Characterization of the Infectious Unit for Man of Two Respiratory Viruses[1] (36260)

BRUCE H. HAMORY, ROBERT B. COUCH,[2] R. GORDON DOUGLAS, JR.,[3] SAMUEL H. BLACK,[4] AND VERNON KNIGHT

Departments of Microbiology and Medicine, Baylor College of Medicine, Houston, Texas 77025

We have previously reported 50% human infectious doses (HID_{50}) of adenovirus, type 4 (adeno 4), and Coxsackie virus A, type 21 (A21) (1, 2). The HID_{50} were determined in normal volunteers and expressed as 50% tissue culture infectious doses ($TCID_{50}$) using the most sensitive assay system available for each virus. Determinations of HID_{50} were made for each virus with two different inoculation methods: nasal instillation of a small volume of virus suspension, which deposits entirely in the nasopharynx; and inhalation of a small particle aerosol, which deposits primarily in the lower respiratory tract. Adeno 4 was more infectious in small particle aerosol than in nasal drops; whereas the reverse was true for A21. While these data suggest important biologic differences between viruses, they do not provide absolute quantitation of infectious units and since the sensitivity of the respective assay systems is not known, they do not allow quantitative comparisons between viruses.

In order to characterize the infectious unit of these respiratory viruses for man, and to provide data with which comparisons could be made between viruses, we made electron microscopic counts of viral particulates and numbers of virions in particulates in the virus suspensions of adeno 4 and A21 which were used for volunteer inoculations. In the present report these data are presented and compared to the previous data on infectivity of the virus suspensions for man and for tissue culture.

Methods and Materials. Virus. The strains used in these studies were obtained from Marine recruits with acute respiratory disease. The adeno 4 volunteer inoculum (strain 78650) had been passaged once in human embryonic kidney tissue cultures (HEK). The harvest was frozen and thawed once, pooled, centrifuged at 1000*g* for 20 min, and filtered through 800 mμ membrane filters (Millipore). Filtrates were stored at —70° until used. The A21 volunteer inoculum (strain 48654) had been passaged twice in strain WI-26 human embryonic fibroblast tissue cultures (HEF). In addition to the above described procedures this inoculum was submitted to vacuum concentration and trifluorotrichloroethane (Genetron) treatment. Details of these procedures, as well as methods of safety testing of inocula have been previously described (3–5).

Inoculation procedures and determination of administered dose. The method used for small particle aerosol inoculation and determination of inhaled dose has been previously reported (6, 7). Calculations on deposition site within the respiratory tract indicated that about 83% of inhaled virus was retained; and of this, about 47% deposited in the lower respiratory tract and the remaining 36% deposited in the nasopharynx (8). Nasopharyngeal inoculations were performed by the instillation of 0.25 ml of virus suspension into each nostril and dose was determined by simultaneous titrations of the virus suspension used.

[1] Supported by Grant RR-00350 from the Clinical Research Centers Branch, National Institutes of Health, Bethesda, MD.

[2] Request reprints from Dr. Couch, Department of Microbiology, Baylor College of Medicine, 1200 Moursund Ave., Houston, TX 77025.

[3] Present address: Department of Medicine, University of Rochester School of Medicine, Rochester, NY 14260.

[4] Present address: Department of Microbiology, Michigan State University, East Lansing, MI 48823.

Infectivity for tissue culture was determined by making serial 10-fold dilutions of the virus suspension and inoculating 0.2 ml of each dilution into each of four tissue cultures. HEK was used for detection of adeno 4; and cultures were observed 60 days for cytopathic effect (CPE), with blind passage as necessary. Longer incubation periods, similar use of Hep II cultures for 60 days, and plaque assay using Hep II or HEK cultures incubated for 10 days failed to detect more virus (unpublished data). HEF (strain WI-38) was used for detection of A21 and cultures were observed 14 days for CPE. In other tests, longer incubation periods, similar tests with HEK and plaque assay using HEF cultures incubated for 5 days failed to detect more virus (unpublished data). All cultures were incubated at 33° on roller drums turning at 12 rpm. Final concentrations of virus were calculated using the method of Kaerber.

Virus particle enumeration. Virus preparation and particle counting techniques used were those of Sharp as modified by Smith and Benyesh-Melnick (9). In brief, the virus suspension was allowed to form a droplet and fall on a large block of 2% agar, which was then placed in an oven at 37°; and the droplet evaporated. A suspension of 0.75% parlodion in amyl acetate was placed on the block and, when a membrane had formed, the block was carefully trimmed to the area of the original spread of the droplet. The membrane, now containing viral particulates, was floated onto a staining solution which was 2% phosphotungstic acid (PTA) for A21; and 0.5% uranyl acetate for adeno 4. Membranes were quickly picked from the stain with a 200 mesh copper grid, dried, and examined in a Sieman's Elmiskop 1A electron microscope at an accelerating voltage of 60 kV.

Particle counting of each membrane was performed on five randomly selected fields photographed at 5000× magnification. A particulate was defined as any single virion or an aggregate of virions. The number of virions in particulates were also counted except for virions which appeared morphologically incomplete. Incomplete virions were counted separately.

The average distribution for all five fields was calculated and formed the distribution for that membrane. Two membranes for the adeno 4 suspension, each from a separate aliquot, and three for the A21 suspension were similarly counted; and the average distribution of each group formed the distribution for that virus suspension. Percentage deviation from the average for total and also for single particles of adeno 4 was 3%. For A21 the deviation of the three membranes from separate aliquots was 13, 14, and 1% for total particles but 9, 60, and 42% for single particles. The latter was obtained from an average count of 176 particulates/membrane. Accuracy of counting was assessed by recounting an A21 membrane at a separate time, and the second count and distribution were almost identical to the first.

Results. Characterization of viral inocula. Table I shows the relationship between particulates enumerated and detectable tissue culture infectious units for each of the volunteer inocula. As shown, the adeno 4 suspension contained 0.11×10^9 $TCID_{50}$ and 1.45×10^9 viral particulates. Assuming a Poisson distribution of viral particulates in suspension, calculations indicated that about 1 in 20 morphologically complete units of adeno 4 is detected in the biological assay. Morphologically incomplete virions comprised about 10% of total virions and are not included in these calculations.

Also shown in Table I are similar data for A21. Calculations based on the same assumptions indicated that about 1 in every 2 or 3 particulates of A21 is detected in the biological assay. It was not possible to identify incomplete virions of A21 with the particle counting methods used.

TABLE I. Relationship Between Quantity of Virus Detected by Tissue Culture and Electron Microscopy.

Virus	$TCID_{50}$[a]/ml $\times 10^9$	VP[b]/ml $\times 10^9$	$VP/TCID_{50}$
Adenovirus 4	0.11	1.45	13.2
Coxsackie A21	1.13	2.60	2.3

[a] 50% tissue culture infectious doses.

[b] Viral particulates.

TABLE II. Distribution of Viral Particulates According to Number of Virions Contained in Each.

Virus	VP[a]/ml × 10^9	Particulates (%) with indicated no. of virions					
		1	2	3	4	5	6+
Adenovirus 4	1.45	67	11	8	4	3	7
Coxsackie A21	2.60	70	9	7	5	2	7

[a] Viral particulates.

The distribution of viral particulates in each suspension according to number of virions in each is shown in Table II. As shown, about two-thirds of particulates in each suspension occurred as single virions and the remaining particulates contained a varying number of virions.

Comparison of HID_{50}. A summary of the results of volunteer inoculations, the calculated HID_{50} for each virus and inoculation method, and the respective confidence limits for each are shown in Table III. As shown, adeno 4 is about 20-fold more infectious for man when given by small particle aerosol than by nasal drops; whereas A21 is about 5 times more infectious by nasal drops.

Using data from Table I, the HID_{50} were recalculated and expressed as viral particulates. The values for an HID_{50} for each virus and inoculation method expressed as $TCID_{50}$ and viral particulates is shown in Table IV. As shown, adeno 4 is about 11 times more infectious for the lower respiratory tract than is A21; whereas A21 is about 9 times more infectious for the nasopharynx than is adeno 4. Furthermore, since only about 83% of the aerosol is retained and of this 47% deposits in the lower respiratory tract, calculations indicate that three or four particulates in the lower respiratory tract are sufficient to induce infection in volunteers free of serum antibody to adeno 4.

Discussion. These studies have provided physical quantitation of two respiratory viruses for comparison to quantitation using biological systems. Comparison of physical viral particulates to infectivity results for tissue culture revealed that the infectivity assay for adeno 4 was about 20-fold less sensitive for detection of virus than was particulate enumeration by electron microscopy. This finding is within the range reported for adenovirus type 5 by Pereira and Valentine (10). One cannot assume, however, that all particulates are infectious, even though virions that appeared incomplete were excluded from counts. It is possible that aggregates of adenovirus virions are inherently less infectious than single virions, although it seems more likely that the failure of the HEK assay to detect all particulates is due to a relative insensitivity of the cell population. This is further suggested by the fact that man appears to be more sensitive for detection than is the HEK system. As noted, enumeration by EM added very little in-

TABLE III. 50% Human Infectious Dose (HID_{50}) for Antibody-Free Volunteers.

Virus	Method of inoculation	No. of volunteers	No. of infections	HID_{50}[a]	95% Confidence limits
Adenovirus 4	Nasal drops	17	7	9[b]	3–25
	Aerosol	21	16	0.5	0.2–1.4
Coxsackie A21	Nasal drops	14	7	6	3–13
	Aerosol	14	8	34	22–52

[a] Expressed as 50% tissue culture infectious doses.

[b] Less than reported in Ref. (1) because more volunteers were tested.

TABLE IV. Characterization of HID_{50}.[a]

Method of inoculation	Adenovirus 4		Coxsackie A21	
	$TCID_{50}$[b]	VP[c]	$TCID_{50}$	VP
Aerosol	0.5	7	34	78
Nasal drops	9	118	6	14

[a] 50% human infectious dose.
[b] 50% tissue culture infectious dose.
[c] Viral particulates.

crease in sensitivity for detection of A21 and for this virus incomplete virions could not be enumerated.

The differing patterns of infectivity of the two viruses for the different inoculation methods is probably relevant to understanding the pathogenesis of the illnesses. The greater infectivity of A21 for the nasopharynx than for the lower respiractory tract is in keeping with the occurrence of nasopharyngeal disease in all volunteers who developed illness in association with infection (2). Calculations of the portion of the HID_{50} by aerosol that deposited in the nasopharynx (10 $TCID_{50}$) indicates that the HID_{50} by aerosol could be accounted for by virus which deposited in the nasopharynx. Infection was probably additionally produced in the tracheobronchial tree, however, since tracheobronchitis was seen in some individuals infected by aerosol (2). Nevertheless, this virus appears to possess a strong affinity for the nasopharynx, and a greater affinity than does adeno 4 since it is about 10-fold more infectious for this site. In contrast, adeno 4 appears to exhibit its greatest affinity for the lower respiratory tract since 20-fold less virus is required to produce infection at that site than in the nasopharynx, and about 10-fold less than for A21. This is further supported by the particle count data, which suggests that three or four particulates are sufficient to induce infection in the lower respiratory tract. Febrile pharyngitis and lower respiratory tract illness characterize the natural illness and it is best reproduced by small particle aerosol inoculation (4). Quantities of virus recovered in sputum would be sufficient to secondarily infect the pharynx if the primary site of natural infection for this virus is the lower respiratory tract (1). Thus, for both these viruses, about the same quantity of virus is required to infect the more susceptible part of the respiratory tract; and this site is also a site of major disease in naturally occurring infections with other strains of the same type (2).

1. Couch, R. B., Knight, V., Douglas, R. G., Jr., Black, S. H., and Hamory, B. H., Trans. Amer. Clin. Climat. Ass. **80**, 205 (1968).

2. Couch, R. B., Cate, T. R., Douglas, R. G., Jr., Gerone, P. J., and Knight, V., Bacteriol. Rev. **30**, 517 (1966).

3. Couch, R. B., Cate, T. R., Gerone, P. J., Fleet, W. F., Lang, D. J., Griffith, W. R., and Knight, V., J. Clin. Invest. **44**, 535 (1965).

4. Couch, R. B., Cate, T. R., Fleet, W. F., Gerone, P. J., and Knight, V., Amer. Rev. Resp. Dis. **93**, 529 (1966).

5. Knight, V., "Progress in Medical Virology," Vol. 6, p. 1. Karger, Basel (1964).

6. Knight, V., Gerone, P. J., Griffith, W. R., Couch, R. B., Cate, T. R., Johnson, K. M., Lang, D. J., Evans, H. E., Spickard, A., and Kasel, J. A., Amer. Rev. Resp. Dis. **88**, 135 (1963).

7. Griffith, W. R., Amer. Rev. Resp. Dis. **89**, 240 (1964).

8. Knight, V., Couch, R. B., and Landahl, H. D., J. Am. Med. Ass. **214**, 513 (1970).

9. Smith, K. O., and Benyesh-Melnick, M., Proc. Soc. Exp. Biol. Med. **107**, 409 (1961).

10. Pereira, H. G., and Valentine, R. C., J. Gen. Microbiol. **19**, 178 (1958).

Received Oct. 27, 1971. P.S.E.B.M., 1972, Vol. 139.