

In Vitro Characterization of an Avian Reovirus Vaccine Strain

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In vitro studies were performed to characterize the vaccine strain, designated P100, derived from the arthrogenic reovirus isolate, S1133, by cold adaptation. P100 appeared to be temperature sensitive, shown by a marked drop in titer and efficiency of plaquing after incubation at 41°. Studies indicated that genomic double-stranded RNA and protein synthesis were severely restricted at the elevated temperature. Differences in the growth behavior of P100 and S1133 at 37° were also noted. The vaccine strain seemed to be more cell associated than S1133. Three outer coat proteins of P100 grown at 37° displayed mobilities different from those of S1133 by PAGE. It is possible that alterations in these proteins may have some relationship to the growth characteristics observed for the P100 strain.

INTRODUCTION

Reoviruses are ubiquitous in nature, possessing a wide range of hosts including man, numerous other mammals, and domestic birds (Ramig and Fields, 1977). The avian reoviruses comprise a serologically distinct group that can be differentiated further from the mammalian reoviruses by their lack of a hemagglutinin, different host range, and ability to fuse cells in culture (Olson, 1978). Moreover, unlike the mammalian reoviruses, avian reoviruses have been naturally associated with a variety of diseases including viral arthritis and tenosynovitis, gastroenteritis, hepatitis, myocarditis, and respiratory illness in chickens (van der Heide, 1980) and infectious enteritis in turkeys (Gershowitz and Wooley, 1973).

Viral arthritis in chickens, first recognized in 1959 (Olson, 1959), has since received great attention from researchers in the poultry industry. The disease, although associated with low mortality, often produces high morbidity rates resulting in significant economic losses (Glass *et al.*, 1973; Olson and Solomon, 1968) and

therefore attempts have been made to produce a vaccine suitable for young chicks. Continued *in vitro* propagation at 31° of an extremely virulent and arthrogenic isolate, S1133, was performed and the resulting pools were tested at various levels of passage for efficacy in terms of immunogenicity and protection afforded upon challenge with wild-type virus (Rau *et al.*, 1980; van der Heide, 1975; van der Heide, 1980). A vaccine has been produced from the 100th passage virus pool and designated "P100." Pathogenicity of P100, when given by different routes of inoculation and at various doses to day-old chicks, has been studied and compared to the parent S1133 (Gouvea and Schnitzer, 1982).

The conditions under which strain S1133 was serially propagated in order to obtain P100 were those of "cold-adaptation." However, the manner in which P100 differs from the parental S1133 has not been examined *in vitro*. The relative ability of P100 to grow at either low or high temperature has not been evaluated nor have any biochemical studies looked for alterations in viral proteins or genes. The aim of this study was to undertake an initial *in vitro* characterization in biological and

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biochemical terms of the vaccine strain P100 in an attempt to detect differences from the parent S1133. Further examination of such alterations could then help identify genetic and biochemical markers that may be useful for studies on factors related to viral virulence in chickens.

MATERIALS AND METHODS

Cells. Chick embryo fibroblasts (CEF) and chick embryo liver cultures (CELi) were prepared from 10- and 14-day-old embryos, respectively, by conventional tissue culture techniques. CEF cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 5% tryptose phosphate broth, 2% newborn calf serum (NCS), and 2% chick serum. The first and second passages of CEF grown in the same medium were used for virus propagation. CELi cultures were prepared in DMEM supplemented with 10% NCS and used within 48 to 72 hr.

Viruses. The 73rd chorioallantoic membrane (CAM) passage of strain S1133 of avian reovirus, originally isolated during an outbreak of severe tenosynovitis in Connecticut, and its attenuated vaccine strain P100 (van der Heide, 1975, 1980) were kindly supplied by Dr. L. van der Heide (Department of Pathobiology, University of Connecticut, Storrs). The P100 virus had been produced by 235 passages in CAM followed by an additional 100 passages in CEF, 40 of which were at low temperature (32°) (L. van der Heide, personal communication). Both S1133 and P100 viruses were plaque-purified three times in CEF and a final pool was made and titrated to be used in all assays.

Virus titration. CEF or CELi monolayers grown in six-well plastic plates were infected with 0.5 ml/well of a series of 10-fold dilutions of virus suspension in phosphate-buffered saline (PBS) and virus allowed to absorb at 37° for 1 hr. The inoculum was then removed and the cells were washed twice with PBS. Two milliliters of overlay medium (1.5% agarose, 2% NCS in minimum essential (MEM)) was added per well and plates were incubated at 37°. When plaques could be eas-

ily detected, after 2 days (CELi) or 4 days (CEF), 1 ml of a second overlay (0.9% agarose, 0.005% neutral red in MEM) was added per well. Plates were incubated at 37° and plaques were counted on the next day.

Efficiency of plaquing (EOP) at 33 and 41°. Determination of the ability of the viruses to produce plaques at high and low temperatures was done as described above for virus titration, except that after adsorption at 37°, plates were incubated at 33, 37, or 41° accordingly.

Propagation and protein labeling of viruses. Confluent monolayers of CEF cells in 90-mm plates were infected with 0.5 ml of virus suspension at a multiplicity of infection (m.o.i.) of approximately 1, and incubated at 37° for 1 hr. After virus adsorption, the inoculum was removed, 5 ml of DMEM-2% NCS was added per plate, and the plates were returned to 37°. When CPE became evident, but infected cells were still attached to the plate, the medium was replaced with DMEM containing 5% the normal amount of methionine and 2% dialyzed NCS. Then 100 μ Ci of [³⁵S]methionine (Amersham, 400-800 Ci/mol) was added per plate and virus multiplication allowed to proceed at 37°. At maximum CPE, usually 48 hr postinfection (p.i.), infected cultures were harvested and either frozen at -70° or immediately processed for virus purification.

Purification of virus. Cellular and supernatant fractions from infected cultures were separated by low-speed centrifugation. Virus was recovered from the supernatant by pelleting, pooled with cellular fraction, extracted with Freon, and purified by CsCl gradient centrifugation as previously described (Schnitzer *et al.*, 1982).

Radiolabeling and immune precipitation of viral proteins in cell extracts. CEF cells in 60-mm plates were infected at a m.o.i. of 10 PFU/cell as described above and incubated either at 37 or 41°. At various times after adsorption, metabolic labeling was done by replacing the medium with methionine-free medium containing 2% dialyzed NCS and supplemented with 25 μ Ci of [³⁵S]methionine/ml. After a 3-hr pulse, a cellular extract was prepared and

viral polypeptides were immunoprecipitated as previously described (Schnitzer *et al.*, 1982).

Radiolabeling of cytoplasmic dsRNA. CEF monolayers in 50-mm plates were infected at a m.o.i. of 10 PFU/cell and incubated at 37° for 1 hr. After adsorption the inoculum was removed and 3 ml of DMEM containing 2% dialyzed NCS, 60 µg/ml actinomycin D (Act. D), and 30 µCi [5-6-³H]uridine (Amersham) were added per plate and the plates incubated either at 37 or 41° in a 5% CO₂ atmosphere. At 18 hr postinfection the cells were harvested and separated from the supernatant by low-speed centrifugation. Virus was pelleted from the supernatant by centrifugation at 50,000 *g* for 90 min and solubilized in Laemmli's sample buffer. Cytoplasmic double-stranded RNA (dsRNA) and cell-associated virus in the cellular fraction were dissolved in Laemmli's sample buffer.

Polyacrylamide gel electrophoresis. Electrophoresis was carried out in 9% acrylamide-0.267% bis-acrylamide slab gels in a discontinuous Tris-glycine buffer system. Samples were boiled for 1 min prior to loading onto gels, and electrophoresis proceeded at 140 V for 15 hr (dsRNA) or

at 200 V for 4 hr (proteins). After the run, gels were fixed and processed for autoradiography as previously described (Gouvea and Schnitzer, 1982) or silver stained as described by Sammons *et al.* (1981).

RESULTS

Growth characteristics. Representative growth curves of virus strains S1133 and P100 incubated at 33, 37, and 41° in CEF cells are shown in Fig. 1. It can be seen that S1133 replicated well at all three temperatures, with peak virus production occurring at 18 hr p.i. The P100 strain yielded titers similar to those of S1133 after incubation at 37°, while appearing to generate slightly more progeny than S1133 at 33°. However, in marked contrast to S1133, P100 seemed unable to multiply at 41°. The efficiency of plating (EOP) of both viral strains at 33 and 41° was determined (Table 1), demonstrating that P100 had also acquired a temperature-sensitive (*ts*) characteristic in the process of becoming cold adapted. At either 33 or 37°, the plaque morphologies of both strains were indistinguishable. Both viruses produced extensive syncytia with complete destruction of the monolayer, although the syn-

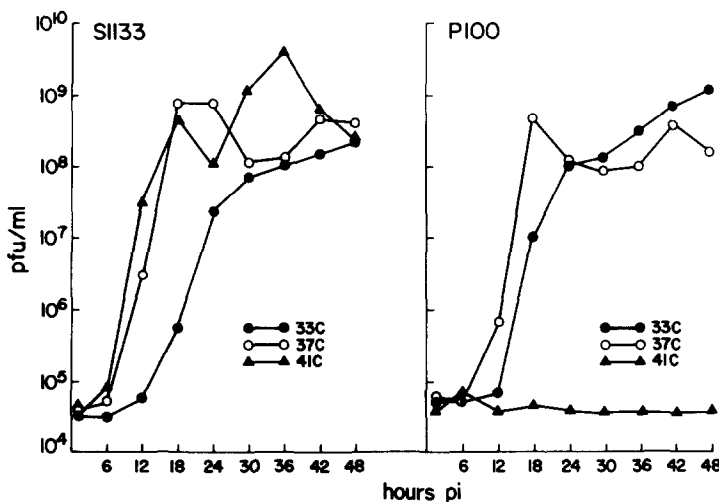


FIG. 1. Growth curves of S1133 and P100 at 33, 37, and 41°. CEF monolayers in 30-mm plates were infected at an m.o.i. of 2 PFU/cell. After virus adsorption for 1 hr at 37°, the cells were washed twice with PBS and 2 ml of DMEM-2% NCS was added. Plates were incubated at 33, 37, or 41°. Every 6 hr duplicate plates were withdrawn from each incubator, submitted to three cycles of freeze-thawing, and the culture suspensions were titrated in CELi monolayers incubated at 37°.

TABLE 1
EFFICIENCY OF PLAQUING (EOP) AT 33 AND 41°

Virus	Cells	Virus titer (log ₁₀ (PFU/ml)) ^a		
		33°	37°	41°
S1133	CEF	6.3	7.2	6.9
	CELi	6.8	7.8	6.8
P100	CEF	7.0	6.5	<2.0
	CELi	7.9	7.5	<2.0

^a Numbers represent the mean of titers obtained in 3 experiments.

cytia in S1133-infected cultures were often significantly larger than those obtained in P100-infected cultures.

Another difference in the behavior of the two strains is emphasized in Table 2. Viral titers obtained at various times after infection from the supernatant of either S1133- or P100-infected cells or from the harvested cell monolayers themselves suggested that P100 particles may be more cell associated than S1133 particles. The titers show that up to 24 hr p.i. the yields of the parent strain, S1133, from the supernatant and the cells follow a similar distribution to that seen in the P100-infected cultures with approximately 100-fold more virus being found in the cellular fraction than in the supernatant. However, by 37 hr p.i. and after, this pattern was changed. In the S1133-infected cul-

tures, roughly equal amounts of virus were shed into the supernatant as were found in the cellular portion of the cultures; in the P100 infections, 100- to 1000-fold more virus was recovered from the cell monolayers than from the supernatant.

Synthesis of genomic dsRNA. Although the P100 strain seemed unable to form infectious progeny at 41°, it was of interest to determine whether any phase of viral replication was taking place within the host cells at the restrictive temperature. CEF cultures infected with P100 in the presence of Act. D were incubated at 41° and labeled with [³H]uridine to see if any genomic dsRNA was synthesized. The autoradiogram in Fig. 2 shows that P100 segments are not present in infected cell lysates after incubation at the restrictive temperature. By subjecting the gel to the more sensitive assay of silver staining, faint bands could be detected which migrated parallel to the genomic segments synthesized during a productive infection (data not shown). These data indicate that a very limited level of P100 replication may take place at the nonpermissive temperature, although the infection is largely inhibited.

The dsRNA segments extracted from supernatant or cell monolayers infected with P100 at 37° migrated identically to those of S1133 analyzed in a similar manner (Fig. 2). There was no evidence of either smaller genomic fragments or dele-

TABLE 2
TITERS OF P100 AND S1133 OBTAINED FROM SUPERNATANT AND CELLULAR FRACTIONS OF INFECTED CULTURES INCUBATED AT 37°^a

hr p.i.	S1133			P100		
	PFU/ml supernatant	PFU/ml cells	Total PFU/ml	PFU/ml supernatant	PFU/ml cells	Total PFU/ml
2	2.3 ^b	2.6	2.8	2.9	<2.0	2.9
7	2.3	2.6	2.8	2.9	<2.0	2.9
12	2.7	4.6	4.6	2.0	2.3	2.4
18	2.9	5.0	5.0	2.0	3.4	3.4
24	4.3	6.0	6.0	2.6	4.0	4.0
37	6.8	7.7	7.8	4.0	7.0	7.0
42	8.0	7.7	8.1	4.1	7.0	7.0
52	7.3	7.0	7.5	5.7	7.3	7.3

^a Supernatant fractions refer to medium alone before the monolayer was harvested. The monolayer was then subjected to 3 cycles of freeze-thawing before sonication. Infection was initiated at an m.o.i. of 2-5.

^b Numbers are expressed as log₁₀ PFU/ml.

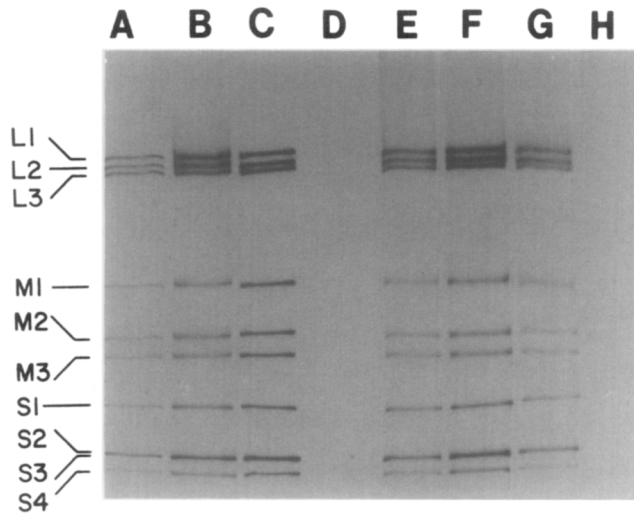


FIG. 2. [^3H]Uridine-labeled dsRNA of S1133 and P100. Supernatant virus dsRNA of S1133 produced at 37° (A) and 41° (B), and of P100 produced at 37° (C) and 41° (D). Cytoplasmic and cell-associated viral dsRNA from cellular fraction of S1133-infected cells synthesized at 37° (E) and 41° (F), and of P100-infected cells synthesized at 37° (G) and 41° (H).

tion of entire genome segments to suggest DI particle formation during infection. Similar results were seen after S1133 infection at either 37 or 41°. In addition, there was no detectable difference in the mobilities of the S1133 or P100 segments, an observation which has been previously reported (Gouvea and Schnitzer, 1982).

Synthesis of viral proteins. Infection with the P100 strain at 41° was also examined for any evidence of viral protein synthesis. Cultures were pulse-labeled with [^{35}S]methionine at various times after infection. At the end of the labeling period, cell lysates were prepared and proteins immune-precipitated to determine which, if any, viral proteins could be synthesized at the nonpermissive temperature. Figure 3 depicts the results of one such experiment. The gel patterns indicate that only trace amounts of some of the proteins of P100 are synthesized after incubation at 41° in comparison to the profile seen after growth at 37°. Although only proteins μ_B , σ_A , and σ_C appear most clearly, it is not possible to say that the other proteins are not being produced. It can be noted that all the S1133 proteins are present in large quantities after infection at either 37 or 41°. Results similar to those obtained from infected CEF cultures are seen when Vero cells are

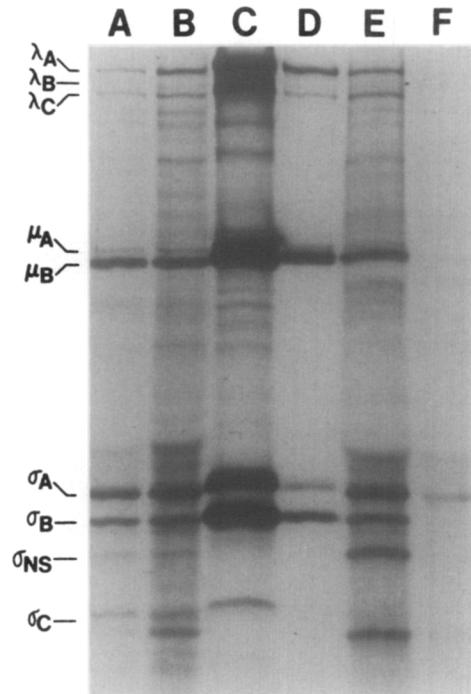


FIG. 3. Autoradiogram of [^{35}S]methionine-labeled polypeptides of S1133 and P100. Cell extracts from S1133-infected cells incubated at 37° (A) and at 41° (B), and of P100-infected cells incubated at 37° (E) and at 41° (F) were pulse-labeled (3 hr) at 12 hr p.i. and subjected to immune precipitation. Comparison was made with CsCl-purified S1133 (C) and P100 (D) viruses.

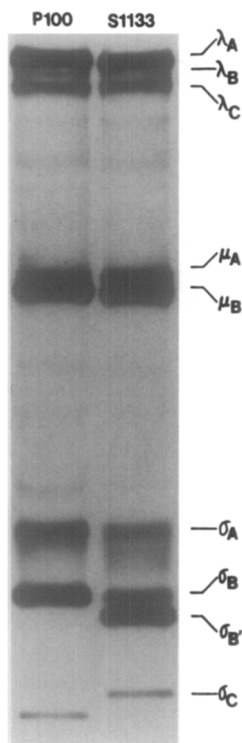


FIG. 4. Autoradiogram of [^{35}S]methionine-labeled structural polypeptides of S1133 and P100-purified viruses. Note the additional S1133 polypeptide $\sigma_{B'}$, a degradation product of polypeptide σ_B .

infected with S1133 or P100 at either temperature (data not shown) suggesting that the severe decrease in synthesis of P100 polypeptides at 41° is not a host-cell-dependent phenomenon.

Comparison of the polypeptides of S1133 and P100 strains. When the proteins of P100 and S1133 are examined by PAGE, it can be seen that at least two polypeptides of P100 have mobilities which differ from those of the parental S1133 (Figs. 3 and 4). The major μ polypeptide, μ_B , appeared to have a slightly slower migration, while a minor σ protein, σ_C , ran well ahead of the S1133 σ_C . Both these avian reovirus polypeptides have been identified as outer capsid proteins (Schnitzer *et al.*, 1982). In addition, repeated examination of the proteins of the same preparation of purified S1133 revealed that upon prolonged storage or repeated freeze-thawing cycles, there was a gradual degradation of the σ_B polypeptide yielding a new, lower-molecular-weight protein $\sigma_{B'}$ (Fig. 4). This oc-

curred repeatedly with several different preparations of S1133. However, preparations of purified P100, treated in the same manner, seldom generated this fragment, and when $\sigma_{B'}$ was seen it appeared in much lesser proportions than the σ_B of S1133. The other P100 proteins appeared identical to those of S1133.

DISCUSSION

The data presented show that the avian reovirus vaccine strain, P100, has acquired some growth characteristics which are not seen in its parental strain, S1133. In the process of becoming cold-adapted, P100 has also become temperature sensitive at 41° , demonstrated by the inability of the virus to multiply or plaque at this temperature. Although cold adaptation and temperature sensitivity are independent properties, as has been shown with many chemically mutagenized viruses, it is not uncommon for viruses adapted to grow at a lower temperature to simultaneously acquire the *ts* characteristic (Maassab *et al.*, 1981).

The analysis of the growth behavior and synthesis of viral genome and proteins indicates that S1133 grows equally well at normal (37°) and "restrictive" temperatures (41°). However, the P100 strain appears severely limited in its ability to replicate at the higher temperature. Minute amounts of genomic dsRNA were detectable only by silver staining and not by autoradiography, and only trace amounts of, at most, three viral proteins could be found by immunoprecipitation after infection at 41° . These observations would suggest that P100 can enter the host cell and is presumably uncoated, permitting initiation of viral replicative events and limited protein synthesis. Amplification of viral replication seems to be restricted afterward, evidenced by the low virus yields at the elevated temperature. It is not possible to determine which defective viral functions contribute to this inhibition.

Acquisition of the *ts* trait in numerous instances has occurred spontaneously, oftentimes accompanied by a loss of virulence. P100 has been demonstrated to be much less virulent for day-old chicks than S1133 (Gouvea and Schnitzer, 1982). Yet

the same experiments demonstrated that lack of virulence is not a reflection of decreased infectivity. P100 virus was still able to infect, spread, localize, and multiply in target tissues. It is worth noting that 41°, the *in vitro* restrictive temperature for P100 virus, is the physiological temperature of the chicken. It is possible, though, that sites with slightly lower body temperature would permit at least low levels of P100 viral replication. Site-specific attenuation has been documented for infections with *ts* mutants of influenza and respiratory syncytial virus (Murphy *et al.*, 1972; Wright *et al.*, 1971).

Differences in behavior between P100 and S1133 have also been noted at 37°. There is an indication that P100 is not capable of shutting off the host as well as the wild-type S1133 (unpublished results). Another interesting observation is that P100 appears to remain cell associated at 37° while S1133 particles are released. These functional changes could relate to the decreased pathogenicity of P100.

Those factors responsible for the differences seen between the parent and vaccine strain are not known. It has been shown in studies with the mammalian reoviruses that upon infection, virions enter cells where they are partially uncoated, triggering the activation of the endogenous transcriptase (Ramig and Fields, 1977; Spandidos and Graham, 1976). Uncoating or unmasking of the transcriptase is initiated by the complete loss of polypeptides σ_1 and σ_3 and partial cleavage of μ_{1C} (Joklik, 1981). Although correlations between the mammalian and avian reovirus polypeptides have not yet been made conclusively, preliminary observations have led to assignments of the avian polypeptides σ_C , σ_B , and μ_B to polypeptides σ_1 , σ_3 , and μ_{1C} of the mammalian reovirus, respectively (Schnitzer *et al.*, 1982). It is of note that the σ_C , σ_B , and μ_B proteins were found in P100 to have altered mobility by PAGE (σ_C and μ_B) or altered stability (σ_B) when compared to homologous polypeptides of wild-type S1133 virus. It is possible that the alterations in these proteins may have contributed to the observations made in this study.

Further work needs to be done to define

with the avian reoviruses the functions of the individual genes and their respective protein products during viral replication. By the production and analysis of reassortant viruses, utilizing both P100 and S1133 in combination with another appropriately selected avian reovirus strain, it should be possible to determine which gene or genes of P100 are important for both the biological as well as biochemical differences seen in comparison to S1133. Furthermore, *in vitro* hybridization analyses can directly examine gene alterations between these two viruses. In this way, the mechanism behind the differences in behavior of S1133 and P100 at 37 and 41° might be determined, yielding better insights into the effects these strains exert on their host.

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