IMMUNOLOGICAL ASPECTS OF AIRBORNE INFECTION: REACTIONS TO INHALED ANTIGENS

GARDNER MIDDLEBROOK

Division of Research and Laboratories, National Jewish Hospital, Denver, Colorado, and Department of Microbiology, University of Colorado School of Medicine, Denver, Colorado

An attempt will be made to review published and some unpublished observations, especially in relation to aerogenic immunization against bacterial and viral agents of infection of animals and human beings. Some references will also be made to studies on immunological reactions to nonviable antigens administered by the respiratory route.

A natural consequence of the development of knowledge concerning airborne infection was to investigate the possibilities of using the airborne route for purposes of immunization against specific pathogenic microbes. The science of airborne infection has slowly become rooted in the physics and chemistry of production and behavior of airborne particles (dust and droplet nuclei) and the biophysics of parasites in such particles in atmospheres of variable composition. It is also based on increasing knowledge of the physiology of inhalation of particles and on the parasitic behavior of microbes after inhalation. Quantitative methods have been devised for the enumeration and control of dusts and droplet nuclei under experimental conditions. Some of these methods have been employed to determine the fate of inhaled microbes and the pathological and immunological responses of animals breathing infected air.

Several investigators during the past 10 years have observed that animals can become immunized as a consequence of inhalation of air containing enormously high concentrations of particles bearing widely variable and usually unknown numbers of living microbial units. The microbial strains employed have been those attenuated strains which were originally developed for immmunization of animals or human beings by other routes. It is interesting that veterinarians were among the first to recognize the possibilities of mass aerogenic vaccination. Living Newcastle disease vaccines were administered to chickens by inhalation in 1952 (11). Definite immunity was achieved by Hitchner and Reising in these experiments. During the next 2 years, Gorham, Leader, and Gutierrez (9, 10) showed that ferrets and mink could be immunized by exposing them to nebulized, living, egg-adapted distemper virus. Their studies were admittedly crude but very provocative (Table 1).

Since that time Russian investigators (3, 4), employing dry vaccine preparations containing living vegetative cells or spores (anthrax) of attenuated bacterial strains, have published data indicating that they could achieve highly effective immunization of experimental animals in short-term experiments against plague, tularemia, brucellosis, and anthrax. However, these Russian investigators claimed that small laboratory animals (guinea pigs and rabbits) were not very suitable models for testing the effectiveness of the dry aerogenic vaccines. Better results were obtained in sheep. Thus, aerogenic anthrax vaccine was compared with subcutaneous and percutaneous immunization in sheep. At various periods after vaccination (from 5 days to 7 months) the animals were challenged by subcutaneous, intracutaneous, and aerogenic infection with pathogenic anthrax spores in doses of 1,000 to 10,000 MLD. The mortality rates were as follows: 73.3% for control animals, 16.3% for percutaneously immunized animals, 5% for subcutaneously immunized animals, and 3.3% for aerogenically immunized animals.

These same Russian investigators carried these studies to human beings. Their results were measured by the development of agglutinins (tularemia, brucellosis), by opsono-phagocytic index tests (brucellosis), allergic tests (tularemia, brucellosis, and anthrax), and complement-fixation tests (plague). The results of aerogenic immunization of humans against brucellosis are presented in Table 2. They have also presented the results of opsono-phagocytic index studies on 41 subjects indicating that aerogenic vaccination is as effective as subcutaneous vaccination with their brucellosis vaccine (Table 3). A total of 363 persons were subjected to aerogenic vaccination with dry spores of attenuated strains of *Bacillus anthracis*, along with similar numbers of in-

TABLE 1. *Response of aerosol-exposed ferrets to challenge with virulent distemper virus*

No. of ferrets in group	Date of aerosol exposure	No. of days before challenge	Day after challenge	
			Onset of Signs	Death
2	8/9/53	32		
1	8/17/53	24		
1	8/25/53	16		
1	9/1/53	9		
2	9/3/53	7		
2	9/5/53	5		
2	9/6/53	4		
			14	20
2	9/7/53	3	14	Recovered
			13	21
2	9/8/53 (AM)	2	8	12
			9	12
2	9/8/53 (PM)	1.5	8	9
			8	11
2	9/9/53 (AM)	1	8	10
			9	11
2	9/9/53 (PM)	0.5	8	11
			10	12
2	9/10/53	0*	8	10
			9	11
2	9/11/53	−1	8	10
			9	11
2	Nonexposed controls		8	10
			9	11

* Simultaneous aerosol exposure and challenge virus.

TABLE 2. *Immunization against brucellosis with viable attenuated vaccine organisms, dry (40 × 10^6 to 500 × 16^6 units, calculated)*

Method of vaccination	No. of persons examined	Times of examination	No. of persons with positive Burnet reaction	No. of persons with positive Wright reaction	
				Total	1:20
		days			
Aerogenic	21	7	4	4	
Subcutaneous	23	15	Not performed	23	1
Aerogenic	42	15	30	40	
Subcutaneous	22	30	10	22	1
Aerogenic	50	30	48	48	3
Aerogenic	42	90	Not performed	36	4
Aerogenic	19	90	16		

Data from Aleksandrov et al. (3).

TABLE 3. *Immunization against brucellosis with viable attenuated vaccine organisms, dry (40 × 10^6 to 500 × 16^6 units, calculated)*

Time of examination	Aerogenic vaccination			Subcutaneous vaccination		
	No. of subjects	Opsono-phagocytic index		No. of subjects	Opsono-phagocytic index	
		Average figures	Maximal/minimal		Average figures	Maximal/minimal
Before vaccination	35	9.7	22/2	35	14.4	23/4
At 7 days	19	21.7	27/14			
At 15 days	41	26.0	31/16	23	22.2	33/13
At 30 days	48	20.6	29/12	23	25.4	31/20
At 90 days	41	24.3	37/14			

Data from Aleksandrov et al. (3).

dividuals vaccinated by the cutaneous and the subcutaneous routes with the same vaccine material (Table 4). In a room with a volume of 40 m^3, these investigators claimed to have succeeded in vaccinating aerogenically, during the course of 1 hr, up to 300 persons, with an exposure time of 5 min (3, 4).

The Russian investigators, it is only fair to point out, stated that the dosage of dried organisms necessary to be inhaled by an individual to achieve definite immunogenic responses was never less than 100,000 viable units. For some vaccines as many as a billion or even more were calculated to have been inhaled by each individual, on the average.

Preoccupation with the biological and biochemical significance of attenuation of isoniazid-resistant mutants of tubercle bacilli led Middlebrook and associates to distinguish, on an operational basis, between "infectivity" and "pathogenicity" of these organisms. As part of this inquiry it became important to know whether or not other attenuated strains of tubercle bacilli, BCG strains in particular, had diminished pathogenicity without diminished infectivity.[1] The airborne route of infection was chosen for these studies. The type of apparatus and the

[1] This work was supported by grants-in-aid from the Committee on Medical Research, American Trudeau Society, Medical Section of the National Tuberculosis Association, and from the U. S. Public Health Service (E-1717).

TABLE 4. *Aerogenic immunization with ST-No. 1 anthrax spores (40 × 10^6 to 600 × 10^6 viable units per dose)*

Time of examination after vaccination	Method of vaccination	No. examined	No. of persons with reactive allergic reaction	No. of persons with ineffective reaction	No. of persons with doubtful allergic reaction	No. of persons with a positive allergic reaction				
						In all	+	++	+++	++++
day										
7	Aerogenic	19	7	1	2	9	9			
	Subcutaneous	25	4	1	3	17	5	5	7	
	Cutaneous	25	10		4	11	3	6	2	
15	Aerogenic	26	4		3	19	4	7	6	2
	Subcutaneous	49	18		8	23	11	7	5	
	Cutaneous	50	20	2	6	22	5	10	7	
30	Aerogenic	64	21	1	11	31	14	9	8	
	Subcutaneous	50	14	4	14	18	8		10	
	Cutaneous	50	22	2	14	12	6	2	4	
90	Aerogenic	52	8	1	8	35	18	9	7	1
	Subcutaneous	52	15	2	8	29	12	4	11	
	Cutaneous	24	7	2	6	9	8	1		
	Control (unvaccinated)	70	50	9	7	4	4			

Note: To the ineffective were referred the reactions where hyperemia was present after the application of both the allergen and the physiological solution.

Data from Aleksandrov et al. (4).

techniques employed were first described in 1952 (16). In Fig. 1 is presented a schematic representation of this device. Figure 2 is a picture of the latest model of this basic design. This larger model has been used to expose 30 guinea pigs or 10 large rabbits simultaneously. It is important to state that in spite of intensive, almost daily use over a period of 8 years, none of the tuberculin-skin-test negative personnel working in the area where the apparatus has been located have become tuberculin-skin-test positive.

The most important facet of the technology involved in experimental airborne infection studies with mycobacteria, in our experience, has been the development of techniques of cultivation and preparation of suspensions of tubercle bacilli in such fashion that nephelometric measurements are reliably correlated with colony counts which, as is well known, take 3 weeks to perform with tubercle bacilli.

For example, during the month of November, 1960, four suspensions of cultures of the H37Rv strain were prepared, filtered through porosity F fritted glass filters, and adjusted to have 10 nephelometric units (NU) per 0.1 ml in a Coleman nephelo-colorimeter. These suspensions yielded the following mean plate counts of viable units in 0.1 ml of 10^{-5} dilutions on albumin oleic acid solid agar medium after 3 weeks of incubation: *76* (Nov. 1); *80* (Nov. 8); *75* (Nov. 11); and *90* (Nov. 17).

As previously pointed out by Lurie et al. (15), there is a close quantal relationship between the number of primary tubercles in guinea pigs and the number of virulent tubercle bacilli inhaled to the pulmonary spaces. The data recorded in Table 7 illustrate how good is the relationship, in experiment after experiment, between the number of viable units of virulent tubercle bacilli per milliliter of suspension nebulized and the average number of primary pulmonary tubercles that have developed per animal at 21 to 28 days after infection.

In Table 5 are compared the numbers of primary pulmonary tubercles in a group of guinea pigs and a group of mice after airborne infection with medium-fritted-glass-filtered suspensions, on the one hand, and fine-fritted-glass-

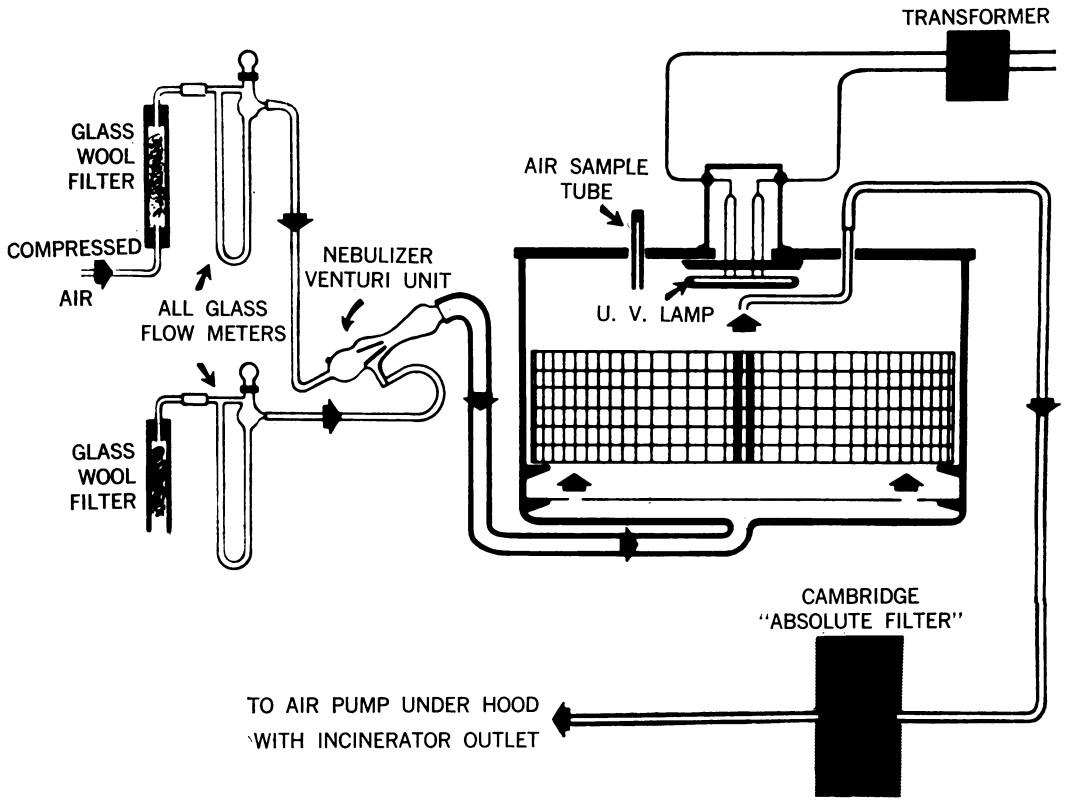

FIG. 1. *Airborne infection apparatus*

filtered suspensions, on the other. Careful examination of the results of this experiment reveals first that there is a remarkable constancy of numbers of primary pulmonary tubercles from animal to animal, the variations being fairly attributable to differences in minute respiratory volumes, the weights of the animals selected having varied from about 200 to 500 g. It is also noteworthy that the medium-fritted-glass-filtered suspension containing many small clumps initiated as many tubercles as the single-cell, fine-fritted-glass-filtered suspension in guinea pigs, whereas in mice, with their much smaller airways, only a small proportion of the clumps were able to reach the pulmonary spaces to initiate development of a lesion.

It seemed important to provide the results of these basic experiments to justify the formula which we have used for calculation of the number of aerogenically infective doses of attenuated tubercle bacilli. The general formula for such calculations is presented in Table 6. The predicted value of x (Table 6) has been confirmed with the use of fully virulent tubercle bacilli which *can* give rise to primary pulmonary tubercles. Furthermore, it is of interest that our data have confirmed the observations of Lurie et al. (15) to the effect that about one in every three or four droplet nuclei bearing single tubercle bacilli inhaled by guinea pigs reaches a susceptible locus, namely some site in the "pulmonary spaces" (in Professor Hatch's terminology).

Inasmuch as the method which we most commonly use to measure immunity against experimental tuberculosis involves challenge with virulent organisms by the airborne route, it seems worthwhile to describe just one set of experiments giving the results of application of our method of measuring immunity after the usual route of vaccination with attenuated tubercle bacilli.

In Table 8 are shown the results in the form of bacterial counts by culture and lesion counts by gross examination at 3 weeks after challenge of guinea pigs immunized by the subcutaneous route with approximately 10^6 bacterial cells of a

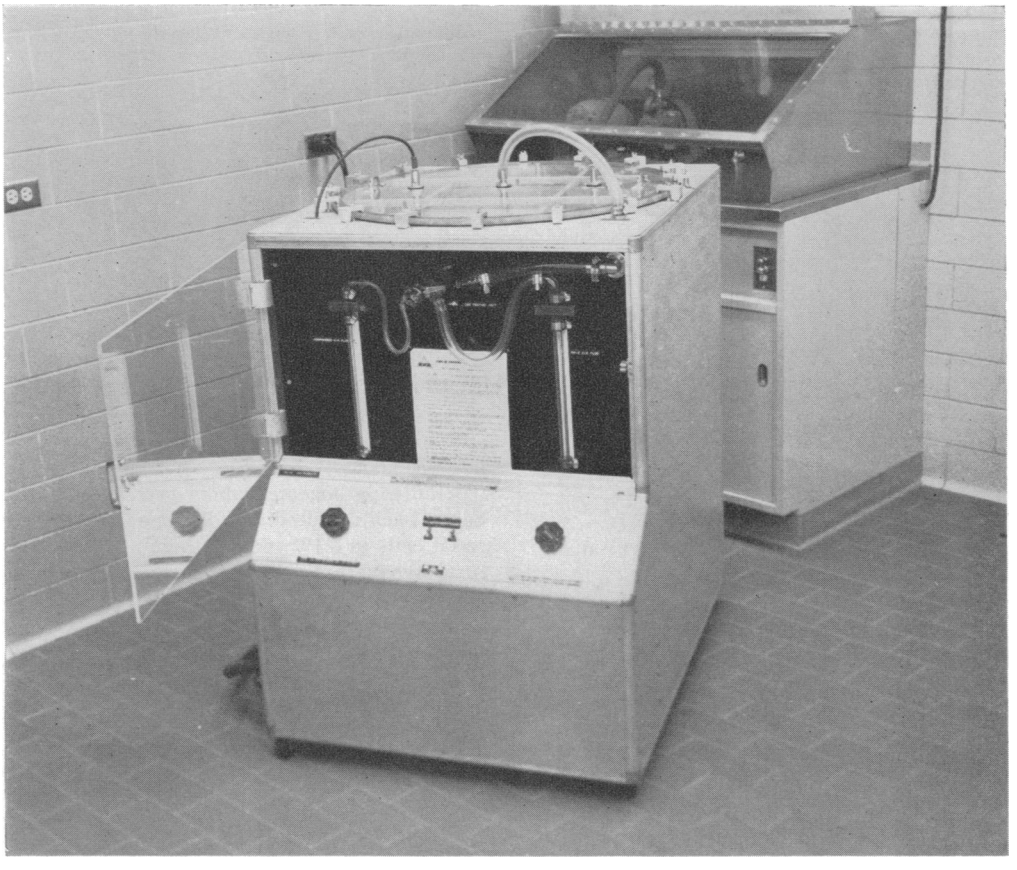

FIG. 2. *Airborne infection apparatus, whole body exposure type, designed by G. Middlebrook and manufactured by the Tri-R Instrument Company, Jamaica, N. Y.*

strain of BCG. The animals of one set, six BCG vaccinated and six controls, were challenged with isolated cells. Animals of the other set were challenged with suspensions containing many small clumps. It is clear from the results that the bacterial counts of virulent tubercle bacilli in both sets of animals are much lower in the vaccinated than in the controls, and there is not much difference between the two sets so far as bacterial counts are concerned. However, challenge with isolated cells of the virulent strain results in a somewhat clearer demonstration of immunity so far as the number of primary pulmonary tubercles is concerned: There are 10-fold fewer visible tubercles in the vaccinated than in the controls in the first set, whereas in the second set there is less than a 4-fold drop in numbers of visible primary tubercles. Nevertheless, we have learned to rely most heavily upon the viable bacterial counts from the lungs and spleens as the simplest and quickest way of evaluating immunity in experimental tuberculosis and as the method most amenable to statistical analysis. Usually we have waited 21 days after virulent challenge before sacrificing the animals. The results shown in Fig. 3 reveal that it might be possible to sacrifice the animals earlier than 21 days, perhaps even as early as 7 days. The upright bars on the 7-day points reveal how narrow were the limits of variation of bacterial counts by culture for the two groups, controls and vaccinated, with five guinea pigs in each group.

Having discussed some of the technological aspects of airborne infection with tubercle bacilli, I turn now to our use of the airborne route for purposes of immunization against experimental tuberculosis.

A) In Tables 9, 10, and 11 are presented the

TABLE 5. *Primary pulmonary tubercles in guinea pigs and mice after exposure to droplet nuclei containing single bacterial cells or small clumps*

Animal and no.	No. of tubercles after exposure to:	
	F-filtered suspension* (>90% single cells)	M-filtered suspension* (>90% small clumps, 4–15 bacilli)
Guinea pig:		
1	28	59
2	42	63
3	63	80
4	63	96
5	122	97
Avg.	64	79
Mice:		
1	3	0
2	4	1
3	5	1
4	6	1
5	8	2
6	11	3
Avg.	6.1	1.3

* Both suspensions from the same culture of H37Rv, adjusted to the same nephelometric density (122 NU/ml) and the same plate count for viable units or 17 (± 2) $\times 10^4$/ml of nebulized suspensions. Guinea pigs and mice exposed simultaneously. F and M = porosity grade of filter.

TABLE 6. *General formula for calculation of number of aerogenically infective doses of tubercle bacilli per individual host*

x = number of $ID_{63g.p.}$ (infective dose for 63% of guinea pigs, or one infective dose per animal, on the average, according to Poisson's law of small chances).
n = number of viable units per ml of nebulized suspension.
R = respiratory volume per 30 min per host, in liters.
f = factor characteristic of the specific nebulizer.
t = time of exposure, in minutes.
V = volume of air flow through exposure chamber, in liters per minute.

Then, $x = \dfrac{n \cdot R \cdot f \cdot t}{7.2 \cdot 10^3}$

This has been calculated from theory, and experimentally substantiated (*see* Table 7).

TABLE 7. *Empirical confirmation of general formula for value of x (in ID_{63} values for guinea pigs)*

Expt no.*	(n) No. of viable units† of virulent tubercle bacilli /ml of nebulized suspension	(x) Predicted value of x	Observed No. of primary pulmonary tubercles
8-153	$8 \cdot 10^3$	8‡	8‡
8-155	$8 \cdot 10^2$	0.8	2
9-91	$1.3 \cdot 10^5$	130	140
9-135	$2.5 \cdot 10^5$	250	182
10-49	$2 \cdot 10^5$	200	210
10-98	$5 \cdot 10^5$	500	240
10-163	$7.5 \cdot 10^4$	75	78

* Guinea pigs, 4 to 6 in each group (genetically heterogeneous).

† Fully pathogenic human type strains of tubercle bacilli diluted and dispersed as single bacterial cells in 0.1% serum albumin, 0.01% Tween 80 (polyoxyethylene sorbitan monooleate).

‡ Means/animal.

TABLE 8. *Effect of size of airborne infective unit on manifestation of immunity in guinea pigs*

Bacterial suspension in nebulized fluid	Bacterial counts and lesions*			
	BCG vaccinated†		Controls	
	Lung	Spleen	Lung	Spleen
Isolated cells (F-sintered-glass filtrate); 2×10^5 viable units/ml	6×10^3	2×10^3	4×10^5	1×10^5
	4 tubercles (pinpoint)		47 tubercles (0.5 to 1 mm)	
Small clumps (M-sintered-glass filtrate); 2×10^5 viable units/ml	1×10^4	1×10^3	7×10^5	2×10^5
	31 tubercles (0.5 mm)		111 tubercles (0.5 to 1 mm)	

* Counts are expressed as averages of: viable units/right lower lobe of lung, viable units/whole spleen, and visible tubercles/entire lung.

† With 0.1 ml of undiluted Tween-albumin culture of "Phipps"—subcutaneous—in *each* groin, 5 weeks before challenge (6 animals in each experimental group) with H37Rv 1:200.

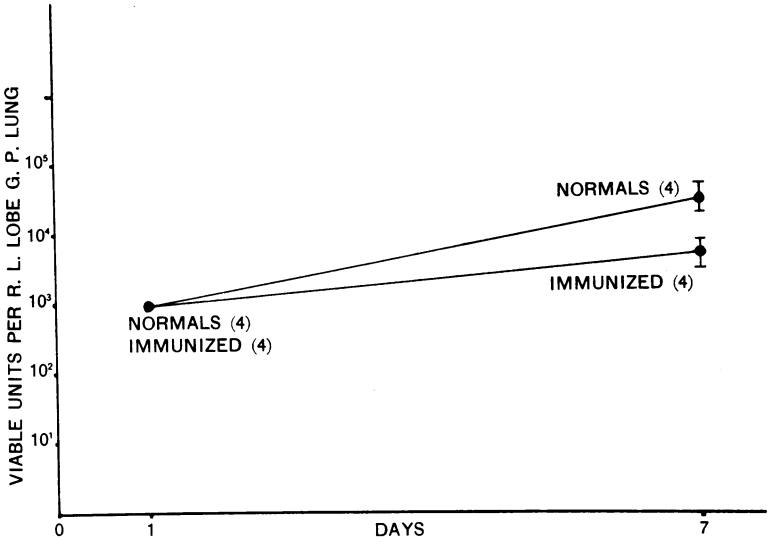

FIG. 3. *Influence of BCG vaccination. Bacterial counts on lungs at 24 hr and at 7 days after airborne infection. Pearson I strain of tubercle bacilli (1:100) (clumps). G.P. = guinea pig; R.L. = right lower.*

TABLE 9. *Airborne BCG immunization of guinea pigs against experimental tuberculosis*

No. of animals	Airborne BCG exposure	Tuberculin reactions* (41st day)	Mean bacterial counts† after virulent challenge	
			Lung right lower lobe/50) (70th day)	Spleen (whole organ/50) (70th day)
6	2.5×10^4‡	(2) Negative	2.5×10^4	1.1×10^4
		(4) Positive	1.0×10^2	2.5×10^1
6	2.5×10^5	Positive	1.7×10^2	6.0×10^1
6	2.5×10^6	Positive	0.5×10^2	3.0×10^1
6	0 (Controls)	Negative	1.2×10^4	1.7×10^4

* With 300 tuberculin units intracutaneously, read after 24 hr.

† Plate counts at 28 days after airborne challenge with about 100 infecting units of virulent tubercle bacilli (H37Rv) per animal.

‡ Number of viable units per ml of nebulizer fluid.

Note: No significant differences in degrees of immunity, as measured by bacterial counts, of animals exposed to 10- or 100-fold more than the smallest vaccinating dose of this strain.

results of experiments which illustrate the following: Inhalation by guinea pigs of very small numbers of viable units of BCG organisms, either as single cells or as clumps, can result in infection and the development of dermal tuberculin hypersensitivity and acquired resistance against subsequent challenge with virulent tubercle bacilli. Statistical analysis of the results presented in Table 10 has shown that 20 viable units of BCG per animal by the airborne route give rise to significantly greater immunity than 10 times that number from the same population of attenuated organisms by the subcutaneous route.

B) Table 12 shows results which are consistent with the conclusion that as few as 10 viable units of BCG per animal give rise to as much acquired resistance against subsequent virulent challenge as one million BCG units induced by the intracutaneous route. The question naturally arose during the course of these experiments whether or not there was any organ-specific immunity of the lungs after aerogenic BCG vaccination. The results presented in Tables 13 and 14 reveal that there is no striking difference between the results of challenge by two different routes, aerogenic on the one hand and intravenous on the other, of

TABLE 10. *BCG vaccination of guinea pigs; results of airborne vs. subcutaneous routes*

Group	No. of viable BCG units/animal	Tuberculin reaction at 44 days	Mean bacterial counts of virulent tubercle bacilli 28 days after challenge*	
			Lungs	Spleens
I (5), airborne	20	++ to +++	5×10^2	0.2×10^2
II (5), subcutaneous	200	+ to ++	30×10^2	1.5×10^2
III (5), controls	0	0	260×10^2	40×10^2

* Challenged by airborne route with about 200 living cells of H37Rv.

Degree of immunity of animals in group I statistically significantly greater ($P = 0.02$) than immunity of animals in group II.

immunity after aerogenic or intracutaneous BCG vaccination of guinea pigs. Thus, aerogenic immunity can be quantitatively, but does not appear to be qualitatively, different from that induced by other routes of inoculation of tubercle bacilli.

C) This state of immunity to infection with virulent tubercle bacilli persists, in effect, for at least 2 years, as illustrated in Table 15. The bacterial counts of virulent tubercle bacilli in the lungs of group I animals were not statistically significantly different from the controls. However, the spleen counts were different ($P = 0.01$). In other experiments it has been observed that the tubercle bacilli of airborne infection do not move in significant numbers from the lungs to the spleen until the seventh day after infection. Therefore, this evidence suggests that the persistence of aerogenic BCG immunity in guinea pigs has the character of an anamnestic response early during the course of the virulent challenge infection.

D) The positive results which we have obtained in measuring immunity of aerogenically vaccinated guinea pigs by sacrificing them at 21 days after virulent challenge and obtaining bacterial counts on their lungs and spleens have been confirmed by the more conventional methods of measuring immunity to experimental tuberculosis, namely by *survival* after virulent challenge. This method, which is most cumbersome, taxing the patience of any investigator who wishes to use small, ecologically realistic numbers of challenge organisms, requires more than 1 year and a larger number of guinea pigs, i.e., more than 20, in each group.

E) In Table 16 are recorded data from an experiment showing what may seem unnecessary to demonstrate in view of the fact that we already have bacteriological evidence of actual multiplication of BCG organisms in the mediastinal nodes in guinea pigs after aerogenic vaccination. Thus, it is obvious that infection—bacterial multiplication—is necessary for the development of immunity when such small numbers of viable units are inhaled.

TABLE 11. *Airborne BCG vaccination guinea pigs*

Vaccine diluted in 0.1% serum albumin and 0.01% Tween 80, 2×10^8 viable units/ml of original suspension, containing clumps as well as single bacterial cells. Exposure in 467-ft³ chamber at Aeromedical Laboratories, University of Illinois (Rosenthal and Middlebrook).

	Tuberculin reaction at month:		
	2	3	6
1:500 dilution:			
No. pigs	4	4	4
No. positive	4	4	4
Avg diameter (mm)	18.4	15.4	15.1
1:2,500 dilution:			
No. pigs	10	10	10
No. positive	4	4	5
Avg diameter (mm)	18	14	10
Control:			
No. pigs	6	6	5
No. positive	0	0	0
Avg diameter (mm)	0	1.4	0

Conclusion: Value of x for 1:2,500 dilution was predicted from the general formula as 0.6 $ID_{63g.p.}$; observed were 50% "takes" at this dilution, or 0.7 ID_{63} according to Poisson's law of small chances.

F) In two experiments with rabbits and three experiments with mice we have been unable to obtain any evidence of aerogenic immunity in either of these animal species. This result in rab-

TABLE 12. *BCG vaccination of guinea pigs; results of airborne vs. intracutaneous routes*

Group	No. of viable BCG units/animal	Tuberculin reaction at 62 days	Mean bacterial counts of virulent tubercle bacilli 21 days after challenge*	
			Lungs	Spleens
I (5), airborne	10	++ to +++ (pale pink)	1×10^2	6
II (5), intracutaneous	1×10^6	++ to ++++ (pink)	3×10^2	14
III (5), controls	0	0	1.7×10^4	4.2×10^3

* Challenged by airborne route with about 200 living cells of Lowenstein I.

TABLE 13. *Effect of challenge route on manifestation of immunity after aerogenic or intracutaneous BCG vaccination of guinea pigs*

Aerogenic Challenge

Group	Tuberculin reactions (61 days)	Mean bacterial counts of virulent tubercle bacilli 21 days after challenge*		
		Lungs	Spleens	Livers
I (6), controls	0	2.6×10^4	5.1×10^3	3.5×10^2
II (6), aerogenic vaccination 2×10^2 ID_{63}	+ to +++	8×10^2	4×10^0	0.3×10^0
III (6), intracutaneous vaccination 2×10^6 viable units	+++	9×10^2	4.8×10^1	1×10^0

* About 370 ID_{63} of single cells of Lowenstein I strain.

TABLE 14. *Effect of challenge route on manifestation of immunity after aerogenic or intracutaneous BCG vacccination of guinea pigs*

Intravenous Challenge

Group	Tuberculin reactions (61 days)	Mean bacterial counts of virulent tubercle bacilli 21 days after challenge*		
		Lungs	Spleens	Livers
I (6), controls	0	1×10^3	8.2×10^4	7×10^3
II (6), aerogenic vaccination 2×10^2 ID_{63}	++ to +++	2.1×10^1	2.1×10^3	5.2×10^1
III (6), intracutaneous vaccination 2×10^6 viable units	++ to +++	3.2×10^1	1×10^5	2.8×10^2

* About 10^6 viable units as single cells of Lowenstein I strain.

TABLE 15. *Persistence of aerogenic BCG immunity in guinea pigs*

Group	Tuberculin reactions (250 TU) at day:				Mean bacterial counts of virulent tubercle bacilli 21 days after challenge*	
	37	92	244	686	Lungs	Spleens
I (5), aerogenic vaccination†	+ to +++	++ to +++	+	+	9.4×10^3	4.9×10^2
II (5), controls	0	—	—	0	4.6×10^4	1.2×10^4

* About 140 ID_{63} of single cells of Lowenstein I strain, on 3/28/60.

† BCG "D" Phipps, about 100 ID_{63}, on 3/26/58.

TABLE 16. *Effect of chemoprophylaxis with isoniazid on BCG vaccination by inhalation*

Animal group	Tuberculin reactions at 50 days	Results at 28 days after challenge			
		Lungs		Spleen	
		Lesions	Bacterial counts	Weights	Bacterial counts
Controls (5), no Rx	Negative	1–3 mm with necrotic centers	Range: 2×10^5 to 2×10^6 Avg: 8×10^5	Range: 1.9–3.0 g Avg.: 2.6 g	Range: 7×10^5 to 3×10^6 Avg: 13×10^5
Vaccinated (5), no Rx	Positive	<0.5–0.5-mm tubercles	Range: 1×10^4 to 8×10^4 Avg: 4×10^4	Range: 0.9–1.4 g Avg: 1.2 g	Range: $<5 \times 10^1$ to 1.6×10^5 Avg: 4×10^4
Vaccinated (4), *but* INH Rx*	Negative	Same as controls			

* With 40 mg/kg of isoniazid per day for 10 days after aerosol exposure.

bits should not be surprising in view of the well-known fact that rabbits are less susceptible than guinea pigs to infection with tubercle bacilli. Indeed, it is not always possible to infect them with small numbers of the most pathogenic human type of *Mycobacterium tuberculosis*. As for mice, it suffices to state that it is much more difficult to immunize these animals than guinea pigs with BCG organisms administered in small numbers by any route. It is only fair to point out that our experience is limited to two strains of mice, CF[1] and C57 black.

G) Different strains of BCG differ widely in their ability to immunize aerogenically (7). This also is not surprising in view of several previous studies which have revealed wide differences in infectivity among BCG strains.

H) In several different experiments it has been shown that the smaller the number of viable units inhaled, the longer it takes for dermal hypersensitivity and immunity to develop in guinea pigs. This, too, is not surprising, but it is noteworthy that an occasional guinea pig, after inhalation of about one viable unit, does not become tuberculin-skin-test positive until after an interval of 3 months (this phenomenon is illustrated in the data of the middle of Table 11).

I) The few experiments which have been carried out on human beings at the University of Illinois by Dr. Sol Roy Rosenthal, who courteously provided the data in Tables 17 and 18, have shown that our species behaves more like guinea pigs than like mice or rabbits in that no more than 10 guinea-pig infective doses of a standard BCG vaccine suffice to render human beings tuberculin hypersensitive. The duration of tuberculin skin hypersensitivity after vaccination by this route is not yet established.

TABLE 17. *Tuberculin conversion rates following airborne BCG vaccination*

Age	5–8 Weeks					
	No. tested		Per cent positive		Average diameter (mm)	
	1:1,000	1:100	1:1,000	1:100	1:1,000	1:100
yr						
1–5	15	15	0.0	46.7	0.0	6.8
6–10	14	13	14.3	76.9	2.1	9.7
11–15	1	1	0.0	0.0	0.0	0.0
Total 1–15	30	29	6.7	58.6	1.0	7.9
16–22	8	8	12.5	87.5	4.9	14.6
Disc method						
Newborns	305	305	94.0	99.0	13.4	21.5
16–22	42	30	78.0	95.0	12.8	27.6

Aerogenic vaccination of human beings in 467-ft^3 chamber at Aeromedical Laboratories, University of Illinois, with 1:2,500 dilution of BCG vaccine, containing many clumps, as well as single cells, in nebulizer (Rosenthal).

Eigelsbach et al. (8) reported on the reactivity and immunogenicity for the *Macaca mulatta*

TABLE 18. *Aerogenic BCG vaccination of human beings*

Age group	"Takes"*/no. exposed	Respiratory volumes per 30 min (estimated)†	$ID_{63g.p.}$ (calculated)
yr		*liters*	
1–5	7/15	60	6
6–10	10/13	100	10
16–22	7/8	180	18

* Became skin hypersensitive to 100 tuberculin units within 5 to 8 weeks after exposure.

† From Committee on Handbook of Biological Data, NAS-NRC (7*a*).

Data from S. R. Rosenthal.

Conclusions: 1 $ID_{63(humans)}$ = or <10 $ID_{63(g.p.)}$.

TABLE 19. *Effect of the route of vaccination on resistance of monkey to respiratory challenge with 750 cells of strain Schu S5*

Animal group	Febrile response observed 3 to 7 days post challenge	*Pasteurella tularensis* isolated from blood during course of infection	Infection ratio	Survival ratio
	No. animals challenged	No. animals challenged		
Inoculated intracutaneously with rehydrated lyophilized vaccine	8/8	4/8	8/8	5/8 (63%)
Inoculated intracutaneously with live vaccine culture	7/7	5/7	7/7	4/7 (57%)
Inoculated aerogenically with live vaccine culture	8/8	1/8	8/8	7/8 (88%)
Nonvaccinated control	7/8	7/8	8/8	1/8 (13%)

TABLE 20. *Febrile response of guinea pig to live tularemia vaccine*

Route vaccination	Vaccine dose	Percentage febrile	Initial febrile response, day post vaccination	No. days febrile
Respiratory	20*	60	8–10	1–3
	10^3	100	6–10	2–5
	10^5	100	6–7	2–4
Subcutaneous	10	0	None	None
	10^3	60	5–8	1–3
	10^5	100	4–5	1–5

* Number of organisms inhaled; approximately 5 organisms retained.

TABLE 21. *Agglutinin response in guinea pig 24 days post administration of live tularemia vaccine*

Dosage	Respiratory route		Subcutaneous route	
	No. animals	Titer	No. animals	Titer
10–20	4	Negative	9	Negative
	1	1:80	1	1:160
	4	1:160		
	1	1:320		
10^3	1	Negative	4	Negative
	2	1:80	2	1:80
	2	1:160	3	1:160
	1	1:320	1	1:320
	3	1:640		
	1	1:1,280		
10^5	6	1:320	1	1:10
	2	1:640	1	1:20
	2	1:1,280	1	1:80
			6	1:160
			1	1:320

monkey and the Hartley guinea pig of a live tularemia vaccine administered by the respiratory, compared with the dermal, route. Their data, as indicated in Tables 19 to 22 and Figs. 4 to 6 (kindly provided for this Conference* by the Fort Detrick workers of the U. S. Army Chemical Corps), led them to conclude that aerogenic vaccination of these animals affords greater immunity against experimental tularemia than does vaccination by the dermal route. Inhalation of as few as 5 to 20 viable organisms by guinea pigs resulted in the development of agglutinins (six out of ten animals) and of some immunity against either respiratory or subcutaneous challenge with pathogenic *Pasteurella tularensis*. Inhalation of larger doses, however, produced significantly greater degrees of immunity in these animals. Doses less than 10^6 vaccine units were not reported in their experiments on

* NAS-NRC Conference on Airborne Infection, Dec. 7–10, 1960.

monkeys. However, inhalation of this number of vaccine organisms produced higher titers to agglutinins and greater immunity than immunization by the dermal route with the same number of vaccine organisms.

Many investigators have attempted to expose immunologically sensitized animals by way of the respiratory tract to shock experiences with homologous and heterologous antigens. A review of the literature in this area has led to the conclusion that most of their experiments and criteria are subject to serious contemporary questioning. In particular, there has been little or no attention to quantitative aspects of the subject. Most investigators have employed powders and have completely disregarded the importance of particle size. Others have instilled liquid or dry antigens by insufflating or spraying them into the nares of experimental animals in attempts to reproduce asthma or pollinosis experimentally. E. Friedberger in 1911 succeeded in producing a kind of anaphylactic pneumonia in animals that had been previously sensitized to horse serum by subjecting them to a spray of this substance. B. Busson did the same thing in guinea pigs with diluted cattle serum. Ratner (17) reviewed the experimental animal data up to the time of his writing, in 1950. At the same time Abramson, Gettner, and Sklarofsky (2) reviewed and investigated this subject with respect to man. Since that time some further work has been performed in human subjects (1, 6, 12–14, 19–22), the most noteworthy being that of Lowell and Schiller. It is especially significant in these semiquantitative studies that the mucous membrane of the lower respiratory tract may or may not be significantly sensitive to allergens which elicit strong reactions in the skin of allergic individuals. But the literature is in dispute as to whether an individual can have airway allergy to an antigen to which his skin gives no reaction. The factors which can influence

TABLE 22. *Immunogenicity of live tularemia vaccine administered aerogenically or subcutaneously to guinea pig*

Vaccine dose	Per cent survival on indicated day post challenge							
	Respiratory challenge (4,000 cells Schu S5)				Subcutaneous challenge (1,000 cells Schu S5)			
	7	15	7	15	7	15	7	15
	Respiratory vaccination		Subcutaneous vaccination		Respiratory vaccination		Subcutaneous vaccination	
Nonvaccinated	0	0	0	0	0	0	0	0
10–20	30	0	5	0	75	15	45	10
10^3	75	45	35	0	100	68	90	11
10^5	100	60	30	5	100	90	90	30

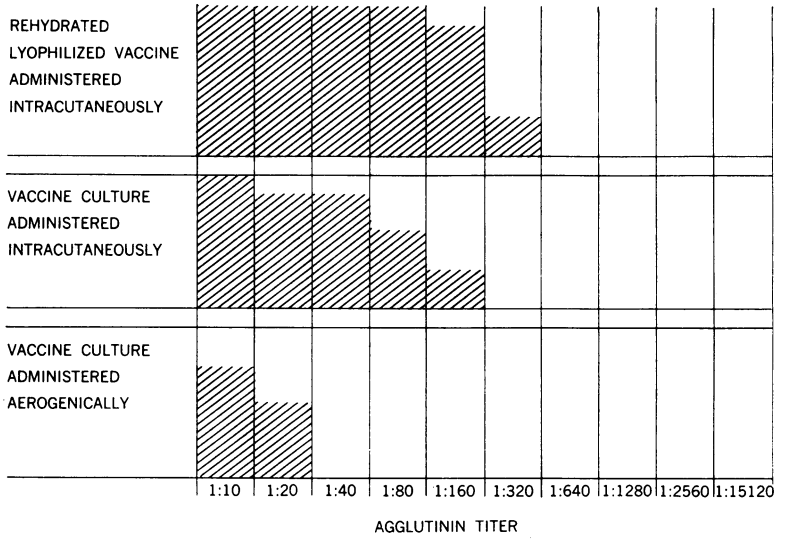

FIG. 4. *Agglutinin response in the Macaca mulatta monkey 1 week after vaccination.*

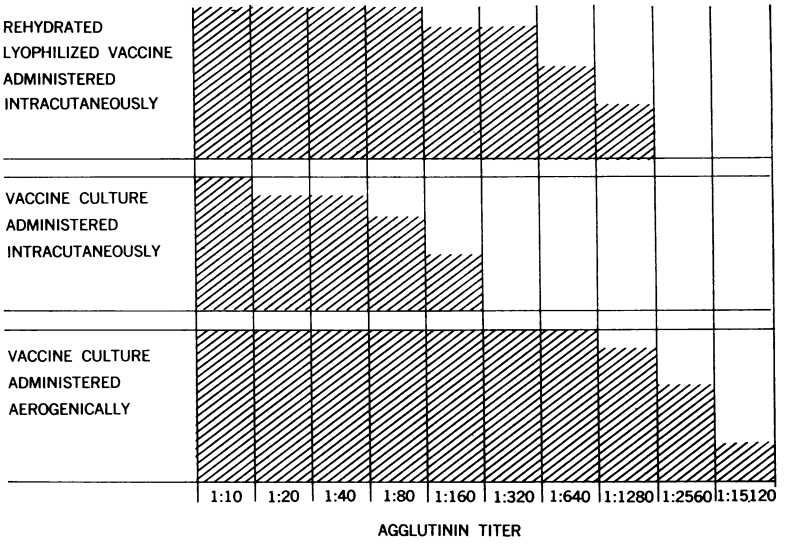

FIG. 5. *Agglutinin response in the Macaca mulatta monkey 3 weeks after vaccination.*

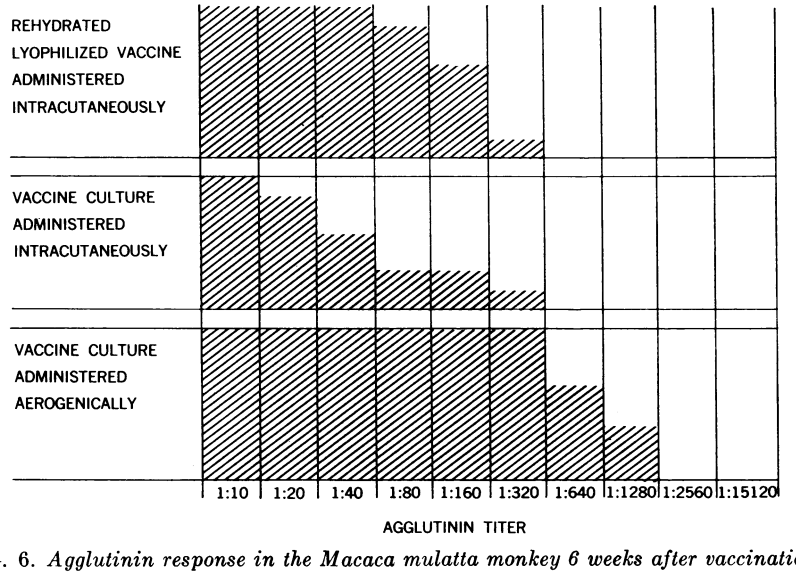

FIG. 6. *Agglutinin response in the Macaca mulatta monkey 6 weeks after vaccination.*

inhalation test reactions, including, in particular, the amount of antigen used, have not been rigorously controlled. Such problems, among others, have suggested that more precise, quantitative approaches to the study of immunological reactions in the airways would be highly profitable.

In collaboration with Drs. I. H. Itkin, S. Anand, and S. Permutt of the National Jewish Hospital, I have devised a new apparatus and carried out some experiments on a human subject which I wish to present to illustrate how more precise quantitation might be brought into this field.

The purpose of this work was to try to devise a method which would permit easy and prompt measurement of the quantity of an antigen inhaled by a human subject in very small amount over a period not exceeding 5 min. Special emphasis was placed upon the development of an apparatus which would yield aerosol droplets only of such size as would penetrate and deposit deep in the respiratory tract, i.e., droplets with

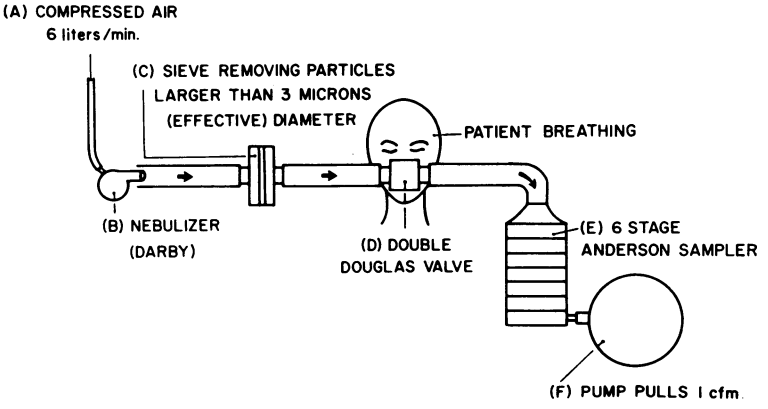

FIG. 7. *Apparatus for measuring deposition of airborne particles in "pulmonary spaces" of human subjects.*

diameters less than 3 μ (expressed in terms of the diameter of unit density spherical particles), preferably in the size range of 0.5 to 3 μ. A large body of evidence, much of which has been summarized at this Conference, shows that particles in this size range escape upper respiratory removal, on the one hand, and, on the other hand, are either retained in the smaller bronchi or are inhaled and exhaled without deposition in the respiratory tract.

A schematic representation of the apparatus used for these studies is presented in Fig. 7. It consists of a source of compressed air (*A*) driving a Vaponephrin or Darby type nebulizer (*B*) with about 6 liters of air per minute. The nebulizer is located at the open end of a Tygon tube (1 in. i.d.) leading to a sieve (*C*) which removes over 95% of particles larger than 3 μ in diameter inasmuch as it is constructed so as to behave like the no. 3 stage sieve of the Andersen sampler (model no. 0200) (5). Another piece of Tygon tubing connects the sieve to a double Douglas valve (*D*) which permits air to move only in one direction (from left to right as diagrammatically represented here). From this site the subject breathes through a rubber mouthpiece. From the double Douglas valve, the patient's sampling point, another length of Tygon tubing connects with an Andersen sampler (*E*), designed for the collection of particulate material on stainless steel plates of a six-stage impinger which has the characteristics previously described (5). Finally, an air pump (*F*) pulls air through the system at 1 ft^3/min. In preliminary studies it was found that the dye phenol red (medicinal quality, Nutritional Biochemicals Corporation, Cleveland, Ohio) could safely be inhaled by the asthmatic subject in an aqueous solution containing 0.5% glycerol (v/v), with the dye at a concentration of 0.4% (w/v). Therefore, this dye was used as a tracing agent after the method developed by Gilman and Phillips and described by Rosebury (18). Thus, the mass of particles on each collection plate of the apparatus was washed off with a solution of 0.01 N NaOH diluted to an appropriate volume and the optical density at 425 mμ was determined in order to establish, from comparison with a known standard, the amount of phenol red collected.

After little training it was possible for a subject, with the rubber mouthpiece in his mouth, either to inhale from and exhale into (with a nose clip) the air stream between the sieve and the sampler or to inhale through the nose and exhale only into the apparatus. It is evident that the difference between the amount of phenol red collected in the apparatus when the patient inhales and exhales, and the total amount when he exhales only, represents the amount deposited in the subject's respiratory tract.

Inasmuch as any water-soluble antigen in known concentration could be dissolved in the nebulizer fluid along with the phenol red, and since it seems safe to assume that the phenol red and the water-soluble antigen would distribute themselves in aerosol droplets in constant proportion, it was possible to calculate from the phenol red data the total amount of allergen actually deposited in the respiratory tract.

In Table 23 are presented the results of two

TABLE 23. *Allergic reaction of human asthmatic subject to inhalation of specific allergen*

Subject: Harvey, 15 years old, specific reaction of skin and airways to extract of *Alternaria* sp.

Collection plate (Andersen sampler)	Expt no. 1		Expt no. 2	
	Inhalation and exhalation (5 min)	Exhalation only (5 min)	Inhalation and exhalation (5 min)	Exhalation only (5 min)
	μg phenol red per plate		*μg phenol red per plate*	
Sieve between nebulizer and Douglas valve	74.8	47.1	117	63
No. 3	<1.0	<1.0	<1.0	<1.0
No. 4	4.4	58.7	1.1	36.5
No. 5	37.4	91.8	15.3	71.3
No. 6	54.4	70.7	31.4	41.3
Totals	171	269	165	213
Calculations:	269–171 = 98 μg phenol red ≅ 12 PNU of allergen FEV_1 : 2.5 → 1.3 liters		213–165 = 48 μg phenol red ≅ 6 PNU of allergen FEV_1 : 2.8 → 2.1 liters	

experiments on a young asthmatic patient, Harvey (NJH no. 14853). This patient had a long history of asthmatic symptoms on exposure to dust, and he manifested both skin and inhalation allergic reactions to aqueous extracts of *Alternaria* sp. Preliminary studies showed that he gave no reaction detectable by measurement of the forced expiratory volume in the first second (FEV_1 on a Stead-Wells respirometer). On the contrary, as illustrated by the results of experiment no. 1, after the calculated deposition of 12 protein nitrogen units (PNU) of alternaria extract from a 500 PNU/ml nebulized solution, his FEV_1 dropped from 2.5 to 1.3 liters within a period of 15 min after the end of the 5-min run and stayed at this level for another 30 min, after which it gradually rose toward normal. The subject suffered all the signs and symptoms of an attack of bronchial asthma. In the second experiment, in which only half as much antigen was calculated to have been deposited, his FEV_1 dropped from 2.8 to a minimum of 2.1 liters (at 15 min). This drop was considered significant in view of many previous control studies on this particular patient.

It would be too risky to attach any significance at this time to the different amounts of dye collected on the different plates of the sampler. But it does seem reasonable to suggest that apparatus and techniques of this kind can permit a more quantitative approach to the study of inhalation of allergenic materials and perhaps of other water-soluble agents such as pharmacologically active substances. With certain modifications, this type of apparatus might also be useful for the investigation of quantitative aspects of the deposition of other types of airborne particles in the respiratory tract of man. Perhaps with better control and measurement of the size of airborne particles this sort of approach to the determination of the "shock loci" in asthmatic subjects can be operationally defined much more precisely than is possible today. Suffice it to emphasize that a very small but indirectly measurable quantity of an allergen (12 PNU, or about 1 μg), deposited in the pulmonary spaces of an asthmatic subject, was enough to elicit an attack of bronchial asthma.

Literature Cited

1. Abdel-Maguid, R. 1957. Direct bronchial testing and hyposensitization in asthmatics using aerosolized dust extracts. Intern. Arch. Allergy Appl. Immunol. **10**:225–233.
2. Abramson, H. A., H. H. Gettner, and B. Sklarofsky. 1951. Experimental asthma in man with aerosols, p. 502–519. *In* H. A. Abramson, [ed.], Somatic and psychiatric treatment of asthma. The Williams & Wilkins Co., Baltimore.
3. Aleksandrov, N. I., N. E. Gefen, N. S. Garin, K. G. Gapochko, I. I. Daal-Berg, and V. M. Sergeyev. 1958. Reactivity to and effectiveness of aerogenic vaccination against certain zoonoses. Voenno-Med. Zhur. No. **12**:34–38.
4. Aleksandrov, N. I., N. E. Gefen, N. S. Garin, K. G. Gapochko, V. M. Sergeyev, M. S. Smirnov, A. L. Tamarin, and E. N. Shliakhov. 1959. Experiment of mass aerogenic vaccination of people against anthrax. Voenno-Med. Zhur. No. **8**:27–32.
5. Andersen, A. A. 1958. New sampler for the collection, sizing, and enumeration of viable airborne particles. J. Bacteriol. **76**:471–484.
6. Arner, B. 1949. Experiments in induction of allergy by inhalation of allergens. Nord. Med. **42**:1257.
7. Cohn, M. L., C. L. Davis, and G. Middle-

BROOK. 1958. Airborne immunization against tuberculosis. Science **128**:1282–1283.

7*a*. Committee on Handbook of Biological Data, NAS-NRC. 1958. *In* Handbook of respiration. W. B. Saunders Co., Philadelphia. 403 p.

8. EIGELSBACH, H. T., J. J. TULIS, E. L. OVERHOLT, AND W. R. GRIFFITH, 1960. Aerogenic vaccination against tularemia. Bacteriol. Proc. **1961**:89.

9. GORHAM, J. R., R. W. LEADER, AND J. C. GUTIERREZ. 1954. Distemper immunization of ferrets by nebulization with egg adapted virus. Science **119**:125–126.

10. GORHAM, J. R., R. W. LEADER, AND B. S. GUTIERREZ. 1954. Distemper immunization of mink by air-borne infection with egg-adapted virus. J. Am. Vet. Med. Assoc. **125**:134–136.

11. HITCHNER, S. B., AND G. REISING. 1952. Flock vaccination for Newcastle disease by atomization of the B_1 strain of virus. Proc. Am. Vet. Med. Assoc. **89**:258–264.

12. LOWELL, F. C., AND I. W. SCHILLER. 1947. Reduction in the vital capacity of asthmatic subjects following exposure to aerosolized pollen extracts. Science **105**:317.

13. LOWELL, F. C., AND I. W. SCHILLER. 1948. Measurement of changes in vital capacity as a means of detecting pulmonary reactions to inhaled aerosolized allergenic extracts in asthmatic subjects. J. Allergy **19**:100.

14. LOWELL, F. C., AND I. W. SCHILLER. 1948. The induction of asthmalike attacks in subjects with "idiopathic" asthma. J. Allergy **19**:172.

15. LURIE, M. B., A. G. HEPPLESTON, S. ABRAMSON, AND I. B. SWARTZ. 1950. An evaluation of the method of quantitative airborne infection and its use in the study of the pathogenesis of tuberculosis. Am. Rev. Tuberc. **61**:765–797.

16. MIDDLEBROOK, G. 1952. An apparatus for airborne infection of mice. Proc. Soc. Exptl. Biol. Med. **80**:105–110.

17. RATNER, B. 1951. Experimental asthma, p. 62–92. *In* H. A. ABRAMSON, [ed.], Somatic and psychiatric treatment of asthma. The Williams & Wilkins Co., Baltimore.

18. ROSEBURY, T. 1947. Dye and dye-tracer studies, p. 54–63. *In* T. Rosebury, Experimental air-borne infection. The Williams & Wilkins Co., Baltimore.

19. RUBITSKY, H. J., J. A. HERSCHFUS, L. LEVINSON, E. BRESNICK, AND M. S. SEGAL. 1950. Evaluation of therapeutic substances employed for the relief of bronchospasm. VII. J. Allergy **21**:559.

20. SCHILLER, I. W., AND F. C. LOWELL. 1947. The effect of drugs in modifying the response of asthmatic subjects to inhalation of pollen extracts as determined by vital capacity measurements. Ann. Allergy **5**:564.

21. SCHILLER, I. W., AND F. C. LOWELL. 1952. The inhalation test as a diagnostic procedure with special emphasis on the house dust allergen. J. Allergy **23**:234.

22. STEVENS, F. A. 1934. A comparison of pulmonary and dermal sensitivity of inhaled substances. J. Allergy **5**:285.