Effect of $\beta$-Propiolactone on Sendai Virus

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The biological properties (infectivity, hemagglutination, hemolysis, cell fusion, neuraminidase) of Sendai virus were dissociated on the basis of sensitivity to $\beta$-propiolactone, by freeze-thawing, by heating at different temperatures, and by adsorption-elution with formalinized chicken erythrocytes. Possible mechanisms whereby $\beta$-propiolactone selectively destroys viral infectivity are discussed.

$\beta$-Propiolactone (BPL) is an alkylating agent that renders Sendai virus noninfective while leaving unaffected the capacity of the virus to fuse cells rapidly in culture (12, 14). Although fusion by inactive Sendai virus has had increasing application in studies involving the use of polykaryons and heterokaryons, the actual mechanism of syncytium formation under these conditions of high input is still unknown. Moreover, the effects of BPL on other properties of Sendai virus [neuraminidase, hemolysis, hemagglutination (HA)] have not been examined quantitatively. This report describes the effect of BPL on these several properties of the virus in relation to infectivity and in comparison with the effects of heat inactivation, freeze-thawing, and adsorption-elution with erythrocytes. Our results provide information on the nature of the interaction between BPL and Sendai virus and on the mechanism of virus-induced syncytium formation.

MATERIALS AND METHODS

Seed Sendai virus diluted $10^{-2}$ was inoculated into the chorioallantoic cavity of 11-day-old chicken embryos. Infective chorioallantoic fluid (CAF) was harvested after 48 hr at 36 C and clarified by low-speed centrifugation. Virus was pelleted by centrifugation for 2 hr at 32,000 $\times$ g, and the pellet was resuspended in 0.5% bovine serum albumin in phosphate-buffered saline (PBS), pH 7.4. A 40-fold concentration of hemagglutinating units was thereby achieved.

Hemolysis and HA were measured as described previously (8). Hemolytic activity was assayed with group O human erythrocytes which had been washed three times in PBS and resuspended to a concentration of 0.5%. Dilutions of virus in 0.2 ml of PBS were mixed with 1 ml of erythrocyte suspension at 4 C for 10 min. The tubes were then incubated at 37 C for 45 min. After being chilled by the addition of 3 ml of cold PBS and centrifuged at 4 C for 10 min at 300 $\times$ g, hemoglobin in the supernatant fluids was measured spectrophotometrically at 540 nm. The highest dilution giving 100% hemolysis was taken as the end point. HA and HA-inhibition assays were performed with chicken erythrocytes by the pattern method as previously described (7). Neuraminidase activity was estimated by incubating virus with excess Colloidal mucoid (7) in 0.1 M acetate buffer (pH 5.5) at 37 C. At various times, periodate was added to stop the reaction, and free sialic acid was assayed by the method of Warren (17).

**BPL treatment.** BPL was tested at various concentrations by the method of Neff and Enders (12). Briefly, a freshly prepared 10% solution of BPL in water was diluted in a saline bicarbonate solution to 10 times the desired final BPL concentration. This 10 $\times$ BPL solution was again diluted 1:10 in a concentrated virus suspension (8,000 HA units/ml), and the mixture was shaken at 4 C for 10 min and then incubated for 2 hr at 37 C, during which time the mixture was shaken every 10 min. Treated virus was used the next day after overnight refrigeration at 4 C to insure complete hydrolysis of BPL.

**Fusion assays.** Virus was diluted in Earle's balanced salt solution containing 0.1% calf serum. A 0.2-ml amount of virus dilution was used to fuse human amnion (FL) cells grown in confluent monolayers on cover slips in Leighton tubes. After 2 hr, cells were fixed in methanol and stained with Lillie's azure-eosin. For quantitative assays, cover slips were placed in 5 x $10^{-4}$ M ethylenediaminetetraacetic acid in PBS for 2 min before fixation and staining to cause the retraction of mononucleated cells from syncytia and thereby to facilitate cell and nuclear counts (11). Fusion indexes were expressed as the number of nuclei counted/number of cells in which they were found. At least 500 nuclei and four different fields were examined on each stained cover slip.

**Infectivity.** Infectivity was assayed by inoculating decimal dilutions of virus into 11-day-old embryos and harvesting CAF after 48 hr at 36 C and assaying for HA activity. Alternatively, virus was inoculated onto replicate tube monolayers of chicken embryo fibroblasts, allowed to adsorb for 2 hr, and incubated under maintenance medium for 3 days at 37 C. End points were determined by hemadsorption by using a 0.5% suspension of guinea pig erythrocytes in PBS.
Adsorption-elution with formalinized erythrocytes.
Sendai virus, pretreated with 0.01% BPL, was adsorbed to and eluted from well-washed formalinized chicken red blood cells by a modification of the method of Kisselov et al. (10). Sendai virus (1,024 hemagglutinating units) was allowed to adsorb, at 4°C with stirring for 1 hr, to a 2% suspension of formalinized erythrocytes in a total volume of 20 ml of PBS. The cells were pelleted and washed twice in PBS at 4°C before elution of virus was allowed to take place at 37°C for 1 hr into a volume of 3 ml of PBS. After pelleting the erythrocytes, the virus-containing supernatant fluid was used for HA, hemolysis, neuraminidase, and fusion assays.

RESULTS
The hemagglutinin of Sendai virus was not affected at any of the BPL concentrations tested (0.0005 to 0.13%). However, Sendai infectivity was completely abolished at drug concentrations of 0.005% and higher. Fusion capacity was unaffected by 0.01% BPL but was inactivated by 0.05%. Hemolytic and neuraminidase activities, in turn, were more resistant, with only partial inactivation occurring at 0.05 and 0.10% BPL. Thus, the rates at which the various biological activities were abolished or substantially reduced by BPL can be ordered in a descending sequence corresponding, respectively, to infectivity, fusion, neuraminidase, and hemolysis (Table 1). Heating noninfective virus at 43°C destroyed its fusing properties but did not affect hemolytic activity. The neuraminidase and hemolytic activities of BPL-inactivated Sendai virus, although still present after heating at 43°C, were largely inactivated at 46°C, a temperature at which hemagglutination titers were reduced by 75%. Freezing and thawing virus up to 10 times greatly increased its hemolytic capacity without affecting fusion titer, neuraminidase, or hemagglutinin. After virus was adsorbed to and eluted from formalinized red blood cells, it failed to fuse cells in monolayer but retained hemolytic, hemagglutinating, and neuraminidase activities equal in potency to the corresponding controls (Table 2).

Treating Sendai virus with 0.01% BPL had no effect on the reactivity of the viral envelope with antibody. Antiserum to whole, pelleted virus gave the same titers of HA inhibition with treated and untreated virus. The high fusion titer of BPL-treated virus was completely neutralized by antiviral antibody after 30 min at room temperature.

### Table 1. Effect of BPL on Sendai virus

| BPL (%) | Hemo-lytic activity | Neura-minidase | HA | CEF | CAF | Fusion titer |
|---------|---------------------|---------------|----|-----|-----|-------------|
| 0.13    | 10                  | 17            | 100| <10⁻¹| <10⁻¹| No fusion   |
| 0.10    | 20                  | 29            | 100| <10⁻¹| <10⁻¹| No fusion   |
| 0.05    | 68                  | 62            | 100| <10⁻¹| <10⁻¹| No fusion   |
| 0.01    | 92                  | 78            | 100| <10⁻¹| <10⁻¹| 200         |
| 0.005   | ND                  | ND            | 100| >10⁻⁴| ND    | 200         |
| 0.0005  | ND                  | ND            | 100| >10⁻⁴| ND    | 200         |
| None    | 100                 | 100           | 100| >10⁻⁴| >10⁻⁴| 200         |

* Abbreviations: BPL, β-propiolactone; HA, hemagglutination; CEF, chick embryo fibroblasts; CAF, chorioallantoic fluid; ND, not done.

| Treatment | Residual<sup>a</sup> | Infection | Fusion titer<sup>c</sup> |
|-----------|-----------------------|-----------|--------------------------|
| Heated 15 min at | | | |
| 37°C | 100 | 100 | 100 | 160 |
| 40°C | 92 | 100 | 100 | 20 |
| 43°C | 81 | 100 | 100 | No fusion |
| 46°C | 12 | 0 | 25 | No fusion |
| 51°C | 11 | 0 | 0.2 | No fusion |
| Freeze-thaw, three times | 145 | 100 | 100 | 160 |
| Freeze-thaw, 10 times | 171 | 100 | 100 | 160 |
| Adsorption-elution with formalinized red blood cells | 100 | 100 | 100 | No fusion |

* A total of 81,920 hemagglutination (HA) units inactivated with 0.01% (β-propiolactone).
* Per cent activity retained by virus heated to 37°C for 15 min.
* Reciprocal of highest dilution of virus giving human amnion cell fusion index > 2.

DISCUSSION
Our data support the suggestion offered by Okada (13) that something more than a virus-specific lytic mechanism underlies both the cell-fusing and hemolytic properties of Sendai virus, since they can be dissociated in several ways. Treatment with 0.05% BPL or heating to 43°C abolished the ability of the virus to form syncytia without appreciably affecting hemolytic activity. Similarly, virus frozen and thawed 10 times in-
increased by 71% in hemolytic activity without detectable change in fusion capacity (Table 2). Virus which had been adsorbed to and eluted from formalinized chicken red blood cells was devoid of fusion activity but retained full hemolytic and neuraminidase activities. The latter finding suggested that, during the process of adsorption and elution, some viral envelope component(s) necessary for syncytium formation but not for hemolysis may have remained attached to the erythrocyte surface. Similar dissociation of Newcastle disease virus envelope functions was demonstrated by Kohn (11), who observed that Newcastle disease virus heated to various temperatures for 15 min lost hemolytic activity before losing fusion capacity and that, contrariwise, virus treated with trypsin retained its ability to fuse cultured cells but could no longer lyse red blood cells. With neither Sendai nor Newcastle disease viruses (1) can the process of syncytium formation be reduced to a simple one-component schema.

The titer of Sendai virus hemagglutinin was not reduced at any of the BPL concentrations tested (0.0005 to 0.13%), nor was there any effect on the capacity of the viral envelope to react with antibody as determined both by HA and fusion inhibition assays. In contrast, BPL was found to lower the infectivity, HA titer, and antigenicity of influenza virus, pari passu (6). These differential effects of BPL on influenza and Sendai viruses may reflect the major divergence in size and conformation of the respective virions as well as other biological properties which distinguish the myxoviruses from the paramyxoviruses.

BPL at low concentrations (0.01%) destroys Sendai virus infectivity while leaving envelope viral properties intact. It has therefore been inferred that the lactone binds preferentially to the viral genome. Accordingly, Roberts and Warwick (15) have shown that BPL reacts with ribonucleic acid (RNA) to yield 7-(2-carboxyethyl) guanine after RNA hydrolysis in 1 N HCl. Extending this observation, Colburn et al. (3, 4) have observed that, if the reaction between BPL and guanine is allowed to continue after formation of 7-(2-carboxyethyl) guanine, alkylation at the N-9 position also occurs to yield 7,9-di-(2-carboxyethyl)guanine.

These considerations leave unexplained just how an alkylating agent can penetrate to the ribonucleoprotein core without binding to and in some way affecting the envelope proteins of the virion. We have shown that Sendai fusing, hemolytic, and neuraminidase activities are affected by concentrations of BPL higher than the minimum necessary to inactivate infectivity. Alkylation must therefore also occur at certain loci on the viral envelope. Dickens and Jones (5) have shown that BPL will react with cysteine to produce S-(2-carboxyethyl) cysteine, and Colburn and Boutwell (3) have indicated that BPL binds well to mouse skin protein and deoxyribonucleic acid as well as to RNA. It would therefore appear that, when Sendai virus is exposed to BPL, the latter may be bound first to N-7 sites in guanine residues of the viral RNA, possibly because more guanine than cysteine sites are available for reaction. Increasing the BPL concentration may lead to alkylation of viral protein, possibly due to S-(2-carboxyethyl) cysteine formation, and reduction in fusing, hemolytic, and neuraminidase activities. Because fusion capacity is most sensitive to BPL after infectivity, this may mean that the viral subunit or subunits necessary for fusion contain more BPL-reactive sites than do either the hemolysin subunit(s) or the viral neuraminidase. Alternatively, relatively little alkylation may suffice to eliminate activity in the first instance but not in the latter two.

RNA-dependent RNA polymerase activity has been found in Newcastle disease virions by Huang et al. (9), in Sendai virions by Robinson (16), and in influenza virions by Chow and Simpson (2). Inactivation of polymerase by BPL would offer an alternative explanation for the killing action of the drug on these viruses. Moreover, inactivation of polymerase in the viral envelope or nucleocapsid might occur at concentrations of BPL lower than those affecting other envelope functions. This possibility is under examination.

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