed much interstitial and intralveolar edema, with congestion and cellular infiltration. Tissue excised from each of these areas produced abnormal isoenzyme patterns, each very different from the other. The red area produced one major isoenzyme band, band 5, with considerably reduced activity in the remaining isoenzyme fractions.

Current theories suggest that the LDH molecule is a tetrameric peptide molecule and that the synthesis of the molecule is controlled by two genes (5). One gene is responsible for the synthesis of LDH isoenzyme 1 and one for the synthesis of LDH isoenzyme 5. The three remaining isoenzymes are merely combinations of isoenzymes 1 and 5. Cahn, Kaplan, and associates (5) suggest that LDH isoenzyme 1 is associated with cells undergoing aerobic metabolism and LDH isoenzyme 5 with cells functioning anaerobically. Brody and Engel (4) have demonstrated that LDH activity is associated with the mitochondrial membrane and is readily dissociated when the tissue is manipulated during fixation. Therefore, rupture of the cellular membrane (cell death) would be indicated by an increase in serum LDH activity.

Altered metabolism induced by stress may be related to increased enzyme activity and altered isoenzyme ratios. Recently, Vesell (27) has published contradictory results. Studies conducted with nucleated red blood cells and normal red blood cells devoid of a nucleus indicated that LDH isoenzyme band 5 is located in the nucleus, and isoenzymes 1, 2, and 3 are located in the cytoplasm. Vesell therefore takes exception to the theories regarding the relationship of aerobic-anaerobic metabolism to isoenzymes 1 and 5.

The preliminary data acquired to date do not permit interpretation of the cellular mechanisms involved. Nevertheless, it is apparent that alteration of the isoenzyme ratios is related to pathology, and may ultimately provide information regarding altered cellular metabolism induced by the nitrogen dioxide and infectious challenge stress.

EFFECT OF CHRONIC EXPOSURE

Exposure to low levels of pollutants over extended periods of time are a threat to heavily populated communities. Air pollution surveys indicate a maximal concentration of 3.5 ppm of nitrogen dioxide. Daily variations in the concentration of nitrogen dioxide in a polluted atmosphere result from varying emission rates, wind velocity and direction, height of inversion layer, etc. The average 8-hr levels of oxides of nitrogen in one urban area on days with significant air pollution ranged from 0.1 to 0.5 ppm (26).

Several investigators have reported on the effect of chronic and intermittent exposures to nitrogen dioxide. Ronzani (24) concluded that repeated daily exposures to 100 ppm had no distinct acute effect in animals. Gray et al. (12) exposed rats to 9 to 14 ppm for 4 hr per day, 5 days per week, for 6 weeks. They observed an inflammatory condition spread throughout the entire respiratory tract. The same authors (13) found no evidence of pathology in rats, guinea pigs, and mice exposed daily for 6 months to 4 ppm.

Wagner et al. (28) exposed dogs, guinea pigs, rabbits, rats, hamsters, and mice to 1, 5, and 25 ppm for periods up to 18 months. At no exposure level did changes in body weight, hematological value, or biochemical index vary significantly from the control data. The respiratory functions in exposed rabbits were equivalent to those in the controls, with the exception of the 25-ppm group, which indicated a slight and transitory elevation in mean oxygen consumption. Detailed histological examination of tissues of animals sacrificed at various time intervals presented no evidence that nitrogen dioxide had any morphological effect. Their studies with a strain of mice susceptible to spontaneous pulmonary tumor suggested a possible tumorigenic accelerating capacity of nitrogen dioxide.

TABLE 6. *Effect of continuous exposure to 0.5 ppm of nitrogen dioxide on mortality of mice challenged with Klebsiella pneumoniae*

NO_2 exposure	Mortality (deaths/total)			P
	Infected controls	Exptl group	Change	
			%	
7 days	187/280	189/280	1.0	
14 days	81/180	92/180	13.6	
1 month	26/60	34/60	30.9	
2 months	68/100	78/100	14.7	
3 months	64/100	92/100	43.7	<0.05
6 months	24/50	44/50	83.3	<0.001
9 months	38/70	49/70	28.9	<0.001

In our studies, Swiss albino mice were exposed continuously, 24 hr per day, to 0.5 ppm of nitrogen dioxide. Three times a week, the mice were removed from the chamber for approximately 1 hr for maintenance and feeding. After various periods of nitrogen dioxide exposure, the mice were challenged with the aerosol of *K. pneumoniae* and maintained in a clean air atmosphere for 14 days after the challenge. Control animals were of the same age as the experimental mice, and were treated identically with the exception of the nitrogen dioxide exposure. The data in Table 6 show an increase in susceptibility to infection after 3 months of exposure to the gas. Some degree of linearity was observed when the arc transformed differences in mortalities were plotted against the duration of exposure to nitrogen dioxide (Fig. 5).

The effect of continuous and intermittent exposure to 0.5 ppm of nitrogen dioxide over a 30-day period was investigated. After 30 days of continuous exposure, Swiss albino mice were challenged by the respiratory route with airborne *K. pneumoniae*. The deaths were recorded during the next 14-day holding period at ambient atmosphere. For intermittent exposure, mice were exposed to 0.5 ppm for 6 hr a day, 5 days a week, for a total of 30 days before the infectious challenge. The data summarized in Table 7 show the significant mortality increase due to the intermittent exposure.

Exposure of Swiss albino mice to 1.5 ppm of nitrogen dioxide for periods ranging from 2 hr to 3 months prior to the infectious challenge resulted in mortality shown in Table 8. The increase in mortality was significant after exposures of 8 hr or longer. A corresponding reduction in

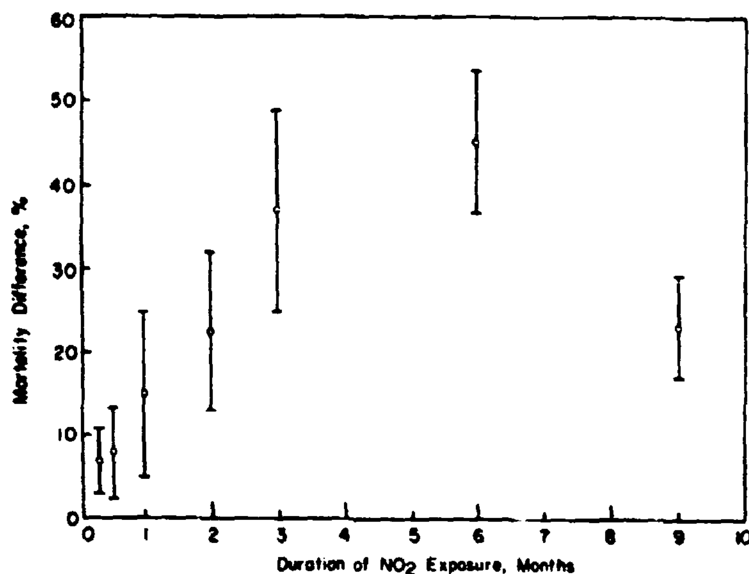

FIG. 5. *Mortality difference versus chronic exposure to* NO_2.

TABLE 7. *Effect of continuous and intermittent exposure to 0.5 ppm of nitrogen dioxide for 30 days on mortality of infected mice*

NO_2 exposure	Mortality		Change	Survival time
	Deaths/total	Per cent		
			%	days
Continuous				
Controls	32/80	40.0		10.8
Experimental group	79/140	56.4	41.0	10.2
Intermittent				
Controls	22/60	36.6		12.2
Experimental group	57/80	71.3	94.8[a]	9.2

[a] Significant at $P < 0.05$.

TABLE 8. *Effect of continuous exposure to 1.5 ppm of nitrogen dioxide on mortality of mice challenged with Klebsiella pneumoniae*

NO_2 exposure	Mortality (deaths/total)		Change
	Infected controls	Exptl group	
			%
2 hr	45/90	51/90	13.4
8 hr	45/90	67/90	48.8[a]
24 hr	45/90	59/90	31.2[a]
7 days	20/40	28/40	40.0[a]
14 days	17/40	39/40	129.4[a]
90 days	23/50	70/100	52.3[a]

[a] Significant at $P < 0.05$.

the survival time occurred in all groups except the one exposed to nitrogen dioxide for 2 hr.

Exposure of infected mice to 1.5 ppm of nitrogen dioxide after the infectious challenge also increased mortality. The mortality in the control group challenged with the infectious agent only was 45%. After exposure to nitrogen dioxide for 2, 8, or 24 hr, the mortality rates were 80.0, 88.3 and 73.3%, respectively. The respective increases in mortality were 77.8, 96.2, and 62.9%, all three values being significant.

The significance of pre-exposure to nitrogen dioxide is further illustrated in Fig. 6. Three groups of mice were used. The one serving as the control was challenged with *K. pneumoniae* aerosol and maintained in clean air after the infection. The second was infected and placed immediately after the challenge in an atmosphere of 1.5 ppm of nitrogen dioxide. The third was exposed to nitrogen dioxide for 24 hr, challenged, and returned to the 1.5-ppm atmosphere. Although the mortality of both groups exposed to nitrogen dioxide was higher than that of the control group, mice exposed to the gas both before and after the infectious challenge died faster, and ultimately the mortality in this group was the highest. The mortality at the end of the 30-day holding period was 58.3% for controls, 78.3% for mice exposed to nitrogen dioxide after the in-

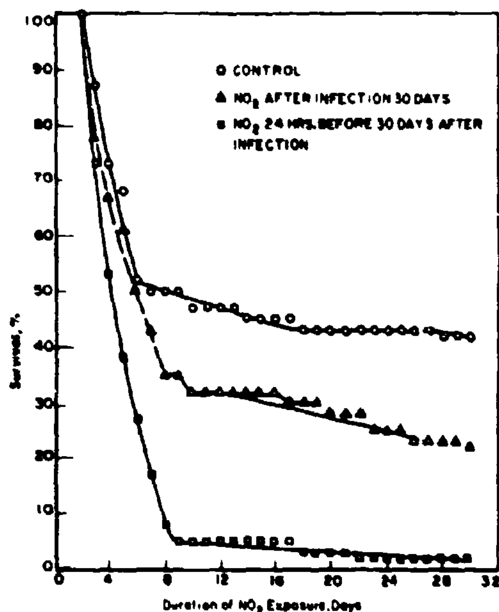

FIG. 6. *Effect of pre-exposure of mice to nitrogen dioxide on resistance to Klebsiella pneumoniae.*

fectious challenge, and 98.3% for mice exposed to nitrogen dioxide both before and after the infectious challenge.

EFFECT OF AGE

The effect of age on resistance to *K. pneumoniae* infection was investigated. Young mice 6 to 8 weeks old and weighing 20 ± 2 g, mice 6 months old and weighing 32 ± 2 g, and mice 9 months old and weighing 39 ± 2 g were maintained at ambient atmosphere and were challenged simultaneously with the infectious agent. Deaths were recorded for 14 days after the challenge. No significant differences were observed in the mortality rates among these groups. The mortality of the 6-month-old mice was 48.0; the 9-month-old mice, 54.2%; and the 6 to 8-week-old mice, 52.5%.

DISCUSSION AND CONCLUSIONS

The effects of exposure to nitrogen dioxide on man and on animals are confined almost exclusively to the respiratory tract. With increasing dosage, the progressive effects of this gas are: odor perception, nasal irritation, difficulty in breathing, acute respiratory irritation, edema, and death. Experimental and epidemiological data pertaining to nitrogen dioxide effects in man are sparse, especially in the low concentration level found in community air pollution.

In most species of laboratory animals, concentrations of nitrogen dioxide above 200 ppm produce death even after a single 5- to 15-min exposure. Continuous 30- to 60-min exposures to 100 to 200 ppm or 8-hr exposures to 50 ppm also produce death. Intermittent exposures of less than 50 ppm, on the other hand, are not fatal. Thus, it appears that the existence of a recovery period reduces mortality.

Lower concentrations, 10 to 20 ppm, produce pathological changes in the lungs. Continuous exposures to 5 or 10 ppm result in changes in the bronchial epithelium; lower concentrations produce only minor changes. Freeman and Haydon (10) observed minor changes in the bronchial epithelium after continuous exposure to 4 ppm for 20 weeks. Balchum et al. (1) showed that exposure of guinea pigs to 5 ppm produced minor pulmonary changes and demonstrated the development of circulating substances capable of agglutinating normal lung proteins.

The work reported in this paper suggests a more sensitive indicator of biological effects of nitrogen dioxide, namely, a synergistic effect or secondary effect, demonstrated by reduction in resistance to infection. A single 2-hr exposure of Swiss albino Webster strain mice or of inbred mice to 3.5 ppm of nitrogen dioxide before or after respiratory challenge with aerosol of *K. pneumoniae* significantly increased mortality. To produce the same effect in hamsters and squirrel monkeys, 35 ppm was required during the 2-hr exposure period. The effect of the single 2-hr exposure was not persistent, and a return to normal resistance to the infection was observed within 24 hr after the exposure to nitrogen dioxide.

Continuous exposures to 0.5 ppm for 3 months or longer as well as intermittent daily exposures over a 30-day period produced the same effect in mice.

Exploratory studies conducted to define the mechanisms responsible for the increased susceptibility to infection suggest that exposure to nitrogen dioxide permits better colonization of bacteria in the lungs of mice and hamsters.

Exposure to 25 to 35 ppm of nitrogen dioxide affected the pulmonary function in squirrel monkeys. Similar observations in guinea pigs were reported by Murphy et al. (19). The respiratory rate increased and the tidal volume decreased in guinea pigs exposed to 5.2 and 13.0 ppm of nitrogen dioxide. The time of onset of the respiratory changes was inversely related to the concentration of the inhaled gas. When the guinea pigs were returned to clean air, the pulmonary function gradually returned to the pre-exposure level.

Extrapolation of the effects of nitrogen dioxide on resistance to *K. pneumoniae* infection of man, or that due to other species of pathogenic miro-organisms, can be speculative only. However, the work is significant in pointing to possible relationships between air pollutants and changes in resistance to respiratory infection.

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Attenuation of Aerosolized Yellow Fever Virus After Passage in Cell Culture

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INTRODUCTION

Reports in the literature, recently reviewed by Mussgay (8), contain numerous examples of alterations in the properties of arboviruses as a result of their passage in various host systems. Countless other observations of this type undoubtedly have been made but not reported. Early accounts of alterations in virulence of yellow fever virus (YFV) as a result of propagation in vitro have been described by Lloyd, Theiler, and Ricci (6) and by Theiler and Smith (11). Studies by Theiler and his associates led to the isolation and eventual use of the well-known 17-D strain for human vaccination. A more detailed description of the development of various attenuated strains of YFV may be found in a review by Theiler (10). More recently, Hallauer (1) reported losses in virulence of the 17-D strain for mice and of the Asibi strain for monkeys after passage in KB cell cultures. Following this, Schindler and Hallauer (9) described additional losses in the viscerotropism of Asibi strain variants obtained after prolonged passage in KB cells. These authors also reported the isolation from human cell lines of a 17-D viral substrain whose virulence for monkeys was reduced to a degree that has apparently not been found in any other 17-D strains. Hardy (2) demonstrated attenuation of the Asibi strain for monkeys with viral isolates obtained after serial passage in HeLa cells. In keeping with the general subject of this symposium, we should like to present some observations on changes in the properties of yellow fever virus that were discernible upon aerosolization of preparations after growth in HeLa cells.

A major consideration in determining our experimental approach was the prevailing lack of information on the behavior of airborne virus that previously had undergone routine passages in cell culture in the laboratory. Information of this kind has broad applications not only for the viral geneticist but for those who are engaged in the problems of laboratory safety. To carry out a meaningful study, it was recognized that a comparison of properties of virus when in suspension with those of virus that was aerosolized was needed. The results will show that the dominant characteristic of virus that was serially passed in cell culture was its decline in virulence. Other properties that were also found to change appeared to vary in parallel with the loss in virulence. It seems probable that some of these alterations may have resulted in, or at least contributed to, the decline in capability of the virus to induce lethal infections.

EXPERIMENTAL APPROACH

Two years ago at Fort Detrick, Hardy (2) demonstrated that his strain of yellow fever virus, which was ordinarily noncytopathic in cell culture and lethal for monkeys, induced cell lysis and became attenuated for these animals after six serial passages in HeLa cells. Subsequently, results reported by Miller et al. (7) indicated the feasibility of performing studies on aerosolized yellow fever virus after it had proliferated in HeLa

TABLE 1. *Properties of typical and atypical yellow fever virus populations obtained after one and three serial passages in HeLa cells*

Population	Titer in culture, $MICLD_{50}$ (log_{10})	Day of maximal titer	Cell lysis in culture	Per cent recovery in aerosol: 40-50% RH[a]	Per cent recovery in aerosol: 70-80% RH	Monkey lethality: 40-50% RH	Monkey lethality: 70-80% RH
Ty-1[b]	7.1	5	Negative	43.2	46.7	Lethal at 10 $MICLD_{50}$	
ATy-1[c]	8.1	5	Positive	9.7	28.2	Lethal at 10 $MICLD_{50}$	
Ty-3[d]	8.8	3	Positive	9.8	31.5		Nonlethal at >52.5 $MICLD_{50}$
Aty-3[e]	6.9	3	Negative	19.7	12.3		Nonlethal at 800 $MICLD_{50}$

[a] Relative humidity.
[b] "Typical" viral population, representative of harvests obtained in 11 of 12 experiments.
[c] "Atypical" viral population, representative of a harvest obtained in 1 of 12 experiments.
[d] "Typical" viral population, representative of harvests obtained in four of six experiments.
[e] "Atypical" viral population, representative of harvests obtained in two of six experiments.

cells. Encouraged by the results of these efforts, we designed experiments to characterize properties of yellow fever virus in greater detail during serial passage in cell culture. An effort was made during this work to study properties of virus in both the pre- and postaerosolized state and to determine whether any correlation existed among the properties that were altered as a result of passage in vitro. It was found that the viral changes that were reported by Hardy, as well as additional previously undescribed changes, actually began to occur as early as the third serial passage in HeLa cells. We observed alterations in the ability of the virus to grow and induce cell lysis in HeLa cell monolayers, confirming Hardy's published work. We also found changes in the stability of infected cell culture preparations upon aerosolization, and in the level of virulence of such products for rhesus monkeys.

ALTERATIONS IN VIRAL PROPERTIES AFTER CULTIVATION IN HELA CELLS

Recovery of "Typical" and "Atypical" First- and Third-Passage Viral Populations

During the course of our first series of studies, viral products that were prepared in the same or similar manner during either one or three passages did not repeatedly demonstrate the same properties. In other words, although the majority of viral populations behaved in a typical fashion, infrequent atypical populations could be recovered. These proved to be fortuitous events, because this not only allowed for an assessment of the differences between typical one- and three-passage harvests, but, by studying the incidence of variation among properties of typical and atypical viral populations prepared at the same passage level, we were able to gain some insight into possible relationships that existed among individual genetic markers. Of considerable interest was the indication that certain of the properties that were encountered in vitro could be correlated with properties displayed by the virus in aerosols. Examples are shown in Table 1.

It can be seen that typical first-passage preparations possessed titers of approximately 10^7 $MICLD_{50}$ that were obtained on day 5 in the absence of cell lysis. This preparation showed maximal stability during aerosolization at 50% relative humidity (RH) and was lethal for monkeys by the respiratory route. In contrast, typical third-passage preparations, represented in the third column, possessed titers of 10^9 $MICLD_{50}$ (or greater) that were obtained on day 3 attended by cell lysis. The viral recoveries obtained at 50% RH were significantly lower (P <5%) than those obtained at 80% RH. These preparations were not lethal for monkeys by the respiratory route. In column two, properties of an atypical first-passage preparation are shown. A 1-log increase in titer to 10^8 $MICLD_{50}$ was obtained at day 5, accompanied by cell lysis. This preparation was significantly less stable (P <5%) at 50% RH than at 80% RH, but it was lethal for monkeys. Atypical third-passage preparations showed maximal titers of about 10^7 $MICLD_{50}$ at day 3 and did not induce cell lysis. This virus was not appreciably affected by changes in RH, although the overall viral recoveries appeared slightly decreased, and it was not lethal for monkeys.

TABLE 2. *Summary of changes in properties of yellow fever virus (Asibi strain) in HeLa cell cultures*

HeLa cell prepn	Maximal MICLD50/ml (log10) titer in HeLa cells	Day of maximal titer	Cytopathic effect[a]	Effect at 50% RH[b]	Attenuation of virus for monkeys[c]
One passage	6.9-7.4	5	Negative	Resistant	Negative
	8 or >	5	Positive	Sensitive	Negative
Three passages	6.8-7.0	3	Negative	Resistant	Positive
	8 or >	3	Positive	Sensitive	Positive

[a] Cell rounding, increase in density, and detachment from glass.
[b] Effect of aerosolization on virus at 50% relative humidity.
[c] By exposure to infected aerosols.

Comparison of Viral Properties After First and Third Passage

Some of the properties that were established for the various viral harvests appeared to be closely related to one another. An attempt to represent this is shown in Table 2 and by the following examples. (i) Virus grown after one passage in HeLa cells (approximately 10^7 MICLD50 in 5 days) was virulent for monkeys. Conversely, virus grown in HeLa cells after three serial passages (10^8 or greater MICLD50 in 3 days) was attenuated for monkeys. (ii) Virus that had shown a cytopathic effect was adversely affected by aerosolization at 50% RH, but virus that did not induce a CPE was unaffected. (iii) Viral harvests that failed to show maximal titers in excess of 10^7 MICLD50, despite some degree of increased adaptation in HeLa cells (maximal titer at day 3), did not induce a cytopathic effect; harvests that contained titers of 10^8 MICLD50 or greater induced a cytopathic effect in culture.

The results of these experiments also suggested that first-passage viral preparations were stabilized more easily than the third-passage preparations; 1 of 12 of the former populations behaved atypically, whereas 2 of 6 of the latter proved to be atypical. The atypical viral harvests shared properties with both the typical first- and third-passage preparations, and appeared, therefore, to represent intermediate viral populations. Additional work toward the further elucidation of such populations was indicated. Since multiple-passage preparations gave the greatest indication of genetic instability, we concentrated our experimental effort chiefly on characterizing the properties of viral harvests of this type.

FURTHER STUDIES ON VIRAL POPULATIONS AFTER ONE PASSAGE IN HELA CELLS

Studies on virulence were expanded to include a comparison between rates of infectivity after administration of virus by the intraperitoneal (ip) as well as the respiratory route. To accomplish this, graded doses of the same viral preparation isolated from HeLa cells were administered to monkeys by either route; survivors were challenged ip 21 days later with a multiple lethal dose of a mouse brain virus seed to determine whether the original administration of virus had subclinically infected the animals. The first series of tests with this experimental protocol was carried out with first-passage material to obtain base-line information with which to compare the third-passage preparations to be reported later.

TABLE 3. *Response of monkeys to yellow fever virus given by intraperitoneal or respiratory routes after one passage in HeLa cells*

Dose (MICLD50)	Lethality		Infectivity[c]	
	Resp[a]	ip[b]	Resp	Ip
50	6/6[d]		—	
40		6/6		..
5.0	3/6		0/3[d]	
4.0		3/4		1/1
0.5	0/3		0/3	
0.4		3/6		2/3
0.05	NT[e]		—	
0.04		1/3		1/2

[a] Virus administered by respiratory route.
[b] Virus administered by intraperitoneal route.
[c] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.
[d] Number of monkeys affected per number treated with virus.
[e] Not tested.

Properties of Pre- and Postaerosolized Populations After One Passage

The results of these tests are represented in Table 3. Doses given by the respiratory route represent average values from several experiments. They show that doses of approximately 50 MICLD50 given by either route were lethal for the

test monkeys. Doses of approximately 5 MICLD$_{50}$ of aerosolized virus were lethal for one-half of the exposed monkeys. A dose of 4 MICLD$_{50}$, however, was lethal for three of four monkeys injected by the ip route. Three of six monkeys succumbed when given a dose of 0.4 and one of three monkeys succumbed with a dose of 0.04 MICLD$_{50}$.

Animals that survived viral aerosols showed no evidence of having been infected. This was shown by the fact that three monkeys that failed to succumb to respiratory doses ranging from 1 to 10 MICLD$_{50}$ also failed to resist the ip challenge with a multiple lethal dose of suckling mouse brain virus. Thus, the dose that was necessary to infect appeared to be very close if not identical to the dose necessary to cause a lethal illness.

Two of three monkeys resisted a lethal challenge of virus after they survived a dose of 0.4 MICLD$_{50}$ given by the ip route; one of two monkeys that survived the ip dose of 0.04 MICLD$_{50}$ was not resistant. Thus, although there was some evidence that infection by the ip route was not invariably fatal with very low doses, it appears that the median lethal dose and the median infectious dose values obtained by the ip route were much closer to one another with first-passage virus than those obtained with multiple-passaged virus.

FURTHER STUDIES ON VIRAL POPULATIONS AFTER MULTIPLE PASSAGES IN HELA CELLS

Scheme of Tests

The next series of experiments was directed toward elucidating the characteristics of viral populations that arose after multiple serial passages in HeLa cells. Results of tests on two preparations obtained after three serial passages in HeLa cells and one other after seven passages will be presented. The following test scheme was devised for each viral harvest. After inoculation of the virus in culture, that is to say, during preparation of the third- or seventh-passage harvests, samples were obtained from the culture and titrated daily for 6 days postinoculation to establish the maximal titer and the time postinoculation of its occurrence. During that time, the cell sheet was examined microscopically for evidence of cell lysis. Both supernatant fluid and the cells were harvested at or near the time of maximal viral yields, and the material was frozen in glass ampoules. A few days later, a sample of this viral material was titrated intracerebrally in mice and by the ip route in monkeys. Then the preparation was aerosolized at 50 and 80% RH. The amount of virus that could be recovered in the aerosol and the extent to which the property of lethality for monkeys was decreased after the virus was airborne were determined. Values for the former were obtained by plotting recovery values at intervals during the 60-min period following aerosolization.

Virulence for monkeys was ascertained by exposing these animals at various intervals after rendering the agent airborne. Differences in dosage were obtained by exposing the monkeys to clouds of various ages, the older the cloud the smaller the dose. Impinger fluids were collected at intervals corresponding to those during which the monkeys were exposed, and these samples were injected ip into monkeys in such a manner that the same theoretical viral dose was administered to duplicate monkeys by either the respiratory or ip route. As in previous experiments, any monkey that survived the administration of the viral preparations by either route was challenged 21 days later with a multiple lethal dose of a mouse brain virus seed. Two uninfected control monkeys also were challenged in the same manner. The data presented in Table 4 show the results obtained with one third-passage preparation.

Properties of Pre- and Postaerosolized Third-Passage Viral Populations

In Table 4, the data are divided into two main sections. On the left are the results of injecting monkeys intraperitoneally with a third-passage HeLa cell preparation prior to its aerosolization. The most striking feature of these data is the lack of a clear-cut end point in the lethality pattern. Doses ranging from 3 to 3,000 MICLD$_{50}$ were lethal for one-half of the monkeys tested. All survivors were resistant to a multiple lethal dose of virus given 21 days later as an ip challenge. Two other monkeys that were originally injected with 0.3 MICLD$_{50}$ evidently were infected subclinically, since they also resisted the lethal challenge. The dose of 0.03 MICLD$_{50}$ apparently failed to infect the monkeys.

On the right side of Table 4 are the results of administering aerosolized virus to monkeys. Shown are the doses expressed as the number of MICLD$_{50}$, the RH employed during aerosolization of the virus, the lethality and infectivity resulting from exposing the monkeys to aerosolized virus and the lethality and infectivity of the impinger fluid into which the various viral doses were collected postaerosolization.

These data show once again that a clear-cut end point in the lethality pattern did not occur with either route; this was especially evident in monkeys exposed by the respiratory route. Moreover, there was no consistent difference in the incidence of lethality at 50 and 80% RH. This corresponds to the lack of any difference

TABLE 4. *Response of monkeys to yellow fever virus given by intraperitoneal or respiratory routes after three serial passages in HeLa cells*

Preaerosolization			Postaerosolization					
Dose (MICLD$_{50}$)	Intraperitoneal		Dose (MICLD$_{50}$)	RH (%)	Respiratory		Intraperitoneal	
	Leth[a]	Inf[b]			Leth	Inf	Leth	Inf
3,000	1/2[c]	1/1	2,304-915	50	1/2	1/1	0/2	2/2
300	1/2	1/1		80	1/2	1/1	1/2	1/1
30	1/2	1/1	210-54	50	1/2	1/1	2/2	—
3	1/2	1/1		80	2/2		1/2	1/1
0.3	0/2	2/2	12-3	50	2/2		1/2	1/1
0.03	0/1	0/1		80	1/2	1/1	0/2	2/2
			1-<1	50	0/2	0/2	0/2	1/2
				50	1/2	1/1	0/2	2/2

[a] Lethal for monkeys.
[b] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.
[c] Number of monkeys affected per number treated with virus.

TABLE 5. *Response of monkeys to yellow fever virus given by intraperitoneal or respiratory routes after three serial passages in HeLa cells*

Preaerosolization			Postaerosolization					
Dose (MICLD$_{50}$)	Intraperitoneal		Dose (MICLD$_{50}$)	RH (%)	Respiratory		Intraperitoneal	
	Leth[a]	Inf[b]			Leth	Inf	Leth	Inf
50,000	2/2		2,531-1,239	50	0/2	2/2	2/2	—
5,000	1/2	1/1		80	0/2	2/2	2/2	—
500	1/2	1/1	111-54	50	0/2	2/2	0/2	2/2
50	1/2	1/1		80	1/2	1/1	2/2	—
5	0/2	2/2	13-6	50	0/2	2/2	0/2	2/2
0.5	0/2	2/2		80	0/2	2/2	1/2	1/1
0.05	0/2	0/2	2-<1	50	0/2	0/2	0/2	0/2
				80	0/2	2/2	0/2	2/2

[a] Lethal for monkeys.
[b] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.
[c] Number of monkeys affected per number treated with virus.

between the per cent viral recoveries that were obtained at either humidity. Despite this, however, the RH effect might have been an influencing factor in the incidence of nonlethal infections in monkeys. For example, in the lowest dose range, no animals became infected when exposed to aerosols at 50% RH. At 80% RH, however, one of two monkeys succumbed, and the survivor was resistant to the lethal challenge. After injecting impinger fluids by the ip route, a slight increase may have occurred in the incidence of infectivity at 80% RH. Additional evidence of this is shown in data presented in Table 5.

In Table 5, the results of tests with a second third-passage preparation are shown. As with the previous findings, data on the left side of the table obtained with preaerosolized virus show once again the lack of a clear-cut end point in the lethality pattern. On the right side of the table, it can be seen, in contrast to the previous third-passage preparation, that no appreciable lethality was obtained by the respiratory route. Some lethality was found, however, after injecting the impinger fluids by the ip route; the lethality that occurred with the ip route appeared to be more pronounced at the 80% RH. Similarly, the incidence of infectivity was greater with very low doses of this viral preparation after it was aerosolized at 80% RH and administered by either route than when it was aerosolized at 50% RH.

TABLE 6. *Response of monkeys to yellow fever virus given by intraperitoneal or respiratory routes after seven serial passages in HeLa cells*

Preaerosolization			Postaerosolization					
Dose (MICLD$_{50}$)	Intraperitoneal		Dose (MICLD$_{50}$)	RH (%)	Respiratory		Intraperitoneal	
	Leth[a]	Inf[b]			Leth	Inf	Leth	Inf
700,000	0/2[c]	2/2	2,108–1,198	50	0/2	2/2	0/2	2/2
70,000	NT[d]	NT		80	0/2	2/2	0/2	2/2
7,000	0/2	2/2	277–93	50	0/2	2/2	0/2	2/2
700	NT	NT		80	0/2	2/2	0/1	1/1
70	0/2	2/2	12–4	50	0/2	2/2	0/2	2/2
7	NT	NT		80	0/2	2/2	0/2	2/2
0.7	0/2	2/2	1–<1	50	0/2	0/2	0/1	1/1
0.07	NT	NT		80	0/2	2/2	0/2	2/2
0.007	0/2	0/2						

[a] Lethal for monkeys.
[b] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.
[c] Number of monkeys affected per number treated with virus.
[d] Not tested.

TABLE 7. *Number of MICLD$_{50}$ necessary to produce infectivity with or without lethality after passage in HeLa cells*

Passage no.	Preaerosolization		RH (%)	Postaerosolization			
	Intraperitoneal			Respiratory		Intraperitoneal	
	Leth[a]	Inf[b]		Leth	Inf	Leth	Inf
1st	0.04	0.04	50	5	5	NT	NT
			80	5	5	NT	NT
3rd (I)	3	0.3	50	3	3	3	<1
			80	<1	<1	210	<1
3rd (II)	50	0.5	50	>1,239	6	>1,239	<1
			80	210	<1	12	<1
7th	>700,000	0.7	50	>2,108	12	>2,108	1
			80	>1,098	<1	>1,098	<1

[a] Lethal for monkeys.
[b] Infective without lethality; resistant to a multiple lethal challenge dose of mouse brain seed given intraperitoneally.

Properties of Pre- and Postaerosolized Seventh-Passage Viral Population

The final test was carried out with a seventh-passage preparation, which resulted from a continuation of serial passages from the first of the third-passage harvests. Results of this test shown in Table 6 revealed that an increase in the attenuation of the virus had occurred; no lethality was encountered with either the pre- or postaerosolized viral preparations. The incidence of infectivity, as shown by immunity to the lethal challenge, however, did not appear to have declined.

Comparison of Properties of Viral Populations After Multiple Passage in HeLa Cells

In Table 7, we have summarily compared the main points of interest of the three viral preparations. All of the values are expressed as the minimal number of MICLD$_{50}$ that were shown to have induced either a lethal or nonlethal infection in monkeys. The data show that the pre- and postaerosolized first-passage HeLa cell preparation proved to be a comparatively efficient inducer of lethality, although somewhat more virus appeared to be necessary to infect monkeys by the respiratory route than by the ip route. There did

not appear to be a significant difference between the viral response to either RH.

The first of the third-passage HeLa cell harvests showed a decreased level of lethality for monkeys when administered by either route. As the data in Table 4 had indicated previously, the assessment of the virulence of this preparation was greatly influenced by an apparent interference phenomenon that was expressed as a partial inability of this virus to cause lethality over a wide range of doses. Since the first-passage preparation did not display this phenomenon during its lethal effect in monkeys, it is tentatively concluded that the behavior of the third-passage harvest in this case indicates a weakening of the virulence character as a result of serial passage in HeLa cells. Information derived from other multiple-passage preparations clearly supports this view. The second of the third-passage preparations contained virus possessing a level of virulence that had declined to the extent that it failed to produce lethality by the respiratory route; moreover, it had markedly declined in its efficiency in producing lethal illness by the ip route. After seven passages, all traces of lethality for monkeys had vanished from the viral harvest. In all of the viral preparations, the loss in lethality was not accompanied by a loss in the capability of the virus to immunize the animals.

DISCUSSION AND SUMMARY

Manifestation of the viral "RH" marker was more subtle than expected in the two third-passage and seventh-passage preparations. In prior tests with this virus in our laboratory (4), the effect of RH was readily evident in experimental data showing that statistically significant decreases in viral recoveries were encountered upon aerosolization at 50% RH. This was similarly found to be the case with recovery values for airborne Colorado tick fever, vesicular stomatitis, neurovaccinia, and encephalomyocarditis viruses as reported by Watkins et al. (12). In the present results, the initial recovery values were not as high as those previously encountered in our laboratory, and it became necessary to use other criteria to demonstrate any difference that may have occurred as a result of aerosolization at 50 and 80% RH. In cases in which conditions were such that differences could be demonstrated, serially passed virus aerosolized at 50% RH was the least active.

The first of the third-passage preparations provided, at first glance, what might appear to be an exception to this. The dose of 210 $MICLD_{50}$ of the impinger fluid at 80% RH shown in Table 7 as that necessary to produce lethal illness in monkeys by the ip route is higher than that necessary to produce lethal illness at 50% RH. Two factors appear to have been responsible for this. The first is that the dose of 210 $MICLD_{50}$ was inadvertently higher than that planned. On the basis of other data obtained under similar circumstances, the same clinical response would be expected to have been achieved with a much lower dose. Events such as these illustrate, perhaps, an important disadvantage in using the small number of animals that is usually necessary when monkeys must be employed.

In summary, the data presented in this paper clearly indicate that a pronounced loss of virulence rapidly occurred when yellow fever virus was serially passed in HeLa cells. By as early as the third passage, viral populations become demonstrably weakened in their ability to induce lethal illness in rhesus monkeys by either the ip or respiratory route. At this passage level, very high humidities were necessary to sustain even some semblance of lethality. Passage of the virus in cell culture, however, did not appreciably reduce the ability of the virus to induce an immunity in these animals.

From a genetic viewpoint, it is of considerable significance that the third-passage preparations were highly unstable. It is not surprising, therefore, that many of these viral populations possessed properties that varied to some extent from each other, not only in their degree of attenuation, but also in their response to 50% RH, and in their ability to cause a cytopathic effect in cell culture. In a previous publication (4), we noted that atypical first-passage and atypical third-passage viral populations that had been studied up to that time shared no properties with each other. This was unexpected, since both of these presumably represented viral forms that were intermediate to the virulent, aerosol-stable form and the attenuated aerosol-unstable form. Studies on third-passage populations that were recovered and studied since then have supported the view that these viral populations do represent truly intermediate forms. Furthermore, the data indicate that when a sufficient number of these unstable viral populations were examined, atypical intermediate forms that shared some of their properties with each other could be revealed. The genetic determinants for the viral properties that we have examined, therefore, are probably in close relationship to one another but obviously not linked.

The results of these studies raise the question of whether viral isolates of reduced virulence may be commonly acquired after passage in cell cultures. Viral mutants with either lowered

virulence or decreased stability, or both, might be easily selected in an in vitro system. The use of such mutant populations could reduce the danger of airborne contamination of laboratory workers, experimental animals, and other viral or cell culture materials. Venezuelan equine encephalomyelitis virus, another arbovirus, has been shown to lose its virulence in vivo as a result of its serial passage in vitro (3, 5). The question of whether this applies to other arboviruses can be determined only after an adequate number of suitable tests have been performed.

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Discussion

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Your supposition that a Dutch bacteriologist would have more experience of yellow fields of tulips than of yellow fever is absolutely correct. It is thus with great respect that I have read the careful and laborious experiments of Dr. Hearn on which he certainly is to be complimented.

Dr. Hearn described variations in yellow fever virus after passage in HeLa cells: (i) variation in growth rate, growth capacity, and the appearance of cytopathic effects; (ii) attenuation of virulence for monkeys; and (iii) variation in aerosol stability. These correlations are obvious and important. The HeLa cultures were passed at high multiplicity so that mixed populations were studied. Consequently, the bulk of the particles in the atypical first passage (aTy 1) might well have lost the lethality for monkeys, but the population might still contain a few per cent of virulent particles. Again, the difference between the Ty 3 and aTy 3 might be due to interference or a von Magnus phenomenon. Thus, though the populations seem unstable, the variants might be quite stable genetically. Admittedly, yellow fever virology is very difficult, but, unless these variants are isolated from single plaques or passed at limiting dilutions, it is difficult to discuss these variations in terms of genetic markers. I sincerely hope that Dr. Hearn will find opportunity in the future to work in this direction.

The aerosol work is again of the highest level.

The monkeys were exposed to aerosols of different ages. The dose was expressed in terms of $MICLD_{50}$. (Let us hope this unit is constant before and after HeLa passage or aerosolization.) This means that, especially at lower doses, the monkeys receive a few viable particles and very many inactivated particles. Whether these inactivated particles still contain active ribonucleic acid is not known. It might well be then that the large number of inactivated particles in these experiments produced some kind of interference. The additional attenuation by aerosolization itself (Table 5) might also point in this direction. It would be interesting to know what happened when monkeys were exposed to various doses of aerosols of the same age.

All this, of course, detracts nothing from the fact that an important step has been taken in the direction of immunization with an avirulent yellow fever virus. In this connection, it would be important to know whether this virus could multiply in mosquitoes.

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Aerosol Vaccination with Tetanus Toxoid

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INTRODUCTION

In recent years, the efficacy of aerosol vaccination against a variety of infectious diseases has been demonstrated in experimental animals and man. These diseases include Newcastle disease (14), distemper (11), tuberculosis (7, 20), tularemia (9), measles (15, 21, 25), Venezuelan equine encephalitis (29), and influenza (28, 30). Combined aerosol vaccination against Newcastle disease and infectious bronchitis (19) and tularemia and Venezuelan equine encephalitis (16) also has been effective in experimental animals. Russian investigators (1-3) have reported highly effective immunization of animals and man exposed aerogenically to dried living vaccines against anthrax, brucellosis, plague, and tularemia.

The vaccines employed in the above studies consisted mainly of viable attenuated organisms or their spores. Our studies were concerned with the immunological response following inhalation of a soluble antigen-tetanus toxoid. Tetanus toxoid is universally accepted as an effective immunizing antigen. Experience during World War II, when active immunization with this vaccine was first employed on a large scale for American and British forces, attests to its almost complete effectiveness in preventing tetanus (26). Although the initial conventional course of three doses provides long-lasting immunity (possibly for 10 years or more), reinforcing doses are invaluable for maintaining the titer of circulating antitoxin.

Studies in the USSR (23) have shown that aerogenic administration of diphtheria toxoid to experimental animals or man is an effective revaccination procedure after subcutaneous vaccination. A marked and prolonged rise in antitoxin titer was seen in guinea pigs and rabbits administered an aerosol booster of fluid toxoid 5.5 and 3 months after subcutaneous inoculation with aluminum hydroxide-adsorbed diphtheria toxoid (Table 1). The experiments were conducted in a 600-liter chamber. Purified concentrated toxoid with a potency of 2,100 units per ml was dispersed by means of a mechanical atomizer for 60 min.

By increasing the cloud concentration with toxoid electrically atomized with ultrasound in a 30-liter chamber, the Russian scientists reduced the period of aerosol exposure (23). A reduction in aerosol-exposure time from 30 min to 10 to 20 min had no effect upon the antibody response in guinea pigs administered an aerosol booster 3 to 4 months after subcutaneous inoculation with 6 units of adsorbed diphtheria toxoid (Table 2). A marked increase in antitoxin titer also was reported to occur in guinea pigs even after an exposure period of 1 to 2 min. A member of their laboratory staff was reported to have shown a marked rise in antitoxin titer after inhalation of diphtheria toxoid for 5 min.

Aerosol revaccination of children after primary subcutaneous inoculation also has been reported to be effective (24); undue systemic reactions were not observed. On the other hand, an allergic reaction was reported to have occurred in exposed adults. Concentrated dry toxoid given only as a primary dose or as a booster after subcutaneous vaccination was reported to produce increased antibody titers in rabbits and man (1).

AEROSOL VACCINATION WITH TETANUS TOXOID

Our current aerosol vaccination studies with fluid tetanus toxoid were performed with Hartley strain guinea pigs. Young adult guinea pigs of both sexes, weighing 300 to 470 g, were exposed to predetermined respiratory doses of fluid toxoid in groups of five to eight animals. The aerosol chamber consisted of an approximately 300-liter plastic hood installed within a biological safety hood. Purified concentrated fluid tetanus toxoid with a potency of 2,405 limit of flocculation (Lf) units per ml was sprayed with a University of

TABLE 1. *Reimmunization by inhalation of diphtheria toxoid with an exposure time of 60 min*[a]

Animals	No. of animals	Subcutaneous immunization: Dose	Subcutaneous immunization: Mean antitoxin titers 1 month after subcutaneous inoculation	Interval between the first immunization and reimmunization	Mean antitoxin titers before the reimmunization	Mean antitoxin titers (units/ml) after reimmunization by inhalation at various times: Weeks 1	Weeks 2	Weeks 3	Weeks 4	Weeks 6	Months 2	Months 4
		units	*units/ml*	*months*	*units/ml*							
Guinea pigs	10	30	2.64	5.5	0.36	74	118	82	71		34	18.2
Rabbits	7	30	1.52	3	0.62	23.9	14.6		8.4	5		1.1

[a] Data from Muromstev et al. (23).

TABLE 2. *Reimmunization by inhalation of diphtheria toxoid with different exposure times*[a]

Group of animals	No. of guinea pigs	Subcutaneous inoculations: Dose	Subcutaneous inoculations: Mean antitoxin titers 1 month after immunization	Interval between inoculations	Mean antitoxin titers before reimmunization	Exposure time for reimmunization by inhalation	Mean antitoxin titers (units/ml) at various times after reimmunization by inhalation: Weeks 1	Weeks 2	Weeks 3	Weeks 4	Months 2	Months 4
		units	*units/ml*	*months*	*units/ml*	*min*						
First	8	6	1.4	3	0.83	30	71.2	88.7	101.5	62		19.1
Second	9	6	1.24	3	0.96	30	93.7	93.2	88.1	59.3		18.1
Third and fourth	11	6	1.16	4	0.97	10–20	83.2		114.2	66.6	27.5	

[a] Data from Muromstev et al. (23).

Chicago Toxicity Laboratory-type atomizer. The vaccine contained no preservatives and was obtained through the courtesy of Eli Lilly & Co. The fluid vaccine was fed to the atomizer by a 50-ml syringe, actuated with a motor-driven piston delivering 0.4 ml/min. Filtered air was supplied to primary and secondary inlets of the atomizer at a flow rate of approximately 1 ft^3/min.

A particle discrimination device, a British preimpinger, was used in the sampler system to obtain information on the particle diameter of fluid toxoid clouds. The preimpinger was fitted to a standard all-glass impinger (AGI-30) to select particles with diameters equal to or less than 5 μ in the collecting fluid. Physiological saline, the collecting fluid, was assayed for toxoid concentration by the flocculation test. Tetanus flocculating serum was furnished by the Division of Biologics Standards, National Institutes of Health. The respiratory dose was calculated as the product of the aerosol concentration, minute volume of respiration (27), and duration of exposure. Precalculated respiratory doses were administered by varying the duration of aerosol exposure.

TABLE 3. *Primary antibody response 5 weeks after aerosol or subcutaneous vaccination with fluid tetanus toxoid and survival 14 days after subcutaneous challenge with tetanus toxin*

Primary vaccination: Route	Primary vaccination: Dose (Lf)	Hemagglutination titer: Geometric mean	Hemagglutination titer: Range	Survival after challenge: Survivors/total	Survival after challenge: Per cent
Respiratory	4[a]	24	<10–320	1/7	14.2
	10[a]	79	<10–1,950	4/13	30.7
	15[a]	200	10–640	7/13	53.8
	19[a]	336	<10–2,560	3/8	37.5
	32[a]	72	<10–1,280	4/7	57.1
Subcutaneous	9	1,158	300–4,800	6/6	100.0
	15	4,609	600–19,200	6/6	100.0

[a] Calculated respiratory dose ($\pm$1 Lf); $\leq$5-μ particles.

PRIMARY AEROSOL VACCINATION AND CHALLENGE

The primary antibody response of guinea pigs after inhalation of various doses of aerosolized fluid tetanus toxoid (particles $\leq 5\ \mu$) and survival data taken after subcutaneous toxic challenge are

TABLE 4. *Serum hemagglutination titer and survival of aerosol-vaccinated guinea pigs after subcutaneous challenge with tetanus toxin*

Hemagglutination titer	Survival after challenge	
	Survivors/total	Per cent
<160	3/29	10.3
>160–<640	5/8	62.5
>640	11/11	100.0

summarized in Table 3. The duration of aerosol exposure varied from 20 to 80 min. Control animals were vaccinated with fluid toxoid by the subcutaneous route. Blood samples for serological analysis were obtained by cardiac puncture 5 weeks after vaccination, and the guinea pigs were challenged 1 week later with 10 guinea pig MLD of tetanus toxin administered by the subcutaneous route. The results demonstrated that mean serum antibody titers, measured by the passive hemagglutination (HA) procedure (31), were lower after inhalation of toxoid than after subcutaneous inoculation with comparable doses of fluid toxoid.

A mean serum HA titer of approximately 526 or greater was protective against subcutaneous challenge with 10 MLD of tetanus toxin. The relationship between HA titer of aerosol-vaccinated guinea pigs and survival after toxic challenge is summarized in Table 4. Guinea pigs vaccinated by the subcutaneous route had titers ranging from 300 to 19,200 and did not succumb to the toxic challenge dose (Table 3).

One possible contributing factor to the range of antibody responses following aerosol vaccination (Table 3) may be variations in the breathing capacity or pattern of individual guinea pigs. Since minute volume of respiration was not determined for the individual guinea pigs and is based on a reference value, variations may be expected to occur in the actual inhaled dose. It is unlikely, however, that the irregularities in antibody response could be attributed wholly to differences in breathing. The response seen with respiratory doses of 15 and 19 Lf units certainly should have been eliminated at 32 Lf units if this were the only explanation.

The position of the animals in the aerosol chamber in relation to the atomizer did not appear to influence the antibody response. Cloud samples, obtained throughout the aerosol exposure period, were quite uniform in toxoid concentration, as determined by the flocculation test. Other factors which can influence the efficiency of aerosol immunization are discussed below.

AEROSOL REVACCINATION

Results from experiments to evaluate the secondary antibody response following inhalation of atomized fluid tetanus toxoid are summarized in Tables 5 and 6. Guinea pigs were bled for HA

TABLE 5. *Secondary antibody response with aerosol booster 6 weeks after inhalation of fluid tetanus toxoid*

Respiratory dose (Lf)		No. of guinea pigs	Determination	Hemagglutination titer			
				Prebooster[a]	Weeks after booster		
Primary	Secondary				1	5	10
8	6	14	Mean[b]	47	3,898	5,099	953
			Range	<10–1,280	10–256,000	<10–128,000	10–48,000
6	14	7	Mean[b]	176	9,696	9,729	3,772
			Range	10–1,920	<10–192,000	1,280–384,000	400–32,000
14	5	7	Mean[b]	732	188,988	31,051	7,463
			Range	10–12,800	12,800–1,024,000	4,000–192,000	2,000–48,000
					Weeks after primary		
					7	11	16
11	0	4	Mean[b]	708	774	235	132
			Range	160–2,560	<10–2,400	<10–9,600	<10–1,920
21	0	4	Mean[b]	80	293	280	140
			Range	<10–5,120	<10–25,600	<10–16,000	<10–4,000

[a] At 5 weeks after primary vaccination.

[b] Geometric mean.

TABLE 6. *Secondary antibody response with aerosol booster 6 weeks after subcutaneous vaccination with fluid or alum-precipitated tetanus toxoid*

Subcutaneous vaccination	Dose (Lf)	Respiratory booster dose (Lf)	No. of guinea pigs	Determination	Hemagglutination titer			
					Prebooster[a]	Weeks after booster		
						1	5	10
Fluid toxoid	7.5	5	3	Mean[b]	29,081	133,126	146,520	66,463
				Range	6,400–102,400	96,000–192,000	64,000–384,000	24,000–192,000
		9	11	Mean[b]	5,335	101,640	74,965	24,490
				Range	1,280–25,600	32,000–384,000	48,000–128,000	8,000–64,000
		0	5	Mean[b]	10,808	4,095[c]	8,386	6,553
				Range	6,000–16,000	2,000–12,000	3,000–48,000	400–25,600
Alum-precipitated toxoid	7.5	3	4	Mean[b]	36,472	627,070	583,560	524,280
				Range	12,000–192,000	384,000–1,024,000	256,000–768,000	256,000–1,024,000
		10	11	Mean[b]	52,050	694,130	286,410	317,490
				Range	8,000–32,000	96,000–3,072,000	32,000–1,024,000	64,000–768,000
		0	8	Mean[b]	6,586	10,024[c]	11,272	8,088[d]
				Range	1,000–16,000	2,000–32,000	2,400–25,600	2,400–16,000

[a] At 5 weeks after primary vaccination.
[b] Geometric mean.
[c] At weeks after primary vaccination.
[d] One guinea pig lost from group.

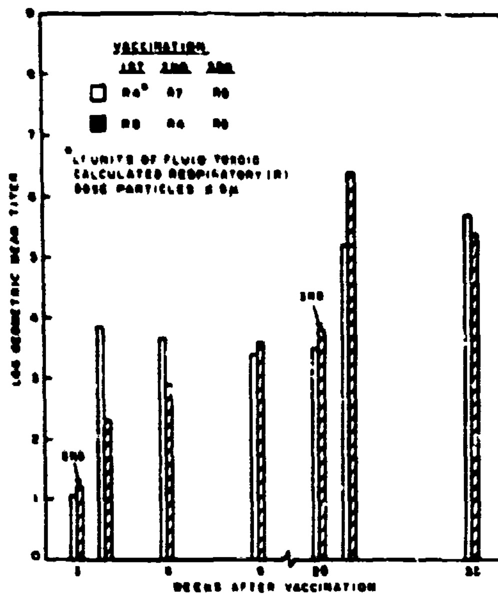

FIG. 1. *Serum hemagglutinating antibody response in guinea pigs after vaccination series with fluid tetanus toxoid administered by the respiratory route.*

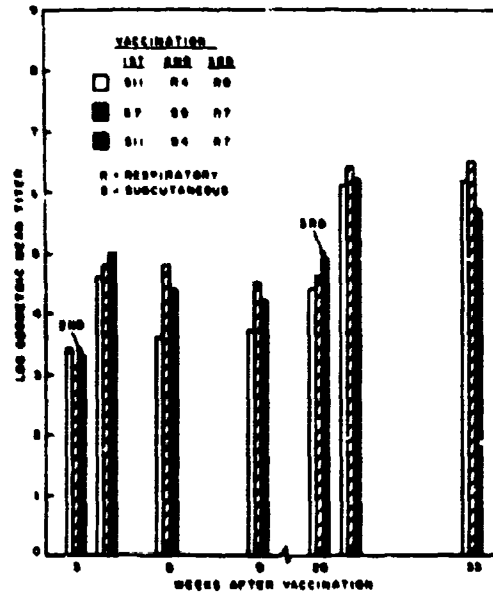

FIG. 2. *Serum hemagglutinating antibody response in guinea pigs after vaccination series with fluid tetanus toxoid administered by combinations of subcutaneous and respiratory routes.*

antibody 5 weeks after the primary vaccination and 1, 5, and 10 weeks after the aerosol booster.

Aerosol revaccination of guinea pigs sensitized initially by the respiratory route resulted in an enhancement of the HA antibody response (Table 5). The range of titers indicates, however, that adequate sensitization was not achieved in all animals. A few guinea pigs showed essentially no serum antibody response after either primary or secondary aerosol stimulation. However, when sensitization was adequate, a marked enhancement of the antibody response occurred in all guinea pigs after secondary aerosol exposure to tetanus toxoid. This is seen with animals administered a primary respiratory dose of 14 Lf units, followed by an aerosol booster with 5 Lf units of toxoid. The control guinea pigs received respiratory doses approximately equal to the total doses employed in the revaccination studies.

Aerosol revaccination of guinea pigs inoculated initially by the subcutaneous route with a standard dose of either fluid toxoid or alum-precipitated toxoid (Table 6) was more effective than respiratory-respiratory vaccination. In this case, adequate sensitization of all guinea pigs was achieved prior to the aerosol booster. The response was superior in animals receiving the alum-precipitated toxoid preparation as the primary sensitizing dose.

In another experiment, the HA antibody response was followed over a period of approximately 8 months, with small groups of guinea pigs revaccinated by the aerosol route after a combination of vaccine treatments with fluid tetanus toxoid. The mean serum HA antibody response was measured at various intervals with pairs of guinea pigs revaccinated 3 and 28 weeks after the primary vaccination.

The mean serum HA antibody response after a vaccination series of three respiratory doses of fluid toxoid is summarized in Fig. 1. Although the response was minimal 3 weeks after primary inhalation of 4 or 8 Lf units of fluid toxoid, revaccination by the respiratory route resulted in a marked enhancement of the antibody response after secondary and tertiary stimulations. The mean titer prior to the tertiary respiratory booster was 4,280 for both groups. After tertiary inhalation of vaccine, the titers of the individual guinea pigs were >192,000. The total calculated respiratory dose for both groups was approximately 20 Lf units. A subsequent challenge to one guinea pig from each subgroup with 10 MLD of tetanus toxin administered by the subcutaneous route did not result in signs of tetanus or death during a 14-day observation period. Unfortunately, one guinea pig from each group died from the intracardial bleeding procedure prior to the toxic challenge.

Figure 2 summarizes the mean serum HA antibody response after respiratory revaccination of guinea pigs initially vaccinated by the subcutaneous-subcutaneous or subcutaneous-respiratory routes with fluid toxoid. The total dose administered in these experiments was approximately 23 Lf units. The antibody response was enhanced in all three groups of animals after secondary and tertiary stimulations by a combination of subcutaneous and respiratory treatments.

Two groups of guinea pigs administered a vaccination series of two subcutaneous doses followed by a respiratory dose showed comparable responses, although the subcutaneous doses were given in different combinations. Prior to inhalation of the vaccine, the mean HA titer of both groups was 50,904. After the respiratory booster, the individual titers of the guinea pigs were >512,000.

Guinea pigs receiving the subcutaneous-respiratory vaccination series showed only a transient increase in titer after the respiratory booster. The titer essentially dropped to its prebooster level by the 6th week and then increased to 25,600 by the 25th week after the secondary booster. This "delayed response," which was also evident to a lesser degree in animals vaccinated with similar doses by the subcutaneous-subcutaneous routes, has been previously reported in guinea pigs after two injections of diphtheria toxoid (5). After the tertiary aerosol booster, the individual titers of the guinea pigs were >256,000.

The long-term antibody response therefore was comparable in all three groups. None of the guinea pigs responded during a 14-day observation period to 10 MLD of tetanus toxin administered by the subcutaneous route.

PARTICLE-SIZE RELATIONSHIP

As reviewed by Langmuir (18), the recognition that respiratory deposition and retention of inhaled particles vary with aerodynamic particle size is perhaps the most significant finding in aerobiology during the past two decades.

Recent studies by several investigators (6, 13, 22) indicate that the critical diameter of particles for deep lung deposition and retention is 1 to 5 μ. Hatch (13) reported that deposition of inhaled particles in the respiratory spaces of the lung occurs with the highest probability with particles 1 to 2 μ in diameter (gravity settlement) and with those in the submicroscopic region below 0.2 μ (precipitation by diffusion). Particles larger than 5 μ may not reach the respiratory bronchioles and alveoli; most between 0.25 and 0.5 μ may be exhaled.

TABLE 7. *Absolute recovery of sodium fluorescein-tetanus toxoid cloud particles at various relative humidities (RH)*

Impactor particle size	Mean recovery (μg ± SD)		
	61-70% RH	71-80% RH	81-90% RH
μ			
16	5.8 ± 0.4	6.5 ± 0.3	5.9 ± 1.3
8	17.7 ± 3.2	19.5 ± 1.3	17.6 ± 4.0
4	9.1 ± 3.0	8.9 ± 0.5	8.5 ± 1.7
2	2.3 ± 0.4	3.1 ± 0.4	2.6 ± 0.6
1	2.7 ± 1.0	1.6 ± 0.3	1.5 ± 0.2
0.5	2.4 ± 1.0	0.5 ± 0.3	0.5 ± 0.1
0.25	2.9 ± 1.2	0.7 ± 0.3	0.2 ± 0.1
Total	42.9 ± 5.0	40.8 ± 1.8	36.8 ± 5.7
No. of trials	4	7	5

The importance of aerosol particle size to airborne pathogenesis is illustrated by the studies reported by Goodlow and Leonard (10). These investigators demonstrated a relationship between particle diameter and LD_{50} in guinea pigs and monkeys exposed to aerosols of *Pasteurella tularensis*. The LD_{50} for guinea pigs increased by almost 4 logs as the particle diameter increased from 1 to 12 μ.

The critical parameters for quantitative characterization of aerosols have been recently summarized by Goodlow and Leonard (10) and Wolfe (32). Basic parameters such as particle-size distribution, physical and biological decay rates, age of aerosol, and technique of aerosolization are critical not only in experimental airborne infection but also in aerosol immunization.

The particle-size distribution of tetanus toxoid clouds was studied with a Battelle miniature cascade impactor (BMCI), with sodium fluorescein as a physical tracer. Fluorometric assay of each stage of the BMCI was performed with a Coleman 12C electronic photofluorometer. Tables 7 and 8 summarize the absolute recovery and the cumulative recovery, respectively, of sodium fluorescein from each stage of the impactor during 16 trials conducted with sodium fluorescein-tetanus toxoid clouds at various relative humidities. The sensitivity of the particle-sizing device did not allow measurement of particles in the submicroscopic region below 0.25 μ. Also, since the sensitivity of relative humidity measurements can vary by approximately 5%, the data are grouped arbitrarily into three humidity ranges.

The data presented in Table 7, expressed as the mean absolute recovery of sodium fluorescein, suggest higher recoveries of particles less than

TABLE 8. *Cumulative recovery of sodium fluorescein-tetanus toxoid cloud particles at various relative humidities (RH)*

Impactor particle size	Determination	Recovery (%)		
		61-70% RH	71-80% RH	81-90% RH
μ				
16	Mean	100.0	100.0	100.0
8	Mean	86.2	83.8	82.4
	Range[a]	79.0-93.4	78.3-89.3	75.9-88.9
4	Mean	45.8	36.1	35.6
	Range[a]	39.9-51.7	31.6-40.6	30.3-40.9
2	Mean	26.0	14.1	12.7
	Range[a]	17.5-34.5	7.7-20.5	5.1-20.3
1	Mean	20.4	6.5	5.7
	Range[a]	13.1-27.7	0.9-12.1	0.0-12.3
0.5	Mean	13.4	2.7	1.7
	Range[a]	8.7-18.1	0.0-6.2	0.0-5.9
0.25	Mean	6.7	1.5	0.4
	Range[a]	3.8-9.6	0.0-3.7	0.0-3.0

[a] Values are 95% confidence limits for the estimate of the mean, with variance pooled across humidities for each particle size.

2 μ in diameter at humidities below 70%. The same data, expressed as cumulative per cent recovery in Table 8, indicate a similar trend toward recovery of smaller particles at humidities below 70%. Since only a few trials were performed for each humidity range, the data are presented only as a preliminary conjecture for possibly relating particle-size distribution of aerosolized fluid tetanus toxoid to degree of immunological response.

In a preliminary communication (33), we reported that, after inhalation of 9 or 15 Lf units of toxoid, three of eight and six of seven guinea pigs, respectively, survived a challenge of 10 MLD of tetanus toxin given subcutaneously 6 weeks after the primary vaccination. The mean HA titers were 71 and 278 for the 9- and 15-Lf doses, respectively, 5 weeks after vaccination. The primary antibody responses at 5 weeks from these and subsequent aerosol immunizations are summarized according to relative humidities above and below 70% in Table 9. The data suggest a possible relationship between particle-size distribution of toxoid as a function of relative humidity and degree of immunological response. Until additional trials are conducted at lower humidities, however, the results are not conclusive.

One of the critical parameters that requires further investigation is the antigenic stability of aerosolized tetanus toxoid at various relative humidity ranges. The problem of aerosol decay is less acute in a continuously generated aerosol than in a static aerosol. As indicated earlier,

TABLE 9. *Relationship between relative humidities above and below 70%, and primary antibody responses 5 weeks after inhalation of various doses of fluid tetanus toxoid*

Relative humidity range	Calculated respiratory dose[a] (Lf)	No. of guinea pigs	Hemagglutination titer		
			Geometric mean	Range	Per cent ≥320
%					
71-80	9	12	37.7	<10-1,280	25.0
	15	17	534	<10-12,800	58.8
	20	5	199	<10-5,120	40.0
61-70	9	24	171	<10-2,560	41.7
	15	15	75	<10-960	40.0
	20	15	561	<10-5,120	86.7

[a] Calculated respiratory dose (±2 Lf units).

TABLE 10. *Antigenicity of aerosolized tetanus toxoid administered subcutaneously*

Fluid tetanus toxoid	Subcutaneous dose (Lf)	No. of guinea pigs	Geometric mean hemagglutination titer		
			1 week[a]	3 weeks	6 weeks
Aerosol[b]	7.5	3	<10	1,280	8,776
Stock	7.5	3	<10	1,846	7,241[c]
Stock	5.0	3	<10	1,016	5,120
Stock	2.5	3	<10	528	1,099

[a] Weeks after primary vaccination.
[b] AGI-30 sample (≤5-μ particles) collected in physiological saline.
[c] One guinea pig lost from group.

tetanus toxoid clouds were quite uniform in their flocculating capacity throughout aerosol exposure. Mean nominal per cent recoveries of toxoid as determined from AGI-30 samplers were 7.6 and 6.6% for humidity ranges from 61 to 70% and 71 to 80%, respectively. The loss of tetanus toxoid immunogenicity was not significant when guinea pigs were inoculated subcutaneously with aerosolized toxoid material collected at a relative humidity of approximately 85%. The antibody response was comparable to that achieved in guinea pigs inoculated subcutaneously with equivalent concentrations of stock fluid toxoid (Table 10).

Many other factors should be considered in relating aerodynamic particle size to degree of penetration and retention in the lungs. The breathing pattern and capacity of the experimental animal at the time of aerosol exposure may be influenced by such conditions as excitability and stress. The physical and chemical nature of

the aerosolized material and its composition considerably influence its passage down the respiratory tree during inspiration. The interaction of small and large particles in a heterogeneous aerosol may determine the particle-size diameter during the passage of an aerosol through the respiratory tract.

Since air in the lungs is nearly saturated with water vapor, particles sensitive to humidity may increase in size immediately after reaching the nostrils or trachea, where the relative humidity is above 90% (8). The activity or function of the mucociliary system in the upper respiratory tract must be considered in determining the fate of an inhaled foreign particle (4, 6). However, in contrast to a particulate substance, soluble material would be expected to be absorbed rapidly from most surfaces within the respiratory tract, thereby obviating the necessity of other physiological processes (6). These and other factors, such as electrostatic charge on the particle and on the body of the experimental animal (22), play a vital role in the effectiveness of penetration and retention of particles in the respiratory tract.

The preliminary data presented, from studies still in progress, serve only to emphasize the potential of immunization by inhalation of a nonviable soluble antigen. That effective primary immunization of guinea pigs can be achieved by aerosol vaccination is evident. An aerosol booster after either primary respiratory or primary subcutaneous vaccination appears to be an effective reimmunization procedure. Attempts to relate discrepancies in the immune response to particle-size distribution of aerosolized fluid tetanus toxoid resulted in data that, although not conclusive, provide a working basis for more definitive studies relating aerodynamic particle size to degree of immunological response. As indicated by Lamanna (17), definitive studies are lacking on the mechanisms of permeability of respiratory tract tissue to substances of high molecular weight.

Of obvious concern during the guinea pig immunization studies was the possible induction of hypersensitivity in the animals by administration of toxoid by the respiratory route. The guinea pig is especially prone to development of the hypersensitive state. Untoward reactions were not observed after primary aerosol vaccination with fluid toxoid or after secondary aerosol treatment in combination with aerosol or subcutaneous vaccination procedures. However, guinea pigs receiving two subcutaneous doses of alum-precipitated toxoid at an interval of 3 weeks, followed by a tertiary aerosol booster with fluid toxoid 26 weeks later, developed signs resembling anaphylaxis, resulting in death of some of the animals. The important consideration here which requires closer and further evaluation is the dosage, interval, and frequency of vaccinations

SUMMARY AND CONCLUSION

The method of aerosol vaccination might confer superior protection against pathogenic microorganisms whose natural portal of entry is the respiratory tract (29). Although inhalation of tetanus toxin per se represents an unnatural circumstance, except as an intentional act of war (17), aerosol vaccination with tetanus toxoid, and possibly other soluble antigens derived from pathogenic microorganisms, may provide a rapid and painless method of immunization.

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Discussion

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It was clearly shown by Dr. Yamashiroya that inhalation of purified tetanus toxoid will induce a well-marked primary immune response in the guinea pig, and that the secondary response following toxoid inhalation by a primed animal is even better. Dr. Yamashiroya's results seem to indicate that the relative humidity, by affecting the particle size distribution of the aerosol, may influence its immunogenic effectivity. It should be observed that the size distribution of droplet nuclei is mainly determined by the concentration of the spray solution, and it would seem worthwhile to study comparatively the immune responses of guinea pigs in Dr. Yamashiroya's experimental model by varying the concentration of the sprayed toxoid solution inversely to the period of exposure.

Site of deposition, in relation to particle size, is generally assumed to be an important factor in determining the infective or immunizing effects of inhaled aerosols (3, 5). However, various other factors, in addition to influences on the retention and site of deposition of the particles, as properly referred to by Dr. Yamashiroya, may affect the mechanisms underlying the immune response after the inhalation of antigens.

In a study on aerosol immunization against tetanus, we have investigated the effect of adding bacterial adjuvants to the toxoid spray solution. Groups of 20 mice were exposed in a modified Henderson apparatus (7) to the immunizing aerosols, sprayed with a Collison nebulizer from solutions containing 7,000 to 10,000 limit of flocculation (Lf) units of toxoid per milliliter. It should be admitted that, in comparison with guinea pigs, mice are less suitable for immunization studies with unabsorbed fluid tetanus toxoid, the results being rather irreproducible (1). A further disadvantage is caused by the fact that it is hardly feasible to follow the serum antibody level over a prolonged period of time in individual animals, as was done by Dr. Yamashiroya in guinea pigs. Nonetheless, in our experimental model, we were able to demonstrate that the primary immune response following toxoid inhalation is strikingly enhanced by the addition of certain bacterial adjuvants.

Immune responses were evaluated, 3 weeks after immunization, by subcutaneous challenge with 50 mouse LD$_{50}$ of toxin, and by antitoxin titrations in the pooled sera of small groups of animals. All sera were titrated in vitro by the indirect hemagglutination (HA) technique, as described by Stavitsky (10); in addition, a number of sera were also titrated in vivo by the mouse protection test on a 0.001 antitoxin unit (AU) level. Toxoid aerosols were sampled in liquid impingers and evaluated by indirect hemagglutination inhibition tests (10). Inhalation doses were estimated from cloud sample assays, periods of exposure, and mouse respiratory volumes as defined by Guyton (4).

The results of a series of experiments with

TABLE 1. *Primary immune response of mice 3 weeks after administration of plain tetanus toxoid by aerosol or by subcutaneous injection*

Route of administration	Dose (Lf)	Survivors 5 days after challenge[a]	Antitoxic serum titer (hemagglutination)[b]
Aerosol	10-12	0/20	ND
	20-24	1/20	ND
	40-48	1/16	<10
Subcutaneous injection	0.5	11/20	128
	1.0	20/20	ND
	2.0	20/20	320

[a] Number of survivors/number challenged.

[b] Hemagglutination titrations of pooled sera of four animals from each group; ND, not determined.

TABLE 2. *Immune response of mice 3 weeks after repeated administration of plain tetanus toxoid by various combinations of inhalation and subcutaneous injection*

First dose (Lf)	Second dose (Lf)[a]	Survivors 5 days after challenge[b]	HA antitoxic serum titer[c]
10-12 (R)	10-12 (R)	1/20	ND
0.5 (I)	10-12 (R)	16/16	4,000
0.5 (I)	0.5 (I)	16/16	4,000
10-12 (R)	—	0/20	ND
0.5 (I)	—	11/20	128

[a] Doses given 3 weeks apart.

[b] Number of survivors/number challenged.

[c] HA titrations of pooled sera of four animals from each group. ND, not determined; (R), given by the respiratory route; (I), given by injection.

aerosols of plain toxoid are presented in Table 1. In preliminary tests, the 50% protective dose by subcutaneous injection had been found to be in the order of 1 Lf; therefore, control animals were given toxoid by the subcutaneous route in doses ranging from 0.5 to 2.0 Lf. The figures clearly demonstrate that under these experimental conditions almost no immune response is elicited in mice by a single inhalation of plain toxoid at dosages up to 40 Lf. On the other hand, a single injection of a dose as small as 0.5 Lf induces partial protection.

Results obtained with aerosol treatment in two doses given 3 weeks apart are presented in Table 2. Also shown are the effects of a booster treatment by aerosol in comparison with that of an injection. It is seen that inhalation of plain toxoid on two occasions did not induce a better immune response than did a single inhalation. However, when given as a booster to mice already primed by injection, the toxoid inhalation provoked a marked anamnestic reaction, similar to that induced by a booster injection.

The strong potentiating effect of a bacterial adjuvant on the immunizing activity of inhaled toxoid is illustrated by the data shown in Table 3. Killed *Bordetella pertussis*, an effective adjuvant (9), was mixed with the plain toxoid solution to a concentration of approximately 4×10^{10} organisms per milliliter. Control animals received adequate doses of the same mixtures by injection. Since no figures are available for the retention of the components of the inhaled mixture, a comparison of the dosages used for immunization by the respiratory route and by injection cannot be made. Nonetheless, it is evident that toxoid given by aerosol together with *B. pertussis* cells conferred a significant degree of immunity (compare Tables 1 and 2), and that, at the given doses, both the primary and secondary immune responses were not inferior to those following injection.

It has been shown by others (2) that, in stimulating the antibody response to protein antigens, *B. pertussis* extracts may be as active as whole-cell preparations. To investigate the effect of such an extract on the immunogenicity of the inhaled toxoid, additional experiments were carried out. Two fractions, which had been derived from *B. pertussis* in the procedure for the preparation of a soluble pertussis vaccine at the National Institute of Public Health, Utrecht (6), were tested. According to data provided by the laboratory for vaccine production of the National Institute of Public Health, one of these

TABLE 3. *Immune response of mice 3 weeks after single or repeated administration of a mixture of tetanus toxoid and Bordetella pertussis cells by aerosol or by subcutaneous injection*

First dose (Lf)	Second dose (Lf)[a]	Survivors 5 days after challenge[b]	Antitoxic serum titer[c]	
			Hemagglutination	Mouse test
16 (R)	—	15/16	128	ND
0.5 (I)	—	13/16	ND	ND
16 (R)	16 (R)	16/16	16,000	1.15 AU
0.5 (I)	0.5 (I)	16/16	8,000	0.80 AU

[a] Doses given 3 weeks apart.
[b] Number of survivors/number challenged.
[c] Titrations of pooled sera of four animals from each group; ND, not determined (R), given by the respiratory route; (I), given by injection.

TABLE 4. *Immune response of mice 3 weeks after single or repeated administration of mixtures of tetanus toxoid and Bordetella pertussis preparations by aerosol*

Mixture	First dose (Lf)	Second dose (Lf)[a]	Survivors 5 days after challenge[b]	Antitoxic serum titer[c]	
				Hemagglutination	Mouse test
T + C	8–10	—	20/20	ND	ND
T + I	8–10	—	20/20	ND	ND
T + II	8–10	—	16/20	ND	ND
T + C	8–10	8–10	10/10	320,000	32 AU
T + I	8–10	8–10	10/10	160,000	32 AU
T + II	8–10	8–10	10/10	80,000	8 AU
T Pl.	16–20	—	0/10	ND	ND
T Pl.	16–20	16–20	0/20	ND	ND

[a] Doses given 3 weeks apart.
[b] Number of survivors/number challenged.
[c] Titrations of pooled sera of 10 animals from each group; ND, not determined; T + C, toxoid + whole cells; T + I, toxoid + extract; T + II, toxoid + cell wall debris; T Pl., plain toxoid.

fractions, the soluble extract, exhibited nearly all the biological activity contained in the original cell suspension, in terms of immunizing potency and histamine-sensitizing factor. In the other fraction, consisting of cell wall debris, no such biological activity could be discovered. As can be seen from the data summarized in Table 4, the extract did indeed show an adjuvant activity similar to that of the whole-cell preparation. However, the cell wall debris also appeared to be an active adjuvant. A further analysis of these fractions will be needed to determine the factors responsible for the observed adjuvant effects.

From these experiments, as compared with those of Dr. Yamashiroya, it may be concluded that mice show a much weaker immune response to inhaled plain tetanus toxoid than do guinea pigs. This may be associated with a lower degree of cellular reactions, in terms of phagocytic activity and lymphoid hyperplasia. Such a difference between mice and guinea pigs has also been observed by Henderson in his studies on the infectious processes developing after inhalation of certain bacterial pathogens (8). The highly potentiating effect of *B. pertussis* preparations in aerosol vaccination of mice has been clearly demonstrated in our experimental model. The histological changes in the respiratory tract and adjacent lymphoid tissues of the mouse, associated with this adjuvant effect, are being studied.

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Aerosol Infection of Monkeys with *Rickettsia rickettsii*

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INTRODUCTION

There are many infectious diseases which are not transmitted via the air-borne route. Such is the case with Rocky Mountain spotted fever. Studies in both man and animals have clearly established that transmission occurs through the bite of an infected tick and less frequently through abrasions in the skin contaminated with infected tick feces or tissue juices.

Recent studies indicate that aerosols of arthropod organisms, under certain conditions, can lead to disease (10, 20, 21, 24, 25). To our knowledge, human infection due to aerosol exposure to *Rickettsia rickettsii* has not been documented. Rocky Mountain spotted fever of man has characteristic features which are remarkably similar to those of monkeys injected with infectious material (1, 27). It remained for experimental studies to determine whether the portal of entry of the infectious agent alters the characteristics of the disease, its pathology, and the response to prophylactic and therapeutic measures. It is the purpose of this paper to describe the results observed in monkeys challenged with *R. rickettsii* by the respiratory route.

RESPONSE OF NORMAL MONKEYS TO PRIMARY CHALLENGE

Clinical Aspects

During the course of these studies (17), rhesus and cynomolgus monkeys were exposed in a model 3 Henderson apparatus (7, 15) to aerosols of the R1 strain (13) of *R. rickettsii* propagated in the yolk sac of developing chick embryos (23). Assuming that the respiratory volume was about 1 liter (4) and that about 50% of the particles were retained (6), the monkeys received challenges varying from 1.5 to 9,000 yolk sac LD_{50}. Clinically obvious disease was observed in 56 of 60 (93.3%) rhesus monkeys, and 42 of 56 (75.0%) died of specific infection 7 to 24 days postchallenge; 38 of 56 (67.9%) exhibited rash.

Clinical findings in the 56 rhesus monkeys that become ill after challenge are summarized in Table 1. Fever was observed in all; rectal temperatures of 105 to 106 F (40.6 to 41.1 C) were common. Monkeys usually became febrile 5 to 7 days after exposure and 1 to 2 days before appearance of other symptoms. Monkeys usually survived if their fever did not persist for more than 2 or 3 days.

Lethargy, anorexia, and weakness were first observed from the 6th to 9th postexposure day (PED). Monkeys sat quietly with heads hanging and arms clasped about the body and were noticeably weak when removed from the cage. In monkeys that eventually died, symptoms became progressively worse during the next few days. Animals would not respond to stimuli, did not resist handling, and could not rise from a lying position. Monkeys usually were in coma for 12 to 24 hr prior to death. Terminally, the pupils were dilated and nonreactive, and patellar reflexes were absent. Respirations were usually deep, abdominal, and at the rate of 4 to 6 per min. The body was cold on palpation, and the rectal temperature was less than 100 F (37.8 C).

TABLE 1. *Clinical findings in 56 rhesus monkeys after respiratory challenge with Rickettsia rickettsii*[a]

Clinical finding[b]	No. of monkeys	Per cent
Fever	56	100.0
Chills or tremors	8	14.3
Lethargy, anorexia, weakness	56	100.0
Heart sounds less intense and muffled	53	94.6
Respiratory signs[c]	12	21.4
Dehydration	9	16.1
Diarrhea	3	5.4
Rash	38	67.9
Peripheral necrosis	9	16.1

[a] Data from Saslaw et al. (17).

[b] Delirium, 2; pruritis (general), 2; epistaxis, 1; hemorrhagic rhinitis, 2; purulent nasal exudate, 1; serous conjunctivitis, 1; suppurative conjunctivitis, 1; suppurative inflammation of rash, 1.

[c] Dyspnea, 10; râles, 4; friction rubs, 3; percussion dullness, 2; coughing, 1; sneezing, 1.

TABLE 2. *Distribution and time of appearance of rash in 38 rhesus monkeys after respiratory challenge with Rickettsia rickettsii*[a]

Site	No. of monkeys	Per cent	Appearance (mean post exposure day)
Thighs	27	71.5	11.1
Legs, below knees	18	47.4	11.5
Feet	5	13.2	15.4
Face	22	57.9	11.7
Ears	24	63.2	12.7
Arms	13	34.2	11.7
Hands	4	10.5	15.3
Genitalia	14	36.8	11.9
Pubic, perineal, perianal areas	9	23.7	11.7
Back	6	15.8	10.9
Tail	7	18.4	11.1
Total	38	100.0	11.1

[a] Data from Saslaw et al. (17).

The earlie t death occurred on the 7th PED, and one monkey died on the 8th day; 29 of 42 (69.1%) died on the 9th through 12th PED, and 11 of 42 (26.2%) survived beyond the 12th day. The median survival time was 11 days, and the mean, 12.3 days.

The course of the illness in 14 surviving monkeys was highly variable. One monkey became acutely ill and was comatose for about 24 hr, but eventually recovered, whereas three monkeys showed only mild lethargy and anorexia. Intermediate gradations of disease were observed in the remaining 10 animals. Surviving monkeys usually were febrile for 5 to 7 days and ill for 7 to 10 days.

Heart sounds became less intense and muffled with, or shortly after, onset of acute illness, and this condition persisted until death of the animal or until recovery was definite.

Only 12 of 56 (21.4%) monkeys showed respiratory symptoms. Dyspnea was observed, respectively, in five, two, and three animals during the early, acute, and late stages of the infection. Similarly, râles were detected in two, one, and one during these stages. The infrequency and irregularity with which respiratory signs were observed suggest that primary rickettsial pneumonia was not established after aerosol challenge, although serous pneumonitis was observed, microscopically, at autopsy (see below).

Rash, similar to that seen in man, was observed in 38 of 56 (67.9%) rhesus monkeys that became ill, and usually appeared 9 to 13 days after challenge. Only 13 of 23 (56.5%) animals that died before the 12th day exhibited rash, whereas 18 of 19 (94.7%) succumbing on the 12th day or later showed skin manifestations. In nonfatal infections, 7 of 14 showed skin lesions. Rash was observed most frequently on the extremities and head, as shown in Table 2, but the anatomical area first affected was variable (17).

Peripheral necrosis was observed 13 to 24 days after challenge in 9 of 56 (16.1%) rhesus monkeys (Table 1). The ears were affected in all nine, and two also had necrosis of the fingertips. Six of the nine had recovered and were otherwise normal when necrosis was first observed. The remaining three died 18 to 22 days after exposure.

Mortality in monkeys exposed to aerosols of *R. rickettsii* was dose-dependent to a limited degree only (Table 3). One of four monkeys retaining 1.5 $YSLD_{50}$ exhibited typical symptoms and rash and died on the 15th PED, whereas the remaining three animals showed no symptoms or laboratory evidence of infection. All of six monkeys retaining 4 to 6 $YSLD_{50}$ were infected, and five of six died. However, although all of seven monkeys retaining 11 $YSLD_{50}$ became ill, only three died. In other experiments involving 24 monkeys and retained doses ranging from 67 to 450 $YSLD_{50}$, there was little or no relationship between retained dose and mortality. This poor correlation between mortality and the number of rickettsiae retained during exposure is shown by the fact that, with varying doses of less than 20, 20 to 200, 200 to 500, and more than 500 $YSLD_{50}$, 9 of 17 (52.9%), 6 of 10 (60.0%), 13 of 14

TABLE 3. *Relationship between retained dose, morbidity, and mortality in rhesus monkeys after aerosol exposure to Rickettsia rickettsii*

YSLD₅₀ retained	Morbidity	Mortality
1.5	1/4[a]	1/4[a]
4	2/2	2/2
5	2/2	2/2
6	2/2	1/2
11	7/7	3/7
67	1/2	1/2
107	2/2	1/2
138	2/2	1/2
156	2/2	2/2
191	2/2	1/2
305	7/7	6/7
333	2/2	2/2
341	2/2	2/2
342	1/1	1/1
450	2/2	2/2
555	2/2	1/2
650	2/2	2/2
1,013	2/2	1/2
2,475	2/2	2/2
2,745	3/3	1/3
3,210	2/2	2/2
3,330	2/2	2/2
7,950	2/2	1/2
9,000	2/2	2/2
Total	56/60	42/60

[a] Number ill/total number exposed.

TABLE 4. *Laboratory findings in 56 rhesus monkeys after respiratory challenge with Rickettsia rickettsii*[a]

Finding	No. of monkeys	Per cent
Positive CRP	56	100.0
Leukocytosis	23	41.1
Left shift in differential	55	98.2
Decrease in hematocrit	40	71.5
Decrease in hemoglobin	48	85.7

[a] Data from Saslaw et al. (17).

TABLE 5. *Isolation of rickettsiae from peripheral blood of monkeys after respiratory challenge with Rickettsia rickettsii*[a]

Days postchallenge	No. of isolations attempted	No. of isolations successful	Per cent
1-5	17	0	0.0
6	1	1	
7	8	5	
8	1	1	
9	5	3	
10	2	2	
11	3	2	
Total, 6-11 days	20	14	70.0
12-18	10	2	20.0

[a] Data from Saslaw et al. (17).

(92.9%), and 14 of 19 (73.7%) animals, respectively, experienced fatal infection.

On the other hand, the incubation period seemed to be inversely related to dose. For example, the means of the days on which monkeys became febrile after receiving less than 20, 20 to 200, 200 to 500, and more than 500 $YSLD_{50}$ were 8.7, 6.4, 6.2, and 5.5 days, respectively. Similarly, the means of the days on which rash was first observed in animals in the four dose groups were 12.6, 11.2, 11.1, and 10.1 days, respectively.

Only eight cynomolgus monkeys were utilized in these studies. Two and six cynomolgus monkeys received 468 and 1,013 $YSLD_{50}$, respectively. All 8 died at 10 to 13 days postchallenge. The acute, rapidly fatal process noted in cynomolgus monkeys was similar to that observed in rhesus monkeys receiving similar doses.

Laboratory Aspects

Laboratory findings in rhesus monkeys exposed to aerosols of *R. rickettsii* included positive C-reactive protein (CRP) reactions, a rather dramatic left shift in neutrophils with or without leukocytosis, anemia, rickettsemia, and the appearance of OX-19 agglutinins and complement-fixing antibodies. CRP usually was first detected on the first day of fever, i.e., 1 to 3 days prior to appearance of other symptoms. All of 56 monkeys that became ill exhibited positive CRP reactions (Table 4). CRP tests were positive until death in fatal infections and became negative 1 to 3 days after clinical improvement was noted in survivors. The left shift in the differential count was also apparent 1 to 3 days before onset of illness in all of 56 monkeys, except for 1 that became only mildly ill. Anemia was observed regularly in acutely ill animals 1 to 3 days after appearance of symptoms (Table 4), but normal values were restored about 1 week after clinical improvement.

Rickettsemia was readily demonstrable 6 to 11 days postchallenge. A total of 47 blood samples taken from 10 monkeys at 1 to 18 days after exposure were inoculated into guinea pigs. As shown in Table 5, none of 17 samples obtained 1 to 5 days after challenge yielded rickettsiae, whereas 14 of 20 (70.0%) samples drawn on the 6th through 11th days produced typical symptoms, febrile response, and complement-fixing

TABLE 6. *Antibody response of monkeys surviving infection after respiratory challenge with Rickettsia rickettsii*[a]

Monkey no.	Complement-fixing antibody titer			OX-19 Agglutination titer		
	Base line	Peak	Day of peak titer[b]	Base line	Peak	Day of peak titer[c]
2	—	320	28	—	160	21
3	—	320	28	—	—	—
4	—	160	28	20	80	21
5	—	640	28	—	—	—
6	—	640	28	—	10	21
7	—	640	14	—	—	—
8	—	320	28	—	160	14
9	—	640	14	5	160	14
10	—	320	14	—	—	—
11	—	80	35	5	160	14
12	—	320	21	5	40	14
13	—	160	28	—	20	14
14	—	320	14	—	20	14
15	—	640	21	—	80	21

[a] Data from Saslaw et al. (17).
[b] Mean, 23.5 days.
[c] Mean, 16.8 days.

antibodies in guinea pigs. Only 2 of 10 samples taken after the 11th day yielded *R. rickettsii*.

Homogenates of tissues taken at autopsy from 13 monkeys with fatal infection were also inoculated into guinea pigs. *R. rickettsii* was isolated, respectively, from spleen, liver, kidney, lung, and brain of 9 of 13, 6 of 10, 6 of 7, 6 of 8, and 5 of 6 animals. Rickettsiae were recovered from heart blood in only one of five monkeys, although they were isolated from other tissues from all five.

All monkeys surviving long enough to allow serial serological studies showed significant rises in complement-fixing antibody titers (Table 6), with peak titers at 14 to 35 days (mean, 23.5 days) postchallenge. In contrast, only 10 of 14 showed significant titer changes in the Weil-Felix agglutination test, with peak titers 14 to 21 days (mean, 16.8 days) after challenge.

Pathology

Gross and microscopic pathology in monkeys dying after exposure to aerosols of *R. rickettsii* were similar to those observed in monkeys infected by other than the respiratory route (11). The lungs consistently showed patchy serous pneumonitis, which was characterized by septal widening associated with serous exudation and swelling and proliferation of capillary endothelial cells. Obvious pneumonia was not observed. Thus, although the respiratory tract was the portal of entry, the pathological findings were consistent with the clinical observation that pulmonary involvement was not a major factor in the outcome of the disease. Severe lesions of the skin, ears, and nasal septae were characterized by vascular thrombosis and necrosis. Other findings included pericardial effusion, localized myocardial capillaritis, glomerulitis and intertubular capillaritis, endarteritis of the pulmonary vessels, fatty degeneration of the liver, and splenomegaly with lymphoid depletion for the first 2 weeks, followed by regeneration. Congestion of the tunica albuginea was a frequent lesion, as was congestion or hemorrhage, or both, of the adrenal cortex and medulla. No gross or microscopic lesions were seen in the brain.

IMMUNITY STUDIES

Response of Vaccinated Monkeys

In an attempt to establish the degree and duration of immunity conferred by vaccination, monkeys were challenged with aerosols of *R. rickettsii* at intervals up to 1 year after three weekly subcutaneous injections of 0.25 ml of commercial vaccine (Lederle Laboratories, Pearl River, N. Y.). In the first of four experiments (Table 7), four vaccinated monkeys showed no clinical signs of illness after challenge with 650 $YSLD_{50}$ at 3 weeks postvaccination, whereas two control monkeys exhibited typical symptoms and died on the 9th and 10th PED, respectively. Results of laboratory studies indicated that subclinical infection had been established in three of four vaccinated monkeys. For example, three of four exhibited positive tests for CRP and characteristic changes in the leukocytes; one of four showed a slight but significant fever, and all four showed significant increases in complement-fixing antibody titer after challenge.

Similarly, four monkeys challenged with 341 $YSLD_{50}$ 6 weeks after vaccination exhibited no symptoms after exposure, but laboratory evidence of infection was obtained in two of four, and all four showed significant rises in complement-fixing antibody titer. Both control animals became acutely ill and died 11 and 12 days, respectively, after challenge.

In contrast, when monkeys were challenged 6 months postvaccination with 2,745 $YSLD_{50}$, one of three became ill, and all three showed significant fever, positive CRP reactions, and characteristic changes in the differential count; however, all three survived. All three control animals became acutely ill, and two of three exhibited typical rash. One of three died on the 9th PED, and the remaining two survived. When two monkeys

TABLE 7. *Comparison of response of vaccinated and previously infected monkeys after aerosol challenge with Rickettsia rickettsii*

Response	Weeks after vaccination				Weeks after first challenge			Vaccination controls	Rechallenge controls
	3 (650)[a]	6 (341)	26 (2,745)	52 (1,980)	8 (264)	26 (300–11,745)	52 (1,980)		
Symptoms	0/4[b]	0/4	1/3	2/2	0/2	0/13	0/4	10/10	13/13
Fever	1/4	2/4	3/3	2/2	0/2	6/13	4/4	10/10	13/13
Rash	0/4	0/4	0/3	1/2	0/2	0/13	0/4	8/10	10/13
Positive CRP	3/4	2/4	3/3	2/2	0/2	11/13	4/4	10/10	13/13
Leukocyte changes	3/4	2/4	3/3	2/2	0/2	8/13	3/4	10/10	13/13
Died	0/4	0/4	0/3	1/2	0/2	0/13	0/4	8/10	10/13
Complement-fixation antibody rise	4/4	4/4	3/3	1/1[c]	0/2	12/13	4/4	2/2[c]	3/3[c]

[a] Numbers in parentheses show the $YSLD_{50}$ retained.
[b] Number showing symptoms over total number challenged.
[c] Survivors.

were challenged with 1,980 $YSLD_{50}$ 1 year postvaccination, both became ill, and one showed extensive rash and died on the 10th day. All three control animals experienced acute illness and died on the 10th or 11th day. The clinical picture in both vaccinated monkeys was almost identical to that seen in control animals.

Response of Monkeys After Rechallenge

Two monkeys that had become ill after aerosol challenge, and that had recovered spontaneously, were rechallenged with 264 $YSLD_{50}$ 2 months later (Table 7). Neither showed any evidence of infection; both remained asymptomatic, and all laboratory tests were negative. Both control animals became ill and exhibited typical rash; 1 of 2 died on the 21st PED. Thus, the first infection apparently conferred a solid immunity. Both rechallenged monkeys had shown peak complement-fixing antibody titers of 1:320 after first exposure. Titers had dropped to 1:80 in both by the time of rechallenge and did not change after the second exposure.

Thirteen monkeys that had been previously infected and treated successfully with antibiotics (see below) were rechallenged 6 months later with doses ranging from 300 to 11,745 $YSLD_{50}$. None exhibited clinical signs of illness, whereas all of eight control animals became acutely ill and six of eight died. However, the rechallenged monkeys were not solidly immune, in that fever, positive CRP reactions, and changes in the differential leukocyte count were noted in 6 of 13, 11 of 13 and 8 of 13, respectively, after the second exposure. Complement-fixing antibody titers ranged from 1:10 to 1:80 at the time of rechallenge, and rose significantly in 12 of 13 monkeys to 1:40 to 1:640 after rechallenge.

In addition, four infected monkeys that had recovered after antibiotic therapy were rechallenged 1 year later with 1980 $YSLD_{50}$. None became ill, but all four exhibited significant fever and positive CRP tests, and three of four showed changes in the differential count. Three additional monkeys that had been vaccinated and challenged by aerosol 6 months later (see above) were challenged a second time in this experiment, i.e., 1 year after vaccination. The combination of vaccination and challenge resulted in solid immunity, in that all three monkeys remained well and all laboratory tests were negative. Three control animals died on the 10th or 11th day.

Thus, it would appear that as in man (29), infection apparently resulted in immunity of longer duration than did vaccination. None of 19 monkeys rechallenged 2, 6, or 12 months after recovery from established infection became ill. No illness was observed in monkeys challenged 6 weeks after vaccination. However, one of three and two of two challenged 6 and 12 months, respectively, after vaccination showed typical symptoms. One monkey exposed 12 months postvaccination exhibited extensive rash and died on the 10th day.

ANTIBIOTIC THERAPY AND PROPHYLAXIS

The response of monkeys infected by the aerosol route to appropriate antibiotic therapy was similar to that observed in man after natural infection. Therapy was not instituted in monkeys until at least 48 hr of significant fever and other signs consistent with well-established disease were present. Monkeys treated with tetracycline, demethylchlortetracycline, and chloramphenicol became afebrile and asymptomatic within 1.5

TABLE 8. *Results of therapy of Rocky Mountain spotted fever in monkeys*[a]

Expt no.	Challenge dose (yolk sac LD_{50})	Drug[b]	Dose	Days	Deaths	
					Treated	Controls
			mg/kg			
1	4	DMC	25.0	7	1/4[c]	2/2
2	5	DMC	25.0	7	1/6	2/2
3	156	DMC	25.0	3	0/3	2/2
			12.5	11		
		DMC	12.5	14	0/3	
4	3,210	DMC	8.5	3	1/3	2/2
		DMC	8.5	5	0/3	
		DMC	8.5	7	0/2	
5	107	TC	14.0	3	0/3	1/2
		TC	14.0	5	0/2	
		TC	14.0	7	0/2	
6	191	DMC	30.0	2	0/5	1/2
			15.0	5		
		TC	50.0	2	0/4	
			25.0	5		
7	2,475	C	75.0	2	0/6	2/2
			50.0	5		
		E	50.0	2	1/4	
			25.0	5		

[a] Data from Saslaw et al. (16).

[b] DMC = demethylchlortetracycline (Declomycin); TC = tetracycline (Achromycin V); C = chloramphenicol (Chloromycetin); E = erythromycin estolate (Ilosone).

[c] Number dead per total number.

to 3.5 days (16). Erythromycin estolate was somewhat less effective, and recovery occurred more slowly from 2 to 6 days after institution of therapy. As can be seen in Table 8, only 4 of 50 treated monkeys died, whereas 12 of 14 untreated controls succumbed. Thus, the effectiveness of broad-spectrum antibiotics in treatment of naturally occurring Rocky Mountain spotted fever was also demonstrable when the respiratory route served as the portal of entry.

Studies on prophylaxis (16) showed that administration of 30 and 50 mg/kg per day of demethylchlortetracycline and tetracycline, respectively, for 3 days prior to challenge merely delayed onset of symptoms for 2 days, and deaths occurred 4 to 5 days later than in control animals. When the same doses of each drug were given daily for 3 or 5 days, beginning with the day of exposure, signs of illness were delayed for about a week, but six of nine died as compared with one of two in controls.

STUDIES ON PERSISTENCE OF *R. RICKETTSII* IN MONKEYS

Limited studies were conducted to determine whether *R. rickettsii* could be recovered from monkeys that survived infection spontaneously or after antibiotic therapy. In addition, previously vaccinated monkeys that were subsequently challenged were included.

Of 4 monkeys that recovered spontaneously and were sacrificed 25 to 75 days after challenge, *R. rickettsii* was isolated by guinea pig inoculation of splenic tissue from one monkey sacrificed on the 27th day. The spleens and other tissues of the other three failed to yield rickettsiae.

The only other monkey from which *R. rickettsii* was isolated had been treated successfully with demethylchlortetracycline for 7 days and sacrificed on the 48th day. The organism was isolated from a bronchial lymph node, but not from other tissues.

No isolates were obtained from 25 other treated monkeys sacrificed 25 to 75 days after challenge. Similarly, no isolates were obtained from 6 monkeys previously vaccinated and then sacrificed 27 to 98 days after challenge.

Although other avenues of approach may have yielded evidence of persistence of *R. rickettsii* in tissues of infected monkeys, these studies via guinea pig inoculation resulted in isolation in only two instances, 27 and 48 days after challenge. Various studies have demonstrated the presence or recrudescence of rickettsiae long after initial infection (9). Attempts to activate infection with cortisone were not successful in 16 monkeys. It is conceivable that other stresses, such as X irradiation, or the use of tissue culture or immunofluorescence, may have resulted in detection of rickettsiae more frequently.

COMMUNICABILITY

During the entire course of these studies, there was no evidence either clinically or serologically of monkey-to-monkey transmission of disease. Normal monkeys kept in the same or adjacent cage showed no evidence of experience with *R. rickettsii* even when contacts were made shortly after aerosol challenge of test monkeys. This is consistent with observations of the nonappearance, in the absence of arthropod vectors, of naturally occurring infection in man (5) or of naturally occurring infection of guinea pigs, as seen in our laboratory.

DISCUSSION

There are occasions in the practice of medicine when the exact source or portal of entry of the infectious agent cannot be established with certainty. Similarly, in laboratory-acquired infections, there has been increasing evidence that infections have occurred in the absence of laboratory accidents or poor technique (26). This has led to a greater appreciation of the potential role of "aerogenic transmission" of a wide variety of diseases. In recent years, studies of experimental tularemia in monkeys (3) and man (18, 19) have demonstrated the infectivity of aerosols of *Francisella tularensis* and have confirmed the suspicion that tularemia had been acquired in laboratories by the respiratory route (26). These observations support other studies which have suggested that the respiratory tract could serve as a portal of entry even in arthropod-borne infections, such as typhus, rickettsialpox, yellow fever (24), Venezuelan equine encephalomyelitis (10, 20, 21), and St. Louis encephalitis (25).

Spencer and Parker (22), among others, recognized that infection of laboratory workers with *R. rickettsii* was frequently observed in the absence of a history of tick bite. Their studies showed that infection in guinea pigs could be induced by instillation of the organism in the conjunctival sac or by placing a drop of infected tick tissue suspension in the mouth. The respiratory route was not investigated. The results of our studies with *R. rickettsii* aerosols in monkeys suggest that some cases of Rocky Mountain spotted fever in laboratory workers could have been due to inhalation of aerosols. Monkeys were highly susceptible to *R. rickettsii* administered by aerosol; one monkey which retained only 1.5 $YSLD_{50}$ became acutely ill, exhibited typical rash, and died, as did five of six receiving four to six $YSLD_{50}$. Of further importance is the fact that the clinical response in monkeys exposed to aerosols of *R. rickettsii* was similar to that seen in monkeys infected by other routes (1, 27). Thus, accidental aerosol exposure of man might be expected to result in disease indistinguishable from that resulting from a tick bite. Of additional significance is the similarity between clinical and laboratory findings in monkeys and those observed in naturally occurring Rocky Mountain spotted fever in man.

As in man, high fever was a constant finding in monkeys exposed to aerosols of *R. rickettsii*, but morning remissions of fever seen in man (5, 29) were not observed in monkeys. The appearance of fever and symptoms 5 to 7 and 6 to 9 days, respectively, after aerosol exposure of monkeys is in agreement with the observed incubation period in man (29). Also as in man, monkeys first showed lethargy and weakness, followed by acute illness terminating in prostration and coma. The abnormal heart sounds heard in monkeys during the acute stages were similar to those in man (2).

Monkeys exposed to aerosols of *R. rickettsii* exhibited rash and peripheral necrosis similar to those observed in the naturally occurring infection in man, but the distribution of the rash was different. In monkeys, rash was observed most frequently on the extremities and head. Involvement of the trunk was limited to the lower back, perianal, and perineal regions. In the classic disease in man, rash begins on the wrists and ankles and spreads to the trunk, so that the entire body may be involved. None of the monkeys exposed to aerosols showed typical rash on the chest or abdomen. However, the rash in man may be minimal and fleeting in mild cases (5). As in man, peripheral necrosis in monkeys usually involved only the ears and digits. In addition, necrosis of the tail, tip of the penis, and skin over the patella was noted in one monkey each.

Laboratory findings in monkeys exposed to *R. rickettsii* aerosols were also similar to those in Rocky Mountain spotted fever in man. Anemia was observed regularly in acutely ill animals. A marked left shift of neutrophils was noted in 55 of 56 monkeys that became ill after exposure; about 50% exhibited significant leukocytosis. Although opinions differ as to the frequency with which changes in the leukocytes are observed in man, Harrell (5) has stated that, as the disease progresses, leukocytosis with a left shift in the differential leukocyte count is noted.

Rickettsemia was readily demonstrable, by guinea pig inoculations, 6 to 11 days after exposure, but not during the first 5 days and only infrequently after the 11th day. In man, rickettsiae can be recovered from the blood throughout the 1st week of illness and during the first part of the 2nd week (5) with sufficient regularity to make the guinea pig infection test a reliable diagnostic procedure.

As in man, surviving monkeys showed OX-19 agglutinins and complement-fixing antibodies. All of 14 exhibited significant increases in complement-fixing antibodies, whereas significant titer changes were noted in only 10 of 14 as measured by the Weil-Felix test. Peak OX-19 agglutination titers were observed 14 to 21 days (mean, 16.8 days) after exposure, whereas peak complement-fixing antibody titers were not attained until 14 to 35 days (mean, 23.5 days) postchallenge. In Rocky Mountain spotted fever

in man, OX-19 agglutinins are almost always present by the 12th day of illness (29), although a few patients never show a positive Weil-Felix test. Complement-fixing antibodies appear during the 2nd or 3rd week of illness, i.e., later than OX-19 agglutinins. Hersey, Colvin, and Shepard (8) have shown that the complement-fixation test is more sensitive than the Weil-Felix reaction in detecting Rocky Mountain spotted fever in man.

Studies of the effectiveness of vaccination in monkeys and the response of monkeys to rechallenge provided further similarities in *R. rickettsii* infections in monkeys and man. None of 19 monkeys rechallenged 2, 6, or 12 months after recovery from established infection became ill. Vaccination was effective in preventing symptoms after challenge 6 weeks later, but one of three and two of two challenged 6 and 12 months, respectively, after vaccination showed typical symptoms, and one of two exposed 12 months postvaccination became acutely ill, exhibited typical rash, and died. It is generally agreed that, in man, recovery from infection confers a higher level of immunity of longer duration than does vaccination.

In addition, the response of infected monkeys to antibiotic therapy was similar to that observed in Rocky Mountain spotted fever in humans. Monkeys became afebrile and asymptomatic 1.5 to 3.5 days after institution of therapy with tetracycline, demethylchlortetracycline, or chloramphenicol. Erythromycin estolate was somewhat less effective, however. Chloramphenicol, chlortetracycline, oxytetracycline, and tetracycline have proved to be effective chemotherapeutic agents in human infections, but erythromycin failed to alter either the febrile or toxic course of the illness in two patients (29).

Thus, throughout these studies of monkeys exposed to aerosols of *R. rickettsii*, remarkable similarities to the naturally occurring infection in man were observed. Additionally, the clinical picture in monkeys exposed to aerosols was similar to that observed by others in monkeys challenged by other than the respiratory route. From these results, it would be predicted that exposure of man to *R. rickettsii* aerosols would result in illness much like that observed after a tick bite. These studies in monkeys would suggest, therefore, that aerogenic transmission should be considered in infections of laboratory personnel who have had no known contact with ticks.

ACKNOWLEDGMENT

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Discussion

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Rocky Mountain spotted fever (RMSF) in man is rarely acquired by means other than tick bite. Nevertheless, a number of instances have occurred in the past, especially among laboratory workers, in which it was possible to infer that some of these rickettsial infections were transmitted aerogenically (3, 4).

Saslaw and Carlisle have presented incontrovertible experimental evidence that the etiological agent of RMSF, in remarkably small doses, can produce a true airborne infection in monkeys. A point to be emphasized is that a disease naturally transmitted by ticks can also be induced by aerosols of the microorganism. During the past decade or more, it has been demonstrated that a number of classical arthropod-borne diseases can be established in laboratory animals exposed to aerosols of the respective microorganisms; Venezuelan equine encephalitis (6), Rift Valley fever, and yellow fever are a few examples (2).

A question that arises is: do aerosols of these infectious microorganisms play any role in the ecology of the diseases they produce? In analyzing the question, one comes to the conclusion that aerogenic transmission has little, if any, influence on the ecological aspects of the arbovirus diseases mentioned. It is tempting to speculate, however, that the aerosol stability of some of the rickettsiae might be of some importance in determining whether they can also be aerogenically transmitted. It can be shown experimentally that the aerosol stability of the rickettsiae of RMSF and typhus is relatively low, considerably lower, in fact, than that of the rickettsiae of Q fever (*unpublished data*), which is characteristically transmitted by aerosol in man. It is interesting to note parenthetically that RMSF and Q fever resemble each other ecologically in b[illegible] involved with the same biotic tetrad—the ri[illegible]ttsiae, the ticks, vertebrates (mainly mammals), and man. The importance of ticks in the maintenance of both diseases in nature is unquestioned; yet, in man RMSF is caused almost

exclusively by infected ticks, and Q fever is transmitted almost exclusively by aerosols. Thus epidemiological disease patterns may be influenced to some extent by the survival capacity of infectious microorganisms in the airborne state.

Another point of interest concerns the treatment of RMSF with certain antibiotics, which are, without question, highly effective therapeutic agents against the disease. Before the advent of antibiotics, mortality was exceedingly high and RMSF greatly taxed the symptomatic and supportive therapeutic resources available. One can see a dramatic reduction in mortality from approximately 20 to 5% since 1949, the year when antibiotic treatment was first introduced. It is a matter of record also that, with the availability of the highly effective therapeutic agents, the utilization of vaccine has correspondingly decreased—except perhaps for those at great risk. Despite the gradual decrease in the total number of cases reported each year since 1949 in the United States, and the reduction in the case-fatality rate, RMSF continues to be an important disease, especially among children in the South Atlantic States (5). Atwood et al. (1) have presented evidence which indicates that "the true incidence of RMSF is currently much greater than the number of reported cases." One reason for the discrepancies in the statistical data is the wide use of broad-spectrum antibiotics early in the course of many febrile illnesses. It was because of these facts and the almost complete reliance on antibiotics in dealing with this disease that an attempt was made to determine whether the rickettsiae of RMSF could be rendered resistant to selected antibiotics.

Employing the procedures of Weiss and Dressler (7) with some modifications, we exposed rickettsiae of RMSF in continuous passage to erythromycin (73 passages), oxytetracycline (55 passages), and chlortetracycline (50 passages). Application of high antibiotic concentration to a large number of infected eggs was also attempted to isolate resistant mutants by subsequent passage in eggs injected with the same three antibiotics. In limited experiments, ultraviolet radiation was also employed as a mutagen. Under these kinds of experimental conditions, no antibiotic-resistant mutants have been isolated. This is not to be interpreted to mean that these rickettsiae cannot become antibiotic resistant. The matter of mutation rate may be involved—one much lower than 10^{-7} or 10^{-8}. The data do suggest, however, that the chance appearance of antibiotic-resistant strains of RMSF is remote.

Although the danger of the rickettsial diseases has diminished, most of these infections remain widespread, constituting an ever-present threat to human health. RMSF will probably continue to be a problem in the United States, affecting about 200 persons or more annually. The number of cases might, in fact, increase because trends in land use seem to be increasing the amount of area suitable for tick habitation, and because many such areas are being suburbanized (1). Thus, in this country, RMSF merits continuing vigilance and research into methods for its treatment, control, and eventual eradication.

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Industrial Inhalation Anthrax

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INTRODUCTION

"Woolsorter's disease," or inhalation anthrax, was a serious problem in Europe in the latter part of the 19th century; at least several hundred cases were reported, with an associated high fatality rate. Governmental inquiries conducted in different countries (9, 10, 15, 17) resulted in a series of effective control regulations, including the establishment in Liverpool of a disinfection station (14), where all "dangerous wool and goat hair" had to be decontaminated before being further processed in England.

In the United States, however, inhalation anthrax has never been a serious problem, although workers have routinely been exposed to naturally occurring aerosols while processing imported wool, goat hair, and hides. Since 1900, there have been fewer than 20 cases of inhalation anthrax reported in the United States (13); approximately half of these occurred among individuals with only fleeting contact with materials known to be contaminated. The only reported epidemic of the disease in this country occurred in 1957 among employees at a goat-hair processing mill in Manchester, N.H. (6, 13). Five individuals developed inhalation anthrax over a 10-week period, with four fatalities. Other cases since 1957 have involved a laboratory employee who was accidentally exposed, a secretary in a goat-hair processing mill who entered a highly contaminated area for only a brief moment, and a 27-year-old man with quiescent Boeck's sarcoidosis whose only known contact was in passing the open receiving door of a tannery on his way home from work (7).

It is not clear why more cases have not occurred in goat-hair and woolen mills and in tanneries, especially among employees working in the dustiest areas where the most concentrated *Bacillus anthracis*-containing aerosols are created. It may be that the dose to which employees are exposed is below the infecting dose for man, or that employees have developed resistance from chronic exposure. It is conceivable that cases have occurred that were not properly diagnosed. Equally unusual has been the sporadic occurrence of cases in people with no industrial exposure. It may be that these individuals are unusually susceptible, as may have been the case with the individual with Boeck's sarcoidosis. More specific information about inhalation anthrax in man is currently difficult to obtain because almost all workers in the high-risk industries within the United States have been immunized (8).

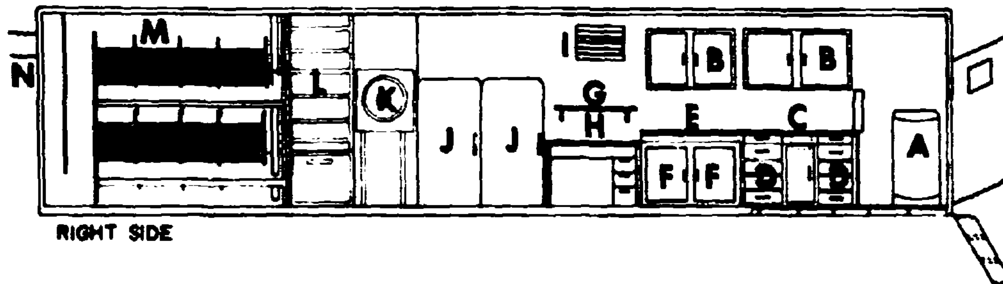
Significant data related to the pathogenesis of the disease and the dose-response relationships have been reported on the basis of animal studies conducted in both this and other countries (1, 2, 4, 16, 19). However, these studies have involved exposures to pure, concentrated aerosols of *B. anthracis* over relatively short periods of time. There have been no reports on the effect of chronic exposure of animals to aerosols containing *B. anthracis*, either homogeneous aerosols, as in laboratory experiments, or heterogeneous aerosols, as in the natural situation in a factory.

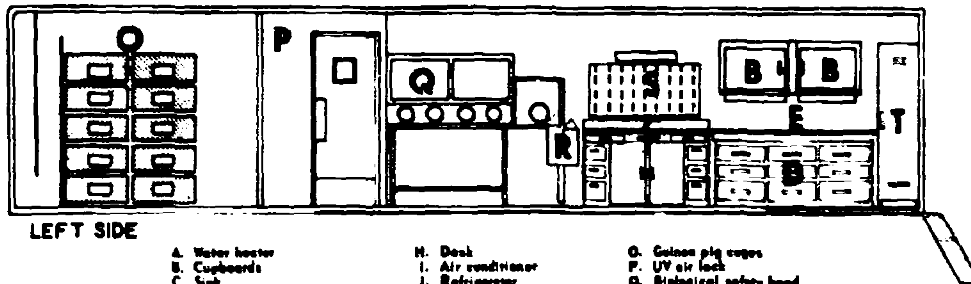
At the Conference on Airborne Infection, Riley discussed his studies on airborne tuberculosis in guinea pigs (11), in which he utilized a holding chamber through which air passed from rooms housing patients with sputum-positive, cavitary tuberculosis. Using this physical arrangement as a model, the authors, with Harold Glassman and Elwood Wolfe of Fort Detrick, developed a protocol to study the clinical course, pathogenesis, and dose-response relationships of experimental animals to a naturally occurring *B. anthracis* aerosol produced in a goat-hair processing mill.

MATERIALS AND METHODS

A 40-foot trailer was outfitted at Fort Detrick (under the direction of Harold Curry) as a com-

LABORATORY TRAILER





SCHEME OF ANIMAL EXPOSURE

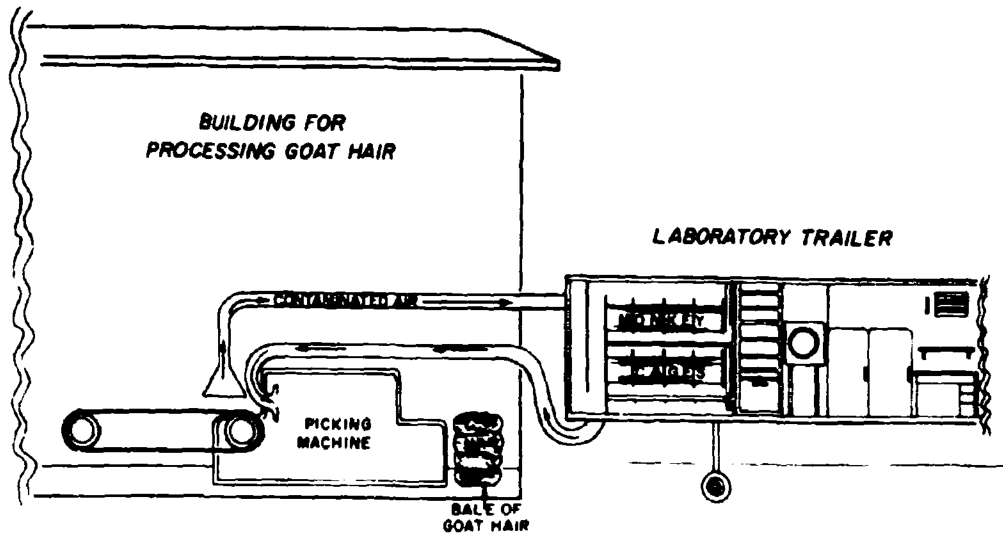


FIG. 1. *Laboratory trailer and scheme of animal exposure.*

bined animal exposure chamber and laboratory and was subsequently located at a mill in South Carolina (Fig. 1). This mill processes goat hair, imported mainly from India and Pakistan, into a woven hair-cloth interlining for suit coats. There were approximately 250 employees, all of whom had been immunized with the Wright anthrax antigen (8). Prior to immunization, 19 cases of cutaneous anthrax had been reported during the 2.5 years the mill had been in operation. Inhalation anthrax had never been reported at this mill.

The aerosol for animal exposure was created around the picking machine, the first machine in the processing cycle, where the clumps of hair were raked apart. The mill usually worked 8 hr a day and 5 days a week, but the picking machine was in operation intermittently during the working day for a total operational time of from 2 to 4 hr. Plastic conduits located in a hood over the picking machine and a suction fan were installed to carry the aerosol from the mill through the animal exposure chamber and back again to the mill (Fig. 1, bottom). A "T" connection made it possible to bring in outside air when the animals were not being exposed to mill air. The trailer was completely self-sufficient except for water and electricity.

As a result of experiences at Fort Detrick, the cynomolgus monkey was selected as the test animal. Preconditioned 3-lb monkeys imported from Asia were used in all runs. All monkeys were tuberculin-negative; if necessary, they were treated for respiratory disease and parasites but not less than 7 days prior to exposure. The monkeys were grouped two or three to a cage and fed a standard diet, fresh fruit, and water *ad libitum*. The temperature in the exposure area was controlled between 22 and 33 C. Monkeys were bled for serological studies before they entered the trailer, at intervals during the exposure period, and at the termination of exposure in the case of survivors. Monkeys were observed at least three

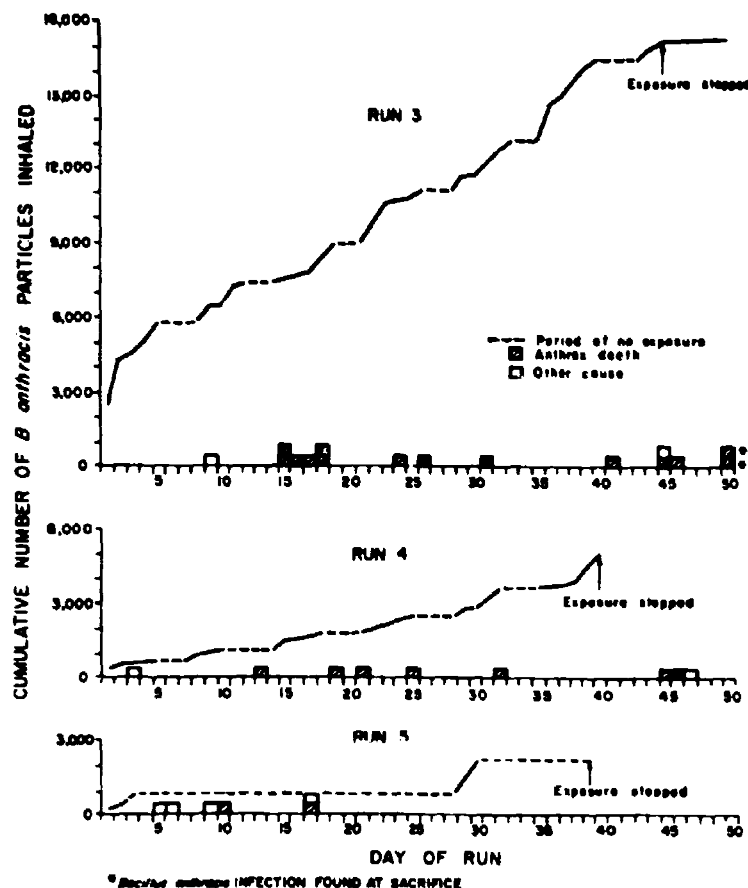


FIG. 2. *Occurrence of deaths by day, from beginning of exposure*

times a day, and autopsies were performed as soon as possible after death. All surviving animals were sacrificed with an intravenous injection of Nembutal. Postmortem examinations were conducted in general accord with the procedures outlined in the veterinary necropsy protocol of the Armed Forces Institute of Pathology, Washington, D.C. Appropriate cultures were obtained, and the tissue blocks in 10% formaldehyde were returned to Fort Detrick, where Frederic G. Dalldorf, Pathology Division, performed the histological examinations.

With the cooperation of Harry Lefkowitz, Fort Detrick, the protocol for obtaining air samples in the exposure chamber was developed. The all-glass impinger with the British preimpinger was selected as the standard air-sampling equipment to be used (18). Each sampler was run for 20 min, and air samples were obtained throughout all periods during which the monkeys were exposed to mill air. The impinging fluid consisted of 20 ml of gelatin phosphate collecting fluid with 3 drops of a 1:10 dilution of Dow-Corning Antifoam A. The bacterial content of the collected samples was determined by streaking 0.1 ml from the reagitated collecting fluid on each of three 5% human blood-agar plates, which were then incubated at 37 C for 15 to 20 hr. All suspicious colonies were counted, and a representative number were examined by routine bacteriological methods. Calculation of the dose of *B. anthracis*-bearing particles less than 5 μ in diameter inhaled by individual monkeys was based on the various dilution factors, the average number of *B. anthracis* colonies per plate during exposure, and an estimated respiratory rate of 1 liter per min. (All further discussion of the calculated, inhaled dose of *B. anthracis*-bearing

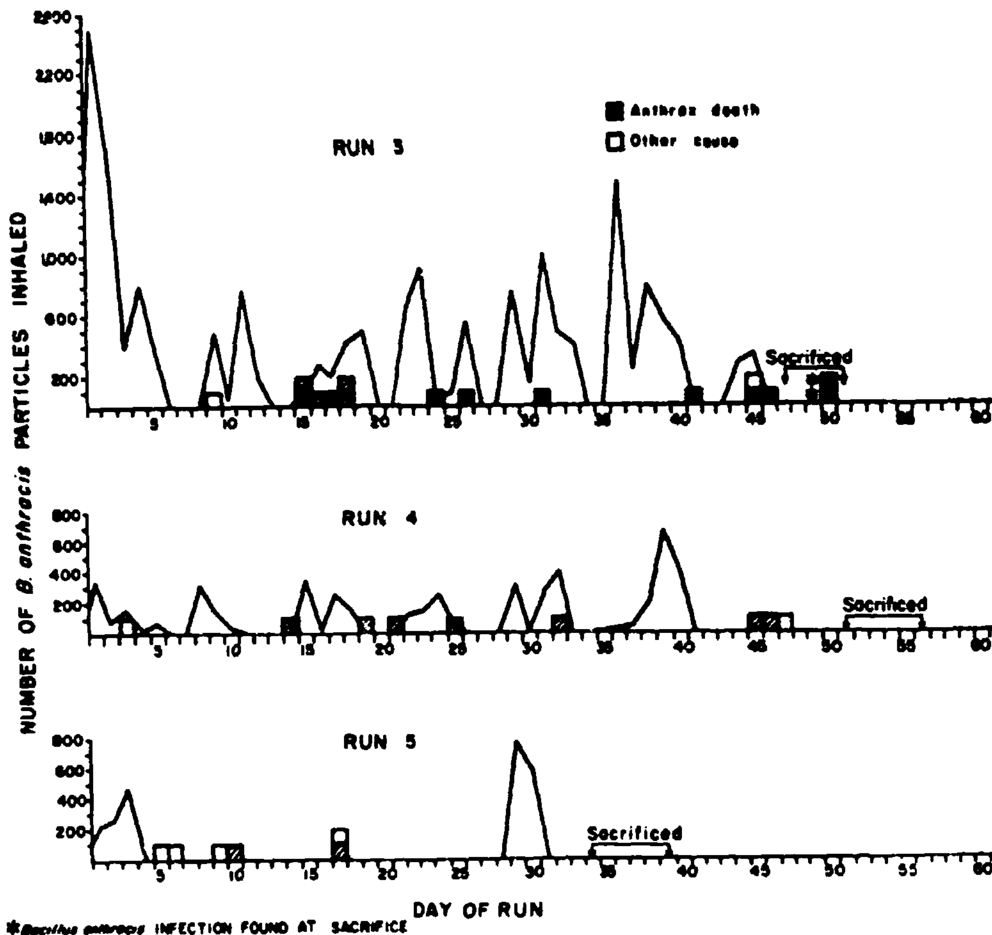


FIG. 3. *Estimated daily dosage (aerosol) per monkey of Bacillus anthracis.*

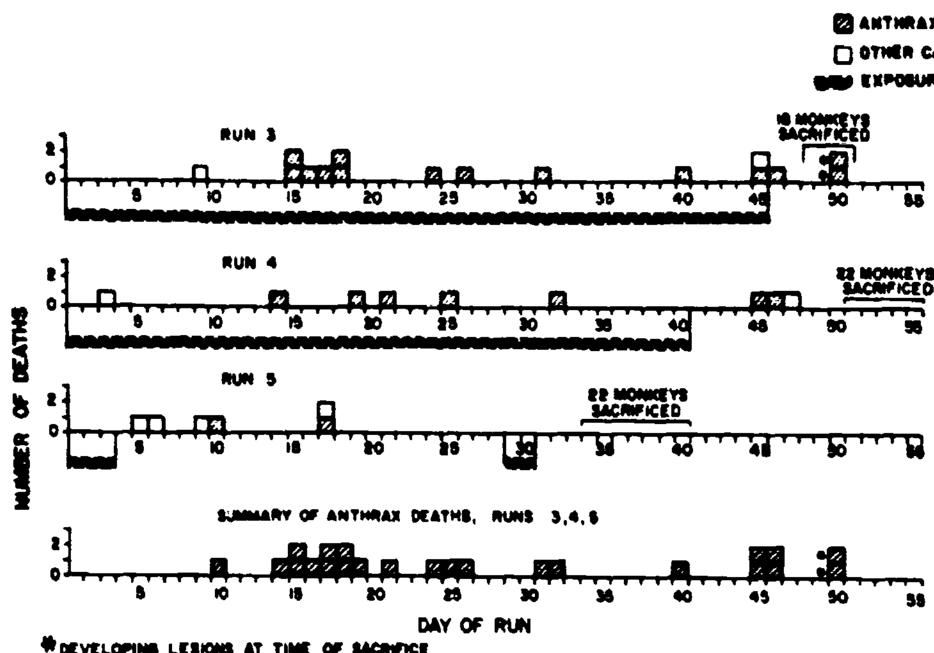


FIG. 4. *Estimated cumulative dosage (aerosol) per monkey of Bacillus anthracis.*

particles refers to those particles less than 5 μ in diameter.)

There were five separate exposure periods extending over 18 months. Because of inadequate preconditioning of the monkeys, difficulties with the environmental control within the exposure chamber, and deficiencies in the collection of air samples and serum specimens, the data from the first two runs were considered to be inadequate for analysis. These technical problems were corrected by changes in the trailer set-up and protocol. The remainder of this discussion will deal with the data developed from the third, fourth, and fifth runs. In the third and fourth runs, the animals were exposed constantly during the working day, regardless of whether the picking machine was in operation, and air samples were collected continuously during this exposure. During the fifth run, exposure was limited to the periods when selected bales of goat hair were actually being picked.

RESULTS

During the third run, 32 monkeys were exposed during 47 consecutive days; 12 of them died of anthrax between the 15th and the 46th day of exposure (Fig. 2, 3, 4, Table 1). Anthrax in 11 of these deaths was diagnosed at necropsy, and, in an additional death, when the microscopic sections were examined. Two monkeys died of other causes. The 18 survivors were sacrificed and examined; two of them evidenced anthrax infection (no. 51 and 50), one by positive blood culture (no. 51), and both by the presence of organisms resembling *B. anthracis* in mediastinal lymph node sections. Fluorescent-antibody studies of sections from these two monkeys confirmed the identification of anthrax organisms in one (no. 51) and were questionably positive in the other (no. 50). The remaining 16 monkeys appeared to be free from any anthrax infection. The final anthrax fatality rate was 43.8%.

Actual exposure occurred during only 32 of the consecutive 47 days of the third run, and the daily mean for these days was 530 *B. anthracis*-bearing particles. Exposure on the 1st day of the third run was greater than any other single day's exposure in all the runs, being calculated at an inhaled dose of 2,500 *B. anthracis*-bearing particles. A cluster of six deaths occurred between 15 and 18 days after this peak exposure. Other deaths were scattered through the remainder of the run. The total calculated inhaled dose for monkeys surviving the exposure was 16,962 *B. anthracis*-bearing particles less than 5 μ in diameter.

During the fourth run, 31 monkeys were exposed over 41 days. Survivors were held for an additional 10 days before being sacrificed. A

TABLE 1. *Summary of the five exposures*

Dates of exposure	Type of exposure	Length of exposure (cumulative days)	Observation period following termination of exposure (days)	Frequency of collection of air samples	Calculated inhaled dose of *Bacillus anthracis*[a]		No. of monkeys	Results					
								Died during exposure		Survivors		Total anthrax deaths	Mortality rate (%)
					Total cumulative dose	Avg 24 hr dose		Anthrax	Other causes	Evidence of anthrax infection	Normal[b]		
1. 28 January through 28 February 1963	Constant	32	None	Sporadic	[c]	[c]	17	2	13	0	2	2	11.8[d]
2. 25 March through 10 May 1963	Constant days 1-6; intermittent days 7-20; constant days 21-35	47	None	Sporadic	[c]	[c]	20	2	15	0	3	2	10.0[d]
3. 14 October through 29 November 1963	Only when "picking" hair	47	2 to 5	Total	16,962	530	32	12[e]	3	2	16	14	43.8[f]
4. 3 February through 13 March 1964	Only when "picking hair"	41	10 to 15	Total	4,959	198	31	7	2	0	22	7	22.6
5. 1 June through 3 June 1964	Only when "picking" hair	55 hr	25	Total	947	413	28	2	4	[g]	[g]	2	7.1
29 June through 30 June 1964	Only when "picking" hair	31 hr	4 to 8	Total	1,347	1,041	22	0	0	0	22	0	0

[a] Particles less than 5 μ in size.
[b] No evidence of anthrax infection.
[c] Data too fragmentary to use for calculations.
[d] Minimal figure
[e] In one monkey (no. 40), anthrax was considered a contributing cause of death.
[f] Two animals survived and found infected with *B. anthracis* at autopsy.
[g] Survivors re-exposed.

TABLE 2. *Summary of gross findings (third, fourth, fifth runs—monkeys that died of anthrax)*[a]

Run	Animal no.	Sex	Cumulative days after exposure began	Mediastinum: Edema	Mediastinum: Nodes	Mediastinum: Paratracheal nodes	Pleural effusion	Parenchymal abnormalities[b]	Adrenal	Spleen	Hemorrhagic meningitis	Culture	Final histopathologic diagnosis
3	52	F	15	+	+	−	+	+	+	+	+	+	Anthrax
	66	F	15	−	−	−	−	+	−	−	−	−[c]	Bronchopneumonia and anthrax pneumonia
	40	F	16	+	+	−	−	−	−	−	−	−	Anthrax; staphylococcal septicemia; cachexia
	63	F	17	+	+	−	+	−	+	+	−	+	Anthrax
	43	F	18	+	+	+	+	+	+	+	−	+	Anthrax
	61	M	18	+	+	+	+	−	+	+	−	+	Anthrax
	44	F	24	+	+	+	+	−	+	+	−	+	Anthrax
	69	M	26	−	±	−	+	+	+	−	+	+	Anthrax pneumonia
	55	F	31	+	+	+	+	+	+	+	−	+	Anthrax
	59	M	39	+	+	+	−	+	+	+	−	+	Anthrax
	49	F	45	+	+	+	+	+	+	+	+	+	Anthrax
	60	M	46	+	+	+	−	+	+	+	−	+	Anthrax
	50[d]	F	50	−	−	−	−	−	−	−	−	−	Anthrax in one lymph node
	51[d]	M	50	−	−	−	−	−	−	−	−	+	Anthrax in two paratracheal lymph nodes
4	80	M	14	−	+	−	−	+	−	?	+	+	Anthrax
	110	M	19	+	+	−	+	+	+	+	?	+	Anthrax
	101	M	21	−	+	−	−	+	?	+	−	+	Anthrax
	100	M	25	+	+	−	+	+	?	+	−	+	Anthrax
	87	F	32	−	+	−	−	+	−	+	−	+	Anthrax
	86	F	45	+	+	−	−	−	+	+	?	+	Anthrax
	92	M	46	−	+	+	−	−	?	+	−	+	Anthrax
5	147	F	10	−	−	−	−	−	?	+	−	+	Anthrax
	151	F	17	+	+	−	+	+	+	+	−	+	Anthrax

[a] Symbols: + = grossly pathologic; − = no significant abnormal findings; ? = questionable abnormal findings.
[b] Hemorrhagic appearance to lungs.
[c] Heavy overgrowth with *Proteus* species.
[d] Sacrificed as normal at end of run.

total of seven monkeys (22.6%) died of anthrax, five during the 41 days of exposure between the 14th and the 46th day, and two during the 10-day postexposure holding period 4 and 5 days after exposure was terminated. Two monkeys died of other causes. None of the sacrificed animals had evidence of anthrax infection. There was actual exposure on 25 days of the total of 41 consecutive days, and the daily mean for these days was 198 *B. anthracis*-bearing particles. The total calculated inhaled dose over the 41-day period was 4,959 *B. anthracis*-bearing particles.

To develop additional specific dose-response data, in the fifth run animals were exposed to as concentrated an aerosol as possible and then held for a prolonged observation period. Accordingly, arrangements were made with the mill to process a maximal number of bales of goat hair through the picking machine as rapidly as possible. Twenty-eight monkeys were initially exposed during three separate periods over three successive days to a calculated aerosol of 947 *B. anthracis*-bearing particles. The animals were then held for an additional 25 days without further exposure to mill air. Two animals died of inhalation anthrax 10 and 17 days after the first day of exposure, and four died of other causes. The limits of the incubation periods for these

TABLE 3. *Summary of microscopic findings—(monkeys that died—third, fourth, and fifth runs)*

Run	Animal no.	Diagnosis	Anthrax bacteremia	Mediastinal lymph nodes[a]	Cervical lymph nodes[a]	Liver necrosis	Splenic necrosis[b]	Anthrax meningitis	Adrenal hemorrhage and necrosis	Anthrax lobar pneumonia	Pulmonary edema[c]	Colonies of bacilli in submucosa of trachea
3	52	Anthrax	+	+++	++	−	+++	+	0	−		−
	66	Bronchopneumonia and anthrax pneumonia	+	++	−	−	+++	−	−	+		−
	40	Anthrax; staphylococcal septicemia; cachexia	−	+	−	−	++	−	−	+		−
	63	Anthrax	+	++	+	+	++	+	+	−		−
	43	Anthrax	+	+++	++	+	+++	+	−	−		−
	61	Anthrax	+	+++	+	−	+	−	0	−		−
	44	Anthrax	+	+++	0	+	+++	−	+	−		−
	69	Anthrax pneumonia	+	+++	−	+	+++	+	+	+		+
	55	Anthrax	+	+++	0	−	+++	−	−	−		−
	59	Anthrax	+	+++	+	+	+++	+	+	−		−
	49	Anthrax	+	+++	+++	+	++	−	+	−		−
	60	Anthrax	+	++	++	−	+++	−	+	−		−
	50	Anthrax in one lymph node	−	++	−	−	+	0	−	−	−	−
	51	Anthrax with two paratracheal lymph nodes	+	++	−	−	−	0	−	−	−	−
4	80	Anthrax	+	+++	+	−	++	+	−	−	+	−
	110	Anthrax	+	+++	+	−	+++	+	+	−	+	−
	101	Anthrax neumonia	+	+++	+	−	+++	−	−	+	++	−
	100	Anthrax	+	+++	+	−	+++	+	−	−	+	−
	87	Anthrax	+	+++	+	−	+++	+	−	−	−	+
	86	Anthrax	+	+++	+	−	++	−	+	−	+	−
	92	Anthrax	+	+++	+	−	+++	−	−	−	−	−
5	147	Anthrax	+	++	+	−	++	−	−	−	−	−
	1[illegible]1	Anthrax	+	+++	+	+	+++	−	−	−	−	−

[a] Key for lymph node morphology: + = anthrax bacilli with follicular necrosis; ++ = bacilli with necrosis and edema; +++ = bacilli with necrosis, edema, and hemorrhage.
[b] Key for splenic morphology: + = sinusoids engorged with neutrophils; ++ = neutrophils in sinusoids plus central necrosis of malpighian bodies; +++ = necrosis of red and white pulp.
[c] Key for pulmonary edema: + = minimal edema; ++ = moderate edema; +++ = marked edema; − = no edema in alveoli. (0 = no tissue submitted.)

two deaths were 7 and 17 days. The fatality rate was 7.2%. Subsequently, the remaining 22 monkeys were exposed during two separate periods over 31 hr to a calculated aerosol of 1,347 *B. anthracis*-bearing particles. No deaths occurred during the following 4-day observation period. The surviving monkeys were sacrificed over a 6-day period, and none revealed evidence of anthrax infection. During the fifth run, 47 guinea pigs were exposed to the same aerosols and held for the same period as the monkeys. None of them died of anthrax.

Dalldorf studied sections from the 91 monkeys on which autopsies had been done. All had lesions attributable to other causes, such as parasites. Twenty-three showed evidence of anthrax infection (Table 2). Twenty died of inhalation anthrax, and in one anthrax was considered a co-primary

cause of death along with staphylococcal septicemia and cachexia due to enteritis. Two monkeys sacrificed at the end of the third run had early infection in the mediastinal lymph nodes only. Nonanthrax deaths were primarily due to pneumonia and enteritis.

The most consistent pathological findings in anthrax-positive monkeys were mediastinal edema, pleural effusion, enlarged hemorrhagic mediastinal lymph nodes, and enlarged, soft spleens. In four instances, gross hemorrhagic meningitis was observed. *B. anthracis* was recovered on culture from all but three infected animals: a sacrificed animal with an early infection, the monkey in which anthrax was considered a contributory cause of death, and a monkey from which the plates prepared at autopsy were heavily overgrown with *Proteus* sp. There was no gross evidence of primary cutaneous or gastrointestinal anthrax, and there were no lesions associated with the oral cavity, including the tonsils.

Histological examination of the tissues showed that infection was largely limited to the reticuloendothelial system, though there was always widespread dissemination of the bacilli through the vascular system at the time of death (Table 3). Tissue response was primarily that of edema, hemorrhage, and necrosis. The mediastinal lymph nodes were infected in all cases, and in a few monkeys the paratracheal lymph nodes were also infected. No primary lesions were found in the trachea or bronchi. In three monkeys, there was evidence of anthrax pneumonia, but this was not considered primary.

Pathological changes noted in other organs as a result of anthrax infection included splenic and hepatic necrosis, adrenal hemorrhage and necrosis, ovarian hemorrhage, and meningitis. The renal glomeruli contained many bacilli, but the kidneys were otherwise normal.

Serological studies were conducted by George Wright, Immunology Branch, Fort Detrick, by use of a micromodification of the Ouchterlony double-diffusion technique. A total of 210 sera, 66 collected before exposure and 144 collected during or after exposure, were tested without demonstration of any antibody titers.

DISCUSSION

The overall fatality rate of 25.3% indicates the susceptibility of the cynomolgus monkey to naturally occurring, industrially produced aerosols containing *Bacillus anthracis* and attests to the feasibility of the experimental design. The objectives initially outlined have been partially attained. The clinical and pathological effects of chronic exposure are not dissimilar to those seen after acute exposure in laboratory experiments.

The pathogenesis of inhalation anthrax after chronic exposure is similar to that postulated after acute exposure of laboratory animals or of man naturally exposed, such as was seen in the Manchester, N.H., epidemic. The necropsy findings of mediastinal edema, mediastinal hemorrhagic lymphadenitis and necrosis, and pleural effusion, without tracheal or bronchial lesions and without primary anthrax pneumonia, support the concept that inhaled *B. anthracis* spores are carried to the mediastinal lymph nodes, where they germinate and produce toxin with development of toxemia and bacteremia. Additional evidence to support this concept is found in the necropsy data from the two sacrificed monkeys in the third run; tissues from these monkeys revealed *B. anthracis*-like organisms primarily in mediastinal lymph nodes. These animals were undoubtedly in the early stages of disease and presumably would have developed systemic disease and died, had the experiment not been terminated. Histological examination of the necropsy tissue from all monkeys that died of anthrax shows widespread dissemination of *B. anthracis* organisms.

The serological studies do not support the development of subclinical infection. Norman et al. (12) studied sera from 72 unvaccinated employees of a goat-hair mill and found 11 who had anthrax antibodies demonstrated by a precipitation inhibition test. Most of the positive reactions occurred among employees who worked in the dustiest part of the mill.

In discussing industrial anthrax, Brachman and Fekety (5) compared the length of employment in goat-hair processing mills of a group of employees who did not have a history of anthrax infection with the length of employment of employees with a history of previous anthrax infection; they found that the two curves were essentially parallel. This suggests that the length of employment did not influence the development of anthrax. They noted that some cases of cutaneous anthrax occurred in employees who had worked in these mills for 15 to 20 years. Their conclusion: "These workers do not develop subclinical infection or immunity to anthrax by prolonged exposure to the organism."

The studies with monkey sera may support these data; however, it is possible that the serological test employed was not sensitive enough to demonstrate antibodies actually present, that the antigen used was not specific for protective antibodies, or that the inhaled dose was too low to stimulate production of demonstrable antibodies.

Data from the fifth run indicate that, with a total exposure to 947 *B. anthracis*-bearing particles intermittently over a 55-hr period, there were two deaths at 10 and 17 days after first exposure, for a fatality rate of 7.2%. The shortest incubation period possible was 7 days, and the longest was 17 days. The second exposure during the fifth run resulted in an inhaled dose of 1,347 *B. anthracis*-bearing particles over 31 hr. The fact that there were no deaths during the 4-day holding period and the 6-day period during which survivors were sacrificed may represent either a lack of susceptible monkeys or, more likely, an inadequate observation period.

Data from the third and fourth runs are more difficult to interpret, because of the irregular, sawtooth pattern of exposures on successive days, without knowing the effect of repetitive exposures on the monkeys. For example, frequent small doses may stimulate an antibody response that increases an animal's resistance to clinical disease, or repeated exposures may build up a level of *B. anthracis* organisms in the body which causes disease when a certain threshold level is reached. Another possibility is that repetitive exposure over a period of several days increases the chances of the animal's acquiring an infecting dose.

As already discussed, the serological studies did not demonstrate the presence of circulating antibodies, which supports the concept that chronic exposure does not lead to development of resistance to infection. The lack of deaths following the second exposure in the fifth run cannot be assumed to represent protection resulting from the first exposure because of the lack of an adequate observation period after the second exposure.

The peak exposure during the first 5 days of the third run, 5,685 *B. anthracis* particles, may be causally related to the six anthrax deaths that occurred from 15 to 18 days after the first day of exposure. If related, the fatality rate was 20% (6 of 30), and the incubation period would then have ranged from 10 to 17 days, which is similar to the incubation period in the fifth run.

Another peak exposure occurred from the 36th to the 40th day, when the surviving 20 monkeys were exposed to 3,525 *B. anthracis*-bearing particles with two deaths occurring from 5 to 11 days after exposure. The two animals found to be infected at autopsy may have become infected as a result of contact with this same aerosol; the incubation period would have been from 10 to 14 days for these two monkeys. If all four deaths are related to the last peak aerosol, the fatality rate would be 20% (4 of 20). It is most likely

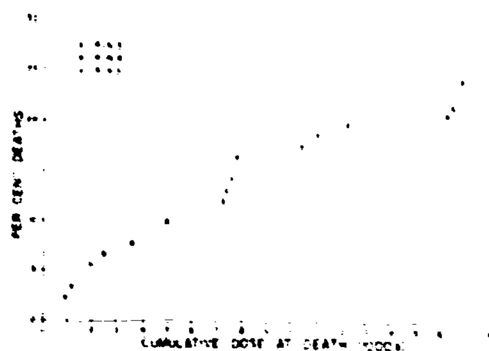


FIG. 5. *Calculated cumulative dose of Bacillus anthracis at time of death.*

that the death on the 41st day was from exposure to the preceding week's aerosol, that is, from days 28 to 33, which would then indicate an incubation period for this particular death of from 8 to 12 days.

The two deaths on successive days at the end of the 4th run (days 45 and 46) occurred 5 to 8 days after a peak exposure to 1,250 *B. anthracis*-bearing particles. If this relationship is correct, then this exposure would be associated with a fatality rate of 8%. The other deaths in the third run and all deaths in the fourth run are harder to associate with specific periods of exposure.

If repeated low-dose exposure results in accumulation of *B. anthracis* organisms until a certain level is reached, after which disease develops, the comparison of the percentage of deaths and the accumulative dose at death should show a straight-line relationship until the critical level is reached, after which there should be a sharp upsurge in the percentage of deaths. As shown in Fig. 5, this is not the case. Additionally, the data were examined to see whether the dose accumulated during specific periods preceding death, that is 7, 10, 12, or 15 days, would suggest an effect of accumulation of *B. anthracis* organisms. Such an analysis is presented in Fig. 6, for which the particles inhaled during the 7 days preceding death are plotted against the day of death of each monkey. Again, the scattering of deaths over a wide range of calculated inhaled doses would tend to be against the theory of accumulation.

Analysis of these data does suggest a dose-response relationship with exposure to approximately 1,000 *B. anthracis*-bearing particles over a 3- to 5-day period, resulting in a fatality rate of approximately 10%. When the exposure is from 3,500 to 5,500 *B. anthracis*-bearing particles over a 5-day period, the rate is from 20 to 25%.

The prolonged incubation periods are un-

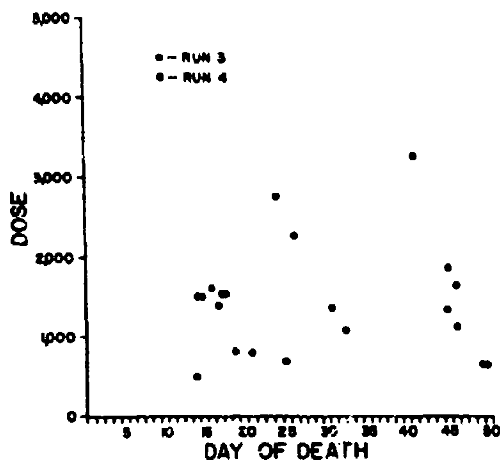


FIG. 6. *Calculated inhaled dose during 7 days preceding death.*

doubtedly related to the low-dose exposure to *B. anthracis*. Exposure to more concentrated, pure aerosols is usually associated with incubation periods of from 3 to 7 days.

Extrapolation of these data to man is difficult. One reason is that man samples a greater proportion of the contaminated mill air because his minute volume is 10 times that of the monkey. There is no reason to suspect any change in the type of goat hair processed or in the method of production from the years before immunization to the present. Thus, the aerosol produced during the three experimental runs is probably representative of the working situation in the picking area at the time when employees were not protected by the anthrax-protective antigen. The lack of cases of inhalation anthrax may therefore represent the lack of exposure to "peak" aerosols as defined above. Also, the monkeys were exposed to a maximal concentration of *B. anthracis* aerosol produced by the picking machine; people, however, are never exposed to the total aerosol produced, but only to a relatively small part of it while they work in the vicinity of the picking machine.

The 1957 epidemic of inhalation anthrax in Manchester, N.H., can possibly be explained by the exposure of the five susceptible individuals to a "peak" aerosol related to a specific batch of hair. In addition, the sporadic cases that have been reported unassociated with the goat-hair processing industry also may represent the chance exposure of susceptible individuals to a "peak" aerosol.

SUMMARY

Exposure of 91 cynomolgus monkeys to naturally produced aerosols containing *B. anthracis* resulted in an anthrax fatality rate of 25.3%. The pathological findings of mediastinal edema and hemorrhagic lymphadenitis and necrosis are similar to findings in animals after acute exposure to pure aerosols of *B. anthracis*, and also to the findings in humans who have developed fatal inhalation anthrax after industrial or sporadic exposure. With the low-dose chronic exposure to natural aerosols, the incubation period appears to range from 5 to 17 days.

Analysis of the data suggests a dose-response relationship with fatality rates ranging from 10 to 25% after exposure to from 1,000 to 5,500 organisms over 3 to 5 days. There is no specific evidence to support the development of subclinical infection, or of an accumulative effect of anthrax organisms. These studies do support previous concepts concerning the pathogenesis and dose-response relationships of anthrax in monkeys.

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Discussion

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Industrially acquired anthrax has been associated with transportation and processing of imported wool, hides, and goat hair. The spores of *Bacillus anthracis* have been cultivated from as many as 50% of samples of the raw products (4, 15, 17). Industrial processing creates dust, so that anthrax spores regularly contaminate the surfaces and air of the factories; up to 66% of surface samples were positive in three mills processing goat hair (5). Respiratory exposure of workers may reach 510 spores in particles 5 μ or less in diameter in a [illegible] working period (9). A significant percentage of anterior nasal swabs and pharyngeal washings from mill workers processing goat hair yielded *B. anthracis* (8).

Despite this, inhalation anthrax is rare in the United States (7). Brachman et al. (6) studied the response of the cynomolgus monkey exposed chronically to the air from the dustiest portion of a goat-hair processing mill in an effort to enlarge our understanding of industrial anthrax. It is my purpose to assist in this objective by relating their observations to selected laboratory studies. Specifically, I will consider the variable incubation period they observed, and the dose-response relationship.

Modern views of the pathogenesis of inhalation anthrax are based on the studies in laboratory animals of the experimentally induced disease by Young et al. (19), Barnes (3), Ross (16), Albrink and Goodlow (2), and Gleiser et al. (10), and the pathological findings in three fatal cases in man reported by Albrink et al. (1). The observations of these investigators agree in defining the role of the lung as a portal of entry in inhalation anthrax; primary anthrax lesions are not found in the trachea or bronchi, at least not in the absence of pre-existing lung lesions. Thus, we may visualize spore-bearing, airborne particulate matter of sufficiently small size (i.e., 5 μ in diameter, or less) after inhalation, penetrating to the deep recesses of the lungs and being deposited there as essentially inert particles. Subsequent removal of the spores is accomplished principally by alveolar macrophages that transport them via the lymphatics to the regional

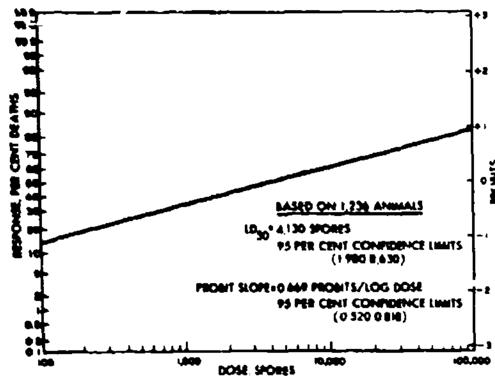

FIG. 1. *Response of the cynomolgus monkey to aerosols of Bacillus anthracis.*

lymph nodes. Neglecting, for our present discussion, the further steps in the pathogenesis of inhalation anthrax, it seems reasonable that a significant period of time will be required for clearance of a large number of spores, even if these are introduced in acute experimental exposures. The respiratory retention of inhaled spores was initially studied by Harper and Morton (12), using radioactively labeled *Bacillus subtilis*. When these were presented to guinea pigs for inhalation as particles 1 μ in diameter, the majority of the radioactivity was found in the lungs (as contrasted with the head or the trachea), and essentially no loss of radioactivity of the lungs was measurable over a 24-hr observation period. Subsequently, in studies of the prophylaxis of inhalation anthrax in the rhesus monkey, Henderson et al. (13) demonstrated that a daily regimen of procaine penicillin, initiated 24 hr subsequent to aerosol exposure, could delay the onset of disease and death, and that this protection ceased promptly on termination of the therapy. They also showed that spores of *B. anthracis* can be detected for as long as 100 days after their deposition onto the lung epithelium. In a more recent similar study, Gochenour et al. (11) provided further direct evidence of prolonged spore retention in the lungs after an acute inhalatory exposure. One of their monkeys died with anthrax meningitis 25 days after completion of an apparently successful course of therapy. Cultures of the lungs of all animals surviving 55 to 84 days after exposure to aerosols of anthrax spores were positive for this organism. Finally, one of my colleagues, Joseph V. Jemski, has made available information he obtained several years ago in making a study of the time to death of the cynomolgus monkey after inhalation of aerosolized anthrax spores. Several of the animals in that study, which was directed toward determining the minimal holding period required to assure statistically valid dose-response data, died of culturally proven anthrax after prolonged incubation periods—one animal succumbed 98 days subsequent to exposure.

The concepts involved in the pathogenesis of inhalation anthrax, as well as the experimental evidence cited above regarding the prolonged retention of spores in the lungs, are completely consonant with the variable incubation periods reported by Brachman et al. (6) in their studies of chronic exposure to varying doses over many days. An additional pertinent laboratory observation has been the dose dependency of the incubation period, with lower inhaled doses of spores resulting in longer incubation times (10).

In considering the dose-response relationship of the cynomolgus monkey in experimentally induced inhalation anthrax, I am again indebted to Dr. Jemski for placing at my disposal hitherto unpublished data. These represent a compilation of the results of several individual experiments in which large numbers of cynomolgus monkeys were acutely exposed (1 to 10 min) to heterogeneously sized aerosols of anthrax spores. The aerosol clouds were sampled with an impinger preceded by a preimpinger, the latter device screening out the majority of particles greater than 5 μ in diameter (14, 18). Thus, the dose reported, after microbiological assay of the collection fluid of the impinger, represents spores present in particles predominantly 5 μ in diameter, or less. This dose, and the mortality of the monkeys from culturally proven anthrax during a 10-day observation period subsequent to aerosol exposure, have been subjected to statistical analysis by the probit method (Fig. 1).

It will be noted that the median lethal dose (LD_{50}) based upon a total of 1,236 animals is 4,130 spores with 95% confidence limits of 1,980 to 8,630 spores. The probit slope is 0.669 probits per log dose, with 95% confidence limits of 0.520 to 0.818. As a consequence of this unusually low probit slope, large changes in the dose of inhaled spores will result in comparatively small changes in the per cent mortality. For example, a 100-fold range of dose (10-fold above and 10-fold below the calculated LD_{50}) will only change the predicted mortality from 25 to 75%.

These laboratory studies of Jemski et al. have many similarities to the experimental epidemiological investigations of Brachman et al. (6). There are, however, several important differences. The former involved a very large number of animals, acutely exposed to laboratory-grown spores, under well-controlled conditions for

experimental airborne infection. The latter represented the chronic exposure, over many days, of a small group of animals to an uncontrolled, industrially generated aerosol of spores that were present as a result of the industrial use of contaminated animal products. Nevertheless, the dose-response relationship derived from the laboratory data was predictive of the field results, if the cumulative dose of spores inhaled by the monkeys is considered the most important factor in the chronic exposures [see particularly Fig. 4 and 5 of Brachman et al. (6)].

Precise comparisons of the laboratory observations and the field studies are not possible for reasons already mentioned. For the same reasons, conclusions from consideration of both sets of investigations must be drawn with caution. With this caveat firmly in mind, it does seem that the following statements are justified. (i) The dose-response relationships determined with the cynomolgus monkey in the laboratory permitted prediction of the outcome when the same species was exposed chronically to a contaminated industrial atmosphere. (ii) The adequacy of the cynomolgus monkey as a model for predicting the quantitative aspects of the response of man in the industrial environment is open to reasonable doubt.

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Epidemiology of Airborne Staphylococcal Infection

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INTRODUCTION

It is a characteristic of the airborne route of infection, in contrast to transfer by food or water, that whenever there is the possibility of aerial transfer there is almost always also the possibility of transfer by other routes. This is perhaps especially true of the forms of staphylococcal infection that have been most extensively studied, namely, those occurring in hospitals. But, during the last few years, there has been a great volume of work based on the assumption that airborne spread is an important route in the spread of the human staphylococcal disease, and there is therefore a considerable body of information for review.

It is logical and convenient to discuss first the studies on dispersal of staphylococci into the air and, second, the survival of the cocci in, and their carriage by, the air. These aspects can be presented in some precise and quantitative detail. When we come to consider acquisition, we enter an area in which extrapolation and analogy loom large, but sufficient quantitative data have now been accumulated to give some factual foundation to the discussion. Nevertheless, the final summing up as to the probable importance of airborne transfer in relation to other modes of spread is of necessity a product of judgment rather than arithmetic.

Material Reviewed

For the most part this paper is based on a selective review of published reports, with special reference to those on a series of investigations (44, 45, 58, 59) carried out with R.A. Shooter at St. Bartholomew's Hospital, London, England (referred to as S.B.H.).

Use has also been made of a recent study of my own at St. Mary's Hospital, London, England (S.M.H.), a report of which is in preparation. In this study, we sampled the air in two surgical wards, one having a total of 14 or 15 patients in four rooms and the other having 22 beds in an open ward. Petri dishes [diameter, 6 inches (15 cm)] of serum-agar containing phenol-phthalein phosphate (2) were exposed in the rooms for 12 hr (or in part of the experiment for 24 hr) on each of 5 days each week. Nasal cultures were examined from each patient weekly.

The total number of staphylococcus-carrying particles on the air plates was recognized by the phosphatase reaction and either all or, when the numbers of colonies on the plate were larger than three to five, a portion were subcultured and tested for coagulase and for phage type. It was thus possible to make some estimate of the number of coagulase-positive staphylococci settling from the air, and of the proportion with various phage patterns, for attempted correlation with

the strains isolated from the patients' nasal cultures.

For airborne particles with a diameter equivalent to those found to carry *Staphylococcus aureus*, that is about 14 μ (38), the settling rate in colonies per square foot per minute is numerically approximately equal to the volume count expressed as colonies per cubic foot. A 6-inch petri dish has an area of approximately 0.2 ft^2; in round figures, therefore, the count on such a plate exposed for 24 hr is about 60% of the number of particles inhaled by an adult person in the same time, since the volume inhaled is ordinarily about 0.3 ft^3 per min.

DISPERSAL OF STAPHYLOCOCCI INTO THE AIR

The frequency with which normal individuals harbor *S. aureus* in the nose and on the skin is now well known (56), and such normal carriers are one important source from which the cocci are dispersed. The other source from which staphylococci may be dispersed, especially in hospitals, comprises patients with infected lesions—of skin, wounds, respiratory tract, or gut.

Nose and Skin Carriers

Hare and his colleagues were among the first to define the frequency with which nasal carriers of *S. aureus* liberate the cocci into the environment; they counted the numbers shed into the air of a very small cubicle during exercise. Hare and Ridley (21) found that all but 6 of 19 carriers liberated staphylococci, and 7 gave substantial numbers; this and subsequent work (41) pointed to the special importance as dispersers of persons who harbor staphylococci on the perineum. On the other hand, White (54, 55) emphasized the relation between dispersal of staphylococci and the total numbers present in the nose and on the skin.

One feature that also emerged from these and other studies was the wide individual variation in the number of staphylococci shed into the air by carriers. The individuals at the upper end of the distribution seemed to differ sufficiently from those at the lower end to justify the use of the term "heavy disperser" for them, and the suggestion that such heavy dispersers might be responsible for epidemics of hospital infection stimulated further study of the mechanism of dispersal.

Mechanism of Dispersal

Hare and his colleagues showed that very few staphylococci are liberated into the air directly from the nose of carriers during ordinary activity; Hare (19) described the liberation by other routes as "outflow" and emphasized the importance of friction with the skin. White (54) had found that the extent to which patients contaminated their bedding was correlated with the numbers of staphylococci found in their nasal cultures. Subsequently, Davies and Noble (14) demonstrated that large numbers of skin fragments are dispersed into the air during the activities known to liberate bacteria; they suggested that most of the staphylococci are carried on such fragments, and they were able to cultivate *S. aureus* from epithelial squames liberated by a known carrier (15).

It thus seemed that the differences among individual carriers in the number of staphylococci that they disperse might be related to (i) the number of cocci on the skin, (ii) the particular area of skin colonized, or (iii) the rate of desquamation. By parallel sampling of air for skin squames and staphylococci, Noble and Davies (37) showed that the last of these was not likely to be the explanation; they thought that the extent of skin carriage was probably the most important determinant. Hare and Ridley (21) had previously suggested that carriage on the skin of the perineum was particularly likely to lead to dissemination, and Solberg (49) not only confirmed this but also showed that, in the absence of perineal carriage, there is a correlation of numbers of staphylococci disseminated with the number found in the nose or on the skin (in his experiments, of the fingers and hand). The importance of the perineal skin as a source for dispersal receives indirect support from observation that, in operating-room clothes, the greatest liberation of skin bacteria seems to be from below the waist and especially through open trouser ends (5, 8). It may be noted that most observers have measured air contamination while the subjects were exercising in some form of cubicle and generally making quite vigorous leg movements; this may perhaps over-emphasize the contribution of the perineum to aerial dispersal.

Frequency and Magnitude of Dispersal

It was clear from the early work of Hare and Ridley (21) that many nasal carriers shed staphylococci into the air while exercising. This has been amply confirmed. On the basis of experiments in small cubicles, Bethune et al. (5) reported that 14 of 38 nasal carriers (from a group of 150 normal people) generated an air contamination level of one *S. aureus* particle per ft^3 or more, corresponding to a total liberation of about 100 particles or more in 2 min of walking-on-the-spot. Noble and Davies (37) examined 127 persons, 54 of whom were normal adults whereas the rest

were hospital patients, many with skin disease. The subjects removed all their clothing and then dressed again in a 100 ft³ cubicle from which the air could be sampled. Of the whole group, 30, including only 2 of the 54 normal adults, liberated *S. aureus* to 1% of the total flora in the cubicle; this corresponded to the liberation of about 25 staphylococcus-carrying particles or more. Eight liberated more than 10,000 *S. aureus* particles.

More precise estimates of the numbers of staphylococci liberated by carriers have been provided by Solberg (49; *personal communication*), who estimated the aerial contamination resulting from a standardized agitation of a group of persistent carriers' bedding in a special chamber. Solberg found the air counts of staphylococci dispersed during the making of the beds of his carriers to be distributed in a log-normal fashion, and at least 20% of the 126 carriers (drawn from 2,014 patients surveyed) dispersed more than 10,000 staphylococcus-carrying particles in the standard test (Fig. 1).

Our own studies of air counts in a hospital ward offer another basis for estimating the frequency of dispersal. In Fig. 2 are plotted the mean daily counts of *S. aureus* of the phage type carried by each of the patients who were carriers on admission to the subdivided S.M.H. ward,

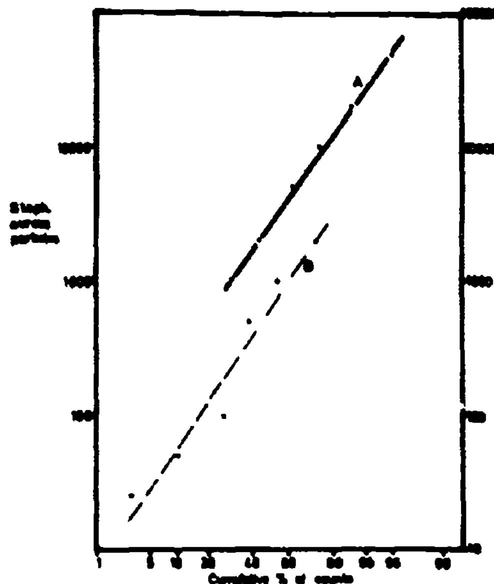

FIG. 1. *Air counts of Staphylococcus aureus. (A) Generated by disturbance of bedding of persistent carriers [after Solberg (49), supplemented by a personal communication]; (B) during undressing and redressing [after Noble and Davies (37)]. Plotted on a probability scale, so that a straight line represents a normal distribution*

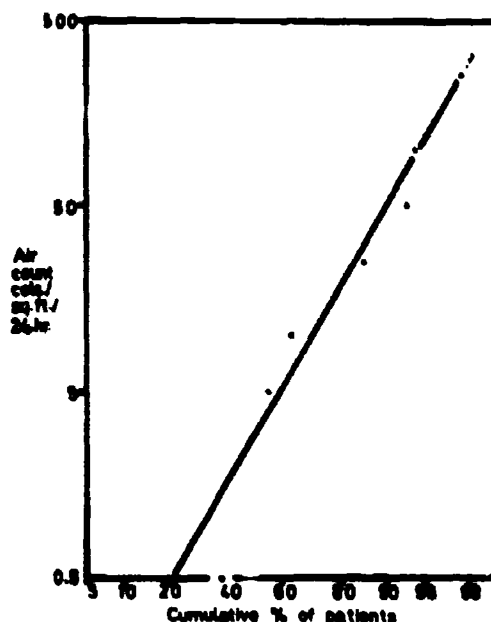

FIG. 2. *Air counts (particles per square foot settling in 24 hr) of Staphylococcus aureus generated by patients admitted as carriers to a hospital ward (S. M. H.).*

excluding those who were carrying strains of types already known to be present in the ward; some of the patients, in contrast to Solberg's subjects, were only transient carriers. The counts are clearly also distributed log-normally. About 50% of carriers generated air counts below 5 colonies per ft² per 24 hr, but 10% generated counts that averaged more than 50 colonies per ft² per 24 hr during their stay in the ward, at times when they were the only carriers known to be present. Some rough estimates as to the ventilation rate of the ward suggest that this implies the liberation of 10^6 to 10^7 staphylococcus-carrying particles in 24 hr.

It seems likely, therefore, that the heavy dispersers of staphylococci represent the top end of a continuous distribution. This is compatible with the idea that the degree of dispersal depends largely on the extent of skin contamination with staphylococci, and that the shedding of the staphylococci into the air is due to the continuous desquamation of skin fragments carrying cocci, which either may be transients recently deposited there from the reservoir area in the anterior nares, or may be actually multiplying in or on the skin. The rate of desquamation is presumably related in part to friction of the skin and clothes or other skin areas.

It is, perhaps, remarkable how many bacteria are shed on exercising, even when the subjects

are naked (50). Also, in a few unpublished observations in coal mines, O.M. Lidwell and I found that nearly naked miners distributed skin bacteria into the air in quite large numbers. It may also seem surprising that carriers liberate as many staphylococci as they do when it is considered how relatively scarce staphylococci appear to be when carriage is determined by swabbing; however, the area of skin generally examined is very small, and most methods for the bacteriological examination of skin are known to be very inefficient (62).

Factors Influencing Dispersal

Blowers and McCluskey (8) commented that they have not yet encountered a heavy disperser of staphylococci among the normal women they have examined, whereas they found nearly 10% of men to be dispersers. None of the other studies has discussed the influence of sex, but, of the 10 heavy dispersers reported by Solberg, 3 were women, and in general his results do not show any significant differences between men and women carriers in the numbers of staphylococci dispersed. In my ward studies, one of the five heaviest dispersers was a woman.

It has been found that treatment of a nasal carrier of tetracycline-resistant staphylococci with tetracycline led to an increase in the number of staphylococci dispersed into the air, presumably as a result of increased nasal carriage (17), or possibly as a result of increased skin carriage resulting from a reduction in the normal flora and a consequent reduction in the fatty acid content of sebum (18). A similar phenomenon was observed in debilitated or dying patients by Solberg: an increase in the number of organisms in the nose and a corresponding increase in the number shed. M.T. Parker (*personal communication*) has made a similar observation. One observation that a concomitant virus infection might increase dispersal (16) does not seem to have been confirmed.

A very substantial increase in the number of staphylococci liberated has been found to follow the taking of a shower bath (4, 62). The increase may be 10-fold or more, and the effect persists for at least 60 min. The mechanism of this increase is not known, though it is presumed that the washing in some way allows an increased loss of the superficial squames.

Within broad limits, clothing makes remarkably little difference to the liberation of skin bacteria, and indeed Speers et al. (50) found that some of their subjects liberated as many bacteria when exercising naked as they did when fully dressed, either in street clothes or in a sterile operating room suit. The only practicable method so far described for reducing the rate of liberation is the use of very closely woven clothing, with a trouser suit tightly closed at the ankles (4, 8).

Infected Lesions

The discussion to this point has been concerned with healthy carriers of staphylococci, without any staphylococcus-infected lesions. As would be expected, patients with staphylococcal infections of the skin tend to be especially heavy dispersers (1, 20, 37). Thom and White (52) found, however, that there was little dispersal from septic wounds during the performance of wound dressing, and it seems likely that the effect of skin lesions is, partly at least, to increase the load of staphylococci on the skin. There may also be an increase in the rate of desquamation, for example, in some patients with psoriasis, and one such has been implicated as the source of an epidemic of surgical wound infection in an operating room (32*a*), though generally in such patients many of the skin particles dispersed are too large to remain airborne (37). Our own observations (59) indicated that carriers can be as important as sources of cross infection as patients with septic lesions. Burke and Corrigan (13), on the other hand, found patients with septic lesions to disperse more staphylococci than healthy carriers; but they studied only 44 carriers. Patients with chest infections have been thought from time to time to be especially dangerous as dispersers (e.g., 44), but there is little direct evidence on this point. The possible effect of antibiotic treatment on dispersal needs to be considered when patients with septic lesions are being compared with healthy carriers.

Air Contamination Resulting from Dispersal

It can thus be concluded that most persons who carry staphylococci in the nose, all of whom must from time to time contaminate their skin, liberate their staphylococci into the air around them. A small proportion of the carriers are especially heavy dispersers and give rise to a high level of aerial contamination. It is not surprising, therefore, that there are considerable variations in the counts of staphylococci in hospital ward air (Fig. 3). The variations in the air counts are, of course, directly related to the presence or absence of heavy dispersers in the ward (34). When the air count in the ward was high, it was virtually always found that the air staphylococci were almost all of one phage type and usually attributable to spread from one person. In the large ward, there were occasions when two dispersers contributed significantly to the air count, but those occasions were relatively uncommon (34).

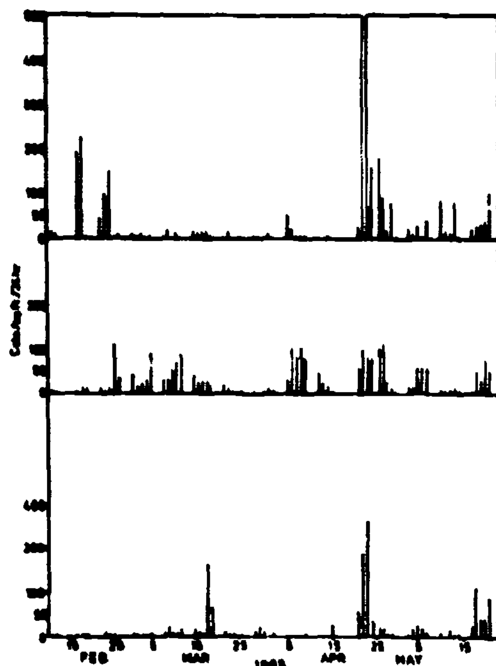

FIG. 3. *Air counts (particles per square foot per 24 hr) of Staphylococcus aureus in three rooms of the divided S. M. H. ward.*

But, as in other situations, the air counts in hospital wards have been found to conform to a log-normal distribution. In Fig. 4, the air counts from the divided ward are plotted as logarithms on a probability scale and are seen to fall close to a straight line. For comparisons between wards, therefore, the median is the most appropriate statistic. Lines depicting the distributions in five different wards are shown in Fig. 5, and the medians from some of them are given in Table 3.

In the S.B.H. open ward, the median count of *S. aureus* was about 0.1 colonies per ft². This count was derived from two periods of 2-hr sampling each week, and it might be thought that this could be no more than generally indicative of the total daily exposure of the particles to airborne staphylococci. However, a very similar median and distribution of staphylococcal counts were observed in the open ward at S.M.H., tested by exposure of 12-hr sedimentation plates.

It is instructive to present the counts in terms of the numbers of staphylococcus-carrying particles that might be inhaled by ward patients in 24 hr. In the two open wards, the median numbers that would be inhaled per day were about 18 and 23 particles; the daily dose exceeded 100 particles on about 15 to 22% of days. In the divided wards at S.M.H. and S.B.H., the median was about 4, and a dose of 100 was exceeded on only about 3% of days.

It is interesting to note that in a small series of tests in a ward at the Queen Elizabeth II Hospital, Welwyn, which consists of four-bed bays opening off a wide corridor, the air counts are intermediate between those of the open and the divided wards (data kindly supplied by R. W. Payne). The explanation of these differences clearly demands further investigation, and it is of obvious relevance to the acquisition of nasal carriage of staphylococci, discussed below.

TRANSFER THROUGH THE AIR

For a proper understanding of the mode of spread of airborne staphylococcal infection, a knowledge of the size of the airborne particles and of their load of staphylococci is needed. Studies with the size-grading sampler devised by Lidwell (26) indicated that the mean "equivalent diameter" of particles carrying *S. aureus* was about 14 μ (the "equivalent diameter" is the diameter of a sphere of unit density settling in air at the same rate as the particle in question); the interquartile range was about 8 to 20 μ (38). A much smaller proportion of large particles was observed by Walter et al. (53), using the Andersen sampler, but this is doubtless attributable to the characteristics of that instrument (29). Earlier work by Lidwell and his colleagues (27) indicated that, on the average, airborne staphylococcus

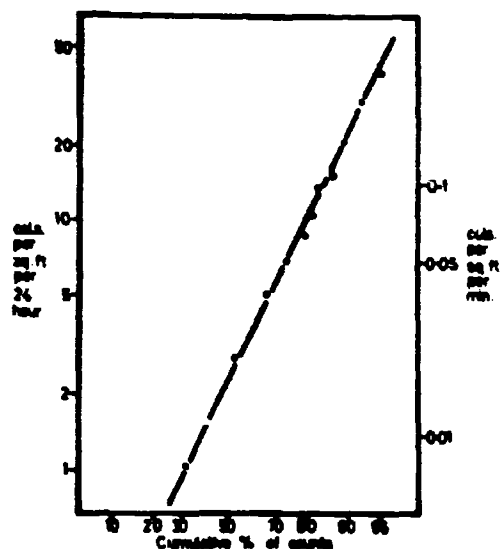

FIG. 4. *Distribution of air counts of Staphylococcus aureus in the divided S. M. H. ward, based on a total of 1,037 12-hr sedimentation plates.*

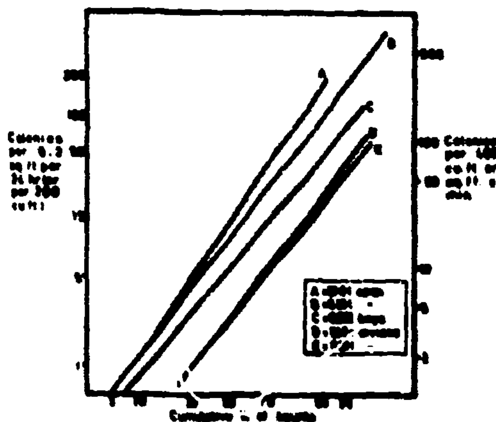

FIG. 5. *Distributions of air counts of Staphylococcus aureus in different wards. A = S.M.H., 22-bed open ward; B = S. B. H., open wards; C = Queen Elizabeth II Hospital, 4-bed open bays; D = S. M. H., divided ward; E = S. B. H., divided ward.*

particles carried about 4 viable cocci, the range being from 6 for the particles greater than 18 μ in diameter to about 1 for those less than 4 μ. These sizes and numbers of bacteria are consonant with the idea that most airborne *S. aureus* cells are associated with desquamated fragments of skin (37).

In normally turbulent air and in a room 10 ft high, particles 14 μ in diameter settle at a rate equivalent to about six air changes per hour, so that 50% of the particles remain suspended for 6 min and 20% for 15 min. Directional air currents of 40 to 50 ft per min are not uncommon in occupied buildings, so that transfer of staphylococci for considerable distances is clearly possible.

In a few studies, in an open surgical ward in which we have found large numbers of staphylococci being dispersed near one sampling point, the mean counts at sampling points about 20 and about 70 ft distant were, respectively, 26 and 11% of the count at the point nearest to the disperser.

When a heavy disperser was present in one of the rooms in the four-room S.M.H. ward, the count in the other rooms has been on average about 5% of that in the room with the disperser. Lidwell and his colleagues (27*a*) have studied a ward divided into nine rooms and found that the count in rooms other than that containing a source of staphylococci is about 10% of that in the source room.

In studies of staphylococcal infection in a surgical operating room, Shooter et al. (46) demonstrated what appeared to be aerial transfer over the distance of 90 ft that separated the wards from the operating room.

The actual extent to which staphylococci can be conveyed within a ward or between rooms must depend on the local circumstances of structure, site, and ventilation, but enough has been said to show that aerial conveyance over considerable distances is quite possible.

The aerial route is not, of course, the only way by which staphylococci can be conveyed from one room to another, and hospital routines commonly prescribe quite elaborate rituals for dealing with potentially infected dust on floors, shoes, trolley (cart) wheels, and the like, which is thought to generate secondary airborne spread. But, though many workers have estimated the bacterial content of floors, few have made any useful studies of actual transfer by this route (see 53).

Viability in Air

There is a considerable amount of laboratory work to show that staphylococci commonly survive in the dried state for periods measured in days or weeks. Whether there is any significant alteration in their infectivity on storage is not certain. Indications of some loss of infectivity were obtained by Hinton et al. (23) and by Taylor et al. (51); other workers have found no such effect (e.g., 28, 42). Noble's (35, 36) experimental work in animals has suggested that any effect on infectivity from desiccation is limited to an extension of the lag period and that, if staphylococci are protected from body defenses immediately after introduction into the tissues, they are as virulent as fresh organisms.

ACQUISITION OF AIRBORNE STAPHYLOCOCCI

There are two important ways in which airborne staphylococci might infect patients in hospitals: by inhalation or by settling directly into some susceptible area, such as a wound, or onto instruments or dressings that subsequently come into contact with the wound. Inhalation infection may occur anywhere and at any time; sedimentation infection is of particular importance in operating rooms and treatment rooms where surgical wounds are exposed, often for long periods of time. It will be convenient to deal with sedimentation infection in operating rooms first.

Operating Room Infection

Airborne transfer from without. Recent studies of air hygiene in surgical operating rooms date largely from the work of Bourdillon and Colebrook (10) in a Burns Unit treatment room, but it was the application of their work to the control of a high incidence of postoperative staphylococ-

cal wound infection by Blowers et al. (6) and by Shooter et al. (46) that brought the subject to general attention. Shooter et al. estimated that, in an 8-month period, the incidence of operating-room infections was 9% of 427 wounds; 0.07 particles per ft³ containing *S. aureus* were found in samples from the air during operations. A simple alteration of the ventilation so as to generate a positive pressure in the operating room and exclude staphylococcus-contaminated air from the wards was followed by a substantial reduction in the general air bacterial count (the number of *S. aureus* was not reported), and by a reduction to less than 1% in the incidence of sepsis of presumed operating theater origin in 532 wounds. It is reasonable to assume that this reduction in sepsis was attributable to a reduction in the number of staphylococci settling from the air into the wounds and onto the sterile instruments and equipment. No other investigation has been reported in which alteration of the ventilation was the only change made, but the published report of Blowers et al. (6) and subsequent unpublished experience (Blowers, *personal communication*) supported the general idea that the prevention of contamination of operating room air with bacteria from other parts of the hospital by the introduction of positive-pressure ventilation has often been associated with a reduction in the incidence of postoperative sepsis. Blowers and Crew (7) recorded a mean *S. aureus* count of 0.6 colonies per ft³ in an exhaust-ventilated operating room, compared with 0.03 colonies per ft³ in a plenum-ventilated operating room.

Locally generated aerial contamination. The work just cited concerned contamination of operating room air with staphylococci from other parts of the hospital, drawn into the operating room by air currents. It is this form of transfer that is controllable by positive-pressure ventilation. But aerial contamination can also be generated within the operating room, either by disturbance of the patients' bedclothes and drapes or from the skin of the operating room personnel, as discussed already.

Air counts and infection rates. The bacterial count observed in the air of an operating room is clearly the sum of that produced by infiltration of contaminated air from without and that generated locally. It would be of great value for the monitoring of operating room hygiene if it were possible to relate the staphylococcal (or even the total bacterial) count in the air to the risk of postoperative sepsis. The difficulties of deriving such a relationship are, however, very great. The incidence of wound sepsis is in any case generally very low perhaps between 1 and 5%. Only a part of the septic cases are infected during operation, and this portion is difficult to estimate, and in any case is not all attributable to sedimentation of airborne staphylococci. In addition, the numbers of staphylococci actually settling onto susceptible areas are so small as to be difficult to measure.

Burke's (11) study is in many ways the most detailed available. By using a very sensitive technique he was able to recover *S. aureus* from 46 of 50 wounds examined at the end of operation; most wounds yielded two or more different strains, and the mean number of viable units of staphylococci was 14 per wound. Potential sources for the staphylococci found in the wounds were: air, 68%; carrier site on patient, 50%; hands or nasopharynx of the surgical team, 20%. (In some cases, there were two or more potential sources.) Only 2 of the 50 wounds developed any clinical sign of postoperative infection; the rate for wounds that had not been carefully washed out for bacteriological examination was not presented.

In a comparable study of the sources of infection in 35 patients who developed wound sepsis apparently resulting from operating room infection, Bassett et al. (3) thought that a member of the surgical team was concerned in 31% and the patient himself in 17%, the source of the remainder being untraced.

There are several other published studies in which an attempt was made to relate postoperative infection to airborne staphylococci found in the operating room (e.g.,24,53,60), but they do not allow easy summary. The general impression is that staphylococci of the type responsible for postoperative infection were rarely found in the air, but this may well reflect the very small air samples generally examined.

In general, it appears that, in reasonably well-ventilated operating rooms with good staff discipline, the *S. aureus* count is of the order of 0.01 to 0.05 colonies per ft³, in a series of operating rooms, we have observed a mean settling count of about 0.01 colonies per ft² per min, while an American cooperative study reported a count as low as 0.001 colonies per ft² per min. (33).

The operating room ought to be a situation in which it would be possible to determine the average infecting dose of staphylococci for man. Taking a figure of 0.01 colonies per ft² per min, and assuming an effective target area of 1 ft² (to include instruments, etc.) and a duration of operation of 2 hr, a frequency of operating room infections of 1%, would imply that the 1% infective dose is about 1 staphylococcus-carrying particle. But, to put any real meaning into the

figures, we need to measure the air count and the sepsis rate in a far greater number of patients than has yet been attempted and to carry out at the same time elaborate bacteriological cultures on the patient himself and on the ward to try to assess the relative importance of routes of transfer other than air. And we should remember Burke's (12) thesis that sepsis is often determined largely by the condition of the actual tissue on which the staphylococcus alights, and on the state of the patient; if his observations are generally applicable, there are usually plenty of staphylococci.

Infection in Wards

There is ample documentation of the rate at which both newborn infants and adult patients become nasal carriers of the prevalent staphylococcus in many hospital wards (56). It was reasonable to postulate in the first place that these staphylococci reached the nose by way of the air. Evidence has been sought on this point in several ways—by examining the order in which different parts of the body are colonized, by attempting to interfere with transfer by one route or another, by trying to identify the source of the staphylococcus more precisely, and by relating the acquisition rate to the staphylococci found in the air.

The most precise investigations in this field concern newborn infants. It was shown, first, that the umbilicus and abdominal skin are generally colonized before the nose (25, 48). Second, Rammelkamp and his collaborators showed that a nurse carrier only conveyed her staphylococci to infants if she handled them (61), and later that the colonization of the infants could be delayed by increasing the precautions against contact infection (31, 32). With very strict precautions against cross infection, the rate of acquisition of staphylococci was reduced from 43 to 14%; the latter infections were assumed to be due to aerial transfer. The relative unimportance of inhalation infection in newborn infants is perhaps hardly surprising when one considers that the infant has a minute volume of only about 500 ml (about 0.02 ft³), and that he has to be handled frequently, usually by nurses who handle a good many other infants. But a 14% acquisition rate in a 4-day hospital stay is equivalent to some 3 to 4% per day, which is of the same order as observed in adult wards.

TABLE 1. *Primary site of colonization in adults**

Carrier sites, etc., positive before nose for staphylococci of same phage type	No. of patients who became nasal carriers in ward: Nose positive twice or more		Nose positive once only		Total	
None	19	25	25	28	44	53
Clothing only	6		3		9	
Dressing or wound	7		5		12	
Hand or other skin site	14		2		16	
Total	46		35		81	

* Patients swabbed daily; apparent acquisitions on first 3 days of hospital stay, and acquisitions of untypable strains, excluded.

With the evidence from the newborn infants in mind, it is pertinent to ask whether the nose or some skin site is the first area to be colonized in the adults who acquire staphylococci in hospitals. It is obviously more difficult to obtain evidence on this for the adult than for the infant, but in a study of surgical patients (22) R. A. Henderson examined swabs daily from the nose, skin of the hands, skin near the wound site, wound, bedclothes, and environment (Table 1). Some 20% of the 81 patients who became nasal carriers yielded staphylococci of the relevant phage type from one or other of the two skin sites before its appearance in the nose, and a further 15% had yielded the staphylococci from the wound. In the remaining 66% of acquisitions, the nose was the first site on the patient found to yield the staphylococcus. Two important provisos have to be entered here: there was a striking dominance of staphylococci of one phage type among the acquisitions in the ward, which means that there is a serious risk of regarding as related two independent acquisitions; and the area of skin examined was very small and perhaps not representative. Additionally, even skin or clothing contamination might result from airborne transfer, which need not operate only to give inhalation infection. However, the evidence, such as it is, does not contravert the idea that direct inhalation infection is important in the acquisition of the nasal carrier state in adults.

In our recent study at St. Mary's Hospital, 53 patients were observed to acquire nasal carriage of *S. aureus* while in the ward. The same phage type had been recovered from the air prior to its recovery from the patient in 64% of cases (Table 2). In this ward, there was no marked dominance of one type, and indeed 25 types are represented among the 53 acquisitions. Again, this is not formal evidence that the nasal carrier state was acquired by inhalation of cocci, but it is consistent with such an explanation.

Further evidence for the importance of aerial transfer comes from studies of different ward

structures. In an open 22-bed ward we found that separation of patients by the full length of the ward (about 50 feet) only reduced the rate of acquisition of staphylococci by about one-half, as compared with the acquisition rate for a patient in a neighboring bed (59). At the other extreme, very low nasal acquisition rates have been found in patients nursed in single rooms opening to fresh air, that is, when the chance of aerial transfer from one room to another is very low indeed (40). There also appeared to be very little spread of tetracycline-resistant strains from patients nursed in isolation rooms fitted with exhaust ventilation, and the acqusition rate for such strains was greatly reduced in a ward in which all patients harboring such strains were isolated (59).

In adult patients, there are technical difficulties in recognizing the acquisition of nasal carriage that are not present with infants, since truly persistent carriers may fail to yield staphylococci on some occasions. However, since carriage of tetracycline-resistant staphylococci is even now relatively rare (at least in Britain) in people outside hospitals, such strains form a convenient indicator of hospital acquisition. Some rates of acquisition of tetracycline-resistant staphylococci in various wards are presented in Table 3. Unfortunately, data for tetracycline resistance of staphylococci isolated from air samples in these wards are not available, so it is only possible to compare the ranking of the wards with respect to the two parameters. The very limited results suggest that the acquisition rate was higher in the wards with the higher counts of air staphylococci. For various technical reasons, it has not yet been possible to test directly the relation of the acquisition rate to the exposure to particular staphylococci, though this clearly needs to be done.

TABLE 2. *Number of patients showing apparent acquisition of nasal carriage in relation to previous air exposure (S. M. H.)*[a]

No. of weeks nose negative for the acquired staphylococcus before acquisition	Staphylococci of acquired type—		Total
	Found in air previously	Not found in air previously	
1	15 (9)[b]	11 (8)	26 (17)
2	9 (6)	2 (2)	11 (8)
3	3 (2)	2 (2)	5 (4)
4	3 (1)	2 (2)	5 (3)
5+	4 (3)	2 (1)	6 (4)
Total	34 (21)	19 (15)	53 (36)

[a] An additional 13 (9) patients were found on admission to the ward to be carriers of a staphylococcus previously found in the air and so may well have acquired their nasal carriage in the ward.

[b] Numbers in parentheses give patients carrying the acquired strain on one occasion only.

Minimal infective dose. If we are to understand the epidemiology of airborne infection, we must know the minimal dose of microbes ordinarily needed to effect colonization or infection; this number is not known, but it is so important that it seems justified to indulge in some extrapolation from the few figures available. Shinefield and his colleagues, in their investigations of bacterial interference, found that they could set up a carrier state in the nose of 50% of newborn infants by the inoculation of between 200 and 400 cocci. As noted already, most airborne staphylococcus-carrying particles appear to contain no more than one to six viable cocci.

In experimental infections, it is generally found that the relation between dose and attack rate is not linear, but conforms to an S-shaped curve. For extrapolation to be possible, it is therefore necessary to apply some transformation to the data, e.g., to plot the logarithm of the dose inoculated against the probit of the percentage attack rate. This has been done in Fig. 6 for the data obtained by Shinefield and his colleagues (43, supplemented by a personal communication from

TABLE 3. *Acquisition of nasal carriage of tetracycline-resistant Staphylococcus aureus in relation to daily exposure to airborne staphylococci*

Reference	Type of ward	Median exposure (particles/24 hr)	Acquisition rate (per cent per day)
Williams et al. (59), Noble (34)	22-24 bed open, S. B. H.	18	0.7
Shooter et al. (47)	22-24 bed divided in two parts, S. B. H.	9[a]	0.6
		8[a]	0.3
Williams (*in preparation*)	14 beds in 4 rooms, S. M. H.	4	0.3
Lidwell et al. (27*a*)	30 beds in 9 rooms, S. B. H.	4	0.1

[a] These values are estimates based on mean counts provided by O. M. Lidwell, converted to medians on the assumption that the distribution was similar to that in the earlier S. B. H. studies.

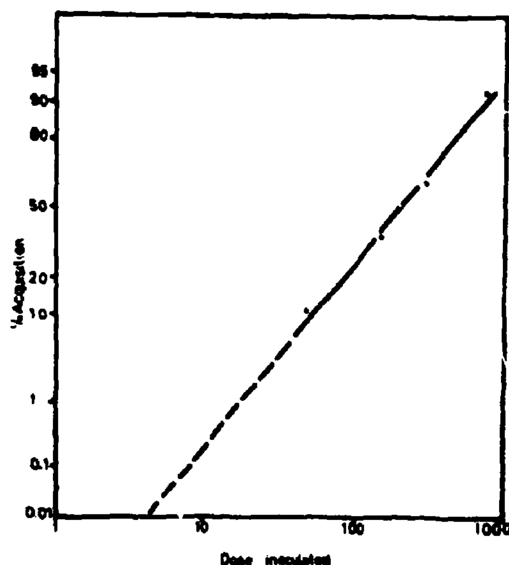

FIG. 6. *Relation between dose of staphylococci inoculated into infants' noses and acquisition rate for nasal carriage. Based on figures kindly supplied by Henry R. Shinefield.*

Dr. Shinefield), and the points lie very close to a straight line. Extrapolation of the line back would suggest an attack rate of about 0.02% for a dose of five cocci. The observations of Shinefield et al. were made on newborn infants who are presumably more susceptible to staphylococcal colonization than adult subjects, but, in the absence of any other figures, the calculation may be worth pursuing.

The data in Fig. 5 suggest that the median number of staphylococcus-containing particles inhaled in the S.B.H. wards may have been about 18. Each of these particles probably contained, on the average, about 4 viable cocci, so that the total daily dose inhaled could be estimated at about 70 cocci; if the dose-response relation observed by Shinefield were applicable to the adults, this dose might be expected to generate a "take-rate" of just over 10% per day if all the inhaled particles co-operated to set up the carrier state, or 0.16% if they acted independently. Unfortunately, we do not know how many of the airborne staphylococci were tetracycline-resistant, but the apparent acquisition rate for tetracycline-resistant strains was about 0.7% per day.

In the S.M.H. divided ward, the median dose of sensitive and resistant staphylococci inhaled was about 16, which on Shinefield's figures would indicate a take-rate of 0.6%, or less than 0.01% if all the particles acted independently; the actual rate of acquisition of tetracycline-resistant strains was 0.3% per day.

These and some other similar data are presented in Table 3. Although quite insufficient to indicate a clear relation, they suggest that the staphylococcal acquisition rate in different wards may well be related to the air count. In fact, the acquisition rates in the wards are, considering the amount of extrapolation involved, clearly of the same order as those predicted from Shinefield's figures. But at least these calculations clearly indicate that there is no wild improbability in the idea that the acquisition of the nasal carrier state in surgical patients results from the inhalation of such airborne staphylococci as can be shown to occur in the wards. The number of complicating factors in any precise analysis is formidable.

In the first place, as already noted, the figure for a median bacterial count conceals enormous variations, and we clearly need to know whether a short exposure to a large number of airborne staphylococci is equivalent to a more prolonged exposure to smaller numbers. A second complication arises from the fact that staphylococci appear to vary in their ability to colonize the nose (57), so that there is reason to think that inhalation of large numbers of cocci of some strains may be less effective in setting up the carrier state than inhalation of others. The third complication arises from differences in the recipients. The phenomenon of bacterial interference, studied in detail by Shinefield et al. (43, 43*a*) in infants, almost certainly operates in adults also. Several workers have shown that patients admitted to hospital as carriers of *S. aureus* are less liable to acquire hospital strains than patients admitted as noncarriers (e.g.,58). The fact that, at least in open wards, patients treated with antibiotics acquire hospital staphylococci in the nose more often than those who are not (e.g., 39) is presumably another example of the same phenomenon, which was well demonstrated experimentally by Boris et al. (9). At the same time, antibiotic treatment probably prevents nasal acquisition in other patients.

Relevance of Nasal Acquisition

In the operating room, we must assume that, whatever the dose-response relation, the aerial transfer of staphylococci to the wound itself is potentially important. It may be asked whether there is any corresponding relevance in the nasal acquisition of staphylococci in the wards. There seem to us to be two ways in which the nasal spread is important.

In the first place, it appears that, at least in some circumstances, nasal carriage of staphylococci predisposes to postoperative infection

(58). There has been some discussion on the significance of these observations (3, 22, 30), but scrutiny of the records of a considerable number of patients leaves no doubt in my mind that the phenomenon is real, even if not generally so frequent as suggested by our original observations.

But nasal carriage is also relevant in that it seems to be the mechanism by which the endemic staphylococci persist in the hospital. Such persistence can often be for a long period. For example, at Saint Bartholomew's Hospital we observed the spread of a staphylococcus of phage type 75/77 which continued from the start of the study in one ward in February 1959 until the end of January 1960. During this period of 1 year, there were only 39 days when there was not present a patient who was either known or reasonably presumed to be a carrier of the strain. A total of 23 patients were infected with the strain, but only 6 of them had any clinically infected lesion.

Conclusion

The commensal association of staphylococci with man is universal (56) and to a large degree harmless. The transfer from one individual to another must, under ordinary circumstances, very often be by direct or indirect contact. But ability to disperse *S. aureus* into the air in large numbers is a characteristic—sometimes temporary and sometimes persistent—of a number of healthy people, and wherever we go indoors there is a chance that we shall inhale staphylococci. [A few observations in two Post Offices in London have given an average sedimentation count of 0.01 colonies per ft² per min, a figure quite similar to that for hospital wards (J. Corse, *personal communication*)]. But it is only in hospitals that any detailed study of the processes of transfer has been made.

Airborne transfer in hospitals gains its special significance from the fact that, if this route is actually operative, a single disperser is potentially able to infect a considerable number of other patients, who need not be confined within the same room, or even perhaps on the same floor; and the transfer of infection cannot be contained by ordinary methods of asepsis.

The evidence that has been reviewed seems to leave little doubt that airborne transfer can be of importance. It suggests that the acquisition of nasal carriage of *S. aureus* by patients nursed in hospital wards can be explained if the dose-effect relationship determined experimentally in infants is approximately applicable to adults. If the results obtained in the studies reviewed can be confirmed elsewhere, we should have a rational basis for assessing one aspect of hospital hygiene in relation to the prevention of staphylococcal infection. We still lack, however, a precise measure of the relative part played by this airborne spread in the etiology of staphylococcal hospital-acquired infection generally.

To take surgical wound infection as an example, we have to recognize that infection can be derived from: (i) staphylococci carried by the patient on admission to hospital; (ii) staphylococci that the patient has come to carry in the nose and on the skin after admission, which subsequently enter the wound; and (iii) staphylococci that reach the wound directly without the prior intervention of the nose or skin carrier state. It appears that aerial transfer plays a major part in the second of these categories and a part—sometimes major and sometimes minor—in the third. But we have insufficient precise evidence on the relative importance of the three categories themselves. The proportion will clearly differ greatly from one hospital to another, and within one hospital, from one sort of surgical operation to another, and from time to time.

The challenge with which we are faced is to provide much more firmly based estimates of the relative frequencies in these categories and the factors that determine them. The practical justification for attempting such an analysis is that it can provide the only basis for judging how best to construct and ventilate hospitals. And the fundamental difficulty of performing the analysis is that in any hospital, where the analysis would be practicable, the overall incidence of infection is probably no more than 1 to 2%, and this small proportion must be distributed over all the various routes and sources.

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Discussion

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Dr. Williams has presented a perceptive review of our knowledge of the occurrence of *Staphylococcus aureus* in the air of hospital wards and surgical operating rooms and of its spread to patients. It is impressive how much work has been reported during the past decade and what a

major contribution Dr. Williams and his colleagues have made to the total body of this knowledge. He has been penetrating in his analyses and careful to limit his rather guarded interpretations and conclusions to areas in which he has substantial microbiological data to support his long experience.

The present discussant has neither these assets nor limitations. As a general practitioner of epidemiology and an early convert to a recognition of the importance of airborne infection in some diseases, I believe I can best contribute by discussing certain broader issues that may be related not only to the subject of staphylococcal infection, but also to the whole purpose of this Conference.

In his introductory paragraph, Dr. Williams points out, "It is a characteristic of the airborne route of infection—in contrast to transfer by food or water—that whenever there is the possibility of aerial transfer there is almost always also the possibility of transfer by other routes." Obviously Dr. Williams was thinking about staphylococcal infection in hospitals, where his concept is crucially important, but, considering the generalization as stated, it needs considerable qualifications.

Until the mid-1940's, our concepts of airborne infection were vague. In fact, to most epidemiologists and microbiologists, the term still carried the stigmata of miasms and malarias of the prebacteriological era. During the past 20 years, however, our concepts of airborne infection have become increasingly sharp and distinctive, owing in no small part to the studies conducted by the U.S. Army Biological Laboratories and the Microbiological Research Establishment in the United Kingdom.

The clinical, pathological, and epidemiological patterns of primary histoplasmosis, coccidioidomycosis, inhalation anthrax, primary pulmonary tuberculosis, Q fever, tularemic pneumonia, pneumonic plague, and a host of laboratory-acquired infections leave no basis for confusion as to their airborne origin. In these diseases, the portal of entry is the terminal bronchiole or alveolus of the lung. Infection arises by the inhalation of small particles 1 to 3 μ in size. The primary pathology appears in the periphery of the lungs or the mediastinum. Epidemiologically, there is a history of direct exposure to an aerosol or to gross aerial contamination of large rooms, whole buildings, and at times the outdoor atmosphere. The airborne character of these diseases can no longer be rationally disputed. When these infections occur by other routes, for example, scrofula from tuberculous milk, tularemia from a tick bite, or bubonic plague from a flea, the clinical and epidemiological patterns are distinctive.

Turning our attention to infections in which the portal of infection is the respiratory epithelium of the nose or the tonsillar tissue of the nasopharynx, the problem of differentiating possible multiple routes of transfer does [illegible]ise. It has long been a challenge to the epidemiologist to distinguish among four related, but distinct, modes of spread: (i) direct contact, as in kissing; (ii) indirect contact, as in the use of contaminated surgical instruments; (iii) droplet infection with direct impingement on the face, mouth, or conjunctiva; and (iv) airborne infection from the inhalation of suspended droplet nuclei or infectious dust particles that have travelled some distance through the air.

In is interesting and pertinent to isolate staphylococci from the air of wards and operating rooms and to show that these organisms have retained their virulence. Likewise, it is highly suggestive to isolate staphylococci from the anterior nares of postoperative patients. But such findings by themselves are insignificant. The human nose is an excellent filter, especially for larger aerosols. The isolation of a few staphylococci from the nose, particularly if only on a single occasion, may be of little or no significance. The infectivity of a dried bacterial particle in metabolically suspended animation almost certainly is less than that of a droplet. Such a moist particle may carry actively metabolizing bacterial cells along with toxic products, enzymes, and receptor substances that may greatly facilitate invasion of tissue and enhance the competitive position of the microorganism vis-a-vis the body's still-to-be-mobilized defense mechanisms.

What is essential and necessary evidence is the demonstration that the elimination of one or more of the means of spread, keeping the remaining ones constant, radically and consistently reduces the incidence of actual disease. The importance of contact, both direct and indirect, was established so long ago that it now tends to be forgotten or ignored. The development of aseptic surgery proved that contact was the dominant mode of spread of surgical sepsis and that airborne infection was of minor consequence. Application of the same aseptic principles to hospital management permitted the development of the modern pediatric hospital and contagious disease services, again establishing the dominant importance of contact-infection.

It is more difficult to distinguish precisely the role of droplet infection, because it is so closely associated with direct contact. The tendency of many persons to equate droplet infection with the airborne route rather than with contact is, in my judgment, ill-advised. The control of droplet infection calls for strict personal hygiene, wearing of masks, and other individual measures regularly

associated with aseptic techniques rather than for the engineering methods applicable to the control of aerosols and dust.

Some rather heroic efforts have been made to control airborne infection in pediatric and surgical wards and operating rooms with controlled ventilation and ultraviolet irradiation. It is a safe generalization to say that, the more carefully controlled these experiments, the less impressive the evidence in favor of airborne infection has been. As emphasized by Dr. Williams, when strict asepsis is enforced to minimize contact and droplet infection, the rate of hospital-acquired infection is reduced to well below 5%, and may reach 1%. It is exceedingly difficult to prove whether this low residual rate results from airborne infection or from failure of the aseptic techniques to eliminate all contact.

Dr. Williams has drawn guarded conclusions regarding the role of airborne staphylococcal infection in hospitals. He has emphasized the multiple sources of infection and modes of spread. He admits that "we have insufficient precise evidence" on their relative importance. The epidemiological evidence, also far from precise and based on more general considerations, supports his caution.

Discussion

Viability of Hospital Staphylococci in Air

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The role of airborne transmission in the spread of staphylococci in hospitals (3) has not been definitively established. Though several authors studied the survival of staphylococci in air and on surfaces (1, 2, 4), exact knowledge of the survival of "epidemic" and "nonepidemic" strains is scarce.

The survival of a number of strains (for description, see Table 2) in air of differing relative humidity (RH) was studied by spraying with a direct spray (FK8) in a static system (4,000 liters) and sampling 10 liters in a slit sampler on blood-agar plates (Fig. 1a). The reference strain no. 1600 (isolated from a nose swab, phage type 187) showed a low decay rate at 50% RH and an increased decay rate at high RH (Fig. 2). This effect was generally found with most strains. During the experiments, it became clear, however, that by varying the growth medium, the age of the culture, the suspension medium, the method of aerosolization, and the composition of the collection plates, any desired result could be obtained. Decay curves could be logarithmical or curved, and the effect of relative humidity could be very marked or nonexistent. This variability was specially marked at high relative humidities, and less so below 50%. The late

[1] Deceased 25 October 1965.

TABLE 1. *Survival in air of strain isolated in hospitals compared with reference strain 1600 (90% RH)*[a]

Strain	Phage type	$K \times 10^{4}$	K/K_{1600}
1600	187	87, 81, 79, 89, 85, 95, 82, 96, 95, 76, 97 } 85	1.00
1	80/81	95	1.09
7	80/81	74	0.94
17	80/81	77	0.81
18	52/80/81	73	0.77
3	52/80/81	77	0.89
8	52/80/81	70	0.80
1330 A	52/80/81	76	0.94
1451 A	52/80/81	70	0.79
2	NS I III	84	0.97
1330 B	NS I III	87	1.07
1451 B	NS I-III	76	0.89
4	III	79	0.98
10	III	102	1.15
11	III	92	1.03
16	III	108	1.14
15	83 A	114	1.27
13	III type A	117	1.38
9	II	79	1.00

[a] Three strains were tested daily together with the reference strain. K/K_{1600} was calculated with the K_{1600} observed on that day. Calculation based on the mean of K_{1600} reduced the differences.

appearance of small colonies on collection plates at high RH (Fig. 1b) indicated that many organisms were damaged and started growth only after a long lag.

An instance of variability with culture age is given in Fig. 3 and 4. Figure 3 gives the growth curve of strain 1600 in nutrient broth (Difco). At the indicated times (↑), the number of single organisms and of clumps of two, three, or more organisms was determined, or a sample of the

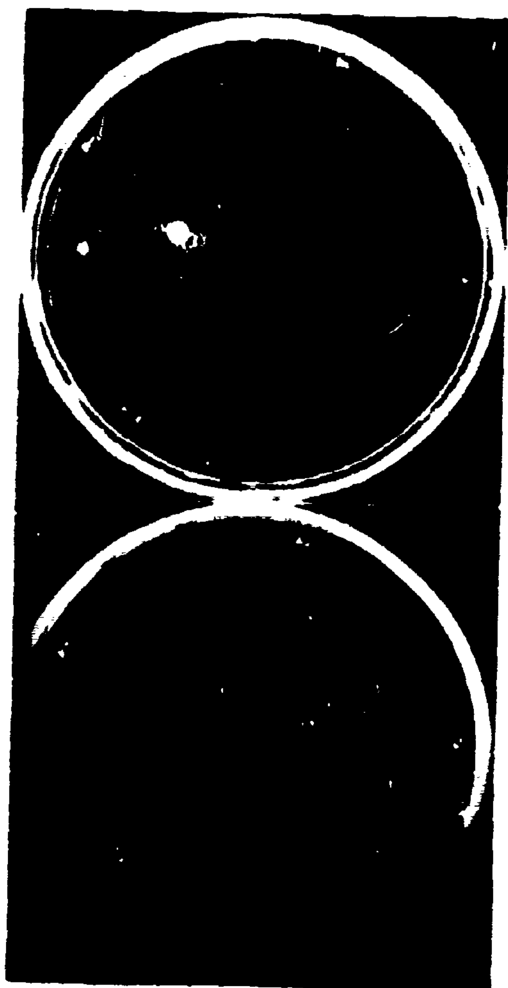

FIG. 1. *Colonies of staphylococci (strain 1600) recovered from aerosols in air of 20.0 C on blood-agar plates in a slit sampler. The four sectors represent 20-min (10-liter) samples 40, 45, 50, and 60 min after aerosolization; (a) 40% RH, uniform colonies; (b) 80% RH, increasing numbers of small colonies due to metabolic damage in later samples.*

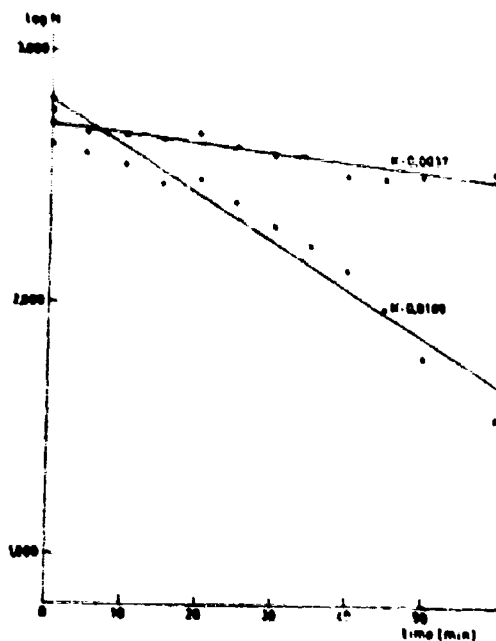

FIG. 2. *Decay curves of Staphylococcus no. 1600 at 39% RH (●) and at 75% RH (○).*

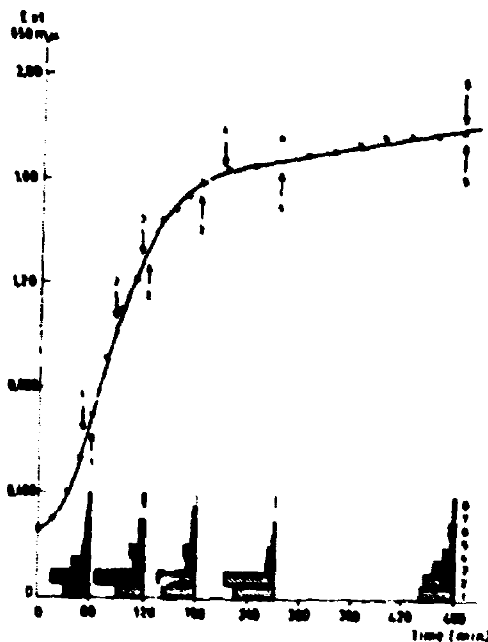

FIG. 3. *Growth curve of strain 1600, with frequency distribution of single organisms and clumps of various sizes shown at the bottom of the figure (right-hand scale). At the indicated times (↓), samples were diluted and aerosolized (compare with Fig. 4).*

culture was diluted ( ) and aerosolized. Figure 4 gives the corresponding decay curves at 70% RH.

Finally, we arrived at an experimental procedure that gave reproducible results and decay curves which were straight on a logarithmic scale (Fig. 5) at all RH levels. The strains were inoculated from stock cultures into 10 ml of meat infusion broth and incubated for 8 hr at 37 C. This culture was diluted 1:500, and a standard droplet (0.03 ml) was inoculated into 20 ml of meat broth which was incubated for 16 hr on a turntable at 37 C. This culture was diluted with meat broth to an extinction of $E = 0.180$ (Unicam). A further dilution of 1:100 was made with meat broth. This suspension containing about 6×10^{4} viable particles per cubic meter was sprayed with a direct spray (FK 8) with 5 atm of nitrogen (0.5 ml in 4,000 liters). Collection was on double-layered blood-agar plates with 10% sheep blood in a meat broth base. Important features seem to be the incubation under slight agitation, the use of metabolically inert organisms after 16 hr of growth, omission of centrifugation and washing, and the use of a direct spray. Recoveries were between 40 and 50%. At low

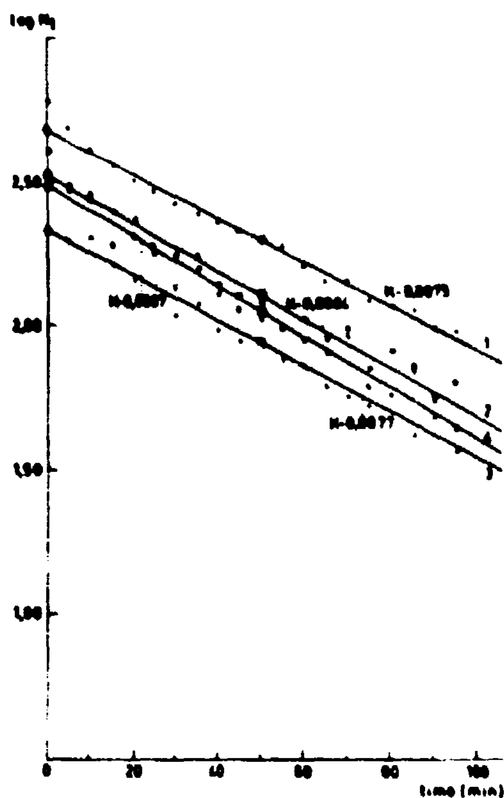

FIG. 5. Decay curves of staphylococci at 90% RH. Standardized experimental procedure. Curves calculated with least squares. Strains 1, 2, and 3 from Table 1. No. 4 = strain 1600.

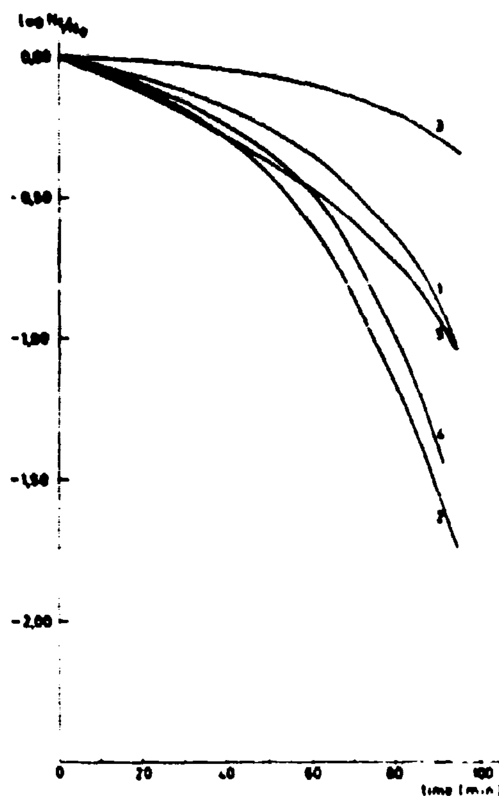

FIG. 4. Variance of decay curves with culture age (70% RH).

TABLE 2. *Survival in air of strains which had caused epidemic events in hospitals, compared with reference strain 1600 (90% RH)*

Strain	Phage type	$K \times 10^{-5}$	K/K_{ref}
1600	187	85	1.00
3827	80/81	87	0.90
3828	80/81	78	1.03
3829	80/81	77	1.01
3830	80/81	76	1.00
3822	52/52A/80/81	74	0.75
3826	6/7/47/53/54/75/83A/81	76	0.79
3824	6/53/83A	91	0.96
3825	NT type A	84	0.88
3823	83A	91	0.96

RH, they were slightly higher. Differences between strains were negligible. In Table 2, the mean recovery was 48%, with $s = 2.4\%$.

The results with a number of strains are given in tables 1 and 2 in terms of total decay rate K ($K = \frac{\Delta \log N}{\Delta t}$, where N = number of organisms and t = time in minutes). Physical fall out in the system, as tested with spores and with fluorescein, was below $K = 0.002$, but was not subtracted. No obvious difference was observed between strains of various phage types, all isolated from patients (noses, lesions, etc.) or between the strains received by the courtesy of M.T. Parker from the Central Public Health Laboratories at Colindale, London, England, which had given rise to epidemics (Table 2).

The data seem to indicate that "epidemic" strains do not survive better than other strains in air. Considering the difficulties in standardization of experimental conditions and our lack of knowledge of the factors causing the reproducibility, it remains possible that differences between strains are masked by the procedure used.

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Experimental Epidemiology of Coccidioidomycosis

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INTRODUCTION

Coccidioidomycosis, a highly infectious but noncontagious disease caused by the dimorphic fungus *Coccidioides immitis*, is limited mainly to the desert regions of southwestern North America and the Grand Chaco-Pampa region of South America (Fig. 1).

This disease, primarily a respiratory infection, manifests itself over a wide range of severity, from acute bronchitis or pneumonia in about 40% of the infections to subclinical or nonsymptomatic disease in approximately 60% of the instances (10, 30). Occasionally, in a few infections (0.5 to 2%), extrapulmonary dissemination takes place, resulting in a fatality rate of approximately 50% in the disseminated cases (Fig. 2). The disease is of great economic importance in the endemic areas, from the standpoint of both human and canine infections (16, 25). As an example, Hugenholtz (19*a*) has reported that at Williams Air Force Base, near Phoenix, Ariz., the cost of man-hours lost and of hospitalization due to coccidioidomycosis approaches $70,000 per year. Other Air Force bases in the area have estimated the cost of these infections at $50,000 to $100,000 yearly. When the number of Air Force Bases, Army installations, etc., in the Southwestern United States is taken into consideration, this could amount to a significant cost to the government.

Respiratory exposure results from inhalation (20) of arthrospores of the saprophytic phase, which grow in the soil and are disseminated by wind during dust storms (12, 30). Although *C. immitis* can readily be isolated from the soil (5-9, 15, 23, 33), isolation by means of air samplers is

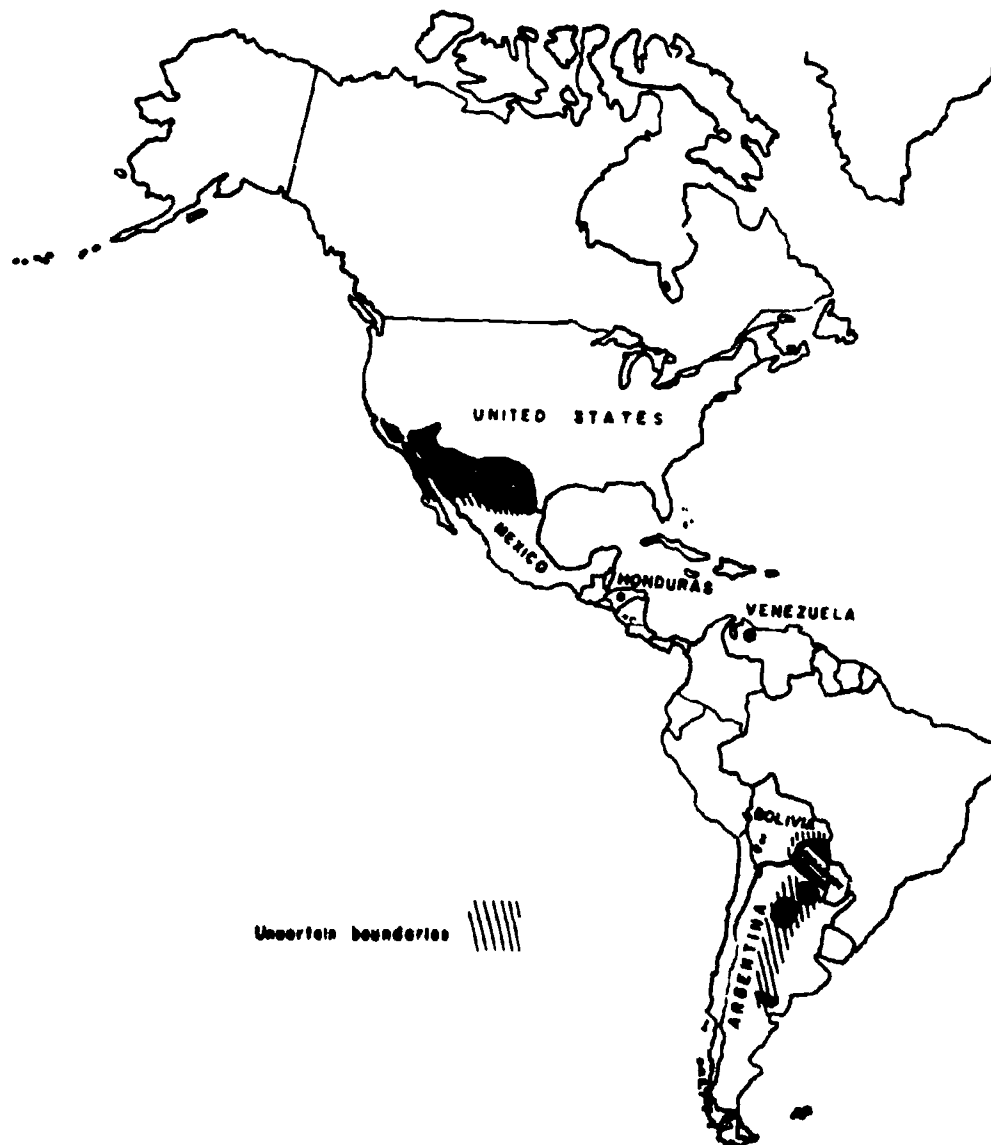

FIG. 1. *Endemic areas of coccidioidomycosis in North, Central, and South America (10). (Courtesy of M. J. Fiese.)*

extremely difficult. Consequently, not much is known concerning the size of the infectious dose in nature.

The purpose of the present study was an attempt to determine the infectious dose by the use of laboratory animals as "biological air-samplers." As a baseline for the study, the U.S. Army Biological Laboratories has a large amount of data (2-4, 28) on the pathogenesis of coccidioidomycosis in monkeys and in dogs (serology, histopathology, X ray, etc.) exposed to graded respiratory doses of 10 to 80,000 *C. immitis* arthrospores.

In our experience, the dog was as susceptible to the disease as the monkey, but was more resistant to its effects, because of its ability to maintain a blood supply to the lesions for a longer period of time and because of a faster and more prolific collagen response to the presence of the organism

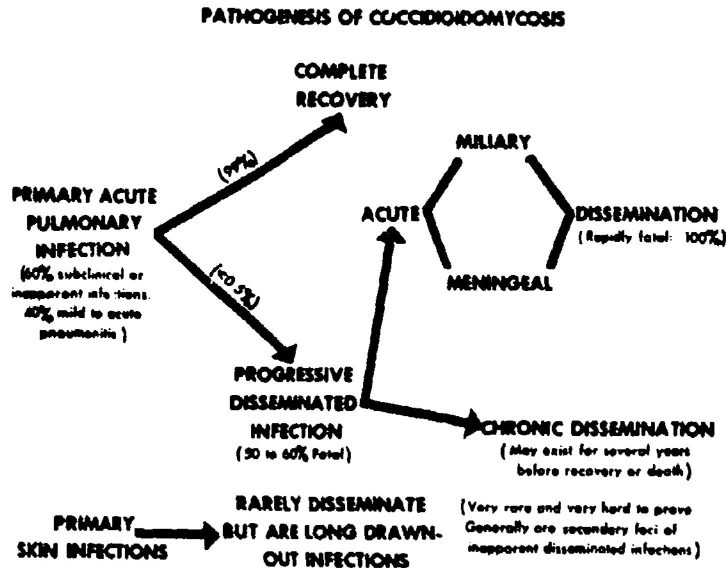

FIG. 2. *Schematic summary of types of infection caused by Coccidioides immitis.*

(3). It was postulated that the monkey is most susceptible to the ravages of the disease, that the dog is least affected, and man is somewhere on the scale between the species, but probably much closer to the dog than to the monkey. Moreover, other experimental studies in dogs by Reed (26) and Hugenholtz et al. (13) have indicated that the pathogenesis of coccidioidomycosis in man and dogs is very similar; the only real difference lies in the bone lesions, which are more destructive in man and more proliferative in dogs (16, 25).

With this type of data available, it seemed logical that observations on monkeys and dogs exposed in the open, in an endemic area, might be compared with observations of animals receiving known experimental respiratory doses, thereby resulting in a valid estimation of the infectious dose of *C. immitis* received by man in nature.

CHOICE OF LOCATION FOR NATURAL EXPOSURE OF ANIMALS

The endemic areas of coccidioidomycosis and the climatic and geophysical conditions necessary for growth of *C. immitis* in the soil have been firmly established. Among the major contributions in this area of study have been: (i) the very thorough epidemiological studies of C. E. Smith and his co-workers (29-32) and Palmer et al. (21), using coccidioidin skin hypersensitivity in man to define the endemic areas, and to correlate rainfall and dry, dusty atmospheric conditions with seasonal morbidity rates (30); (ii) similar studies by Maddy et al. (17, 18) through widespread skin testing of home-grown cattle; (iii) Hugenholtz's study of the optimal climatic factors for growth of *C. immitis* in the soil (12); (iv) the extensive soil studies by Egeberg (6, 7) and others (8, 15, 22), associating soil types and salinity of the soil at various seasons with optimal growth conditions for *C. immitis*; and (v) a demonstration of the close association of the boundaries of the Lower Sonoran Life Zone (Fig. 3) with those of the known endemic areas of coccidioidomycosis, by Maddy (17).

These investigators have suggested that ideal conditions for the fungus to maintain itself in the soil include an arid or semiarid climate, hot summer months, mild winter temperatures, light, slightly alkaline, uncultivated soil with sparse vegetation, and 5 to 20 inches (13 to 51 cm) of annual rainfall. They postulate that the hot summer temperatures sterilize the upper 4 to 6 inches (10 to 15 cm) of soil, eliminating all competitive organisms, and leaving this layer a good medium for growth of *C. immitis* after the next rainfall; the arthrospores from this growth are then carried off by wind during the next dry spell.

For the purpose of the present epidemiological study, the Tucson area in southern Arizona, lying in the heart of the endemic area for coccidioidomycosis, was chosen as the exposure site. The infectivity for man in this area (Fig. 4) approaches 70% in long-time residents, and for cattle is closer to 80% (18). The facilities of the College of Agri-

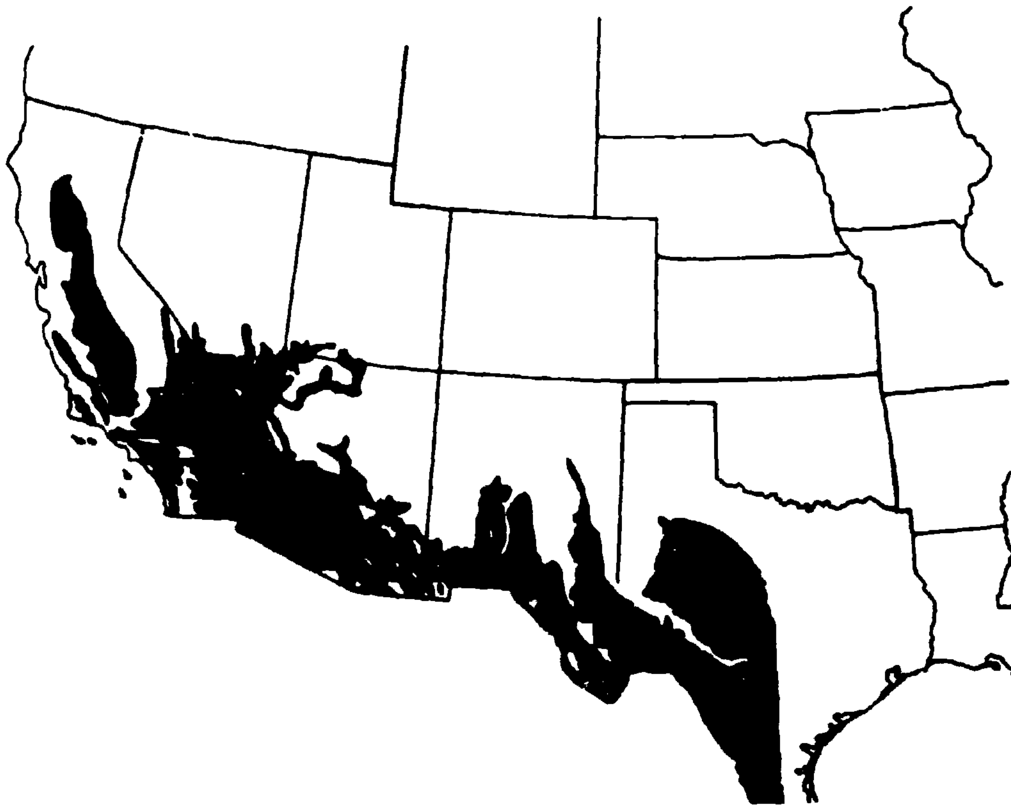

FIG. 3. *Area of the United States covered by the Lower Sonoran Life Zone* (17). (*Courtesy of Keith T. Maddy.*)

culture, The University of Arizona, Tucson, were made available for the project, under direction and cooperation of the Department of Animal Pathology.

PHYSICAL SET-UP OF ANIMAL EXPOSURE SITES

Three fenced-in (chain-link) areas, approximately 30 by 40 ft in size and approximately 100 ft apart, were constructed in a shallow arc arrangement, affording each enclosure exposure to the prevailing wind (Fig. 5-7). The pens were located at the University's Casa Grande Farm in the Santa Cruz River basin (a venturi-like geographical site, the local hills of which funnel the prevailing wind through the area). This farm contains feed-lots in which practically all cattle imported from nonendemic areas eventually become infected with *C. immitis* (19).

In each of the three enclosures (Fig. 8), eight mixed-breed dogs were allowed free run of the area, and eight monkeys (*Macaca mulatta*) were confined in open cages (Fig. 9) approximately 26 inches (66 cm) above ground level, under appropriate shelter. All animals remained at the open exposure sites for a period exceeding 1 year, unless they became infected with *C. immitis*. As the animals became infected, they were immediately removed from the exposure site and were replaced with reserve, susceptible animals.

PROCEDURES FOR DETERMINATION OF NATURAL INFECTION

Two dogs and two monkeys from each exposure pen (one-fourth of the population of each species) were subjected to coccidioidin dermal sensitivity tests, agar-gel immunodiffusion precipitin tests (24), and thoracic radiographs each week. This provided a population observation turnover once every 4 weeks. Each time an infection was noted, tests were immediately repeated on all animals. In addition, all animals were critically observed, several times a day, for clinical signs of infection.

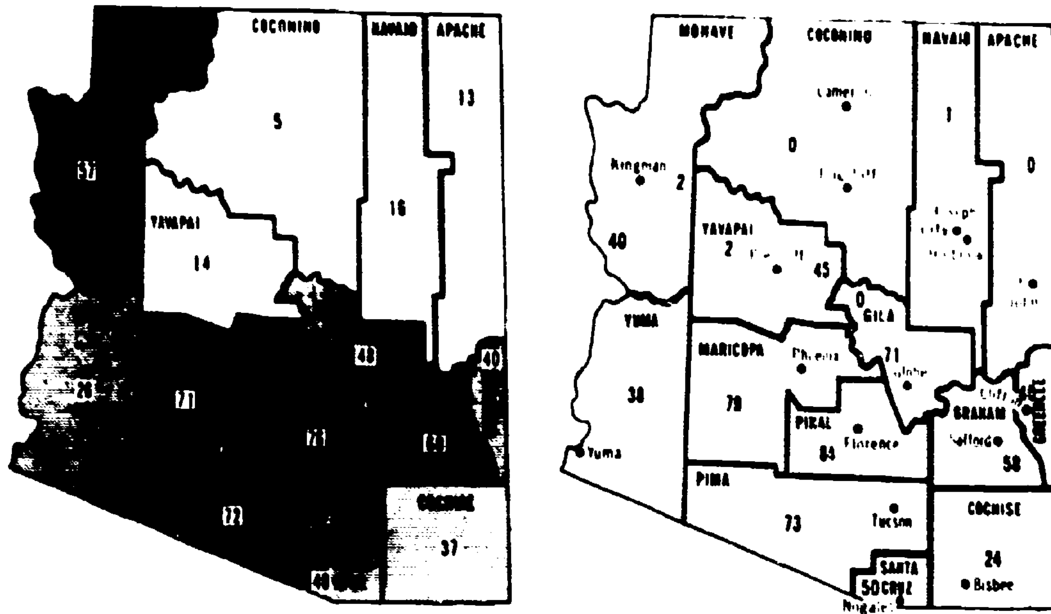

FIG. 4. *Maps of Arizona, showing infectivity (per cent) for man (left) and cattle (right, 18). (Courtesy of Keith T. Maddy.)*

CLINICAL AND LABORATORY OBSERVATIONS OF NATURALLY INFECTED ANIMALS

As each infection was noted, the animal in question was immediately removed from the exposure site and placed in air-conditioned quarters at another location (The University's Campbell Avenue Farm, noted for its low infectivity rate; R. E Reed, *personal communication*) to lessen the chance of further exposure to the organism. It remained under observation until the termination of the study. Changes in erythrocyte sedimentation rate, packed cell volume, per cent hemoglobin, total and differential leukocyte count, development of complement-fixation antibodies, and immunodiffusion precipitin titer were recorded weekly. Rectal temperatures were taken daily, and thoracic radiographs were made at 4-week intervals.

CONTROL ANIMALS

Controls for this study consisted of four types.

Ground Controls

Five monkeys were caged at ground level (physical contact with the soil) for 6 months (June to December 1964) of the 1-year period, at one of the exposure sites, to equate any differences in the monkey infection rate (housed several feet above ground level) and the dog infection rate (having free access to the soil).

Environmental Controls

Uninfected, susceptible monkeys and dogs were maintained at the Campbell Avenue Farm (where reserve susceptible animals were housed) and examined during and at termination of the study to assure that the naturally infected animals were not receiving any further exposure to *C. immitis* after removal from the exposure sites (Casa Grande Farm).

Experimentally Infected Controls

Ten monkeys and eight dogs were inoculated intratracheally with 10 or 100 *C. immitis* arthrospores from a culture isolated from the soil of the area under study. These animals received the same clinical and laboratory tests as the naturally infected animals. In addition, determinations were made each week of serum amylase, serum glutamic-oxaloacetic and serum glutamic-pyruvic transaminases, total serum protein, and serum protein fractions.

Animals from Former Experimental Studies

As a further comparison of naturally and experimentally infected animals, similar data from

FIG. 5. *Photograph of the Tucson area. Note dust cloud visible at the base of the mountain.*

former studies (2, 4) of monkeys exposed, via the respiratory route, to aerosols of *C. immitis* arthrospores (10 to 10,000 organisms) and dogs (R. E. Reed, *personal communication*) inoculated, via the intratracheal route, with 10 to 100,000 organisms were assembled for use at termination of the present study.

PATHOLOGICAL STUDIES

At termination of the study (52 to 54 weeks), complete necropsies were performed on all of the naturally exposed animals (both infected and noninfected) and all of the controls. Gross pathology was recorded and photographed; the lungs and any suspicious lesions were examined by impression smears for presence of *C. immitis* and were cultured on Mycobiotic agar (Fisher Scientific Co., Pittsburgh, Pa.) for recovery of the organism; tissues for histological study were fixed in 10% buffered formaldehyde, impregnated with paraffin, serially sectioned, and stained with the Giemsa and Gomori silver methenamine stains.

GEOPHYSICAL STUDIES

Climatic Factors

Throughout the 12-month period (October 1963 to October 1964), continuous wind speed and direction (anemograph) and relative humidity and temperature (hygrothermograph) recordings were made. Total precipitation was measured each day.

Soil and Aerobiological Studies

Soil in the area of the exposure site was analyzed to determine percentages of sand, silt, clay, and organic content, the hydrogen ion content, and both qualitative and quantitative analyses of salinity (Na, Ca, Mg, K, Cl, SO_4, CO_3, HCO_3, etc.). In addition, four to eight surface and subsurface soil samples were collected, bimonthly, in and around the exposure pens. These were plated directly on Mycobiotic agar, and aqueous suspension (1:10 dilutions) were injected intranasally and intraperitoneally into mice (six per sample) for recovery of *C. immitis*. Mycobiotic agar plates

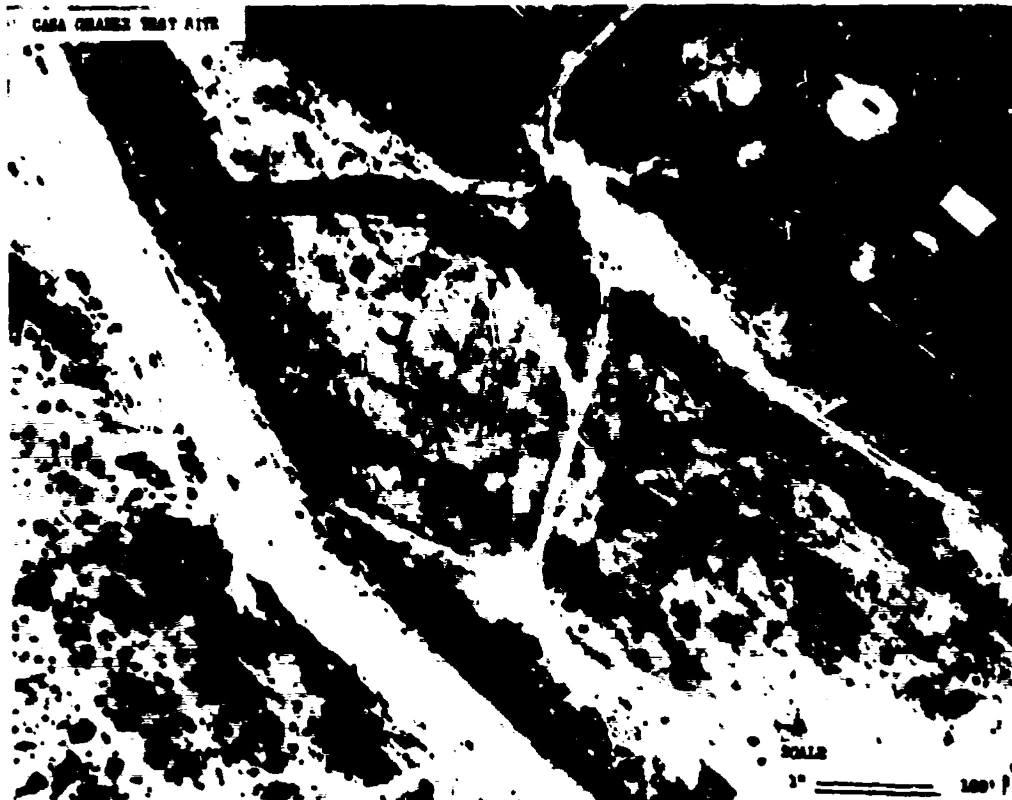

FIG. 6. *Aerial photograph of the Casa Grande exposure area. The three exposure sites (location of pens) are indicated by arrows. Note dry bed of the Santa Cruz River.*

were exposed to the atmosphere each day for recovery of the organism from the air.

COMPARISON OF METEOROLOGICAL DATA FROM THE PRESENT AND OTHER EPIDEMIOLOGICAL STUDIES

The meteorological factors (Table 1) were very similar to those of former studies in the same general region (South Central Arizona). Maddy (17) and Hugenholtz (12), after analyses of weather data covering periods of 10 to 20 years in several locations in South Central Arizona, reported mean July temperatures of 80 to 90 F (26.7 to 32.2 C), compared with 88 F for the present study. Their mean maximal and peak temperatures were recorded as 105 and 110 F, respectively, compared with 101 and 110 F in our study. Mean January temperatures of 50 to 55 F in the former studies were slightly higher than the 47 F mean temperature at Casa Grande Farm. The average yearly rainfall of 9 inches reported by Maddy, and 6 to 10 inches by Hugenholtz, was somewhat less than the 12.5 inches we recorded. The only climatic factors that might have affected this study adversely were mean winter temperatures about 10 degrees below normal (from January on), with a very late, cold spring and an excessive amount of rainfall during August and September (total of 9 inches).

RECOVERY OF *C. IMMITIS* FROM AIR AND SOIL

Prevailing winds throughout the area were not very consistent during this study. Wind direction varied greatly, but analysis of the anemograph data indicated that most wind came from the west, through the north quadrant, rather than from the south through west as expected. Wind speeds sometimes as high as 34 mph were recorded. All attempts to isolate *C. immitis* from the air met with failure; however, two soil samples collected in August 1964 and two in October 1964 were positive for the fungus. The animal exposure area was well bracketed by these four positive soil samples (Fig. 10); one was actually collected from within exposure pen 3.

SEASONAL MORBIDITY OF MONKEYS AND DOGS

Five of 34 monkeys and 29 of 50 dogs became infected with *C. immitis* during the 12 months (Fig. 11). The majority of these infections (20 of the 29 infected dogs and all 5 of the infected monkeys) were diagnosed during the cooler months of November through March. Infections in an additional group of seven dogs and one monkey were

FIG. 7. *Ground view of the same area as in Fig. 6. Note Santa Cruz River meandering through center of photograph and exposure pens in upper left, near horizon.*

FIG. 8. *View of one of the three exposure pens. The solid-appearing structure extending up the fence from the ground is made of louvered aluminum, and permits the entry of wind and dust. The roofed structure visible inside the fence is the monkey shelter.*

FIG. 9. *Close-up view of the monkey shelter. The battery consists of four cages (housing two monkeys each), completely open on four sides and partially open on a fifth side. Note one of the oil-drum dog shelters in the background. The dogs have free run of the fenced-in area (30 by 40 ft).*

TABLE 1. *Comparison of weather conditions in three epidemiological studies*

Observation	Maddy (17)	Hugenholtz (12)	Present study
Mean July temperature	90	80–90	88
Mean maximum Temperature..	105		101
Peak temperature		110	110
Mean January temperature...	50	50–55	47
Minimum temperature			14
Rainfall (inches per year)	5–20 (Avg 9)	6–10	12.5

classed as equivocal. These eight animals may have received an exceedingly small exposure to the fungus. In these instances, however, either an equivocal dermal sensitivity was never corroborated by serological or histological reactions, or else the serological titers were very low and inconsistent. Seven of these eight questionable infections also occurred from November to March; the other, in one of the five monkeys housed at ground level and having access to the soil, was noted in October 1964.

PATHOGENESIS IN MONKEYS

Natural Infections

Only three of the five naturally infected monkeys developed precipitin titers, although all five eventually became complement fixation (CF)-positive (Table 2). Only two monkeys exhibited histological lung changes indicative of coccidioidomycosis; these were very minor. Lung cultures of all monkeys were negative for *C. immitis*. The five infected monkeys remained in apparent good health during the 12-month period, showing no clinical signs of disease; their serological titers (both precipitin and CF) were comparatively low (mean maximum of 1:8, with a range of negative to 1:64); very little evidence of infection was noted in X-ray studies.

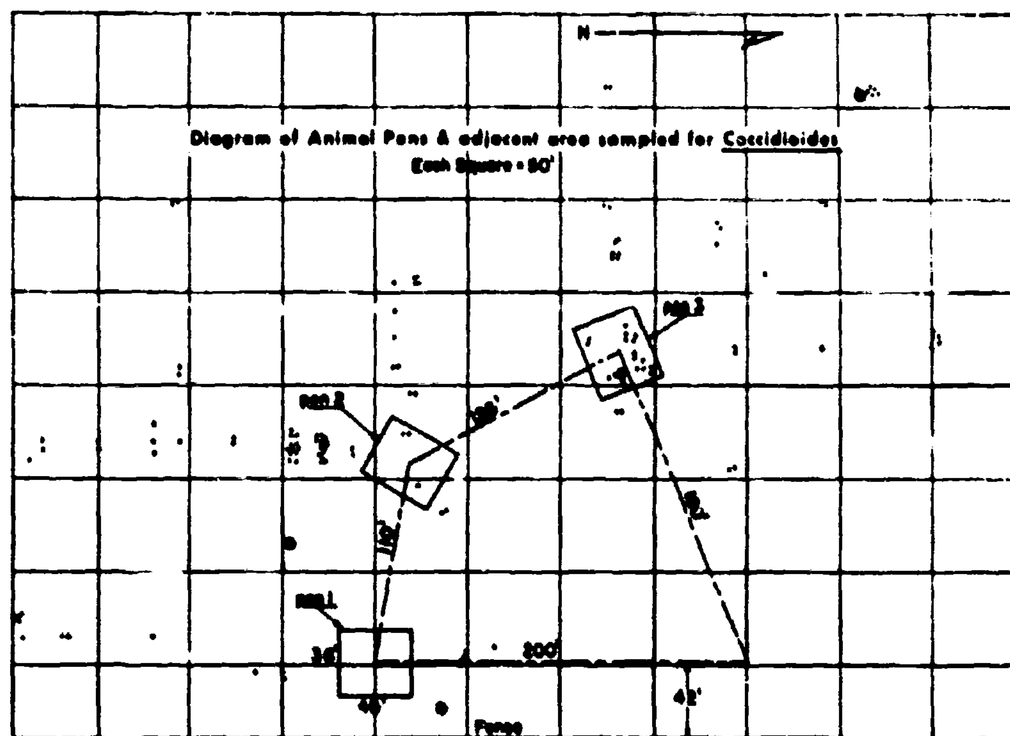

FIG. 10. *Pattern of soil samples collected in the exposure area. Dots indicate samples (the four positive samples are circled). Note bracketing of exposure pens by the positive samples (one collected from within pen no. 3).*

Comparative Pathogenesis of Naturally Infected and Intratracheally Inoculated Control Monkeys

Table 3 indicates that the 10 monkeys experimentally infected via the intratracheal route with 10 *C. immitis* arthrospores developed more serious infections than the five monkeys that became infected through natural exposure. All animals in the inoculated group developed precipitin titers, compared with only 60% of those infected naturally. Although the mean titer was the same (1:8) in both groups, no precipitin data were available for the three inoculated monkeys with the most extensive infections. Such data should have substantially increased the mean titer of the inoculated group.

Of the inoculated group, 40% were clinically ill, as indicated by weight loss, listlessness, and coughing. This included one animal that died from the infection 24 days after inoculation and two animals that, upon autopsy, were judged unable to have survived. All animals in the naturally exposed group were considered to have subclinical infections.

Upon autopsy, 80% of the inoculated monkeys exhibited histological changes in the lung indicative of coccidioidomycosis, including presence of the organism, compared with only 40% of those developing natural infections. The fungus was recovered from 60% of the lung cultures from animals in the former group, but all cultures from the latter group were negative. Figures 12 and 13 graphically illustrate the more extensive lung involvement in the inoculated group.

Comparative Pathogenesis of Naturally Exposed Monkeys and of Monkeys from Former Experiments Receiving Respiratory Challenges by Inhalation of C. immitis Aerosols

Again, as indicated by data in Table 4, less severe disease was noted in monkeys developing natural infections than in those infected experimentally. Monkeys in several former studies, receiving calculated inhaled doses of from 10 to 300 arthrospores, developed mean serological titers ranging from 1:128 to 1:512, respectively, compared with a mean of 1:8 exhibited by the

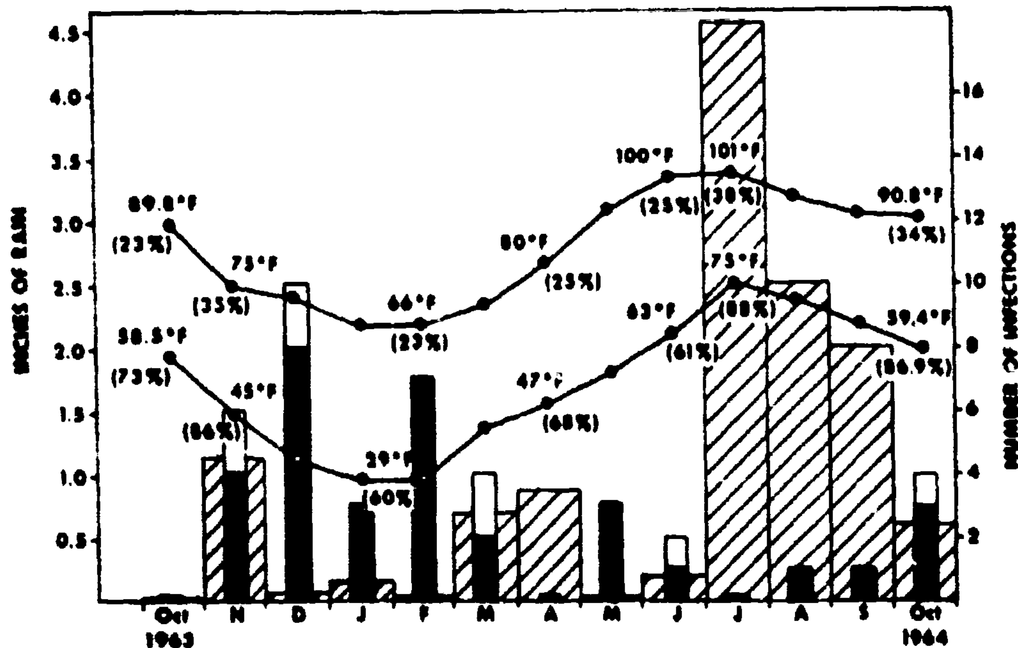

FIG. 11. *Seasonal morbidity of experimental animals. Cross-hatched bar indicates rainfall in inches; solid bar, number of infections; and solid lines, mean maximal and minimal temperatures for each month. The figures in parentheses represent per cent relative humidity at the temperature indicated. The open areas shown at the top of several of the solid bars indicate suspected infections that were never subsequently proved.*

TABLE 2. *Natural infections in monkeys and dogs*

Animal	No. exposed	No. infected	No. with positive skin test	No. with positive serology	No. showing histopathological changes	No. with positive lung cultures
Monkey	34	5	5	3	2[a]	0
Dog	50	29	20	22	18	3

[a] Very minor.

naturally exposed animals in this study. Moreover, a mortality of 30 to 40% in the former studies contrasted to the lack of mortality in naturally exposed animals in the present study.

PATHOGENESIS IN DOGS

Natural Infections

The extent of the disease, as well as the infection rate, was much greater in the naturally exposed dogs than in the naturally exposed monkeys (Table 2). Of the 29 naturally infected dogs, 20 developed dermal hypersensitivity to coccidioidin, and 22 exhibited positive serological titers (21 of these 22 were positive for both the precipitin and CF tests). Upon autopsy, 18 showed histological lung changes due to *C. immitis*. In three instances, *C. immitis* was isolated from the lungs by culture. In 4 of the 29 infections, the disease was diagnosed by histological methods alone, since all clinical and laboratory tests on these four animals were negative.

The majority of the 29 infected dogs remained healthy in appearance throughout the observation period. Of the 29, 7 exhibited clinical signs of illness (weight loss, listlessness, and coughing), but these appeared to have recovered before the end of the experiment. From this standpoint, the remaining 22 infected dogs could be classed as subclinical cases of the disease.

Comparative Pathogenesis of Naturally Infected and Intratracheally Inoculated Control Dogs

In contrast to the findings for monkeys, the disease was more extensive in dogs infected by natural exposure than in those inoculated intratracheally (Table 5). None of the dogs in the inoculated groups (10 or 100 arthrospores) developed precipitin titers, compared with 75% of those infected naturally (mean titer of 1:16, with a range of negative to 1:512). The majority of the

TABLE 3. *Comparison of naturally and experimentally infected monkeys*

Exposure	Positive skin test	Positive serology: PPN	Positive serology: CF	Clinical signs of illness	Lung culture positive	Histologically positive
10 arthrospores (intratracheal inoculation)	100%	100%; mean titer,[a] 1:8 (±1:2 to +1:32)	100%, 1:256 I[b] 1:2; 1:256 I 1:2; 1:4 I 1:2; 1:4 I 1:2; 1:2 I 1:2	40% (one died at 24 days; two judged unable to survive)	50%	80%
Natural exposure	100% (one equivocal)	60%; mean titer, 1:8 (negative to 1:64)	100%; 1:32; 1:8; 1:4; 1:4; I 1:2	None	None	40%

[a] No data for the three animals with the most extensive infections, which should have increased the mean titer of this group.
[b] I = incomplete reaction.

FIG. 12. *Comparative X rays of naturally infected (top) and experimentally infected (bottom) monkeys. Note areas of pulmonary infiltration in the majority of animals receiving intratracheal inoculations of 10 arthrospores, as compared with the relatively clear lungs of the naturally infected monkeys.*

FIG. 13. *Comparative histological sections of naturally infected (top) and experimentally infected (bottom) monkeys. Note particularly the lung tissue (comparatively normal appearing in the naturally infected monkeys, as compared with the large areas of consolidated lesions in animals inoculated intratracheally with 10 arthrospores).*

animals in all three groups eventually developed CF titers; however, titers for the inoculated groups were negligible (possibly equivocal) compared with a mean titer of 1:8 and a range of negative to 1:256 in the naturally infected group. Clinical signs of illness were evident only in the naturally infected group (approximately 25%), but these were relatively mild.

Upon autopsy at termination of the observation period, *C. immitis* was not isolated by culture from the lung of any of the inoculated dogs, but was found in the lungs of 3 of the 29 dogs infected by natural exposure. Of the inoculated dogs, 38% exhibited histological changes in the lung indicative of coccidioidomycosis, compared with 62% of those exposed naturally. Although not too much difference was noted on X-ray examination of inoculated and naturally exposed dogs (mainly pulmonary lymphadenopathy in both groups), Fig. 14 illustrates graphically the difference in histological changes in the lungs of the two groups. Although the disease in naturally infected dogs was more extensive than that in naturally infected monkeys, the 29 dog infections were considered to be relatively mild illnesses.

Comparative Pathogenesis of Naturally Exposed Dogs and of Dogs in Former Studies Receiving Intratracheal Inoculations of C. immitis

In former studies (R. E. Reed, *personal communication*) of pathogenesis of coccidioidomycosis in dogs, approximately 100 animals of various age groups (from 6 weeks to 6 months) were given intratracheal inoculations of graded doses (from 10 to 100,000) of *C. immitis* arthrospores. Unfortunately, no serological data are available on these dogs, but it may be seen in Table 4 that inoculation of 10 or 100 arthrospores resulted in 10 or 20% mortality, respectively, as compared with no mortality in the 29 naturally infected dogs.

ENVIRONMENTAL CONTROLS

There was no clinical, serological, radiological, histological, or cultural evidence of coccidioido-

TABLE 4. *Comparison of naturally exposed infected animals with experimentally infected animals*

Monkey			Dog		
Dose[a]	Mean[b] maximal titer	Mortality	Dose[c]	Mean maximal titer	Mortality
		%			%
Natural exposure (unknown)	1:8 (negative to 64)	0	Natural exposure (unknown)	1:16 (negative to 512)	0
10	1:128 (64:256)	40	10	—[d]	10
50-100	±1:256 (128:256)	30	100	—	20
300	1:512 (128:1,024)	40	1,000	—	47

[a] Numbers refer to aerosol arthrospore dose (experimental; references 4, 14). These data represent a total of 50 monkeys, at 5 to 10 monkeys per dose group.
[b] Immunodiffusion precipitin test. Numbers in parentheses indicate spread.
[c] Numbers refer to intratracheal arthrospore dose (R. E. Reed, *personal communication*).
[d] Test not made.

TABLE 5. *Comparison of naturally and experimentally infected dogs*

Exposure	Positive skin test	Positive serology[b]		Clinical signs of illness[c]	Lung culture positive	Histologically positive
		Precipitation	CF			
	%			%	%	%
100 arthrospores (it)[a]	100	None	100% 2 + 1:4 ±1:2 ±1:2 ±1:2	None	None	50
10 arthrospores (it)[a]	100	None	75% +1:4 ±1:4 ±1:2 Negative	None	None	25
Natural exposure	69	75%; mean titer, 1:16 (negative to 1:512)	74%; mean titer, 1:8 (negative to 1:256)	24	10	62

[a] Intratracheal inoculation.
[b] Figures in parentheses indicate span.
[c] Weight loss, listlessness, cough.

mycosis in any of the monkeys or dogs maintained at the Campbell Avenue holding area as environmental control animals.

DISCUSSION AND CONCLUSIONS

Previous epidemiological studies (12, 17, 31) have covered longer periods of time (10 to 20 years) and have included many more infections than the present study. These factors would tend to smooth out any inconsistencies, such as those possibly caused by freak weather conditions affecting growth of the fungus in the soil, or its dispersal by the wind, during any one year. Most observers have reported two peaks in the human infectivity rate for coccidioidomycosis: one during the hot, dry summer months, and another occurring in late fall to early winter. The lack of a summer infectivity peak in this study may have been due to the unusual amount of rainfall during July, August, and September 1964 (75% of the total for the year: Fig. 11). This period was noted for the frequency of rains, lack of drying between precipitation, wet, packed soil, and lush growth of weeds, all of which would discourage dissemination of the fungus by the wind.

The peaks in the infectivity rates for both species of animals in the present study were consistent in that all of the monkey infections, and

FIG. 14. *Comparative histological lung sections of naturally infected (top) and experimentally infected (bottom) dogs. The first two sections on the left (bottom row) received 100 arthrospore intratracheal inoculations; the next two, 10 arthrospores; and the section at the extreme bottom, right, was from an uninoculated control dog. Note visible lung lesions in all naturally infected animals, and in only one of the four inoculated dogs shown here.*

20 of the 29 dog infections, were diagnosed from November to March. Although the infectivity rate was much higher for dogs than for monkeys, this undoubtedly was the result of their free run of the exposure pens (direct contact with the soil), their habit of fighting among themselves, and their constant digging (sometimes burrowing as much as 1 ft in the ground); all of these tended to stir up the dust and to uncover the fungus during the seasons when it would be expected to be 6 to 12 inches below the surface of the ground (8). Infection of dogs from contact with the soil was further indicated by the infectivity pattern; 19 of the 29 dog infections occurred in pen 3, the remaining 10 were divided between pens 1 and 2. Not only has it been pointed out before that growth of *C. immitis* in the soil is spotty rather than universal, even in heavily endemic areas (8), but also one of the few positive soil samples (Fig. 10) in this study was collected from within pen 3.

For the purpose of this study, the monkey was considered a more valid indicator ("biological air sampler") of the human infectious dose than the dog, because, being housed several feet above ground level, the monkeys undoubtedly became infected by normal airborne arthrospores rather than from close contact of the nose with the soil. However, an analogy in man to dog infectivity higher than that of the monkey might help to explain the more severe disease found in ground-construction workers and people engaged in agricultural pursuits (10), who also turn over the soil and stir up large amounts of dust in their daily occupations. Possibly man also contributes to the peak infectivity period shown in human infections during the summer months by his outdoor recreational activities, such as hiking, picnicking, digging for fossils, etc. (27).

In analyzing the present study, we must not lose sight of the fact that virulence differs among various strains of *C. immitis* (11, 11*a*), and also that the natural infections in the monkeys and dogs may have resulted from repeated exposure to extremely small doses (one to two arthro-

spores) before being detected by dermal sensitivity or serology, thereby building up a certain amount of immunity. However, a presumption that the infections originated from *large doses* of arthrospores of *low virulence*, would not be in accord with the *extreme* difficulty in isolating the organism from the air, since low virulence and low viability are not synonymous, and the presence of large numbers of arthrospores in the atmosphere should have been indicated by air sampling.

The authors realize, fully, the limitations of an experimental epidemiological study of this type, but considering the marked differences in histopathology, the lack of mortality in either species, and the fact that clinical signs of illness were lacking in the monkey (an extremely susceptible animal) and were relatively mild in the few dogs that exhibited them, we feel that the naturally acquired disease in these animals resulted from very small infectious doses; 10 or fewer arthrospores in the monkeys, and from 10 to more than 100 arthrospores in the dogs, although the individual dose range received was probably much greater in the dogs than in the monkeys.

The pattern of infections in the monkeys, dogs, and in man might well be compared: (i) there are large percentages of subclinical or asymptomatic infections in all three species (70% of the infected dogs, 100% of the infected monkeys, and 60% of infected human subjects); and (ii) the more severely affected dogs are analogous to the more severely affected construction and agricultural workers, since they all actively stir up the dust during their daily existence, whereas the less severely affected monkeys might be compared with the average human infections.

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Discussion

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The preceding paper reveals the difficulties of field experiments in contrast to experiments conducted in the laboratory. The authors have constructed an experimental exposure chamber in the open to look for natural infections in a highly endemic area in Arizona. Their experimental design was quite satisfactory, although one could suggest they might possibly have used cynomologous monkeys rather than mulatta. Their previous experiences with the mulatta, however, does indicate its sensitivity to infection.

Having set up this natural infection experiment, the authors awaited the infections. It turned out that 15% of the monkeys and 58% of the dogs were infected during the course of 1 year. Unfortunately, the year encompassed a year of exceptionally heavy rainfall, during which 12.5 inches fell, an amount much higher than that customary in the area (6 to 10 inches). They found the expected number of infections in the early part of the year, but there were very few infections in the latter part of the year, during which the heavy rainfall occurred. If the observations had continued for another year, it is quite probable that after the period of heavy rainfall they would have encountered a period of heavy infection.

On the whole, one is inclined to think that the

authors' data are perhaps more satisfactory than they claim. Since, for the area studied, Hugenholtz had reported (2) a yearly infection rate of 5.4% and an illness rate of 2 to 3 per 1,000 population, one should not be at all discouraged by finding that almost 15% of the monkeys were infected and more than half of the dogs. The results, therefore, would indicate that the area in which they were working was exceptionally heavily infected and that the results were quite in line with those expected from the conditions of the experiment.

The observations on the serological and clinical response of the infected animals do suggest low dosage infection. None of the 5 monkeys and only 3 of the 29 dogs showed positive cultures at autopsy.

Perhaps it would be well to consider separately the results of the exposure in monkeys and in dogs. The clinical picture found in the monkeys which became infected after natural exposure was less severe than that of animals infected artificially with 10 organisms. On the other hand, experimentally infected animals showed a higher mortality and higher antibody titers.

With dogs, the frequency of clinical symptoms was higher, as were positive cultural and histologic findings, among the naturally infected animals than among those artificially infected. This is probably to be expected, inasmuch as the dogs were allowed to roam free and dig in the soil, which would probably mean they would secure heavier exposure.

It is interesting to note that, although no illness occurred among the infected monkeys, 35% of the infected dogs had illness; this agrees very well with the observations made by Smith et al. (3) among naturally infected man.

When one considers the wards full of acute coccidioidomycosis in the air bases at Arizona, one would expect that large numbers of naturally exposed animals would develop infection and illness. One is, therefore, somewhat disappointed until one considers that the large numbers of cases reported by Hugenholtz (2) really represent a low infection rate per 1,000 persons stationed at the base, since fewer than 3 per 1,000 population were admitted to the hospital during any one month.

When one considers again the clinical implications of this work, one is struck by the lack of severity of disease in the monkeys and dogs versus man. This must be related to dosage, since there is evidence that both dogs and monkeys do develop severe disease after experimental infection. If one recalls the high frequency of severe clinical disease reported in the epidemics of coccidioidomycosis where exposure was known to be heavy, one is forced to conclude that clinical severity reflects in part at least heavy dosage of fungi. This is supported by the fact that the dogs, which had closer contact with the soil, had higher infection rates and more severe disease than the monkeys whose cages were elevated off the ground. A correlary of this observation would appear to be that the exposures in the Army personnel who became ill must of necessity have been heavy. Similar relationship of degree of exposure to degree of illness has been reported in histoplasmosis epidemics.

Still another factor is the difference in clinical symptoms related to the age of the individual at the time of infection. This is still largely an unknown factor and was not explored in these trials.

Finally, one comes to the contribution of these studies to the basic epidemiological question as to where one does get infected with *Coccidioides*. Is it by walking down the streets of Phoenix or Tucson or Bakersfield and inhaling the air, or is it necessary to go to a point source where the organisms are growing and stir up an aerosol there? As most of you know, the evidence in histoplasmosis points to point source visits as the mechanism of infection. The studies reported here suggest that this mechanism is also operative in coccidioidomycosis and that the animals were infected from the local focus. This is supported by the soil isolations which were made in the cage area. These data, therefore, support the "local focus" theory of infection rather than the "generalized windblown" theory.

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Concluding Remarks

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Modern reviews on microbial aerosols and airborne infection were initiated by the publication of the monograph, "Aerobiology," in 1942, by the American Association for the Advancement of Science (1). In the following decade, William F. Wells summarized his life-long investigations of these topics in an important volume (6). Some 6 years later, his pupil, Richard Riley, and Francis O'Grady reconsidered the problems of the transmission of respiratory infection (5).

These publications, and the numerous studies during that same period of the pulmonary deposition and retention of inhaled aerosols (2), provided a much firmer foundation than existed previously for the experimental investigation of airborne infection and the interpretation of the resultant observations. In addition, there have been extensive advances during the past two decades in the apparatus and techniques for quantitative experimentation in these fields.

A consequence of these improvements has been the emergence of a wealth of novel information on many aspects of aerobiology, and the need for periodic reassessments of our progress, both experimental and interpretive. To meet this need, a series of conferences has been organized—the first held at Miami Beach in 1960 under the sponsorship of the National Academy of Sciences (3), the second held in Berkeley in 1963 under the sponsorship of the Office of Naval Research and the Naval Biological Laboratory (4), and the present meeting being sponsored by the Illinois Institute of Technology Research Institute and the U.S. Army Biological Laboratories.

The presentations at this year's conference already have been thoroughly discussed by experts. It is not our intention, in these concluding remarks, to review again, in detail, the findings reported by each of the speakers. Instead, we will try to focus attention on some of the broad subdivisions of our overall theme.

The proceedings of previous conferences in this field, as well as several individual scientific publications, have provided information on equipment and techniques for the generation, containment, and quantitative characterization of microbial aerosols. These are at present sufficiently reliable that aerosols can be formed reproducibly with a content of infectious organisms as dilute as 1 to 10 cells per liter. Thus, with one exception, experimental equipment, per se, has not been a topic in this year's conference program. The exception is the subject of sampling. A notable deficiency in the past has been the absence of dependable, high-volume samplers for determining the organisms suspended in the air of hospital rooms, barracks, school rooms, etc. In these environments, transmission of infection is frequently observed, but the role of the airborne route is still questioned. The overall problem of the epidemiology of airborne staphylococcal infection in hospitals has been thoughtfully reviewed by Williams. May, Perkins, and Gerone, in their individual papers, have provided evidence that improved high-volume air samplers are practical. Future improvements in design and aditional experience in the use of such samplers will make substantial contributions to forthcoming studies of the transmission of infection in environments where the aerial content of organisms is quite low.

The impact of physical and chemical stresses on microbial aerosols has been discussed separately by Zentner and by Hatch. It is amply apparent from these reports, as well as several on the same topic in earlier conferences, that microbial aerosols are quite sensitive to changes in their water content, i.e., dehydration or rehydration, and alterations in the gaseous composition and temperature of their environment. Despite the progress made in these studies, it is not presently possible to formulate a theory which will permit the prediction and understanding of the changes in viability and infectivity such aerosols undergo when subjected to alterations in their environment.

The physical and physiological features of the respiratory tract have been reviewed by Proctor, Kass, Dalhamn, and Rylander. Proctor's development of novel techniques for measuring the air flow in the nasal passages gives promise of providing information of considerable value in studies of air pollution, as well as of the airborne transmission of infection. The clearance mechanisms of the respiratory system are often circumvented in experimental studies where highly infectious organisms are administered to the host, in dilute form and as micron-sized particles. Under natural circumstances, the role of the respiratory

tract as a portal of entry for airborne infectious organisms is probably influenced to a significant extent by these mechanisms.

The resistance of the host to infection may be significantly altered by metabolic disorders, debilitation, extensive surgery, immunosuppressive or radiation therapy, etc. This altered resistance may play an important role in the epidemiology of hospital-acquired infections, which Williams has discussed. The so-called normal individual may also be subjected to environmental stresses, such as the inhalation of air pollutants. Ehrlich has demonstrated an alteration in susceptibility to airborne infection of the mouse, when exposed to small amounts of nitrogen dioxide. It is too soon to comment on the relationship of these experimental observations to public health problems of man.

Saslaw, Hearn, and Miller, in individual presentations, have provided considerable additional evidence for the susceptibility of laboratory animals to infection by inhalation of aerosols of viruses or rickettsiae that, in nature, are transmitted by insect vectors. Saslaw has made a particularly thorough study of Rocky Mountain spotted fever in the monkey and has demonstrated the similarities in the disease induced through the respiratory portal of entry to the naturally occurring disease.

Aerogenic immunization is a commonly used technique in poultry husbandry, but has not been sufficiently well evaluated, thus far, to receive general acceptance in human medicine. Two studies have been reported at this conference, that of Yamashiroya using tetanus toxoid and that of Hornick using a live tularemia vaccine. The latter investigation is particularly noteworthy, since it has been possible to use both experimental animals and human volunteers as subjects, and to evaluate immunization by direct challenge with fully virulent organisms. Further work will be required to determine the relative merit of the aerogenic as compared with more conventional modes of vaccine administration. In addition, careful consideration should be given to defining the medical and physical conditions which would contraindicate use of the aerogenic technique.

There are a number of infectious diseases whose airborne characteristics can be studied only in laboratory animals. Investigations in man are precluded, since adequate means of control of the disease are not available. The present conference has heard two such investigations—that of Brachman on industrial inhalation anthrax and that of Converse on coccidioidomycosis. Both field investigations were preceded by thorough laboratory studies of the airborne disease in the same species of animal that was later used in the field. As a result, an extensive body of knowledge was available from laboratory investigations on dose-response relationships, incubation period, histopathological and immunological alterations, etc., which permitted a comprehensive correlation and interpretation of the field results.

Tularemia has been a most useful model of a bacterial disease that may be investigated in man. Studies reported in earlier publications, as well as the present conference, have presented a wealth of information on the susceptibility of man to experimental airborne infection, the use of attenuated strains of *Francisella tularensis* as a live vaccine, and the efficacy of vaccines, prepared in a variety of ways, in protecting the host against respiratory tularemia. Sawyer has extended our knowledge of this disease by investigating the conditions under which a bacteriostatic antibiotic (tetracycline) is effective in its management. A regimen has been found which may be employed for prophylactic or therapeutic purposes.

One of the direct benefits of the 1960 Miami Beach Conference was the subsequent initiation of a cooperative program of research on human viral respiratory diseases by the National Institute of Allergy and Infectious Diseases and the U.S. Army Biological Laboratories. This collaboration has provided an opportunity to apply quantitative and reproducible techniques for the generation, sampling, and characterization of viral aerosols to the exposure of human volunteers, and the study of their laboratory and clinical evidences of infection and illness.

Gerone has commented on some properties of laboratory-generated viral aerosols used for inoculation purposes and has presented early results in evaluating the output of viral aerosols into the environment of an infected human host.

Couch summarized the dose-response relationships observed in exposure of human volunteers to aerosols of coxsackievirus A-21, rhinovirus NIH 1734, and adenovirus type 4, and has compared the results obtained with this mode of inoculation with those obtained after intranasal instillation of virus. He has provided evidence for the production of airborne virus by coughs and sneezes of the infected volunteers—probably sufficient contamination of the environment to transmit infection to other susceptible individuals. In an interesting preview of a field study still in progress, Couch has provided preliminary information on the transmission of coxsackievirus A-21 infection among men in a controlled barracks environment, with evidence that the airborne

route played a significant role in the high level of transmission observed.

These thorough and extensive laboratory and clinical studies, in which the response of man is quantitatively related to the dose of virus inhaled, have provided a firm base for the design and interpretation of the experimental epidemiological studies, in which the mode of transmission of the virus under natural circumstances is the main focus of the investigation.

It is amply clear that this conference has served a valuable function in providing a critically prepared summary of progress in our knowledge of microbial aerosols and airborne infection. At the same time, the conference and the discussion it has stimulated will unquestionably influence the course of future investigations in these fields. The plan for prompt publication of the Proceedings in *Bacteriological Reviews* is an important mechanism for assuring the attainment of this objective. We congratulate all who have contributed to the success of this conference.

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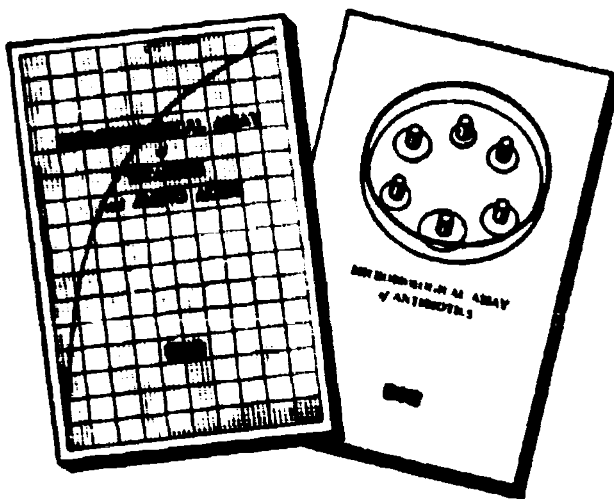

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