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Noncytopathic Mutants of Newcastle Disease Virus

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We have isolated a novel class of mutants of Newcastle disease virus which are less cytopathic than their virulent parent but are still capable of infectious virus production. Unlike wild-type virus, the mutants did not form plaques after 2 days of incubation; they did, however, make hemadsorbing spots. The mutants range in production of infectious virus from 10 to 200% of that of the wild type. They were less cytopathic in a single cycle of infection by light microscopy, loss of protein from the plate, and inhibition of total protein accumulation. All of the mutants exhibited extended mean embryo death times, a correlate of virulence in the adult animal.

We are interested in the molecular basis of cell killing by paramyxoviruses and the role of cell killing in the pathogenesis of these viruses in vivo. Newcastle disease virus (NDV) is an avian paramyxovirus which is particularly suitable for the study of viral pathogenesis (15). This virus is represented in nature by a variety of strains which differ extensively in the organ systems that they affect and the severity of the symptoms which they produce. They range from the avirulent or "lentogenic" strains—some of which are employed as live vaccines—through the "mesogenic" strains, which produce asymptomatic or mild infections, to the virulent or "velogenic" strains, which cause death within a few days (7). These differences in virulence for adult birds are reflected in the speed with which a standard dose of virus kills 10-day-old chicken embryos—measured as mean embryo death time (MDT). Lentogenic and velogenic strains also exhibit consistent phenotypic differences in tissue culture. For example, in contrast to virulent strains, avirulent strains produce noninfectious virus in certain cell types and, therefore, do not grow in multiple cycles or form plaques (13); in addition, in single cycles of infection, avirulent strains are consistently less cytopathic than virulent strains (15; Madansky and Bratt, unpublished data), and cells infected by avirulent strains accumulate less virus-specific mRNA than those infected by virulent strains (1, 11). The relationships, if any, among these phenotypes, as well as their possible relationships to virulence in vivo, are as yet unknown. Moreover, unknown genetic differences among these strains make it impossible to establish causal relationships on the basis of strain comparison alone.

An alternative to comparing strains is to isolate and characterize mutants altered in their cytopathology. Plaque formation in tissue culture provides a potential means of selecting such mutants; specifically, one can select mutants of a virulent strain which have lost their ability to form plaques. One source of such mutants is the temperature-sensitive mutants of NDV which have been isolated in our laboratory (14). These mutants, however, are also uniformly temperature sensitive for the production of infectious virus. Thus, whereas they are potentially useful in studying cell killing during single cycles in tissue culture, we feel they are of limited value in studies designed to investigate the contribution of cell killing to virulence in vivo. Furthermore, Karzon and Bang (10) have shown that a mesogenic strain of NDV grows to titers comparable to those obtained with a velogenic strain in most of the organs in infected adult chickens, while causing little if any disease. Thus, differences in pathology need not result exclusively from differences in virus growth, but may also reflect differences in cytopathology per se.

We therefore developed a nonconditional procedure to screen for mutants which fail to produce plaques, but are nevertheless capable of infectious virus production. The technique is based upon the ability of NDV-infected cells to be detected by hemadsorption in the absence of cell killing. This procedure is applicable, in theory, to any virus which is capable of inducing both hemadsorption and plaque formation. We now report the isolation and preliminary characterization of six independent, noncytopathic mutants of the virulent Australia-Victoria strain of NDV.

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Annual Meeting of the American Society for Microbiology, New Orleans, La., 8–12 May 1977. This study constitutes a portion of a thesis to be submitted by C.H.M. in partial fulfillment of the requirements for a Ph.D. degree at Harvard University.)

MATERIALS AND METHODS

Cell culture. Preparation and cultivation of primary and secondary chicken embryo cell cultures have been described (2). All experiments were performed on secondary cultures seeded at 7×10^4 cells per cm^2 and incubated at 39°C for 2 days.

Solutions and chemicals. Standard medium consists of Eagle minimal essential medium, 2.5% calf serum, 2.5% tryptose phosphate broth, and 0.07% sodium bicarbonate ($NaHCO_3$). Standard agar consists of standard medium made 0.9% (wt/vol) with respect to agar (Difco). *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was a gift of M. Berman. Embryonated hen's eggs were obtained from Spafas, Inc., Storrs, Conn.

Virus strains. The velogenic Australia-Victoria (1932) strain of NDV was used as wild type (AV-WT). Mutants were derived from a cloned stock of this virus, which was grown and purified approximately 40-fold as previously described (8). All stocks were grown in a humidified egg incubator at 38.5°C.

Mutagenesis. In vitro mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was performed as previously described by Tsipis and Bratt (14). A 1-h exposure to a final concentration of 1 mg of mutagen per ml resulted in approximately a 100-fold reduction in titer as indicated by plaque assay.

Selection of noncytopathic mutants. The technique for the selection of noncytopathic mutants is based on the ability of chicken embryo cells infected by NDV to adsorb erythrocytes and, thus, follows in principle the procedure of Hotchin et al. (9) for measuring the infectivity of noncytopathic viruses. It is designed to enrich for, clone, and assay mutants which produce infectious virus, but do not form plaques. Secondary cultures, grown on 100-ml plastic tissue culture plates (Lux), were inoculated with 0.2 ml of a dilution of mutagenized AV-WT to give 50 plaques per plate. After adsorption for 30 min at room temperature, cultures were overlaid with standard agar and incubated for 2 days at 39.5 or 41.8°C or for 3 days at 36°C. (Any temperature of incubation which allows virus growth can be used to screen for such mutants.) Plaques were then located with the naked eye in diffuse light and marked on the bottom of each plate. The bottom of each plate and the overlying agar were marked with a series of symbols to allow subsequent realignment. The agar overlay was carefully removed, intact, with a bent stainless steel spatula, so that the surface which had been in contact with the cell culture now faced upward. The culture was then washed gently by immersing the entire dish in pH 7.4 Dulbecco phosphate-buffered saline (6) at 4°C, flooded with 4 ml of a 2% solution of heparinized chicken erythrocytes (Colorado Serum Co.) in phosphate-buffered saline, and incubated at 4°C for 20 min. Cultures were then washed with phosphate-buffered saline as above and examined for hemadsorbing spots. Any spot which had not previously registered as a plaque was marked as possibly infected with a noncytopathic mutant. The plate was then inverted, and the agar overlay was realigned with the plate using the symbols as guides. The agar overlying the spots of interest was then picked using the mouth end of a sterile, plastic, 1-ml pipette (Falcon Plastics, Inc.). Virus was eluted from the agar in Hanks balanced salts (pH 7.4) containing 2% calf serum for 24 h at 4°C and then stored at −20°C. New 100-mm cell cultures were then inoculated with 0.2 ml of the eluate, and the entire process was repeated to screen for, clone, and thereby enrich for mutants capable of releasing infectious virus from infected cells. After the second round of cloning, clones of each potential mutant were diluted to 10 spot-forming units (SFU) per ml, and 0.1 ml was applied to each of a series of 35-mm cell cultures. After adsorption, cultures were overlaid with standard agar and incubated at the appropriate temperature. (All potential mutants were incubated throughout the selection process at the temperature at which they were originally isolated.) The agar overlay was then removed intact from plates which showed no plaques after 2 to 3 days of incubation, and hemadsorption was performed as described above. The agar from plates which showed only one hemadsorbing spot was washed with 1.5 ml of Hanks balanced salts plus 2% calf serum at 4°C for 24 h, and 0.2 ml of the eluate was used to infect 10-day-old embryonated hen's eggs. After incubation for 48 h, the amnioallantoic fluid was harvested and centrifuged at $12,000 \times g$ for 10 min at 4°C, and the supernatant (seed stock) was stored at −70°C. The seed stocks provided the inocula for the generation of all stocks used for subsequent experiments.

Mutants were considered to be independent because the mutagenesis involved no amplification of virus (4), and only mutants derived originally from separate 100-mm plates were finally used. Mutants were obtained at a frequency of 3%; no spontaneous mutants (less than 0.2%) were found.

Titration of infectivity. Mutants were titrated by hemadsorption as described above, and titers were expressed as SFU per milliliter. AV-WT was titrated by plaque assay as previously described (2). (For AV-WT, PFU per milliliter and SFU per milliliter were equivalent.)

Hemagglutination titrations. Hemagglutination titrations were performed by the method of Clavell and Bratt (5).

Viral growth curves. Cells were infected at a multiplicity of 5 for 30 min at either 36, 39.5, or 41.8°C and washed five times with prewarmed standard medium to remove unadsorbed virus. A 3-ml amount of prewarmed medium per 60-mm plate was added, and the cultures were incubated at the appropriate temperature in a 5% CO_2 atmosphere, maintaining a pH of 7.2. Samples (0.2 ml) of the culture medium were removed at 3-h intervals and stored at −20°C for subsequent titration as described above.

Light microscopy of infected cells. Cells infected as for viral growth curves were incubated for 12 h at either 36, 39.5, or 41.8°C. Culture medium was removed, and the cells were washed with Hanks balanced salts, fixed with 95% methanol, and stained with Giemsa. Stained plates were examined with a Nikon

microscope fitted with a Nikon camera back, and photographs were taken using Kodachrome-64 film (Eastman Kodak Co.).

Total protein accumulation. The method of Hightower and Bratt (8) was used, except that trichloroacetic acid-precipitable counts per minute were corrected for variations in both uptake of radioactive precursor (acid-soluble counts per minute) and total protein content of the plate, which was determined by the method of Lowry et al. (12).

MDT. MDTs were determined as described by Waterson et al. (15). Ten-day-old embryonated hen's eggs were inoculated with 10^3 PFU or SFU per egg and incubated at 38.5°C. Eggs were examined every 8 h by candling; integrity of the circulatory system and movement of the embryo were the criteria of viability. Any embryos which were dead at 24 h post-inoculation were discarded as nonspecific deaths and did not figure in the calculation of MDT. MDT signifies the hour at which half of the embryos were observed to be dead.

RESULTS

Figure 1 illustrates the appearance of cultures infected with small numbers of AV-WT (panel A) or a noncytopathic mutant, nc9 (panel B), overlaid with standard agar and incubated at 36°C for 3 days. Plaques are easily discernible in the culture infected with AV-WT. In contrast, no plaques are visible on the culture infected with nc9, even in the presence of neutral red, which increases the visibility of the AV-WT plaques. Figure 1C shows the hemadsorbing spots obtained after removal of the agar overlay from the culture shown in Fig. 1B. Table 1 summarizes the spot-forming phenotypes of the six noncytopathic mutants which form hemadsorbing spots of characteristic and reproducible size. Although each mutant was isolated at the indicated temperature, the relative spot size for each of the mutants varied little between 36 and 41.8°C. For mutants nc9 and nc12, however, plaques sometimes became discernible after 2 days of incubation at 41.8°C. In fact, all of the mutants eventually made plaques, even at 36°C, after 4 days of incubation. These late-forming plaques were not due to plaque-forming revertants, because the virus isolated from the plaques maintained its noncytopathic phenotype upon subsequent passage (data not shown).

Table 1 also shows that the titers of purified stocks of each of the mutants (SFU per milliliter) and AV-WT (PFU per milliliter) grown in embryonated hen's eggs are similar, except perhaps mutant nc16, whose titer is slightly lower than the others. Hemagglutination titers for each stock—an indirect measure of relative quantities of physical particles—are also presented in Table 1, along with the hemagglutinin/infectivity ratios. There appeared to be no significant differences between the mutants and AV-WT in their production of noninfectious virus particles in eggs.

Examples of growth curves obtained for each of the mutants in a single cycle in tissue culture at 39.5°C are shown in Fig. 2. Each panel represents a separate experiment in which the wild type and a mutant were compared. Mutants nc9 and nc12 characteristically reached titers 2-fold higher than AV-WT, whereas values for nc16, nc17, and nc4 ranged between 30 and 80% of those for wild-type; nc7 reached titers at least 10-fold lower than AV-WT. Growth curves obtained at 36 and 41.8°C and for as long as 24 h postinfection show essentially similar results (data not shown). Table 1 shows the growth of each of these mutants, expressed as an average relative to AV-WT.

Light microscopy of cells infected for 12 h provides further evidence that the mutants are

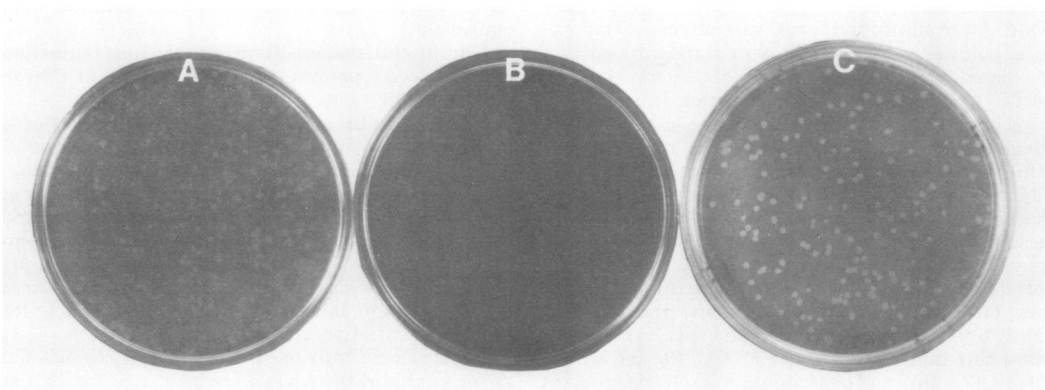

FIG. 1. *Cultures infected with small numbers of AV-WT (A) or the noncytopathic mutant nc9 (B), overlaid with agar and incubated for three days at 36°C. (C) shows the hemadsorbing spots obtained after the agar was removed from the culture in (B), and an erythrocyte solution was added as described in the text.*

TABLE 1. *Properties of the noncytopathic mutants*

Virus	Temp of isolation (°C)	Relative spot size[a]	Growth in eggs: Infectivity (PFU/ml or SFU/ml × 10^{-10})[b]	Growth in eggs: HAU/ml[c]	Hemagglutinin/infectivity ratio (×10^7)	Growth in a single cycle (% AV-WT)[d]	CPE[e]	Total protein accumulation (% uninfected)[f]	Protein/plate (% uninfected)[g]	MDT (h)[h]
AV-WT		○	1.3	1,040	0.80	100	+++	20	55	44
nc9	36	●	1.6	1,920	1.2	164	−	100	97	64
nc12	36	●	2.1	1,760	0.85	196	−	100	100	64
nc17	39.5	•	2.3	1,440	0.63	60	−	44	95	64
nc4	41.8	•	0.95	1,920	2.0	85	−	40	90	77
nc16	41.8	•	0.39	960	2.5	30	−	44	100	86
nc7	36	·	1.2	2,880	2.4	12	−	60	98	77

[a] Relative sizes of hemadsorbing spots at 39.5°C were determined as described in the text.

[b] Values represent the average infectivity titers of virus grown for 48 h in eggs infected with 10^3 PFU or SFU of virus, harvested, and purified 40-fold as described in the text.

[c] Hemagglutinating units (HAU) per milliliter were determined on egg-grown stocks which had been purified 40-fold as described in the text. Values represent the average of several determinations.

[d] Figures represent the average titers of virus released at 6, 9, and 12 h postinfection at 39.5°C from cells infected for growth curves as described in the text.

[e] Cytopathic effects (CPE) measured by light microscopy include the following criteria: parallel array, spindle shape, presence of vacuoles, and number of cells attached to plate. Cells were infected and processed as described in the text.

[f] Cells infected at a multiplicity of infection of 5 were labeled for 30 min at 39.5°C at 12 h postinfection with medium containing a ^{3}H-amino acid mixture as described by Hightower and Bratt (8). Total protein accumulation is expressed as a percentage of uninfected cultures.

[g] Values are expressed as a percentage of the protein remaining on uninfected cultures and represent the results of determinations made on cultures used for total protein accumulation studies at 12 h postinfection at 39.5°C.

[h] MDT was measured as described in the text. Values represent the average of several determinations.

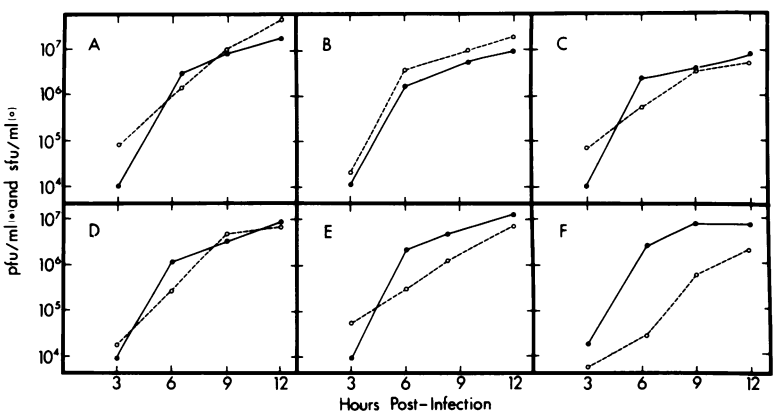

FIG. 2. *Single-cycle growth curves. Titers are expressed either as PFU per milliliter for AV-WT* (●) *or as SFU per milliliter for the mutants* (○). *(A) nc9; (B) nc12; (C) nc17; (D) nc4; (E) nc16; (F) nc7.*

reduced in cytopathogenicity. Figure 3 shows the appearance of uninfected cells (panel A) and cells infected at a multiplicity of 5 PFU per cell with AV-WT (panel B) or 5 SFU per cell with mutant nc9 (panel C). Panel A shows uninfected secondary cultures of chicken embryo cells which exhibit a characteristic spindle shape, parallel array, and lack of vacuoles. Each of these properties has been altered in the AV-WT-infected cultures (panel B). It should be noted that fusion from within by AV-WT was suppressed under the conditions used in these experiments (multiplicity of infection of 5, pH 7.2 [3]). Cultures infected by nc9 (panel C) have the appearance of the uninfected cells shown in panel A. Similar results were obtained with the other nc mutants (Table 1) and several naturally occurring avirulent strains (Madansky and Bratt, unpublished data).

Figure 4 presents two further illustrations of

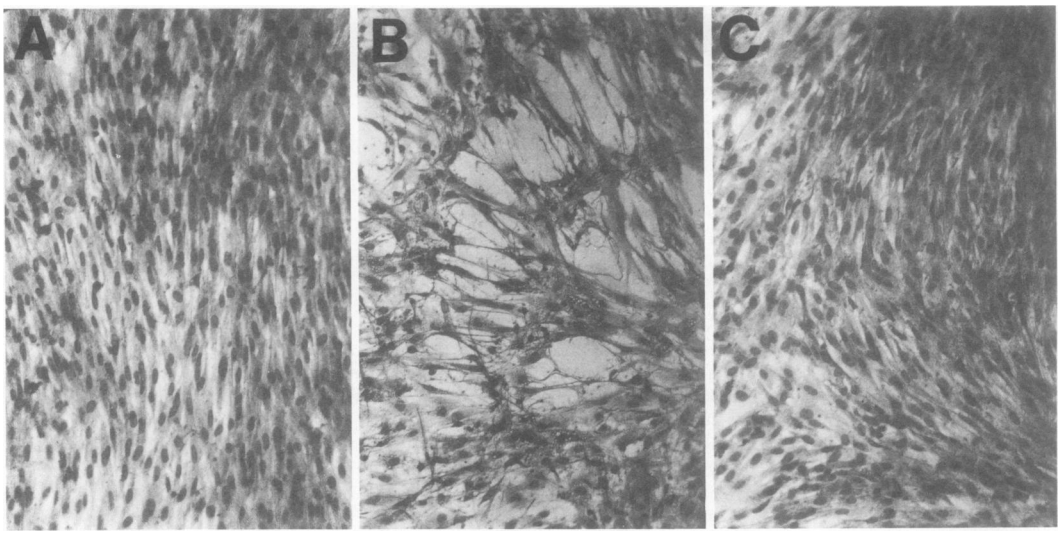

FIG. 3. *Morphology of cells in cultures either uninfected (A), infected with AV-WT (B), or infected with the noncytopathic mutant nc9 (C) as visualized by light microscopy. ×200.*

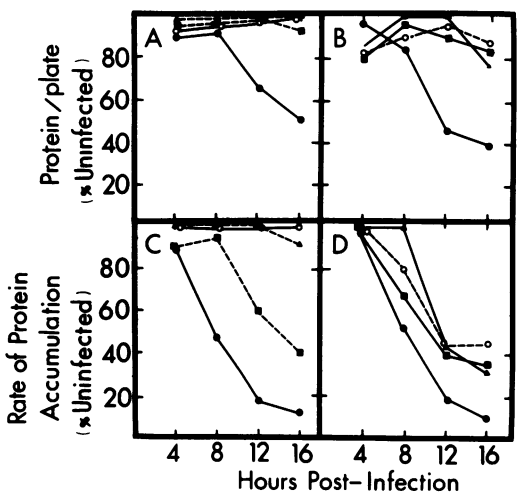

FIG. 4. *Loss of protein from plate (A and B) and inhibition of total protein accumulation (C and D) in cultures infected with AV-WT or the noncytopathic mutants. Panels A and C and panels B and D represent separate experiments. Total protein accumulation is corrected for differences in uptake of radioactive precursor and loss of protein. AV-WT* (—●—); *nc9* (—○—); *nc7* (- - -■- - -); *nc12* (- - -▲- - -); *nc17* (- - -○- - -); *nc4* (—■—); *nc16* (—▲—).

the reduced cytopathogenicity of the mutants: protein remaining attached to the plate after infection and rate of intracellular protein accumulation. Panels A and B show the percentage of protein remaining on plates for cultures infected with AV-WT or the noncytopathic mutants. By 12 h postinfection, only AV-WT-infected cultures show a significant loss of protein. Panels C and D show the effects of infection on total intracellular protein accumulation in the same cultures. Mutants nc9 and nc12 show no inhibition, whereas the other mutants appear intermediate relative to AV-WT. These data are summarized in Table 1.

Finally, we assessed the MDT for each mutant. Table 1 summarizes these results. AV-WT had an MDT of 44 h, consistent with its velogenic classification. Mutants nc9, nc12, and nc17 had MDTs of 64 h, whereas mutants nc7, nc4, and nc16 had MDTs of 77 to 86 h. The latter value approaches those obtained with naturally occurring avirulent strains, such as N.J.-LaSota and B1-Hitchner (90 and 95 h, respectively [data not shown]).

DISCUSSION

In this study, we describe a novel class of mutants which are less cytopathic than their virulent parent but are still capable of infectious virus production. The selection procedure screens for mutants which fail to make plaques in the same period of time in which plaques are formed by the parental strain. Theoretically, one could select mutants with even longer delays in the onset of plaque formation by extending the time after which cultures are screened for mutants.

In addition to their defect in plaque formation, the mutants were less cytopathic in single cycles of growth by several criteria, including light microscopy, inhibition of total protein accumulation, and loss of protein from infected cell cultures. The mutants were selected to be less

cytopathic on cells cultured from 10-day-old chicken embryos, predominantly fibroblasts. Whether they are noncytopathic in other types of cells, both in tissue culture and in ovo, is currently under investigation.

The mutants differ somewhat in their production of infectious virus in single cycles, but only mutants nc7, and possibly nc16, were significantly reduced in yield (10-fold and 3-fold, respectively) relative to the wild-type virus. It is interesting to note that, in most cases, the yield of infectious virus appeared to correlate with spot size.

All of the mutants exhibited extended MDT, suggesting they are reduced in virulence. It is possible that the variation in MDT among the mutants results either from "leakiness" of the noncytopathic phenotype or from different types or numbers of lesions. For instance, the mutants with the longest MDTs (i.e., nc7, nc4, and nc16) made the smallest spots and may have been selected for lesions both in reduced cytopathogenicity and in factors which affect the spread of virus from cell to cell. In support of this notion, Nagai et al. (13) have suggested that, in certain types of cells, the cleavage of the F_0 polypeptide into the F polypeptide—a phenotype which controls infectious virus production and thus virus spread—is directly related to the virulence of the strain of NDV used for infection. Although MDT is positively correlated with virulence for adult chickens (7), neither the molecular basis for this correlation nor the actual cause of death of the embryo is known. Thus, although a change in MDT is likely to reflect a change in virulence in vivo, only tests in adult chickens would give a definite answer.

Studies to be reported elsewhere include characterizations of other phenotypes—cell fusing ability, RNA and protein species and synthesis, etc.—of the noncytopathic mutants and independent plaque-forming revertants of each mutant. These studies should make it possible to determine: (i) whether each mutant possesses one or more lesions; (ii) whether each mutant possesses similar or different types of lesion; (iii) which phenotypes are directly related to cell killing and which to virus spread; and ultimately (iv) the extent to which the cell-killing and virus-spread phenotypes contribute to MDT and virulence in vivo.

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