However, the available data are merely suggestive and further experiments are needed to prove this supposition. If this hypothesis is correct, adrenalectomy (with substitution for lack of mineral corticoids) should increase the incidence of non-thymic lymphomas in neonatally thymectomized hosts.

The incidence of myeloid leukemias was markedly increased by thymectomy in our earlier experiment(1) and in those by Gross (12). In the present experiment only one case of myeloid leukemia was found in 106 thymectomized virus-infected rats. It occurred in a thymectomized rat with an implanted empty Millipore chamber. The lower incidence of myeloid leukemia in the present experiment (less than 1%) compared with 12% in our earlier experiment(1) is puzzling. We expected a higher incidence because more rats escaped early deaths from non-thymic lymphomas (cf. 6). Failure of thymectomized rats to develop myeloid leukemia in the present experiments may be explained by low concentration of the virus. The thymus is a favored organ for replication of the virus(2). If so, neonatal thymectomy prior to virus-infection as done in the present experiments, eliminates this effect of thymus and neonatally thymectomized rats might be expected to have less virus than rats infected with virus first and thymectomized one month later.

Summary. 1. Neonatal thymectomy reduced the induction rate of leukemia by virus from 74.3% to 13.2% in one experiment and from 93.0% to 19.0% in another. 2. This indicates that many non-thymic lymphomas occurring in rats thymectomized one month after virus-infection(1) originated in cells which have been transformed in the thymus and seeded in other lymphoid organs prior to thymectomy. 3. The data also suggest a) that non-thymic lymphomas may be induced by the virus and b) that stressful agents which cause corticoid discharge might lower not only the incidence of the thymic, but also the non-thymic lymphomas.

We acknowledge the able assistance of Miss Sandra DeLuca and Miss Janice Williams.

1. Kunii, A., Furth, J., Cancer Res., 1964, v24, 493.
2. Okano, H., Kunii, A., Furth, J., ibid., 1963, v23, 1169.
3. Levinthal, J. D., Buffet, R. F., Furth, J., Proc. Soc. Exp. Biol. and Med., 1959, v100, 610.
4. Dischler, W., Rudali, G., Rev. franç. Ét. clin. biol., 1961, v6, 88.
5. Levey, R. H., Trainin, N., Law, L. W., J. Nat. Cancer Inst., 1963, v31, 199.
6. Furth, J., Okano, H., Kunii, A., In The Thymus in Immunobiology, Good, R. A., Gabrielsen. A. E., Ed., Harper & Row, 1964, p595.
7. Law, L. W., Dunn, T. B., Trainin, N., Levey, R. H., in The Thymus, Wistar Institute Monograph 2, 1964, p105.
8. Miller, J. F. A. P., in Ciba Found. Symp. on Tumour Viruses of Murine Origin, J. & A. Churchill, London, 1962, p262.
9. ———, Nature (Lond.), 1961, v191, 248.
10. Levinthal, J. D., Eaton, M. D., Proc. Am. Assn. Cancer Res., (Abst.), 1963, v4, 38.
11. Rudali, G., Acta Unio Inter. contra Cacrum, 1963, v19, 252.
12. Gross, L., Proc. Soc. Exp. Biol. and Med., 1963, v112, 939.

Received November 11, 1964. P.S.E.B.M., 1965, v118.

Preparation and Properties of a Small-Particle Aerosol of Coxsackie A_{21}. (29981)

ROBERT B. COUCH, PETER J. GERONE, THOMAS R. CATE, WILLIAM R. GRIFFITH, DAVID W. ALLING AND VERNON KNIGHT

U. S. Department of HEW, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboraory of Clinical Investigations, Bethesda, Md., and U. S. Army Biological Laboratories, Ft. Detrick, Md.

A variety of inoculation methods have been used in previous volunteer studies with respiratory viruses(1). While these studies often resulted in virus infection and illness, they were limited by the uncertainty regarding the location and dose of virus delivered to the

respiratory mucosa and the resultant inability accurately to assess dose-effect relationships.

It is known that the site of deposition of aerosol particles in the respiratory tract and number of particles deposited is a function of particle size(2,3). Thus, when defined aerosols are inhaled it is possible to estimate accurately both the site of virus deposition and the amount of virus deposited in the respiratory tract. The availability of aerosol generating equipment(4) suitable for human inoculations prompted a study of the preparation and properties of a small-particle aerosol containing Coxsackie A_{21}, an agent which causes respiratory disease in man(5,6). This report concerns the results of this study.

Materials and methods. Virus. The strains of Coxsackie A_{21} used in these studies were obtained from Marine Corps recruits at Camp Lejeune, N. C. who were ill with acute respiratory disease(5,6). The harvest of primary human embryonic kidney (HEK) or Wistar-26 human embryonic lung fibroblast (HEF)(7) tissue cultures was pooled to provide sufficient volume for the atomizer.

Preparation of the aerosol. Virus aerosols were generated in a Collison atomizer which was attached to a 7-foot by 6-inch tubular chamber containing apertures for human inoculation and sampling. This equipment and other necessary auxiliary components were contained in a mobile truck semi-trailer,* and have been previously described(4). During experiments, the aerosol was continuously generated in the Collison atomizer by 8 l/min of filtered air and on entering the chamber, it was diluted with 200 l/min of secondary filtered air. All aerosol studies were performed at 25°C and 50-60% relative humidity.

Aerosol sampling for virus. Modified Shipe impingers(8) were used to sample the aerosol for determination of virus concentration, and 3 to 7 impinger samples of each aerosol were collected. Each Shipe impinger contained 10 ml of Eagle's basal medium containing 2% inactivated chicken serum and antibiotics. The sampling rate was 9.5 l/min and 2-minute (19 l) samples were collected.

* Designed and built by the U. S. Army Biological Laboratories, Ft. Detrick, Md.

Virus distribution in the aerosol according to particle size was determined with the use of an Andersen sampler(9). To maintain the sampling characteristics, each petri dish must contain 27 ml of solid medium. To avoid dilution of the samples in this great a volume, a 21 ml base of hard agar was prepared in each plate and then overlaid with 6 ml of 12% gelatin. After sampling, the dishes were warmed to 37°C and the liquified gelatin assayed for virus.

Virus assays. Virus concentration in aerosol samples and in the virus suspension from the Collison atomizer were determined simultaneously for each experiment by inoculating aliquots of serial 10-fold dilutions into tissue culture tubes, 4 tubes per dilution, and observing 14 days for cytopathic effect. HEK cultures were used initially but HEF cultures were substituted when they were shown to be equally sensitive. Maintenance media consisted of equal parts of Eagle's MEM and medium 199 with 2% inactivated calf serum and added antibiotics. When enterovirus cytopathic effect was seen and progressed to involve the entire sheet, the tissue culture was considered positive. Final concentrations of virus in the samples were estimated using Karber's method and expressed as the dose of virus that infected 50% of tissue cultures ($TCID_{50}$)(10). The minimum detectable difference in virus concentrations provided by this assay procedure is 0.25 $\log_{10}$ $TCID_{50}$/ml.

Particle size. The distribution of particle sizes in the aerosol was determined by evacuating a sample of the aerosol into a 64-oz mason jar and allowing the aerosol to settle on glass slides coated with Permount®. The diameter of 500 particles was then measured by light microscopy using an eyepiece containing a calibrated scale.

Results. Quantitation and control of aerosol dose. To determine the ratio of the virus concentration in the aerosol to that of the atomizer suspension, a series of aerosols containing Coxsackie A_{21} were generated, impinger samples collected and both suspension and impinger fluid assayed simultaneously for virus. The mean impinger sample concentration of duplicate assays was used as the basis for calculating aerosol concentration. The mean of duplicate assays of the original sus-

TABLE I. Aerosol Assay Results.

Sample		Virus conc (log_{10} $TCID_{50}$/ml) Assay 1	Assay 2
Suspension	Pre	4.70	4.45
"	Post	4.45	4.95
"	Mean	4.575	4.70
Impinger	1	2.20	1.70
"	2	1.70	2.20
"	3	1.95	1.20
"	4	1.45	1.20
"	5	1.20	1.70
"	6	1.70	1.95
"	Mean	1.70	1.66

pension before and after atomization was used for suspension concentration. As an illustration of this type of determination results of one such experiment are shown in Table I.

As can be seen in each impinger assay, there is variation in the concentration of virus in impinger samples collected during an aerosol run. Comparison of the results of assay No. 1 and assay No. 2 suggests that although part of this variability is due to the assay procedure, the remaining part is due to fluctuation in aerosol virus concentration. Analysis of variance of the data from a series of aerosol runs such as that shown in Table I revealed a standard deviation (SD) among impinger samples of 0.25 log_{10} $TCID_{50}$/ml (11).

In Fig. 1 virus concentration in each of a number of aerosols is plotted against virus concentration in the corresponding atomizer suspensions. A range of virus suspension concentrations of 10^6-10^{11} $TCID_{50}$ per liter produced a virus aerosol concentration of $10^{0.3}$-$10^{4.3}$ $TCID_{50}$ per liter. The straight line that best fits these observations has a slope of 0.87 (95% confidence limits 0.70-1.06) and a y intercept of −5.2(12). The average difference in the suspension and aerosol virus concentrations is $10^{6.3}$ $TCID_{50}$ per liter. In most experiments, this difference can be accounted for by dilution of the aerosol particles in air, although in some instances loss of virus viability, slight inefficiency of sampling, or sedimentation and wall impaction of particles in the chamber may have contributed to the difference.

Physical and virologic properties of the aerosol. The distribution of particles in the aerosol according to measured diameters is shown in Fig. 2. As can be seen, the majority of the microscopically visible particles (85%) are less than 1 μ in diameter and the high frequency of particles of small diameter suggests that particles below the measurable limit may be present. Calculation of the proportion of suspension fluid represented in each measured particle size range indicates that although the majority of particles are less than 1 μ in diameter, 54% of the total volume is represented by particles in the 1 to 2 μ diameter range. The mean particle diameter was calculated to be 0.656 μ and the mean particle volume to be 0.368 μ^3.

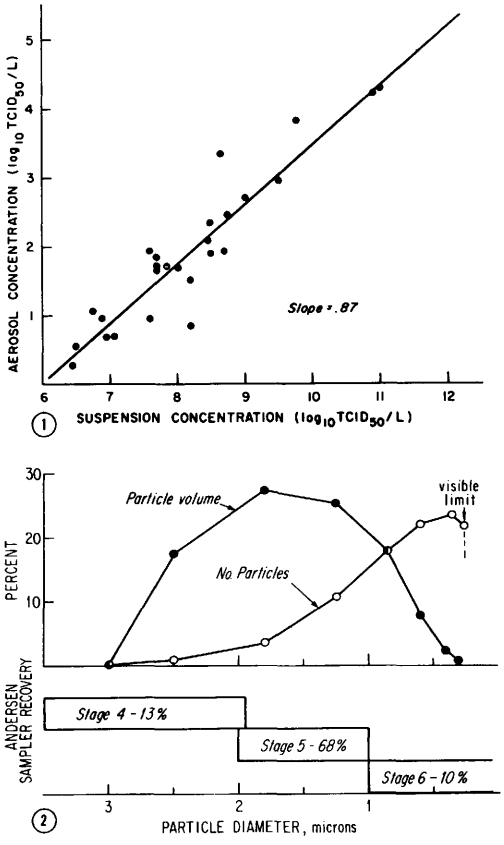

FIG. 1. Relation of virus concentration in atomizer suspension to virus concentration in aerosol. Each point represents an aerosol experiment and the line represents the slope that best fits these points.

FIG. 2. Distribution in aerosol of particles, particle volume, and virus according to particle diameter.

To determine virus distribution according to particle size, 5 aerosols produced by different concentrations of virus in the atomizer suspension were collected in the Andersen sampler. Over 90% of the virus was recovered in stages 4-6. The average recovery and particle size range impacting in each stage are shown in Fig. 2. The greatest proportion of virus, mean of 68%, was recovered from stage 5, which is estimated to retain 95% of particles in the 1.0 and 2.0 μ diameter size range if they reach that stage.

Upon inhalation into the human respiratory tract, the aerosol particles are exposed to moisture-saturated air so that enlargement of the particles by absorption of water is likely to occur. To obtain some information regarding the possible change in particle size, similar aerosols were generated, collected and measured at 50% and at 90% relative humidity. In addition, the same aerosol was collected and measured at 50% relative humidity and then exposed to 90% relative humidity and again collected and measured. These studies revealed a 2.0-2.5-fold increase in median particle diameter (0.5-1.0 or 1.5 μ) following the changes in relative humidity.

Discussion. This study has described the preparation and aerobiologic properties of a virus-containing aerosol suitable for inoculation experiments using normal volunteers. Significant properties of the aerosol are as follows: it has a particle size distribution ranging from the lower limits of light microscope visibility of 0.2 μ diameter to an upper limit of 3.0 μ; virus is distributed in the aerosol according to particle volume; and the concentration of virus in the aerosol bears a nearly fixed relationship to the concentration of virus in the liquid suspension from which the aerosol is generated.

The slope of the relationship of virus concentration in aerosol to that of the suspension on a log scale was 0.87 and the 95% confidence limits for this slope include 1.0. Assuming the true slope is less than 1.0, this would indicate a decreasing proportion of recovery of infectious virus from the aerosol with increasing concentration in the spray suspension. There is no present explanation of this phenomenon but aggregation of virus-containing particles with increasing concentration would seem to be one possibility. In any event, using this relationship, the concentration of virus in the aerosol can be predetermined with sufficient accuracy to perform experiments with volunteers in which the influence of relatively small differences in virus dose can be discerned.

There was an SD of 0.25 $\log_{10}$ $TCID_{50}$/ml in Shipe impinger samples of the aerosol collected during an experiment. Since the minimal detectable difference in concentration provided by the assay procedure was 0.25 $\log_{10}$ $TCID_{50}$/ml, it is possible that aerosol virus concentration over a period of time may be more uniform than the variance tests indicate. However, for aerosols of *P. tularensis,* direct counts of organisms revealed a similar variance(4), suggesting that the variation in aerosol concentration of Coxsackie A_{21} during an aerosol run closely approximates the inherent variation in performance of the equipment.

Since both the site of deposition and degree of retention of aerosol particles within the respiratory tract are related to particle size(2,3), it is important for interpretation of volunteer experiments to know the virus distribution in the aerosol according to particle size. It was found that distribution of virus and suspension fluid in the aerosol were approximately the same and that almost 70% of the virus was present in the particle size range of 1 to 2 μ in diameter. These findings substantiate assumptions by Duguid(13) with reference to the distribution of bacteria in aerosol.

It was not possible to obtain direct information concerning the presence in the aerosol of particles below the microscopically visible limit (0.2 μ in diameter), but since virus appears to have a distribution in aerosol similar to that of the suspension fluid extrapolation of the curve for particle volume to this size range suggests that the amount of virus that might be contained in such particles is negligible.

Inhaled aerosol particles may conceivably increase in size prior to deposition as a result of rehydration in the moisture-saturated air of the respiratory tract. This may affect both the site and extent of deposition of particles. Studies have revealed that change from 50%

to 90% relative humidity results in a 2.0-2.5-fold increase in particle diameter. If such a change occurred with the present aerosol before particle deposition had taken place, the majority of virus would be presented to the respiratory tract in particles 2-4 μ in diameter. This increase in particle size would lead to deposition somewhat more proximally in the respiratory tract and in greater proportion than calculations based on initial particle size would have indicated. Such changes have not been assessed *in vivo,* but the limited extent to which they might occur as suggested by the above experiments would probably not alter interpretation of volunteer studies, since in either case substantial deposition would occur in lower respiratory sites.

Summary. A method of generation and a description of the aerobiologic properties of small-particle aerosols containing the picornavirus, Coxsackie A_{21}, have been described. The size of particles in the aerosol ranged from 0.2 to 3.0 μ in diameter. Particles 1-2 μ in diameter comprised 54% of the total particle volume and contained 68% of recoverable virus. The SD among aerosol virus concentrations during periods of administration was 0.25 $\log_{10}$ $TCID_{50}$. The log concentration of virus in the aerosol expressed as a function of the log concentration of virus in the suspension used to generate the aerosol was a straight line with a slope of 0.87. Virus concentration in the liquid suspensions tested ranged from 6.4 to 11.0 $\log_{10}$ $TCID_{50}$/liter, and in aerosols from 0.3 to 4.3 $\log_{10}$ $TCID_{50}$/liter. The mean difference in concentration between liquid and aerosol through the range of values tested was $10^{6.3}$ $\log_{10}$ $TCID_{50}$/liter.

Gratitude is expressed to Mr. Holly A. Smith, Mrs. Carol Uhlendorf, Mr. James Turner, Mr. Leonard P. Durocher, and Mr. Donald L. Hollabaugh for their technical work; to Mr. Edward B. Derrenbacher and Mr. Charles O. Masemore, U. S. Army Biological Laboratories, Ft. Detrick, Md., for assisting with the aerosol inoculations, and to the following who materially assisted in the program: Mr. James Bennett, Director, and Dr. Charles E. Smith, Chief Medical Officer, Bureau of Prisons, U. S. Dept of Justice; Dr. Clifton K. Himmelsbach, Associate Director, and Mr. Franklyn Gray, Assistant Supervisor, Normal Volunteer Program, Clinical Center, Nat. Inst. Health.

1. Knight, V., Progress in Medical Virology, E. Barger, J. L. Melnick, Ed., S. Karger AG, Basel, 1964, p2.

2. Hatch, T. F., Gross, P., Pulmonary Deposition and Retention of Inhaled Aerosols, Academic Press, New York, 1964.

3. Davies, C. N., Industrial Pulmonary Diseases, E. J. King, C. M. Fletcher, Ed., Little, Brown & Co., 1960, p44.

4. Griffith, W. R., Am. Rev. Resp. Dis., 1964, v89, 240.

5. Johnson, K. M., Bloom, H. H., Mufson, M. A., Chanock, R. M., J.A.M.A., 1962, v179, 112.

6. Bloom, H. H., Johnson, K. M., Mufson, M. A., Chanock, R. M., ibid., 1962, v179, 120.

7. Hayflick, L., Moorehead, P. S., Exp. Cell Res., 1962, v25, 585.

8. Public Health Monograph No. 60, Sampling Microbiological Aerosols, U.S.P.H.S. Publication 686, 1959.

9. Anderson, A. A., J. Bact., 1958, v76, 471.

10. Finney, D. J., Statistical Method in Biological Assay, Charles Griffin & Co., London, 1952, p524.

11. Scheffe, H., The Analysis of Variance, John Wiley & Sons, Inc., New York, 1959.

12. Halpern, M., J. Am. Stat. Assn., 1961, v56, 657.

13. Duguid, J. P., J. Hyg., 1946, v44, 471.

Received December 2, 1964. P.S.E.B.M., 1965, v118.