Inactivation of Encephalomyocarditis Virus in Aerosols: Fate of Virus Protein and Ribonucleic Acid

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After aerosolization at relative humidities of 50% or lower, encephalomyocarditis virus is rapidly inactivated. In this process the protein coat of the virion is damaged. This appears as a loss of hemagglutination activity and loss of affinity for hemagglutination inhibiting antibodies. The ribonucleic acid of the virus retains its infectivity but it becomes susceptible to ribonuclease. It sediments in sucrose gradients when centrifuged at high speed with the same velocity as free infectious ribonucleic acid extracted with phenol from intact encephalomyocarditis virus.

Inactivation of picornaviruses by physical factors often involves extrusion of the ribonucleic acid (RNA) from the virus particle, either as naked RNA or associated with some virus protein. This has been reported for poliovirus and rhinovirus heated at 50 to 60 °C (5, 12, 18) and for poliovirus subjected to ultraviolet irradiation (13). The infectivity of the RNA is generally more heat-stable than that of the whole poliovirion (14). The conformational changes in the poliovirus coat at heating produce changes in antigenic specificity (15).

When aerosolized in dry air, picornaviruses are often inactivated at a rapid rate (1, 8, 10). Little is known of the nature of the inactivation process. Infectious RNA (iRNA) extracted with phenol from mengovirus was found to be stable in aerosols, even at a relative humidity (RH) where the infectivity of the virus dropped 3 to 5 logs. When the RNA was extracted from aerosol-inactivated bacteriophages φX174 or MS2 it was found to have retained its original infectivity (8). The same was found with poliovirus (J. C. de Jong, M. Harmsen, and T. Trouwborst, manuscript in preparation). With phage T1 CsCl-gradient-analysis of aerosol-inactivated 32P-labeled virus showed separation of the deoxyribonucleic acid (DNA) from the protein coat (20).

This investigation deals with the inactivation of encephalomyocarditis (EMC) virus (a picornavirus) in aerosols. Attention was focused on four biological activities of the virion: infectivity of the intact virus, infectivity of the RNA, hemagglutination (HA) activity, and antibody-blocking activity.

MATERIALS AND METHODS

Virus. The EMC virus was obtained from W. J. C. Bogaerts (3). The virus was grown in L cells, suspended in Eagle minimal essential medium, without serum or bicarbonate, and buffered with 0.01 M HEPES (N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid). After 20 h at 37 °C the infected cell suspension was freeze-thawed and clarified by low-speed centrifugation. The virus was titrated by conventional plaque techniques on L-cell monolayers. For aerosol experiments virus was concentrated at 105,000 × g in a Spinco ultracentrifuge (Beckman, Palo Alto) rotor 30 during 2 h. The pellet was taken up in 0.005 of the original volume of Hanks balanced salt solution without bicarbonate or phenol red, pH 7.2. The titer of the suspension was about 107 plaque-forming units (PFU) per ml.

Assay of iRNA. iRNA was extracted by the phenol-deoxycholate method (19) with slight modifications. Virus suspensions in phosphate-buffered saline (PBS in grams per liter: NaCl, 8.0; KCl, 0.2; KH₂PO₄, 0.2; and Na₂HPO₄, 1.15, pH 7.2), with ethylenediaminetetraacetic acid (EDTA) (0.1 g/liter) and sodium deoxycholate (2 g/liter) were shaken vigorously by hand for 4 min with an equal volume of phenol saturated with PBS plus EDTA. The extraction was then repeated twice, shaking for 1 min with fresh phenol. Finally, the phenol was removed from the water phase by shaking four times for 20 s with a double volume of ethyl ether. The ether was evaporated by bubbling nitrogen gas through the solution for 5 min. Then 100 µg of diethylaminoethyl (DEAE)-dextran (Pharmacia, Uppsala; molecular weight 2 × 10⁶) per ml was added to the extract. A 2-ml portion of this solution was mixed with 20 × 10⁶ L cells and was incubated for 2 min at 37 °C. Dilutions of this cell suspension in PBS plus EDTA (0.1 g/liter) were added to L-cell monolayers without removal of the growth medium. After 1 h the growth medium was
replaced by nutrient agar. The plates were then incubated and plaques were counted as for virus. The infectivity of the extracted RNA was about 0.3% of the infectivity of the virus suspension.

Ribonuclease treatment. In some experiments impinger fluids were incubated with ribonuclease (Boehringer, Mannheim), 30 μg/ml, for 10 min at 37 C. This treatment did not influence the infectivity of intact virus for L cells.

Assay of HA activity. HA activity was measured by using sheep erythrocytes according to Martin et al. (16). Virus dilutions in PBS with CaCl₂ (0.1 g/liter), MgCl₂ (0.1 g/liter), glucose (36 g/liter), gelatine (2 g/liter) (– hemagglutination medium = HAM), and erythrocytes (0.01%) were incubated in plastic HA trays (Linbro, Gateway International, Los Angeles) for 18 h at 4 C.

Aerosol equipment. A 1-ml amount of virus suspension was aerosolized in 6 s with an FK8 direct type spray gun, operated by nitrogen at a pressure of 5 atm. The aerosols were produced and stored in a double-walled tank of stainless steel with a volume of 2,000 liters of air, which was sterilized beforehand by ultraviolet radiation. The air was homogenized by a fan and was kept at 20.0 ± 0.1 by water circulating through the double wall of the tank. Temperature and RH were continuously monitored by a liquid expansion thermometer and a LiCl dew cell, respectively. RH was lowered by pumping air out of the tank and refilling with dry air, and was raised by spraying distilled water into the tank. Aerosol samples were taken by using the lower stage of May’s multistage liquid impinger (17) filled with 10 ml of PBS with EDTA (0.1 g/liter). In 5 min 275 liters of air passed through the impinger. The sampled air was automatically replaced in the tank by sterile air of the same RH and temperature.

Resulting infectivity titers were adjusted to compensate for physical losses during spraying, storage (physical fall-out), and sampling (dilution of the aerosol, sampling efficiency). The total physical loss was measured in separate experiments by using ³²PPO₄ as a tracer and was expressed as Δ log titer: 0.23 (first sample 0 to 5 min), 0.50 (second sample 15 to 20 min), 0.66 (third sample 30 to 35 min) and 0.80 (fourth sample 60 to 65 min). Calculated titers were based on the number of PFU sprayed, the volume of the tank, the size of the aerosol sample, and the volume of the impinger fluid.

Sucrose gradients. Linear sucrose gradients of 5 to 20% were formed in 5-ml cellulose nitrate tubes (Beckman, Palo Alto) by mixing solutions of 20 and 5% of ribonuclease-free sucrose (Serva, Heidelberg). With 0.3 ml of impinger fluid layered on top, the gradient was centrifuged in an SW39L rotor of a Spinco ultracentrifuge at 15 C. Fractions of 0.3 ml were obtained through the bottom of the tubes and assayed for infectivity. A similar gradient, loaded with an artificial mixture of virus and phenol-extracted rRNA, was run together with the impinger fluid gradient as a reference. Sedimentation constants were calculated by adopting Burness’ S values for EMC virus (163.9 ± 4.5) and its rRNA (37.0 ± 0.5) (6).

For RNA-like sedimenting material (rRNA') formula (1) was used; for virus, formula (2) was used.

\[ S_{RNA} = \frac{d \text{ of rRNA'}}{\text{mean } d \text{ of rRNA}} \times 37.0 \]  
\[ S_{virus} = \frac{d \text{ of virus'}}{\text{mean } d \text{ of virus}} \times 163.9 \]

where \( d \) = distance of peak of infectivity from meniscus.

The original virus suspension was analyzed in the same way but 0.05 ml was applied instead of 0.3 ml.

RESULTS

Inactivation of EMC virus in aerosols at different relative humidities and temperatures. The stability of the infectivity of EMC virus in aerosols is strongly dependent on the RH (Fig. 1), being very low below 45% RH and high above 60% RH. Most of the inactivation of low RH was already observed in the first sample.

Effect of prehumidification on the recovery of EMC virus infectivity from aerosols at low RH. The inactivation at low RH, as shown in Fig. 1, could have been the consequence of the rapid rehydration during collection rather than of the aerosolization. Evidence for such a mechanism was found with some viruses, where humidification of the aerosol prior to sampling raised the virus recovery (2, 9; see Discussion).

EMC virus was sprayed at 35% RH and 5-min
samples were taken starting at 0, 15, 30, and 60 min. Water was nebulized into the tank 65 min after spraying, raising the RH to 78%. Five to 10 min later the final sample was collected. Its titer was in line with the titers of the preceding samples (Fig. 2). Apparently, prehumidification has no effect on the recovery of infectivity.

**HA activity of aerosol-inactivated EMC virus.** Inactivation could affect the protein coat, the RNA, or both. The protein coat is responsible for the HA activity of the EMC virus. According to Martin et al. no hemagglutinating particles other than the infective particles are present in EMC virus preparations (16). During our purification procedures (ultracentrifugation in growth medium, in CsCl or in sucrose gradients; ECTEOLA-cellulose chromatography) HA activity was indeed always found together with infectivity and at a constant ratio.

To investigate the fate of the virus protein in aerosols, the HA titers of impinger fluids were determined. Figures 3 and 4 show that for aerosols at 35 and 50% RH the HA property was inactivated in parallel with the infectivity. Experiments at 15% RH yielded the same results. This points to the protein coat as a target of inactivation.

**Antibody blocking by aerosol-inactivated EMC virus.** The HA property of the virion could be lost by disintegration into intact sub-
units which retained only one combining site (univalent) or were too small to link two erythrocytes. This situation was found with influenza virus: the proteins that were detached from the virion by bromelain did not hemagglutinate but blocked HA-inhibiting antibodies (4). Aerosol-inactivated EMC virus was tested for such a blocking activity.

An impinger fluid was obtained from a 0- to 5-min-old aerosol at 35% RH. It contained 1.2 × 10^4 PFU of virus and 3 × 10^4 PFU-equivalents of aerosol-inactivated virus per 0.3 ml. This fluid, supplied with additives to conform to HAM was used as a diluent for antiserum. The serum dilutions were in the range where one HA unit (= 6 × 10^4 PFU) was inhibited when pure HAM was used as a diluent (Table 1A). The dilutions were incubated for 30 min at 37°C to allow virus proteins to bind to antibodies. Different amounts of active EMC virus, in the range of one HA unit, were then added. After a second incubation for 30 min at 37°C to allow remaining free antibodies to react with the active virus, sheep erythrocytes were added and HA was read after 16 h at 4°C.

One HA unit (= 6 × 10^4 PFU) was inhibited by the antiserum dilution of 1:6.4 × 10^4, irrespective of whether the antiserum was diluted in impinger fluid or in pure HAM (Table 1B, line 2 and Table 1A, line 2, respectively). The slight increase in inhibition after dilution in impinger fluid is within the experimental error. It can be concluded that an amount of aerosol-inactivated virus, originating from 50 HA units, did not block an antibody concentra-

**TABLE 1. Blocking of HA-inhibiting antibodies against EMC virus by aerosol-inactivated EMC virus**

| Dilution of antiserum | EMC virus PFU/0.3 ml | 5 × 10^4 | 2.5 × 10^4 | 1.2 × 10^4 | 6 × 10^4 | 3 × 10^4 |
|-----------------------|---------------------|---------|------------|------------|---------|---------|
| A. Control            |                     | +       | +          | +          | +       | +       |
| 1:3.2 × 10^4          |                     | +       | -          | +          | +       | +       |
| 1:6.4 × 10^4          |                     | +       | -          | -          | +       | +       |
| 1:1.28 × 10^4         |                     | +       | -          | -          | +       | +       |
| No antiserum          |                     | +       | -          | -          | +       | +       |
| B. With inactivated EMC |                    |         |            |            |         |         |
| 1:3.2 × 10^4          |                     | +       | +          | +          | +       | +       |
| 1:6.4 × 10^4          |                     | +       | -          | -          | -       | -       |
| 1:1.28 × 10^4         |                     | +       | -          | -          | -       | -       |
| No antiserum          |                     | +       | -          | -          | +       | +       |

* EMC antiserum diluted in HAM.
+*, HA block; −, no HA.
1 EMC antiserum diluted in impinger fluid with CaCl₂, MgCl₂, glucose, and gelatine as in HAM. The impinger fluid contained 1.2 × 10^4 PFU virus and 3 × 10^4 PFU-equivalents of aerosol-inactivated virus per 0.3 ml.

**A. Control**

**B. With inactivated EMC**

**Infectivity of the RNA of aerosol-inactivated EMC virus.** The RNA in impinger fluids, obtained from aerosols in which extensive virus inactivation had occurred, was extracted with phenol and plated for infectivity. At 35% RH (Fig. 3) a large loss of complete virus was observed in the first few minutes, followed by slow inactivation during further storage of the aerosol. In contrast, the virus RNA apparently retained its full complement of infectivity. Experiments at 15% RH yielded the same results. At 50% RH (Fig. 4) there was a smaller initial loss of virus and a larger decay in the 15 min thereafter. Despite this difference in the kinetics of the inactivation of the whole virus the virus RNA was again stable. Apparently, most of the virus in the aerosol had lost its infectivity, but still contained iRNA.

**Infectivity of aerosol-inactivated EMC virus after addition of DEAE-dextran.** To investigate whether the surviving RNA was present in the impinger fluid as free RNA or still linked to protein it had to be studied without phenol extraction. It was determined, therefore, whether its infectivity was enhanced by adding DEAE-dextran to the impinger fluid before titration.

As shown in Table 2A the titer of intact virus (in log PFU), in the presence of DEAE-dextran, was 1.23 lower than without the polymer (line 1). In contrast, the titer of the impinger fluid was raised from 4.21 to 5.44 (line 5). The latter value equals, within the experimental error, the titer of 4.97 calculated for free iRNA.

**Infectivity of aerosol-inactivated EMC virus after treatment with ribonuclease.** To determine whether the surviving RNA or even the surviving virus was sensitive to RNase, impinger fluids were treated with 30 μg of RNase per ml and assayed as before (Table 2B).

The titer of the impinger fluid, when assayed without DEAE-dextran, was not affected by RNase treatment (compare lines 6 and 7, first column). The surviving virus, therefore, was not sensitive to the enzyme. On the other hand, when titrated after addition of the polymer, the titer dropped from 5.44 to 3.13 logs (compare line 6 and 7, second column). The latter titer can be completely accounted for by the surviving intact virus in the impinger fluid (expected: 4.29 - 1.23 = 3.06 logs). Apparently, the infectivity of the surviving RNA in the impinger fluid was fully sensitive to RNase.
TABLE 2. Effect of DEAE-dextran and RNase on the infectivity of aerosolized EMC virus

| Virus suspension                  | Titer in log PFU/ml | Without DEAE-dextran | With DEAE-dextran |
|-----------------------------------|---------------------|----------------------|-------------------|
| A. Original virus suspension      |                     |                      |                   |
| After phenol extraction           | 10.00 (±0.13)       | 8.77 (±0.12)         |                   |
| Calculated for impinger fluid     | 3.74 (±0.69)        | 7.43 (±0.47)         |                   |
| After phenol extraction           | 7.54 (±0.13)        | 4.97 (±0.47)         |                   |
| Impinger fluid                    | 4.21 (±0.16)        | 5.44 (±0.21)         |                   |
| After phenol extraction           | ND                  | 5.14 (±0.18)         |                   |
| B. Original virus suspension      |                     |                      |                   |
| After phenol extraction           | 10.00 (±0.13)       | 8.77 (±0.12)         |                   |
| After RNase treatment             | 3.74 (±0.60)        | 7.43 (±0.47)         |                   |
| After phenol extraction followed by RNase treatment | ND | 5.14 (±0.18) | |
| Impinger fluid                    | 4.21 (±0.16)        | 5.44 (±0.21)         |                   |
| After RNase treatment             | 4.29 (±0.17)        | 3.13 (±0.18)         |                   |

* Standard virus titration on monolayer.
* After addition of 100 μg of DEAE-dextran per ml the fluid was tested for plaque-forming activity with the cell suspension method for phenol extracts.
* Titers calculated, assuming no biological decay, from the number (n) of PFU sprayed (virus, log n = 10.00; iRNA, log n = 7.43), the fraction of aerosol sampled (Δ log n = 0.89) and the volume of impinger fluid (10 ml) and corrected for physical losses (Δ log n = 0.80). EMC virus was sprayed at 35% RH and sampled 60 min afterwards. Titers are the means of eight experiments.
* ND, Not determined.

Sedimentation of infectivity of aerosolized EMC virus in sucrose gradients. To further clarify the physical state of surviving RNA and surviving complete virus the sedimentation velocities of both infectivities through sucrose gradients were measured. Sedimentation through sucrose was preferred to banding in CsCl, since the high osmotic values of the latter could dissociate slightly damaged virus particles into protein and RNA.

Sucrose gradients were loaded with impinger fluid and with artificial mixtures of virus and iRNA. Fractions were titrated for virus and surviving RNA or iRNA without or with DEAE-dextran. It follows from Table 3 and Fig. 5 that surviving RNA sedimented with a velocity not differing significantly from that of phenol-extracted RNA in the reference gradient (P > 0.05). This indicates that no significant amounts of protein had remained attached to the virus RNA. Also, the surviving complete virus sedimented like unaerosolized virus, suggesting that no major physical changes such as disintegration or clumping had occurred in this fraction. The nature of this resistant virus is the subject of another study.

To ensure that the peak observed in Fig. 5 at the place of iRNA was a consequence of aerosolization, the original virus suspension was aerosolized also in this way. As Fig. 6 shows, no infectivity was found in the iRNA region when samples were titrated with the technique suitable for iRNA infectivity. Moreover, precautions of the virus with RNase did not change the distribution of infectivity over the gradient fractions (Fig. 6). It was concluded that the original virus suspension did not contain free iRNA.

Stability of phenol-extracted iRNA of EMC virus in aerosols. To investigate whether free iRNA is really stable in aerosols phenol-extracted iRNA was sprayed and sampled as described for whole virus. The results are plotted in Fig. 3 and 4 and show that the iRNA is stable in aerosols at 35 and 50% RH. Experiments at 15 and 80% RH yielded the same result. This stability is in agreement with the work of Akers and Hatch (1).

TABLE 3. Sedimentation of the infectivity of aerosolized EMC virus through a sucrose gradient

| Gradient | S of RNA in± | S of virus in± |
|----------|-------------|---------------|
| Impd     | Ref         | Impd          | Ref          |
| 40.3     | 32.8        | 160           | 155          |
| 35.9     | 38.3        | 155           | 171          |
| 40.5     | 42.9        | 165           | 160          |
| 39.1     | 33.8        | 168           | 168          |
| 38.4     | 35.5        |               |              |
| 35.4     | 39.2        |               |              |
| 37.5     | 37.0        |               |              |
| 37.4     | 36.7        |               |              |
| Mean     | 38.1        | 37.0          | 162.0        | 163.5
| SD       | 0.7         | 1.1           | 2.9          | 3.7 |

* Mean total recoveries from the gradient fractions were for whole virus 25% (impinger fluid) and 20% (reference gradient), for surviving RNA 60% (impinger fluid), and for iRNA 40% (reference gradient) of the input. Sedimentation rates are expressed as sedimentation constants S.
* Gradients run for 3 h at 51,000 × g, 15 C.
* Gradients run for 3 h at 40,000 × g, 15 C.
* Impinger fluid obtained from a 0- to 5-min-old aerosol at 35% RH.
* Reference gradient with a mixture of virus and phenol-extracted iRNA.
* SD, Standard deviation of the mean.
myelitis virus higher recoveries were recorded with prehumidification (2). In the present study it was demonstrated, however, that prehumidification did not change the recovery of EMC virus from aerosols at low RH (Fig. 2). Apparently, the rate of rehydration did not influence recovery. However, the possibility that the inactivation does occur at rehydration, rather than in stages 2 and 3, still cannot be excluded.

This investigation shows that in aerosols the HA activity of EMC virus decreased in parallel with the virus infectivity, whereas the infectivity of the virus RNA was unaffected (Fig. 3 and 4). Moreover, the antigenic structure of the hemagglutinin seemed to be destroyed (Table

**DISCUSSION**

When aerosolized at low RH the infectivity of EMC virus is decreased by 2 to 3 logs. In principle, this inactivation could occur at any step of the experiment: (i) fragmentation of the liquid into droplets, (ii) evaporation of the droplets to equilibrium with the ambient air, (iii) storage of the dried particles in the aerosol, (iv) rehydration when collected, (v) mechanical forces during collection, i.e., penetration in the impinger fluid, shearing, and surface forces in the operated impinger.

The high recoveries found in experiments at high RH demonstrate that mechanical injury by aerosolization (stage 1) or at sampling (stage 5) are not responsible for the inactivation process.

Hatch and Warren reported that recovery of infectivity of bacteriophage T3 from aerosols at low RH could be raised by humidification of the aerosol prior to sampling. The recovery increased by a factor $10^2$ to the recovery found at high RH (9). This pointed to the possibility that the inactivation observed at low RH without prehumidification occurred at rehydration rather than in the aerosol. Also with polio-

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**Fig. 5.** Sucrose sedimentation analysis of aerosolized EMC virus. The gradients of 5 to 20% sucrose were centrifuged for 3 h at 51,000 × g, 15 C. One gradient (AIR) was loaded with impinger fluid from a 0- to 5-min-old aerosol at 35% RH and the fractions were assayed for virus (○) and surviving RNA (△). Another gradient (REF) contained a mixture of virus (●) and phenol-extracted iRNA (▲).

**Fig. 6.** Sucrose sedimentation analysis of the original EMC virus preparation. One gradient was loaded with 0.05 ml of virus and run for 2.5 h at 51,000 × g, 15 C. Samples were titrated without DEAE-dextran on monolayers (MONO, ○), with infection in the presence of DEAE-dextran in suspension (SUSP, ●). Also, the samples were treated with ribonuclease, then DEAE-dextran was added and the sample was titrated with infection in suspension (Susp after RNase; ▲). Another gradient received 0.05 ml of phenol-extracted iRNA and was centrifuged for 2.5 h at 51,000 × g, 15 C. Samples were titrated with infection in suspension in the presence of DEAE-dextran (Extr. iRNA, △). The experiment was done three times with similar results and the figure represents the results of one experiment.
1. The infectivity of the surviving virus RNA was dependent on the presence of DEAE-dextran, was sensitive to RNAse and sedimented in sucrose gradients as free iRNA (Tables 2 and 3 and Fig. 5). No infectivity sedimenting like free iRNA was detectable in the original virus suspension (Fig. 6). From these data we conclude that, during virus inactivation in aerosols, the virus RNA is released in a free, infectious form. The factors that are responsible for the virus inactivation will be dealt with in the second paper of this series.

Apparently, inactivation of EMC virus in aerosols is similar in several aspects to that of other viruses described in the Introduction. The process also has some features in common with thermal inactivation of poliovirus between 50 and 60 C. After heating poliovirus for 10 min at 50 C Breindl (5) observed, besides 35S RNA, an intermediate particle. This particle, like iRNA, was sensitive to RNAse and only infectious in the presence of DEAE-dextran. It sedimented at 80S, however. No such particles were found in our sucrose gradients (Fig. 5). Whether they arise during thermal degradation of EMC virus is currently being investigated.

After thermal inactivation of poliovirus the antigenicity changes from N to H (15). Similarly, after inactivation of EMC virus in aerosols the virus protein has lost the affinity for HA-inhibiting antibodies (Table 1). This suggests that aerosol-inactivated EMC virus cannot elicit such antibodies when administered to an animal. This phenomenon is relevant to techniques of immunization per aerosol against EMC virus or related viruses. Such a conclusion is premature, however, as Hinuma et al. showed that H-reactive poliovirus particles, although not reacting with neutralizing antibodies in vitro, could give rise to neutralizing antibodies after injection into rabbits (11).

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