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Replication of Transmissible Gastroenteritis Coronavirus (TGEV) in Swine Alveolar Macrophages

By H. LAUDE,* B. CHARLEY AND J. GELFI

Institut National de la Recherche Agronomique, Station de Virologie et d'Immunologie,
78850 - Thiverval-Grignon, France

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SUMMARY

Several strains of the enteropathogenic coronavirus transmissible gastroenteritis virus (TGEV) have been shown to replicate in alveolar macrophages maintained *in vitro*. A distinct cytopathic effect was observed at a multiplicity of infection ≥ 0.1 . Infected cells released infectious virus. The extent of both virus production and cell destruction was highly dependent upon the virus input. At low input, cell viability was affected only slightly, and a delayed and persistent virus production could be observed. TGEV infection of macrophages also led to a marked synthesis of type I interferon. Thus, the possibility that alveolar macrophages act as an extra-intestinal target for TGEV must be considered.

INTRODUCTION

Cells of the mononuclear phagocyte system often play a key role in determining susceptibility to virus infection (Mogensen, 1979; Morahan & Morse, 1979). One of the best defined systems for these studies employs the coronavirus mouse hepatitis virus where a positive correlation between growth of virus in macrophages and susceptibility of the host to the infection has been established (for references, see Virelizier, 1981). In contrast, only limited data are available concerning the biological effects of other members of the coronaviridae on macrophage-like cells (Pedersen, 1976; Patterson & Macnaughton, 1982).

The interaction of transmissible gastroenteritis virus (TGEV), a porcine coronavirus, with its natural host has been studied in great detail and serves as a model for the pathogenesis of enteric virus infections. The main targets of the virus are the absorptive epithelial cells covering the small intestinal villi (Pensaert *et al.*, 1970). The villous atrophy observed in infected animals leads to severe intestinal disorders, which are frequently lethal during the neonatal period (Haelterman, 1972). However, there are indications that TGEV may also be harboured in the respiratory tract, although clinical symptoms could not be unequivocally demonstrated at this level. Virus recovery and transmission of the disease using lung extracts or pharyngeal swabs from naturally or experimentally TGEV-infected animals have been reported by different authors (Underdahl *et al.*, 1974; Kemeny *et al.*, 1975). Moreover, studies on the distribution of cell-adapted virus strains in infected newborn piglets showed that virus titres were as high in the respiratory as in the digestive tract (Furuuchi *et al.*, 1978/9).

In view of these findings, we decided to investigate the interactions between TGEV and the alveolar macrophage. We present evidence that, *in vitro*, this cell type supports the replication of TGEV.

METHODS

Collection and culture of swine alveolar macrophages. Macrophages were obtained from 5- to 7-month-old pigs that were conventionally reared and kept in our facilities. All animals were devoid of antibodies against TGEV. Collection of cells was performed by washing the lungs of pigs that had been sacrificed and exsanguinated, as described previously (Charley, 1982). The recovered fluid was centrifuged at 600 g for 15 min, and the cells were washed twice. Cell suspensions contained $90 \pm 5\%$ macrophages (esterase-positive cells), the contaminating cells

being lymphocytes. Adherent cells were cultivated on plastic supports at a density of about 10^6 cells/cm² in RPMI 1640 medium supplemented with 20% foetal calf serum, 2 mM-glutamine and antibiotics. Following overnight incubation at 38 °C in a 5% CO₂ incubator, the medium was changed. Almost 100% of adherent cells were shown to phagocytose zymozan particles (Charley, 1982).

Determination of macrophage viability. Monolayers were rinsed and stained by adding neutral red (RAL, Biolyon, France) diluted 1:10⁴ in Eagle's medium buffered by Tris-HCl (160 mM, pH 7.6). After 30 min at 37 °C, supernatants were discarded and the monolayers were rinsed. Then, 0.1 ml/cm² of 90% ethanol buffered to pH 7 with 10 mM-Tris-HCl was added in order to solubilize incorporated dye, and the absorbance at 460 nm was measured. Validity of this procedure had been determined by comparison with a conventional eosin staining technique. Results were expressed as % of control, i.e. (A_{460} of infected cells/ A_{460} of control cells) × 100. A_{460} values of uninfected cultures ranged from 0.5 to 0.6 in the different experiments.

TGEV strains and infectivity assays. Two low-passage strains, D₅₂-5 and 6386-5, and one high-passage strain, Purdue 115, were used. These cell-adapted strains are partly attenuated for piglets. Their origin and propagation have been described elsewhere, as well as the plaque assay on the porcine cell line RPTG (Laude *et al.*, 1981). The virulent strains Miller and D₅₂ were obtained from E. H. Bohl (Wooster, Ohio, U.S.A.) and P. Vannier (Ploufragan, France) respectively. Both strains have undergone one passage in 2-day-old specific-pathogen-free piglets in the laboratory. Virus suspensions consisted of clarified small intestine fluid from animals sacrificed at 20 h post-infection. A limiting dilution technique was used for infectivity assay of the latter strains: confluent monolayers of the ST cell line (obtained from E. H. Bohl), established in 96-well plates (Falcon) were maintained after infection in Eagle's minimum essential medium supplemented with 20 µg/ml pancreatin (grade 6, Sigma). After 3 days of incubation at 38 °C, the cell sheets were fixed and processed for immunofluorescence as described below. Titres (p.f.u./ml) of wild-type viruses could be estimated by reference to an internal standard consisting of an aliquoted suspension of Purdue virus of known titre.

Indirect immunofluorescence test. Macrophage cultures in 24-well trays (Costar) were fixed at intervals after infection with cold acetone-ethanol (75/25%), and processed for immunofluorescence using (i) a porcine hyperimmune serum to TGEV (D₅₂ strain) diluted 1:50, and (ii) a rabbit fluorescein conjugate against porcine immunoglobulin diluted 1:50 (Institut Pasteur, Paris, France). Both reagents had been adsorbed with fixed RPTG cells. The plates were examined using an epi-fluorescence apparatus (Ploem).

Interferon (IFN) assays. Samples were centrifuged for 10 min at 10⁵ g in an air-driven ultracentrifuge (A100, Beckman) prior to the assay. Porcine IFN activity was determined in the MDBK cell line using vesicular stomatitis virus as a challenge, as previously described (La Bonnardière & Laude, 1981). Antiviral titres were expressed in MDBK units/ml by reference to an internal standard IFN, previously titrated by the 50% reduction method.

RESULTS

Effect of TGEV infection on cell viability

Cell-adapted strains of TGEV induced a rapid and distinct cytopathic effect (c.p.e.) in alveolar macrophage cultures. The cytopathic alterations consisted of degenerative changes followed by cell detachment from the support (Fig. 1). No syncytium formation was observed. Inoculation at a multiplicity of infection (m.o.i.) of about 2 p.f.u./cell of both the low-passage strains, D₅₂ and 6386-5, or the high-passage Purdue strain consistently resulted in extensive cell destruction, frequently reaching 50 to 75% within 24 h post-infection. No such effect was seen using a virus suspension, either u.v.-inactivated or mixed with neutralizing antibodies (Table 1). Some variation was seen in the time course of c.p.e. depending on the cell batch used. Cultures observed for 1 week after infection showed a correlation between virus input and the c.p.e. level at any time after infection (Fig. 2). At a m.o.i. < 0.1 the c.p.e. remained limited.

Antigen and virus production

To determine whether the cytopathic changes were associated with viral replication, immunofluorescence and infectivity experiments were performed. In cultures stained 24 h post-infection at a m.o.i. of 2, more than half of the attached cells showed a specific cytoplasmic fluorescence. Infectivity titres between 5×10^4 and 5×10^5 p.f.u./ml were obtained regardless of the virus strain used (Table 1). Maximum virus production at 38 °C was reached about 12 h post-infection at a m.o.i. from 0.1 to 5. Although virus yields were low, it could be assumed that

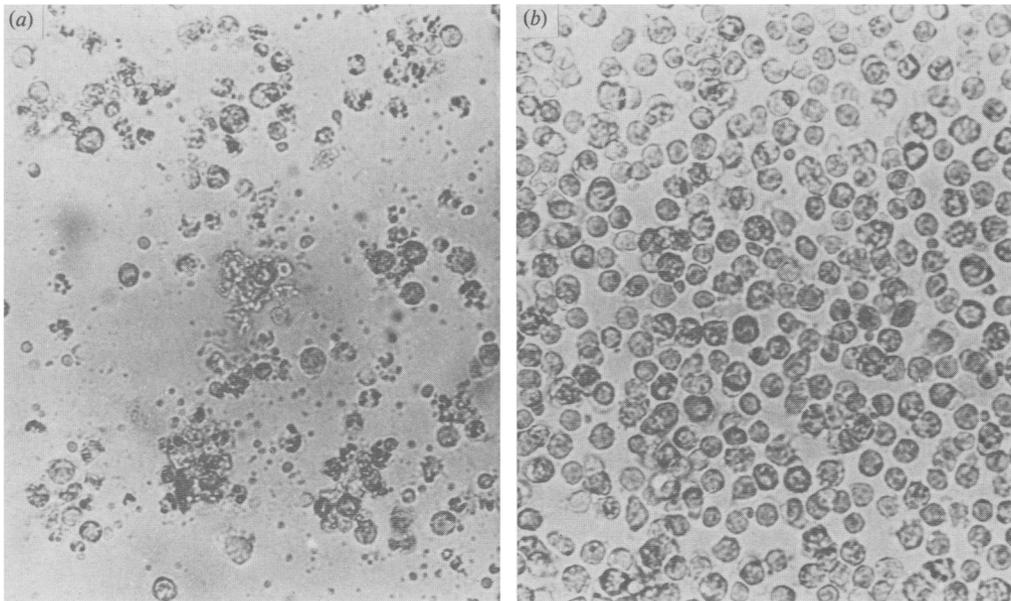


Fig. 1. TGEV-induced cytopathic effect in alveolar macrophages. (a) Typical aspect under light microscope of a culture 24 h post-infection using 5 p.f.u./cell of Purdue virus; (b) uninfected control culture. Magnification $\times 297$.

Table 1. Multiplication of different cell-adapted strains of TGEV in two preparations of macrophages*

Virus strain (1 to 3 p.f.u./cell)	Cell viability (% of control)		Infectivity titre (p.f.u./ml)		IFN titre (MDBK units/ml)	
	Prep. 1	Prep. 2	Prep. 1	Prep. 2	Prep. 1	Prep. 2
D ₅₂ -5	39	42	1.2×10^5	9.5×10^4	470	250
6386-5	37	48	4.5×10^4	7×10^4	270	170
Purdue 115	26	35	4.5×10^5	1.1×10^5	1400	510
Purdue 115 + antiserum†	>95	NT§	$< 10^1$	NT	NT	NT
U.v.-inactivated‡	>95	NT	$< 10^1$	NT	≤ 10	NT

* Cell cultures were established in 25 cm² plastic flasks (Falcon). After 1 h adsorption at 38 °C, the inoculum was discarded and the monolayers were carefully rinsed, then maintained in RPMI 1640 medium plus 5% foetal calf serum. After 24 h incubation, cultures were submitted to one cycle of freezing-thawing prior to infectivity and antiviral activity assays. Cell viability was measured in duplicate cultures by neutral red uptake as described in Methods.

† Immune serum anti-D₅₂, used 1:20 (neutralizing titre 4 log₁₀).

‡ 6 W/m² for 10 min.

§ NT, Not tested.

the infectivity recovered essentially corresponded to newly synthesized virions, rather than to residual virus from the inoculum, since titres consistently increased between 4 h and 12 h after infection. This was particularly evident when using low input multiplicities.

The subsequent evolution of virus production as a function of the m.o.i. was followed in two experiments, of which typical findings are illustrated in Fig. 3. Infectivity curves corresponding to the highest m.o.i. (1 and 0.1 p.f.u./cell) exhibited a biphasic profile. At the lowest m.o.i. (10^{-5}), a delayed and transient virus production was seen. At an intermediate m.o.i. (10^{-3}), a continuous release of virus was observed for 7 days after infection. Curves obtained by separately titrating cell-free and total infectivity were quite similar in shape. Nearly 60% of the

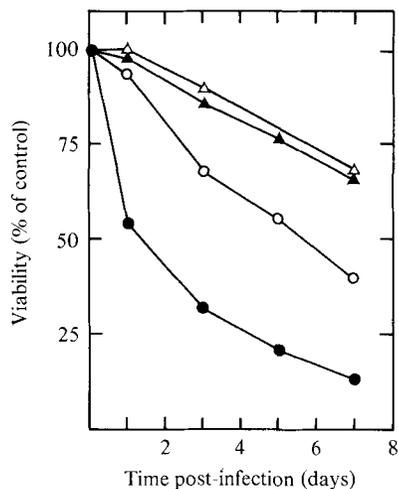


Fig. 2

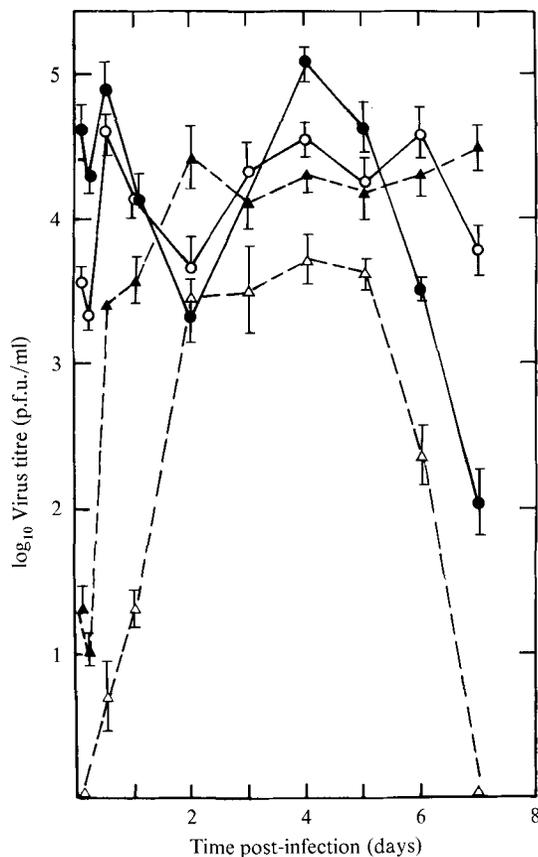


Fig. 3

Fig. 2. Effects of TGEV (Purdue strain) on the viability of macrophage cultures as a function of m.o.i. The experiment was carried out in duplicate with cultures established in 6-well trays. At the indicated times post-infection cell viability was measured by neutral red uptake. M.o.i. values were 1 (●), 10^{-1} (○), 10^{-3} (▲) and 10^{-5} (△).

Fig. 3. TGEV growth on macrophage cultures as a function of m.o.i. The experiment was carried out as described in Table 1. (Horizontal bars represent the titre range of duplicate cultures.) M.o.i. values were 1 (●), 10^{-1} (○), 10^{-3} (▲) and 10^{-5} (△).

virus produced was found to be cell-associated (results not shown). This kind of experiment could not be continued for more than 10 days of cultivation because of the appearance of fibroblastic cell clones.

Interferon (IFN) synthesis

Supernatant fluids of infected cultures contained a marked antiviral activity characterized as type I interferon (La Bonnardière & Laude, 1981), which was absent in control cells. Maximum IFN titres were reached about 16 h post-infection at a m.o.i. of 1 to 3, and ranged from 200 to 1500 units/ml (Table 1). In the experiments such as the one presented in Fig. 3, variations in virus titres could not be correlated with variation in the level of antiviral activity, which remained almost stable throughout the observation period.

Do wild-type TGEV strains replicate in macrophages?

We subsequently performed the same set of experiments with the wild-type strains of TGEV, Miller and D₅₂. Inoculation of virus suspensions at a m.o.i. of 0.3 to 0.001 caused no appreciable

change in the viability of the different macrophage preparations tested. Besides, our attempts to detect virus replication by infectivity tests or by immunofluorescence tests remained negative.

Finally, no IFN activity could be detected in infected cultures, strongly suggesting that no significant replication took place at the m.o.i. used. M.o.i. >0.3 were not assayed due to a non-specific toxic effect of intestinal viral suspensions.

DISCUSSION

Our study provides evidence for the replication of TGEV in cultured alveolar swine macrophages. The infection is cytotoxic and induces a marked IFN synthesis. Comparison of the outcome of TGEV-macrophage interaction with that of other swine viruses is of interest. Thus, the same macrophages do not support the replication of haemagglutinating encephalitis coronavirus (H. Laude *et al.*, unpublished results). Concerning IFN production, we did not detect significant activities in pseudorabies virus-infected or hog cholera virus-infected cultures using the same antiviral assay, although both viruses replicated efficiently (infectivity titres $>10^6$ p.f.u./ml). The same situation has been reported in the case of African swine fever virus (Wardley *et al.*, 1979). Therefore, TGEV appears to be an exceptionally good IFN inducer in this cell system.

Growth of TGEV in macrophages also displays some interesting features when compared to growth in conventional cell systems. As previously reported for other viruses (see Mogensen, 1979), the yields of infectious virus obtained from TGEV-infected macrophages are rather low: ≤ 0.1 to 0.2 p.f.u. per infected cell as compared to about 20 in the pig kidney cell lines tested. However, the duration of the virus cycle, i.e. 12 h, is comparable to that observed in a fully permissive cell system (10 h in the RPD cell line; Laude *et al.*, 1981). Several observations indicate that these cells partially restrict multiplication of TGEV. At low m.o.i., a minimal c.p.e. was shown to be associated with a persistent production of virus during at least 7 days after infection, after which the virus eventually disappeared. Also, the biphasic production observed at higher m.o.i. might reflect an uneven level of permissiveness within the cell population, some cells acquiring susceptibility to TGEV only after some days of cultivation. Accordingly, no virus antigen production could be detected in a significant proportion of macrophages infected for 20 h at a m.o.i. of ≥ 5 (up to 20% in certain preparations).

As TGEV effectively replicates in cultured alveolar macrophages, one could speculate that such cells would act as a target also in the respiratory tract. In support of this view, titres averaging 10^5 p.f.u./ml were consistently associated with cells collected by lung washes (3×10^6 cells) performed on newborn piglets infected orally with either Purdue or D₅₂-5 strain (H. Laude & C. La Bonnardière, unpublished results). Therefore, infection of macrophages could explain the presence, and perhaps the persistence (Underdahl *et al.*, 1975), of TGEV within the respiratory tract. The possibility remains, however, that additional cell types are involved since it has been claimed that TGEV replicated in alveolar and bronchiolar epithelial cells (Underdahl *et al.*, 1974).

It would be worth investigating whether other phagocytic cells offer a target for TGEV. Preliminary experiments *in vitro* indicated that porcine blood monocytes are not infected by TGEV. In contrast, Kupffer cells are likely candidates since in our hands the liver is one of the organs from which TGEV is most regularly detected in infected animals, whereas no virus can be isolated from the kidney (H. Laude & C. La Bonnardière, unpublished results). The possible interaction of TGEV with intestinal macrophages in the initial stages of infection also deserves attention. This problem is largely unexplored in the case of enteric viruses in general (Lefevre *et al.*, 1979). The presence of coronavirus-like particles in macrophages of the intestinal mucosa from TGEV-infected animals has been described (Chu *et al.*, 1982).

Another interesting point emerging from this work is the apparent differential behaviour of virulent wild-type viruses as compared to attenuated viruses: we failed to demonstrate any multiplication of the former in macrophage cultures. On the other hand, preliminary results indicated that alveolar macrophages isolated from neonate piglets infected with the wild-type TGEV strain produced virus and IFN (H. Laude & C. La Bonnardière, unpublished results). As

the macrophage cultures prepared for experiments *in vitro* were derived from older animals, we checked the susceptibility to wild-type virus D₅₂ strain of macrophages from 2-day-old piglets but found no evidence of infection. It is possible, however, that replication would be observed if a higher input of wild-type virus, than cell-adapted virus, were used, as reported for African swine fever virus (Wardley *et al.*, 1979).

Whether attenuated strains actually exhibit an enhanced tropism for macrophages, as indicated by our results, requires further investigation because of the virtual implications of this finding. Mononuclear phagocytic cells may be involved in virus-induced immunosuppression (Isakov *et al.*, 1982). It is now well known that TGEV strains adapted in culture, even after a low number of passages, lose the capacity to induce a reliable production of IgA in milk, responsible for the so-called lactogenic immunity (Bohl *et al.*, 1972). The mechanism responsible for such a phenomenon is not yet clearly understood and it may be questioned as to whether the deleterious effects of TGEV on macrophages could, in some way, impair the IgA response.

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