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Characterization of a Paramyxovirus Isolated from the Brain of a Piglet in Mexico

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With 4 Figures

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Summary

A virus morphologically resembling members of the family Paramyxoviridae has been isolated from the brain of a piglet with a central nervous disorder accompanied by pneumonia and corneal opacity. The virus, designated LPM, grows in a large variety of cultured cell types and elicits a cytopathic effect including formation of syncytia and cytoplasmic inclusion bodies. The virus has hemagglutinating, neuraminidase and hemolytic activities. Studies on experimental transmission showed that young pigs are susceptible to infection which induced a disease similar to that in natural cases. The virus killed mice and chicken embryos. The structural proteins of LPM virus, as resolved by polyacrylamide gel electrophoresis are similar to those described for other paramyxoviruses. Serologically the virus proved to be distinct from the paramyxoviruses tested so far.

Introduction

In 1980 an outbreak of encephalitis in piglets occurred in farms around the town La Piedad, district of Michoacan, Mexico. The disease affected piglets between 4 to 10 days old with a morbidity of about 20 per cent and mortality of about 90 per cent.

Stephano *et al.* (8) described the disease which was characterized clinically by disorders of the central nervous system (CNS) and also by corneal opacity in 1 to 10 per cent of cases. In 1 to 4 per cent of pigs more than about three weeks old, corneal opacity was generally the only sign observed. In pregnant sows, apart from corneal opacity, reproduction was also affected,

resulting in a reduced fertility rate, increase in stillbirths and mummified fetuses.

A hemagglutinating virus was isolated from the brains of suckling pigs with clinical symptoms and microscopic lesions of an acute encephalomyelitis. Electron microscopy indicated the presence of a paramyxovirus-like agent. In transmission studies STEPHANO *et al.* (9) showed that the isolated virus produced clinical symptoms and pathological changes in piglets and sows, similar to those which had been observed in natural outbreaks. Pigs exposed to the virus when they were more than ten days old only rarely showed nervous disorders. Moreover, animals with passively acquired immunity were resistant to infection with the isolated virus.

The present report is concerned with a partial characterization of a virus isolated from the brain of a piglet with symptoms of nervous disorder, corneal opacity and pneumonia. The virus strain designated LPM (La Piedad, Michoacan, Mexico) and used in this study, was isolated from an outbreak in 1984.

Materials and Methods

Source of the LPM Virus

The brain of a 5 days old piglet showing typical symptoms of the disease was removed under aseptic conditions and sent immediately after the autopsy to the virus laboratory in Palo Alto, México, D.F. The material was sent in 50 per cent sterile glycerol. A 10 per cent cell-free suspension was prepared in Eagle's Minimal Essential Medium (MEM) supplemented with antibiotics. The suspension was maintained at −70° C until using it for experimental transmission in pigs. Part of the brain material was sent to the Department of Veterinary Microbiology in Sweden and used as the source for virus isolation.

Cell Cultures and Embryonated Hens Eggs

The types of cell cultures used are listed in Table 1. All cells were grown as monolayers in MEM supplemented with 10 per cent fetal calf serum (FCS). The same medium without FCS was used for maintenance of the infected cells. Five and eleven days old embryonated eggs were used for inoculation with supernatants of infected cell cultures into the allantoic cavity, yolk or amniotic sac.

Virus Propagation and Titration

The LPM virus was propagated in PK-15 cells in bottles until the culture showed maximum cytopathic effect (CPE). Small aliquots of the virus pool were stored at −70° C until use. For virus titration, serial 10-fold dilutions of virus were made in MEM. The infectivity of the virus was titrated in cell cultures in roller tubes or Petri dishes and the titer was expressed in $TCID_{50}$/ml or plaque forming units (PFU/ml), respectively.

Hemagglutination (HA) and Hemadsorption (HAD) Tests

These were carried out in a conventional way. In HA tests erythrocytes from chicken, guinea pig, cattle, swine, horse, rabbit, sheep, goat, rat, turkey and human type 0 erythrocytes were used. Hemadsorption (HAD) tests were carried out in roller tubes and with the same types of erythrocytes as above.

The lectin neuraminidase (LNA) test. This was performed according to the method described by LUTHER *et al.* (6).

The hemolytic (HL) activity. This was measured after concentration of virus harvest according to the method described by DINTER *et al.* (3).

Plaque test. This was performed in 35 mm Petri dishes after overlaying infected cell monolayers with MEM containing 5 per cent FCS and 1 per cent agar.

Serological Tests

Hemagglutination-inhibition (HI), complement fixation (CF) serum neutralization (SN), immunofluorescence (IF), and hemolysin-inhibition (HLI) tests were carried out according to standard procedures. In indirect IF tests, cell cultures grown on cover slips and infected with LPM virus were first incubated with antiserum and then with goat anti-swine IgG conjugated with fluorescein isothiocyanate. The Ouchterlony double immuno-diffusion (ID) method as described by WADSWORTH (10) was used. In ID tests, antiserum to LPM virus and other sera modified below were directed against semi-purified LPM virus. Antiserum to this virus was also tested against semi-purified bovine parainfluenza-3 virus (PIV-3) or Newcastle disease virus (NDV). Two antisera to LPM virus have been used, one prepared by hyperimmunization of rabbits and another from an experimentally infected pig. Guinea pig antisera to PIV-3 virus and chicken antisera to NDV were prepared in our laboratory. Guinea pig antisera against human parainfluenza virus types 1, 2 and 3, were obtained from the National Bacteriological Laboratory (SBL, Sweden) and human positive serum against mumps virus, measles virus and respiratory syncytial virus (RSV) were obtained from the Laboratory of Viral Diagnostics, Uppsala, Sweden. This sera were used in HI, CF, ID and SN tests. Sera from convalescent pigs as well as sera obtained from experimentally infected pigs, before and after infection, were used in different serological tests.

Virus Purification

Virus infected cell culture fluid was clarified by low speed centrifugation and the virus was precipitated by polyethylene glycol (7). The virus precipitate was suspended in TEN buffer (0.1 M NaCl, 0.01 M Tris HCl, 0.001 M EDTA) and then layered on a discontinuous gradient of 50 per cent (w/w) sucrose overlaid by 25 per cent sucrose in TEN buffer. Following centrifugation at 100,000 g for 1 hour at 4° C, the virus band was harvested, diluted 1 to 10 in TEN buffer and layered on a linear 10 to 60 per cent sucrose gradient. This gradient was centrifuged at 100,000 g for 4 hours, the virus band collected, diluted 5 × with TEN buffer and concentrated by sedimentation onto a cushion of 30 per cent sucrose. Virus concentration was estimated by the microtiter HA test. Viral protein determination was made by the method described by BRADFORD (2).

SDS-PAGE of Viral Proteins

Polyacrylamide gel electrophoresis (PAGE) was carried out in slab gels of 10 per cent acrylamide and 0.27 per cent N'N-methylenebisacryl-amide in the presence of sodium dodecyl sulphate (SDS) in a discontinuous buffer system (5). The Pharmacia Electrophoresis Calibration Kit (LMW) was used as molecular weight marker. The samples were prepared with 1 per cent SDS and 1 per cent 2-mercaptoethanol and heated to 100° C for 1 minute. Electrophoresis was run at 50 V over night. The gels were stained with 0.1 per cent Commassie Brilliant Blue.

Electron Microscopy

The LPM virus propagated in PK-15 cell cultures was clarified by centrifugation, one drop of the supernatant placed on a Formvar carbon coated copper grid and then negatively stained with 2 per cent ammonium molybdate. The grid was examined in a Philips EM 300 microscope at an accelerating voltage of 60 KV.

Transmission Experiments

Pigs

Two pigs, three weeks old and apparently healthy were inoculated intravenously and intranasally with 3 ml of the original bacteria-free brain suspension diluted 1 to 10 in MEM. Temperature was taken and blood serum samples were collected prior to an every week after inoculation. The animals were sacrificed after 22 days of observation. One out of three pigs 3 months old was inoculated intravenously, and a second pig orally and intranasally with 3 ml of the 5th passage of the virus in PK-15 cells. The inocula contained about 10^4 $TCID_{50}$/ml. The 3rd pig was maintained as a control but in contact with the inoculated animals.

Mice

The original bacteria-free brain suspension diluted 1 to 10 in MEM was inoculated intracerebrally or intraperitoneally into adult mice. Infectious cell cultures from the 5th passage in PK-15 cells were filtered through a 0.45 μ Acrodisc disposable filter and diluted 1 to 10 in MEM. The virus in amounts of 10^4 $TCID_{50}$/ml was inoculated intracerebrally (0.01 ml) or intraperitoneally (0.2 ml).

Cytology and Histology

Virus-infected cell cultures grown on cover slips were fixed in Bouin's solution and stained with hematoxilin and eosin. Selected tissues were collected from naturally and experimentally infected pigs for histological examination. The specimens were fixed in neutral-buffered 10 per cent formalin, and sections stained with hematoxylin and eosin.

Results

The Cytophatic Effect (CPE) of LPM Virus

The results are summarized in Table 1. Pig kidney (PK-15) cell line and bovine turbinate (BT) cells were used for virus isolation. The CPE in both cell types was first observed on day 2 after inoculation and was complete on the 5th day. The CPE in BT cells consisted of rounded, refractile cells forming grape-like clusters; some syncytial formation was observed and many dead cells were detached from the monolayer. In PK-15 cells, the CPE consisted of vacuolated cells and massive syncytial formation. The CPE developed fast, resulting in the destruction of the monolayer within 16 hours. No or only slight CPE was observed when the virus was inoculated into PK-15 cells from another source. In both cases the inoculation dose was 10^5 $TCID_{50}$ per tube culture. In other cell types the CPE was similar to that in BT cells except in pig choroidplexus and human fetal cells in which a network of fibroblast-like cells was seen. The infectivity titers are given in Table 1.

The LPM virus could be titrated by the plaque technique. Distinct but irregularly shaped plaques with a diameter of 1 to 2 mm were seen. In cultures of PK-15 cells the titer of supernatants was 10^7 PFU/ml.

Growth in Embryonated Eggs

The virus inoculated into the yolk sac killed the embryos within 3 or 4 days, but not when it was inoculated into the allantoic cavity or amniotic sac.

Table 1. *Cell types susceptible to the LPM virus*

Cell types[a]	CPE	Syncytial formation	Cytoplasmic inclusion bodies	HAD[b]	Virus titer[c] $TCID_{50}$/ml
Porcine:					
Turbinate	+++	++	ND	+++	10^6
Kidney	+++	+++	ND	+++	10^6
Choroidplexus	+++	–	ND	+++	10^9
IBRs*	+++	++	ND	+++	10^7
PK-15, source A*	+++	+++	+	+++	10^7
PK-15, source B*	+	+	ND	++	10^3
Bovine:					
Turbinate	++	+	ND	++	10^6
Kidney	++	+	ND	++	10^6
Testicle	++	–	ND	++	10^6
Skin	++	–	ND	++	10^4
Palatine	++	–	ND	++	10^6
Choroidplexus	++	–	ND	++	10^6
Simian:					
Vero*	++	+	ND	++	10^5
GMK*	++	–	++	++	10^5
Other cells:					
Mink lung	++	–	ND	++	10^5
Human fetal	++	–	ND	++	10^3

[a] Secondary cell cultures at low passages were used. Asterisks indicate established cell lines. Cultures of each cell type were inoculated with the 5th passage of LPM virus in PK-15 cells. CPE, syncytial formation and HAD are graded from + to +++. ND = Not done

[b] The test was carried out with guinea pig erythrocytes

[c] From infected cell-culture supernatants sampled on post-inoculation day 3 or 4

Since no HA activity could be detected in egg fluids, the yolk and amniotic sacs were chosen for virus adaptation. Hemagglutination activity could not be detected in suspensions of yolk or amniotic sac from each of 7 consecutive passages. However, virus replication measured by HAD and CPE could be detected when egg fluids from each passage were inoculated into PK-15 cells. The HA activity of these cell culture supernatants was inhibit by homologous antisera in HI tests. After 10 serial passages, the LPM virus was adapted to growth in the yolk and amniotic sacs showing HA titers of 8 to 64.

HA, HAD, NA and HL Activities

In all this tests, viral cell cultures harvest were used as antigens. The hemagglutinating activity of the virus was tested using erythrocytes from different animal species including man (see Materials and Methods). Hemagglutination (HA) was obtained with all types of erythrocytes tested

and the highest HA titer was seen with chicken, guinea pig and turkey erythrocytes, i. e., 128 or 256. Hemadsorption (HAD) with guinea pig erythrocytes was seen in cultures of all cell types tested (Table 1). Elution of erythrocytes occurred within a few minutes of incubation at 37° C and is ascribed to NA activity with the highest titer of 64 measured by the LNA test. Concentrated virus exerted hemolytic activity (HL) which was inhibited by homologous antisera.

Structural Properties

Electron microscopy of LPM virus showed particles with the morphology of a paramyxovirus (Fig. 1). In SDS-PAGE, the pattern of LPM virus proteins were compared to those of PIV-3, strain U-23 and NDV, strain Montana (kindly supplied by Drs. B. Sundqvist and B. Klingeborn at our laboratory). At least four proteins were resolved from the LPM virus by SDS-PAGE; their profile was similar to that of PIV-3 but somewhat different from that of NDV (Fig. 2).

Experimental Disease in Pigs and Mice

Pigs

The two apparently healthy pigs, 3 weeks old became ill on day 4 after inoculation with the suspension of an infected brain. The most pronounced

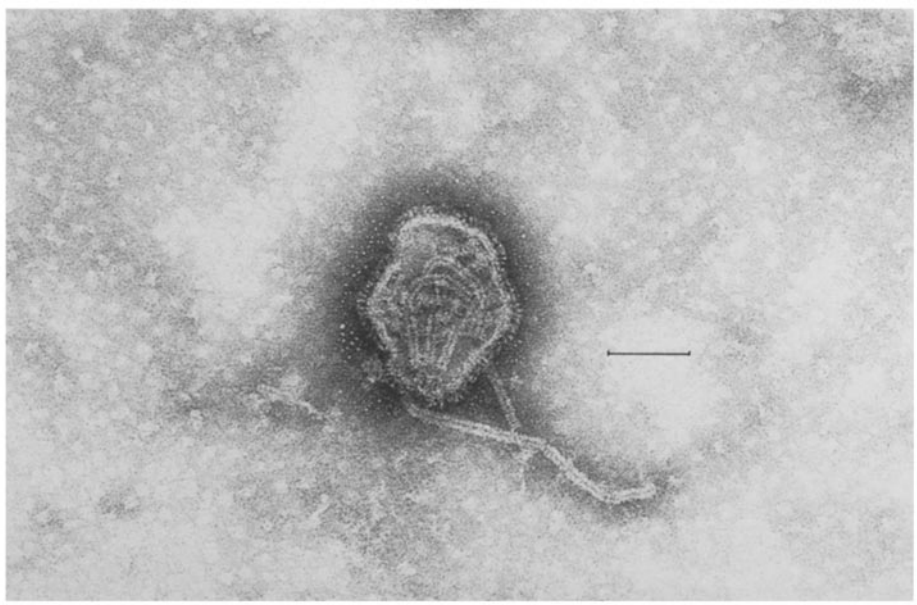

Fig. 1. Electron micrograph of a partially disrupted LPM virus particle. The bar indicates 100 nm

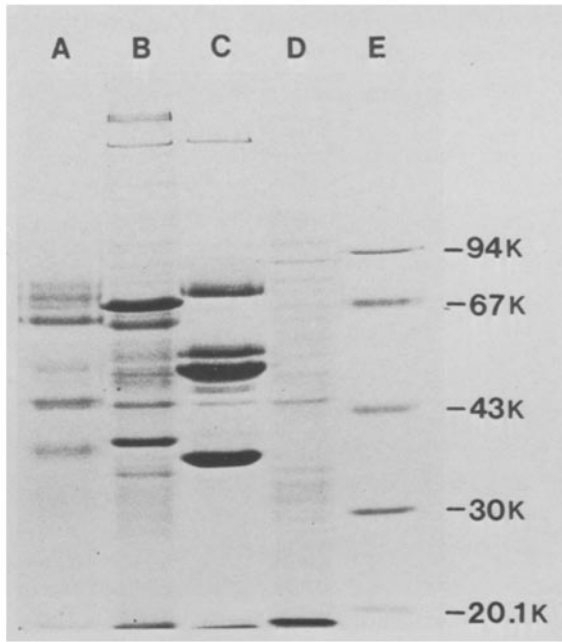

Fig. 2. SDS-PAGE of LPM virus proteins. The molecular weights markers are indicated (Phosphorylase b, Albumin, Ovalbumin, Carbonic anhydrase, Trypsin Inhibitor). *A* PIV-3, strain U-23. *B* The LPM virus. *C* NDV, strain Montana. *D* uninfected and sonicated PK-15 cells. *E* Molecular weight markers

signs of the disease were fever (41° C), anorexia, conjunctivitis and nasal and ocular discharges. On the 9th day the animals showed mild corneal opacity, torticollis, muscular tremor, lack of coordination, running movements and protration. The animals were killed after 22 days of observation. No virus could be recovered from the organs. Sera obtained on days 9, 15 and 21 p. i. showed HI titers of 16 to 128. One serum from the 21th day p. i. neutralized the LPM virus and was positive in the immunofluorescence test.

The additional two pigs, 3 months old, inoculated with the 5th passage of LPM virus in cell culture developed respiratory symptoms on day 3 p. i. and showed fever, conjunctivitis and nervous disorder. In general, the symptoms in these two animals were mild. The one uninoculated animal in contact with these inoculated animals developed only mild respiratory symptoms.

Mice

All mice inoculated intracerebrally with the suspension of an infected brain or cell culture suspension showed tremor and excitation and died on days 3 to 5 after inoculation. The LPM virus was isolated from the brain, lung, lever and spleen. The mice inoculated intraperitoneally remained healthy.

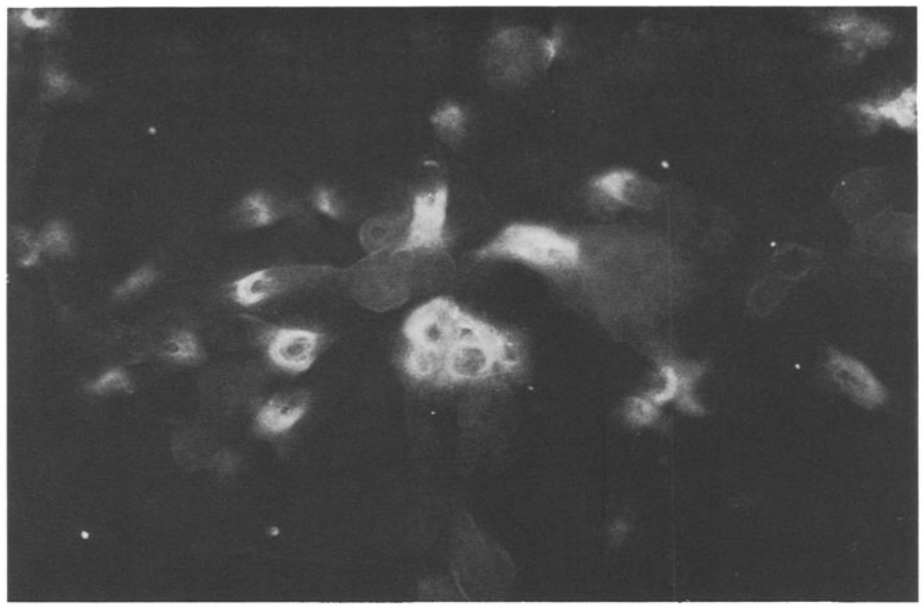

Fig. 3. Cytoplasmic fluorescence in PK-15 cells infected with the LPM virus

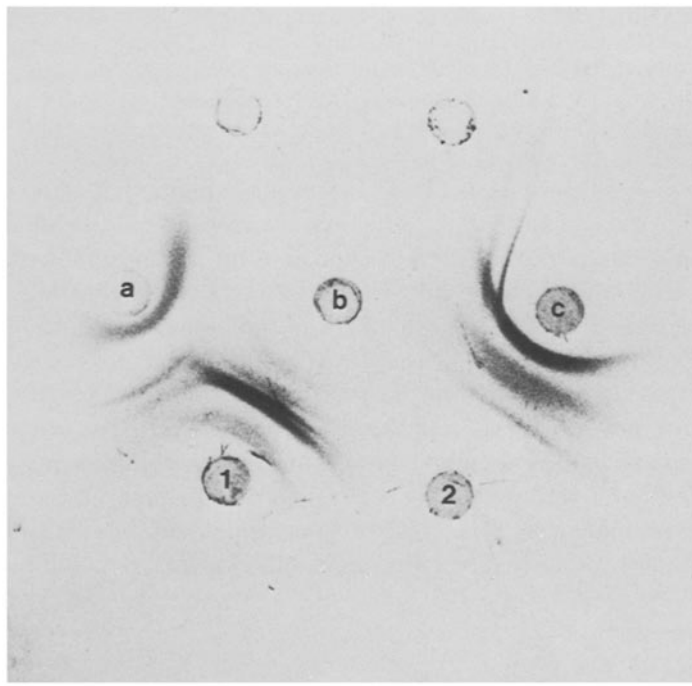

Fig. 4. Immunodiffusion test. *a* Serum from a pig, experimentally infected with the LPM virus; *b* Serum from a rabbit, hyperimmunized with the LPM virus; *c* Serum from a guinea pig, hyperimmunized with a bovine PIV-3 strain. *1* LPM virus; *2* PIV-3. There is no cross-reactivity between *2* and *b*

Immunological Studies

Convalescent sera from 20 naturally and 5 experimentally infected pigs were examined for the presence of HI and SN antibodies to LPM virus. These sera had HI titers of 16 to 128 and SN titers of 4 to 32. Hyperimmune sera against some other paramyxoviruses—human parainfluenza virus types 1, 2, and 3, mumps virus, measles virus, RSV and NDV—failed to react with the LPM virus in SN, CF, ID and HI tests. Infected PK-15 cells were examined by indirect immunofluorecence with sera from naturally infected or non-infected pigs. A diffuse cytoplasmic fluorescence was observed (Fig. 3) with the former sera. In ID tests there was only a homologous reactivity, i. e., LPM virus did react with anti-LPM serum but not with the other antisera tested. Furthermore, the anti-LPM sera did not react with the PIV-3 or NDV. Fig. 4 shows an example of the results.

Cytopathology

PK-15 cells, inoculated with the LPM virus and subsequently stained with hematoxylin and eosin, showed vacuolation of the cytoplasm within 10 hours followed by formation of massive syncytia which often contained more than 50 nuclei. Numerous cytoplasmic inclusion bodies surrounded by a halo were observed in monkey (GMK) cells but only a few in PK-15 cells. Cells with acidophilic cytoplasm, pycnotic nuclei, cariorhexis and cariolysis were seen within 24 hours after infection. The histology of the brain of diseased animals showed a non-supurative meningoencephalitis with a marked perivascular lymphocytic infiltration, gliosis and necrosis. Neuronophagia, neuronal and glial necrosis, meningitis and choroiditis were also seen. The most affected areas were the thalamus, midbrain and cerebral cortex. An interstitial pneumonia, affecting the apical and cardial lobes was also observed.

Discussion

The present report deals with the characterization of a virus isolated from the brain of a piglet with CNS disorder, pneumonia, and corneal opacity. This virus, named LPM, has basic properties in common with members of the genus Paramyxovirus.

The LPM virus has an unusually broad spectrum of hemagglutinating activity and it grows in a large variety of cell types with the formation of extensive syncytia in some of them. The virus prefers cell culture systems instead of embryonated eggs for cultivation. By this latter property the LPM virus resembles more the group of parainfluenza viruses than NDV and mumps virus (for references, see 1). Even the profiles of viral structural proteins, shown in Fig. 2 confirm the resemblance to a parainfluenza virus. However, no serological relationship has been detected so far between the

LPM virus and the parainfluenza viruses types 1, 2 and 3. Thus, it appears that the LPM virus may be a hiherto unknown paramyxovirus.

Heretofore, no studies have appeared concerning a paramyxovirus which infect pigs with affinity to the CNS. After natural infection, the virus kills piglets less than 10 days old. The animals show disturbances of the CNS, different from those previously observed in pigs in Mexico. Corneal opacity is pathognomonic and presumably due to precipitation of immune complexes. Corneal opacity is the only sign of the disease in older and adult animals even if they also can develop nervous symptoms. In sows, the reproduction system can be affected.

It is not known whether the LPM virus can naturally infect and kill other animal species. To our knowledge the pig is the only natural host. Since the first described outbreak in Mexico in 1980, there has been no report on the disease in other animal species or man either in Mexico or elsewhere. Following experimental infection of pigs 3 weeks old, the pathogenicity of the LPM virus indicates that the virus was the agent responsible for the disease. The clinical signs were similar to those reported in natural cases. However, experimentally infected animals at the age of 3 weeks survived the observation period of 22 days. This is in accord with the observation that lethality is more or less restricted to the age of up to 10 days, as reported by STEPHANO *et al.* (9).

The experimental infection of mice was fatal when the LPM virus was inoculated intracerebrally. The symptoms were those deriving from the CNS and death occurred 3 to 5 days after inoculation. The LPM virus could be recovered from different organs after propagation in PK-15 cells. It appears that the pathogenicity of the LPM virus in mice depends on the route of inoculation and that the virus has to reach the CNS in order to induce a lethal disease.

Summarizing, the LPM virus proved to be highly infectious and lethal for very young pigs with a tropism centered upon the CNS and respiratory tract (lungs). Such a combination is not unusual for at least one paramyxovirus, the NDV, the mesogenic isolates of which attack young chickens causing pneumoencephalitis (4).

The prevalence of infection and disease caused by the LPM virus has not been studied. Such studies could be performed by serological screening if this virus occurs as a single serotype. Therefore, strains isolated from different outbreaks should be compared. At present, the origin of the virus and how it spreads mong the farms in Mexico is unknown. Further epidemiological and virological studies are in progress.

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