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Cytomegalovirus excretion in gnotobiotic pigs

By N. EDINGTON, R. G. WATT and W. PLOWRIGHT
Department of Pathology, Royal Veterinary College
London, NW1 0TU

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SUMMARY

Germ-free piglets were infected intranasally with porcine cytomegalovirus (PCMV) at 1 day (group A) or 3 weeks of age (group B). Viraemia and virus excretion by the nasal, pharyngeal and conjunctival routes was studied up to the time of death or to 12 weeks. Virus was also sought in tissues at death or at slaughter, as well as in a few urine samples.

Viraemia was detected in group A between days 5 and 19 after infection and in group B between days 14 and 16 inclusive. The chief route of virus excretion was the nasal mucosa, followed by the pharynx and conjunctiva; the maximal duration of excretion by these routes was 32, 25 and 14 days for pigs of group A and 9, 7 and 4 days for group B. The quantity of virus was also greater in the former group, of which 3 died of generalized PCMV infection.

A viruria was demonstrated in 2 animals.

Antibody detectable in indirect immunofluorescence (IIF) tests appeared towards the end of the third week, reaching maximal titres at 5 to 7 weeks after infection. The mean peak titre of antibody in group B was lower than in group A.

Corticosteroid treatment at days 56–62 after infection resulted in some recrudescence of virus excretion, accompanied in group B by about a twofold increase in IIF antibody. PCMV was isolated in cultures of lung macrophages from 4 of 7 animals killed at about 12 weeks after inoculation.

INTRODUCTION

Cytomegaloviruses have been identified in many species (Plummer, 1973) but only in mice and infants has the pattern of virus excretion and antibody production been studied (Rowe, Hartley, Cramblett & Mastrota, 1958; Medearis, 1964; Levinsohn *et al.* 1969). Porcine cytomegalovirus is ubiquitous in its distribution (Corner, Mitchell, Julian & Meads, 1964) and, in Britain, can be readily isolated from the nasal excretions of piglets 5 to 8 weeks old and less frequently from younger animals (Plowright, Edington & Watt, 1976).

Experimental infection of gnotobiotic pigs allowed a more detailed analysis of the pattern of virus excretion, which could be compared both with that in conventional pigs and with the behaviour of cytomegaloviruses in other species. It also provided an opportunity to investigate the possible exacerbation of infection induced by corticosteroids.

MATERIALS AND METHODS

Inoculum

The B6 strain of porcine cytomegalovirus (PCMV) was used after 5 passages in gnotobiotic pigs (Edington, Plowright & Watt, 1976) and 3 in pig lung macrophage (PLM) cultures. Clarified culture fluid having a titre of $10^{4\cdot7}$ TCD 50/ml. was employed as an inoculum.

Experimental animals

A litter of 10 gnotobiotic pigs was obtained and reared as described by Trexler (1971); they were at first housed in pairs in small isolators.

Experimental procedure

Six pigs (group A, nos. 1–6 incl.) received 1 ml. of virus intranasally when they were 1 day old and the remaining 4 (group B, nos. 7–10 incl.) were similarly inoculated at 3 weeks.

At 6 weeks of age the 2 groups of surviving pigs were transferred to 2 large isolators and, at 8 weeks after infection, they were injected with 2·0 mg./kg. of dexamethasone and 6·0 mg./kg. prednisolone, the preparations being divided equally between the intravenous and muscular routes. Altogether, 3 such injections were given to each animal at 3-day intervals.

Throughout the experiment, nasal, pharyngeal and conjunctival swabs were taken thrice weekly from all animals, while blood for leucocyte separation was obtained at the same time from 2 pigs in each group. Serum was collected once a week and urine whenever possible. Each group of pigs was killed 12 weeks after virus inoculation and portions of turbinate, lung and kidney tissue were taken for virus isolation and histological examination; in addition, direct cultures were made from pulmonary macrophages of each pig (Watt, Plowright, Sabo & Edington, 1973).

Virus isolation

Cultures of PLM cells were prepared and maintained as described previously (Watt *et al.* 1973; Plowright *et al.* 1976). Swabs were expressed in 2·0 ml. of phosphate-buffered saline (PBS) containing 10% fetal bovine serum and 1000 units/ml. penicillin, 1000 μg./ml. streptomycin, 1000 units/ml. kanamycin and 25 μg./ml. fungizone. The fluid was either stored until required at $-70°$ C. or 0·2 ml. samples were inoculated immediately into 3 PLM cultures and adsorbed at $37° \pm 0{\cdot}5$ °C. for 1 hr.

Using 7·5 mg./ml. EDTA as an anticoagulant, leucocytes were separated from 2·0 ml. of blood, which was centrifuged at 90 *g* for 5 min. The leucocyte fraction was washed twice with PBS, resuspended to 0·6 ml., and 0·2 ml. of the final suspension was inoculated into 3 PLM cultures. These were incubated for 1 hr. at 37° C. and, after rinsing with PBS, maintenance medium was finally added.

Urine samples were spun at 1400 *g* for 10 min. and the deposit was inoculated into PLM cultures, using a final washing with PBS to reduce acidity.

Table 1. *Leucocyte counts following the administration of corticosteroids to gnotobiotic piglets*

Days after virus inoculation*	Mean total leucocyte counts × 10^3/mm.^{3}	
	Group A	Group B
42	15·5	9·9
49	13·3	12·2
56	12·1	14·4
63	19·9	20·2
70	17·2	13·8
77	13·1	15·6

* 2·0 mg./kg. dexamethasone and 6·0 mg./kg. prednisolone were administered on days 56, 59 and 62 after virus inoculation.

Tissue homogenates, prepared in Ten-Broek grinders, were treated as described elsewhere (Plowright *et al.* 1976).

Antibody determinations

Indirect immunofluorescence (IIF) tests for serum antibody were performed as already described (Plowright *et al.* 1976).

RESULTS

Clinical events

Three of 6 piglets in group A died within the first 5 weeks; no. 5 died on day 16 after infection, with widespread subcutaneous oedema and petechiae at necropsy; no. 3 died on day 22 without any macroscopic lesions, and pig no. 4 died on day 34 as a result of torsion of the colon and caecum. The remaining 3 piglets in this group (1, 2 and 6) remained smaller and more uneven than those in group B, all 4 of which remained healthy throughout the experiment. No clinical reaction followed the administration of dexamethasone and prednisolone and mean total leucocyte counts are recorded in Table 1; they were not significantly affected.

Virus recovery

Isolations of virus from leucocytes and various swabs are recorded in Figs. 1 and 2, for groups A and B respectively.

In group A, minimal quantities of virus were present very rarely in nasal and conjunctival swabs between days 2 and 12 after inoculation, but some of the earliest isolations may have been accounted for by persistence of the inoculum, rather than local proliferation or excretion. Viraemia was detected in these animals between days 5 and 19 inclusive and towards the end of this time the first continuous recoveries were made from nasal, pharyngeal or ocular secretions (Fig. 1). The maximum duration of nasal excretion, excluding sporadic isolations before viraemia, was 32 days, whilst pharyngeal and conjunctival shedding persisted for 25 and 14 days respectively. Urine samples were obtained very infrequently, but 2 animals were positive at 14 days and one at 19 days, probably with a moderate titre.

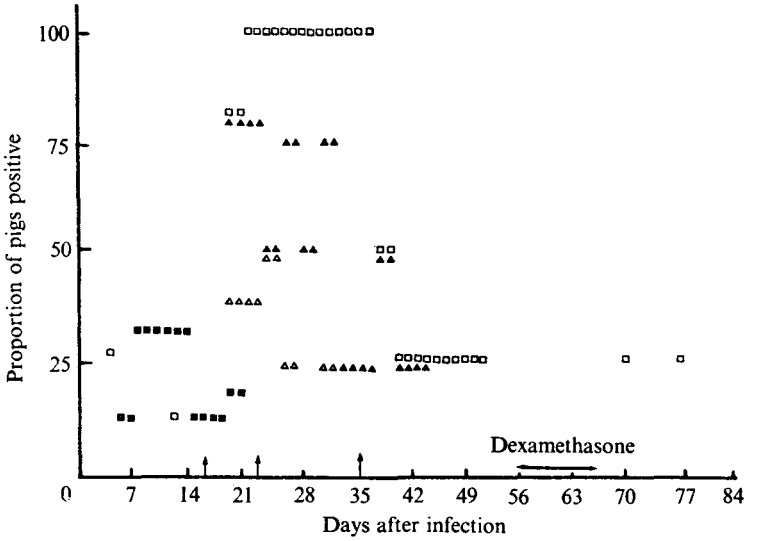

Fig. 1. Recovery of virus and the effect of corticosteroids on pigs infected with PCMV at one day of age. □□, Nasal excretion; ▲▲, pharyngeal excretion; △△, conjunctival excretion; ■■, viraemia; ↑, death of one pig.

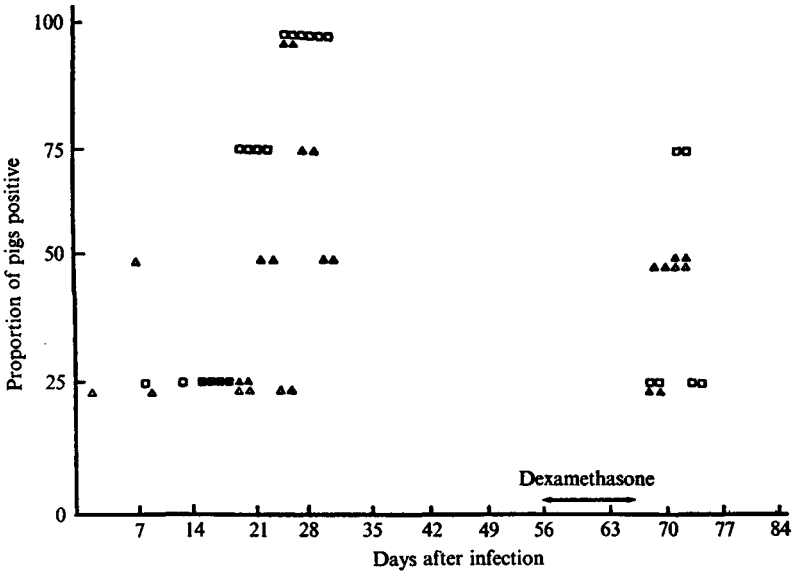

Fig. 2. Recovery of virus and the effect of corticosteroids on pigs infected with PCMV at 21 days of age. □□, Nasal excretion; ▲▲, pharyngeal excretion; △△, conjunctival excretion; ■■, viraemia.

In pigs of group B (Fig. 2) virus was again recoverable occasionally from swabs taken between days 2 to 12, but this was never continuous and the quantities, as judged by the number of infected cells in stained cultures, remained small. The viraemia was brief and later in onset, (days 14–16) whilst virus excretion was detected for shorter periods, namely 9 days from nasal swabs (19–28 days after

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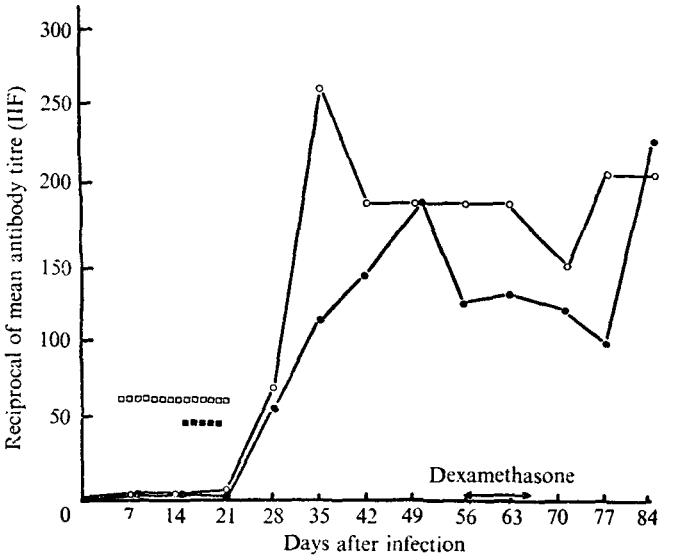

Fig. 3. The duration of viraemia and the development of antibody in pigs infected with PCMV. □ □, Viraemia in pigs inoculated at 1 day of age; ■ ■, viraemia in pigs inoculated at 21 days of age; ○ ○, antibody titre in pigs inoculated at 1 day of age; ● ●, antibody titre in pigs inoculated at 21 days of age.

infection), 7 from throat swabs (days 21–28) and 4 days only from the conjunctiva (days 19–23).

Cytomegalovirus was recovered from the kidney and nasal mucosa of nos. 3 and 5, the first 2 pigs to die in group A, but isolation was not attempted from the third animal (no. 4).

After corticosteroid treatment, virus was isolated at about 70 days after infection from nasal, pharyngeal or conjunctival swabs of 3 or 4 pigs in group B (Fig. 2). This was 38 days after the last previous isolation from nasal secretions. Direct cultures of lung macrophages were also positive from these 3 pigs at 83 days and also from one animal in group A. It was concluded that the corticosteroid treatment did probably result in low-level recrudescence of the PCMV infection.

Antibody development

In both groups A and B, antibody detectable at a serum dilution of 1/4 or higher first appeared in the third week after infection, its detection thus coinciding with the termination of viraemia and the commencement of virus recovery from secretions (Fig. 3). Antibody attained maximal individual titres of 1/64 to 1/256 by the end of the 6th week at which time excretion of virus was detectable in only one pig. High mean titres were maintained during the remaining 6 weeks of the experiment.

In pigs of group B from which virus was isolated at days 68 and 70 after infection there was a twofold increase in the mean titre at 82 days, thus reinforcing the suggestion from late virus recoveries that some reactivation of infection followed corticosteroid treatment (Fig. 3).

Pathology

Histological examination of tissues taken at 12 weeks after inoculation of virus showed no inclusion bodies in the 7 surviving pigs. Small red infarcts were seen along the margins of the spleen in two pigs (nos. 2 and 9). Focal interstitial aggregations of lymphocytes and plasma cells occurred in the kidney cortex and were also associated with involution of the nasal mucous glands in all 3 animals of group A and 2 of 4 (nos. 9 and 10) in group B. Foci of perivascular, lymphocytic infiltration and neuronophagia were recorded, predominantly in the brain stem, in 2/3 pigs in group A and in 2/4 in group B.

The widespread distribution of cytomegaly and basophilic intranuclear inclusions in capillary endothelial cells, particularly in the lung, kidney, spleen and liver tissue of pig no. 5 which died on day 16, was consistent with previous observations in very young animals (Edington *et al.* 1976). Typical cytomegalic inclusion-bearing cells were found in pigs 3 and 4, dying at days 22 and 34 respectively; they were commonest in epithelial cells of the nasal mucous glands and kidney tubules. There was also a diffuse encephalitis in pig no. 3 but the torsion of the colon and caecum which occurred in pig no. 4 was apparently unrelated to PCMV infection.

DISCUSSION

After intranasal infection of day-old gnotobiotic pigs with PCMV the first unequivocal recoveries of progeny virus were from the blood, at 5 to 12 days particularly. However, when 3-week old piglets were infected there was somewhat clearer evidence of a primary phase of multiplication in tissues associated with the respiratory tract or conjunctiva, since excretion by these routes was detected more frequently at days 7 to 12, whereas viraemia was not demonstrable until days 14 to 16. Evidence for a phase of primary replication in the nasal mucosa was also obtained in an earlier experiment (unpublished) in which day-old pigs showed nasal excretion of PCMV at day 6.

Generalization in younger animals led to death of half of them and was accompanied in all by a greater duration and intensity of viraemia and virus excretion by all the routes investigated. This confirms earlier observations on the effect of age on susceptibility to PCMV infection (Edington *et al.* 1976).

The predominance of nasal over pharyngeal excretion is not perhaps surprising if the main source of virus is infected mucous glands in the nasal mucosae, as the pathological and virological evidence suggests. It also helps to explain the success of epidemiological investigations using nasal excretion studies in commercial herds (Plowright *et al.* 1976); PCMV was demonstrated in the majority of animals tested between 5 and 8 weeks of age. If the period to nasal excretion is regularly about 19 days, as found in the present experiment, then the field observations point to infection occurring normally at 2 to 3 weeks of age, which was the time when several litters of piglets and their dams were mixed together for the first time. The group of pigs in the same field study which showed massive excretion of virus at 3 weeks of age must, by the same criterion, have been infected congenitally or in the immediate postnatal period.

The excretion of PCMV by experimental pigs lasted a maximum of 32 or 10 days according to their age at infection and for piglets in commercial herds a maximum of 3 weeks; these times are considerably shorter than those for cytomegalovirus in other species, such as 2 years for the mouse (Medearis, 1964) or several months for man (Levinsohn *et al.* 1969; Numazaki *et al.* 1970) but shortcomings in the PLM culture system for virus isolation could account for some of the disparity, as already discussed (Plowright *et al.* 1976).

It was noticeable in this study that antibody production was first detected in both groups by 3 weeks but increased more rapidly in group A, for which the mean titre reached a maximum (1/266) at 5 weeks, compared with a lower maximum (1/168) attained only after 7 weeks in group B. Nevertheless nasal excretion of virus continued to day 51 in group A and it would be interesting to know whether antibody is present in the nasal secretions and if, like serum antibody, it has a very poor neutralizing activity (Plowright *et al.* 1976). Another possibility is that more virus infectivity in later swab samples was cell-associated and not, therefore, vulnerable to antibody.

The administration of corticosteroids, whilst it did not produce leucopenia, did appear to result in re-excretion at low levels and it may have facilitated isolation of virus from pulmonary macrophages of 4 pigs slaughtered 82 days after first infection. Such reactivation or recrudescence of cytomegalovirus infection is well recognized after immunosuppressive treatment of man (Lang, 1972) and the mouse (Henson, Smith, Gehrike & Neapolitan, 1967), as well as being readily induced with other herpesvirus infections such as infectious bovine rhinotracheitis in cattle (Sheffy & Davies, 1972).

Recrudescence of infection under conditions of stress, without clinical signs, may account for the transfer of PCMV infection from adult breeding stock to young piglets or, alternatively, might precipitate transplacental infection of fetuses. These proposed mechanisms may account for the reported ability of breeding animals, whether female or male, to introduce PCMV infection into hitherto unaffected herds.

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