Lack of Interaction Between Sendai Virus and Bacterial Cells (36469)

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The basic approach to cell fusion induced by Sendai virus *in vitro* was first described by Okada (1) and later modified and applied to interspecific cell fusion by Harris *et al.* (2). Since its introduction, this technique has become a very useful tool for the production of heterokaryons and in the study of various aspects of cell biology. No reports have appeared in the literature concerning the possible effects of Sendai virus on bacterial cells. The fact that the cell fusion phenomenon does not require infective virus particles suggested that it might be possible to use Sendai virus as a reagent for inducing bacterial heterokaryons.

This investigation was directed toward answering two basic questions. Would the cell fusion factor (CFF) contained in Sendai virus cause detectable fusion of bacterial cells? If not, could it be determined at what step(s) the fusion process was blocked? The present report describes our efforts to assay for bacterial cell fusion by direct physical methods and by recombination analysis. Cells used in the fusion procedures included normal cells and protoplasts of *Bacillus megaterium,* normal cells and spheroplasts of *Escherichia coli,* and *Mycoplasma laidlawii.* HeLa cells were used as a control on the activity of the virus.

Materials and Methods. Sendai virus. Sendai virus carried in our lab stock was propagated in the following way. Infected allantoic fluid (AF) with a titer of 5,000 hemagglutinin units (HAU)/ml was diluted to 10^{-3} with beef heart infusion broth. A 0.1 ml of the diluted suspension was injected into the allantoic sac of 10–11-day-old embryonated chicken eggs. The eggs were incubated at 38° for 48 hr, chilled at 4° overnight, and the AF collected aseptically. The infected AF was clarified by centrifugation at 1,025*g* for 10 min at 4°. This crude AF (titer of 3,000–5,000 HAU/ml) was used directly or after a further concentration step was performed by centrifuging at 32,000*g* for 30 min. The resulting pellet was resuspended in phosphate buffered saline (PBS) and stored at 4°. Because of the reported lability of Sendai CFF to cold storage (3), all virus was prepared fresh each week and used within 2–3 days of collection. Virus was inactivated by exposure to UV light for 3 min (5.2×10^3 ergs/cm^2/sec).

Hemagglutinin titrations of Sendai virus were done employing a Microtiter Kit (Cooke Engineering Co., Alexandria, VA). Titrations were done in U-bottom plastic trays using doubling dilutions of virus in 25 μl of PBS. An aliquot of 25 μl of 0.5% chicken red cells suspended in PBS was added to each well. The highest dilution of virus giving complete hemagglutination after 2 hr at room temperature was defined as one hemagglutinin unit.

Assay of N-acetylneuraminic acid. Cells to be assayed for *N*-acetylneuraminic acid (NANA) were treated with 0.05 *N* H_2SO_4 for 1 hr at 80°. The digests were centrifuged at 1,085*g* for 10 min and the supernatant fluids removed for assay. NANA was determined by the thiobarbituric acid assay (4) and by the direct-Ehrlich assay (5). A commercial preparation of NANA (Calbiochem, Los Angeles, CA) was utilized as a standard.

Labeled virus. A sample of 100 μCi of ^{32}P orthophosphate was inoculated into the allantoic sac of 8-day-old embryonated chicken eggs. The eggs were incubated for 48 hr at 38° and then 0.1 ml of a 10^{-3} dilution of Sendai virus was inoculated into the 10-day-old eggs. After 48 hr incubation at 38°, the eggs were chilled at 4° for 18 hr and the infected AF containing labeled virus was har-

vested. The labeled virus was partially purified and concentrated two-fold by two cycles of adsorption onto an elution from chicken red blood cells (2% suspension in PBS).

Bacterial cultures. Escherichia coli and *B. megaterium* were stock cultures carried in our laboratory. *Mycoplasma laidlawii* was obtained from Dr. Harold Morowitz of Yale University. *Bacillus megaterium* was grown in a basal salts medium consisting of 1 g NaCl, 1.5 g K_2HPO_4, 0.5 g KH_2PO_4, 3 g sodium citrate, 4 g $(NH_4)_2SO_4$, and 0.7 g $MgSO_4$ per liter of distilled water. Protoplasts were prepared by adding lysozyme to a final concentration of 50 μg/ml and incubating the cells in medium containing 20% sucrose at 35° for 30 min.

Escherichia coli normal cells were cultured in Penassay broth (Difco). For the formation and reversion of *E. coli* spheroplasts, the method of Hirokawa (6) was used. A minimal medium containing 100 μg/ml of streptomycin was used for the detection of recombination between *E. coli* cultures. This medium consisted of 100 mg streptomycin, 4 g dextrose, 15 g agar, 7 g Na_2HPO_4, 0.5 g NaCl, 1 g NH_4Cl, and 3 g KH_2PO_4 per liter of distilled water.

Mycoplasma laidlawii was grown in a medium consisting of 20 g Fisher tryptose, 5 g NaCl, 3.75 g Fisher Tris, 10 g dextrose, 10 ml Difco PPLO serum fraction, and 500,000 units Penicillin G per liter of distilled water.

Results. Cells to be fused were mixed with UV inactivated virus at 4° for 30 min. During this period virus particles adsorb onto the cell surface by attachment to NANA receptors and cell agglutination occurs. After incubation with shaking at 37°, adjoining cells fuse, and at the points of fusion the membranes disappear, resulting in the formation of multinucleate cells. Using the optimal conditions for fusion described by Guggenheim *et al.* (3), we were consistently able to obtain approximately 30% fusion of HeLa cells with our Sendai virus preparations. In these experiments cells which contained three or more nuclei were scored by direct microscopy after fixation and staining. With mammalian cells, the process of cell fusion can easily be followed. Agglutination of cells, changes in membrane surface morphology, and the formation of multinucleate cells are very distinct phenomena as seen under the microscope. As might be expected, we found that with bacterial cells direct observation was of little value as a means of detecting cell fusion. The small size of the cells and their procaryotic nature were major hindrances. Nuclear staining revealed no obvious differences between Sendai-treated cells and control cells to which uninfected AF had been added. We could also find no difference in the banding of Sendai-treated cells and control cells in sucrose gradients.

Dewitt and Zell (7) have demonstrated that the receptor for the virus (NANA) is present on the surface of *E. coli*. Their studies have also shown that bacterial NANA will act as a substrate for viral neuraminidase and the *E. coli* endotoxin containing NANA will inhibit hemagglutination of myxovirus. We conducted experiments with spheroplasts of *E. coli* since these were considered more likely candidates for fusion than normal cells that possessed a restricting cell wall. Figure 1 is an outline of the procedure used to detect fusion of *E. coli* spheroplasts by recombination analysis. The two strains used in these experiments were an *E. coli* B prototroph which is streptomycin sensitive (SR5), and *E. coli* K12F$^-$, a leucine, thymine auxotroph which is streptomycin resistant (SR4). Using this procedure we were unable to detect any recombination between the spheroplasts after virus treatment and reversion to rods. Also no recombinants were found with normal cells or ethylenediaminetetraacetic acid treated normal cells. These experiments were carried out with virus concentrations ranging from 1,000–12,000 HAU/ml and cell concentrations varying between 10^7 and 10^9/ml.

We later found that by removing the cell wall in the formation of spheroplasts, we were also removing most of the virus receptor. In an attempt to produce cells with both exposed portions of membrane and intact receptor, spheroplasts containing various amounts of cell wall material were used. The

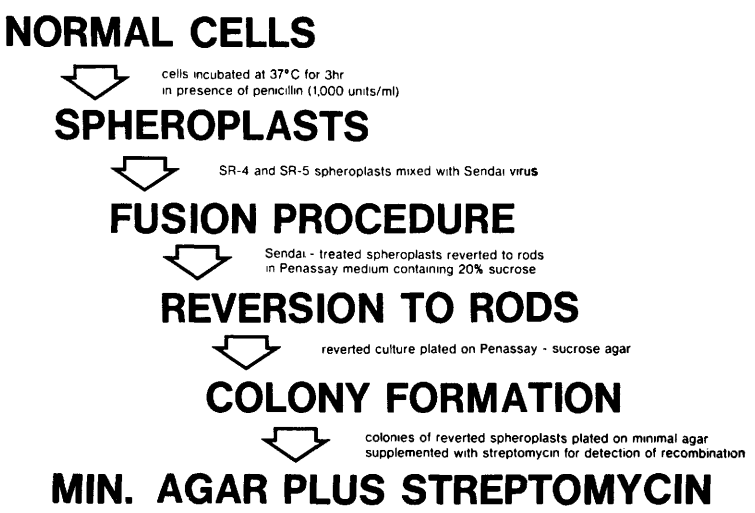

FIG. 1. Procedure used to detect fusion of *E. coli* spheroplasts by recombination analysis.

time of reversion to rods and the time of penicillin treatment were used to vary the cell wall content. Although we felt that these cells were likely candidates for fusion, we were unable to recover any recombinants indicating that either cell fusion did not occur or it occurred at a very low level which we could not detect (less than one in 10^9).

Because cell fusion could not be demonstrated by either direct or indirect means, we decided to look at the degree of adsorption of Sendai virus by bacterial cells. Table I shows the results of a hemagglutination assay to test for virus adsorption. A sample of virus was allowed to undergo three cycles of adsorption with the various cells and the hemagglutinin titer was determined after each adsorption period. None of the *E. coli* cells were able to lower the virus titer by a detectable amount while both HeLa cells and chicken erythrocytes caused a 98–100% drop in titer after three adsorptions.

To determine if there was a low level of adsorption not detectable by the hemagglutination assay, the ability of *E. coli* cells to adsorb ^{32}P-labeled virus was tested. Table II shows the comparative adsorption of labeled virus by the different cells. All the *E. coli* cells showed some adsorption but it was low as compared to HeLa cells. Normal cells showed more adsorption than the spheroplasts.

Table III shows the amount of NANA in the two *E. coli* strains as compared to HeLa

TABLE I. Adsorption of Sendai Virus to Different Cell Types by Determination of Change in Hemagglutinin Titer.

Cells	Original titer[a] (HAU/ml)	Titer after adsorption		
		1	2	3
SR-4 normal	5120	5,120	5,120	5,120
SR-5 normal	5120	5,120	5,120	5,120
SR-4 spheroplasts	5120	5,120	5,120	5,120
SR-5 spheroplasts	5120	5,120	5,120	5,120
HeLa	5120	1,280	320	0
RBC[b]	5120	1,280	160	80

[a] Reciprocal of titer dilution.
[b] Adult chicken erythrocytes.

TABLE II. Comparative Adsorption of ^{32}P Labeled Sendai Virus by HeLa Cells and Spheroplasts and Cells of *Escherichia coli.*

Sample	cpm × 10^4	% Adsorption of total counts[a]
HeLa cells	81.1	97.0
HeLa cell supernatant[b]	0.8	
SR-4 cells	3.5	4.5
SR-4 cell supernatant	78.4	
SR-5 cells	2.7	3.2
SR-5 cell supernatant	77.7	
SR-4 spheroplasts	1.4	1.6
SR-4 spheroplast supernatant	73.3	
SR-5 spheroplasts	0.1	0.1
SR-5 spheroplast supernatant	80.0	

[a] Total counts = counts obtained with unadsorbed virus.
[b] Supernatant = unadsorbed counts.

cells and chicken erythrocytes both before and after virus treatment. Almost all of the NANA was released from the animal cells by the Sendai neuraminidase while we could detect no change in the amount of NANA in *E. coli* cells after virus treatment.

Discussion. Since fusion of bacterial cells was difficult to demonstrate, we felt that the recombination experiments would show a degree of cell fusion not detectable by other procedures. However, the method depends not only on cell fusion but on the ability of the fused cells to recombine their genetic material and then multiply.

Studies on the various strains of *E. coli* by Dewitt and Zell (7) showed that NANA was found in the surface lipoprotein layer of the cell. They further showed that the NANA released from the cells by acid hydrolysis was susceptible to viral neuraminidase although untreated live cells would not serve as substrate. Our results support these earlier observations. Normal cells of *E. coli* were shown to contain more NANA than spheroplasts indicating that there is a direct correlation between NANA content and the presence of an intact cell wall. Furthermore, it was shown that NANA was not released from *E. coli* cells by the action of Sendai neuraminidase.

The adsorption studies indicate that fusion of the bacterial cells may be blocked by insufficient adsorption of the virus. This may be a reflection of too little NANA on the surface of the *E. coli* cells or of the inaccessibility of NANA to the virus particles. Since there did not seem to be a correlation between the amount of NANA and adsorption of the labeled virus by *E. coli* and HeLa cells, it may be that the bacterial NANA is partially masked by other cell wall components. *Escherichia coli,* which contained more than one half the NANA of HeLa cells showed

TABLE III. Action of Sendai Neuraminidase on Cells Containing Bound *N*-Acetylneuraminic Acid.

Cells	μg NANA[a]/mg cells nontreated	μg NANA/mg cells Sendai-treated	% NANA released
HeLa	3.4	0.2	94
RBC[b]	5.6	1.0	82
SR-4	1.9	1.9	0
SR-5	2.5	2.5	0

[a] *N*-Acetylneuraminic acid.
[b] Adult chicken erythrocytes.

only a fraction of the amount of adsorption demonstrated with HeLa cells. The fact that spheroplasts showed less adsorption than normal cells seems to rule out nonspecific adsorption.

Summary. Fusion of *E. coli* cells and spheroplasts, *B. megaterium* cells and protoplasts, and *M. laidlawii* by UV-inactivated Sendai virus could not be demonstrated by direct methods or indirectly by recombination analysis. Studies show that *E. coli* cells and spheroplasts adsorb, in contrast to HeLa cells, only small amounts of Sendai virus, indicating that fusion may be blocked at the adsorption stage. The NANA in *E. coli* appears to be inaccessible to the virus neuraminidase.

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