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HOG CHOLERA VIRUS: ART AND FACTS

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Résumé

VIRUS DE LA PESTE PORCINE CLASSIQUE: FAITS ET DIFFICULTÉS. — Les données accumulées au cours de deux dernières décennies sur l'agent de la Peste Porcine Classique (genre pestivirus) sont passées en revue. L'auteur analyse divers aspects de l'interaction virus-cellule, puis décrit les propriétés structurales et antigéniques du virion lui-même. En dépit de nombreux travaux, les connaissances acquises sur ce virus comportent d'importantes lacunes, que la mise en œuvre de nouvelles techniques permettra sans doute de combler.

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Introduction

Scientists who have experience of working on hog cholera virus (HCV) can testify that study of this virus requires a certain dose of art, whereas the path leading to the facts remains scattered with artifacts. There are few other economically important animal viruses for which the knowledge has advanced so slowly, particularly despite the intense research efforts invested. Investigations on HCV structure and replication have progressed even slower than that of bovine viral diarrhea (BVD) virus, its co-member within the pestivirus genus. The Commission of the European Community had sponsored studies on this important porcine pathogen from 1964 to 1976. Since then, the rhythm of publications concerning in vitro properties of HCV has decreased dramatically (fig 1). However, a new lease of activity - and of interest - on pestiviruses in general can be noticed during the last few years, and 1986 may well prove to be a turning point in basic research on HCV. This review has been undertaken upon the request of the organizers of the European Meeting on Pestiviruses, who felt the need of a survey dealing specifically with the experimental works done on HC virus host-cell interactions and the properties of the virion. For more informations, the readers are refered to several existing monographs on hog cholera (Mahnel and Mayr 1974) or on non arthropod borne togaviruses, including the pestivirus genus (Horzinek 1981a and b).

1. Virus host-cell interactions

Virus cultivation

Growth of HCV has been reported to occur in various primary or established mammalian cells, including of bovine and ovine origin (Pirtle and Kniazeff 1968). However, HCV usually replicates at higher rate in homologous cells, like other pestiviruses. Porcine kidney or testicle epithelial cell lines, such as PK15, RP-TG, ST, remain the most suitable system for its propagation. HCV has also been found to multiply at satisfactory titres levels

in the heterologous cell line FLM (foetal lamb muscle fibroblasts), which is highly susceptible to BVD and BD viruses (Laude 1979). The FLM line may therefore be used as a common cell system, for instance to analyze possible host related discrepancies between the three pestiviruses.

Immunofluorescence assay

As a notorious fact, cytopathology is generally absent in HCV-infected cultures. Actually, establishing steady state chronically infected cultures can be mentioned as one of the few things which are easy to do with HCV... Spontaneously infected cell lines have been described at several occasions (Shimizu et al 1969, De Castro 1973). Due to the lack of CPE, accurate titration of virus infectivity was, and still is, mainly based on the enumeration of microscopic fluorescent foci, a technique initially developped by Carbrey et al (1965). This technique has progressively replaced earlier infectivity assays, which were based on the exaltation by HCV of the cytopathic effect of a challenge virus, NDV (Kumagai et al 1958) or Teschen encephalomyelitis virus (Kubin 1965). A detailed review of HCV immunofluorescence litterature has been written by Avnaud and Bibard (1971), who also outlined the conditions for isolation of low virulent strains from field isolates.

Plaque formation assay

Very few laboratories have routinely used plaque formation under agar overlay for HCV titration. Van Bekkum and Barteling (1970) were the first to describe a direct plaque assay which depended on the use of secondary pig kidney cells: 5-6 days post infection, the plates were stained with neutral red, then incubated in the dark. A comparable technique has been proposed for titra-



Fig. 1. — Number of publications dealing with the in vitro properties of HCV from 1970 to 1985. The histogramm has been draft according to the references listed in the present paper.

tion of non-cytopathogenic strains of BVDV (Straver 1971). Later, just confluent monolayers of several pig kidney cell lines were found to be suitable for assaying HCV infectivity by this method: infected foci appeared 2-3 days p i as hazy plaques when viewed against a dark back-ground, or as white plaques after neutral red staining (Laude 1978a). These findings suggest that HCV induces some destabilization of the lyso-somes, which are known to be the target of the neutral red dye.

An alternative procedure, based on an heterologous interference phenomenon, was initially developped by Fukusho *et al* (1976) for the titration of the GPE- strain. It was subsequently found that pig kidney cell lines infected by any HCV strain tested, exhibit a transient refractory state to the highly cytopathogenic vesicular stomatitis virus (VSV), which leads to the formation of reverse plaque (Laude 1978b). The appearence of direct and of reverse plaques produced by HCV is shown in figure 2.

Cytolytic strains

Only two cytopathogenic strains of HCV have been described so far: the PAV strain (isolated from a persistently viremic pig; Gillespie et al 1961) and the CAP strain (isolated from the widely used IBRS2 cell line; Laude 1978c). The CPE is characterized by the appearence of cytoplasmic granulations, loss of refraction and progressive cytolysis. The CAP strain exhibits a small plaque phenotype, is antigenically close to the virulent Alfort strain and is attenuated for the pig. Such cytolytic strains have been suitable in developping convenient seroneutralization tests. As the PAV strain in the USA, the CAP strain has been used in France for large scale serologic investigations, with results similar to those obtained with the classical micro-IF technique (Vannier et al 1984).

Morphogenesis

Maturation of HCV in PK15 cells has been described in the classic study of Scherrer et al (1970). Newly formed particles were detected only from 10 hrs p i onwards, within the Golgi system and in cytoplasmic vesicles, but mostly in extracellular sites (fig 3). This was rather unexpected since, as stated below, major portion of the cultured virus remains cell-associated. In contrast with alphaviruses, convincing evidence of a budding process in HCV has not been reported so far. In addition, it was concluded from the small number of particles seen that the ratio of physical/infective particles should be very low. The relatively low amount of viral material produced by HCV infected cells is one of the factors which render its purification so troublesome.

Cell cycle

HCV growth in a pig cell line at 37 °C is characterized as follows: after a 5-7 hrs eclipse phase, a logarithmic increase in total virus harvest is observed until 15-16 hrs. Afterwards, the titre still tends to rise, resulting in maximum values of 10⁷-10⁸ PFU/ml (Aynaud 1968, Mengeling and Drake 1969, Danner and Bachmann 1970). In single step growth conditions, maximum titres are reached at 15 hrs and the yields do not exceed 50 PFU/cell (Laude 1977). As previously found by Danner and Bachmann, the titre of cell associated virus far exceeds that of released virus (fig 4). A viral RNA synthesis was detected in actinomycin D treated cells from 6-8 hrs p i onwards (Zeegers and Horzinek 1977).

Virulence markers

It is well established that HCV strains greatly vary in their virulence. Careful studies by Aynaud *et al* (1978) have led to an in vitro differenciation, by defining several temperature markers: the heat-resistance of infectivity and the optimum or supraoptimum temperature at which a strain produced maximum yields (under single step growth conditions). Fully virulent strains are heatresistant and multiply optimally at 39-40 °C, i e the mean body temperature of the pig. On the basis of these virulence markers, cold mutants of HCV were selected and used successfully as vaccine strains (Izawa *et al* 1971, Launais *et al* 1971).

Replication in lymphocytes

In vitro multiplication of the virus in lymphocytes and in mononuclear phagocytes was first described by Korn and Lorenz (1976). Van Oirschott (1980) has examined in detail the replication of HCV in cultured lymphocytes. High titers were recorded 6 to 7 days p i in culture stimulated at the time of infection, whereas resting cells appea red not to be permissive. A cytoplasmic fluorescence was shown in lymphoblastic cells only. Replication occurred irrespective of whether T or B cells mitogens were used. Despite the high degree of infection, neither the responsiveness of lymphocytes to mitogens nor cell viability were affected.

The same author has demonstrated the ability of HCV to grow in endothelial cells derived from aorta, again in the absence of cytopathic damages. Interestingly he failed to demonstrate viral antigens on the membrane of infected epithelial, endothelial or lymphoblast cells. So, the severe regressive alterations in endothelial and lymphoid tissues observed in vivo (Cheville and Mengeling 1969, Ressang 1973) remain to be explained.

Interferon induction

Several workers have mentionned the appearent lack of interferon (IFN) induction by pestiviruses in vitro (Van Aert *et al* 1969, Singh 1969, Diderholm *et al* 1974). It has been confirmed, using the MDBK cell line, which is the most sensitive system for porcine IFN assay, that HCV is a poor IFN inducer in epithelial cells and in macrophages (Laude and La Bonnardière, unpublished results). However an IFN activity was clearly demonstrated in the blood and organs of experimentally infected pigs (Torlone *et al* 1965). Hence, it can be hypothetized that the lymphocytes are main contributors to the IFN synthesis in vivo.

2. Physico-chemical and antigenic properties

Virion morphology

There has been no significant advance in this matter since the pionering studies of Horzinek et



Fig. 2. — Two procedures allowing plaque formation with HCV. A): direct plaques visualized by neutral red staining of an infected RPTG cell monolayer 48 h p i B): reverse plaques observed following an additional 24 h challenge with VSV (see text).



Fig. 3. — A): One step growth curve of HCV in PK15 cell monolayers. Infectious titres of released (●---●) and of cell-associated (●●) virus. B): Estimation of intracellular virus yield per hour per cell. (Reprinted from Laude 1977. Arch Virol, with permission).



al (1971). As a member of pestivirus genus, HCV represents the smallest lipid-containing virus known so far. The reported diameter of negatively stained particles varies between 42 ± 8 nm and 49 ± 8 nm (Enzman and Weiland 1978, Frost et al 1977). Undegraded virions are spherical and look rather amorphous (fig 5). In contrast to that observed with alphaviruses, no peplomers are visible, which would mean that they protrude little out of the membrane, or that they are masked by cell components intimately associated with the latter. However a fuzzy fringe (6-8 nm), indicative of surface projections, has been described on particles purified after formaldehyde fixation (Enzmann and Weiland 1978). This indicates that the peplomers might be lost during a conventional purification process. Isometric core particles of 27-29 nm have been shown to be released after urea or NP40 treatment (Horzinek et al 1971, Frost et al 1977).

Effects of physico-chemical agents

Among the enveloped viruses, HCV appears to be rather resistant to heating and pH changes. The

mean inactivation rate at 56 °C is 1.6-2.7 log units in 30 min (Aynaud and Asso 1970). Differences in thermolability of HCV strains, first noted by Kubin (1967), were used as a genetic marker for virulence (Aynaud et al 1972). HCV infectivity is stable over a large range of pH: 4 to 11 (Aynaud et al 1972). It has been reported to be slightly augmented in a zone of pH roughly corresponding to the isoelectric point of the virus (pHi = 4.8; Laude 1977). HCV is highly susceptible to the action of hydrolytic enzymes such as trypsin or phospholipase C (Van Bekkum and Barteling 1970, Laude 1977). The noticeable loss of infectivity after freezing-thawing can be reduced by the addition of 5 % dimethyl-sulfoxyde (Aynaud unpublished results).

Hydrodynamic parameters and purification

An important variation exists in buoyant density data reported in different laboratories: 1.13 to 1.17 g/ml, the lower figures corresponding to the more recent estimates (see Horzinek 1981 for references). This discrepancy may be related to salt-induced density changes, association of cellu-



Fig. 4. — Ultrathin section of HCV infected PK15 cells. (Scherrer, 1970; unpublished pictures). Viral particles were seen most frequently in extracellular (A) or in intercellular (B) spaces (bars = 100 nm).

lar fragments to the particles, or absence of internal standard. In any case, it appears that no other virus family incorporates such a large proportion of lipids. The host cell system in which the virus is produced may also significantly affect the buoyant density of the particles (Laude 1979). Sedimentation coefficient values in sucrose gradient of 179 and 140 S20w have been reported (Zeegers and Horzinek 1977, Laude 1979).

Overall, the hydrodynamic parameters of the pestivirus appear to be clearly distinct from that of «other togaviruses» (fig 6). Besides, these values are very closed of those of cellular components, probably smooth membrane vesicles, thus explaining the unusual difficulties encountered by different authors in their attempts to purify the virions efficiently (Ushimi et al 1969, Frost et al 1977, Zeegers and Horzinek 1977, Laude 1977, Enzmann and Weiland 1978). As a matter of fact, a cell contaminant has been found to be still present in virus-containing fractions after a twocycle ultracentrifugation in rate zonal followed by isopycnic gradients (Laude 1977). Hence, semipurification of the crude and/or of the concentrated suspension is a necessary requirement, unless one proceeds exclusively with supernatants fluid from infected cells as starting material (Wensvoort et al 1986). A means repeatedly employed for pestiviruses was to include a fluorocarbon extraction step in the purification schedule (Horzinek et al 1971, Laude 1977).

Protein composition and RNA

Due to the above-mentionned difficulties, the authors who attempted to elucidate the polypeptide structure of HCV generally met with little success. In the studies from P Enzmann's group, purified HCV labelled with ³⁵S-methionine resolved in three main radioactive peaks in SDS acrylamide gel. Only three bands - corresponding to mw 55, 46 and 36 kilodaltons - were detected by autoradiography after immunoprecipitation of the above material (Enzmann and Weiland 1978). Structural polypeptides of the similar mw have been published for BVD virus. Furthermore, by applying the galactose oxidase method of external labelling, it was concluded that only the 55 kd and the 46 kd species were glycosylated. This is in agreement with a later study showing that galactose residues, but not mannose-like sugars would be exposed to the outside of the particles, based on the fact that HCV is agglutinated by CBA and not by ConA lectin (Neukirch et al 1981). Therefore, gp55 and gp46 very likely correspond to envelope proteins. The gp46 protein, usually the most abundant, was shown to be excreted in the supernatant of infected cells (Enzman and Weiland 1978).

As a candidate for the capsid protein, the 36 kd species is probably associated to the genomic RNA. For the latter, few features are known: it is infectious, it sediments at 40-45 Svedbergs, its mw is 4 megadaltons in denaturing gel (Zeegers and Horzinek 1977, Frenzel and Meyer 1980, Enzmann and Rebberg 1977). Further interesting results have been obtained by examining the effect of a protease inhibitor (trasylol), added during virus replication. In its presence, the 36 kd protein was only present in small amount, but two polypeptides with mw of about 65 kd and above 90 kd became dominant. Besides the particles produced were no longer infectious, which suggests that a processing of polypeptide precursors is necessary to acquisition of the infectivity (Enzmann 1986).

Viral antigens

The discovery by Darbyshire in 1960 of a precipitating antigen common to HC and BVD viruses provided a historical basis for the pestivirus genus. The identification of a non-sedimentable antigen produced in infected cells was first achieved by Pirtle and Gutekunst (1964). A 58 kd protein purified to homogeneity from pancreas of infected pigs, has been shown to react both with HCV and BVDV antisera (Van Aert 1970). Subsequently, two protease sensitive antigens of about 60 and 30 kd were characterized after immunoelectrophoretic separation (Matthaeus and Van Aert 1971). Matthaeus 1971).

Confirming the earlier results, Dalsgaard (1976) observed three HCV-specific precipitation lines in two dimensional immunoelectrophoresis, using Triton-X100 solubilized infected culture as a material. One of which bound to con A lectin and exhibited a reaction of identity with a BVD «soluble» antigen, suggesting that the common pestivirus antigen is a glycopeptide. Moreover, this product protected the pigs against an otherwise fatal challenge (Dalsgaard and Overby 1977). Wether this antigen is able to induce a neutralizing antibody response is still conjectural. In that case this would imply that it shares common determinants with one of the membrane glycoprotein, that is quite plausible. However, the involvement of a non-structural glycoprotein in immunity cannot be excluded so far. In the case of vellow fever virus (flavivirus), the non-structural gp48 protein has been shown to exert a protective function in mice (Schlesinger et al 1985). In conclusion, an attempt to correlate precipitating as well as neutralizing antigens with virion structure is highly difficult to date. The advent of hybridomas directed against pestiviruses will certainly allow to solve this problem in a near future.

Serological variation

Although the concept of antigenic unicity of HCV strains was never disputed, the existence of serological variations has been reported repeate-

dly. In 1974, Corthier *et al* have published a detailed study on this point, based on the use of refined seroneutralization techniques. Several conclusions could be drawn from the model they proposed (fig 7):



Fig. 5. — Typical appearence of a negatively stained preparation of purified HCV particles (1 % uranyl acetate pH 4.5; Laude, unpublished picture). Bar = 100 nm.

i) whereas the BVDV strains exhibited an antigenic heterogeneity, only two serological subgroups could be distinguished among HCV strains. The first comprised virulent and attenuated vaccine strains and the second, the 331 strain, isolated in the USA by Pirtle and Mengeling (1971), and other strains of low or moderate virulence. Members of the subgroup 331 have frequently been isolated from inapparent or low noise cases of HC in France (Aynaud *et al* 1974). Very similar results have been independently obtained by





Kamijo et al (1977). Since the publication of this work, however, several chronic strains isolated in France (Plateau and Ursache 1977), as well as the Glentorf strain (Liess et al 1976), have been found of the Alfort virulent type. Accordingly, no antigenic markers of the virulence are available so far. ii) Virus strains belonging to the 331 subgroup appeared more closely related to BVD virus, as they were generally neutralized by antiBVDV sera at a higher rate than those of the other group. iii) Crossneutralization tests between HCV and BVDV revealed an appearent antigenic dominance of the latter. However, in such neutralization tests, the residual infectivity is assayed on a different cell system for each virus, and this might be relevant to this observation.

Major advances concerning the antigenic relationship between pestiviruses should be expected from the use of monoclonal antibodies (see the paper of Wensvoort *et al* 1986).

Conclusions

As illustrated along the review, the knowledge of this agent is still in infancy. Little is known on the structure of the genome of HCV and encoded structural and non-structural polypeptides. Similarly, the precise antigenic constitution of the virion has not been unrevealed. However, a page has probably been turned over in the story of HCV basic research. A progress is expected to occur at a much faster pace henceforth, with the easy accessibility of new technological tools now. A complete genomic sequence of the closely related BVD virus has already been established. Monoclonal antibodies have been derived against both HC



Fig. 7. — Schematic representation of the antigenic relationship between HCV (VPP) and BVDV (VMM), as deduced from crossneutralization studies using polyclonal antibodies (reprinted from Corthier *et al* 1974. Ann Rech Vét, with permission).

and BVD viruses. The use of molecular probes derived from recombinant DNA or cell fusion technics will certainly help to overcome a major portion of the obstacles which have hindered investigations on HCV. Any advancement in that field is a step not only to a better understanding of the pathogenesis of the disease, which is still largely unexplained, but also to an improvement of the methods of diagnosis necessary to achieve its eradication.

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Abstract

The aim of this review is to summarize the informations accumulated during the two last decades on hog cholera virus, a member of the pestivirus genus. Different aspects concerning the virus-host cell interactions, and the structural and antigenic properties of the virion itself are successively analyzed. Despite numerous works, many basic informations are still lacking, which can be explained by the difficulties inherent to the study of this virus.

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