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edited by

B. Liess
Institut fur Virologie der Tierarztlichen
Hochschule Hannover
Hannover, Federal Republic of Germany

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LIST OF CONTRIBUTORS

J.M. AYNAUD
Institut National de la
Recherche Agronomique
Laboratoire de Pathologie
Porcine - Immunologie
37380 Nouzilly, F

H.J. BENDIXEN
Commission des Communautés
Européennes
Division VI/B/4
Rue de la Loi 86
1049 Bruxelles, B

P. BIRONT
Institut National de
Recherches Vétérinaires
Groeselenberg 99
1180 Bruxelles, B

E.A. CARBREY
United States Department of
Agriculture
National Veterinary Services
Laboratories
P.O. Box 844
Ames, Iowa 50010, USA

F. EHRENSPERGER
Institut für Veterinär-
pathologie
Universität Zürich
Winterthurer Strasse 260
8057 Zürich, CH

P.J. ENZMANN
Bundesforschungsanstalt für
Viruskrankheiten der Tiere
Paul-Ehrlich-Strasse 28
7400 Tübingen, D

J.W. HARKNESS
Ministry of Agriculture,
Fisheries and Food
Central Veterinary Laboratory
New Haw, Weybridge
Surrey KT15 3NB, GB

J. LEUNEN
Institut National de
Recherches Vétérinaires
Groeselenberg 99
1180 Bruxelles, B

B. LIESS
Institut für Virologie
Tierärztliche Hochschule
Hannover
Bischofsholer Damm 15
3000 Hannover 1, D

V. MOENNIG
Institut für Virologie
Tierärztliche Hochschule
Hannover
Bischofsholer Damm 15
3000 Hannover 1, D

P.L. ROEDER
Ministry of Agriculture,
Fisheries and Food
Central Veterinary Laboratory
New Haw, Weybridge
Surrey KT15 3NB, GB

C. TERPSTRA
Centraal Diergeneeskundig
Instituut
Vestiging Virologie
Houtribweg 39
8221 RA Lelystad, NL

G. TRAUTWEIN
Institut für Pathologie
Tierärztliche Hochschule
Hannover
Bischofsholer Damm 15
3000 Hannover 1, D

J.T. VAN OIRSCHOT
Centraal Diergeneeskundig
Instituut
Vestiging Virologie
Houtribweg 39
8221 RA Lelystad, NL

PREFACE

The history of research on hog cholera (HC)/classical swine fever (CSF) can be roughly divided into three phases which are characterized by the methods available at the time for demonstrating the causative agent. Phase I covered the period before the viral etiology of HC was discovered by de Schweinitz and Dorset (1904)*. Thereafter (Phase II) the detection of HC virus (HCV) was accomplished by laborious, time-consuming and costly pig inoculation experiments. This explains the extensive search for methods not only for detection but also for accurate infectivity titration as well as for applicable serological techniques to solve urgent problems concerning the pathogenesis, diagnosis, epidemiology and prophylaxis of HC. It was not before the late fifties that HC research entered Phase III when fluorescent antibody techniques offered not only the means for detection and titration of HCV in porcine cell cultures but also for more intensive research on hog cholera and its virus. And yet, there are a number of questions to be answered, e.g. on the genetic and antigenic relation of HCV to bovine viral diarrhea (BVD) virus. There are indications that Phase IV of HC research will bear the stamp of biotechnology. In view of this development it appears appropriate to give an up-dating and summarizing account of HC/CSF including comparative aspects of infections caused by structurally related viruses.

The edition of the present volume would have been impossible without the cooperation of several known scientists who instantly agreed when asked for contribution. Thanks are also due to Miss Andrea Gliessmann for preparing the manuscript in cooperation with Dr. Julia Hadar.

CLASSICAL SWINE FEVER AND RELATED VIRAL INFECTIONS

1

DESCRIPTION OF THE VIRUS INFECTION

J.T. VAN OIRSCHOT

1. INTRODUCTION

Classical swine fever or hog cholera (HC) is a virus infection of swine caused by a member of the family of Togaviridae, genus Pestivirus. The virus causing bovine virus diarrhoea (BVDV) and border disease of sheep also belongs to the Pestivirus genus, and is antigenically and structurally closely related to hog cholera virus (HCV).

The pig is the sole animal known to be susceptible to HCV under natural conditions. HCV infections can range from acute disease with high mortality at the one extreme to a harmless subclinical course at the other extreme. The interplay between viral and host factors determine the course and outcome of an HCV infection.

2. VIRAL AND HOST DETERMINANTS OF VIRULENCE

The word "virulence" indicates to what degree a virus strain induces disease in a susceptible host. The virulence of a particular virus strain is best determined by experimental infection of animals, under standardized conditions. Whereas highly virulent strains of HCV produce lethal infections, low-virulent strains give rise to mild disease or asymptomatic infections. However, not only viral virulence but also host characteristics contribute to the outcome of infection. Essentially, the severity and outcome of infection is the result of the composite interaction between viral virulence and host factors, such as age, genetic background, nutritional condition and immune competence.

2.1 Viral virulence

The virulence of strains of HCV varies greatly. Because there is a continuous spectrum of virulence, it is difficult to classify HCV strains according to virulence. Although there is thus no sharp distinction, it is helpful to distinguish the following categories of virulence. High-virulent

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strains kill nearly all pigs, irrespective of age. Moderate-virulent strains generally induce subacute or chronic illness, eventually leading to death or recovery. Piglets postnatally infected with low-virulent strains show few or no signs of disease, subsequently recover and commonly develop immunity. Such strains can, however, cause abnormalities in porcine foetuses. Avirulent strains are usually attenuated and are virtually apathogenic for foetuses. They are used as vaccine, for example the "Chinese" strain.

Our understanding of the virulence of viruses at the molecular and genetic level is beginning to increase. However, nothing is known on the viral properties of HCV involved in determining virulence. The degree of virulence of HCV appears to be related to some "in vitro" characteristics. Virulent strains grow optimally at about 39 to 40 C, whereas the optimal temperature for low-virulent strains is 33 to 34 C. Strains of moderate virulence replicate optimally at intermediate temperatures, between 35 and 38 C. Virulent viruses usually multiply more rapidly and to higher titres in PK-15 cells than less virulent strains. With immunofluorescence techniques large fluorescent foci are visible in PK-15 cells after infection with virulent strains of HCV. Low- or moderate-virulent strains induce small weakly fluorescent foci under the same conditions. In addition, virulent strains are more resistant to heating at 56 C than strains of reduced virulence (1).

In addition to differences in "in vitro" properties, an association may exist between virulence of HCV and antigenicity. Strain 331, which produces chronic infections in experimentally infected pigs, differs antigenically from the virulent Ames strain. These strains are neutralized to a greater degree by homologous than by heterologous antiserums (2). Corthier et al. (3) distinguished 2 serological sub-groups of HCV based on seroneutralization tests.

The first sub-group is composed of virulent and attenuated vaccine strains and the second sub-group of strain 331 and other strains of low to moderate virulence. Strains belonging to the second sub-group are serologically more closely related to BVDV than those of the first sub-group. Members of the sub-group "331" have frequently been isolated from inapparent cases of HC in France (4). Kamijo et al. (5) also divided field strains of HCV into 2 groups based on the degree of neutralization by an antiserum against the T20-5 strain of BVDV. The virus strains that were neutralized readily induced a chronic type of illness in pigs, whereas the

poorly neutralized strains produced acute HC. More recent Japanese isolates could be classified in the same manner (6). Thus, strains that are serologically more related to BVDV seem to be less virulent.

The virulence of HCV seems to be an unstable property, although there are conflicting data on virulence stability. Enhancement of virulence after one or more experimental passages in pigs has repeatedly been reported (7,8,9,10,11,12,). Enhancement of virulence has also been observed after transplacental passage of HCV (13). Others, however, failed to demonstrate changes in virulence of HCV by serial passages in pigs (14,15). The observations on enhancement of virulence leave undefined the mechanisms involved.

It is well-known that in regions where HC in enzootic, chronic or inapparent cases of HC gradually appear more frequently. Such cases are mainly associated with viruses of reduced virulence. Although the mechanisms underlying the evolution of HCV strains of reduced virulence remain far from clear it may be best explained by selection events. In pigs chronically infected with HCV, the virus replicates in the presence of antibody. This may result in a selection of variants either less antigenically related to the original virus population or refractory to neutralization (16). This seems consistent with Korn's observation (17) that weakly virulent HCV emerges in pigs during the recovery phase of a chronic infection. Aynaud et al. (4) hypothesized that serological variants isolated from chronic or subclinical cases are derived from vaccine strains following serovaccination, during which a selection under immunological pressure occurs. An important selection event is the control of HC, which is mainly based on stamping out in most European countries. Thus, easily recognizable cases of HC are eliminated primarily, whereas chronic and atypical outbreaks caused by strains of reduced virulence are more difficult to recognize, which favours spread of those strains. In this context it is worth noting that 55% of the 135 field isolates collected during the last ten years of eradication in the USA were characterized, by pig inoculation, as being strains of reduced virulence (18).

Virulence of HCV appears to be related to the height and duration of viraemia and this in turn is probably dependent on the extent of virus growth in tonsil and other secondary lymphoid organs. Whereas virulent virus thoroughly infects both epithelial cells, reticular cells and macrophages in the tonsil, growth of virus of lowered virulence is mainly restricted to cells of the epithelial crypts (19, 20). Thus, the virulence may well be

determined partially by the interaction of virus and phagocytic cells in these peripheral lymphoid organs. The observation that virulent HCV strains replicate to higher titres in porcine alveolar macrophage cultures than less virulent strains (21) suggests the probable importance of HCV-macrophage interactions in determining course and outcome of an HCV infection.

The dose of virus used to inoculate pigs may be a factor influencing the course of infection. Chronic illness has been produced in pigs given a minimal dose of virulent virus (22, 23).

2.2. Host factors

That host factors play a role in outcome of HCV infections is best illustrated with strains of reduced virulence. With a rabbit-passaged strain a persistent infection was induced in pigs of 6 weeks of age, but not in 3-month-old pigs (7). Mengeling and Cheville (24) reported that 22 of 69 pigs, between 2 and 4 months of age, inoculated with strain 331, developed chronic HC. From the remaining 47 pigs 33 died of acute to subacute HC and 14 recovered. Weaning pigs infected with the Glentorf strain recovered or died several weeks post-infection. This strain induced no signs of disease in sows, but did produce malformations in foetuses (25). Van Oirschot (26) described a strain giving rise to persistent infections in foetuses and subclinical infections in young pigs. Thus, marked individual differences exist in susceptibility to HCV strains of lowered virulence. From the above observations it can be concluded that age is an important host factor in outcome of infections. Foetuses and young pigs are more sensitive than older pigs. Such an age-dependent resistance to disease is a general phenomenon in virus infections. The mechanisms underlying the age-dependent differences in HC are not known, but it is conceivable that the maturity of the immune system and the susceptibility of macrophages play a role.

Age is obviously not the only host determinant involved, because pigs of the same age can respond quite variable to exposure to HCV of moderate virulence. However, most host factors in determining the course of HC are still undefined. Nutritional condition appears to influence the severity of disease. Whereas in other infections malnutrition commonly predisposes for a more severe illness, malnourished pigs develop chronic rather than acute HC after infection with virulent virus (27). Immunosuppression, by administration of corticosteroids, enhanced spread of virus through the body (28) and raised the mortality of pigs given attenuated strains of HCV(29).

3. INFECTIONS WITH HIGH-VIRULENT VIRUS STRAINS

HCV enters the pig into the oral and nasal cavity. Uncommon sites of entry of HCV are conjunctiva, urogenital tract and abraded skin.

In acute HC, caused by high-virulent strains, the spread of virus through the pig is characterized by a lymphatic, viraemic and visceral phase. The virus primarily multiplies in epithelial cells of tonsillar crypts, and then invades the underlying lymphoreticular tissue, where it is found predominantly in reticular cells and macrophages. After entering the lymphatic capillaries the virus is carried to lymph nodes draining the tonsillar region. HCV replicates in the regional lymph nodes and enters the efferent blood capillaries giving rise to a viraemia. The virus then reaches and subsequently replicates to high titres in secondary target tissues, such as spleen, visceral lymph nodes, lymphoid structures lining the intestine and bone marrow. The growth of HCV in leucocytes may contribute to the high level of viraemia, which, in acute HC, is maintained till death. It is presumably late in the viraemic phase that HCV invades the parenchymatous organs (30). The infection of organ epithelial cells appears to be mediated by phagocytosis of cells of the reticuloendothelial system, and by growth of HCV through vascular endothelial cells. Vascular endothelium is considered to be a major site of virus replication (31,32). The spread of virulent HCV throughout the pig is usually completed in 5 to 6 days (30). Virus is shed into the environment from oronasal and lacrimal secretions, from urine and faeces.

Although HCV induces no or low cytopathology in vitro, pathological lesions do develop in many tissues and organs of pigs with acute HC. The pathological picture is mainly characterized by multiple haemorrhages of various sizes, spread throughout the body, most frequently in lymph nodes and kidney. In addition, inflammatory reactions develop in digestive and respiratory tracts. Several explanations have been put forward as to the pathogenesis of the haemorrhagic diathesis. The most likely is that this condition is caused by degeneration and necrosis of vascular endothelial cells, in conjunction with severe thrombocytopenia and disturbances in the blood coagulation mechanism (33). HCV has an affinity for endothelial cells, but it is not known whether the damage to these cells is caused by direct action of the virus. "In vitro", HCV replicated to high titres in cultured endothelial cells derived from the aorta, without inducing a cytopathic effect (26). However, cultured endothelial cells from large vessels may

behave differently from capillary endothelial cells "in vivo". It is interesting to speculate that endothelial cells are damaged as a result of the combined action of virus and proteolytic enzymes and/or oxygen intermediates, released by epithelial cells, activated macrophages and/or leucocytes or other cells infected with the virus. This would be in keeping with the concept that HC may be considered a virus-induced disorder of the enzyme system (34). Korn and Matthaeus (34) reported that pigs given normal pancreas suspensions or the enzyme chymotrypsin reacted with a HC-like syndrome, whereas HC-immune pigs were protected. In addition, it was described that a protease inhibitor significantly reduced severity of clinical signs and mortality from HC (35). They suggested that the enzyme, a chymotrypsin-like protease, is induced in HCV-infected cells and functions by cleaving a virus protein precursor. This enzyme would induce pathophysiological disturbances in, for example, the circulatory system. Their remarkable hypothesis might indirectly be supported by the observation that infected cells, among which macrophages, from pigs with HC have a markedly increased number of lysosomes (36). These cells might produce and release proteolytic enzymes and/or oxygen intermediates; macrophages are a pivotal source of enzymes. In addition, activation of lysosomal enzymes may lead to chromosomal aberrations (37); the latter abnormality has been described in cells infected with HCV (38,39). South-American haemorrhagic fevers have characteristics in common with HC and it has been suggested that circulating leucocytic enzymes are critical in the pathogenesis of these virus infections (40). In Behcet's disease, endothelial cell damage may result from the generation of high levels of oxygen intermediates by stimulated neutrophils (41). The growth of Newcastle disease virus is enhanced in pig testicle cells in which HCV is present (42). The mechanisms responsible for this phenomenon are not completely clarified (43). Although there is no evidence for this, one could imagine that the exaltation of Newcastle disease virus by HCV is partially due to a virus-induced trypsin-like protease that cleaves the F glycoprotein of Newcastle disease virus resulting in enhanced infectivity (44). Avirulent HCV strains then may not produce high levels of the virus induced protease. It is unclear whether circulating antigen-antibody complexes contribute to the endothelial cell damage, or give rise to the disseminated intravascular coagulation condition, observed in acute HC (45).

HCV has a predilection for cells of the immune system. Virulent virus replicates to high titres in tonsil, lymph nodes and spleen and virus

antigen can be detected widespread in histiocytes, reticular and endothelial cells in these organs. The virus infection initially results in proliferation followed by necrosis and depletion of lymphocytes along with hyperplasia of histiocytes and reticular cells (32). The mechanisms responsible for the destruction of lymphocytes remain speculative. HCV is probably not cytolytic for lymphocytes (26), thus, factors other than a direct viral action may be involved. Perhaps enhanced levels of corticosteroids, which have pronounced effects on lymphoid tissues (10), or a soluble cytotoxic factor (proteolytic enzyme?) produced by infected reticular cells or histiocytes, as has been suggested for lactic dehydrogenase virus (another nonarbo togavirus) of mice (46) may play a role. It is noteworthy that in pigs with chronic HC, the intensity of lymphoid necrosis correlated directly with the intensity of histiocytosis and plasmacytosis (36). Shortly after infection with virulent HCV a severe leucopenia develops that persists till death. Concurrently, peripheral blood and spleen lymphocytes are virtually unresponsive to T and B cell mitogens, whereas lymphocytes from lymph nodes have an enhanced rather than a depressed reactivity. In these lymph nodes, lymphocytes are present that bear both B and T cell markers (47,48). A depressed secondary antibody response to lysozyme has been reported in acute HC (49), whereas Mengeling (50) observed a normal antibody response to Brucella abortus antigens in pigs with fatal HC. Thus, an infection with virulent HCV appears to affect certain functions of the immune system.

Pigs with acute to subacute HC can mount an antibody response to the virus (50,51), but a virus-specific cell-mediated immune response has not (yet) been detected in acute HC (48). Interferon has been detected in the blood of pigs infected with virulent HCV (52), but not in pigs given attenuated virus (53), while the reverse would be expected.

Pigs with acute HC show, after an incubation period of 2 to 6 days, severe signs of illness. They develop fever, anorexia and depression. Conjunctivitis, nasal discharge, diarrhoea or constipation, vomiting, weak hind quarters and purplish discoloration of the skin may also be noticed. The mortality rate of infections with virulent HCV approaches the hundred percent. Most pigs die between 8 and 20 days post-infection. The actual cause of death is not known, but the severe disturbance of the circulatory system is the most likely cause (10). Dunne (12) distinguished acute from subacute HC; in the latter case, pigs generally develop less severe clinical

signs and succumb within 30 days.

A virulent HCV infection usually will spread fast in a herd, allowing a rapid clinical recognition and laboratory diagnosis of HC. Consequently, a swine herd with acute HC can be eliminated rapidly and is therefore not highly important in the epizootiology of HC.

4. INFECTIONS WITH MODERATE-VIRULENT VIRUS STRAINS

An infection with HCV strains of moderate virulence can give rise to a wide variety of clinical pictures. Pigs may die from acute to subacute disease, they may recover or may survive the acute phase only to succumb later from chronic HC, or they may develop only mild signs of disease. Pigs that have overcome the initial acute to subacute phase of infection can develop chronic HC. Based on experimental infections with the 331 strain of HC, Mengeling and Packer (16) divided chronic HC into 3 phases: 1) an early acute phase, 2) a period of clinical improvement, and 3) an exacerbation of acute disease. In the first phase of chronic illness the spread of virus through the body resembled that in acute HC, but it was slower and virus titres in serum and organs tended to be lower. Viral antigen was present in reticuloendothelial, lymphoidal and epithelial tissues. In the second phase virus titres in the blood were low or absent. Viral antigen then tended to be limited to epithelial cells of tonsil, ileum, salivary glands and kidney. During the terminal phase virus again spread throughout the body. In lymphoid tissue viral antigen was mainly present in reticular cells and macrophages. The reduction or transient disappearance of HCV from the serum was probably the result of the formation of specific antibody. In addition, there was morphological evidence of exaggerated plasma cell formation and the serum immunoglobulin level increased. The exacerbation of acute disease may have been promoted by the immune exhaustion that developed in chronically infected pigs and made them more susceptible to secondary bacterial infections. The glomerulonephritis may be explained by deposition of antigen-antibody complexes in the kidney and subsequent attraction of neutrophils (54). Leucopenia developed in the first phase, persisted in the second and leucocytosis was associated with the terminal phase of illness (16). The most outstanding lesion in these pigs was a general depletion of the lymphoid system, characterized by a complete atrophy of the thymus and lymphocyte depletion in peripheral lymphoid tissues. A histiocyte hyperplasia with phagocytosis of lymphocytic debris also took place in

lymphoid tissues (36).

Other HCV strains of moderate virulence can also give rise to infections, in which the virus persists during the host's lifetime. Such pigs may initially show moderate to mild signs of disease, such as fever and loss of appetite, followed by a long period, during which the pigs are relatively free of disease, although they may not gain much weight. Severe signs may not be observed until shortly before death. Such pigs usually have a persistent viraemia of high titre and mount no or low antibody responses to HCV (7,18).

A consistent observation in pigs infected with strains of moderate virulence is the great variation in clinical responses among individual pigs given the same virus strain. This indicates that host determinants influence course and outcome of such infections. There are pigs that die from acute HC, whereas other pigs given the same strain of HCV develop inapparent infections with a persistent viraemia (25,55). Duration of fever varied from 1 to 19 days in pigs inoculated with a French moderate-virulent strain of HCV. Eight out of 20 pigs died and the remaining ones recovered (56). Littermates exposed to HCV strains of moderate virulence had widely varying clinical signs and large differences in duration of viraemia (26).

It is not uncommon that infections with moderate-virulent HCV are associated with an abnormal antibody response to the virus. The neutralizing antibody response can remain completely absent, severely impaired or delayed, or only be transiently detectable (18,25,26,51,56). A poor immunogenicity of the virus and/or an infection-induced immunosuppression may account for the defective antiviral response. However, infected pigs that do not produce neutralizing antibody can develop antibodies detectable by other tests (57,58).

In pigs that do not mount a normal antibody response after a primary contact with HCV, two phenomena have repeatedly been observed. Re-exposure of such pigs to HCV can result in a kind of hyperreactivity, characterized by a shorter incubation period, a more severe illness and higher mortality than in primarily infected pigs. Such a sensitizing effect has been reported in pigs inoculated with leucocyte samples collected 5 to 8 hours after a primary infection (59) and subsequently challenged, and in pigs given twice, with intervals of 1 to 3 months, HCV of reduced virulence (9). When piglets were infected around the time of ingesting colostrum from their HCV-immune dams, they did not produce antibody to HCV and appeared also sensitized when

challenged three months later (58,60). Sensitization has also been observed in pigs which were challenged with virulent HCV one month after infection with a moderate-virulent strain of HCV (26). The exact nature of this sensitization phenomenon is still undefined. It is conceivable that immunological mechanisms, comparable with the enhancement of infection by non-neutralizing antibodies as described for Dengue haemorrhagic fever (61), are involved. The finding that pigs which do not elicit neutralizing antibodies to HCV can, however develop HCV antibodies detectable by other tests (57,58), may be relevant in this respect. In contrast with the above described increased susceptibility, an enhanced resistance can occur. In this case, pigs persistently infected with HCV have a markedly prolonged survival upon inoculation with virulent HCV (18,62). An explanation might be that cells infected with HCV are resistant to superinfection with other strains of HCV. The virulent HCV seemed to persist in the superinfected pigs, because a contact pig died from HC within 14 days (62). Presumably, the above phenomena can also occur in the field. "In vitro", HCV-infected cells are resistant to BVDV (63) and vesicular stomatitis virus (64). Such "in vivo" and "in vitro" interference phenomena have also been shown in lymphocytic choriomeningitis virus infections of mice (65,66).

Many pigs infected with strains of moderate virulence recover from the infection, eliminate the virus and are immune to subsequent infection with HCV. The immune response will certainly play a role in the recovery process from HC, but the exact mechanisms still remain undetermined. In pigs that survive HC, specific antibodies generally appear in the blood 2-3 weeks post infection, which is relatively late in comparison with other virus diseases of pigs (26,67,68). In addition, pigs producing antibodies to HCV may nevertheless die from HC (25,50,51,68). The antibody response, therefore, seems not to be of decisive importance in the recovery process from HC. The failure of colostral antibody to eliminate the virus from piglets with high-titred viraemia (62), and the "in vitro" growth of HCV in the presence of HC-immune serum seem consistent with the above concept. On the other hand, when HCV-antibody is given before the infection is well established, it can prevent further virus spread. HCV-antiserum was able to prevent acute disease provided it was given within three days after infection with virulent HCV and in sufficient amounts (69). Piglets born with a low level of viraemia before the uptake of colostrum were apparently free of virus at two weeks after birth (62). The question then arises

whether cell-mediated immune mechanisms play a role in recovery from HC. However,T-cell cytotoxicity and antibody-dependent cytotoxicity are not likely to occur, because HCV-infected cells appear to induce no or very small amounts of viral antigens in their membrane surfaces (26). Korn et al. (68) considered the rise in leucocytes after the leucopenic period as the primary event in the recovery process.

The widely varying clinical picture and the possibility that infections with strains of moderate virulence may involve only a few pigs in a herd, can lead to a delayed recognition of pigs being infected with HCV. As a result, moderate-virulent strains may usually be disseminated during a longer period than virulent HCV strains and therefore tend to be of high significance in the epizootiology of HC.

5. INFECTIONS WITH LOW-VIRULENT VIRUS STRAINS

Whereas the pattern of infection with virulent HCV consists of a lymphatic, viraemic and visceral phase, a postnatal infection with low-virulent HCV seems to be mainly confined to the lymphatic phase, with a slight extension into the viraemic phase. In the tonsil viral antigen is predominantly present in epithelial crypt cells, but virus may spread to surrounding reticular and lymphoid tissues. Virus can be detected in lymph nodes and a low-titred viraemia of short duration can occur. As a result, virus has been found in spleen and some other organs (5,20,47,53). Histopathological changes may be restricted to focal necrosis of lymphatic tissue (70). Pigs infected with low-virulent HCV usually show a transient leucopenia. Concomitantly with the leucopenia, a depressed response of peripheral blood and spleen lymphocytes to B and T-cell mitogens has been detected, whereas lymph node cells did not lose their capacity to respond (47,48). The antibody response to non-HCV antigens may be enhanced rather than depressed in such pigs (71). Pigs infected with low-virulent HCV commonly develop an antibody response to the virus and have lifelong immunity. Corthier (72) could detect a very brief lymphocyte response to HCV in pigs infected with low-virulent virus, whereas others could not (48).

Pigs postnatally infected with low-virulent HCV show no or few signs (fever, slight depression) of disease and subsequently recover. Such pigs probably shed virus for a short period. In a herd, the infection may spread slowly and as a result the diagnosis will usually not be made rapidly. Such herds may therefore act as a source of virus dissemination.

Whereas low-virulent strains of HCV do not produce severe clinical signs in piglets, they can induce a wide spectrum of abnormalities in porcine foetuses. Infection of the pregnant sow can result in intra-uterine transmission of the virus, in all stages of pregnancy. The virus usually spreads haematogeneously and grows across the placenta. There is some evidence indicating that HCV passes the placenta at one or more sites and subsequently spreads from foetus to foetus (13,26). The virus finds a favourable environment for replication in the metabolically active foetal cells undergoing rapid mitosis and differentiation. Porcine foetuses often become viraemic and the distribution of antigen appears to be similar to that in pigs postnatally infected with virulent HCV. Thus, viral antigen is present throughout the foetus in reticulo-endothelial, lymphoidal and epithelial tissues (73,74). The ultimate outcome of foetal HCV infections depends on a number of factors, such as developmental age of the foetus at the time of infection, the virulence of the virus, the genotype of the host and the foetal ability to repair damage. Foetuses infected late during pregnancy are more likely to eliminate the virus than pigs infected before or around the onset of immunocompetence (62). Prenatal death and foetal malformations are more common when maternal infection takes place during early stages of pregnancy than late in pregnancy. Although the precise mechanisms of this age-dependency are not clear, it is probably related to the maturation of foetal tissue and immune system. The virulence of HCV may influence the postnatal course of a congenital infection, because piglets prenatally infected with a moderate-virulent virus were more severely affected and died earlier than piglets exposed to a low-virulent strain (26). There are HCV strains that induce a high rate of congenital tremors (75,76), whereas others do not (26,77). The viral properties determining the involvement of the central nervous system are still undefined.

A congenital HCV infection can range from extremely severe to clinically inapparent. It can result in prenatal death, foetal malformation or neonatal death. In addition, apparently healthy offspring can be delivered that develop a late onset disease months after birth. Early embryonic death with subsequent complete resorption may occur after infection in the early stage of pregnancy, when the skeleton has not developed fully, as suggested by the increase of barrenness in sows infected with HCV (13,78). Stillbirth and mummification rather than abortion can be the consequence of a congenital HCV infection during the second and third

month of pregnancy. The most pronounced lesions in stillborn pigs are excessive accumulations of fluids in body cavities (79). Infection of sows during the last month of pregnancy will usually not result in prenatal foetal mortality (61,80). Malformations may arise when infection occurs during organogenesis, but they are not very common in pigs congenitally infected with HCV. The virus can interfere with the development of the central nervous system, which can lead to cerebral defects, cerebellar hypoplasia and dysmyelination (75,81), resulting in congenital tremors. Head deformation and defects in front legs have also been observed in piglets exposed to HCV "in utero" (82,83). In addition to malformations, HCV can cause lesions in the foetus, such as haemorrhages in several organs and necrosis in the liver (84). Lesions are the result of infection in already fully formed tissues. Many prenatally infected piglets that are born alive, succumb in the first days after birth. Congenital tremor and haemorrhages of skin and internal organs are frequently noted in pigs dying in the neonatal period (62,77,81,85).

A congenital HCV infection may lead to precocious maturation of lymphoid tissue, i.e. germinal centre formation and plasma cell development, and this correlates well with elevations in serum levels of IgG and to a lesser degree to IgM. The elevated immunoglobulin levels have no or low specificity for the causative virus. The immunoglobulins may be directed against altered host antigens or may be incomplete molecules of smaller size and low avidity (74,84). In spite of the fact that porcine foetuses gain immunocompetence around the 60th to 70th day of pregnancy (86), they only appear to produce neutralizing antibody to HCV when infected beyond the 90th to 100th day of pregnancy (58,87). The reasons for this delayed response may be the same as those suggested with regard to postnatal infections. Some piglets infected "in utero" can recover from HC. Such pigs have either a low virus titre or specific neutralizing antibody in precolostral serum, and their leucocytes contain virus at that time. When two weeks old they have no longer virus in serum or in leucocytes. An ongoing specific immune response in conjunction with the ingested colostral antibody may have effected the virus disappearance in these piglets (58).

Piglets infected "in utero" may be born healthy and clinical signs may remain absent until they are several months old. Such piglets have a high-titred viraemia in the precolostral serum that is transiently suppressed by colostral antibody. At two weeks of age the virus titres are again at

precolostral levels and they persist at high levels during the entire host's lifespan (62,73,88). HCV is associated with the peripheral blood mononuclear cell population (62). The viral antigen persists widespread in numerous tissues, especially in epithelial cells and cells of the reticuloendothelial system. These persistently infected pigs develop a late onset disease characterized by runting often associated with leucopenia, and eventually die several months after birth. At post-mortem examination, thymus atrophy, a severe depletion of lymphocytes in peripheral lymphoid organs, proliferation of reticuloendothelial tissue, and hydropic degeneration of vascular endothelium are the most pronounced lesions (89,90,91). There is a marked adrenal hyperplasia, perhaps indicating an enhanced production of glucocorticosteroids. This may be partially responsible for the severe lymphocyte destruction, especially for the thymus atrophy. Notwithstanding the severe morphological changes in lymphoid tissue, found at post-mortem, the pigs have a normal antibody response to sheep red blood cells and parvovirus, except perhaps in the terminal stage of infection. The mitogenic lymphocyte response of peripheral blood lymphocytes of these pigs appeared to decrease gradually with time, but generally remained within the normal range (58,92). The normal immunological responsiveness in these pigs may indicate that the severe lymphoid depletion only develops in the last days of the host's life. Lymphoid depletion may develop rapidly, since pigs treated with dexamethasone and infected with low-virulent HCV had a marked atrophy of the thymus already 7 days later (28). In spite of the normal immune reactivity of persistenly infected pigs, they do not produce an antibody response to HCV. An "in vitro" lymphocyte response to HCV could not be detected either. Thus, an immunotolerant state exists in pigs with congenital persistent HC (58,92,93). The absence of a specific immune response in all probability contributes to the persistence of HCV.

Sows that deliver stillborn or weak piglets, piglets with congenital tremor, or piglets with skin haemorrhages which die shortly after birth, are suspected of having been infected with HCV during pregnancy. In areas where HC is prevalent such piglets should be examined for HC. It is obvious that cases of congenital persistent HC are difficult to recognize in the field and as a consequence the definite diagnosis will often be made months after introduction of HCV on the farm (94). Pigs with congenital persistent HC have a healthy appearance for a long period, but they shed virus in the environment continuously. These infections are therefore of the utmost

significance in the epizootiology of HC.

Interestingly, the congenital HCV infections of swine have a number of features in common with the congenital pestivirus infections of cattle and sheep (95). The latter infections can also give rise to prenatal and neonatal mortality, and to foetal malformations. In all three infections, the virus can interfere with the development of the central nervous system. Cerebellar hypoplasia and dysmyelination are disorders frequently seen, and they are often associated with congenital tremor in the newborn. Skeletal defects and skin abnormalities are also common features of congenital pestivirus infections. In addition, it is striking that two pestiviruses, HCV and BVDV, induce in three different host species a congenital persistent infection that basically has the same characteristics. After an "in utero" infection with BVDV calves and lambs can be born with a healthy appearance. The infection in these animals is generalized, as shown by a lifelong viraemia and the presence of viral antigen in epithelial and reticuloendothelial tissues. As a consequence, they shed virus into the environment continuously. They often do not produce antibody to the virus, whereas they are able to mount an antibody response to unrelated antigens. Thus, also in calves and lambs a state of specific immunotolerance exists, which is neither absolute nor permanent. Unlike pigs, cattle and sheep with congenital persistent pestivirus infections can remain healthy for years and deliver, in consecutive years, healthy yet infected offspring. In comparison with BVDV, HCV may perhaps not (yet) be sufficiently attenuated in order to achieve a, for the survival of the virus very favourable, well-balanced interaction with its natural host. Compared to other persistent virus infections, the interaction between pestiviruses and their respective hosts appears to be unique in several respects.

Under field conditions HCV only infects swine. BVDV, however, is less restricted in its host range, it can infect cattle, sheep and swine. BVDV infections of pigs usually run a subclinical course, and they have relevance in the diagnosis of HC, where they may yield false positive results (69, Terpstra, unpublished observations).

Virus persistence is a prominent characteristic of infections with HCV of low to moderate virulence. Pigs infected with moderate-virulent strains can develop chronic infections lasting for more than 100 days and piglets infected "in utero" with low-virulent HCV can produce a late onset disease. Although in both infections the virus persists for the host's reduced

lifetime some differences are noted (Table 1). The mechanisms underlying the persistence of HCV may therefore be different.

Table 1. General features of chronic (16,24,36,54) and late onset HC (73,90,91,92)

	Chronic	Late onset
Virulence of virus	Moderate	Low
Time of infection	Postnatal	Prenatal
Course of illness	Short incubation period, 3 phases of illness: 1)depression, fever, anorexia, 2) clinical improvement, 3)terminal exacerbation of disease	Late onset of disease, gradually aggravating depression and anorexia, normal to slightly elevated body temperatures, conjunctivitis, dermatitis, locomotion disturbances
Viraemia	Temporary reduction or disappearance	Persistent high level
Leucopenia	Develops quickly, followed by leucocytosis	Develops late during infection
Immune response to HCV	Present	Absent
Death	1-3 months	2-11 months
Gross lesions	Thymus atrophy, ulcers of caecum and colon, infarction of spleen, rib lesions	Lymph node swelling, thymus atrophy
Microscopic lesions	Degeneration of endothelial cells, severe lymphocyte depletion, histiocyte hyperplasia, immune complex glomerulonephritis	Degeneration of endothelial cells, severe lymphocyte depletion, histiocyte hyperplasia

In general, virus persistence may be regarded as a failure of the host defence system to eliminate the infectious agent or as the capacity of the virus or infected cell to elude the immunological attack of the host. It is likely that the dynamic interplay between host and viral factors determines the induction and subsequent maintenance of persistent infections. Several viral factors may be relevant for the initiation and maintenance of HCV

persistence. HCV is not cytocidal for the infected cell and "in vitro" persistent infections are therefore readily established. Although persistently infected cells survive, some of their functions may be mildly affected. They have a slightly extended period of DNA synthesis (96) and may show some chromosomal alterations (38). The virus can spread from cell to cell and from mother to daughter cells in the presence of immune serum (97,98). In addition, the virus appears to mature at intracytoplasmatic membranes (99,100) and viral antigens are either absent or present at low density on the outer cell membrane (26), which renders the cell insusceptible to various mechanisms of immune lysis. The paucity of viral antigen on the cell surface may also be partly responsible for the poor immunogenicity of some moderate-virulent HCV strains. Another key property, which HCV has in common with other persistent viruses, is the capability to infect lymphoid tissues and macrophages. In persistently infected pigs this does not profoundly influence general immunological functions (58,92), but it may play a role in the antigen-specific unresponsiveness or immunological tolerance, often associated with persistent HCV infections. HCV has a special tropism for cells of the reticuloendothelial system. Infection of these cells may affect functions of the reticuloendothelial system, which is essential for clearance of micro-organisms.

A critical factor in the establishment of persistent infections is the age of the host. The younger the pig, the more readily persistence of HCV is induced. This age-dependency may be primarily based on the degree of maturity and competence of the immune system.

6. INFECTION WITH AVIRULENT VIRUS STRAINS

At present, HCV attenuated by serial passages in rabbits ("Chinese" strain) or in cell cultures (Japanese GPE-negative strain, French Thiverval strain) are widely used as vaccines. After parenteral inoculation the attenuated virus appears to replicate mainly in tonsil and lymph nodes (28,101,102). However, the GPE-negative strain had been recovered from faeces and urine within 10 days post-vaccination (101) and virus antigen of the "Chinese" strain could be demonstrated in kidney up to 15 days post-vaccination (103). All three attenuated viruses can occasionally spread to contact pigs. The "Chinese" strain can cross the placenta of pregnant sows and invade foetal tissues, but it generally does not induce adverse reactions in infected foetuses (104,105). There are, however, indications

that the "Chinese" strains of HCV cannot be considered completely innocuous. Overby and Eskildsen (106) found mummified, stillborn and weak piglets after vaccination of a sow inoculated at the 40th day of pregnancy, and they were able to isolate the virus from some piglets. In addition, it has been reported that this strain interfered with the secretion of bacteriostatic substances by the mucociliary apparatus of the lung (107) and affected the bacteriolytical capacity of alveolar macrophages (108). Differences in (passage) history of the "Chinese" strains used may perhaps explain the different results on innocuity of the "Chinese" strain. Neutralizing antibody appears around 2 to 3 weeks after vaccination and generally persists lifelong. A brief lymphocyte response to inactivated HCV antigen has been detected after vaccination (109). Vaccination confers a rapid and long lasting immunity.

7. CONCLUDING REMARKS

HCV has an extremely wide spectrum of virulence. The virulence of HCV appears to be related to some "in vitro" properties, but little, if any, knowledge is available on viral proteins and viral properties involved in the expression of HCV virulence "in vivo", and on how they interact with host factors to determine severity of disease and outcome of infection. The encounter of HCV with phagocytic cells underlying tonsillar epithelial cells may play a central role. An HCV infection may also be regarded as a race between the virus and the pig's immune defence. In the case of a virulent infection, the virus would then have reached and damaged the vulnerable tissues before the immune response is well developed. In the case of a low-virulent HCV infection, virus replication may proceed slower and the immune response may prevent thorough viral spread through the body. It is also conceivable that the balance between proliferation and regression processes in lymphoid tissues is of decisive importance in course and outcome of HCV infections.

The virulence of HCV largely determines the pathogenesis of the infection. Again nothing is known on mechanisms of tissue damage and disease production. The discrepancy between the near absence of HCV-induced cytopathic changes "in vitro", and the extensive lesions "in vivo" leads one to consider the participation of mechanisms other than direct viral action in the production of lesions in HC. Mediators, or proteases produced by virus-infected cells may perhaps be involved in the pathogenesis of HC.

It needs no further argument to demonstrate that numerous gaps exist in our present knowledge on various aspects of HC. Much complicated, but fascinating research will be required to gain some insight into viral and host determinants involved in the expression of virulence, and in the pathogenesis of this multifarious virus disease.

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2

PATHOLOGY AND PATHOGENESIS OF THE DISEASE

G. TRAUTWEIN

1. INTRODUCTION

In this chapter the gross pathology and the light and electron microscopical changes occurring in the acute and chronic form of Classical Swine Fever (CSF) will be described and their pathogenesis discussed. It will then be shown that in recent years considerable progress has been made in the understanding of the pathogenesis of consumptive coagulopathy and disseminated intravascular coagulation. A special problem presents the transplacental infection of the developing foetus. These infections may result in prenatal death, the development of a number of foetal abnormalities, stillbirth and, most important, congenital persistent HC virus infection. The chapter concludes with a discussion of the viral pathogenesis of this disease.

2. PATHOLOGY OF CLASSICAL SWINE FEVER

2.1. Peracute disease.

It has been known for considerable time that in a non-immune swine population CSF may run a peracute course. In the peracute form infected animals show little more than a rise in body temperature of about 41°C before they unexpectedly die 2-5 days post-infection (1, 2, 3). In these cases post-mortem changes are essentially those of shock with pulmonary congestion and oedema, congestion of the liver and gastro-intestinal tract, and serous effusions into the intestinal lumen. Usually, there is little evidence of haemorrhage.

2.2. Acute disease.

In the acute disease affected pigs have fever (41°C) and appear less active within two to six days after exposure

B. Liess (ed.), *Classical Swine Fever and Related Viral Infections*.

to HC virus. Some animals may show staggering movements of the hind legs; in others there is evidence of CNS involvement with convulsions, grinding of the teeth, difficulty in locomotion and finally posterior paresis. Other signs are cyanosis of the skin, particularly of the ears, petechial haemorrhage in the skin, lacrimation and nasal discharge. Usually, mild constipation precedes diarrhoea. Most animals die 10-14 days after the onset of illness. Depending on the immune status of the swine population affected as well as the virulence of the causative virus strain, mortality may be high (90-100%) or low (30-40%).

Characteristic gross lesions seen in acute CSF are those of haemorrhagic diathesis with petechial haemorrhages present in most organ systems (1, 4, 5, 18). Petechial haemorrhages are found most constantly in the kidneys, urinary bladder and lymph nodes, followed by the spleen, larynx, skin, mucosal membranes (nose, trachea, conjunctiva, stomach, intestine, gall bladder) and serous membranes (pericardium, pleura, peritoneum). Since it is known that secondary infections with bacteria may intensify and even modify the pathological picture, gnotobiotic piglets have been experimentally infected with HC virus (6). These experiments clearly confirmed that petechial haemorrhages primarily occur in the kidneys and lymph nodes.

The skin lesions consist of erythema, cyanosis, and petechial haemorrhage. In 3% of CSF-affected pigs the skin may appear icteric. It is not clear, however, whether this jaundice is a sequela of red cell destruction as originally assumed (7).

In lymph nodes including the parotid, submaxillary, cervical, bronchial, iliac and those of internal organs there is evidence of congestion, haemorrhage and hyperplastic swelling. Obviously, the severity of lymph node changes will be determined by several factors, mainly the susceptibility of the pig and the virulence of the HC virus strain. The pathogenesis of the haemorrhagic infiltration of lymph nodes has not been completely clarified. The typical

mottling of the lymph nodes seen on the cut surface may be due to resorption of blood from regional haemorrhage or haemorrhage within the lymph node.

Lesions in the oral cavity seen in some CSF-affected pigs are erosions and ulcers at the tongue, pharynx, and tonsils. The ulcers are believed to be caused by infarction following occlusion of terminal vessels. Secondary infection may aggravate the primary tonsillitis by bacterial invasion of necrotic tissue.

Infarction of the spleen is a lesion which is considered almost pathognomonic of CSF. Grossly, the infarcts are sharply outlined, dark red or pale in color, irregular in shape and elevated. With 50-60% the incidence of infarcts in outbreaks of CSF used to be high; but the lesion is less frequently seen in current epizootics.

Approximately half of the pigs with acute or subacute CSF demonstrate some degree of congestion and interstitial oedema of the lung which may be associated with bronchopneumonia and subpleural petechiae.

The most obvious changes in the kidneys are petechial haemorrhages on the surface and in the renal cortex; the kidneys may appear pale and light brownish. The pale color of the renal parenchyme is thought to be due to vaso-constriction (5).

Lesions frequently seen in the stomach are congestion and haemorrhage in the fundus area of the gastric mucosa, sometimes associated with erosions. Early changes in the small intestine are diffuse hyperaemia of the mucosa and enlargement of Peyer's patches, later followed by diphtheroid inflammation which often is associated with the gut-associated lymphoid tissue.

In the large intestine lesions start with hyperaemia of the mucosa, followed by catarrhal enteritis. In advanced stages fibrinous to diphtheroid enteritis may occur, associated with necrosis of lymphoid follicles at the ileo-caecal valve. Lesions considered pathognomonic for subacute CSF are the so-called bouton-ulcers, which represent spherical,

sharply circumscribed ulcers of few millimeters in diameter associated with solitary follicles. Bouton ulcers are usually seen if the disease takes a protracted course. The pathogenesis of this peculiar intestinal change has not been fully elucidated. Originally, bouton ulcers were thought to arise due to vasoconstriction; but it is more likely that the lesions develop following occlusion of arterioles by swelling of endothelial cells, degeneration of the vascular wall and formation of microthrombi.

Other lesions infrequently seen in CSF-affected pigs are focal necrosis in the mucosa of the gall bladder (8) and an irregular widening of the epiphyseal line at the costochondral junction of the ribs due to a disturbance of calcium and phosphorus metabolism in young growing pigs (9, 10, 11).

2.3. Chronic disease.

According to the definition of Mengeling and Cheville (12), chronic CSF is a lethal disease with a duration of at least 30 days. The factors which allow for prolonged survival of infected pigs are not known with precision. Usually, the disease runs a slow, protracted course, and a single organ system (lung, gastro-intestinal tract, CNS) may predominantly be affected. Secondary bacterial infections are frequently involved. Thus, the clinical picture may often be uncharacteristic and misleading; therefore, chronic CSF was erroneously called "atypical swine fever" (1).

In chronic CSF there is often little evidence of CSF in terms of petechial haemorrhage; but several organ systems may be involved. In the large intestine bouton ulcers are occasionally seen which are followed by a more diffuse diphtheroid-necrotizing enteritis. The lymph nodes in some cases only show hyperplasia, and the typical haemorrhagic changes with mottling of the cut surface seen in the acute disease may not be present. The widening of the epiphyseal line at the costochondral junction of the ribs which was reported as a lesion of acute CSF may also be seen in chro-

nic CSF. This lesion thus appears to be a useful clue in the diagnosis of CSF, particularly with chronic cases in which most other lesions of CSF are absent.

3. LIGHT AND ELECTRON MICROSCOPICAL CHANGES

3.1. Vascular system.

The histopathological changes of acute CSF have been described adequately in the past (1, 5). HC virus exerts a direct effect upon the vascular system, and the characteristic gross lesions, i.e. congestion, haemorrhage, and infarction result from changes in arterioles, venules, and capillaries. It is in these structures where most of the vascular lesions are detectable histologically. Some of the terminal vessels may be closed, others dilated and congested. Frequently, there is a swelling due to hydropic degeneration of endothelial cells, but proliferation of this cell type may also be present. The enlarged endothelial cells may eventually occlude the vascular lumen. Basement membranes of small vessels appear homogeneous and eosinophilic, and the vascular wall eventually is completely hyalinized, contributing to the partial or complete occlusion of the lumen.

Thrombosis of small and medium-sized arteries is another feature. Depending on the severity of the vascular lesions the sequelae are plasma diapedesis and perivascular haemorrhage. Vascular changes are most severe in the lymph nodes, spleen, kidneys and gastro-intestinal tract.

The other important effect of the HC virus is upon the lymphoid system, i.e. thymus, lymph nodes, spleen, tonsils, and gut-associated lymphoid tissue. In these organs the morphological changes are characterized by depletion of lymphocytes and proliferation of histiocytes.

3.2. Lymph nodes.

In the lymph nodes vascular changes like proliferation of endothelial cells, subendothelial oedema, and occlusion of the lumen are observed in arterioles, venules and capillaries. An other lesion seen in arterioles is fibrinoid

necrosis of the vascular wall. Depending on the stage of disease lymph nodes may be markedly depleted of lymphocytes and germinal centers. In these cases the germinal centers contain necrotic lymphoid cells and show varying degrees of histiocytic proliferation (13, 14, 15).

3.3. Spleen.

The morphological changes present in the spleen of CSF-affected pigs have been described in detail (4, 5, 13, 15). In the central artery there is evidence of vascular degeneration with swelling of endothelial cells, oedema and fibrinoid necrosis of the media and occlusion by thrombotic material. These vascular lesions are considered responsible for the development of splenic infarction. Furthermore, there is lymphoid depletion, especially in the thymus-dependent periarteriolar lymphoid sheaths due to degeneration and necrosis of lymphocytes. Other changes include proliferation of histiocytes and hyperplasia of the reticular cell sheaths of the penicillar arterioles and plasmacytosis in the red pulp. Finally, there may be evidence of extramedullary haematopoiesis with presence of megakaryocytes and erythroblastic cells in the red pulp.

3.4. Thymus.

In studies of the pathogenesis of CSF marked atrophy of the thymus was observed in chronic cases (13). Histologically, the thymic remnants show severe to total cortical depletion of lymphocytes. The presence of tingible bodies within large macrophages suggests phagocytosis of necrotic lymphocytes.

3.5. Kidney.

The evolution of morphological changes in the kidneys was clarified in a sequential study (13). Pigs surviving the experimental infection more than 10 days have in their kidneys focal areas of tubular degeneration with accumulation of hyaline droplets in the cytoplasm of tubular cells. In chronic cases there are additional lesions such as interstitial lymphocytic infiltrations, chiefly perivascular in location.

Renal glomeruli have hyaline changes suggestive of an immunological process, i.e. thickening of the glomerular capillary basement membrane. In pigs surviving 70 days post infection, there are deposits of fibrin in the intercapillary space and fibrinoid degeneration of the glomerular capillaries. Arterioles show panarteritis with medial degeneration and necrosis of the vascular wall. Ultrastructural studies of the kidneys reveal electron-dense material deposited irregularly within the glomerular mesangium, predominantly along the adjacent basement membrane (16). Furthermore, slight glomerular damage is characterized by markedly swollen vacuolar endothelial cells which may obliterate the capillary lumen. In more advanced stages massive dense granular deposits are present in the mesangium. Mesangial cells contain numerous dense residual bodies and are surrounded by fibrils of fibrin. Dense granular deposits are also present on the epithelial side of the glomerular basement membrane. There is focal fusion of foot processes, and some capillary lumina contain neutrophilic granulocytes. The granular, irregular electron-dense deposits were thought to represent antigen-antibody complexes. However, conclusive evidence for this assumption is lacking. As an other pathogenetic mechanism the removal of fibrinogen-fibrin-monomer-complexes from the circulation in the course of disseminated intravascular coagulation was suggested (17).

3.6. Adrenals

The adrenals play a central role in the pathogenesis of CSF; therefore, extensive histological studies have been performed (1). The adrenal gland undergoes structural changes characterized by hypertrophy of the adrenal cortex with broadening of the zona fasciculata and narrowing or atrophy of the remaining zones. Changes in the adrenal medulla are focal haemorrhage, lymphocytic infiltration, and degeneration of chromaffine cells with almost total loss of the chromaffine substance.

3.7. Central nervous system.

The histological changes in the brain and spinal cord

have been described in detail by Röhrer (19) and Seifried (20). Their findings were confirmed later by other workers (1). The essential features are non-suppurative meningoencephalitis, characterized by swelling and degeneration of endothelial cells, thrombosis, vascular and perivascular cellular infiltration with accumulation of lymphocytes in the perivascular space, and perivascular haemorrhage especially in the cerebellum and spinal cord (Fig. 1 and 2). Of much less significance are lesions in the neurons, i.e. necrobiosis, necrosis, and neuronophagia. The large pyramidal cells in the cerebrum are more likely to be damaged than other neurons. In addition, there may be small foci of proliferated microglia. The incidence of encephalitis varies between 70-90%.

Immunofluorescence specific for HC viral antigen is first seen at post infection day 4 and occurs in endothelial cells, perivascular astrocytic cells and in few neurons.

The functional significance of the perivascular accumulation of lymphocytes in the CNS is not clear. Unless the phenotype of lymphocytes in these infiltrations has been determined applying modern techniques (immunofluorescence, immunoperoxidase, monoclonal antibodies), it is uncertain whether these immunocytes are significant in exerting as T cells a cytotoxic effect upon CSF-infected target cells or are as B cells an expression of local antibody formation.

3.8. Eyes.

Detailed histological studies of the ocular lesions in CSF revealed intraocular inflammatory changes including retinitis, uveitis, and in a few cases choroiditis (21).

4. HAEMATOLOGICAL FINDINGS

A characteristic haematological finding in CSF is the occurrence of leukopenia which often is recorded before the first rise in temperature (1, 2). Total white cell counts of 9,000 to as low as 3,000 per mm^3 may be demonstrated 4-7 days post infection. Sequential studies revealed 24 hours post infection a moderate leukocytosis which was

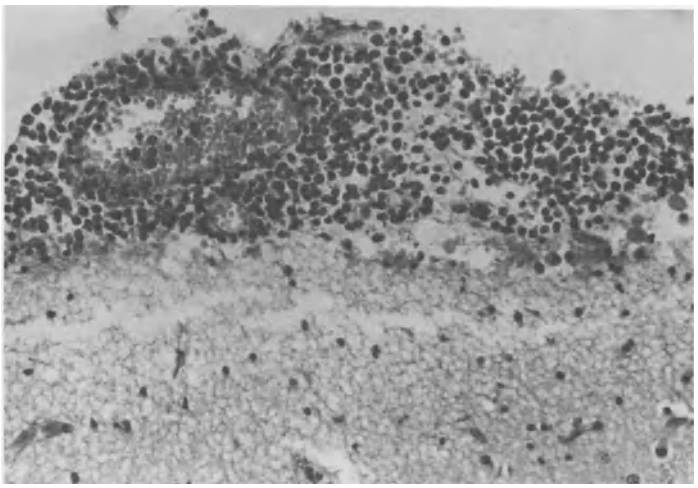

Fig. 1. Meningoencephalitis in the cerebrum with lymphocytic infiltration of the leptomeninges.

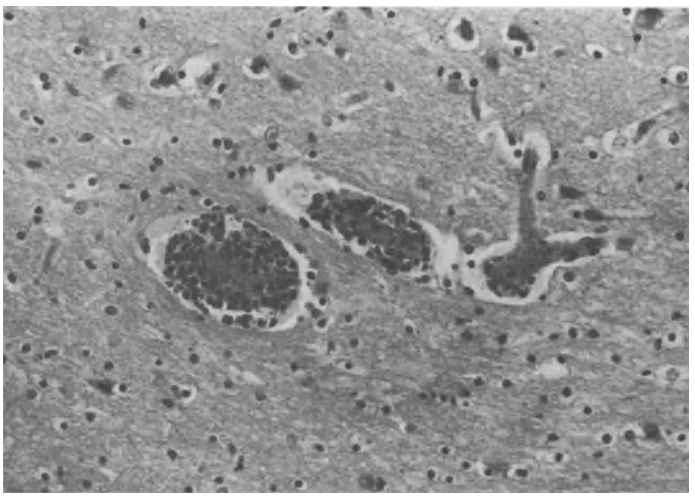

Fig. 2. Meningoencephalitis in the cerebrum with accumulation of lymphocytes in the perivascular space of vessels.

followed by marked leukopenia 2-4 days post infection (1, 22, 23). While in the majority of animals leukopenia persists until shortly before death, a small percentage (28%) of animals shows leukocytosis on the 5th post infection day.

Other haemotological changes are the progressive disappearance of eosinophilic and basophilic granulocytes from the blood which is thought to be due to migration of these cells into the tissues.

Furthermore, anaemia with anisocytosis, poikilocytosis, and polychromasia is considered an essential feature of CSF. An increase of Heinz's bodies was demonstrated in erythrocytes (7, 24). While in the normal blood 15%. of red blood cells have Heinz's bodies, animals with CSF prove to have 80-100%. affected erythrocytes.

In the pathogenesis of the disease equally important like leukopenia and anaemia is thrombocytopenia which was first observed by Dunne (4). Diseased pigs had 5,000-50,000 platelets/mm^3 as compared to 200,000-500,000 mm^3 in healthy pigs. Thrombocytopenia was found to occur in CSF simultaneously with the onset of viraemia and fever (2). Thrombocyte counts were observed to drop from normal levels of about 550,000 per mm^3 to below 100,000 per mm^3.

5. BLOOD COAGULATION DISORDERS

During the course of acute CSF multiple defects of haemostasis develop, the pathogenesis of which has been elucidated in recent years. The diffuse activation of the blood coagulation system may be caused by release of procoagulant substances from cells in lymphoid tissues, endothelial cells in vessels, and epithelial cells of the gastro-intestinal tract, directly damaged by the HC virus (25). A consequence of massive activation of the coagulation system through both the intrinsic and extrinsic pathway is the consumption of platelets and certain coagulation factors (consumptive coagulopathy) resulting in disseminated intravascular coagulation (DIC), severe disturbance of

microcirculation and eventually the occurrence of petechial haemorrhages.

In experimental studies it was shown that HC virus induces an early decrease in platelet counts beginning already on the second day post infection (25). The most severe thrombocytopenia was recorded on post infection day 6 when platelet counts were less than 10,000/mm^3. Other changes of coagulation parameters were decrease of one-stage prothrombin time values, the activities of Factors II (prothrombin) and X (Stuart-Prower factor) and the maximal amplitude in thrombelastography. The fibrinogen concentration in the plasma proved to be constant until post infection day 6; afterwards an increase of this coagulation factor was recorded.

In more recent studies a number of haemostatic defects were observed in pigs experimentally infected with virulent HC virus (26). With the onset of fever 2-3 days post infection the platelet counts began to fall and progressively decreased, reaching their lowest levels (1-2 x 10^5 platelets/mm^3) by post inoculation day 6. The activated partial thromboplastin time (APTT) began to increase on post inoculation day 3 and remained elevated until post inoculation day 6 when it decreased to near preinoculation values. While there were irregular increases in the thrombin clotting time (TCT) from post infection day 4 onward, the one-stage prothrombin time (PT) remained unchanged. Furthermore, fibrin degradation products and a transient decrease in plasma fibrinogen were demonstrated 2-5 days post infection, indicating that early fibrinolysis or fibrinogenolysis may be involved in the inhibition of the clot formation.

The morphological manifestation of consumptive coagulopathy is a disseminated intravascular coagulation (DIC) with the occurrence of microthrombi in small vessels, i.e. arterioles, venules, and capillaries. Microthrombi and accumulation of fibrinous material in vessels were described occasionally in the older literature (1); however, their significance and pathogenesis was only appreciated when

systematical studies were carried out (17, 25, 27). Microthrombi may be formed in almost any organ; but they are seen to occur most frequently in the liver, kidneys, spleen, lymph nodes, lung, intestine, and intestinal lymph nodes (Fig. 3 and 4). Intravascular coagulation may appear morphologically as fibrinous clot, hyaline thrombus, and platelet aggregation. Hyaline thrombi predominate in glomerular capillaries of the kidney and in capillaries of the lung.

In electron microscopic studies of the kidney electron dense substances considered the ultrastructural equivalent of microthrombi were demonstrated in the capillary lumen, mesangial matrix and under the capillary endothelium (17). A similar material was detected in capillaries of the renal cortex. It was concluded that phagocytic mesangial cells remove by phagocytosis fibrinogen-fibrin monomer complexes and their degradation products from the circulation.

In CSF thrombocytopenia develops simultaneously with the onset of viraemia and fever. Since marked thrombocytopenia occurs as early as two days post infection, it can not be explained by consumption of clotting factors during disseminated intravascular coagulation (DIC) alone (28). At this early stage of disease disturbance of the coagulation system other than thrombocytopenia is not yet detectable. Likewise, there is no morphological evidence of DIC. Electron microscopic studies have revealed morphological evidence of primary, HC virus-induced damage to circulating platelets, which begins two days post infection. Sequential bone marrow biopsies in infected pigs show a progressive degeneration of megakaryocytes which is evident four days post infection and in the last stage of disease affects the entire cell population (28). Thus, thrombocytopenia may primarily be due to direct destruction of platelets, followed by peripheral platelet consumption. Finally, the megakaryocyte destruction in the bone marrow is an important pathogenetic factor. The causes of megakaryocyte destruction could be a direct effect of HC virus on this cell

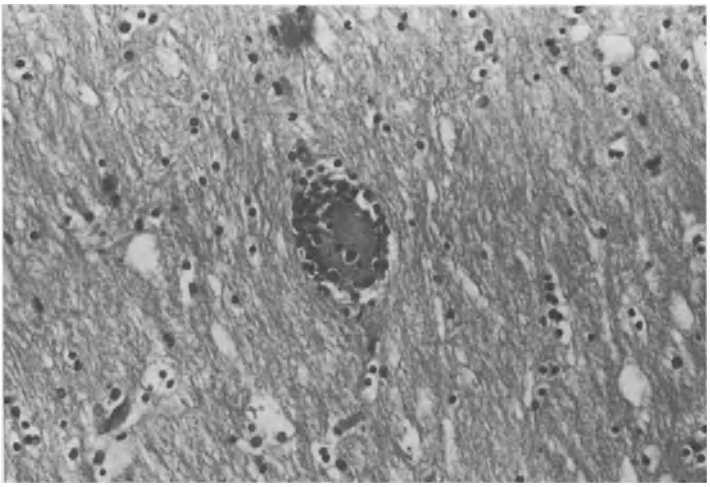

Fig. 3. Microthrombus in a small cerebral artery and perivascular lymphocytic infiltration. Cerebrum of a pig with acute CSF.

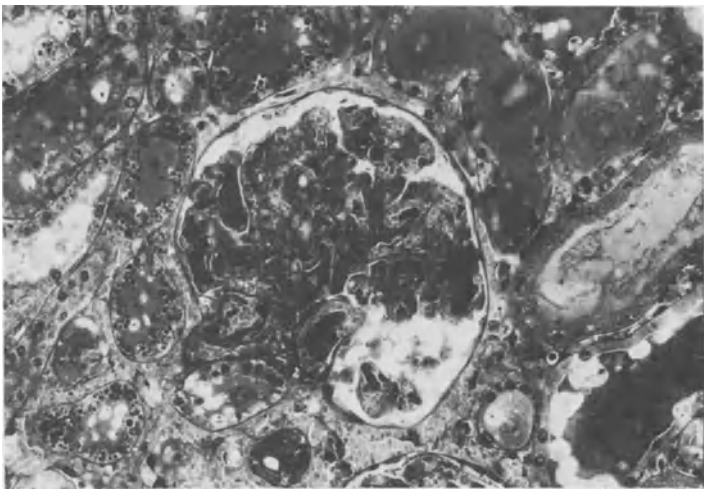

Fig. 4. Multiple hyaline microthrombi in glomerular capillaries and storage of hyaline droplets in tubular epithelial cells. Kidney of a pig with acute CSF.
(Photomicrograph courtesy of Prof.E. Weiss, University of Giessen, FRG).

population, local circulatory disturbances or failure of the bone marrow to regenerate its megakaryocyte pool. Unfortunately, an infection of megakaryocytes by HC virus has not been demonstrated.

It is of considerable comparative interest that in African Swine Fever ASFV can clearly be demonstrated in platelets and megakaryocytes (29). In experimental infections with the ASFV the state of the megakaryocyte was studied. When experimental pigs were infected with ASFV of low virulence, they became acutely thrombocytopenic four to five days after the onset of viraemia and fever. It was shown by immunofluorescence that 2 to 10% of the megakaryocytes were infected.

6. FOETAL AND NEONATAL PATHOLOGY

Several foetal and neonatal abnormalities have been attributed to natural exposure of the dam to HC virus or vaccination with attenuated life virus during pregnancy (2, 30, 31, 32, 33). Transplacental infections may result in prenatal death, anasarca, ascites, a rather large spectrum of malformations, congenital tremors, death of the neonate shortly after birth or later in life. For each one of these abnormalities a critical period of gestation when the developing foetus is exposed to the HC virus is to be assumed; however, precise data are only partly available from experimental infections.

Obviously, HC virus crosses the placenta of the non-immune or insufficiently immunized dam and may induce embryonic or foetal death with resorption of the embryo or mummification of the foetus (31, 34, 35). Several workers observed that foetal death with mummification, malformation or stillbirth occurred in sows vaccinated with attenuated HC virus of low virulence (2, 31, 36).

Experimental infection of pregnant sows with the low virulent HC virus strain Glentorf on the 65th and 67th day of gestation resulted in 30% foetal mortality (37, 49, 50). Exposure of gilts on the 85th day of gestation

led to infection of foetuses but not to virus isolation from the new-born piglets. There was no mortality in foetuses when the gilts were infected between the 94th and 101st day of gestation.

6.1. Teratogenic effect of HC virus.

Transplacental infection of the porcine foetus with both field and vaccine strains of HC virus may induce a spectrum of abnormalities including hypoplasia of the lungs, malformation of the pulmonary artery, micrognathia, arthrogryposis, fissures in the renal cortex, multiple septa in the gall bladder, and malformations of the brain (32, 38, 39, 40). The teratogenicity of HC virus clearly depends on the stage of gestation. In general, the earlier the infection occurs the more severe the abnormalities are likely to be.

In the central nervous system the transplacental infection of the foetus at a critical stage of gestation (30 days) induces retardation in growth and maturation of the brain with the manifestation of microencephaly in the most severe cases (38). More common, however, is cerebellar hypoplasia which in some cases is the cause of congenital tremor in the new-born piglet. Experimentally, cerebellar hypoplasia of varying severity has been induced by infecting pregnant sows with field or vaccine strains of HC virus (38, 41, 42, 43, 54).

6.2. Congenital tremor.

Tremors (trembles) in new-born piglets were postulated to be due to intrauterine infection of the foetus with HC virus (44). Subsequently, cerebellar hypoplasia, hypomyelogenesis, and congenital tremor were observed in new-born piglets from 35 of 63 litters of sows vaccinated with tissue culture-attenuated virus vaccines (32, 42). Other studies indicated that transplacental infection of the foetus with HC virus would cause failure of myelin and cerebellum to develop. It was estimated that in Great Britain 12% of the cases of congenital tremor were due to HC virus infection (45). A specific strain of HC virus

was believed to be responsible for the development of tremors (46). This form of congenital tremor (Type A I) is characterized by dysgenesis, hypoplasia, local cortical dysplasia of the cerebellum, and by the defective myelination of the CNS generally (41, 68). Furthermore, the spinal cord is reduced in size and shows marked dysmyelination.

6.3. Stimulation of the foetal immune system.

Depending on the stage of gestation transplacental HC virus infection of the developing foetus may induce early maturation of lymphoid tissue. When pregnant gilts were infected at days 65 and 85, respectively, there was premature formation of germinal centers and proliferation of plasma cells in lymphoid tissues like lymph nodes and tonsils but not in the spleen (47). This morphological finding of virus-induced stimulation of the immune system correlates with elevation of foetal serum immunoglobulins (IgM and IgG) (48).

6.4. Stillbirth.

Stillbirth is also a feature of transplacental infection of the porcine foetus with HC virus. It usually occurs after maternal infection during the last third of gestation (36, 38, 49, 50). At necropsy, characteristic macroscopic lesions are petechial haemorrhage, subcutaneous oedema, ascites, and hydrothorax, sometimes associated with mottling of the liver (51).

7. CONGENITAL PERSISTENT HC VIRUS INFECTION

One of the sequelae of transplacental HC virus transmission to the porcine foetus is congenital persistent HC virus infection with the evolution of a runting-like syndrome during the first months of life (52, 53). At birth affected piglets appear healthy although they are viraemic. Viraemia may persist throughout the life of the pigs. The first signs of disease appear at the age of 9 weeks, but in some animals as late as 28 weeks. At the onset of disease leukocytes fall below 10,000/mm^3. Clinical signs include growth retardation, increasing anorexia and depression,

conjunctivitis, dermatitis, intermittent diarrhoea, and locomotor disturbance with posterior paresis eventually developing. At necropsy, the most important lesion is a marked atrophy of the thymus which often is associated with swelling of lymph nodes due to oedema. However, lesions characteristic of CSF, particularly petechial haemorrhages, are not present (52). Pigs with congenital persistent HC virus infection are not capable of producing specific antibody against HC virus, suggesting a state of immunological tolerance (53).

A similar syndrome was produced experimentally, especially if the dams were infected at 65 days of gestation (37, 49, 51, 54, 55). In one series of experiments pregnant sows were infected with a low-virulent field strain of HC virus at 40, 65 and 90 days of gestation (51).

Prenatal mortality was highest in litters from sows infected at gestation day 40; postnatal death was most frequently recorded in litters from sows infected at 65 days. A number of surviving piglets born from sows infected at days 40 and 65, respectively, developed a persistent infection. In these piglets a runting syndrome began to appear about one week after weaning. Affected animals are persistently viraemic, show growth retardation, intermittent diarrhoea, and weakness of the hind legs. Similar to spontaneous congenital persistent HC virus infection characteristic lesions at necropsy are gastroenteritis, thymic atrophy, swelling of lymph nodes and in few animals bouton ulcers in the colon.

Serological studies in pigs with congenital persistent viraemia reveal that they neither have antibodies to HC virus, nor virus-antibody complexes. The lymphocyte response to phytohaemagglutinin is slightly depressed, whereas the immune response to pokeweed mitogen is not altered. These findings taken together suggest a state of immunological tolerance in pigs with chronic persistent CSF which were infected in utero (51).

In a similar series of experiments pregnant gilts

were infected intranasally with the low-virulent strain Glentorf of HC virus at days 40, 70, and 90 of gestation, respectively (49, 50, 54). While the experimental transplacental infection resulted in foetal death in some of the foetuses, the majority of new-born piglets born from sows infected at 40 and 70 days of gestation, respectively, proved to be viraemic at birth. These piglets either died within the first week post partum or survived up to eight weeks. Clinical signs included tremor in few piglets. In an other small group there was evidence of growth retardation and runting.

An important feature of both spontaneous and experimental congenital persistent HC virus infection is a severe depletion of lymphocytes in the thymus and peripheral lymphoid organs (54, 56, 57a). The histological examination reveals marked depletion of thymus lymphocytes in the cortex, frequently associated with necrosis of lymphoid cells (56). Much less affected by atrophy is the medullary area of the thymus. Generally, the B-cell-dependent areas in the lymphoid follicles of spleen and lymph nodes are more severely depleted than the thymus-dependent areas (56). In the spleen lymphoid follicles may no longer be visible in the most severe cases. Likewise, superficial and internal lymph nodes show marked regression of lymphoid follicles, and there is proliferation of reticulo-endothelial cells and fibrosis.

Morphological findings in the adrenal cortex are hyperplasia of the zona fasciculata with atrophy of the adjacent zona glomerulosa and reticulata (56).

The pathomechanism underlying the marked depletion and destruction of lymphocytes in lymphoid organs is not well understood. Although in congenital persistent HC virus infection the virus is widespread in lymphoid tissues, it is debatable whether the destruction of lymphocytes is due to a direct cytotoxic effect of the virus. In a working hypothesis it was postulated that lymphocyte depletion may be caused by enhanced release of glucocorticos-

teroids from the hyperplastic adrenal cortex (56).

The pathogenesis of congenital persistent HC virus infection is incompletely understood. Obviously, the stage of gestation at which the virus reaches the foetus is an important factor. Data derived from experimental HC virus infection of pregnant sows indicate that the foetus is to be infected before day 65 of gestation in order to induce a persistent infection (49, 51, 54, 55). If the transplacental infection occurs around or after the onset of immune competence it is likely to be controlled or the virus to be eliminated during pregnancy or early in life (54). An other pathomechanism may be that the virus eludes the humoral and cellular immune response of the host. This view is supported by the finding that cells infected with HC virus bear no or very little viral antigen on the cellular surface ; thus, infected cells may escape the immunological attack of the host (57b). Finally, HC virus by infecting and destroying lymphoid cells of the immune system may induce defects in immunological functions, that is long-lasting immunological tolerance in the developing foetus.

8. PATHOGENESIS OF HC VIRUS INFECTION

Experimental studies have clearly shown that the tonsil is the primary site of virus invasion following oral exposure (1, 58, 67). Interestingly, after intramuscular and subcutaneous infection, HC virus was also found consistently and in high concentration in the tonsil (59). In a sequential study the quantitative distribution of a virulent strain of HC virus in various tissues covering a range of three hours to seven days after oral exposure has been determined (60, 61). HC virus was demonstrated in the tonsil as early as seven hours post exposure. As virus has never been detected in other tissues of the oral cavity within 24 hours following oral exposure, the tonsil appears to be the organ of preference. Virus titers in the tonsil were found to remain at a high level up to post-infection

day 7. Primary multiplication of virus in this organ commences between 7 and 48 hours post-infection. During this time the virus appears to be limited to epithelial cells of the tonsillar surface and crypts. Most likely, the virus is then transferred through lymphatic vessels to the mandibular, retropharyngeal, parotid, and cervical lymph nodes where it can be detected at 16 hours post infection. The virus, possibly associated with cells, enters blood capillaries, thus giving rise to initial viraemia at approximately 16-24 hours (58, 60, 61). At this time the virus can be demonstrated in the spleen and thereafter in many other secondary organs. Further sites of virus multiplication are mainly superficial and visceral lymph nodes, bone marrow, and the solitary lymphoid follicles and Peyer's patches in the intestinal mucosa.

As determined by cell culture and immunofluorescence, the blood, spleen, and lymph nodes attain the highest virus titers (62, 63, 64, 65).

The HC virus exerts its pathogenetic effect on different types of cells including (1) endothelial cells (2) lymphoreticular cells and macrophages and (3) epithelial cells. Since CSF viral antigen can be demonstrated in these cells with the immunofluorescence method, they appear to be the major target cells for virus multiplication (13, 48, 52, 61, 63, 64, 65, 66, 67).

From immunohistological studies it is evident that the HC virus replicates in endothelial cells (13, 25). However, the pathomechanism of damage to endothelial cells eventually leading to plasma and erythrocyte diapedesis is not clear. The endothelium is the major source of synthesis of the so-called plasma-factor VIII-related antigen (VIIIR:Ag). If a significant proportion of endothelial cells is injured, a decrease in plasma levels of VIIIR:Ag would be expected. However, in HC virus-infected pigs neither an increase, nor a decrease of VIIIR:Ag was detectable (26).

There is abundant evidence that the HC virus has a

distinct affinity to cells of the lymphoreticular organs. In the tonsils immunofluorescence specific for the HC viral antigen is seen in the cryptal epithelial cells and later in macrophages of germinal centers as well as in scattered reticular cells. Similarly, viral antigen is detectable in germinal centers of Peyer's patches of the intestine, in germinal centers of lymph nodes, and in the white and red pulp of the spleen.

Important epithelial cells with evidence of HC virus multiplication are the submandibular salivary gland, mucosal cells of the small intestine, especially the crypt epithelial cells and renal epithelial cells.

In persistently viraemic animals infectious HC virus can be isolated from mononuclear cells of the blood (51) and from tissue and blood lymphocytes (33).

9. CONCLUDING REMARKS

The HC virus exerts its pathogenetic effect on different types of cells including endothelial cells, lymphoreticular cells and macrophages, and certain epithelial cells. These cells appear to be the major target cells for virus multiplication. While it is evident from immunohistological studies that the HC virus replicates in endothelial cells, the pathomechanism of damage to these cells is not clear. So far, changes in the plasma levels of the so-called plasma-factor VIII-related antigen indicating injury of endothelial cells, have not been demonstrated.

From immunofluorescence studies there is abundant evidence that the HC virus has a distinct affinity to cells of the lymphoreticular organs. In these organs the virus causes severe depletion of lymphocytes which affects both B-cell and thymus-dependent areas. The pathomechanism underlying the marked depletion of lymphocytes is not well understood. Working hypotheses suggest that the destruction of lymphocytes may be due to the direct cytotoxic effect of the HC virus or that it may be caused by enhanced release of glucocorticosteroids from the hyperplastic adrenal cor-

tex.

During the course of acute CSF multiple defects of haemostasis develop; considerable progress was made in the understanding of the pathogenesis of disseminated intravascular coagulation. However, it is not yet fully understood whether the diffuse activation of the blood coagulation system is caused by release of procoagulant substances from cells in lymphoid tissues, endothelial cells in vessels or epithelial cells of the gastro-intestinal tract following damage by the HC virus. The important consequence of massive activation of the coagulation system is the consumption of platelets and certain coagulation factors resulting in disseminated intravascular coagulation and petechial haemorrhage. Thrombocytopenia appears to be an essential factor in the pathogenesis of haemorrhagic diathesis in CSF. Since marked thrombocytopenia occurs early after infection, it cannot be explained by consumption of clotting factors during disseminated intravascular coagulation. Sequential experimental studies have provided evidence that the HC virus causes damage to circulating platelets beginning two days post infection. But destruction of megakaryocytes in the bone marrow has also been demonstrated as an important pathogenetic factor.

Transplacental infection of the developing foetus with the HC virus may result in prenatal death, anasarca, ascites, a large spectrum of malformations, congenital tremor, and death of the neonate shortly after birth or later in life. It is reasonably clear that these foetal and neonatal abnormalities are either due to natural exposure of the dam to the HC virus or vaccination with attenuated life virus during pregnancy.

Considerable effort has been put into the elucidation of the pathogenesis of congenital persistent HC virus infection. Obviously, the stage of gestation at which the virus reaches the foetus is an important pathogenetic factor, but the virulence of the causative HC virus strain may be equally important. Data derived from experimental HC

virus infections of pregnant sows indicate that the foetus is to be infected before day 65 of gestation in order to induce persistent infection. Several pathogenetic pathways have been considered, including the possibility that the HC virus may, by infecting and destroying lymphoid cells, induce defects in immunological functions, that is long-lasting immunological tolerance in the developing foetus. An other pathomechanism may be that the virus eludes the humoral and cellular immune response of the host; this view is supported by the finding that cells infected with the HC virus bear no or little viral antigen on the cellular surface and thus may escape the immunological attack of the immune system.

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3

CHARACTERISTICS OF THE VIRUS

V. MOENNIG

1. VIRION MORPHOLOGY

1.1. Filtration

In 1903 de Schweinitz and Dorset (1) described the serial transmission of hog cholera (HC) using body fluids of diseased pigs. The fluids had been filtrated through 'finest porcelain filters', thus indicating that the causative agent of hog cholera was a filtrable substance rather than the 'hog cholera bacillus'. These first attempts to characterize hog cholera virus (HCV) particles were followed by experiments using more advanced filtration techniques, electron microscopy, electrophoresis, and ultracentrifugation. Probably due to technical difficulties early results were inconsistent. Table 1 summarizes the attempts to assess the size of HCV particles (2-24). Kernkamp (2) calculated a size of smaller than 35 nm after filtration of the virus through collodion membranes. An even smaller size of 15-25 nm was determined by Pehl and Gralheer (4). However, in more recent filtration studies (6, 9, 11) particle sizes varied from approximately 29 nm to 50 nm. In experiments by Kubin (9), e.g., pigs only seroconverted after being inoculated with filtrates from membranes with a pore size of 35-100 nm. Hantschel (17) calculated a size of 40 ± 5 nm for HCV isolates from serum of experimentally infected pigs using a method that correlates size with electrophoretic mobility of virus particles in agar gels of 2-6%. Horzinek (7) calculated a size of 28-39 nm by determining the sedimentation coefficient of the virus.

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TABLE 1 Determination of whole particle and core sizes by different methods

Particle	Core	Method	Source of virus	Author	Ref.
< 35		filter	serum	Kernkamp 1929	2
22-30		EM	serum	Reagan et al. 1951	3
15-25		filter	serum	Pehl & Grahlher 1956	4
20-44		EM	blood/serum	Ageev 1958	5
< 50		filter	prim. kidney	Dinter 1963	6
28-39		UC	prim. testicle	Horzinek 1967	7
40 ± 3	29 ± 3	EM	prim. testicle	Horzinek et al. 1967	8
> 35		filter	prim. testicle	Kubin 1967	9
29-36	28-29	filter	PK(15)	Mayr et al. 1967	10
39-40	28-29	EM	PK(15)	Mayr et al. 1968	11
40-50		EM	prim. kidney	Cunliffe & Rebers 1968	12
40-50		EM	PK(15)	Ritchie & Fernelius 1967	13
40-50	40	EM	PK(15)	Ritchie & Fernelius 1968	14
46 (41-54)	33	EM	prim. testicle	Ushimi et al. 1968	15
53		EM	tonsil	Cheville & Mengeling 1969	16
40 ± 5		AGE	serum	Hantschel 1969	17
44	36	EM	PK(15)	Scherrer et al. 1970	18
40		EM	lymphnode	Schulze 1971	19
53 ± 14	27 ± 3	EM	PK(15)	Horzinek et al. 1971	20
47 ± 5	27 ± 3	EM	PK(15)	Frost et al. 1977	21
40-45	30-35	EM	div. organs	Rutili & Titoli 1977	22
30-55		EM	prim. kidney	Scott et al. 1977	23
42 ± 8		EM	PK(15)	Enzmann & Weiland 1978	24

AGE - agargelelectrophoresis
EM - electronmicroscopy
UC - ultracentrifugation

1.2. Electron microscopy

Early electron microscopic studies on HCV were hampered by the fact that an efficient 'in vitro' propagation of the virus was not possible. Investigations were confined to tissues and serum samples from infected pigs, possibly contaminated accidentally with viruses unrelated to the etiological agent of HC. Reagan (3) and coworkers found 22-30 nm (mean 27 nm) particles in serum of infected pigs. Ageev (5) detected spherical particles with a diameter ranging from 20 to 44 nm on the surface of erythrocytes and in the serum of pigs infected with HCV. Early reports on HCV particles isolated from tissue culture (25, 26) revealed sizes from 20 to 80 nm. However, it was found later that the viral strains investigated were contaminated with viruses other than HCV (27, 28). Later investigations on purified HCV grown in tissue culture consistently revealed spherical, enveloped viral particles with diameters between 37 and 67 nm (8, 11-16, 20, 21, 23). Occasionally irregularly shaped protrusions (8) or projections (13-15, 24) were found on the viral envelope. The latter structures were shear-sensitive (14) and as a consequence they were easily lost during the process of virus purification. Similar projections, which were only loosely attached to the virus surface have been found on several mammalian oncornaviruses and they represented the major glycoprotein of these viruses (29, 30). Whether the surface structures of HCV represent the viral glycoprotein and whether this structure is identical with the 'soluble antigen' produced in infected organs and cells is not yet clear (14, 31). The mean thickness of the envelope was calculated to be 6 nm (8). The envelope surrounds a hexagonally shaped, electron dense inner core structure. Measurements of core diameter have been shown by some investigators to vary between 24-33 nm (8, 10, 15, 20, 21) and by other investigators to range between 36-40 nm (14, 18). The tadpole- or sac-like appearance of particles negatively stained with potassium phosphotungstate (12-14) (PT) may probably have been artefacts due to the staining procedures with PT. The phenomenon has been observed with a number of

enveloped viruses (H. Frank, personal communication). The remarkable heterogeneity of particle size is attributed to the viral envelope. Studies with rubella, equine arteritis and the closely related bovine viral diarrhoea virus (BVDV) showed that these other non-arbo togaviruses shared morphological features with HCV (6, 20, 32).

Electronmicrographs of ultrathin-sections of HCV-infected PK(15) cells showed enveloped viral particles with a mean diameter of 44 nm and a hexagonally shaped electron dense core of 36 nm thus confirming the more recent observations made with purified HCV particles (18). The majority of mature virus particles was found in extracellular spaces near or closely attached to the cellular membrane. First viral particles were detected 10 hours after infection. Some virus was found in the Golgi apparatus and in intracytoplasmic vesicles (18). In rare instances Scherrer and coworkers (18) were able to demonstrate that virus was released from cells by budding as observed with other enveloped viruses. Electronmicroscopical studies on viral pathogenesis revealed HCV particles with diameters between 40 and 53 nm in ultrathin sections of tonsillar epithelium and lymphnodes (16, 19, 22).

2. PHYSICO-CHEMICAL PROPERTIES

Prerequisites for the efficient study of physicochemical properties of a virus are the availability of 'in vitro' propagation methods, assays for measuring infectivity and effective procedures for concentration and purification of the virus. In contrast to other togaviruses, e.g. alphaviruses, the propagation and purification of HCV presented considerable difficulties (33).

2.1. Nucleic acid

The use of halogenated pyrimidines and other chemicals interfering with DNA synthesis were classical methods to determine the nucleic acid composition of unclassified viruses. Kinetic studies of HCV replication in cell cultures treated with halogenated deoxyuridine derivatives (6, 9, 34) or

aminopterine (6) gave evidence that the virus had a RNA genome. Later investigations showed that viral RNA was infectious (35), as had been shown previously for the genome of BVDV (36). The RNA sedimented at $S_{20,w}$ = 40-45 in linear 10-30% sucrose gradients (37). Infectious BVDV RNA was shown to sediment at a slightly lower rate of about $S_{20,w}$ = 38 in linear 10-30% sucrose gradients (38). Whether these discrepancies in sedimentation behaviour reflect true differences in molecular weight of viral RNAs or whether they can be attributed to modifications of the methods used is not clear. Contrary to other non-arbo togaviruses (39, 40) little else is known about the structure and composition of HC viral RNA.

2.2. Inactivation

Attempts to classify viruses involve treatment of the virus with chemicals and enzymes and exposure to extreme pH values and elevated temperature. Resulting inactivation kinetics are characteristic for the virus and facilitate its classification. Due to its lipoprotein envelope HCV was readily inactivated by chloroform, ether and detergents like deoxycholate, Nonidet P40 (NP 40) and saponin (6, 9, 34, 41). The virus was also sensitive to the action of ultraviolet radiation (9) and to a pH between 3 and 4 (34). As observed with other togaviruses high pH values of about 9.0 stabilized HCV, and the pH of 11 only partially inactivated the virus (9, 42). Storage of HCV at -30^{o} C did not affect its viability, whereas temperatures above freezing influenced the survival of the virus, depending on temperature, time of exposure and the composition of the surrounding medium (6, 9). However, storage for several weeks at refrigerator temperatures in tightly sealed glass flasks appeared not to affect the infectivity of HCV markedly (B. Liess, personal communication). A complete inactivation was achieved within 10 minutes at 60^{o} C (9). Proteolytic enzymes like trypsin had a moderately inactivating effect on HCV compared with trypsin sensitive non-enveloped viruses like foot-and-mouth disease virus (6, 9).

2.3. Density and sedimentation behaviour

Since the propagation of virus in cell culture only yielded moderate or low titres and since the virion seemed to be fragile and somewhat heterogeneous in size and composition, purification presented - as mentioned above - technical difficulties. HCV is released from the cell with its lipid envelope containing host cell components. Due to this structure virions are labile and they vary in size, shape and density depending on their environment. The heterogeneity of viral particles was reflected by a relatively wide range of sedimentation coefficients (84-132, mean 108) as calculated by Horzinek (7). A similar, unusually low sedimentation coefficient ($S_{20,w} > 90$) was observed for BVDV (43), whereas Laude (33) reported a sedimentation coefficient of $S_{20,w}$ = 150 for HCV. Unlike many other togaviruses HCV does not band at a closely defined density region in cesium chloride or sucrose density gradients. There is rather a relatively broad density range observed by using equilibrium centrifugation of HCV (Table 2).

TABLE 2 Physical properties of the virus

Density	Gradient Medium	$S_{20,w}$	Author	Ref.
1.15-1.20	CsCl		Horzinek 1966	44
		84-132	Horzinek 1967	7
1.14-1.20	CsCl		Mayr et al. 1967	10
1.15-1.17	Sucrose		Ritchie & Fernelius 1967	13
1.14-1.15	CsCl		Cunliffe & Rebers 1968	45
1.15-1.16	CsCl		Ushimi et al. 1968	15
1.14	Urografin		Frost et al. 1977	21
1.16	Sucrose		Frost et al. 1977	21
1.13	Sucrose	150	Laude 1977	33

CsCl - cesium chloride

The average density of HCV in sucrose is lower than the density observed after equilibrium centrifugation in cesium chloride. Laude (33) reported that HCV which was harvested from extracts of virus-infected cells banded in sucrose gradients very close to membraneous material of cellular origin at 1.13 g/ml.

3. ISOLATION AND CULTIVATION

3.1. 'In vivo' cultivation

For many years the serial passage of HCV in pigs was the only efficient and reliable method of virus propagation. Thus viral strains retained their immunological and pathogenic properties. Adaptation of viruses to other than their natural hosts often resulted in a change of pathogenicity and thereby led to adequate sources of vaccine viruses. Successful attempts to adapt HCV to other animal species were first performed using ruminants. Jacotot (46) transmitted the virus to goats and Zichis (47) used sheep and calves for his experiments. In Japan the transmission of HCV to goats was repeated (48) and the virus was passaged for 209 times. Thereafter the adapted virus was used for the infection of pigs. The experimentally infected animals developed HC with no apparent change of pathogenicity compared to the original virus, except for a prolongation of the disease for a few days (49). More transmission experiments showed that the 'in vivo' host spectrum of HCV also included peccaries, deer and rabbits. It was noted that experimentally infected animals other than pigs did not transmit the virus naturally to penmates of the same species or to susceptible pigs. Other animal species tested like wild mice, cottontail rabbits, rats, racoons, sparrows and pigeons did not support the propagation of the virus (50). Among all heterologous hosts only the rabbit gained major importance, especially for the production of attenuated vaccine strains. For several mammalian viruses, among them poliomyelitis, dengue and rinderpest viruses (51-53), rabbits had proven to be suitable for virus adaptation. After initial difficulties it became possible to cultivate HCV in rabbits by first using alternate passages in

rabbits and pigs. Thereafter the virus was passaged only in rabbits (54, 55). The only clinical sign produced by HCV infection in rabbits was a short febrile period on the third day after inoculation. At that time most organs, especially spleen and peripheral blood contained substantial amounts of infectious virus (56). Whereas first viral passages in rabbits hardly resulted in any loss of pathogenicity for pigs, high passage levels altered HCV in a way that allowed the widespread use of lapinized virus as a live vaccine (56-58). An alternative method for the attenuation of virulent virus included first passages in chick embryos and chick embryo cells before transmission to rabbits (59). To date a number of lapinized HCV strains of different origins, including the widely used China (C) strain, are used for vaccine purposes. It was noted however, that in spite of their attenuation, some lapinized virus might have a residual pathogenicity for pregnant sows by causing malformations and fetal death (60). The 'in vivo' host range of HCV is summarized in Table 3.

TABLE 3 'In vivo' host range of the virus

Host Animal	Author	Ref.
Goat	Jacotot	46
	Hashiguchi & Hika 1942	48
Sheep	Zichis 1939	47
Calf	Zichis 1939	47
Rabbit	Baker 1946	54
	Koprowski et al. 1946	55
Peccaries	Loan & Storm 1968	50
Deer	Loan & Storm 1968	50

3.2. Cultivation in tissues and primary cells

Due to the tedious and expensive procedures involved with the 'in vivo' multiplication of HCV, relatively early attempts were made to propagate the virus 'in vitro'. Hecke (61)

succeeded in cultivating HCV in several organ explants like plexus chorioideus, lymphnode, bone marrow and spleen. Further attempts for 'in vitro' cultivations were mainly focused on the use of blood cells from spleen (62, 63), bone marrow (64) and cells from the peripheral blood (65-68). Ten Broeck (69) successfully used minced testicle for the 'in vitro' propagation of HCV. Virus titres obtained varied considerably depending on the method of cultivation and the origin of the cells.

With the availability of primary cells from trypsinized organs, studies were conducted to propagate HCV in monolayer cell cultures. It soon became apparent that the virus replicated in embryonal porcine cells (70-73). Although primary kidney cell cultures of different species like guinea pig, fox squirrel, rabbit, striped skunk and American badger supported the growth of HCV (74), primary porcine kidney or testicle cells were most commonly used for the propagation of HCV. The 'in vitro' host range of HCV even included dolphin, whale and mink cells, when low passage cell lines of these animals were used (74).

3.3. Cultivation in permanent cells

The 'in vitro' propagation of HCV soon shifted to the use of permanent cell lines, which offered the advantage of better reproducibility and a standardized virus replication. Initial experiments involved the use of porcine kidney cells which had been transformed by SV40 (75). A variety of permanent cell lines was shown to be susceptible to the infection with HCV, including cell lines from porcine, bovine, canine, primate and guinea pig origin (74-76). Currently several permanent pig kidney cell lines, especially the PK(15) cell are commonly used in laboratories for the cultivation and 'in vitro' propagation of HCV.

3.4. Use of cell culture in the production of vaccine virus

Hopes that the attenuation of HCV in cell cultures could lead to the establishment of viral strains suitable for vaccine

production did not materialize fully, and tissue culture derived vaccines did not completely replace attenuated vaccines produced in rabbits. It was reported in several publications that HCV not only lost its pathogenicity (73, 77, 78) after serial passages 'in vitro', but in some instances it also lost its genuine antigenicity (65). The latter virus was poorly antigenic and, used as a vaccine, failed to induce effective protection in pigs against challenge with virulent HCV (65). These effects are not generally described for all HCV strains passaged in cell cultures. They probably depend on the virus strains and the cell systems used. Rabbit cells (79), ovine cells (59, 80, 81) and guinea pig kidney cells (76) are now used for the production of commercial tissue culture derived HCV vaccines.

3.5. The problem of viral contaminations

All 'in vivo' and 'in vitro' techniques for the cultivation of HCV imply the risk of contamination with viruses other than HCV. The use of non-permanent cell cultures bears a considerable risk of contaminations with other viruses or mycoplasma present in the donor organism. Contaminants, e.g. passenger viruses had initially been the cause for some misleading reports on the properties of HCV (25, 26).

Generally HCV replicated in tissue culture without producing a cytopathic effect and infection of cultured cells had to be monitored by serological methods, e.g. immunofluorescence rather than virus-induced morphological alterations of cells. Only a few strains of HCV were proven to be cytopathogenic (82, 84-86). Some reports on the cytopathogenicity of HCV were questioned later and the cytopathic effects were shown to be associated with contaminating viruses like adenoviruses, parvoviruses or cytopathogenic BVDV (27, 28, 83, 84, 87, 88). The use of permanent cell lines reduced the risks of accidental uptake of other viruses considerably although contaminations with retroviruses, porcine parvoviruses (89) and other pestiviruses namely BVDV still play an important role. Retroviruses were

occasionally found in permanent porcine cell cultures when ultrathin sections of cells were examined electronmicroscopically (90, 91). These viruses can be distinguished from HCV according to their size in electron microscopic studies (Gelderblom, personal communication). C-type particles may also interfere with the purification of HCV since they band in the same density region in sucrose gradients as HCV particles (92). It is not yet clear whether these viruses represented endogenous C-type particles of pigs or whether they originated from outside sources. No interference of C-type viruses with the replication of other viruses, namely HCV, had been reported.

Probably the most serious contamination of permanent cell lines and viral strains which has been frequently observed is caused by BVDV (93-95). Since BVDV is a widespread infection of cattle and since it crosses the placenta of infected animals (96), the common sources for these contaminations are infected fetal and newborn calf sera (97) as well as primary bovine cell cultures. The use of ovine sera and cells is not a suitable alternative unless contamination with border disease virus, a virus closely related to BVDV, is excluded. It is safe to assume that the full extent of damage caused by occult BVDV contaminations in permanent cells and HCV strains has not yet been fully assessed. BVDV contaminations can only be avoided by careful examination of cells and sera and the use of well characterized and purified virus (98).

4. VIRAL STRAINS

No major antigenic differences have been observed among HC viruses. However from early reports it became clear that there were variants, which differed from standard viral strains in both antigenic structure and pathogenicity. These variant viruses were observed in the United States (99, 100) and other countries (101, 102) especially during, but also after mass immunizations of pig herds employing the method of serovaccination formerly introduced by Dorset (103). Whether these variants were the result of a natural variability of HCV or

whether they emerged under the selective pressure of sero-vaccination was subject to speculation (104). Such antigenic differences among HC viruses can be detected by neutralization, since neutralization by homologous antisera is more efficient than neutralization by heterologous sera. Similar results were obtained employing other serological methods, e.g. direct immunofluorescence (105) using conjugates prepared from strain specific antisera and applied to cell cultures infected with one of these HCV strains (Ames and 331 respectively). Aynaud and coworkers (104) distinguished two groups of HCV strains which differed with respect to pathogenicity and serological behaviour. The highly pathogenic Alfort strain, the attenuated Thiverval (106) strain and the lapinized Chinese strain (56) were considered reference strains for the first group of viruses comprising the larger number of HC viral strains. The prototype strain for the second group was the American 331 strain (107), which had been isolated from a pig herd with endemic HC. The latter strain displayed a closer serological relationship to BVDV than members of the first mentioned group (108). A similar classification of HCV strains was proposed by Japanese investigators (109). Pathogenic strains were included in a group designated 'H' with the ALD strain as reference. Strains of low pathogenicity and a close serological relationship to BVDV were classified in group 'B'. In addition intermediate types of HCV strains were described (110). Neukirch and coworkers (111) questioned the validity of classifying HCV strains. They demonstrated that antigenic differences between the Alfort and the 331 strain were expressed in neutralization tests by a difference of only two $\log_2$ steps. Furthermore they were able to show that the BVDV strain A1138/69 was only distantly related to both the Alfort and 331 strains.

Despite relatively minor antigenic variations among HC viruses, a number of strains have been established. Origin and history of these strains was more or less well documented. Some of the more commonly used HCV strains are listed in Table 4. In addition many strains and isolates exist, which are used in diagnostic laboratories and for commercial purposes.

TABLE 4 Some viral strains of hog cholera

Strain	Country of Origin	Authors	Ref.
Michigan A	USA	Dunne et al. 1952	112
Sprankle	USA	Dunne et al. 1957	65
Ames	USA		113
PAV-1	USA	Gillespie et al. 1960	86
ALD	Japan		73
Miyagi	Japan	Sato et al. 1964	73
München-1	Germany	Mayr & Mahnel 1964	25
		Mayr et al. 1968	11
Alfort	France	Aynaud 1968	114
331	USA	Mengeling & Packer 1969	107
Washington	USA		17
Thiverval	France	Launais et al. 1972	106
Loud	France	Aynaud et al. 1974	104
Glentorf	Germany	Pittler et al. 1972	115
Eystrup	Germany	Liess and Schurian 1973	116
Hannover	Germany		117
China	China		56

5. SEROLOGICAL RELATIONSHIP TO OTHER PESTIVIRUSES

5.1. Immunoprecipitation with soluble antigens

Precipitating antibodies against HCV were detected in sera of convalescent pigs by immunodiffusion (ID) tests using antigen preparations from organs of animals infected with HCV (118). The pancreas was the most suitable organ for the extraction of diagnostic antigens. Darbyshire (119, 120) established a similar ID test for the diagnosis of BVD utilizing antigens from several organ extracts of animals suspected of having the disease. When BVDV and HCV antigens were applied to adjacent wells Darbyshire observed a single continuous precipitation line between both antigens suggesting that a very close serological relationship existed between both

agents. Historically this finding has led to the formation of the genus pestivirus within the family togaviridae (121). A similar crossreaction with antisera against HCV was demonstrated using a soluble antigen from supernatants of tissue culture cells infected with BVDV (122, 123). Cells infected with HCV yielded a comparable soluble antigen (31, 124) and a line of identity was observed when tissue culture derived antigens of both viruses were reacted against a HCV antiserum (125). The relationship between both viruses was confirmed by later reports, although there was some disagreement on its extent and the nature of crossreactive antigens.

The cell culture derived soluble antigens of BVDV and HCV and the pancreas-derived antigen of HCV have been thoroughly studied with respect to common antigenic determinants (126, 127). According to these reports three polypeptides, all derived from a single protein and isolated from the pancreas of HCV-infected pigs, crossreacted with the soluble antigen from BVD-infected cells. The soluble antigen from HCV-infected tissue cultures also formed a line of identity with soluble antigen of BVDV cultures (125), suggesting that the pancreas antigen and the tissue culture derived soluble antigen were identical. Based on its binding to the lectin concanavalin A the common soluble pestivirus antigen was identified as a glycoprotein with protective properties when used as an immunogen (128), suggesting that the antigen represented the major viral glycoprotein on the surface of pestiviruses. The above mentioned fact that surface projections on HCV readily detach from the virion (14) strengthened this notion. Similar observations have been made with retrovirus-infected cell cultures, e.g. bovine leukemia virus, where the supernatant contained large amounts of viral surface glycoproteins (129). However, the finding that infectious HCV particles were readily agglutinated by the lectin of the castor bean rather than concanavalin A (130) was not in support of the above mentioned hypothesis. Alternatively the soluble antigen could be one of the nonstructural proteins produced during the replication of pestiviruses (40).

5.2. Complement fixation, immunofluorescence and monoclonal antibodies

Further studies using the methods of complement fixation and immunofluorescence (IF) confirmed the antigenic relationship between BVDV and HCV. Using a direct IF test Mengeling and coworkers (131) were able to demonstrate serological crossreactions between both viruses. This serological technique detects antibodies reacting with most viral antigens, regardless of whether they are located on the viral surface or whether they represent an internal component. The soluble antigen of BVDV was shown to react in complement fixation tests with antisera against HCV (132). Recently monoclonal antibodies against BVDV had been produced (133). Several of these antibodies were specific for all BVDV and HCV strains tested so far, suggesting that there are conserved epitopes shared by both viruses. The location of the common antigenic determinants on viral structural proteins is not yet clear.

5.3. Crossneutralization and crossinfection

The first report on a serological relationship between HCV and BVDV initiated the search for crossneutralizing antibodies and potentially crossprotective surface constituents in both agents. However, early results were inconsistent and the evidence is still not completely conclusive. Sheffy and coworkers (134, 135) failed to induce neutralizing antibodies against BVD in calves previously inoculated with HCV. These animals were not protected against a challenge infection with virulent BVDV. In analogous experiments it was not possible to raise neutralizing antibodies to HCV in pigs that were previously infected with BVDV. However, the latter animals were protected to a limited degree to the challenge with virulent HCV. The results of these experiments seemed to indicate that antigenic homologies between BVDV and HCV were able to mediate a heterologous 'one way' protection. In contrast to the above described results Dinter (6) successfully demonstrated - partly by using the same virus strains as Sheffy and coworkers (134) - crossneutralizing antibodies against BVDV in sera of pigs which

had been hyperimmunized with HCV. Moreover, in some instances he found neutralizing antibodies against HCV in cattle with a history of natural BVDV infection. Using rabbit hyperimmune sera against both HCV and BVDV he confirmed the existence or crossneutralizing antibodies against both viruses. Further evidence for crossneutralization has been documented by other authors (108, 136-139). Instead of the above mentioned 'one way' protection some authors described an antigenic dominance of BVDV over HCV (6, 107, 136). Other authors questioned this kind of antigenic relationship between both viruses (111, 127, 140). A possible explanation for the discrepancies may be the fact that crossneutralizing antibodies are demonstrable only with a delay of few weeks after first detection of homologous antibodies. Furthermore the choice of virus strains for immunization and testing and the titer of homologous viral antibodies play important roles. The titer of crossneutralizing antibodies is almost always considerably lower than the titer against the homologous virus strain (140).

The above mentioned protective effect of BVDV inoculation of pigs prior to challenge with HCV led to attempts to utilize live BVDV experimentally or commercially as a heterotypic vaccine against HC (117, 134, 141-144). However, some authors failed to induce a reliable protection by administering BVDV as a vaccine to pigs (136, 145-147).

Initial experiments for the establishment of crossprotection against HC by immunization of pigs with BVDV provided no information on a possible replication of BVDV in pigs. From observations by Snowdon and French in Australia (139) it became apparent that BVDV or a closely related agent different from HCV naturally infects pigs. Field sera of pigs routinely screened for antibodies against HCV often showed titers against BVDV exceeding those against HCV. Further evidence by Stewart and coworkers (148) proved that BVDV readily infects pigs by natural routes, and that there is a viremia during which virus can be reisolated from infected animals. Antibodies against BVDV in pigs are widely distributed and represent a major problem for the serological diagnosis of

HCV infection (140). There is no evidence that HCV naturally infects cattle although the latter can be infected experimentally with a short-termed viremia after intranasal inoculation (149).

The full extent of the relationship among pestiviruses can only be evaluated by future investigations using monoclonal antibodies and by comparison of nucleotide sequences of viral genomes.

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4

MOLECULAR BIOLOGY OF THE VIRUS

P.-J. ENZMANN

1. INTRODUCTION

The integration of alphaviruses, Rubivirus, pestiviruses and Arterivirus (1) into the common family Togaviridae seems to be justified in many respects regarding the structure and the biochemical data on virus replication. But on the other hand, if biological aspects of the virus multiplication are reviewed, mainly transmission of the arbo-togaviruses (alphaviruses) and their capacity to replicate in arthropods, the nonarbo-togaviruses (Rubivirus, pestiviruses and Arterivirus) seem to build up a separate grouping within the family. Nevertheless, similarities predominate and the molecular events of Pestivirus replication will therefore be described in the community of the togaviruses which is built up by all members of the family. Molecular biology of the alphaviruses will be described comprehensively because for the other genera only a few data exist. Within the family Togaviridae, alphaviruses, Rubivirus and pestiviruses are very similar for example in their composition of structural proteins (described in detail later): mainly two membrane proteins exist with molecular weights in the range of 42 to 63K and one nucleocapsid protein in the range of 30 to 35K (2, 3). Since a great number of molecular events during Alphavirus replication seem to be clear and similarities of alphaviruses with pestiviruses are documented, the molecular biology of alphaviruses will be described in detail. The known data for pestiviruses will be mentioned separately. It seems to be justifiable to proceed in that way since the replication strategy of both groups, arbo- and nonarbo-togaviruses shows similarities. Recently it was demonstrated that in rubella virus replication a subgenomic mRNA acts in synthesizing structural proteins as it was established for arbo-togaviruses (4), and that the gene order for the structural proteins of rubella virus is the same as for alphaviruses (5).

B. Liess (ed.), *Classical Swine Fever and Related Viral Infections.*

It is generally acknowledged that the work with pestiviruses is very difficult and results are scarce. Nevertheless, it would be of interest to push the activities with this important virus group, especially to use the modern methods of molecular biology.

It has been attempted to make the references selective rather than comprehensive, to cite review articles whenever possible, and to include primary articles if necessary.

2. TAXONOMY

The term Togavirus was introduced for the group A and B arboviruses in 1970 (6); later on it was formally approved as a family name (7). Togaviruses were defined as follows (8): "Togaviruses are spherical, 40-70 nm in diameter, and consist of an isometric, probably icosahedral, nucleocapsid, tightly surrounded by a lipoprotein envelope; the viral membrane contains host cell lipid and one to three virus-specified polypeptides, one or more of which are glycosylated. The nucleocapsid, constructed from one nonglycosylated polypeptide, contains a single colinear molecule of single-stranded RNA (molecular weight about $4x10^6$), which is infectious when extracted and assayed under appropriate conditions. Togaviruses multiply in the cytoplasm and mature by budding".

Now the Togavirus family is composed of four approved genera: Alphavirus, (formerly group A arboviruses) as well as Rubivirus, Arterivirus and Pestivirus comprising the so-called nonarbo-togaviruses (9). In the Pestivirus genus the viruses of bovine virus diarrhoea (BVDV), hog cholera (HCV), and border disease (BDV) are hold together by similar physicochemical characteristics. Serological cross-reactions occur within the genus but not with other members of the family Togaviridae (10).

As it will be shown in detail later, the structural proteins and the nucleic acids of pestiviruses are similar to those of alphaviruses.

3. MOLECULAR BIOLOGY OF THE TOGAVIRUSES

Most knowledge on the molecular biology of togaviruses has accumulated within the Alphavirus genus, and only little is known about the other genera. Therefore, the overview on this topic is given for alphaviruses. Alphaviruses belong to the group of positive-strand RNA viruses (plus stranded). This means that the virion RNA is able to function as a messenger RNA and as a consequence, virion RNA is infectious.

Initiation of infection is performed by translation of the parental genomic RNA to produce the viral replicase and transcriptase enzymes. By this enzyme complex minus-strand templates are transcribed and plus-strand genomes are replicated, and in addition, plus-strand subgenomic mRNA's for virion structural proteins are generated (11).

3.1 Processing and modification of viral mRNA's

Alphavirus mRNA's are polyadenylated (about 50-200 nucleotides long) at their 3'-termini. The 3'-polyadenylation in mRNA can arise by transcription of 5'-terminal polyuridylate sequences in complementary negative strands or by posttranscriptional addition. Alphavirus mRNA's are capped at the 5'-termini principally with the methylated cap 1 structure $m^7G(5')ppp(5')N^mNp$ (12, 13). Virus RNA of BVDV did not bind to oligodeoxythymidylate cellulose, suggesting that it does not contain a polyadenylate tail. The BVDV genome possesses a high degree of secondary structure which leads to inefficient translation in vitro (14, 15).

3.2 Early events in virus replication

The first phase of Alphavirus replication is started by absorption of the virus to susceptible cells which occurs within a few minutes. Absorption of alphaviruses is highly specific in its salt requirement, in that maximal binding occurs only in the presence of defined concentrations of monovalent cations. The presence of divalent cations in the medium appears to inhibit virus attachment (16). But absorption is more than a nonspecific electrostatic interaction between negatively and positively charged groups on the virus and cell surfaces. This highly specific process is mediated through the recognition of receptors on the cell membrane by defined regions of the viral glycoproteins. The number of receptors has been determined for alphaviruses in chick and BHK cells to be about 10^5 per cell (17). The nature of the receptor is still unknown. Different lipids have been suggested by several investigators (for a review see 17, 18). The data on the mode of host cell entry for alphaviruses are controversial. The findings of Helenius et al. (19) and Fan and Sefton (20) support the view that adsorptive endocytosis is the way to enter the cell. On the other hand fusion of the viral membrane with the cell plasmalemma (21) or pinocytosis (22) may be the initial event in virus penetration.

3.3 Viral protein synthesis

When the alphaviral genome is uncoated, about two-thirds of it, starting from the 5'-terminus, is translated into one large protein which is cleaved into four proteins with molecular weights of about 86K, 72K, 70K and 60K some or all of which form the Alphavirus RNA polymerase. This enzyme is necessary to transcribe the viral genome into minus-strands which act as templates for genome and 26S-RNA synthesis, a mechanism which is described in detail later. Protein produced from the nonstructural segment of the genome is also required for formation of the subgenomic 26S RNA. Both RNA types act as mRNA for further protein synthesis. Translation of 42S RNA does not produce structural proteins. Thus, in common with other eucaryotic polycistronic mRNA's, the internal site for the initiation of synthesis of structural proteins is not functional in 42S RNA. Therefore, translation of 42S RNA terminates in the region of RNA prior to the structural protein RNA sequences. The 26S RNA represents the 3'-terminal one-third of the genome RNA molecule and codes as a polycistronic messenger for the structural proteins which are translated in the form of a 130K precursor protein. The gene sequence of 5'-C-PE2-5K-E1-3' on the 26S RNA has been firmly established. C is the capsid protein, PE2 is a precursor for the envelope proteins E2 and E3 (E3 is not found in the virions of all viruses), 5K is a further peptide with a molecular weight between 4.5 and 6K; it does not appear in the virion.

The capsid protein C is the first protein synthesized by the 26S RNA. Soon after completion of its synthesis, it is cleaved from the polyprotein (precursor). This cleavage seems to be catalyzed by the capsid protein itself (23). C-protein associates rapidly with the viral RNA to form nucleocapsids. The remaining structural proteins are two (3 in Semliki Forest virus) membrane glycoproteins which traverse the lipid bilayer by short stretches found at or near the C-terminus of the proteins (24, 25). The cleavage between the envelope proteins PE2 and E1 takes place at the time when most of the polyprotein has been translated. The first product is PE2, the precursor of E2 and then follows E1. This processing takes place after anchorage of the precursor in the endoplasmatic reticulum. For generation of PE2 and E1 two proteolytic cleavages on the large precursor are necessary. A short peptide with a molecular weight of 4.5K to 6K, depending on the virus strain, is cut out between PE2 and E1, the function of which is not yet clear. Generation of E2 and E3 from PE2 seems to take

place as a morphogenetic cleavage during budding of the viral particle. It depends on the virus strain whether E3 is incorporated as integral part of the virion (Semliki Forest virus) or it is shed into the medium (Sindbis).

For effective cleavage of Alphavirus polyproteins at least two proteases are necessary, a virus-coded activity present on the capsid protein which acts autocatalytically and one or possibly two cellular proteases. In an excellent review by Strauss and Strauss (11) the processes for generation of structural proteins are described.

In the Pestivirus system there is no indication on the existance of a subgenomic mRNA. Upon disruption of its secondary structure BVDV-RNA programs 'in vitro' the synthesis of several polypeptides of 50K to 150K, all of which were immunoprecipitated by specific antiserum. Therefore, BVD-virion-RNA seems to serve as the message for all viral proteins, both structural and nonstructural, which will be synthesized as precursors with concomitant cleavage (14).

3.4 Viral RNA synthesis

After uncoating of the infecting Alphavirus, the incoming RNA serves as the initial messenger molecule to form nonstructural polypeptides, some or all of which are components of the virus-specified RNA-dependent RNA polymerase. Replication and transcription of viral RNA involves an initiation process followed by an elongation phase. It was postulated by Strauss and Strauss (11) that four activities are involved in replication and transcription of Alphavirus RNA: an elongation enzyme which synthesizes the RNA chain once initiated, and three initiation activities which initiate minus-strands, plus strands, and the subgenomic mRNA. These activities could reside in the four different polypeptide chains which are synthesized in the first biochemical process. This hypothesis is supported by the fact that, in the case of Sindbis virus, four complementation groups are required for normal RNA synthesis after infection with temperature-sensitive mutants (26). The genes for viral RNA replicase (nonstructural functions) are encoded near the 5'end and require about two thirds of the viral RNA (42S RNA). The genes for the viral structural proteins reside in the 3'-terminal one-third of the molecule. There is no evidence for early translation of the structural gene products.

After translation of the RNA replicase the incoming 42S RNA is transcribed by this enzyme as an early function in infection. Apparently,

the minus-strand replicase recognizes a structure in the genomic RNA and then starts minus-strand RNA synthesis (27). This species of RNA is not found free in the infected cell but it is rather tightly associated with the RNA-synthesizing apparatus where it functions as template for plus strand synthesis. This apparatus is a multistranded complex consisting of a 42S negative-strand RNA and several nascent plus strands of varying length. During the whole replication cycle only this type of negative-strand RNA appears. It contains a 5'-terminal tract of poly(U) complementary to the 3'-terminal poly (A) tract in 42S virion RNA. The maximum rate of synthesis of negative-strand RNA is reached at about 150 minutes after infection, declining rapidly thereafter. The rate of synthesis of positive 42S RNA increases exponentially up to 3 hours after infection, remaining constant thereafter up to 6-7 hours. Regulation of minus-strand synthesis seems to be a viral function but the nature of this process is still unclear. It is possible that plus stranded RNA once synthesized is readily used as mRNA for protein synthesis or for nucleocapsid formation. Therefore, only a very limited amount of plus strands are used for transcription. Synthesis of plus stranded 42S RNA is paralleled by synthesis of 26S RNA, a subgenomic mRNA which codes for the structural proteins. As mentioned above, the 26S RNA represents the 3'-terminal one-third of the genome. This type of RNA is transcribed directly from 42S negative-stranded RNA, therefore, there must be a special replicase recognizing an internal initiation signal on the 42S RNA (reviewed by Kennedy (28)), or the function of a special protein (reviewed by Kääriäinen and Söderlund (18)). Nevertheless, regulation of 26S RNA is a viral function. It is an efficient mechanism of reiteration of a portion of the viral genome and allows excess synthesis of the viral structural proteins independently of the synthesis of the nonstructural proteins by the 42S RNA molecule.

3.5 Assembly of the nucleocapsid

Papers reviewing the assembly of Alphavirus nucleocapsid have been published recently (11, 29-31).

The capsid protein of alphaviruses is the first protein to be translated from the subgenomic 26S RNA which functions as a polycistronic mRNA for all structural proteins. Cleavage of the capsid protein from the nascent precursor appears to be a process catalyzed by the C-protein itself (reviewed by Schlesinger and Kääriäinen (23)). Newly synthesized

C-protein associates immediately with 42S RNA to form nucleocapsids in the cytoplasm. Major interactions during nucleocapsid assembly seem to take place between protein and RNA rather than between the protein molecules, since neither empty nucleocapsids nor capsid protein aggregates without nucleic acid are formed during infection. Alphavirus RNA is therefore an integral part of the nucleocapsid structure, a fact which is also indicated by the sensitivity of the nucleocapsid towards treatments with RNase and low concentrations of SDS (32).

How the actual packaging of viral RNA into the nucleocapsid proceeds is proposed by Strauss and Strauss (11): There is a conserved sequence of 51 nucleotides within the genome of alphaviruses, located near the 5'-terminus of the RNA which forms a stable hairpin structure. One or both of these loops may be a binding site for C-protein which then acts as a nucleation site for encapsidation. If the C-protein is present, this process is favoured above binding of the same structure to minus-strand replicase. Therefore, minus-strand synthesis would be regulated by nucleocapsid formation. On the other hand, this theory might explain, why 26S RNA is never encapsidated; it represents only the 3'-terminal one-third of the genome and the double hairpin structure is situated near the 5'-terminus.

Nucleocapsids accumulate in the cytoplasm and migrate to the cytoplasmic side of the host cell membranes to get enveloped.

3.6 Formation of viral envelope

In the Alphavirus system next to the C-protein a precursor of E2 protein, called PE2 is translated from the 26S RNA, followed by E1. In contrast to C-protein which is needed freely in the cytoplasm for assembly of nucleocapsids, the envelope proteins have to be inserted into the membranes of the endoplasmatic reticulum for normal processing which includes glycosylation and transport. Removal of the capsid protein from the nascent precursor polyprotein allows a signal sequence of about 19 residues at the N-terminus to function and results in the integration of precursor PE2 in the endoplasmatic reticulum with concomitant glycosylation (11). A second signal sequence with about 5K is situated between PE2 and E1. After completion of PE2 this signal sequence is integrated into the endoplasmatic reticulum giving thereby the signal that a membrane protein (E1) is translated. Removal of this signal sequence, which separates PE2 from E1 requires two proteolytic cleavages. Glycosylation of E1 occurs on the

nascent protein-chain. The glycoproteins once synthesized and inserted into the endoplasmatic reticulum, migrate to the plasma membrane by way of the Golgi apparatus. The cleavage of PE2 to form E2 and E3 has been postulated to occur in the Golgi vesicles (33, 34). But there remains another way to process PE2. Cleavage of PE2 can be prevented by antiserum against E1 (35, 36), and antigenicity of PE2 is changed during maturation (37, 38). Cleavage of PE2 occurs only partially in infected mosquito cells treated with actinomycin D (39). The only other circumstance in which cleavage of PE2 occurs in the absence of virus maturation is when infected cells are placed in media of low ionic strength (40). Therefore processing of PE2 may be interpreted as a morphogenetic process including a reorganization of the glycoprotein which takes place after budding is initiated by the underlaying nucleocapsid.

3.7 Budding process

The final step in virus maturation is the coating of the nucleocapsid with the viral envelope at the plasma membrane of the infected host cell. The viral glycoproteins are synthesized and transported using the normal cellular mechanisms for these processes (reviewed by Compans and Klenk (41)). Virus capsids attach themselves to the inner surface of the cell membrane after modification of that membrane by insertion of the virus specific glycoproteins and exclusion of host proteins. This final stage in the virus assembly involves a specific interaction between the nucleocapsid and the modified cell membranes in that capsid proteins bind to the cytoplasmic domains of transmembranous glycoproteins. From the equimolar ratio of glycoproteins and nucleocapsid proteins in the mature virions it is suggested, that each glycoprotein subunit interacts with a single capsid protein. As this association is repeated, the plasma membrane is wrapped around the nucleocapsid. From biochemical studies this process seems to be only driven by the sequential interactions of transmembranous glycoproteins E2 and E1 with capsid proteins (reviewed by Garoff et al. (31); Strauss and Strauss (17)). Authors who favour this view of events assume that PE2 is cleaved to E2 prior to these events during transport in Golgi vesicles. Another school of thought holds that reorganization of at least PE2 in the cell membrane, which at this point is not yet cleaved, is a further step necessary for budding (reviewed by Brown (30)). Mainly based on electron microscopic observations it was suggested that after the first contact of a

capsid protein with PE2, conformational changes occur on PE2 which allow cleavage to take place. In this case energy necessary to drive the budding process to completion comes from both processes, binding and conformational changes. Evidence for conformational changes comes also from immunological studies using antibodies against viral envelope proteins (36-38, 42, 43). The transitions in membrane morphology during budding suggest that processing of PE2 to E2 is a morphogenetic cleavage induced by binding of nucleocapsids to the membrane.

There is obviously a contrast in formation of pestiviruses. In a recent study Mahnel (44) described the development of viral particles which takes place near the nucleus as well as in other regions of the cytoplasm, especially in regions with enriched rough endoplasmatic reticulum and in cisternae. Formation of virions by budding through intraplasmatic membranes could not be found.

In the following sections pestiviral RNA's and proteins are described.

4. RIBONUCLEIC ACID OF PESTIVIRUSES

The viral genome consists of single-stranded RNA as it was assessed by use of DNA inhibitors as well as by incorporation of [^{3}H]uridine into viral RNA, by ribonuclease treatment, or by sedimentation characteristics, and by buoyant density determination. Furthermore, no inhibition of Pestivirus multiplication has been noted in the presence of actinomycin D (for reviews see Horzinek (8) and Brinton (45)). Infectivity of Pestivirus RNA has been demonstrated for BVDV by Diderholm and Dinter (46), and by Moennig (47), for HCV by Zeegers and Horzinek (48), and by Frenzel and Meyer (49). HCV RNA sedimented at 40-45S in a sucrose gradient, and a molecular weight of $4x10^6$ was established for this RNA after electrophoresis in polyacrylamide agarose gels (50). A RNAse-resistant RNA sedimenting at 20S was isolated from infected cells (49). Based on the sedimentation coefficient of 37-40S, the molecular weight of BVDV RNA has been calculated to be approximately $3x10^6$. Lowering of the salt molarity resulted in a reduction in sedimentation coefficient, wherease Ca- und Mg-ions increased the velocity of the molecules in sucrose gradients indicating a secondary structure of the essentially single-stranded RNA (47, 51). Pritchett et al. (52) found three different viral RNA's in BVDV, a major component with 38S and two minor components with 31S and 24S. At present it is not known, whether these minor RNA species represent

possibly subgenomic RNA's or only degradation products. Because of reasons which are shown in the chapter "molecular biology" it may be concluded, that these minor RNA's don't represent a subgenomic RNA since in the Alphavirus system 26S RNA is never incorporated into the virion. Several other reports on size determination of pestiviral RNA are reviewed by Horzinek (8) and Brinton (45).

Table 1 Overview on parameters of Pestivirus RNA

Virus	Sedimentation-Coefficient	Molecular-weight	Infectivity found	References
HCV	40-45S	$4x10^6$	----	Enzmann and Rehberg (50)
	----	----	infectious	Zeegers and Horzinek (48)
	42S	----	infectious	Frenzel and Meyer (49)
BVDV	----	----	infectious	Diderholm and Dinter (46)
	27-38S	$3.0x10^6$	infectious	Moennig (47)
	38S	$3.2x10^6$	----	Pritchett et al. (52)
	31S	$2.09x10^6$	----	Pritchett et al. (52)
	24S	$1.27x10^6$	----	Pritchett et al. (52)
	33S	$2.9x10^6$	----	Purchio et al. (15)

5. STRUCTURAL PROTEINS OF PESTIVIRUSES

The proteins of HCV have been analyzed by polyacrylamide gel electrophoresis (3, 53). Gradient purified HC virions grown in PK(-15) cells and labelled with [^{35}S]methionine contained 3 main species of virus-specific proteins with molecular weights of 54-56K, 45-47K, and 35-37K. By labelling concentrated and purified HCV with sodium [^{3}H]borohydride, it was shown that the two larger polypeptides are glycoproteins located on the surface. The viral origin of these polypeptides was demonstrated by radioimmune precipitation. In analogy to Alphavirus proteins HCV polypeptides were named E1(gp55), E2(gp46), and C(p36) (8). Electron microscopic and sedimentation data indicated that HCV envelope is very fragile, since E2 is lost during excessive purification of the virion (unpublished data). In addition, E2 is the predominant virion protein in cell-culture supernatant from which HC virions had been removed by ultracentrifugation. By shifting to a lower buffer osmolarity, it was shown, that the virus particles lost their spikes; E2 could be demonstrated in the supernatant after this

treatment; simultaneously, viral density was shifted to lower values (54). From these data it can be concluded, that the viral spikes are built up from E2 protein. Further interesting results on the structural proteins of HCV were obtained by using an inhibitor of proteases (Trasylol), during virus replication in cell culture. According to Korn (55), there is an accumulation of proteases during virus replication in pig. In Table 2, the results of experiments using the protease inhibitor in cell culture during virus replication are summarized (unpublished results). In the presence of the inhibitor the C protein of HCV was only present in small amounts but two polypeptides with molecular weights of about 65K and above 90K were dominant. These virus particles are not infectious. The processing of a viral precursor protein seems to be inhibited by the drug. The mechanism of virus formation under such conditions is not yet clear, since the C protein would contribute to infectivity in this case. Polypeptides with molecular weights above 55K were already described but were regarded as uncleaved precursors (54). Nevertheless, these results need to be discussed in connection with BVDV proteins.

Table 2 Characteristics of HCV grown in the presence of a protease inhibitor

	90K	65K	55K	46K	36K	Infectivity	EM:particles
HCV	(+)	(+)	+	+	+	+	+
HCV-I	+	+	+	+	-	-	+

Legend: HCV-I, HCV grown in the presence of an protease inhibitor; +/- indicates presence or absence of viral protein in the virion, or other characteristics.

The structural proteins of BVDV were determined by several authors with different results. The proteins may be distributed into two groups with apparently low and high molecular weights. The first report was given on three viral peptides (56). Later on four peptides were found (57): 93-110K, 70K, 50-59K, and 27K. In a further study (58) three isolated peptides that were named gp 57, gp 44, and p 34, resembled those of HCV.

Further data (59, 60) together with a summary are given in Table 3. The problem was also reexamined by Akkina (61) and Purchio et al. (14) who found values which may be a link between the other widely diverging results that means between the two groups of low and high molecular weight. The data were confirmed on three proteins with low molecular

Table 3 Molecular weights of structural proteins of BVDV

Pritchett and Zee (57)		93-110*	70	50-59				27
Matthaeus (58)					g57	g44	34	
Akkina (61)	115		80		g54	g45	35	
Coria et al. (59)			g75	g66	g54			26
Kårsnäs (60)	140		g75	60				g28
Purchio et al. (15)		115	80		g55	45	38	
Enzmann, unpubl.		100	85		57	46	35	

* x1.000

weights (58), as well as on high molecular weight peptides found by the other authors (Table 3). Analogous to HCV, the peptides with molecular weights above 57K were regarded as uncleaved precursors since they did not appear regularly. Interestingly non-cytopathogenic strains of BVDV were unable to process the high-molecular weight peptides (61). By fractionation of polyribosomes from infected cells into free and membrane-bound polyribosomes, BVDV-specific polypeptides synthesized by membrane-bound polyribosomes were never detected (14). The low-molecular weight peptides 45K and 38K were only found in small amounts, and there was no precursor-product relationship between the high-molecular weight peptides and the 55K protein which, as a glycoprotein, is presumably synthesized on membrane-bound polyribosomes. From these data it can be concluded that the precursor of the 55K protein was not detected and that the large molecular weight peptides may be uncleaved precursors of the other structural polypeptides which were incorporated into the virion, as it was also stated for HCV (54). In our studies HCV and BVDV polypeptides were analyzed by radioimmune precipitation. In contrast to former reports (62) that HCV antibodies failed to react with p34 of BVDV, all HCV and BVDV peptides were precipitated by HCV antibodies and BVDV antibodies, but with different reactivities. E2 of HCV was best precipitated by the

heterologous serum. Since radioimmune precipitation is much more sensitive than the analysis of immune complexes without radioactive label, the presence of the BVDV C-protein in the precipitate was possibly unnoticed in the former report (62). Reactivity of the high-molecular weight peptides of HCV with antibodies depends on the source of immune serum. Sera from reconvalescent pigs usually precipitated high-molecular weight peptides to a higher degree than hyperimmune serum produced by inoculation of purified virus by which low-molecular weight peptides were detected. As in the BVDV system (61), an anti HCV serum produced by repeated injections of purified virions into pigs exhibited a good reactivity with a high pathogenic strain of HCV and gave only a poor reaction with a low pathogenic strain. Here again reactivity of the virus with antibodies of different sources seems to depend on the presence or absence of high- or low-molecular weight peptides in the virion. The cleavability of the precursors in the infected cells determines whether the antigen is well or only poorly recognized by the antibodies.

The soluble antigen of BVDV appears early in infected cultures and is liberated concomitantly with infectious virus. Various data were given for the molecular weight of that protein ranging from 28K to 82K in the BVDV- as well as in the HCV system. Although there was no definite report on that protein to be a structural constituent of the virion, it has been shown that pigs could be protected against an otherwise fatal challenge infection with HCV by immunization with soluble antigen prepared from BVDV infected cell cultures (for a very detailed review on the soluble antigen see (8)). From this aspect it may be concluded that the absence of this protein from the virion is artefactual rather than real. Possibly it is a cleavage product from a precursor or an uncleaved precursor itself. Both possibilities are derived from the widely diverging data on the molecular weights. In the pestivirus genus it seems to be a normal event, depending on the virus strain used, that uncleaved precursors of the structural proteins are incorporated into the virion. By this hypothesis, it would be possible to explain the protective effect of the soluble antigen.

6. REPLICATION, STRAIN DIFFERENCES AND MUTANTS OF PESTIVIRUSES

Kinetics of Pestivirus replication is strongly dependent on virus and cell strains used. Studies on the replication of HCV were mostly performed

in PK(15) cells but also in other cell lines or primary cell cultures of porcine tissues, such as spleen, kidney, testicle, and leucocytes obtained from peripheral blood. Adsorption of the virus to the cell surface is slow. First newly synthesized virus is found by fluorescent plaque assay 4 to 7 hours p.i. with an exponential growth up to about 15 hours p.i. Thereafter, titers still tend to rise up to 50 hours p.i. resulting in maximum values of 500 to 1000 PFU/cell (for reviews see Liess (63); Horzinek (8); Brinton (45)).

Characteristic growth curves obtained with different HCV strains at various temperatures have been used as genetic markers of their virulence for the pig (64).

Izawa et al. (65) isolated a cold mutant using a subline of pig kidney cells persistently infected with HCV and kept at 28-29°C. This strain was immunogenic for pigs without inducing clinical signs.

The data reviewed on the kinetics of BVDV growth in cell culture are widely divergent (8). After a latent phase of 4 to 10 hours exponential growth starts and stops at 12 to 24 hours p.i., then a plateau is reached. Obviously conditions for virus adsorption or single growth curves are not clearly defined. On the other hand a large number of BVDV-strains exist. The duration of the latent phase also depends on the virus strain and the cell culture system. However, cytopathogenicity does not seem to be correlated with the duration of the latent phase nor exponential growth phase.

7. CONCLUSIONS

There remain several questions to be solved in the HCV-BVDV system. First of all the relationship of pestiviruses should be further elucidated not only between the HCV group and the BVDV group, but also within these groups. The picture emerges that there exists a gradient of HCV strains from high to low virulence that is connected with a gradient of BVDV strains (including BDV) from low to high virulence. This view comes from virological and biochemical work, for example from neutralization studies using several HCV strains but only one BVDV serum. To be complete, neutralization tests using BVDV sera against low- and high- virulence virus strains seem to be necessary. But this view comes also from precipitation tests (described in the section "structural proteins of pestiviruses") which indicated that sera from reconvalescent pigs precipitated uncleaved

precursors of the viral structural proteins to a higher degree than hyperimmune sera produced by inoculation of purified virions. These experiments need further expansion, in the HCV group as well as in the BVDV group of pestiviruses. In addition, these experiments refer to further unsolved questions in the BVDV-HCV system: The contribution of uncleaved or partially uncleaved precursors of the structural proteins to the structure of the virions must be solved for virulent and avirulent strains of HCV and BVDV, perhaps the role of the soluble antigen and its origin will also be elucidated. It would be of interest to combine the knowledge on protein structure and on viral strains to discuss the relationship which seems to exist on the basis of these different viral characteristics. As it was already stated above it seems to be necessary to push the activities with this important virus genus, especially to use the modern methods of gene technology.

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5

DIAGNOSTIC PROCEDURES

E.A. CARBREY

1. INTRODUCTION

A tentative diagnosis of hog cholera (HC) may be made in the field if a thorough history is obtained and clinical signs and necropsy lesions are carefully observed (1, 2). In many instances there have been recent additions to the herd, or there is illness in herds in the neighborhood. With acute HC, deaths occur, and usually there have been unexplained deaths in advance of the outbreak. The recent use of HC vaccines or the feeding of uncooked garbage may be important points in the history (3).

Rectal temperatures should be taken and total leucocyte counts performed on at least six pigs. A marked leucopenia is associated with the initial rise in body temperature in acute HC infection. The leucopenia persists until death or recovery and is found in chronic HC infection as well. The pigs with the lowest total white blood cell counts should be selected for specimen collection regardless of the severity of clinical signs present (4). In addition, recently dead or moribund pigs should be necropsied, examined for the lesions of HC, and specimens collected. The tonsils, mandibular lymph nodes and spleen should be submitted to the laboratory for virus detection. Tonsil biopsies may be obtained from the pigs when the herd owner is reluctant to provide additional candidates for necropsy. A blood sample may be taken at the same time for serum antibody tests while the pig is restrained for the tonsil biopsy.

It is important to consider the differential diagnosis of other diseases and collect specimens as appropriate. African swine fever should always be considered and is quite difficult

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to differentiate from HC on the basis of clinical signs and lesions. Salmonellosis produces lesions, especially petechial and ecchymotic hemorrhages, that may be mistaken for those of HC. Erysipelas, pasteurellosis, transmissible gastroenteritis, pseudorabies, and acute salt poisoning must also be considered.

The clinical signs and lesions will be less severe when the infecting strain of HC virus (HCV) is of reduced virulence. In some cases modified live virus vaccines have spread to baby pigs and caused losses while the older pigs remained healthy. However, when HC has been enzootic in a country for many years, low-virulence strains will be present and will cause baby pig losses, abortions, stillbirths, and fetal abnormalities and the disease will remain undiagnosed unless laboratory studies are conducted.

2. LABORATORY DIAGNOSIS

2.1. Viral antigen detection in tissue sections

The first application of the fluorescent antibody tissue section test (FATST) was made to detect HC viral antigens in the infected tissues of malformed embryos and fetuses (5). However, it was quickly accepted as a rapid and useful technique for the laboratory confirmation of HC. The same workers reported on an impression smear technique for the detection of HC antigen with fluorescent antibody conjugate using mandibular and parotid lymph nodes, salivary glands, spleen, kidney and lung (6).

Sections for the FATST were cut at 4 μm, and complement was added to the conjugate to enhance the union of antigen and antibody. HC antigens were detected in tissues from infected pigs three to ten days after exposure. Viral antigen was detected in brain, spleen, kidney, lymph nodes, salivary glands, and lung. However, spleen and lymph nodes were consistently positive in all infected pigs.

The FATST was standardized by a committee of the American Association of Veterinary Laboratory Diagnosticians and used extensively in the HC eradication program in the United States (7). Workers in other countries also found the FATST to be a

rapid and accurate procedure for detecting HC viral antigen in tissues (8, 9, 10).

Only three major pieces of laboratory equipment are required for the FATST: microtome cryostat, incubator, and fluorescence microscope. With the aid of a small truck or large station wagon, the basic equipment can be moved to a field location and a temporary laboratory can be set up. The ideal specimen for the FATST is the tonsil, however, spleen and mandibular lymph nodes should also be taken at necropsy. A small biopsy of tonsil suitable for the FATST may be taken using a human rectal biopsy forceps (11). Pigs have flat-ended areas of tonsillar tissue located on each side of the posterio-dorsal part of the oral cavity. Using a speculum to force open the mouth, the forceps are inserted along the hard palate and onto the soft palate a little to either side of the midline. The forceps are opened and the jaws are pushed into the tonsil and closed. The instrument is removed, the jaws are opened, and the biopsy is teased from the forceps with a needle to avoid macerating the tissue fragment. It should be examined for the pores of the tonsil crypts to confirm that tonsillar tissue was otained. Tissues, including the biopsy, may be sent to the laboratory refrigerated or frozen.

An alternative method for collecting tonsillar material from a live pig has been desribed using a specially designed instrument (12). Equipped with a battery as a power source, a light for observation and a rotary blade were used to slice off sufficient tonsillar tissue to prepare a smear for fluorescent antibody staining.

In performing the FATST in the laboratory, blocks of tissue 10 mm square by 3 mm thick are cut and frozen on microtome chucks with water or preferably special solutions such as OCT Compound. Biopsy specimens are mounted intact. Tissue sections 8 μm thick are cut, mounted on glass slides, fixed with acetone for 10 minutes and dried. The slides are covered with HC fluorescent-antibody conjugate and incubated in a moist chamber at 37°C for 30 minutes (13, 14). For control purposes, additional sections may be stained with a mixture of normal

(HC-negative) serum and HC conjugate for comparison. Another useful control is to stain duplicate sections with a conjugate prepared from serum obtained from a specific pathogen-free pig.

After staining, the conjugate is decanted from the slides, the sections are rinsed with phosphate-buffered saline pH 7.2-7.4 (PBS), and coverslips are applied with buffered glycerine (equal parts glycerine and PBS). Upon examination with a fluorescence microscope, the cytoplasm of cells infected with HCV stains bright green while the nuclei do not stain. Uninfected cells are dark green or brown but, if necrotic, may take up conjugate non-specifically. This may be checked by viewing the necrotic cells with incandescent light. These cells and other debris will usually stand out from the background when viewed with incandescent light while infected cells that have viral antigen combined with conjugate will drop in background. Usually, infected cells are found in clusters or lining a tonsillar crypt, but occasionally the whole section will fluoresce. In this case, a careful comparison with control slides should be made. The specific viral fluorescence is most easily confirmed in the epithelial cells of the tonsillar crypts. Fluorescence in the germinal centers may be due to the uptake of stain by macrophages. Occasionally tonsils from healthy pigs will have a germinal center fluorescence that appears to be genuine. In such cases, additional sections should be cut until specific fluorescence is observed in epithelial cells before calling the case positive. It is also a rule in our laboratory never to make a diagnosis of HC if the spleen is the only tissue found positive.

The use of tonsil biopsies has been an effective way of detecting HCV infection in herds where owners are uncooperative and conceal pig losses (11). By collecting tonsil biopsies from 20 to 30 pigs in a suspect herd, it is possible to confirm HC infection and justify depopulation.

2.2. Virus isolation in cell culture

Although it was well established by many workers that HCV will replicate in porcine cell cultures, the failure of the

virus to produce a cytopathogenic effect limited the use of cell cultures for laboratory confirmation (15). Cytopathogenic strains of HCV have been reported but are exceptions to the rule (16). The cytopathogenic virus was useful for titrating neutralizing antibody, but it was difficult to maintain in sufficient titer for routine testing. The application of immunofluorescence to detect viral antigen in infected cells provided a rapid and convenient diagnostic and assay method for HCV (17). Viral antigen is detected only in the cytoplasm of the infected cells and may be observed as early as six to eight hours after inoculation (Fig. 1). Attenuated strains as well as virulent strains propagate in porcine cell cultures. However, rabbit passage strains may replicate more slowly.

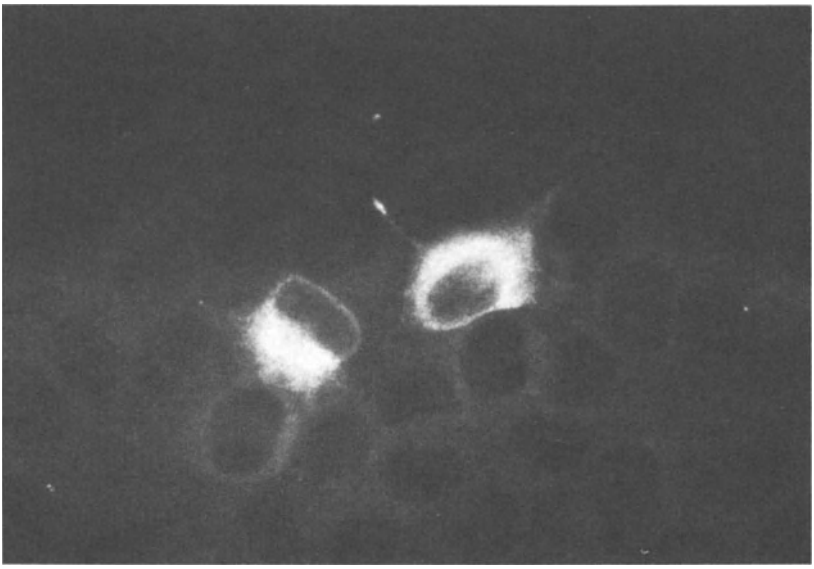

FIGURE 1. HC virus (strain Alfort) replication in PK(15) cell culture 6-8 hours post-cultural inoculation (courtesy Liess, Hannover Veterinary School).

A suspension of spleen, tonsils, or lymph node tissue prepared in cell culture medium is inoculated into coverslip cell cultures. Heparinized blood may also be used, but it is a less consistent source of virus. The PK(15) (pig kidney) cell line has been used extensively, but primary embryonic swine kidney cells or other pig cell lines may be used.

After incubation at 37°C for one hour, the cultures are washed, the medium is replaced and the cultures are placed at 37°C. After incubation overnight, the coverslips are removed, fixed with acetone and stained with conjugate. Using fluorescence microscopy, HCV-infected cells are readily detected by their bright green color in contrast to the darker green or brown of the uninfected cells in the cell sheet. Duplicate cultures should be maintained at 37°C for an additional day to confirm negative findings or to verify a small number of fluorescing cells detected on the first day. The few fluorescing cells observed initially will become clusters of positive cells by the second day of incubation (Fig. 2).

Control cultures, both inoculated and uninoculated, must be stained and observed to make certain that the cell cultures used are susceptible and have not become adventitiously contaminated with bovine viral diarrhea virus (BVDV). In the author's experience, the PK(15) cell line will, without warning, suddenly lose its susceptibility to HCV. If the cell cultures are transferred a few times, usually susceptibility will be regained. It is more convenient to start a new set of cultures from frozen stocks.

The serum used as enrichment in the propagation of the cell cultures must be free of antibodies against BVDV or there will be interference with the isolation of HCV. If bovine fetal serum is used, there is the risk that BVDV contamination may occur from intrauterine infection of the donor calf. The use of BVDV contaminated serum will result in a false positive confirmation of HCV since the HC conjugate will stain BVD infected cells. It is standard practice in our laboratory to culture each lot of BVD antibody-negative bovine fetal serum

and have the virus-negative lots exposed to 2.5 to 3.5 megarads of gamma radiation to destroy any undetected BVDV.

In addition to contamination problems with BVDV, one must recognize that pigs may be naturally infected with BVDV (18). Specimen tissues from BVDV infected pigs will be positive on FATST and by cell culture using HC conjugate. Differential examination using bovine cell cultures and BVD conjugate may be helpful. However, it may be necessary to resort to pig and calf inoculation to identify the BVDV isolate.

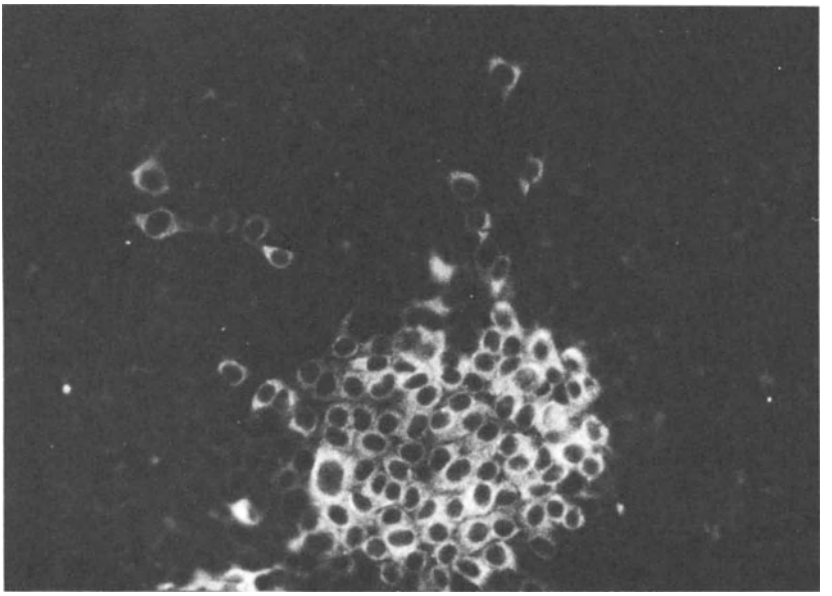

FIGURE 2. HC virus (strain 331) 2 days post inoculation of PK(15) cell cultures (courtesy Liess, Hannover Veterinary School).

The cell culture and tissue section techniques for the confirmation of HCV depend on the availability of a good HC conjugate and initially on the preparation of a potent

hyperimmune serum. A variety of methods have been used, however, it may be worth describing the commercial procedure used in the era of simultaneous vaccination (virulent virus-hyperimmune serum) for HC. Pigs were vaccinated with live virus and serum, given virulent virus 14 days later, maintained for 60-90 days, and then inoculated i.v. with 10 ml of viremic HC blood per kg body weight. After another 14 days, the pigs were bled out for hyperimmune serum. The viremic blood was obtained from donor pigs three to five days after infection, defibrinated, chilled overnight and inoculated into an ear vein of the serum pig under pressure.

2.3. Pig inoculation

The most sensitive method for the detection of HCV is the inoculation of the natural host, the pig (19). When comparisons are made between the titration of virus in cell culture and pigs, the virus is found up to one tenfold dilution higher in the latter. The inoculation of tissue suspensions directly into the pig may produce confusing results. If the pig sickens and dies, it is necessary to confirm HC by either tissue section or cell culture inoculation. If the pig survives, its immunity should be challenged by the inoculation of virulent HCV, or its serum should be tested for neutralizing antibodies.

In our laboratory, pigs have died following inoculation with a spleen suspension that yielded both HCV and Salmonella cholerae suis on culture. When either of these agents were administered seperately, the pigs survived after a mild course of illness. If it is desired to check the virulence of an isolate, the original tissue specimen should be filter-sterilized to remove bacteria.

A differential pig inoculation system that might have application under isolated field conditions was proposed before the development of the tissue section and cell culture technique (20). Four pigs are inoculated: One must be previously vaccinated against HC, a second receives 15 ml of HC hyperimmune antiserum, and two receive only the specimen tissue suspension. A fifth pig is kept in isolation from the others as

a challenge control. After 28 days all surviving pigs are inoculated with virulent virus.

A word of warning is appropriate concerning the difficulties of avoiding security breaks when working with virulent HCV. Air movement must be perfectly controlled to avoid inadvertent infection by aerosol.

2.4. Relative efficiency of the diagnostic test procedures

HCV isolation in cell culture was compared with the inoculation of susceptible pigs and found to be sensitive and specific (19). Samples were examined from 225 herds, 84 of which were negative by both tests. Only 13 of the remaining 141 herds were negative by cell culture and tissue section procedures. In a controlled study, specimens were collected from 122 pigs and were coded to conceal their identity (21). The pigs were divided into two groups according to the treatment given: 48 were classified as HCV exposed and 73 as HCV free. In the HCV exposed category, 32 pigs were found positive and three negative by both techniques. Of the remaining 13, eight were negative by tissue section but positive by cell culture inoculation and five were positive by section and negative by cell culture. Of the 74 HC free pigs, all were found negative by tissue section and all but two were negative by isolation of HCV in cell culture. The two sets of tissues found positive by cultural isolation were from pigs exposed to BVDV. The differences in the two fluorescent antibody techniques were not statistically significant. The tissue section was positive for 77% of the HCV-infected pigs and the cell culture was positive on 83%. In the HCV-free group of 74 pigs, the tissue section accuracy was 100%, while the cell culture was 97%.

3.CHARACTERISTICS OF ISOLATES FROM FIELD OUTBREAKS

3.1. Serotype characterization

Although minor antigenic variations my be detected among isolates of HCV, it continues to be recognized as a monotypic virus. Pigs surviving infection with any strain of HCV are

protected against exposure to other strains. Minor variations are distinguishable but distinct serologic groups do not exist (22). A significant antigenic relationship exists between HCV and BVDV. Using the immunodiffusion precipitin test, a continuous line of identity was found using HCV-infected tissue and BVD and HC antiserums (23). Using the neutralization test, HCV may be identified by the specific envelope antigen. Field infections with BVDV and HCV in pigs can be distinguished by the relative levels of neutralizing antibodies (22).

With much time and effort HCV has been cloned using an endpoint dilution fluorescent antibody technique (24). However, the antigenic and pathogenic characteristics of the cloned virus were the same as those of the parent.

The first report of antigenic variation in HCV was related to problems using the simultaneous vaccination method in the forties (25). The use of certain lots of HCV and antiserum resulted in sickness six to ten days after vaccination. In some cases, all animals in the herd were affected while in other herds only a few. The clinical signs and lesions observed were those of HC. Other pathogenic bacteria or viruses were not isolated from the vaccine or antiserum. When pigs were given the vaccine virus and the antiserum dosage was increased by 50%, they remained healthy. On the basis of the above information, it was decided that a variant of HCV had developed during virus propagation. However, in retrospect, the phenomenon may have been due to poor antiserum quality. At that time, the neutralization test was not available for titration of the antibody content of an antiserum.

Minor antigenic differences in HC viral isolates have been detected using the neutralization test and also by comparing cross neutralization titers obtained with antiserums prepared against strains of BVDV.

A strain of HCV, '331', found to cause persistent infections in experimentally infected pigs, was compared with the virulent 'Ames' strain (26). Using strain-specific antiserums, the two strains were neutralized to a much greater degree by their homologous than by their heterologous antisera.

A 64-fold difference was observed in the cross-neutralizing titers.

A slight antigenic difference was detected between an attenuated (Chinese) strain of HCV and the virulent ALD strain by using specific antiserum prepared against the attenuated strain (27).

Other workers have associated virulence and low immunogenicity with the antigenic properties of different strains (28).

A relationship between the virulence of HCV strains and their neutralization by BVDV antiserums has been reported (29). The strains were inoculated into pigs to determine virulence and reacted against BVDV antiserum to determine the degree of cross neutralization. The BVDV antiserums neutralized the low-virulence HCV strains more strongly than the HCV strains of high virulence. The strains studied were classified into two groups according to their neutralization by BVDV antiserums. Most of the virulent strains were in the group neutralized minimally by BVDV antiserums.

A confirmation of the above observation was found when 23 field isolates of HCV from recent outbreaks were tested against antiserums to the T20-5 strain of BVDV and the ALD strain of virulent HCV (30). Five HCV isolates were weakly neutralized by BVDV antiserum and strongly neutralized by the HCV antiserum, while the remaining 18 reacted conversely. When two isolates from each group were inoculated into pigs the two strains that were neutralized most strongly by HCV antiserum caused death in 10 to 12 days while the two isolates from the second group caused death in 17 to 20 days.

3.2. Pathotype characterization

During the HC eradication program in the United States, 135 field isolates were characterized by inoculation into specific-pathogen-free pigs (31). The isolates were classified as follows:

1) High virulence - pig sickened and died.
2) Low virulence - pig had chronic illness and recovered,

or died after a protracted course.

3) Avirulence (immunizing) - pig had little or no reaction and remained healthy following subsequent inoculation with virulent virus.

4) Persistent infection (immune tolerant) - pig had clinical signs of HC but developed a persistent infection during which it was relatively free of illness but had a high concentration of virus in its blood.

Using the above system 61 (45%) of the 135 isolates were of high virulence, 37 (27%) of low virulence, 29 (22%) avirulent, and 8 (6%) caused persistent infections.

The eight isolates that caused persistent infections in the test pigs were recovered sporadically from 1965 through 1976. Seven of the persistently infected pigs survived for 30, 32, 66, 9, 114, 127, and 152 days, respectively, while the eighth pig was killed at postinoculation day 53. Since all of the pigs had weight gains of 1 to 2 kg per week and appeared healthy, these isolates were considered to be different from low-virulence strains. Six of the pigs were given virulent HCV, five intentionally and one inadvertently. Two of these six pigs died after the administration of the virulent HCV. Although the number of isolates causing persistent infections was small, it was considered that this characteristic was unique to these strains. It is quite certain, however, that the specific host pig contributed to the development of persistent infection since one of the isolates was inoculated into several pigs but not all of the pigs developed a persistent infection.

The persistent infection has been produced experimentally by exposure of pregnant sows to HCV; specifically, the 'Bergen' strain (32, 33). One persistently infected pig survived superinfection with virulent HCV for 45 days. It was proposed that the 'Bergen' strain was a low-virulence strain that was capable of producing congenital infections experimentally and in the field. The baby pigs were born alive with persistent infections. The pigs were viremic and failed to develop serum antibodies or cell-mediated immune responses to HCV, even

though they had normal immunologic responses to other antigens. It has been postulated that certain field strains of low virulence are most suited to produce this persistent or chronic infection (34, 35). The infections occur most often following intrauterine infection since the virus does not kill the sow but passes through the uterus into the fetuses (34, 35).

Other markers for virulence other than serological differences have been reported. Fully virulent viruses were characterized by rapid multiplication and the formation of large cellular plaques when incubated at 39 to 40^{o}C (36). Also, virulent viruses were more resistant to heat at 56^{o}C. Conversely, avirulent strains of HCV had optimum growth at 33 to 34^{o}C, multiplied slowly, formed small plaques, and lost infectivity at 56^{o}C.

Another marker reported was the ability of HCV strains to replicate in porcine alveolar macrophage cultures (37). The macrophages were obtained by lavage of the pulmonary alveoli. Of eight HCV strains tested, five virulent strains replicated well to $\log_{10}$ titers of 4.75 to 6.25 $TCID_{50}$ per ml. Three avirulent strains grew poorly: strain '331' had a titer of 3.75; GPE(+), 1.50 and GPE(-) did not replicate. A positive correlation was established between a severe reaction and mortality in seven days in the pig and replication in porcine alveolar macrophage cells.

4. CONCLUDING REMARKS

Although the clinical signs and lesions of acute hog cholera (HC) are easy to recognize, many field strains of HC virus (HCV) are of low virulence and produce chronic disease or abortions, stillbirths, and baby pig losses. The identification of HC viral antigen in frozen tissue sections using the fluorescent antibody test is a rapid and accurate method of laboratory confirmation. Tonsil biopsies may be taken without sacrificing the pig. As an additional confirmation, the virus may be isolated and identified in cell culture. The inoculation of susceptible pigs for the detection of HCV is the most sensitive confirmatory procedure but must be carefully

controlled to eliminate the presence of other pathogens such as Salmonella and Erysipelothrix. The characteristics of field isolates are considered in terms of antigenic and pathotypic variation. The monotypic nature of the HCV serotype is emphasized while reports of minor differences in HCV strains are considered. The natural variation of the virulence of HCV is reviewed, and the unusual characteristics of strains that cause the persistent infection following pre- or postnatal contact with HCV are described.

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6

SEROLOGY

B. LIESS

1. INTRODUCTION

Animal protection tests for the assessment of immunogenicity and safety of vaccines (1,2) or potency of hyperimmune sera (3,4) against hog cholera (HC) were indispensable as long as in vitro methods for the determination of anti-hog cholera antibodies were not available.

The need for such a serological procedure came also from the growing understanding of some epizootiological features related to HC. Low virulence strains of HC virus (see chapter 11) accounting for subclinical infection, e.g. in pregnant sows, can give rise to immunity and antibody formation which by serology may be detected on a herd basis. Positive reactions would indicate the possible presence of HC virus due to transplacental transfer in persistently infected piglets even in the absence of typical signs of HC in any of the breeding animals present (5).

Furthermore, a serological assay for the qualitative and quantitative determination of HC antibodies was desirable in order to facilitate studies on the host response in the course of infections with various strains of HC virus (6).

Despite the successful propagation of HC virus in several tissue and cell culture systems, no cytopathic effects (CPE) were observed except with one strain of virus at low titres (approximately 100 $TCID_{50}$) and only when lamb serum was incorporated into the culture medium (7). This cytopathogenic strain A of HC virus not only produced HC when inoculated into susceptible pigs, but was also neutralized by immune sera. Nevertheless the use of cytopathogenicity as indicator in a neutralization test was restricted to this particular strain of

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HC virus and was also not easy to handle.

A more versatile test became available when a direct fluorescent antibody technique was first described by Solorzano (8). This procedure allowed the detection of HC viral antigen in cell cultures after inoculation with HC virus for diagnostic and assay purposes (9). The fluorescent antibody cell-culture test (FACCT) also formed the basis for a serum neutralization test (FASNT; 10, 11).

The need for such a test procedure was best illustrated by numerous efforts to develop other methods for the identification of HC virus infections in pigs in order to replace the former pig inoculation test. The latter was not only costly and time consuming but for various reasons was also limited in its applicability as a routine diagnostic test and in addition was unsuitable for precise quantitative studies (12, 13). Many of the HC laboratory techniques for diagnostic and assay purposes as reviewed by Dunne (14) did not find a wide application and will be mentioned in the present chapter only if comparative results are available in the literature. Emphasis has been layed upon the basic antigen-antibody reaction rather than the *in vitro* indicator system and the techniques used for the demonstration of the reaction. Otherwise differing results would give reason for misinterpretation. This could be falsely attributed to the kind of technique used instead of the differences between the components involved in the reaction.

2. SEROLOGIC REACTIONS USED FOR HUMORAL ANTIBODY DETECTION

2.1. Precipitation

In order to confirm the diagnosis of 'swine fever' as hog cholera, the injection of a susceptible pig was considered necessary as long as a satisfactory simple specific test was not available (13). The 'gel-diffusion precipitin test' of Ouchterlony (15) was first used by Molnar (16) to demonstrate the precipitation reaction induced in the presence of HC virus-containing substances and HC-specific immune sera of high titre. The conditions influencing the sensitivity of the

test have been a matter of intensive investigations (17 - 23). Other reports dealt with the reliability and specificity of the test (24 - 28). The HC specificity of the precipitation reaction between swine serum and homologous pancreas extracts has been questioned (29) but can be clearly attributed to a specific antigen-antibody reaction by other methods (27, 30). The nature of the antigen formerly termed 'disease-specific' (31, 32) was confirmed to be specific for HC virus by infectivity tests in pigs inoculated with immunoprecipitates containing antigen-antibody complexes (33) and was found to be possibly identical either with the virus itself or merely a 'soluble antigen' in serum of pigs inoculated with HC virus 5 days prior to bleeding (21).

In the literature most reports described the evaluation of the agar-gel precipitation test (AGPT) as a diagnostic tool for the detection of precipitin antigen mainly in pancreas (17) and spleen (20) of experimental or field cases of HC (19, 26, 36, 37). Intensive consideration was given to the production of a suitable serum or serum concentrate from hyperimmunized pigs or rabbits to provide the most reliable precipitation reaction. When investigating the value of the AGPT for the diagnosis of mucosal disease (MD) in cattle a continuous precipitation line of identity formed on the same Ouchterlony plate between the homologous HC and MD system. This suggested a relationship between the two (34, 38), probably having soluble antigens 'with closely similar molecular arrangements with one or more common groups available for combination with antibody' being

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Synonyma: plate gel diffusion precipitin test (17); gel-diffusion precipitin test (18); double-diffusion in agar gel (34); Agar-Gel-Diffusion (25); agar diffusion-precipitation test (ADP) (19); agar double-diffusion precipitation test (20); agar-gel diffusion-precipitation test (26); Agar-Gel-Test (AGT) (21); Agargel-Präzipitation (35); agar-gel precipitation test (AGPT) (14)

responsible for the cross-reaction (39).

In contrast to the use of the AGPT for the detection of antigen in tissue organs, little information is available on the value of this test as a serological tool for the detection of antibodies in field sera from pigs. The possible value of such a test has been mentioned above (section 1). Following comparative investigations on the presence of HC viral antigen in organ tissues and antibodies detectable in sera of pigs in a herd with high mortality amongst piglets, serological testing of clinically healthy breeding pigs appeared to be an important issue (40). Demonstration of precipitating antibodies using the AGPT correlated in most cases with the presence of neutralizing antibodies with only a few pigs circulating HC virus in the blood and having low neutralizing indices (N.I.) but no detectable precipitating antibodies. This situation occurred in herds with chronic HC virus infections as also shown by other investigators (5, 6, 41 - 44).

All the results accessible in the literature need to be considered in relation not only to the stage of infection but also to the type of infection: (i) virus persistence due to immune tolerance, (ii) acute/subacute infection resulting in typical clinical signs of HC or (iii) subclinical transient infection with recovery from infection. Without differentiation according to the type of infection in pigs of various age groups and conditions (fattening or breeding), comparison of serological results obtained with AGPT and detection of antigen by the same method is of rather limited value. In other words a pig without detectable antibodies might well be infected and contain virus and viral antigen as a consequence of HC virus persistence, and yet the results do reflect the true situation and are not contradictory. With this in mind, former comparative investigations that distinguish between positive and negative results and calculate the diagnostic applicability of the AGPT on this basis appear somewhat misleading. A negative serological reaction obtained with AGPT does not preclude the possibility of HC being present (18) where even more sensitive methods like neutralization tests fail to give a

positive reaction.

Another reason for a negative reaction is an early stage of infection although this is in most cases impossible to determine under field conditions. On the other hand low virulence strains of HC virus might account for a higher percentage of positive reactions (45) compared with other strains of different virulence (18, 37, 40, 46, 47). In this respect the few reports in the literature describing results of testing pig sera for precipitating antibodies against HC viral antigen were based on rather heterogenous or even obscure materials leaving ample room for interpretation. Intriguing contributions were those on the HC specificity of the precipitation reactions with pancreas extracts (29) or proteases (33) and at the same time on the pathogenesis of HC (48). All these factors add to the uncertainty over the reliability of the AGPT although a number of investigators did consider this method as a valuable tool provided that strict requirements governing the test are closely followed (20).

In order to accelerate the immunoprecipitation reactions obtained by diffusion in agar gel, electrophoresis has been additionally applied for distinguishing within 25 to 40 minutes between the sera of healthy pigs and pigs five days post-infection (49). The pig sera served as precipitation antigen which in 'precipitation electrophoresis' migrated towards the anode in contrast to corresponding antibodies moving to the cathode by electroosmosis thus forming a precipitation line at the point of impact. This test was also evaluated as a diagnostic tool for the detection of antibodies against HC in pig sera (50). Immunoelectro-osmophoresis (IEOP) was applied to field sera and sera from pigs experimentally infected or vaccinated using either tissue culture-derived antigens or spleens from HC-infected pigs. Precipitating antibodies could be demonstrated two to three weeks after the inoculation of HC virus strains. The technique proved to be 2 to 6 times more sensitive than the AGPT but did not discriminate between antibodies against HC virus and BVD virus and for this purpose required the more specific neutralization reaction.

2.2. Complement-fixation

There were many attempts to make use of complement-fixation for the detection of antibodies against HC Virus. Ultracentrifuged supernatant fluids of tissue cultures infected with cytopathogenic strains of bovine viral diarrhea (BVD) virus contained a common soluble antigen (51) which was used for the detection of complement-fixing and neutralizing antibody to BVD virus and HC virus in bovine and porcine sera (52). The ultracentrifuged supernatant tissue culture fluid was in addition concentrated before use in the complement-fixation (CF) test employing sera sequentially collected from calves inoculated with BVD virus or BVD soluble antigen, respectively. Hyperimmune HC antisera prepared in pigs were included in the CF tests which resulted in fixation of complement to the antigen-antibody system. The fixation of complement was enhanced by the addition of normal untreated bovine serum which also resulted in significantly higher titres.

Interestingly neutralizing antibodies were not demonstrable in the HC antisera when tested against BVD virus whereas such antibodies were present in all calf sera where they appeared after CF antibodies. This led to the possible interpretation that different types of antibodies are produced at different stages of infection or that different antigens are concerned in the tests where soluble antigen may represent a precursor or by-product of viral synthesis. Furthermore it was concluded that the CF test is a useful aid in studying the relationship between BVD and HC viruses but deficient as a single test for HC because pigs infected with BVD virus could develop CF antibodies without being exposed to HC virus. If pig sera contained CF antibodies against BVD soluble antigen in the absence of BVD virus-neutralizing titres this would be presumptive evidence of earlier exposure to HC virus.

For the detection of HC viral antigen in spleen from experimentally infected pigs Boulanger et al. (53) applied various techniques of the CF test in order to overcome the precomplementary activity often thought responsible for the difficulties in detecting complement-fixing antibodies in pig

sera. Only minimal quantities of precomplementary and lytic substances were present in the immune sera tested. More important was the procedure for the extraction of the antigen and the stage of infection when the spleens were collected from the pigs. The results communicated reflected also the possibility of using the CF test for the detection of HC antibody in immune sera from pigs which previously had proven by AGPT to contain precipitating antibodies (20).

By applying the Fulton-Dumbell microtechnique the CF test was found positive with sera from pigs 20 - 23 days after experimental inoculation with high virulence HC virus but not after another group of pigs received lapinized HC virus vaccine (54). In most of the investigations reported, 5 % normal pretested bovine serum was found a necessary supplement of the guinea pig complement used. Otherwise pig serum antibodies did not fix the complement when the sera were heat-inactivated before tested (53, 55). Attempts were made to overcome this situation by the application of the conglutination complement absorption test (CCAT) based on former experience with certain antisera which did not fix haemolytic complement but would fix horse conglutinating complement. The CCAT was, however, not readily applicable and required selective inactivation to remove inhibitors normally present in porcine sera which interfere with conglutination (56).

Similar to CCAT, in the CF test a haemolytic prozone phenomenon is often encountered with porcine immune sera and can be eliminated by mercaptoethanol (57). The addition of porcine C1q had an enhancing effect on complement-fixation with IgG from porcine pseudorabies immune serum (58). Similarly this resulted in highly improved complement-fixation reactions when sera obtained from pigs inoculated intravenously or in contact with low virulence strains of HC virus were tested against an HC antigen prepared from infected cell cultures (59, 60). In block titration tests the CF antibody response to the low virulence strains of HC virus was rapidly reaching maximum titres two weeks after inoculation. The neutralizing antibodies (plaque reduction test) again developed more slowly. However,

it is worth noting that in a pig exposed by contact to two HC-virus inoculated pigs, complement-fixing or neutralizing antibodies in serum could not be demonstrated during the observation period of 13 weeks.

Using the same CF test (59), sera from experimentally infected pigs were additionally tested for neutralizing antibodies which were detected at the same stage of infection or earlier than complement-fixing antibodies. The latter could not distinguish between antibodies against HC virus and BVD virus while the neutralization test based on peroxydase-linked-antibodies (NPLA; section 2.3.) did so with a high degree of reliability (61).

2.3. Neutralization

The isolation of a cytopathogenic strain of HC virus by Gillespie et al. (7) made it possible to demonstrate antibodies which would neutralize the infectivity of HC virus in tissue culture. Neutralization tests in vitro allowed the identification of HC viral isolates, evaluation of vaccine efficacy and virus dose determinations as well as studies on active immunity in relation to antibody production and maternally derived immunity. A test procedure based on the constant virus varying serum dilution technique was developed and the accuracy established (62). Further studies were designed to show that the neutralization test was an accurate indicator of immunity (63).

After Kumagai et al. (64) like many other investigators had failed to find cytopathogenic effects of HC virus in various cultured cell types they applied the 'exalting effect of HC virus on Newcastle Disease (ND) virus (END)' for the detection and measurement of HC virus and its neutralizing antibodies (65). The END method was based on the observation that ND virus (strain Miyadera) exerted no cytopathic effect when inoculated into swine testicular cell monolayer cultures within 4 or 5 days of cultivation but did so after 6 to 8 days, and that ND virus would produce marked cell changes before the fifth day of cultivation if such cell cultures were infected in

the initial stages of cultivation with HC virus (66). For titration of HC virus as well as the measurement of neutralizing antibodies inhibiting the exalting effect of HC virus 50 % endpoints were determined. The END neutralization test was found simple enough for routine use, highly reproducible and specific (67).

Using the Sato strain of ND virus instead of strain Miyadera, HC virus was observed to inhibit the cytopathic effect of ND virus in pig kidney monolayer cell cultures and to increase the haemagglutination titre of Sato virus (68). The sensitivity of the 'haemagglutination exaltation and inhibition of cytopathic effect' (HEIC) method was improved by extending the culture incubation to nine days and was used for the measurement of neutralizing antibodies in a potency test of crystal violet vaccine (69).

The 'exaltation' methods including the test with high culture passage HC virus rendering Teschen virus cytopathogenic in porcine testis cells (exaltation-Teschen-virus: ETV; 70, 71) and used for the detection and quantification of neutralizing antibodies combined reproducibility with high specificity and sensitivity (72). Nevertheless all these tests including the neutralization test developed and standardized on the basis of the CPE produced under certain conditions by HC virus strain PAV-1 (62, 73) were laborious and time-consuming and, therefore did not achieve wide application. Results however, on the serologic host response following HC virus inoculation are basically valuable and require further consideration in the respective section (3.3.).

For diagnostic purposes all the neutralization techniques mentioned were outranged by tests based on serologic criteria, e. g. immunofluorescence, for the infectivity of HC virus in cell cultures. Following the introduction of the fluorescent antibody (FA) method for diagnosing HC virus infections (8), and its application to cell cultures inoculated with HC virus (74, 75), the fluorescent antibody-cell culture test (FACCT) was adopted for the evaluation of anti-HC sera (9) and modified for use in determining the serologic status of test pigs

(4, 76). Standardized FA procedures using the PK(15) cell line for the multiplication of indicator strains of HC virus were evaluated by using the 50 % endpoint method and widely applied with various equipment, macro- or micro-techniques being introduced (10, 40, 77 - 81). Other procedures were based on the constant virus varying serum technique using a 50 % or 90 % reduction, respectively, of fluorescent micro-plaque counts for the determination of the endpoints (9, 11, 82, 83). Thereafter a variety of modifications of the FA micro-plaque reduction test (PRT) was introduced (84, 85) and either used to determine the serum dilution reducing the micro-plaque (micro-foci) counts by 90 % compared with controls or for the calculation of the neutralization index (86, 87).

Although the use of FACCT as indicator system for the detection and measurement of HC virus-neutralizing antibodies has proven to be not only specific but also highly sensitive giving reproducible results, several attempts were made in order to overcome the immunofluorescence step by a less laborious technique. Other neutralization assays were based on a cytolytic HCV strain producing micro-plaques in a micro-plate (88), or formation of macro-plaques under agarose overlay (89) but did not find great echo. Formerly, a cytopathogenic strain of BVD virus (Oregon C24V) had been used for titrating commercial HC-immune sera (3). However, all of the assays mentioned were strictly bound to cytopathogenic viruses and had thus a low value as compared with test procedures that also permitted titration of noncytopathogenic viruses and homologous (strain-specific) as well as heterologous neutralizing antibodies.

Assays using fluorescein- or enzyme-labeled antibodies had the advantage of not only permitting detection of the indicator viral antigen but also of being independent of the appearance of a cytopathic phenomenon. In this respect a neutralization test carried out in micro-plates using the 'chessboard' principle and read by means of peroxidase-linked antibody (NPLA) innovated some years ago by Holm Jensen (61) proved to be a valuable tool for the detection of neutralizing antibodies

against HC virus on a large-scale (90). It also permitted distinction between antibodies induced by either HC virus or BVD virus (61). The latter aspect is important in view of the difference in antibody titres against HC virus and BVD virus after pigs were infected with one of these viruses under field conditions. The degree of cross-reaction depends on the variant strains of BVD virus or HC virus involved as shown experimentally (91).

Unfortunately not every strain of HC virus can be used in such neutralization tests for the detection of homologous antibodies. The lapinized Chinese strain (see Chapter 10) of HC virus does not multiply readily in cell-culture systems and if it does suspicion arises as to the nature of the virus or to the degree of adaptation to rabbits. In order to determine neutralizing titres in rabbit hyperimmune sera or sera derived from pigs vaccinated with lapinized HC virus, hyperthermic reactions in rabbits have been used as an indicator for the presence or absence of antibodies. The rabbits were injected intravenously with virus-serum mixtures and the neutralizing titre calculated as 50 % endpoint (92, 93). Although it was found that the protective titre in rabbits is comparable to the protective titre in pigs and that the neutralizing titre in rabbits is higher than the protective titre, this test is no longer justified and should be replaced by neutralization tests in cell cultures using FA or enzyme-labeled antibodies (ELA).

2.4. Inhibition

Apart from precipitation, complement-fixation or neutralization, there are additional serologic reactions which may be summarized under (i) inhibition and (ii) mediation of reactions (sandwich reactions).

Inhibition is characterized by blocking of antigenic binding sites by specific antibodies in a way that they are unaccessable for antibody reagents which otherwise detect the respective antigen by a serological reaction. The inhibiting (blocking) antibodies might have properties in common with other antibodies as was shown for the binding affinity in the

direct immunfluorescence technique (94). Therefore inhibition can be ascribed to steric hindrance irrespective of the type of antigen-detection method. A typical example would be a haemagglutination-inhibition test on the basis of a passive haemagglutination reaction (95) or by using treated erythocytes to which HC virus had been adsorbed (96, 97). However, these tests have not found any wider application certainly because of the introduction of more specific and sensitive methods.

The direct FA technique was used for the detection of antibodies which inhibit the specific immunofluorescence (98). Realizing the significance of a serological diagnosis of subclinical HC in eradication and control programs, Have (99) developed an antibody-blocking assay in which test sera mask HC viral antigen so that it will no longer be able to react with a reference rabbit antiserum against HC virus. The antibodies of the latter if bound to the HC viral antigen were detected by swine-anti-rabbit IgG conjugated with horseradish-peroxidase and the antibody-reactivity of test sera was calculated from percentage of inhibition by reading the optical density. This inhibition test based on an indirect enzyme-linked immunosorbent assay (ELISA) appeared to closely parallel a neutralization test (61) in detecting an early antibody response in experimentally infected pigs. The sensitivity of the indirect ELISA was determined by comparison with the neutralizing dose (ND_{50}) and the latter found to correspond to approximately 20 % inhibition (cut-off level) by a reference antiserum against HC virus strain Alfort (99).

2.5. Sandwich reactions

In principal a sandwich reaction results from the overlaying of antigen-antibody complexes with a labeled (conjugated) antibody produced against purified gammaglobulin of the species from which the antigen-bound antibody was derived, e.g. HC convalescent or vaccinated swine. Such reactions are known since Weller and Coons (100) reported on *in vitro* studies with varicella and herpes zoster viruses using the indirect fluorescent antibody assay (IFA). Later IFA was

applied for the rapid detection of HC-specific antibodies in test sera in view of large-scale screening (101). This test seemed to be a promising candidate for such purposes also because of its specificity, simplicity and rapidity. Examination of groups of sera from pig herds suspected of subclinical HC revealed within three hours antibodies specific for HC virus. However, cross-reactivity was shown to occur with antibodies specific for BVD virus and therefore possibly to interfere with this test. This was also observed with an indirect enzyme-labeled antibody (ELA) technique in which horseradish-peroxidase was used for conjugation to antispecies gammaglobulin instead of FITC (102). Similar to the IFA, the ELA 'sandwich technique' was developed for use as a screening method for the rapid detection of HC-specific antibodies following the Hog Cholera Emergency Declaration 1972 in the United States (103). The test was evaluated on blood samples of different classes of experimental pigs including those infected with HC virus and BVD virus, respectively, and a positive correlation of more than 99 % was found with the neutralization results obtained with the same sera.

A slightly modified ELA technique (PLA: peroxidase-linked antibody) was used by Holm Jensen (61) to examine porcine sera for antibodies against HC virus and BVD virus by comparison of the PLA technique, neutralization assay (NPLA) and a CF test. It was concluded that CF and PLA are sensitive tests but not quite as sensitive as the NPLA assay.

In much a similar way as antibodies against HC virus and BVD virus can be detected by any of the 'sandwich' techniques in polyclonal sera, such techniques have been used for the screening of hybridoma supernatant fluids for monoclonal antibodies against HC virus by an ELA technique (104) as well as against BVD virus in comparison to HC virus by IFA (105).

3. APPLICATION OF SEROLOGICAL METHODS

3.1. Potency testing of immune sera

Sixty years ago Benner (106) stressed the importance of administering 'ample doses of potent serum' simultaneously with

a potent HC virus to pigs weighing fifty pounds or more in order to confer a solid and lasting immunity. In the absence of any serological test except experimental pig inoculation, the estimation of potency of hyperimmune serum depended largely on 'how soon after (simultaneous) vaccination' the pigs died. This situation called for an in vitro potency test for anti-HC antibodies in order to replace the animal inoculation assay which provided only approximate information on the immune serum value. For this purpose Simonyi (3) developed a titration procedure for HC immune sera based on the neutralization of the cytopathogenic BVD virus strain Oregon C24V after unsuccessful attempts to produce a cytopathic effect with the strain of HC virus propagated by Gillespie et al. (7) in tissue culture. Since the introduction of the lapinized Chinese ('C') strain of HC virus (see Chapter 10) the use of hyperimmune sera for simultaneous vaccination of pigs decreased gradually. Nevertheless, as late as 1968 a neutralization test based on the FA technique was applied to evaluate 137 commercially produced antisera for antibody content against HC virus (4). This test was also used to determine the serologic status of pigs in which some serials of anti-HC serum were tested for their antibody levels and also to select HC-susceptible pigs for HC vaccine safety testing. This indicated a shift from potency testing of hyperimmune sera to vaccine potency and safety testing which required pigs not immune to HC virus.

3.2. Determination of immune status

Previous studies on the evaluation of vaccine efficacy, colostral protection, experimental HC virus infection in pigs, and antibody production were mainly hampered by the lack of information on the immune status of the pigs prior to use. Therefore neutralization tests as indicators of immunity became very important (1, 4, 6, 12, 62, 76, 85, 107 - 109). Actively acquired neutralizing antibody titres of 1/2 (77), 1/4 (75), 1/5 (90, 110) or higher were taken as threshold and thus of immunity due to former HC virus infection or vaccination. It was realized that titres below these values may occur in spite

of an HC virus infection during incubation or in case of persistently infected immunotolerant pigs. This risk can be reduced to nearly zero by testing sera twice at three to four week interval for antibodies and additional examination for HC virus by cultural isolation.

3.3. Host humoral immune response

No disagreement seems to exist on neutralization being the most sensitive and specific reaction of HC virus with immune sera derived from pigs (or ruminants: see Chapter 13) following experimental or field infection with HC virus or vaccination. Therefore most reports dealing with host humoral immune response to HC virus refer to the development of neutralizing antibody in a more or less precise dose-and-time-dependent manner.

Next in sensitivity to the neutralization peroxidase-linked antibody (NPLA) assay ranged the sandwich techniques ELA (103) or PLA (61), respectively. An indirect ELISA used as an antibody-blocking (inhibition) test displayed a similar sensitivity (99).

Because of low sensitivity (low titres) other reactions than those mentioned above (precipitation, complement-fixation) were mainly used in screening for antibodies (111) without offering means for differentiation of antibodies against HC virus and BVD virus (60). Antibodies against the latter virus appeared following infections with HC virus in pigs usually to lower titres and at a later stage of infection than antibodies against the homologous and heterologous HC virus strains (43, 61, 90, 99).

In an attempt to define the conditions under which the host humoral immune response should be investigated, not only methods and techniques used for the qualitative and quantitative demonstration of antibodies need consideration. Studies on the immune status of the host organisms requires the absence of any former experience with HC or BVD virus infections as well as passive immunity. Virus dose and route of infection are also important factors. They influence to a

considerable extent the serologically measurable response and are due to wide individual variation. Moreover, the serological testing demands the use of the homologous HC virus strain in view of distinct titre differences as compared with results obtained with heterologous as well as BVD virus strains (43, 91, 112). Even if all of these requirements are fulfilled it might be presumptuous to conclude that experimental HC virus infections cover field conditions exactly. Differences in virulence of HC virus strains occuring in the field change not only the disease pattern but also contribute to delayed or suppressed immune responses (see Chapter 8).

In a systematic study using the END neutralization test Ikeda et al. (108) investigated the immunological response of pigs as well as sheep, goats and rabbits, following intravenous or subcutaneous infection of virulent HC virus strain ALD (sheep, goats) or lapinized HC virus (pigs, rabbits). Serological testing at weekly intervals revealed neutralizing antibodies in all but one goat after two weeks and within another two weeks, an increase in titre ranging between 2 to 8 (- $\log_2$) ultimately reaching 10 (- $\log_2$) serum dilution.

Coggins and Sheffy (62) using the CPE neutralization test followed the development of antibodies against a presumably rabbit-adapted HC virus vaccine strain by plotting the average antibody titre of sera taken twice in the second week after vaccination. Antibody increase was noticed between the seventh and eleventh day in two pigs reaching serum titres of 2.0 (- $\log_{10}$) on the fourteenth day. Patterns of neutralizing antibody formation against HC virus strains under experimental as well as field conditions have been reviewed elsewhere (43).

A more intrinsic picture of the serologic response was obtained when groups of pigs were inoculated with HC virus strains of different virulence and tested for neutralizing antibodies against the homologous as well as heterologous strains of HC virus. Plots of mean geometric titres similar to former experiments (62) exhibited distinct and reproducible profiles characteristic for each strain of HC virus including

BVD virus strains used in the neutralization tests on the same sera (43, 112). With the low virulence HC virus strain 'Glentorf' antibodies were detectable in the majority of pigs within the range of 14 to 21 days after contact with infected pigs while pigs intranasally inoculated with the same strain of virus exhibited neutralizing antibodies 10 to 14 days post-inoculation.

Individual variation of the time before neutralizing antibodies appeared after intranasal inoculation or contact with HC virus-infected pigs necessitated the correction of the kinetics to the first date of antibody detection (43, 91).

Absence or low-level neutralizing antibody production has been found in baby pigs after in utero HC virus infection or in young pigs infected with HC virus of reduced virulence (6, 41, 43, 44, 113 - 116.) The impaired antibody response concurrent with persistent HC virus infection has been extensively studied and demands careful consideration whenever the 'normal immune response' is discussed in view of persistently infected pigs as a potential source of HC virus and herd surveillance for the presence or absence of past exposure to HC virus (12).

3.4. Serologic herd diagnosis and surveillance

The epidemiological implications of knowledge gained through the application of FA techniques on the various clinical forms of HC have been excellently described by Young (12). Particular emphasis was layed upon the slow spread of the virus in herds with undetected or unreported cases of HC due to disease caused by less virulent HC virus, contrary to former thinking in terms of acute clinical forms. The chronic or atypical form of HC is characterized by persistence of HC virus with little or no neutralizing antibody detectable among young pigs. On the other hand sows infected with the same virus do respond immunologically with no signs of illness except anorexia and fever (11, 109, 117). However, non-immune pregnant sows transmit HC virus to the fetuses possibly resulting in persistent infections with virus excretion and late onset of disease in the young or adult pig (41) so that perpetuation of

HC virus may be guaranteed in certain herds without giving immediate reason to suspect HC. Slow spreading within such a chronically infected herd is demonstrated by the simultaneous finding of both seropositive and seronegative animals.

For eradication in countries where HC is endemic, early detection of every clinical form of HC is essential. Usually no diagnostic difficulty is encountered in acute disease. Chronic forms of the infection because of the above reasons are far more important in this respect and also more difficult to detect. Demonstration of neutralizing antibodies against HC virus has been proposed as a diagnostic aid and for epidemiological tracing of chronically infected herds (12, 118) and elimination of suspect animals. The same applies to testing formerly negative herds for the absence of neutralizing antibodies (119). However, the results of neutralization tests may be confusing if the vaccination history is not known. In addition antibody titres may result from infection with BVD virus. They can be differentiated on the basis of differences disclosed by neutralization tests with HC virus and BVD virus in parallel (91, 120).

The neutralization test based on the direct FA technique has been recommended in the United States to be used on a herd basis rather than on an individual basis (12). Arbitrarily a minimum of 10 % of the herd, or 10 animals ('whichever is greater') was proposed to be bled since it is not unusual to find seronegative animals and animals with antibody titres in the same group of swine. This as a matter of fact was taken into consideration when a random sampling program was elaborated on the basis of 95 % probability that animals with HC antibodies (k) amongst the total number of antibody carriers (K) would be detected in a herd of particular size (N) by a random sample (n) of a certain size (121) by using the formula

$$P = \left(\frac{N - K}{n}\right)\left(\frac{N}{n}\right)$$

Accordingly the probability of not detecting an animal with HC antibodies (if k = 0) was 5 % in a random sample of not less

than 25 % of animals from a herd containing at least 40 % HC antibody carriers (K). Although the actual mean herd size proved to be 12 animals and the random sample size 35 to 50 % when a minimum of 5 animals was bled there remained the question to be answered for the number of antibody carriers actually present in herds with subclinical or chronic HC virus infections.

In the example mentioned above the confidence level was calculated under the assumption that 40 % of the animals present in a given herd were antibody carriers (121). However this figure is in many cases unrealistic and may be not higher or even far less than 10 %. The sample size required in order to reach a 95 % or 99 % confidence level has been tabulated in order to calculate the risk of not detecting an antibody carrier in a herd with such animals (122). As it was shown the size of the random sample was to be fixed according to the herd size. However, it may come out that the practical size limit will be overdrawn and therefore requires a repetition of the sampling after a certain time interval, e.g. 4 weeks. Calculations of this nature are getting increasingly important in attempts not only to find 'positive' herds but also to be certain that a herd is free of antibody carriers, meaning not suspected of subclinical/chronic HC virus infection. In this way the neutralization test or any other comparable test can be utilized for HC surveillance in order to assure regional or national freedom of HC in a country where HC was enzootic before an eradication program was launched.

4. CONCLUDING REMARKS

During the past two decades a variety of serological methods and techniques have been introduced possessing the sensitivity and specificity required to detect pigs with humoral antibodies against HC virus. Assays allowing quantitative determination of antibody were mainly those based on neutralization or 'sandwich'-reactions with detection by fluorescein- or enzyme-labeled antibodies. For purely qualitative screening of large numbers of sera other tests

based on inhibition, complement-fixation or precipitation are quite feasible. However, it is necessary to distinguish between methods which can be used for assay purposes in research work and those which are recommendable for official testing of blood samples collected with the ultimate goal of HC eradication by tracing pig herds with subclinically infected animals or for surveillance programs. For the latter it can firmly be stated that the serological tools required are available if needed by governmental authorities. Future research work should focus on the comparative analysis of structural and nonstructural viral proteins by use of monoclonal antibodies and the characterization of the HC viral genome. The results of this research may ultimately lead to the development of monospecific reagents and vaccines based on synthetic oligopeptides.

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7

IMMUNOLOGICAL ASPECTS OF THE INFECTION

F. EHRENSPERGER

1. INTRODUCTION

This chapter deals with various aspects of the humoral and cellular immune defense mechanisms as well as with some possible immunopathological events associated with hog cholera virus (HCV) infection in pigs.

The immune response to an infection with HCV is highly variable depending essentially on the strain of virus, dosage and route of infection, as well as on the age and condition of the pigs.

Generally, antigenicity and immunogenicity appear to be related to the degree of virulence of the particular virus (1-3): High-virulence strains usually induce higher levels of neutralizing antibodies than low-virulence strains, possibly due to the amount of virus produced in infected animals (3). Serial passage in pigs of low-virulence nonimmunizing HCV results in an increase in pathogenicity and immunogenicity (2).

The age dependence of HCV infections, predominantly of low-virulence strains, appears at least partly to be due to the maturity, or immaturity of the immune system (4-6). The susceptibility of pig fetuses for high and low-virulence strains (7-16) as well as for attenuated strains of HCV (9,12,17-20) is well documented.

Immunocompetence of pig fetuses to HCV, compared with other viruses, occurs relatively late. Piglets from sows infected before day 85 of gestation have been found to produce no antibodies (21-23). The majority of piglets however do not achieve immunocompetence before term. HCV vaccines (lapinized Chinese strain) were found to be effective already in 7-day-old piglets born from nonimmune sows, and in 5-week-old piglets born and nursing from immune sows (24,25). On the other hand in some instances weaners which were contact infected with

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low-virulence strains of HCV did not exhibit a humoral immune response up to death at 5 to 10 weeks after contact (26).

Among dietary factors low protein intake results in a deficient immune response to HCV vaccination (27-30).

Variability and complexity of the immune response to HCV infections and vaccinations, as well as possible immunopathological mechanisms involved in the pathogenesis of the disease may be evaluated in the light of interactions between the virus and the cells of the immune system. From immunohistological studies it became evident that HCV has a distinct affinity for lymphoreticular cells. Viral antigen can be demonstrated within macrophages and in reticular and endothelial cells (14, 22,31-33). These cells appear to be the major site of the "in vivo" virus replication in acute and persistent HCV infections, whereas in chronic classical swine fever (CSF) viral antigen tends to be limited to the epithelial cells of sites such as the tonsils, ileum and kidneys (31). Infectious HCV has been isolated from peripheral blood mononuclear cells (34) and from tissue and blood lymphocytes (35) of persistently infected animals. On the other hand, HCV has been propagated in porcine buffy coat cell cultures (36,37) as well as in blood monocyte and lymphocyte cultures (38,39). In the presence of mitogens, virus replication in lymphocyte cultures was enhanced. Both B- and T-cell mitogens appeared equally effective. No cytopathic effect occurred in the infected cell cultures.

These data confirmed the affinity of HCV to lymphoreticular cells as well as the susceptibility of lymphocytes and other leucocytes to HCV infection. Natural and experimental fatal HCV infection of pigs resulted in regressive histological changes in lymphoid tissues and in lymphopenia; the exact pathogenic mechanism however remains unclear. Direct or indirect interactions between viruses and lymphocytes and mononuclear phagocytes may lead to various dysfunctions of the immune system, particularly in association with persistent infections (40).

2. HUMORAL IMMUNE RESPONSE

2.1. Types of antibodies

Basically pigs that are vaccinated or recover from HCV infection develop different kinds of antibodies. Based on their experimental par-

enteral immunization studies in rabbits and pigs, Matthaeus and Korn (41-43) proposed a distinction between the following antibody types:

2.1.1. Neutralizing antibodies. These were demonstrated in both rabbits and pigs after HCV infection or vaccination. They appeared as early as 9 days post-infection in recovering pigs, after the 15th day in fatally affected pigs. By gel filtration (Sephadex G-200), ion exchange chromatography (DEAE-Sephadex A-50) and immunoelectrophoresis 19S, 7S and 4S antibodies were identified (42). Neutralizing antibodies are supposedly the most important antibodies in terms of protection (24).

2.1.2. Precipitating antibodies. In sera from pigs recovering from infection with certain immunogenic HCV strains, precipitating antibodies were detected which were directed against "cell pancreas antigen", i.e. chymotrypsin. Antibodies precipitating with viral antigen were only found in sera from HCV-infected rabbits but not in pigs (42,44). On the other hand Terpstra (45) proposed immunoelectroosmophoresis (IEOP) as a diagnostic test for HCV serology in pigs. Antigen extracted from HCV-infected PK-15 cells (porcine kidney cell line) was shown to precipitate with sera from pigs 2 to 3 weeks after inoculation of low-virulence HCV as well as from pigs vaccinated with C-strain HCV.

2.1.3. Antibodies detectable by immunofluorescence. In porcine immune sera antibodies can be detected by using indirect immunofluorescence on preinfected cells or tissues (24,41). Experimentally infected rabbits however failed to produce antibodies detectable by immunofluorescence in spite of the presence of neutralizing and precipitating antibodies (41). These findings confirmed the diversity of HCV antibodies although in other instances rabbits which were repeatedly immunized by C-strain HCV did develop considerable amounts of antibodies useful for immunofluorescence (46).

2.2. Correlation of antibodies to course of infection

In acute CSF, neutralizing antibodies do not appear in the blood until the leucopenia is overcome (47). In acute fatal disease only minimal amounts of neutralizing antibodies are produced later than 13 to 16 days after infection. Ressang (32) demonstrated anti-CSF-antibodies in tissues of fatally affected pigs, predominantly IgM.

Pigs recovering from acute CSF develop a prominent immune response, reaching maximum antibody levels 3 to 4 weeks post-infection. Antibody levels may persist indefinitely but at least for 6 months (42).

In chronic CSF neutralizing antibodies were transiently detectable during the phase of "partial recovery", between 3 and 6 weeks after infection (31,48).

Low virulent HCV strains may cause inapparent infections and were described as often poorly immunogenic (2,49). In other instances however low-virulence HCV strains induced considerable titres of neutralizing antibodies in immunocompetent pigs (26). Specific immunological unresponsiveness, interpreted as immune tolerance and associated with virus persistence, was observed in piglets after intrauterine or early postnatal infection (see below).

3. CELLULAR IMMUNE RESPONSE

Information on the cell-mediated immune response to HCV is sparse and partly controversial.

Corthier (50) observed a short transient specific lymphocyte reactivity on day 17 after infection with the "Loud strain", which is known to cause chronic CSF. UV-irradiated cell culture supernatant was used as "mitogen" in a lymphocyte proliferation assay. No cellular immune response was detected in vaccinated pigs.

No effect on the proliferation rate of lymphocytes was noted by Van Oirschot (23,35) using virulent or UV-irradiated HCV as lymphocyte-stimulating antigen. They investigated lymphocytes from infected as well as from vaccinated pigs.

A distinct effect on the proliferation rate of lymphocytes from pigs vaccinated with C-strain of HCV was reported by Remond et al. (51) using glycidaldehyde-inactivated HCV as stimulating antigen. The specific reaction occurred around the 28th day after vaccination and persisted for 8-12 days. No data are available concerning the role of specific cell-mediated cytotoxicity reactions to HCV-infected cells.

4. EFFECTS OF HCV INFECTIONS ON IMMUNE FUNCTIONS

The majority of viruses that infect cells of the immune system are capable of inducing defects in immune functions. Such defects, speci-

fic or nonspecific, play an important role in the establishment of non-arbo-togavirus persistence (35).

In acute CSF there is evidence of widespread necrosis of lymphocytes. Piglets persistently infected after congenital or early postnatal infection with low-virulence HCV strains develop a runting syndrome accompanied by severe atrophy of the thymus and other lymphoid tissues (46,52,53) (Fig. 1). Thymic atrophy also occurs in children with congenital rubella (54), in calves with congenital bovine viral diarrhea (55) and in lambs with border disease (56).

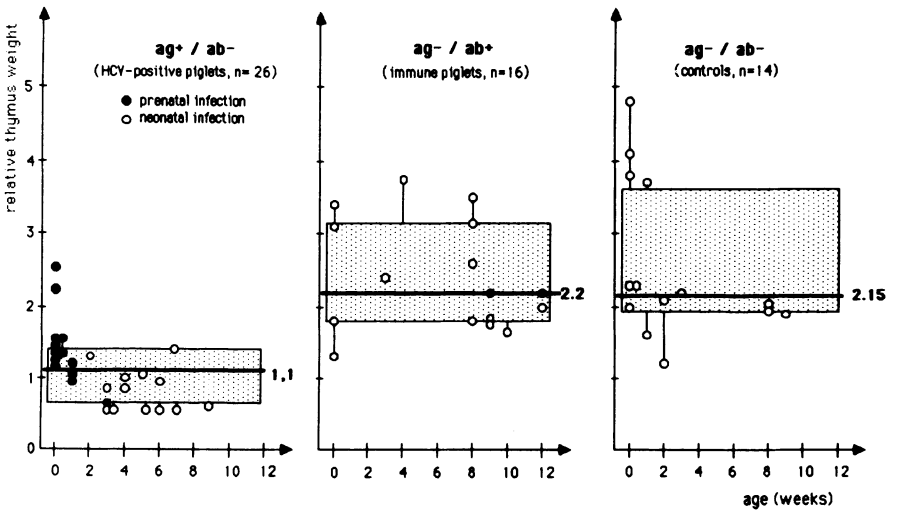

FIGURE 1. Relative thymus weight (mg/g body weight) of piglets after prenatal and neonatal infection with low virulent HCV "GLENTORF"

●○ individual values

— median value ($\tilde{x}$)

▩ boxplot, representing 25-75 % values

4.1. Effects on the specific humoral immune response

The intrauterine infection of piglets with HCV strains may induce a state of specific immunological unresponsiveness (8,16,20,22,23,34,46,57,58). The affected animals are persistently viraemic, and may continue to live for months. The majority of congenitally infected piglets, however, die within the first 3 weeks after birth. The proportion of immunotolerant piglets is dependent on the time of infection as well as on the particular HCV strain (Table 1, Fig. 2). Failure of antibody production associated with persistent HCV infection was occasionally observed after postnatal infection with partly attenuated (59) and low-virulence (46,60) HCV.

TABLE 1. Persistent HCV infection in piglets induced by experimental transplacental transmission.

Virus	Infection		Relative number of persistently infected piglets			Reference
	Route	gestational age	at birth	at 1 wk of age	at 6 wks of age	
"Bergen"	i.m.	40 days	21/33	11/15	11/15	
"	"	65 "	34/38	22/26	11/18	23,34
"	"	90 "	9/21	7/16	1/15	
"Glentorf"	i.nas.	40 days	15/15	0/0	0/0	
"	"	68 "	16/19	7/8	3/4	21,58
"	"	90 "	12/22	8/14	2/8	
"FIN 3086"	i.m.	22 days	18/20	2/2	2/2	
"	"	43 "	12/12	3/3	3/3	16
"	"	72 "	17/24	0/0	0/0	

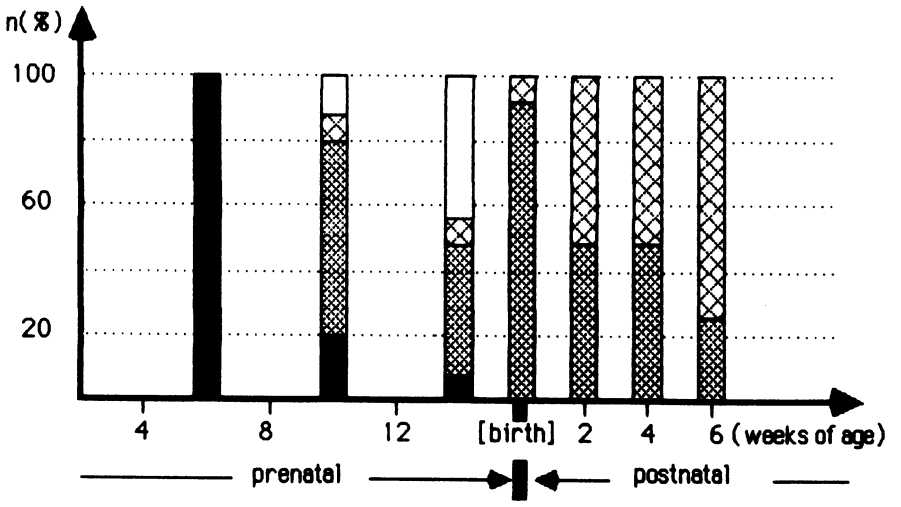

FIGURE 2. Simplified histogram representing the development of immunocompetence and immunotolerance to low virulent HCV strain "GLENTORF"

- ■ stillborn piglets
- ▩ immunotolerant piglets
- ▨ piglets mounting an immune response
- □ piglets without evidence of infection

The mechanisms involved in the initiation and maintenance of immune tolerance and virus persistence in congenital and perinatal HCV infections are unknown. Since HCV replicates in cells of the immune system, it is conceivable that defects in the host defense system would be induced. Furthermore, cells infected with HCV appear to bear no or very little viral antigen on their surface allowing them to elude the immune attack (39). On the other hand the immunological immaturity of the unborn and newborn piglet appears to be an important factor. In only a few instances small amounts of neutralizing antibodies against HCV were

found in precolostral serum samples of piglets from sows infected between the 85th and 90th day of gestation (22,23). In some instances immunological unresponsiveness was observed in weaning piglets (26). Immunocompetence was only achieved several weeks postnatally as expressed by demonstrable antibody formation.

There is indirect evidence that the absence of neutralizing antibodies is only partially responsible for virus persistence. Ingestion of high titers of colostral antibodies seems to prevent, but not terminate or interrupt, viraemia. "In vitro" experiments confirmed that HCV can replicate in cell cultures in spite of the presence of antibodies (61).

In comparison with CSF it is interesting that human fetuses are able to mount an immune response to intrauterine rubella infection by producing IgM-antibodies (62). In congenital bovine viral diarrhea virus infection in calves immune reactivity appears to be variable, its onset being around the 200th day of gestation (63). A late postnatal immune response as well as lifelong virus-specific immune tolerance had been observed in sheep infected with border disease virus (64).

4.2. Effects on globulin synthesis

Piglets infected transplacentally with HCV exhibit a depression of IgG synthesis, an enhanced IgM production and a shortened half life of passively acquired colostral immunoglobulins (Fig. 3a,b) (46,52). Immune complexes in sera of persistently infected piglets could only transiently and sporadically be demonstrated. They were considered not to be of pathogenetic significance (23).

In acute CSF a "disturbance" of globulin synthesis coincident with leucopenia has been reported (47).

Chronic CSF is accompanied, at least in the stage of partial recovery, by increased serum gamma globulin levels. A deposition of immune complexes was detected in the renal glomeruli of these animals (31,48).

Similarly elevated IgM and depressed IgG and IgA levels have been observed in congenital rubella in children (65). Suppression of IgG (T) synthesis in horses persistently infected with equine infectious anemia virus was reported (66).

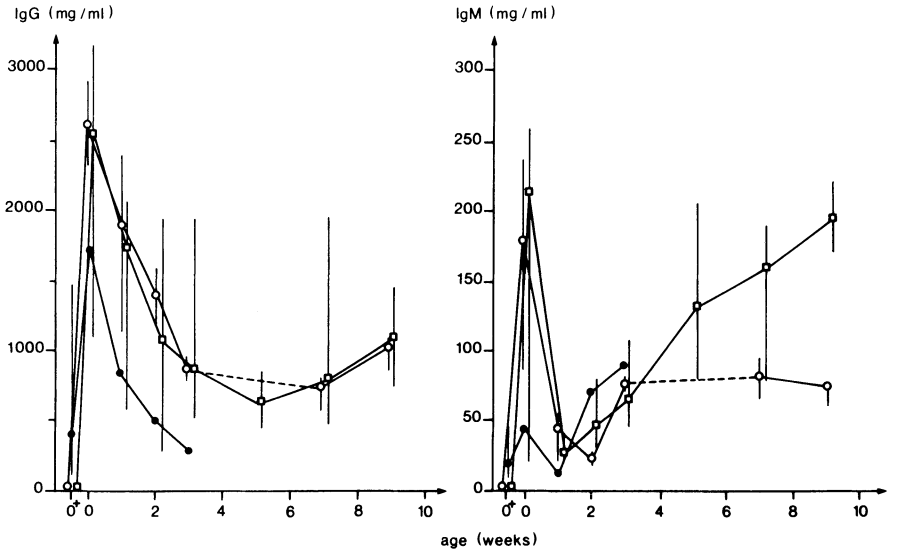

FIGURE 3a. Kinetics of immunoglobulins G and M (median values) in piglets prenatally infected with low virulent HCV: HCV-positive piglets exhibit a depression of IgG-synthesis as well as low levels of colostral IgG and IgM.

0^+ precolostral blood samples
● ag+/ab- (HCV positive piglets)
○ ag-/ab+ (immune piglets)
□ ag-/ab- (controls)

In contrast elevated gamma globulin levels are known to occur in other persistent viral infections such as Aleutian disease of mink (67, 68), Marek's disease in chickens (69) and African swine fever (70).

4.3. Effects on specific cell-mediated immunity

It is not known whether tolerance of the cell-mediated immune response exists in persistent HCV infections. As mentioned above observations on cell-mediated immunity in CSF are controversial and sparse.

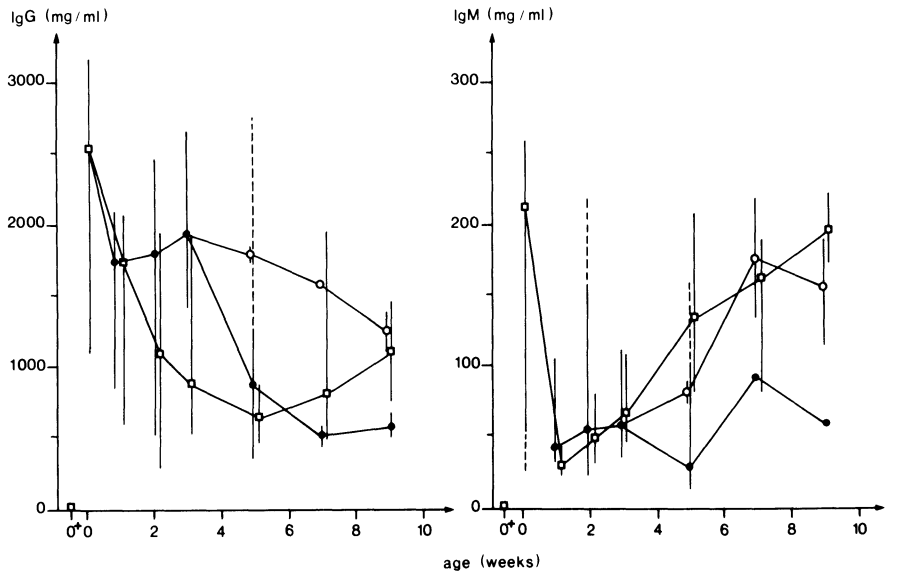

FIGURE 3b. Kinetics of immunoglobulins G and M (median values) in piglets neonatally infected with low virulent HCV: Persistently infected piglets exhibit a continuous depression of IgG- and IgM-levels.
(For legend see Fig. 3a)

4.4. Nonspecific effects on cell-mediated immunity

"In vitro" HCV infection of lymphocytes did not alter the mitogenic proliferation rate (39). However, pigs which are fatally affected by acute CSF had a depressed PHA- (phytohaemagglutinine) response (71). Peripheral blood lymphocytes from pigs with congenital persistent HCV infection responded to PHA and PWM (poke weed mitogen) in a normal way, except for the terminal stage of the disease. B-cell reactivity, assessed by anti-IgM and staphylococcal protein A as mitogens, appeared to be slightly depressed (14,23). NK- (natural killing) effector cell activity, tested in a nonspecific assay using K-562 and PDe-B-1 as tar-

get cell lines, revealed a slight depression in the PDe-B-1-system in persistently infected piglets (46).

In summary, the nonspecific effects in cell-mediated immunity caused by persistent infections with HCV appear to be minimal as far as "in vitro" lymphocyte culture tests are concerned.

In congenital rubella it has been shown that the infection acquired within the first 2 months of pregnancy resulted in an impaired PHA-response (72). A slight inhibition of mitogen reactivity was reported from calves infected persistently with bovine viral diarrhea virus (73, 74).

Some other virus infections with special affinity to lymphoid tissues are known to have a suppressive effect on cell-mediated immune functions: prolonged allograft survival and a depressed tuberculin reaction were observed in chickens infected with Marek's disease virus (75). Similar findings were reported in Aleutian disease in mink (76), canine distemper (77) and lymphocytic choriomeningitis in mice (78). A depression of the "in vitro" mitogen response of lymphocytes occurs in Aleutian disease (79,80) and in Marek's disease (81). In dogs with distemper (82) and in horses with equine infectious anemia (83) a transient inhibition of "in vitro" lymphocyte reactivity is reported.

Cell-mediated immune functions are severely affected in the viral acquired immune deficiency syndrome (AIDS) in man (84) and rhesus monkeys (85) and in feline leukemia (86).

4.5. Immune response to unrelated antigens

The humoral immune response to antigens which are unrelated to HCV is apparently not altered in HCV infected pigs. Van Oirschot (57) investigated the immune response of congenitally infected piglets to sheep red blood cells and porcine parvovirus (PPV) with no effect as compared with uninfected animals.

This again is in agreement with comparable infections: an unaltered immune response to unrelated antigens was also seen in congenital persistent bovine viral diarrhea and border disease virus infections (64,87).

4.6. Sensitization

Pigs which are infected with low-virulence nonimmunizing HCV are able to develop hypersensitivity to the causative agent (88). Following a second infection with high- or low-virulence HCV they suffer from

clinical signs which are more severe and have a shorter incubation period than in control animals. Similar observations were made by Dunne and Luedke (89). Pigs which had been preinfected experimentally with a noninfectious and nonimmunizing "incomplete" HCV were sensitized to a subsequent challenge with virulent virus. The inoculum for the first infection had been prepared from blood samples of pigs in the very early phase of infection.

This phenomenon of sensitization by nonimmunizing HCV was interpreted as allergic. Precipitating antibodies have been demonstrated in spite of the absence of neutralizing antibodies (88). In these experiments the infection was performed by subcutaneous injection. The possibility of sensitization to other constituents of the inoculum, i.e. blood proteins, therefore cannot be finally excluded.

In pigs with congenital persistent HCV infection, both prolonged (34) as well as reduced (23) resistance to superinfection (interpreted as sensitization) with virulent HCV were reported. Similar observations have recently been reported in cattle persistently infected with a non-cytopathogenic bovine viral diarrhea virus superinfected with cytopathogenic virus (90). These animals showed a delayed humoral immune response and a prolonged lymphocytopenia after the superinfection.

5. DISCUSSION AND CONCLUDING REMARKS

The interaction between HCV, which has a distinct affinity for cells of the immune system, and immune functions are complex and not fully understood.

Lymphocytopenia associated with regressive changes in lymphoid tissues are manifestations of acute fatal as well as persistent HCV infections. It is not known whether these are direct or indirect effects of the infection. A direct viral destruction of lymphocytes seems unlikely since HCV has no cytopathic effect on lymphocytes and other cells "in vitro". During the lymphocytopenic phase pigs are usually not able to mount an immune response to HCV.

The ability of pigs to respond immunologically to HCV is dependent mainly on the antigenicity of the virus as well as on the immunological maturity of the host. The immune response is demonstrated by the de-

tection of neutralizing antibodies; the role of cell-mediated immune functions has not yet been sufficiently defined.

The most striking immunological alterations occur after prenatal and neonatal infections with HCV of low virulence. The majority of these piglets fail to mount a specific immune response to HCV and remain persistently infected. Infectious virus can be recovered from the blood, both serum and cells, as well as from most tissues. Persistently infected piglets may live for several months and continuously excrete infectious virus. Clinical signs may be absent, minimal or non-characteristic. Runting, thymus atrophy and a generalized lymphoid depletion can be observed at postmortem.

It is obvious that these piglets play an important role in the transmission and thus the control of HCV infections in the field.

In addition to the specific unresponsiveness, persistently infected animals appear to be susceptible to opportunistic infections such as bacterial respiratory disease (46). This is not surprising and might well be explained by the lymphocytopenia, by the impaired immunoglobulin synthesis and possibly other immunosuppressive effects. "In vitro" attempts have failed to demonstrate severe impairment of lymphocyte reactivity; only a moderate inhibition of the B-cell mitogen response and a transient hyporesponsiveness to T-cell mitogens have been observed (91). These findings, however, have not been confirmed by others (23, 46,56). Experiments have been conducted to investigate the immune reaction of piglets, which are persistently infected with HCV, to unrelated antigens. There was a normal humoral immune response to the antigens tested (56).

Persistent infections associated with specific immunological unresponsiveness appear to be characteristic manifestations of intrauterine nonarbo-togavirus infections before and around the onset of immunocompetence (35). Similar to CSF this condition has been reported in bovine viral diarrhea virus infection in calves (85,92,93) and in border disease virus infection in lambs (64). In congenital rubella in children persistent infections occur despite the presence of circulating antibodies. This difference might be due to the passive intrauterine transfer of maternal immunoglobulins in the human placenta haemo-

chorialis but not in the porcine and bovine placenta epitheliochorialis or the ovine placenta syndesmochorialis.

The mechanisms responsible for the failure of piglets to eliminate HCV or to respond immunologically are poorly understood. The observation that HCV-infected cells bear no, or very little, viral antigen on their surface might explain the ability of the virus to escape the cell-mediated immune attack (39).

In contrast to chronic CSF as described by Cheville and Mengeling (31), in persistent HCV infections neither specific antibodies nor antigen-antibody complexes can be demonstrated (23). This also favours the suggestion that prenatal or early postnatal HCV infections may induce immune tolerance (8,18).

Immune tolerance, generally, can be the result of a blockade, inactivation or dysfunction of specific antigen-reactive lymphocytes. It can be located at the level of B-cells, T-cells or both.

From experimental studies in mice it is known that B-cell tolerance is usually induced by supraimmunogenic doses of antigen ("high zone tolerance") (94). B-cell tolerogens are either thymus-independent or thymus-dependent antigens. Typical thymus-independent antigens have polymeric structures with repeating determinants, e.g. polysaccharides or polypeptides of high molecular weight which characteristically persist in the body for a long time. B-cells of neonatal animals do not appear to be more susceptible to tolerance than cells from adults. Various B-cell tolerance models have shown preferential suppression of IgG antibody formation which suggests a difference in susceptibility of B-cell subpopulations to tolerance. Susceptibility to tolerance seems to be related to the avidity of B-cells to bind thymus-independent antigen. The essential mechanism of the induction of B-cell tolerance probably is a blockade and immobilization of B-cell antigen receptors followed by a inhibition of receptor resynthesis (94).

Thymus-dependent antigens are known to induce both T-cell and B-cell tolerance. B-cell tolerance requires higher doses, has a longer induction period and a shorter duration whereas T-cell tolerance can be readily induced by low concentrations ("low zone tolerance") of a thymus-dependent antigen (95).

Induction of "low zone tolerance" is often accompanied by activation of suppressor T-cells which suppress the IgG antibody response (96) as well as a severe reduction of cytotoxic T-lymphocyte precursors (97,98).

Among immunoregulatory factors which might play an important role in induction of immune tolerance, antibodies and antigen-antibody complexes are probably of some importance (99). This was for instance demonstrated in lymphocytic choriomeningitis virus infection in mice (100).

With regard to HCV infection the mechanisms responsible for immune tolerance are far from clear. A possible explanation for immune tolerance may be that HCV acts by infecting CSF-specific lymphocytes, possibly regulatory suppressor or helper T-cells, and in that way interferes with the production of specific antibodies. Immune complexes as immunoregulants do not seem to be of significance since they are not consistently present in animals with persistent HCV infections. Hypothetically CSF immune tolerance might be classified as thymus-dependent B-cell and possibly T-cell tolerance. However, tolerance of the cell-mediated immune response has not been confirmed so far.

In order to obtain more information on the mechanisms of immune response and immunological unresponsiveness it would be necessary to investigate additional aspects of CSF immunity such as specific and non-specific immune functions, skin allograft survival, reactions to intradermal antigen injection, specific "in vitro" cytotoxicity and macrophage functions. In addition to the epidemiological and economic significance of persistent HCV infection this might be a useful model for the study of other persistent viral infections associated with immune tolerance in man and animals.

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8

PRINCIPLES OF VACCINATION

J.M. AYNAUD

1.INTRODUCTION

A great number of vaccines against hog cholera (HC) have been reported and used in the control of the disease. Two major types of vaccines are the inactivated and the attenuated live virus vaccines. In the past, inactivated virus vaccines were widely used but their low efficacy contributed to the development of live virus vaccines which are known to be more effective. However, the live virus vaccines were constantly reproached on account of their lack of safety. Advances over the last 20 years in our knowledge of hog cholera virus (HCV) and the immunology of the swine species has created a new basis for the development of several live virus vaccines. These are now considered as being effective and totally safe.

In the present review, we attempt to summarize the recent knowledge about principles of vaccination against HC. We try also to report the 'in vitro' and the 'in vivo' properties of three attenuated HC strains used until now as live virus vaccines and the main features concerning the post-vaccinal immunity.

2. TYPES OF VACCINES

2.1. Inactivated virus vaccines

2.1.1. Conventional inactivated virus vaccines.

1) Formalin (1) or crystal violet were used in the past (2,3,4) for preparing inactivated virus vaccines. Killed virus vaccines are usually prepared of tissues and blood of pigs inoculated with virulent HCV. When administered subcutaneously, the safety is excellent, but unfortunately the post-vaccinal immunity is usually mediocre (4,5). Immune response is delayed and weak, and lasts only a few months, even after several booster injections (6). Efficacy of inactivated virus vaccines was greatly affected by maternal passive antibody. Vaccinated animals may exhibit good clinical resistance to

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challenge with virulent virus, but it is not certain if the induced immunity protects them effectively against infection and prevents persistence of the virus in the host. It is known that sows vaccinated with inactivated vaccine and challenged during pregnancy with a fully virulent HCV, withstand the disease well. However, the virulent virus is found regularly in the foetuses, and various disorders and malformations are observed in the newborn piglets born to these sows (7,8). These features are epidemiologically important because they indicate that the vaccinated sow, though resistant to the disease, is still susceptible to the virus infection, and that the virulent virus penetrates, multiplies in maternal tissues and infects the foetus. It may therefore be asserted that the use of inactivated virus vaccine in a country where HC is still active can create conditions favourable for the development of virus carriers. These vaccines were therefore not considered to be effective enough to control HC in many of the countries where there were used.

In countries suffering both from HC and African Swine Fever (ASF), there is a risk of ASF virus contamination of porcine tissues used for preparation of inactivated virus vaccines against HC, especially if ASF virus is resistant to the treatment carried out to inactivate HC virus.

2) Glycidaldehyde (9) and crystal violet (10) were also used in the past for preparing inactivated virus vaccines from HCV prepared in cell cultures.

Preparation of HCV immunogen from photodynamically inactivated virus produced in cell cultures was also reported. But, in spite of these technical improvements, these vaccines did not exhibit an increasing immunogenic activity in pigs, compared with previous inactivated virus vaccines prepared with tissues from infected pigs.

Theoretically, the main advantage of inactivated virus vaccines was judged to be primarily its safety. However, some vaccines were not uniformly inactivated, producing foci of infection by residual virulent virus in vaccinated herds. For these reasons, inactivated vaccines are now rarely used.

2.1.2. Modern inactivated virus vaccines. Since previous inactivated virus vaccines were of no great value for the control of HC, a new type of vaccine was proposed (11). It was based on disruption of HCV infected cell cultures with a detergent (Triton X-100). Detergent split HCV in Freund's incomplete

adjuvant, or in a saponin (Quil A) solution protected pigs against a virulent challenge. A heterologous detergent split vaccine was also prepared with bovine viral diarrhea virus (BVDV) but the protection against HCV challenge was only partial. From a practical point of view, the most interesting feature of this new vaccine is that the application of only 100 ul of HCV antigen given as two injections resulted in complete protection of pigs. This modern approach could be considered as the first step with a new synthetic vaccine based upon use of a protective viral glycoprotein associated with an efficient adjuvant of immunity.

2.2. Live virus vaccines

2.2.1. Homotypic live virus vaccine. Attenuation of HCV by serial passages in rabbits (12, 13) was the starting point of all research done on vaccination using various attenuated strains of HCV. Since then, several methods to modify and to attenuate HCV were proposed: serial passages in rabbits or in cell cultures were carried out by different laboratories. Attenuated strains prepared according to these methods were widely used as live vaccines for active immunization of pigs against HC in the United States and in some other countries (14). They induce high levels of immune protection: immunity usually develops within 4-7 days and lasts several years. But the degree of attenuation was not sufficient. Safety and genetic stability were the main problems from a practical point of view (15). After vaccination, pigs manifested hyperthermia and various symptoms. The residual virulence of these live virus vaccines became apparent mainly in young piglets which showed clinical reactions, hoemorrhagic lesions and sometimes mortality (16, 17). Vaccination of susceptible sows with such live virus vaccines with or without anti-HCV serum at 24 to 60 days of pregnancy resulted in reproductive disorders (small litters, mummified foetuses, stillbirths, etc) caused by transplacental virus infection of foetuses 'in utero'. At birth, the newborn piglets which were infected 'in utero' retained the HCV and became foci of infection in a susceptible herd (18,19).

After a few serial passages through susceptible piglets, these strains can recover their virulence. Their low degree of safety is associated with insufficient attenuation and genetic instability of their acquired characteristics due to the heterogeneity of the virus population which is present in the live virus vaccine. The reason for this genetic heterogeneity is that these attenuated strains were not cloned when passaged through rabbits or

tissue culture. Furthermore, nothing is known about their genetic markers so that there is no possibility to distinguish between a low virulent strain derived from a live virus vaccine and a low virulent HCV from the field.

For these reasons, live virus vaccines have acquired a negative reputation especially in the United States. In 1969, vaccination with live virus vaccines was eliminated to facilitate eradication of HC.

2.2.2. Heterotypic live virus vaccines. Because of the antigenic relationships to HCV, BVD virus was proposed for immunizing swine against HC (20,21,22). However, when BVD-immunized pigs were challenged with virulent HCV, protection was only partial (hyperthermia, viremia...). Moreover, BVD virus is able to cross placenta and to infect the foetuses. In the field, there is evidence of breeding problems associated with BVD infection of pregnant sows, causing interference with diagnostic tests for HC (23). Therefore, the BVD virus vaccine was never approved for use in swine.

2.2.3. Modern live virus vaccines. From 1952, in Far East and in Europe, efforts were made to improve the safety of live virus vaccines against HC. In this perspective, two main approaches were carried out with success:

1) By means of very high number of passages in rabbits, HCV acquired an advanced status of lapinization e.g., the "chinese" lapinized strain (LPC strain of Taiwan).

2) Selection of attenuated strains in cell culture at low temperature ("cold" strains) was carried out: the GPE(-) strain in Japan and the Thiverval strain in France.

3. PROPERTIES OF MODERN LIVE VIRUS VACCINES

3.1. The Chinese lapinized strain.

The so-called Chinese lapinized strain (CLS) is also named "C" or "K" (East European countries) or "LPC" (Taiwan). The CLS has acquired an excellent reputation because of its successful use in practice. But there is no criterion based on a specific character or genetic marker for identification of one original CLS. It is not sure if all the CLS virus used in the world at present has the same origin. An interesting overall report published in 1981 (24), covers findings of numerous experiments conducted by researchers in Taiwan about the successful development of a safe and potent live virus vaccine from the chinese "LPC" strain during 1952-1978. According to this

report, the LPC strain derived from a lapinized HCV ("Rovac") had already undergone 250 serial passages in rabbits in the United States and was again serially passaged through rabbits in Taiwan for more than 800 generations. This "highly" lapinized LPC strain is suspected to be derived from the original CLS which is now widely used as live virus vaccine on a large scale in the control of HC in Taiwan and also in many other countries including Europe. Numerous papers brought evidence that CLS proved to be highly safe for pregnant sows and newborn piglets, as well as being effective (25, 26, 27, 28, 29, 30, 31). The high degree of safety was proven by the good genetic stability of the CLS which was unable to regain its virulence even after 20-30 serial back passages in 6-8 week-old pigs (32,33). Immunity was established 3-4 days after vaccination and lasted for at least 18 months.

Only a few reports deal with the 'in vitro' properties because CLS is not easily workable in cell culture (34). Using immunofluorescence techniques, after virus inoculation of PK(15) cells, the CLS usually induced a very low and delayed specific immunofluorescence. Even after several passages in susceptible cell cultures in optimal conditions, the immunofluorescence remained weak and the infectivity titer of the CLS was constantly low (10^5plaque-forming units/ml). Immunofluorescent microplaques developed at 32-41°C, as compared to 36-43°C for virulent HC strains. Tested for its thermostability, the CLS is moderately less resistant to 56°C, than virulent HC strains. 'In vitro', the CLS can be characterized by slow growth (34,35). The relationship between these peculiar properties and absence of any virulence for pigs is noteworthy. But, on the other hand, the 'in vitro' properties of the CLS are similar to those of some low virulent HCV strains isolated in herds having reproductive failures (36).

The CLS was subsequently adapted to lamb kidney cell cultures. From tissue culture fluids a live virus vaccine was prepared and used successfully in France and in other countries (31).

3.2. The GPE(-) strain.

This attenuated strain was developed by Sasahara in Japan (37,38). The virulent ALD strain of HCV was subjected to a selection process by means of serial passages at low temperature (30°C) in three different cell culture systems including swine, bovine and guinea pig cells, and alternated with virus cloning. As a result, a clone was isolated and designated GPE(-). As compared with the Chinese lapinized strain, the GPE(-) strain is easily

workable because virus growth and infectivity titration in cell cultures are carried out without difficulty. As compared with the virulent ALD strain, GPE(-) clone was characterized by different 'in vitro' properties (genetic markers) which are good criteria for strain identification (39).

T marker: When the GPE(-) strain is inoculated into guinea pig kidney cell cultures and incubated either at 30°C or at 40°C, the virus titer of culture fluid is higher at 30°C than at 40°C. In contrast for the virulent ALD strain, virus titer is higher at 40°C than 30°C.

G-marker: When the GPE(-) and virulent ALD strains are inoculated into guinea pig kidney cell cultures respectively, and incubated at 30°C, the differences in virus titer of culture fluid of GPE(-) and ALD strains are 10^2 or greater.

E-marker: When the GPE(-) strain is inoculated into swine testicular cell culture and when the culture is challenged by Newcastle disease virus (NDV) the GPE(-) strain exhibits an infectivity reduction of about 3-4 $\log_{10}$. In contrast, virulent ALD and Alfort HC strains are only slightly affected (1.5 $\log_{10}$ reduction) (35). Numerous experiments brought evidence that GPE(-) strain is highly safe for pregnant sows and newborn piglets, and effective in control of HC in Japan (39). No virulence recrudescence was found when the GPE(-) strain was passed for 20 serial back passages in piglets (40). After vaccination, GPE(-) strain was detected consistently in tonsils, and also recovered from feces and urine within 10 days post-injection (41). Immune protection was established 3 days after vaccination, but neutralizing antibody started to increase 2-3 weeks after vaccination and persisted for more than two years (38).

3.3. The Thiverval strain.

The Thiverval strain is a clone which was isolated from the virulent Alfort strain in PK(15) cell culture after more than 170 serial passages at 29°-30°C, including 65 passages performed using the limiting dilution method for virus cloning (42,43). As the GPE strain, the Thiverval strain is easily workable because virus multiplies without difficulty in various cell culture systems including pig, calf and lamb cells. Infectivity titration is carried out easily in PK(15) cells using the immunofluorescence microplaque technique. As compared with the virulent Alfort strain, the Thiverval strain is characterized by several genetic markers connected with attenuation for the pigs (43).

The optimal temperature for maximum virus yield in cell culture under one-step-growth-cycle condition, is 33°-34°C for the Thiverval strain, 39°-40°C for the virulent Alfort strain.

The supra optimal temperature ("rt") for virus growth in a one-step-growth-cycle which causes a reduction of 90 % in virus yield is 39°-39.5°C for the Thiverval strain and 42.5°C for the virulent Alfort strain.

Following incubation at 56°C during 30 minutes, the strain exhibits an infectivity reduction of about 3-4 $\log_{10}$. In contrast, the virulent HCV strains are only slightly affected (1.5 $\log_{10}$ reduction) and therefore relatively thermostable.

'In vivo' experiments (44) brought evidence that the Thiverval strain proved highly safe for pregnant sows and newborn piglets, and effective in control of HC in France and other countries where used. Genetic stability of attenuation was demonstrated by reverse back passages in cell culture at supra-optimal temperature (37°C) and by serial passages in piglets. Neutralizing antibodies are detectable at 7 days post-vaccination. The maximum antibody level in serum is observed one month after vaccination and persists constantly for several years (45).

Considering the findings about viral properties, these three attenuated strains and especially GPE(-) and Thiverval strains, are composed of cloned virus which may be characterized as "cold" and "fragile". In contrast, virulent HC strains may be characterized as "hot" and "solid" virus.

These three attenuated strains are considered at present as really safe especially for pregnant sows and newborn piglets. They proved genetically stable even after serial back passages in cell cultures and in susceptible piglets. The genetic stability of these attenuated strains may be explained in that. The risk of reversion to virulence is probably very low because of the existence of two apparently independant mutants, one "cold" and the other one "fragile". The "cold" character may control the level of intracellular virus multiplication. On the other hand, the "fragile" character may control the diffusion and the survival of the vaccine virus outside the host cell. However, there is no unanimity in the literature about spreading of vaccine virus from vaccinated pigs to susceptible contact pigs. But under field conditions, there is evidence of horizontal transmission of vaccinal virus of each these three attenuated HC strains in a limited percentage of pigs (24, 30, 39, 40, 44).

No adverse effects were observed in vaccinated pigs which were treated

with an immunosupressive drug indicating that the live virus vaccine has no adverse effects under cortisone treatment (34, 44, 46).

In contrast to the CLS, the GPE(-) and Thiverval strains have been cloned and especially draw profit from the establishment of several genetic markers in cell culture which are good tools for strains identification. It is perhaps the main reason why Thiverval and GPE(-) strains can be considered as the new generation of modern live virus vaccines as compared to the CLS.

4. IMMUNITY INDUCED BY MODERN LIVE VIRUS VACCINES

4.1. Post-vaccinal immune response in absence of passive immunity.

4.1.1. Systemic immunity. Whatever the strain, modern live virus vaccines have in common their excellent immunogenicity (38, 44, 47). We shall describe immunity elicited by these vaccines: the immune response following a single intramuscular injection of vaccine, measured by resistance to virulent challenge or by the antibody kinetic, is quick, intense and long lasting (24, 39, 40). Following vaccination, animals become resistant to virulent virus infection starting on the 5th day and neutralizing antibodies are detectable in serum from the 7th to 10th day. The antibody reaches its maximum level at the end of a month and maintains the same high level throughout the economic life of pig (more than three years in breeding animals vaccinated with Thiverval strain) (45). There is a close relationship between the presence of neutralizing antibodies in serum and protection against virulent challenge but not the reverse. Therefore booster vaccination turned out to be unnecessary because of the duration of the immunity conferred by a single injection.

4.1.2. Local immunity. The main portal of entry for HCV being the nasopharynx, induction of a post-vaccinal immune response in the upper respiratory tract can be expected to result in an early shut-off of virus infection. Kinetics of neutralizing antibody in serum and in buccopharyngeal secretions were studied in pigs vaccinated with the Thiverval strain (48). Whatever the vaccination route (intranasal or intramuscular) a good systemic and local immune response was induced and pigs resisted the virulent challenge. However, the highest local immune response was obtained by intranasal vaccination with large vaccine dosage (10^7p.f.u./pig). After the virulent challenge using the intranasal route, no clear anamnestic

immune response was detected in bucco-pharyngeal secretions. Even when pigs were vaccinated by intramuscular route (using the Thiverval strain), a neutralizing antibody response occurred in the lung secretions collected by means of a lung washing process (49). Administration of vaccine by aerosol resulted in immunity of 50% of pigs. Oral and conjunctival routes failed to induce immunity (50).

4.1.3. Cell-mediated immunity. Antibody-mediated immunity is suspected to play a major role in the protection of pigs against HC, because the passive transfer of sufficient amount of immune serum provided good protection against virulent HC infection. On the other hand, the role of cellular-mediated immunity in protection was not well documented. Pigs infected with virulent HCV developed a depression of responsiveness of blood and spleen lymphocytes to anti-Ig (a polyclonal B lymphocytes activator) which persists till death (51). In contrast, pigs vaccinated with CLS did not show such depression of the anti-Ig reactivity of lymphocytes. After vaccination with the Thiverval strain, or with CLS, pigs exhibited a strong neutralizing antibody response in serum but a cellular immune response to HCV antigens as measured by the lymphocyte stimulation test was not detectable (52). Virulent and attenuated HCV replicated 'in vitro' in stimulated lymphocytes, in endothelial cells and also in epithelial cells without cytopathic changes and without induction of specific antigens in the plasma membrane of the host cell (51). This property may imply that HCV in infected cells is not sensitive to cytotoxic mechanisms, and also may account for the failure to demonstrate a cell-mediated immune response to HCV in infected or vaccinated pigs.

Virulent HCV infection induces a permanent alteration in recirculation of lymphocytes resulting in persisting blood leucopenia (53). Absence of leucopenia after vaccination with modern live virus vaccines may imply that attenuated HCV strains do not disturb the lymphocyte traffic (51).

4.1.4. Passive immunity conferred on the piglet by colostral antibodies. After uptake of colostrum piglets become resistant to virulent HC infection. The level of passive protection conferred by the maternal immunity depends mainly on the concentration of passive antibodies in serum of piglets at the time of virulent challenge. Experiments with the Thiverval strain (54) and CLS (55, 56, 57, 24) brought evidence that a minimum colostral antibody

level is required to assure passive protection. In other words, piglets are passively protected if their serum has a certain minimum level of colostral neutralizing antibodies which is called "passive protection threshold". When born from immunized sows, piglets are passively protected against a virulent challenge till 7-8 weeks after birth. After this period, serum antibody concentration reached a level lower than the passive protection threshold and piglets became susceptible. But colostral antibodies were still detectable in piglet serum when 90 days old (54).

There is also evidence that duration of passive protection and level of passively acquired immunity depend on the conditions of sow vaccination, especially on the interval between vaccination and farrowing (54,55). It was shown that the passive protection is all the more efficient as this interval is longer. Furthermore, according to length of interval between vaccination and farrowing time, differences were observed in three biological properties of colostral antibodies present in the piglet serum: intensity of suppressive activity on active immune response following vaccination, 'in vitro' avidity for HCV, and mean value of half-life (55).

In piglets having high levels of colostral immunity, antibody was not detectable in bucco-pharyngeal and lung secretions suggesting that transudation of antibody from serum to local secretions does not occur (49,58).

4.2. Active immunization of the piglet in presence of colostral passive immunity.

Antibody-mediated immune suppression occurs when young piglets with naturally or artificially acquired passive antibodies are vaccinated with live virus vaccines. Several approaches have been tested in the past to overcome the passive antibody inhibitory effect on active immunization:

- piglets were successfully vaccinated just after birth and before first colostral absorption. Unfortunately, from the practical point of view, this method could not be easily applied under field conditions.
- piglets were vaccinated successfully with one hundred times the normal vaccine dosage. Active immunity was induced in piglets still protected by passive maternal immunity (54). Again, because of economic reasons, this method was not applied because it is too expensive.

Extensive studies have been performed with modern live virus vaccines. Vaccination of piglets in the presence of maternal antibodies resulted in a variable immune status depending on the concentration of passive antibody

at the time of vaccination.

In the presence of high concentrations of passive antibodies, piglets are not immunologically stimulated: no primary antibody production occurs and the piglets are subsequently susceptible to challenge. In the presence of moderate concentrations of passive antibodies, piglets are immunologically "primed" ; that is, above a certain level of maternal antibodies, vaccination does not induce the production of detectable antibodies but merely "sensitizes" the immune system so that, in case of challenge carried out after complete disappearance of maternal antibodies, pigs may be protected by a rapid secondary antibody response. Experiments brought evidence that it is not necessary to await complete disappearance of maternal antibodies to undertake vaccination in piglets born from immunized sows (54). Vaccination with CLS or with Thiverval strain is effective when serum neutralizing antibody of colostral origin is below a certain level. This value, the "efficient vaccination threshold" is observed when piglets are 30-35 days old. Consequently it is possible to vaccinate successfully 30-35 day old piglets still passively protected by maternal immunity (54, 57).

5. USE OF MODERN LIVE VIRUS VACCINES

When born from non-immune sows, piglets can be vaccinated during the first week of life. When born from immune sows, piglets can be successfully vaccinated 30-35 days after birth, while they are still protected by maternal immunity. A booster injection given to future breeders when 5-6 months old, reinforces the protective immunity which will cover all their economic life.

6. VACCINATION FAILURES

Under experimental conditions, 100% of vaccinated pigs are protected against challenge. In the field, when vaccination is correctly carried out with a well-tested vaccine, failures are rare. However, when vaccination failures occurred, post-vaccinal immunity was not induced because of the following reasons:

1/ the vaccine bottle did not contain live virus,

2/ the bottle contained live virus vaccine, but the virus was inactivated on account of incorrect preservation (heating, freezing...),

3/ the bottle contained live virus vaccine, but the virus became inactivated in the syringe which was sterilized with disinfectant just before use,

4/ normal dosage of live virus vaccine is correctly given to piglets but the viral infectivity is neutralized in the body by residual colostral antibodies.

Vaccination failures can be avoided by vigorous checking of vaccine quality and by appropriate education of staff charged with vaccination in the field.

7. APPRAISAL OF VACCINATION : Epizootiological and economic impact.

Should one vaccinate ? If so, under what conditions ? What is the short and long term impact ?

- If HC is rare in a given country or region, vaccination is unnecessary. However, the diagnostic laboratory involved in serological surveys must pay attention to make sure that the pig population exhibiting no positive serological reactions is really free of HC.

- If HC is prevalent, vaccination should be carried out under the following conditions: if the disease is so prevalent that the laboratory has difficulty in diagnosing the subclinical form of infection in breeding animals, health measures, particularly a "stamping out" policy will prove both expensive and ineffectual. They eliminate only the few foci of acute infection identified by the laboratory, while unidentified ones persist and tend to spread. For both economic and scientific reasons, live virus vaccines with no residual pathogenicity must be used. Stockmen should be able to rely on vaccination because in the short run it is the only method they can easily afford which can prevent the disease and thus avoid heavy economic losses. Regular vaccination provides the pig population with a protective umbrella of immunity that effectively checks the invasion of herds by pathogenic viruses of low virulence introduced through the purchase and sale of animals. Vaccination is found to be followed by a decline in the frequency of breeding disturbances in large suspected herds in which the presence of a low virulent HCV has been detected by serological tests (Aynaud, unpublished data).

The size of the herd is an important parameter because the appearance of a focus of HC infection in a herd of several hundred, or even several thousand, inflicts enormous losses compared with those caused by a focus of infection in a small family-type operation.

Because of the length of the economic life of a sow, her frequent role as a virus carrier and the passive protection provided for 40 to 60 days in

piglets following ingestion of immune colostrum, breeding animals should be the first to be vaccinated regularly.

Protection of breeding animals in a pig population by immunization with the live virus vaccine offers the following advantages :

- low cost of administration because it is administered to 10 to 15 percent of the total pig population, and only once in the economic life of the sow.
- solid and lasting immunity from a single vaccination.
- elimination of virus carriers and reduction of losses from reproductive disorders caused by chronic or subclinical HC.
- passive protection by the colostrum of piglets intended for fattening for 40 to 60 days, or one fourth to one third of their economic life.

Effective and harmless, the recently developed Chinese, GPE^- and Thiverval strains of live virus vaccines are being increasingly and successfully used in the field. Their use will reduce economic losses due to HC and in the short run will restore the normal productivity of sows in herds in which mildly virulent strains responsible for asymptomatic or subclinical forms of the disease insidiously spread. At a more practical level, new cell culture vaccines (the GPE^- and Thiverval strains) are relatively easy and inexpensive to produce and titrate.

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9

VACCINES

P. BIRONT, J.LEUNEN

1. INTRODUCTION

It is not surprising that a large number of vaccines have been developed against such an economically important disease as classical swine fever (CSF). The production methods as well as the properties and quality of these vaccines vary considerably. Some vaccines have become very useful instruments for fighting CSF. Other vaccines have been taken out of production for various reasons : pathogenic character of the virus strain, ineffectiveness, excessive expense, out-of-date technology, impractical to use.

The demands made on a hog cholera virus (HCV) vaccine rise with the development of production techniques and increase in knowledge about the disease and the causative HCV. The purpose of this chapter is not to give a detailed historical review of the origin, production techniques and properties of all CSF vaccines which have ever been put to practical use. Moreover, technical details are often regarded as manufacturing secrets. Therefore, this chapter will deal with only general production principles of present day HCV vaccines. There follows a discussion of the properties which these vaccines should possess according to current scientific standards. The European Pharmacopeia will be used to illustrate some legislative requirements on HCV vaccines.

What the ideal vaccine should look like is reflected by a list which summarizes a whole series of properties, many of which still appear utopian but nevertheless are being aimed at.

2. PRODUCTION OF HCV VACCINES

In order to produce a vaccine, the first thing needed is a suitable virus strain. For inactivated vaccines, the choice can be made quickly. Nothing prevents the use of a virulent strain. However, for live attenuated vaccines the situation is different. In this case, a virus strain

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is required which, on the one hand is as attenuated as possible, while on the other hand retaining most of the original antigenic properties. When using heterologous living viral vaccines, at first glance the problem of a suitable virus strain is not so great since it is assumed that the virus concerned as in this case bovine viral diarrhea virus (BVDV), has the advantage of being non-pathogenic for pigs. Due to the antigenic relationship with the HCV, protection against swine fever can be induced. The assumption that BVD strains are apathogenic for pigs may need to be amended, as will be shown elsewere.

2.1. Inactivated vaccines

Formerly inactivated vaccines were used a great deal. Usually pigs were infected with a virulent virus and the spleen, lymph nodes and blood from these animals were then used as a virus source for the vaccine, either directly or after one passage on pig kidney cells. The HCV was inactivated with one of the following agents : toluidine blue, hydroxylamine, ethylene oxide, β-propriolactone or formalin (1, 2). Some of these vaccines contained aluminium hydroxide as immunity adjuvant. These vaccines are no longer used because :

- the large volume of vaccine to be injected (sometimes up to 20 ml),
- the necessity of injecting twice at a 2 to 4 weeks interval for the first vaccination,
- the slow development (2-3 weeks) and the short duration of the immunity,
- the cost of the vaccines due to the virus origin and the necessity of having high virus titers,
- the difficulties to obtain uniform inactivation,

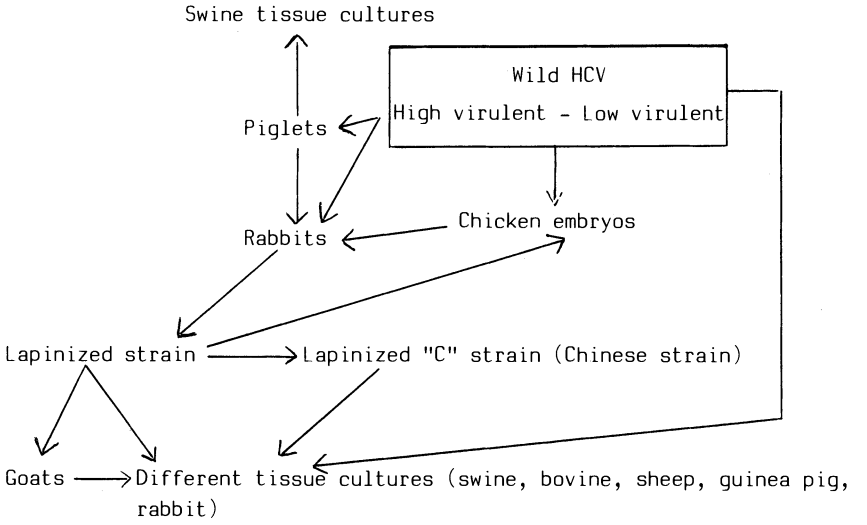
The last (experimental) inactivated vaccines dated from the 1970's. In this case, a virulent virus was grown on PK(15) cells. The virus was concentrated using an ultrafiltration technique (final virus concentration : $10.^{7.5}$ - 10^{8} PFU/ml). Inactivation was effected with glycidaldehyde, an alkylating agent, and an oil adjuvant was added. The dose which had to be used varied between 2.5 ml and 5 ml. Unfortunately, the other disadvantages specified above still applied to this vaccine (3).

2.2. Live attenuated vaccines

2.2.1. Homologous vaccines

In order to avoid the disadvantages of inactivated vaccines and

thanks to the development of new virological techniques, production of live attenuated vaccines has begun. The development of an attenuated vaccine strain has sometimes occurred via many roundabout ways, as the diagram below shows (4):



The virus strains obtained at almost every step in the diagram were tested for use as a vaccine and, to a greater or lesser extent, put to practical use. The diagram also shows that a distinction is made between a "lapinized strain" and a "lapinized "C" strain". Both strains are the final product of several passages in rabbits. However, some lapinized strains lost all virulence for pigs of any age (including pregnant sows) (5, 6, 7). This, then, is also the definition of what will be referred to in the text as "C"-strain. There are a large number of virus strains which can fall under the heading "C" virus without it being possible to check precisely whether or not they have the same origin. It seems almost certain that the virus was finally attenuated in the People's Republic of China and then further cloned in Europe and eventually adjusted to cell cultures (8).

The development of a homologous attenuated HCV vaccine often occurred as follows:

An initial modification of the virus strain was obtained by alternating rabbit-pig passage until the virus was altered in such a way that further passages could only be carried out in rabbits (9) e.g. lapinized strains,

lapinized "C" strains. Usually, the rabbits were slaughtered 3 days and the pigs 5 days after inoculation. One of the first trials for the transmission of the wild virus to rabbits were probably made by Vechiu (Bucarest) (10).

Some strains have been attenuated using the continuous cell virus propagation technique : after incubation with the virus, the culture medium was renewed every 3 to 7 days. In this manner, the virus culture was maintained until cells declined in activity (11) : e.g. PSC_2 strain.

A further cloning then followed using the limiting dilution method, e.g. GPE (-) strain, Thiverval, CR20, CL, PSC_2 strain, and/or by passages on cell cultures carried out at low temperatures, e.g. GPE (-) strain, Thiverval strain (12, 13, 14).

Several examples of the number of passages the field virus underwent before being used as attenuated vaccine virus are presented below:

- "C" strain:

  different wild strains (People's Republic of China) ⟶ 300-478 passages in rabbits or Rovac strain (USA) (lapinized strain not harmless for suckling and pregnant sows) ⟶ x* passages in rabbits (6, 7, 8)

- CR20 strain:

  "C" strain (received from Dr Bognar, Hungary : no longer pathogenic) ⟶ 20 passages in rabbits.

  Used by Smith Kline / RIT (C R 20 = Chinese strain R.I.T.)

- C4 (strain Leunen):

  "C" strain (Pasteur Institute, Bucarest; no longer pathogenic) ⟶ 4 passages in rabbits.

  Used by Syntex Agribusiness, Hoechst, some state laboratories in South America.

- IFFA strains:
  - IFFA/A/22 : SFA strain (wild virus)
    ⟶ 525 passages in rabbits, then
    ⟶ 22 passages in rabbit kidney cells (15)
  - IFFA/A/49 : IFFA/A/22
    ⟶ 49 passages in lamb kidney cells (16)
  - CL-strain:C strain (received from Dr Bognar)
    ⟶ 17 passages lamb kidney cells. Used by Rhône-Mérieux (17)

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* x = number of passages unknow, unclear or contradictory

(CL = Chinese strain Lyon)

- Thiverval strain:SFA strain ——> more than 170 passages swine testicle cells (18) (used by Cogla, Sanofi)
- G.P.E.(-)strain :
  ALD-strain (wild virus)
  ——> 142 passages in swine testicle cells,
  ——> 36 passages bovine testicle cells,
  ——> 41 passages guinea pig kidney cells (13, 14)
- LPC-strain (Taiwan):
  wild virus ——> x passages rabbits (Philippines strain or Rovac strain ? still pathogenic) ——> 800 passages rabbits (19)
- PAV1-strain :
  virulent virus ——> 29 passages porcine kidney cells (20)

2.2.2. Heterologous vaccines

Heterologous live virus vaccines were never developed to the same extent as homologous live attenuated vaccines. Various BVDV strains were used (NY, Oregon, C24V, VJ6M, TOB). Originally, these vaccines were made using spleens of infected calves. Then production on cell cultures was started (bovine kidney). Most of these vaccines were first developed to vaccinate cows (21, 22).

2.2.3. Present day vaccines

The HCV vaccines still produced on an industrial scale (commercially or locally by the states) are the following:

- production in cell cultures:
  - GPE(-)strain(Asia, Central America)
  - CL strain (Europe, Asia, South America)
  - PAV strains (Central America) = PAV 250
- production in rabbits:
  - "C" strains (Asia, Central and South America, Europe):
    CR20, C4 and others ?
  - LPC strain (Asia)

As will be shown later in the chapter on safety, it is important that the vaccine virus which is incorporated into the final vaccine differ as little as possible or preferably not at all from the original

"master seed virus". However, mass production unavoidably means that first a number of virus passages must be carried out before enough virus is available. By using the seed batch system, the number of passages is kept to an absolute minimum. Between the master seed and the seed batch, or working seed, there are 1 or 2 passages. Usually 2 more passages are added to this before the final product is obtained. With 30 ml seed virus, a manufacturer can produce approximately 500,000 doses (GPE(-)vaccine) (14). Vaccines produced on cell cultures contain 10^2-10^4 PFU or 10^3 $TCID_{50}$/ dose. This virus titer can be calculated using the direct immunofluorescence technique on suitable cell cultures.

The titer of the vaccines produced in rabbits can only be calculated on rabbits or pigs (see section "efficacy"). The production methods are also quite different from those of the cell culture vaccines. Rabbits are injected intravenously with the "C"-virus. After a number of days, these rabbits are slaughtered. The organs which contain most virus are homogenized, if necessary purified and finally lyophilized. The time at which the rabbits are slaughtered and the organs (spleen, liver, blood, lymph nodes) finally used vary from manufacturer to manufacturer. After the injection, the virus can be found in the blood from the 3rd day onwards. Spleen and liver contain virus between the 3rd and 7th day. From the 12th day onwards, no more virus is to be found. The amount of virus injected does not have a very great effect on the final virus concentrations in the organs (23). With one ml of the original master seed virus, 25 million doses can be produced. Normally one rabbit is equivalent to 2,500 doses. Some manufacturers calculate the number of doses that can be made from one rabbit by the weight of the infected organs (x gr infected organs = y doses).

The lyophilisation of the live attenuated vaccines must be carried out in such a way that the properties of the vaccines are maintained for a certain length of time. One stipulation often made is that the quality remains more or less unchanged after the lyophilized vaccine has been stored for 1 week at 37°C. In ideal circumstances (low temperature and darkness), the lyophilized vaccine can be kept for 2 years.

3. PROPERTIES OF HCV VACCINES

A vaccine must meet various requirements concerning safety and efficacy. These properties are measured by certain tests. If different

vaccines are to be compared then it is necessary to standardize these tests. However, it is not sufficient to aim only at the uniformity of these tests: the final aim should be to obtain a standardized vaccine. If CSF is to be stopped and eventually eradicated, the properties of the vaccine must be known. When all the vaccines used have the same qualities, then the epidemiological situation in vaccinated areas is easier to compare, at least if the same vaccination program is used.

An evolution can be noted in relation to the required safety and efficacy. When the first HCV vaccines were developed, most of the users were satisfied when inoculated pigs survived a challenge with a virulent virus. Now it is no longer enough to protect animals 100%, the vaccination should also prevent the occurrence of virus carriers.

Since inactivated vaccines are no longer used, the following only concerns live attenuated vaccines.

3.1. Safety

3.1.1. Specific safety. In no case should vaccine virus itself cause any damage. Certain vaccine strains possess a residual pathogenic effect. In order to detect this residual virulence, the following tests must be carried out :

- Inoculation of animals of various ages.

Some strains still cause clinical symptoms in pigs and even to such an extent that they cannot be used without hyperimmune serum (sero-vaccination). Other strains which are already more attenuated are no longer pathogenic for adult animals and piglets but they are still pathogenic for embryos and fetuses. Some cell culture viruses as well as some rabbit-adapted viruses can indeed cause intrauterine infections (24, 25, 26), but other vaccine viruses seem completely safe for pregnant sows and therefore appear to have lost all virulence for the animal type concerned:C4, CL, CR20, Thiverval, GPE(-)strain (14, 26, 27, 28, 29, 30).

So it is evident that, when testing the specific safety, extra attention must be paid to the effect of the virus on the fetus. In practice, this is done as follows:ten pregnant, non immune sows each receive a double dose of vaccine in one injection, between the 25th and 35th day of gestation. Pregnant non-immune sows of the same age and origin receive a placebo (physiological saline solution). The

vaccine virus should not interfer with gestation or be harmful to the piglets (European Pharmacopeia). It should be added that the piglets must be observed for several weeks, not only clinically but also virologically, to check whether we are dealing with "persistently infected piglets" which will only develop symptoms when they are older (31).

- Inoculation of 10 doses of vaccine per animal at one time. After inoculation, the animals are clinically observed for 21 days. Their temperature should remain normal; the animals should show normal growth (European Pharmacopeia). With regard to the GPE(-)vaccine:in this case, even 100 doses are inoculated per animal. The inoculated animals are then observed for 14 days (14).
- Inoculation of animals after treatment with immunosuppressive drug. Virus propagation is probably indirectly stimulated by e.g. costicosteroids because phagocytosis and certain inflammation mechanisms are slowed down (7). The residual pathogenic effect may be revealed more easily if the virus is able to develop well in the organism. In fact, the following can be done: 12 serologically negative piglets weighing about 20 kg are divided into two groups of 5 piglets. The remaining two animals are the non-vaccinated controls. The piglets from one group receive an injection of prednisolone (2 - 5 mg/kg) on 5 or 6 consecutive days. The third day after treatment has started, the two groups are inoculated with one dose of the vaccine. All animals are clinically observed for 21 days (European Pharmacopeia).

  Some publications even refer to a challenge of vaccinated animals treated with cortisone. In these cases, the corticosteroid treatment did not appear to affect the development of immunity (32).

  Although the cortisone test is a generally accepted test, it should still be interpreted with the necessary caution. Corticosteroids can stimulate certain latent infections and possible symptoms of illness, not necessarily caused by the vaccine virus (32). The Thiverval, GPE (-), CR20, C4 and CL strain pass the cortisone test (27, 32, 33, 34, 35, 36).
- Absence of leucopenia in inoculated pigs.

  A characteristic of virus attenuation is the limited development of the vaccine virus in the pig. Therefore, the presence or absence of leucopenia could be a mean of measuring the virulence. This could eventually be combined with a histopathological examination of various

organs of inoculated pigs. For example, the GPE(-)vaccine causes localized focal necrosis in lymphatic tissue (14). It may be assumed that most "C"strains (CL, C4, CR20) and the Thiverval and GPE(-)strains do not cause leucopenia or at least only mildly.

If it is necessary to compare the various vaccines for the absence of leucopenia, then it is important that the same virus titer be used. Low virus concentrations might not result in leucopenia (35).

3.1.2.Non-specific safety. The vaccination may not cause any adverse secondary reactions which would hamper normal breeding and fattening : reduced appetite, poor growth rate, local or general reactions. In general, vaccines based on the CL, CR20, C4, Thiverval or GPE(-)strains satisfy this point (28, 37). However, it is always possible that animals show certain reactions after vaccination and among these, the allergic reactions are the most common. The allergens, as well as the type of antibodies involved (IgM, IgG, IgE) possibly vary so that the symptoms and the time of appearance are not always the same. When using attenuated HCV vaccines, more specifically those produced in rabbits, serious anaphylactic reactions have been described. The phenomon occurs in piglets inoculated for the first time. Two to fifteen minutes after inoculation, the piglets become cyanotic, show abdominal respiration, begin to vomit and cough. The mortality can reach 100%. This phenomenon is possibly caused by the reaction of passively transferred antibodies with an allergen present in the vaccine. These allergens are supposed to be certain rabbit proteins and the antibodies in question are built up by the sow after (repeated) vaccination (38, 39). The only way to avoid this reaction is either to inoculate the piglets when they get older after passive immunity decreases or to use a vaccine which contains either no or as few rabbit proteins as possible. On the whole, vaccines grown on cell cultures contain less non-viral proteins:rabbit-grown "C" strain: 3.8 - 5.9 mg/protein/dose; CL strain grown on cell cultures: 0.3 mg protein/dose.

3.1.3.Genetic stability. The safety of attenuated vaccines not only includes the problem of specific and non-specific safety, but also the question of whether the attenuation of the vaccine virus is genetically stable.

A live attenuated virus vaccine can regain its virulence via the same mechanism by which it was attenuated i.e. cloning of mutants. If, consequently, the vaccine virus is capable of spreading from a vaccinated to a non-vaccinated animal, a selection of more virulent clones - by means of several passages in pigs - can take place. Whether this occurs or not, depends on the degree of attenuation and on the degree of homogenicity of the vaccine virus.

If pigs are inoculated with the CL, C4, CR20, GPE(-) and Thiverval strain under laboratory conditions and are kept together with non-inoculated pigs for a certain period of time, there is no serological evidence for transfer of vaccine virus (14, 28). However, und normal working conditions, "C" virus spreading has been noticed (40, 41). It is worth mentioning in this connection that the GPE(-)virus can spread more easily from vaccinated animals with respiratory problems to non-vaccinated animals (14).

Perhaps the best way of checking how many passages a vaccine virus can undergo in pigs, is as follows: pigs are slaughtered during the first week after vaccination. A suspension of blood, lymph nodes and spleen from these animals are injected together or separately into other pigs (1st passage). To check whether the vaccine virus can be found in the pigs infected in this way, various techniques must be applied, according to the vaccine virus used:

- inoculation of the organ suspension into cell culture for vaccine viruses grown on cell cultures,
- inoculation of rabbits with an organ suspension for "C" virus grown on rabbits and for the CL strain,
- inoculation of pigs with an organ suspension followed by a challenge and/or serological examination; if the virus is present it will result in immunity (can be used for all vaccine strains).

In order to check the absence of vaccine virus dissemination, the European Pharmacopeia suggests the following : 2 pigs are inoculated each with 1 dose of vaccine. Seven days after inoculation, 5 ml blood is drawn for both animals. This is mixed together and this mixture is injected into 2 new pigs. A total of 6 passages are carried out in this way. All animals should remain clinically healthy. All these tests have two aims : to check how many passages in pigs are possible and also to discover whether a return to virulence is possible. Therefore, some

of the pigs who are inoculated with an organ suspension from vaccinated animals should be clinically observed for at least 10 days.

Obviously, more importance is attached to the return of virulence than to the eventual number of passages possible in pigs. 30 passages can be carried out with the "C" virus from which the C4 and CR20 strain originated (24). The CL strain cannot undergo more than 3 passages in pigs before it is lost (32). Passages in pigs with the Thiverval strain would be impossible if blood taken from animals 5 to 7 days after they are inoculated was used (29). Passages in pigs are possible with the GPE(-)strain. After ten passages, virus provokes a slight fever and leucopenia is detectable. However, even after 20 passages, virulence does not increase any more when tested on SPF animals (Shimizu, personal communication). It must be emphasized that in most cases the regaining of virulence was tested only in piglets and not in pregnant sows.

3.1.4.Contamination with other viruses. Viruses other than HCV can contaminatethe vaccine in various ways. The original master seed virus can be infected. With the complicated procedures sometimes used in order to obtain attenuated vaccine virus, it would not be surprising if the HCV became contaminated with other viruses during all those passages on various cell cultures and/or various animal species.

However, even if the master seed virus is pure, the vaccine production itself can carry considerable risks. The problem already begins with the use of serum necessary for the cell cultures. Foetal calf serum, in particular, is a possible source of BVDV infection. Preliminary treatment with β-propriolactone or γ irradication, reduces the risks.

If virus production is carried out in primary cells, these cells shoud also be examined with particular care. Parvo and adeno viruses as well as BVDV (30, 42) can cause infections if cells from piglets, calves or sheep are used. That means that primary cells must be examined very carefully before production of vaccine virus starts. Often, laboratory animals used for making primary cell cultures are placed in quarantine for at least 1 month before use. If an autopsy reveals pathological lesions, the organs are not used (14). Measures like this are not enough to guarantee that primary cells are free of virus. Therefore, the following virological techniques should be applied: immunofluorescence, haemadsorption with erythrocytes from various animal species, inoculation

of certain cell cultures and susceptive animal species with cell extracts from the cell cultures to be used. The animals can be serologically surveyed to check for possible virus infection.

In order to control whether the final product is free of contaminating viruses, the European Pharmacopeia recommends the following procedure : first, the vaccine is treated with a monospecific immunserum and then it is added to cell cultures. These cultures are then tested for CPE, haemadsorption and haemagglutination (chicken erythrocytes). These techniques cannot be used in order to detect a BVDV infection because some strains do not give a CPE, which necessitates the use of immunofluorescence techniques. Possibly the "monospecific" serum will also neutralize the BVDV to some degree. The European Pharmacopoea also included a mouse test for the detection of a possible rabies virus contamination: ten mice (11 - 15 g) are inoculated intracerebrally with 0.03 ml (1 dose = 1 ml). The animals are observed for 21 days. If more than 2 mice die within 48 hours, the experiment must be repeated. An adverse effect caused by the vaccine may not appear from the 3rd to 21st day.

3.1.5.Markers. This section is included here because for a correct diagnosis it is very important to be able to differentiate between a virulent virus and a vaccine virus. The techniques used to arrive at a diagnosis are, on one hand, detection of antibodies and, on the other hand, identification of antigens or isolation of the virus.

At present, it is still impossible to differentiate between antibodies produced against a vaccine virus and those produced against a virulent virus. It has been established that pigs inoculated with a "C" virus grown in rabbits develop antibodies against rabbit proteins (indirect ELISA). This antibody titer varies considerably from one animal to another and is not detectable for more than 3 months after a single inoculation (43).

As Table 1 shows, it is possible to differentiate between vaccine virus strains and wild virus. CSF vaccine viruses possess certain markers which vary according to the virus strain.

Table 1. Differentiation between vaccine strains and virulent strains

Method / Vaccine strain	D.I.F. pig organs	Virus growth					E.N.D. - test	Multiplication in rabbits
		porcine cells	bovine cells	ovine cells	guinea pig cells	optimal temperature (29°-30°)		
"C" virus rabbit (CR20, C4)	-	-	-	-	N.T.	-	N.T.	+
"C" virus cell cultures (CL)	-	+	+	+	N.T.	-	N.T.	+
Thiverval	+	+	+	+	N.T.	+	N.T.	-
G P E (-)	+	+	+	+	+	+	-	-
Virulent strain	+	+	+	+	+	-	+	-

(N.T. = not tested)

According to our experience, when using the direct immunofluorescence technique (DIF), the "C" virus (CR20, C4, CL strain) cannot be found in the tonsils of piglets vaccinated under laboratory conditions. This problem is not normally encountered in practice either. However, a few cases have been experienced in which CR20 virus was detected in tonsils and kidneys from vaccinated pigs (41, 44). In these cases the virus propagation was limited in time (to 15 days after vaccination) and in localization (a few epithelial cells from the crypt of the tonsil epithelium, a few tubuli cells in the kidneys).

Detection or non-detection of "C" virus in cell cultures and organs from pigs and rabbits using the immunofluorescence technique remains rather confusing. There are not many specific publications on this subject and reports are mutually contradictory. Ressang (personal communication, 1969) concluded that C4 virus cannot be detected in PK(15) cell cultures. However, organs from rabbits injected with this virus were positive between 5 and 8 days after vaccination. The C4 virus could be found by immunofluorescence neither in the tonsils nor the lymphnodes of vaccinated pigs (Korn, personal communication, 1971). Aynaud (personal communication 1969) did not succeed in cultivating the CR20 virus on PK (15) cells; the result was negative up to the second passage, doubtful in the third, and negative in the fourth and fifth passages.

Weak immunofluorescence was observed in PK(15) cells with the Suvac "C" strain. This "C" virus also grows more slowly and causes small plaques, unlike the virulent virus. In addition it grows better at a temperature of 32° C (27). A "C" virus from Budapest (Dr Bognar) and a "C" virus from Peking also gave immunofluorescence in frozen sections of lymphnodes from pigs 5 to 7 days after inoculation (45).

With the present day vaccines (C4 and CR20 strains) frozen sections of organs from infected rabbits remain negative with the direct immunofluorescence test (Bironf unpublished).

These contradictory results may indicate that "C" viruses differ in their behaviour. The FITC conjugates used may also play a role. The serum used for producing the conjugate varies considerably because of other virus strains used for immunization and because of different immunization programs.

There is no doubt that the "C" virus grows in rabbits and that this virus propagation is correlated with an obvious rise in temperature. To check this, it is best to take the temperature twice a day starting 24 hours after inoculation and during the five following days. A 1.5° C increase in temperature should be regarded as fever. If the titer of the inoculated material is too low or if the "C" virus is adapted to cell cultures (e.g. CL-strains), then the fever reaction can be less pronounced. In this case, these rabbits can be challenged with a known "C" virus. If the original material contained "C" virus, these rabbits should not develop a fever after the challenge.

The GPE(-)strain has 3 markers: G, T and E. G marker (guinea pig cells): the difference of virus titer is 10^2 $TCID_{50}$ or more when the vaccine virus and the virulent virus are inoculated into guinea pig cells.

T marker (temperature): when the vaccine virus is inoculated into guinea pig kidney cells the virus titer obtained will be higher at 30°C than at 40°C.

E marker (enhancement): if the vaccine virus is inoculated into swine testicle cells and if the culture is challenged by Newcastle disease virus, the virus shall not enhance the cytopathogenic effect of Newcastle disease virus as with virulent HCV (E.N.D. test) (14).

The Thiverval strain has one important marker: its sensitivity to temperature. The optimal temperature for this virus is 30° - 33°C (for wild virus : 39° - 40°C). In addition, this virus strain, contrary to

the wild virus, can be quickly inactivated at 56°C (12).

Vaccine viruses can therefore be distinguished from virulent viruses. In the future, it will perhaps be possible to make this distinction by using monoclonal antibodies.

3.2. Efficacy of HCV vaccines

The quality of a vaccine is determined by its safety and in addition by its efficacy.

It is no longer sufficient that a CSF vaccine can protect pigs from possible signs of disease. Vaccination should also prevent the animals becoming carriers. In this case, carrier means a vaccinated animal in which the wild virus still can multiply and, spread to other animals without causing any clinical symptom in the vaccinated animal.

The tonsils are regarded as the primary target organs of the swine fever virus after oronasal infection. Propagation of the virus in the tonsils can be responsible for the first spreading of the virus. If, during the incubation period, the virus is not found in the tonsils, then the chance that the infection has taken place is low. This explains the importance which must be attached to the presence of virulent virus in the tonsils of vaccinated animals when assessing efficacy.

The factors which determine the efficacy of the live attenuated vaccine are mainly the virus strain used and the virus titer. The amount of virus present in the vaccine can be titrated but that is not always easy. For "C" virus grown on cell cultures, the latter can be used for titration. However, the amount of "C" virus grown in rabbits must be measured in rabbits; rise in temperature as parameters for virus multiplication. The results of such a titration vary considerably according to the rabbits used. SPF rabbits are more sensitive to the "C" virus than rabbits reared conventionally. The amount of γ-globulins appears to indicate the quality of the reaction. SPF rabbits have an average γ-globulin level of 5.35 %. Approximately the same amount is also found in non-SPF rabbits who are free of pasteurella, bordetella, coccidiosis and ear scabies. Male SPF γ rabbits have a lower γ-globulin level (3.7 %) than female rabbits (6.3 %). Traditionally reared rabbits have a γ-globulin level of 12.8 %. All this means that virus titration should be carried out on standardized animals. In addition, the results obtained in such a test are easy to reproduce (46,47).

HCV vaccines can also be titrated in pigs. A number of pigs are divided into 3 groups. The first two groups each contain at least 5 animals The third group is the control group (at least 3 animals). The first group is inoculated with 1/40 dose, the second group with 1/160 dose. Fourteen days later the animals are challenged intramuscularly with $10^4 ID_{50}$ of a virulent strain and are then clinically observed for 14 days. The animals of the control group die between 4 - 10 days after infection. Of the inoculated animals, the following should be regarded as unprotected: those who die as a result of the challenge and those who show clinical symptoms (the European Pharmacopeia only refers to death).

A number of comments should be made in relation to this titration in pigs. It is very important that the challenge occurs 14 days after vaccination. When challenged later, the difference between a "good" and "bad" vaccine is less obvious. A "bad" vaccine can be a vaccine which contains less virus than normal. Less virus means a delayed but not necessarily poor development of immunity. It is not always easy to interpret clinical symptoms. Some pigs will only display fever after challenge. This fever can last one or several days. Should an animal which has a fever for only one day be regarded as protected or not?

In order to surmount these difficulties, the tonsils of the infected animal can be examined for the presence of virus. There is a good correlation between the presence of viral antigen in the tonsils and the appearance of clinical symptoms. In addition, this is a more objective criterion than the interpretation of symptoms (Biront, unpublished observation).

A titer of 8 protective doses (PD_{50}) should be sufficient to give protection against clinical symptoms. If a vaccine is used which contains 20 PD_{50}, the replication of virulent virus in the tonsils cannot be prevented (development of carriers). The vaccine should contain 100 PD_{50} if one wants to be reasonably certain of preventing these carriers (48, 49).

These results and conclusions were obtained after intramuscular infection experiments, although it would have been more logical to calculate the number of protective doses necessary to prevent carriers by using an oronasal infection. Precise data after oronasal infection are still not available. A vaccine (CL strain) which contained more than 160 PD_{50} was able to prevent virulent virus multiplication in the tonsils of piglets challenged oronasally one week after the vaccination. The

same results were obtained if piglets were vaccinated in the presence of low maternal antibody titers (Biront unpublished observation).

A titer of 100 PD_{50} for "C" virus grown in rabbits would correspond to 400 infectious doses in rabbits. In practice, many vaccines are found which contain far more than 100 DP_{50}. In these cases, rabbits react even to vaccine dilutions of 1600 and more (Desmecht, personal communication).

The European Pharmacopeia requires every vaccine to contain at least 100 DP_{50}. The GPE(-) vaccine contains 1000 PD_{50} (Shimizu, personal communication).

4. CONCLUSIONS

Today there are different C.S.F. live virus vaccines available on the market. As the quality of the different vaccines and batches may vary, an efficient control is necessary. As the knowledge about the virus and the disease is advancing, new vaccines will possibly be developed. They must meet the following requirements recommended by the E.E.C. :

- low cost
- lifelong immunity for fattening pigs after only 1 dose and within a few days
- availability "offthe shelf"
- genetic stability
- no leukopenia
  - no adverse affect on corticosteroid-treated pigs
  - no reproductive wastage
  - no spreading horizontally and vertically
  - no interference with diagnosis
- has adequate genetic, biological and biochemical markers
- prevents occurrence of carriers following subsequent infection

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10

EPIZOOTIOLOGY OF HOG CHOLERA

C. TERPSTRA*

1. INTRODUCTION

The pig appears to be the only domestic animal species, which is naturally infected by the virus of hog cholera (HC). All breeds and ages are considered susceptible, although adults generally stand a better chance to survive the infection. Natural outbreaks of HC also may occur in European wild boar (Sus scrofa ferus).

2. DISTRIBUTION AND ECONOMIC IMPACT

HC has been responsible for heavy losses to the pig husbandry ever since its first appearance in the United States and the subsequent spread of the infection to Europe and the rest of the world. According to the FAO-WHO-OIE Animal Health Yearbook 1984 (1), the disease is present in 47 countries, suspected to be present in one, while vaccinations are continued in 10 countries, where eradication of HC has been achieved. The disease is enzootic in most countries of continental Western Europe, South America and the Far East. The information on the geographical distribution of the disease, however, is not equally valid for all countries, particularly those with extensive systems of pig husbandry and/or lacking the facilities for laboratory confirmation of clinical and pathological diagnosis. For details on the distribution and prevalence of HC in the EEC countries the reader is referred to Chapter 12 of this book.

The economic importance of HC is closely associated with the size of the pig population and the standards of pig farming. The financial losses in countries with an industrialised pig production system may be enormous. For example in the Netherlands, the direct costs of transport and destruction of infected herds, disinfection of premises, indemnities to farmers, vaccination, and identification and registration of pigs in behalf of the control of HC totalled *f* 127 million in 1983 and 1984. Converted to the numbers slaughtered, the loss amounted to *f* 3.8 per pig per annum or 0.9% of the gross slaughter value. The additional, indirect,

* Department of Virology, Central Veterinary Institute, P.O. Box 365, 8200 AJ Lelystad, the Netherlands.

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damage as a result of loss of production on infected farms, standstill of pig movements in affected zones or regions and restrictions on export is difficult to evaluate.

The economic losses in countries with a valuable pig population have stimulated control and eradication schemes. An eradication programme in the United States was started in 1962 and successfully completed in 1976 at a total cost of $140 million (2). Elimination of the disease was achieved in the United Kingdom in 1966 after a 3½ year effort and an investment of £12 million. Cost-benefit analysis of the latter eradication programme indicated a revenue of nearly £4 for every £1 spent (3), whereas Lee (4) calculated a benefit-cost ratio of 12.7 for Taiwan. At present HC is no doubt economically the most important pig disease in the EEC and a common programme of eradication in the member states was started in 1982.

3. TRANSMISSION

3.1. Routes

Under experimental conditions pigs have been infected by the oral, nasal, aerogenic, conjunctival, genital and various parenteral routes (5,6,7,8,9). Most of these routes are likely to occur in one way or another under natural conditions as well. Infected pigs may already shed virus during incubation. In case of infection with a virulent virus strain, high levels of virus are present in the blood and tissues and large amounts are excreted with oral fluid and smaller quantities with urine, faeces, nasal and lacrimal fluids (7,10). Viral excretion continues till death or in case of survival until specific antibodies have developed. On the other hand, postnatal infection with strains of low virulence are characterised by short periods of virus multiplication and excretion, followed by an antibody response. As a consequence, virulent strains of hog cholera virus (HCV) usually spread faster in a herd and induce a higher morbidity than low virulent strains (11,12). Chronically infected pigs shed virus continuously or intermittently till death (13).

3.2. Virus carriers

When pregnant sows are exposed to strains of low or moderate virulence, the infection initially will often go unnoticed, but the virus can be transmitted to the foetuses 'in utero'. Depending on the virulence of the virus strain and the stage of gestation such sows may later on abort or produce mummified, stillborn and/or weak piglets (14,15). Because HCV persists in foetuses and foetal placentae, large amounts of virus may be disseminated at farrowing. Wensvoort and Terpstra (12),

investigating gravid uteri of sows from infected herds, found that this so-called "carrier sow syndrome" may occur in up to 43% of the pregnant sows in one herd. Congenital infections in which the piglets are born "healthy" are from an epidemiological point of view the most insidious of all (16,17). These piglets may be shedding large quantities of virus for 4-6 months without showing signs of disease or developing an immune response (16). Postnatal infections with strains of low or moderate virulence may also induce a carrier state (8, 18). Chronic and persistent infections constitute the major mechanism of virus perpetuation in epizootics caused by strains of low virulence.

3.3. Pig movements

The spread of virus from one herd to another can be effected in various ways. Movement of pigs, incubating the disease or persistently infected, is the most common mode. During 1969, 75% of the 1,481 cases of HC in the USA resulted from either direct movements or associated spread from movements (11). The infection usually originates directly from infected breeding farms. Close contact with virus-excreting pigs at markets or during transport may cause considerable dissemination of the virus. Most feared in this respect are road transports of weaners, collected from different breeding farms, sorted and regrouped at markets, and reloaded for distribution to various fattening farms. Injection of a sedative to facilitate transport will further enhance the efficacy of the virus spread. Such transports, often over long distances, result in many non-traceable contacts. The situation will be aggravated if strains of low virulence are involved. Due to the slow spread the virus introduction may go unnoticed for weeks or even months and upon investigation few animals may prove to be infected (12). Movement of pregnant "carrier sows" will introduce the infection in the receiving herd at the time of farrowing and cause a corresponding or even longer delay in diagnosis.

3.4. Role of pork and pork products

HCV is rather resistant in a protein-rich environment and can survive in pork and pork products beyond processing (19,20). Survival can be prolonged for months or even years when the meat is stored cooled or frozen, respectively (21,22). In this way the virus can be transported over long distances and be introduced in hitherto free areas or countries. Susceptible pigs may contract the disease when contaminated slaughterhouse offal or kitchen leftovers are fed without proper heat treatment. Feeding of virus-containing garbage was responsible for 18% of

the HC outbreaks in the USA in 1972 and for 22% of the cases in 1973 (23).

3.5. Transmission by man

Transmission of HC by man is of great significance in areas with a high density of pigs and pig herds. In this respect, farmers, castrators, inseminators, vaccination teams and veterinarians who can transmit the virus by contaminated instruments and drugs, should be mentioned. Man was held responsible for 40% of the spread in Hessen (Germany) over the years 1971-1974, followed by feeding of swill and slaughter offal (19.7%) (24). Farmers often spread the disease within the herd by treating sick animals or by routine prophylactic measures e.g. iron inoculation of newborn piglets. The common practice in veterinary medicine not to change syringes and needles or discard partly used bottles in between herds, constitutes an enormous risk when viraemic animals are around.

3.6. Contaminated clothing

Contaminated clothing and footwear seem of little significance in the epizootiology of HC, as the amounts of virus transferred will be marginal and likely below the minimum infective dose for pigs.

3.7. Unsafe vaccines

Unrestricted use of insufficiently attenuated "modified live" vaccines in the late fifties and the sixties has been a major factor in the spread of HCV. In 1967/68, no less than 21% of the HC outbreaks in the USA were attributed to vaccination (25). These vaccines have been replaced by safe and stable products without residual virulence, which do not contribute towards the spread of infection.

3.8. Arthropod vector transmission

Although of minor importance, haematophagous arthropods may contribute under certain conditions to the spread of HC. As early as 1919, Dorset et al. (26). described the contamination of house flies (Musca domestica) and stable flies (Stomoxys calcitrans) with HCV by allowing them to feed on eye secretions or blood of affected pigs. Transmission was achieved by inoculating a suspension of homogenised house flies or by the bite of stable flies up to 24 h after exposure. More recently Miller et al. (27) isolated HCV from house flies and stable flies up to 72 h after exposure to an infective blood meal. Face flies (Musca autumnalis), which tend to live longer than the former two species, harboured large quantities

of virus for up to 7 days. Tidwell et al. (28) reported the successful transmission of HCV with two species of horse flies (Tabanus lineola and Tabanus quinquevittatus) within 2 h after biting an infected pig, but failed to transmit the virus with six species of mosquitoes. On the other hand, Stewart et al. (29) isolated HCV by pig inoculation from pools of mosquitoes trapped at infected farms up to 3 days after depopulation. Furthermore they were able to transmit the disease with Aedes aegypti up to 3 days after ingestion of viraemic blood. Although biological transmission has never been shown, it is conceivable that the aforementioned Diptera species occasionally could play a role in the mechanical dissemination of virus from infected to susceptible herds, especially where these are in close proximity and where the potential vector species is in abundance. Persistently infected pigs with a livelong, high-titered viraemia constitute an ideal reservoir for vector transmission. Since depopulation may stimulate resident fly populations to migrate, appropriate vector control measures should be initiated in advance on the infected farm.

3.9. Airborne transmission

Reports about airborne transmission are scarce (9). Conclusive proof for this mode of infection has been obtained recently (Terpstra, unpublished observation). Transmission was achieved between infected donor pigs and susceptible recipients, housed in isolation cubicles. The cubicles were connected by a pipe (700 cm x 7.5 cm Ø) and held at a slightly different pressure, resulting in a flow rate of 4.5 m^3/h. It is conceivable that aerogenic transmission could play a role in the spread of virus between mechanically ventilated units at close proximity.

3.10. Role of liquid manure

The amounts of virus excreted with urine and faeces are low as compared with the titres in blood, kidney and intestinal tissue (7). In addition, preliminary results indicate that the virus is readily inactivated in manure under aerobic conditions (30). Consequently, landspreading of liquid manure is unlikely to be a factor in the epizootiology, the more as only negligible quantities will stay airborne.

3.11. Worms, birds and rodents as vectors

A cycle of HCV via lungworm eggs ingested by rainworms, which in turn are eaten by pigs (31,32), could not be confirmed (9) and for certain will not occur in areas where pigs are kept indoors. Except for sparrows which were able to transmit the disease over a distance of 6 feet (9), there is no experimental support

for the alleged role of birds, dogs, cats or rodents (33). On the contrary, transmission could not be effectuated when rats, trained to feed in close contact with infected pigs, were transferred directly to HC-susceptible pigs, not even when the rats had eaten just before from animals which succumbed to the disease (Terpstra, unpublished observation). On the other hand, predators and scavenger birds may transfer the virus with the prey in HC epizootics in wild boar.

4. RESERVOIRS

The infected pig is by far the most important virus reservoir. Animals incubating the disease or infected either inapparently, atypically, chronically or persistently, and "carrier sows" constitute the major sources of infection.

Pork and pork products from infected slaughter pigs kept in freezers and cold stores are the second most important virus reservoir and form a protracted potential risk for farms feeding abattoir offals or swill. Frozen placentae or foetuses stored by farmers for prophylactic "treatment" of reproduction failures fall within the same category.

Other, often overlooked, reservoirs of local importance are contaminated syringes, needles and partly used medicine bottles, especially when kept cool. Inadequately cleaned and disinfected stables or vehicles are sources of minor importance, as putrifaction and other environmental influences rapidly inactivate the virus. In West European countries, epizootics of HC in wild boar appear to extinguish spontaneously (Liess, Terpstra unpublished observation), whereas in East European countries the infection seems more persistent. This difference might reflect differences in climatic conditions (survival of virus in frozen carcases during the winter) and/or the size of the wild boar population. Nonetheless, an infected population of wild boar is a virus reservoir and a potential risk of infection for domestic swine, either through the food chain or by contact. The latter applies particularly to countries, where domestic pigs are held on free or semi-free range.

5. FACTORS INFLUENCING THE EPIZOOTIOLOGY

The epizootiology of HC is influenced by a large number of divergent factors e.g. the production system, prices for pigs and pig meat, density of the pig population, size and density of the farms, type of farm, trading and transport systems, standards of pig husbandry, virulence of circulating virus strains and last but not least, the measures applied to control or eradicate the disease, including vaccination regimes and methods used for laboratory diagnosis.

In countries where pigs are kept on free range or in small holdings the disease may be self limiting. In areas where the infection persists, the incidence is closely linked to the production system. In enzootically infected countries of Western Europe, a certain periodicity in the number of outbreaks has been observed. Ellis et al. (34) distinguished seasonal peaks in the spring and autumn and a 3-4 year periodicity. The seasonal variation, which was again observed in the 1982-1985 epizootic (35), can no longer be explained by larger numbers of pregnant gilts and sows in certain periods of the year (34), but might be associated with climatic conditions favouring a higher stability of the virus. The long-term variation is associated with the so-called "pig cycle". An increase or expected increase in pork prices causes a demand for weaners to be fattened, which in turn stimulates breeders to increase the production of piglets. As a result movements of live pigs expand and consequently the rate of virus spread.

The relationship between the number of outbreaks and the density of breeding farms in a certain area is an accepted fact. In addition, there are indications for a positive correlation between numbers of outbreaks in an area and the proportion of pig holders owning breeding stock (34). In such areas of high risk, the virus tends to hold out in years with a low incidence of disease. Liess et al. (36) have shown that in as much as 5% of the breeding units serologically positive pigs may be present in inter-epizootic periods. Circumstantial evidence indicates that the "carrier-sow" syndrome together with chronically and persistently infected piglets are responsible for maintaining the infection, especially in medium to large size herds.

Breeding farms are no doubt the most important source of infection for fattening herds. Supply and trading systems are of paramount importance and vary between areas according to the prevalent size of the herds. Larger units with a regular intake and overlapping age groups obviously stand a higher risk of becoming infected than smaller units with an all in-all out system. On the other hand large units often compensate for the risk by establishing steady trade relations with a few breeders. Closed and fully integrated chains of top quality breeders, commercial breeders for production of piglets for fattening and fatteners have proven to be the safest in this respect, whereas individual fattening farms supplied via markets are the most vulnerable.

The relationship between the risk of infection on the one hand, and the type of farm and the size of the herd on the other hand in areas with intensive pig production may well be illustrated by an analysis of the 1982-1985 HC-epizootic in the Netherlands. Out of 441 outbreaks that were diagnosed between January

1982 and January 1986, 223 occurred on breeding and mixed (breeding-fattening) farms and 218 in fattening units. Taking into account the number of herds present, the risk of breeding herds of becoming infected amounted to 1.17% against 1.28% for fattening herds (Table 1). In 1982 and 1983, breeding farms accounted for 44.3 and 45.6% of the oubreaks, whereas in 1984 and 1985, when vaccination of areas at risk was started after smaller and smaller numbers of outbreaks, this percentage increased to 54.3 and 66.7, respectively. From these figures it is apparent that breeding farms stand a relatively high risk of becoming infected, which is the more remarkable as one breeding unit is often responsible for several downstream outbreaks in fattening herds.

TABLE 1. The prevalence of HC-infections in relation to herd size in the Netherlands during the 1982-1985 epizootic.

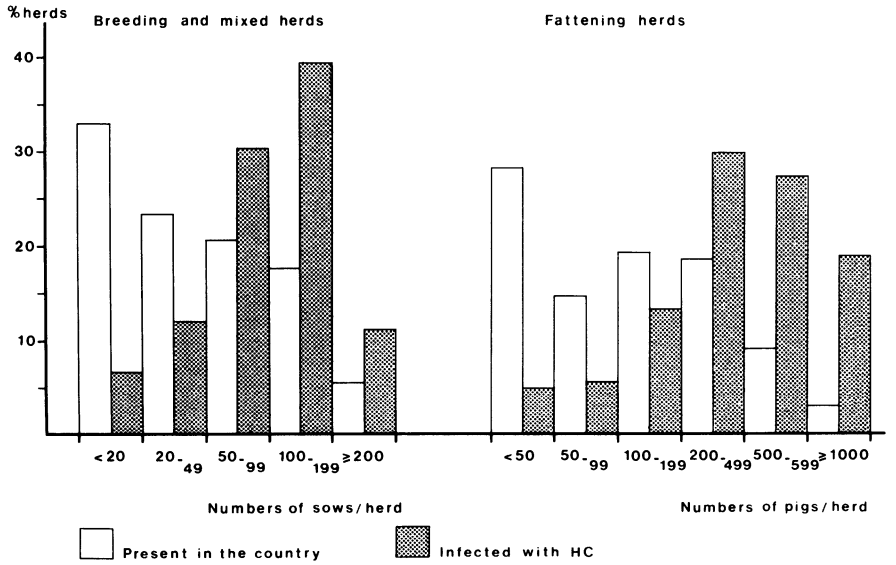
Breeding and mixed herds				Fattening herds			
Number sows per herd	Number of herds[1)]	Number HC-infected herds	% herds infected	Number pigs per herd	Number of herds[1)]	Number HC-infected herds	% herds infected
< 20	6294	15	0.24	< 50	3635	11	0.30
20-49	4447	27	0.61	50-99	2831	12	0.42
50-99	3952	68	1.72	100-199	3939	29	0.74
100-199	3394	88	2.59	200-499	4317	65	1.51
≥ 200	1054	25	2.37	500-999	1701	60	3.53
				≥1000	615	41	6.67
Total	19141	223	1.17	Total	17038	218	1.28

1) Source: Stock census 1984, Agricultural Statistical Yearbook 1985.

When the outbreaks are grouped according to the size of the herds, the frequency distribution for breeding units rises from 15 infected herds in the group with < 20 sows to 88 in the group with 100-199 sows and then drops back to 25 infected herds with ≥ 200 sows (Table 1). Taking into account the number of herds that, according to the 1984 stock census, falls into each group, it can be calculated that 0.24% of the herds with < 20 sows became infected during the 1982-1985 epizootic against 2.59% of the herds with 100-199 and 2.37% of the herds with ≥ 200 sows. Consequently, the latter two groups stood a 10-fold higher risk of infection than the small herds. In fattening units the percentage that

became infected rose almost linearly with the size of the herd from 0.30 in herds with < 50 to 6.67 in herds with ⩾1000 pigs (Table 1). Thus, fattening units with ⩾1000 pigs stood a 22-fold higher risk than units with < 50 pigs. Fig. 1 compares the percentage distribution of outbreaks with herd size distribution of the national pig population. Both in breeding and in fattening herds the ratio of the two parameters reverses as the size of the herd increases.

FIGURE 1. Comparison frequency distribution of percentages HC-infected herds versus herds present in the Netherlands.



In proportion to their numbers, farms feeding garbage or swill stand a statistically significant ($p < 0.01$) higher risk of infection than other fattening farms (Terpstra, unpublished observation). Swill farms are usually situated in areas with a low density of pigs, while trade connections, as in other fattening farms, are restricted to the intake of weaners and the sale for slaughter. Consequently upstream and lateral spread of infection by this production system is rare, leaving the risk of recontamination of the food chain.

Highly virulent strains generally cause death within 2-3 weeks regardless of age or condition, whereas strains of very low virulence do not produce disease unless piglets have been infected 'in utero'. Epizootics produced by strains of low

virulence differ in three respects from those caused by virulent strains:

- Low virulent strains induce a relatively high proportion of inapparent, atypical and chronic infections and thus betray their presence not as readily as virulent strains. An infected breeding herd may thus transmit the virus unperceived to a large number of customers, while infected fattening herds may, also unnoticed, contaminate the food chain.
- As discussed under "transmission", a low virulent strain tends to spread slowly through the herd, which also contributes to a less alarming course of disease.
- The "carrier-sow syndrome" plays a highly significant role in epizootics caused by strains of low virulence. While pregnant sows infected by virulent strains die, or in case of survival either abort or produce weak and diseased piglets which succumb shortly after birth, infection of such sows by low virulent strains generally will pass unnoticed. The healthy-looking progeny however may be congenitally infected and immune tolerant, and shed large amounts of virus for weeks or months without being detected.

Due to the principle of selection, outbreaks caused by low virulent strains are likely to be the terminal cases to be encountered in any HC eradication programme.

Finally, the control measures adopted also greatly influence the epizootiology of the disease. Closure of markets and standstill orders temporarily arrest the spread of virus, while stamping out eliminates the foci of infection. A total stamping out policy, supported by veterinary police and zoo-sanitary measures, has been successful in eradicating the disease in the Scandinavian countries, Ireland, United Kingdom, Switzerland, Australia, Canada and the United States. In various West- and East European countries, Japan and the State of Singapore with areas of intensive pig farming, the aforementioned measures have proved inadequate. Consequently vaccination has been applied on a large scale, either with the aim to eliminate the disease or to reduce the number of outbreaks to a level at which eradication by sanitary measures alone would be feasible (37). Although the modern "modified live" HC-vaccines do not produce undesired side-effects (38,39,40,41), under certain conditions they may have an unexpected influence on the epizootiology. Vaccination of inapparently infected pigs may lead to the spread of virus by needle and initiate an outbreak in the herd (42). On the other hand animals inoculated with vaccines of insufficient potency may develop a subclinical infection, when exposed to field strains of HCV, while pregnant sows may transmit the virus both horizontally and via the placenta (43). Furthermore, offspring from vaccinated sows are protected to a large extent against a lethal

infection during the early weeks of life, but not against multiplication and excretion of virus (44). Such piglets may experience a subclinical infection even with a strain of high virulence (45). In large breeding units, the perpetuation of HCV may also be favoured by a continuous weaning system at weekly or biweekly intervals, and by the custom of the farmer to place unthrifty weaners together and keep them longer in order to deliver batches of uniform weight for fattening. In this way HCV has been found to persist in breeding farms for up to eight months after the start of a vaccination regime.

Experience in Europe has proven, however, that HC can be eradicated from enzootic areas, if a systematic and strict vaccination regime, supported by veterinary police and zoo-sanitary measures, is continued for a certain length of time (42,46,47). Conversely, the EEC programme for eradication of HC has demonstrated that in regions with a non-systematic or incomplete vaccination regime supported by the same supplementary measures, the number of outbreaks already may start to rise when such a regime gets more slack in anticipation of a ban on vaccination (see Chapter 12 of this volume). Indeed the disguising effect of vaccination tends to be overlooked by national and international veterinary authorities responsible for the control of notifiable diseases.

6. TRACING OF HERDS INFECTED WITH HC

Although the modes of transmission nowadays are well understood, the actual pathways by which HCV is spread in the field often remain obscure. Conscientious tracing of an outbreak to its origin is of utmost importance for any notifiable disease and vital for the sake of its control and final eradication. Any unknown source of infection is likely to continue the dissemination of virus and to cause further outbreaks sooner or later.

6.1. Epizootiological tracing

In most infected countries tracing of HC is hampered because pigs are not identified and registration of movements is lacking. Even where these facilities exist, as in the Netherlands, the majority of the outbreaks could not be traced to their origin. Out of 223 outbreaks diagnosed in the 1982-1985 epizootic of HC on breeding and mixed farms, 33 (14.8%) were attributed to purchase of infected stock, 4 (1.8%) to known indirect contacts, whereas 65 (29.1%) and 121 (54.3%) were recorded as "neighbourhood infection" and "source unknown". The source of infection of 218 outbreaks in fattening herds in the same period were recorded as purchase of infected piglets (45.9%), swill (2.3%), known indirect contacts (1.8%),

neighbourhood infection (21.6%) and reason unknown (29.4%). As a matter of fact, the latter two categories have to be regarded as one, since neither group provides any lead as to the origin of the infection. With the help of the identification and registration system movement of pigs could be ruled out as the cause of infection in the two groups, which leaves man as the suspected principal transmitter of the virus in 83% of the outbreaks in breeding herds and of 51% in fattening herds. Reports from Germany and France (24,48,49) indicate that the problems faced in tracking the source of infection is common to areas with intensive pig farming. Vannier et al. (49) tried in vain to resolve the pathways of spread with the aid of a computer in a cluster of outbreaks in Brittany. A comprehensive epizootiological questionnaire undoubtedly would help the Field Veterinary Service to collect all relevant information, required for tracing the source of an outbreak more successfully.

On the other hand, attention to downstream contacts should not be limited to farms which have received pigs from the infected herd, but should also include farms which were visited on the same day and subsequent to the infected premise by persons handling pigs. In case of transport to the abattoir, an attempt should be made to recover and condemn the suspect carcasses.

6.2. Identification of transmitters

Human contacts between farms are frequent in areas with a high density of pig units. Apart from contacts between farmers or their personnel, it is not uncommon in such areas that several farms are visited by the same consultant, trader, transporter, inseminator, castrator or veterinarian on the same day. Grouping of the contacts, which infected farms have in common, might yield an indication as regards the "vector" most likely involved. The problem of tracking is aggravated in case of outbreaks caused by strains of low virulence and becomes virtually insolvable, when the diagnosis may have been delayed for weeks or even months due to absence of the usual signs of disease.

6.3. Virological and serological tracing

The position of herds that have been in contact, either directly or indirectly, with a confirmed outbreak of HC should be clarified by virological and/or serological investigations. The methods to be chosen somewhat depend on the nature of the contact and the time elapsed since. Due to the suppressive effect of the virus on the immune system, neutralising antibodies cannot be detected with certainty till 4 weeks after the suspected contact, while the testing itself requires

another 2-4 days before results are available. Virus isolation from heparinised blood of febrile pigs or detection of viral antigen by immunofluorescence are quicker and hence more compatible with the necessity of the earliest possible diagnosis. In case of a direct animal contact, it has been found useful to sacrifice for diagnostic purposes a proportion of the pigs, which had been moved from the positive herd (12). Care should be taken to check a fair percentage of the traded pigs, since only few may have been infected. In most countries a total ban is enforced on the movement of pigs in an area around an outbreak of HC. In the EEC Member States, this so-called "protective zone" has a radius of 3 km and lasts for 30 days. Unknown contacts may be traced successfully, if the tonsils of all pigs dying in the "protective zone" are examined by immunofluorescence.
Serological methods are very valuable in inter-epizootic periods or periods with a low incidence of disease (36) and are imperative if a country desires to become internationally recognised as free from HC.

7. CONCLUDING REMARKS

In countries with an intensive pig production system, HC, where present, may be regarded as one of the economically most important pig diseases. Although reservoirs of virus and pathways of transmission are well understood, field experience has shown that the disease is extremely difficult to eradicate, especially in countries with intensive pig breeding.

The virulence of the strains circulating in the field and the measures applied to control the disease determine to a large extent the course of an epizootic. Outbreaks by highly virulent strains are readily recognized. Epizootics caused by low virulent strains are characterised by indistinct signs of disease, slow spread of virus through the herd and a relatively important role of the "carrier sow syndrome". The latter phenomenon, with the possible birth of healthy-looking but persistently infected and immune-tolerant piglets, and chronic infections are largely responsible for the perpetuation of the virus in the pig population.

The 1982-1985 epizootic of HC in The Netherlands showed an almost equal prevalence of outbreaks in breeding and fattening herds. The proportion of breeding herds increased as mass vaccination of areas at risk was started earlier in the epizootic. In proportion to the number of herds present in the country, there was in both categories an approximately linear relationship between the risk of infection and the size of the herds.

In close-knit communities with large, modern units, man appears the single most important factor in the herd-to-herd transmission of the virus, whereas

transport and introduction of infected pigs accounts for the majority of outbreaks in areas with small pig farms. Farms visited subsequent to contact with an infected herd by persons handling pigs should, therefore, be included in the epizootiological investigations. Virological and serological methods are indispensable in order to confirm or to rule out the possibility of infection of suspected contacts, especially in outbreaks caused by strains of low virulence. Permanent education of farmers to take and maintain precautionary measures against introduction of contagious diseases and, in the case of an advancing epizootic, special instructions to all people entering piggeries, might contribute to reduce the untraceable pathways of spread.

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11

CONTROL OF CLASSICAL SWINE FEVER

H.J. BENDIXEN

1. INTRODUCTION

Classical swine fever (CSF) appears to have caused losses in many pig herds already during the first part of this century. Although the etiologic agent was not identified it is most likely that CSF virus was present among pigs in Europe and North America, where the production of pig meat increased during this period. It is interesting that control measures were already introduced a long time before the etiology and epidemiology was fully recognized; thus UK banned imports of live pigs from the European continent as early as 1882, because a disease called 'swine fever' had been discovered. In Denmark the feeding of pigs on uncooked swill in garbage waste areas was forbidden in 1928.

As the knowledge of the nature of CSF increased many countries managed to eliminate the infection by rather simple restrictive measures (stamping out and import ban), e.g. Denmark in 1933, Ireland in 1958, Great Britain in 1966 (apart from one outbreak in 1971), Hungary in 1972 and USA in 1976.

Other countries preferred to protect the pig population against CSF by routine vaccination, and especially after the introduction of the Chinese 'C' strain vaccine, this type of prophylaxis has been the strategy of choice in many European countries.

2. THE EPIDEMIOLOGIC SITUATION

For those who wish to study the problems related to control of CSF it is interesting to look into the situation as it is in Europe. CSF appears as a series of epizootics. As can be seen from Table 1 there is a certain regularity as the outbreaks

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TABLE 1 NUMBER OF OUTBREAKS OF CLASSICAL SWINE FEVER IN MEMBER STATES 1960 to 1985*

COUNTRY	1960	'61	'62	'63	'64	'65	'66	'67	'68	'69	'70	'71	'72	'73	'74	'75	'76	'77	'78	'79	'80	'81	'82	'83	'84	'85*
BELGIUM	109	215	1705	679	508	337	184	283	317	402	510	93	40	90	85	3	0	1	0	0	7	37	102	26	9	12
DENMARK	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0	0
FRANCE	392	735	1350	662	722	123	44	32	132	161	132	15	84	62	119	97	47	17	39	28	19	6	8	13	19	1
GERMANY	404	2823	2366	1553	769	341	1908	517	142	139	343	396	961	3936	1226	200	68	202	349	87	18	4	19	535	1041	209
IRELAND	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0	0
ITALY	900	525	407	838	770	439	310	455	99	38	3	17	14	51	11	3	5	48	62	7	0	5	34	48	13	7
LUXEM-BOURG	0	0	7	2	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	8	1	1	0	0
NETHER-LANDS	1519	1729	512	974	782	1117	473	333	283	144	917	338	164	904	162	38	42	11	3	0	0	11	65	161	176	17
U. K.	-	-	-	-	-	-	-	-	-	-	-	3	-	0	0	0	0	0	0	0	0	0	0	0	0	0
GREECE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20	4	2	3	0
TOTAL	3324	6027	6347	4708	3551	2357	2919	1620	973	884	1907	863	1263	5043	1603	341	162	279	453	122	44	91	233	786	1261	246

* 1st Quarter 1985

develop in waves at intervals of 3-4 years and each time of a duration of 1-3 years. Earlier the number of affected herds amounted to about 20,000 during one epizootic, now the number can be kept on the level of 2,000, which is still too high.

Another feature is that CSF tends to concentrate and settle in certain parts of Europe, ie. the pig populations in Northern France, Belgium, southern parts of the Netherlands, and western parts of the Federal Republic of Germany. The geographical pattern of the latest epizootic which started in 1981/82 can be seen in Fig. 1. It is evident that CSF virus is almost permanently present in some parts of the European pig population. Where these reservoirs of virus are may be different, but it is suggested that CSF virus may have a relatively moderate or low virulence, which means a number of animals may be infected

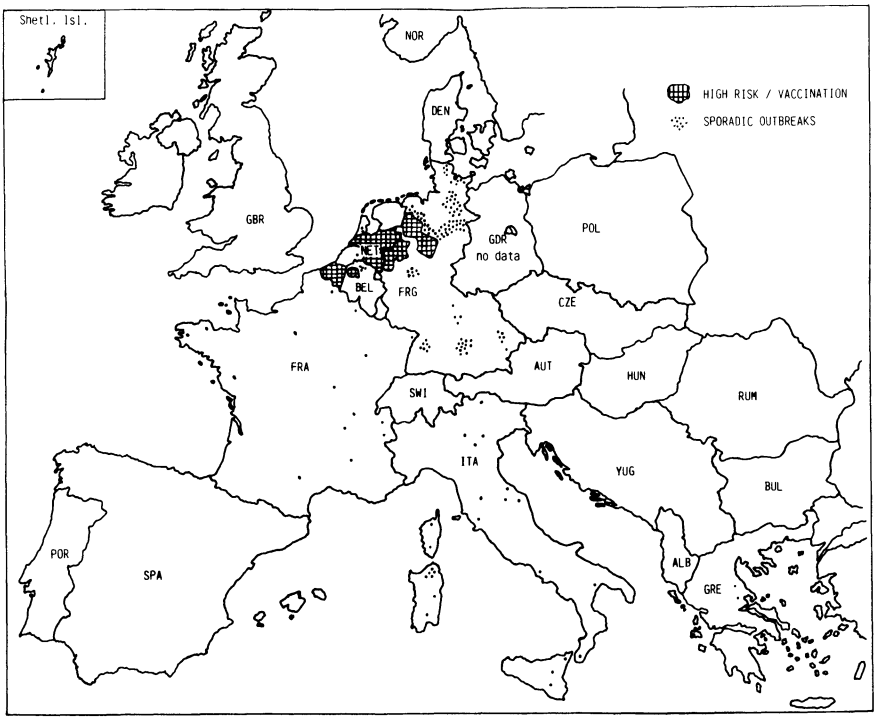

FIGURE 1. Geographical distribution of the CSF outbreaks in the EEC member countries during the epizootic 1981-1985.

subclinically and shed the virus for some time. Another source of virus infection may be the immunotolerant piglets born by infected sows. Ellis et al. (1) have mentioned that the epizootics could often be related to the periods when the number of guilts and sows was increased in order to produce more piglets. This appears also in waves, the so-called sow-cycle.

3. THE STRUCTURE OF THE PIG PRODUCTION

It is typical that most of the epizootics are concentrated in those parts of Western Europe where the density of the pig population is high (Fig. 2).

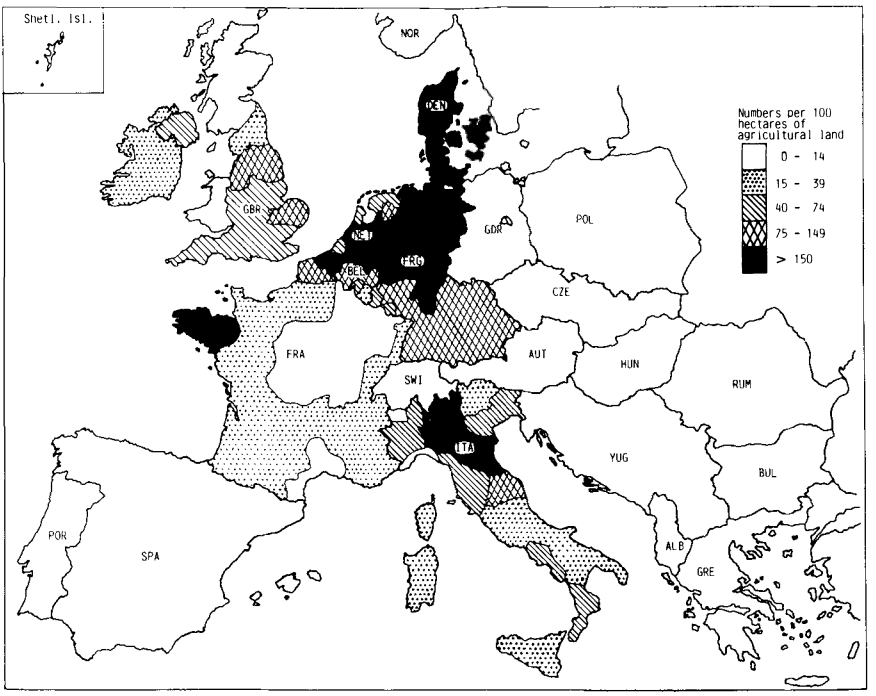

FIGURE 2. Density of the pig population (total pigs) within the Member States of the EEC in December 1981. (From: EEC livestock holdings and structures, May 1984, European Booklet 84/1, Meat and Livestock Commission, Queensway House, Bletchley, Eng.).

If the density of the sows (Fig. 3) is compared with the density of the total pig population as shown in Fig. 2, it is evident that there is a long-distance transport of weaned piglets from, eg. the Netherlands to Northern Italy, which also explains the pattern of many epizootics.

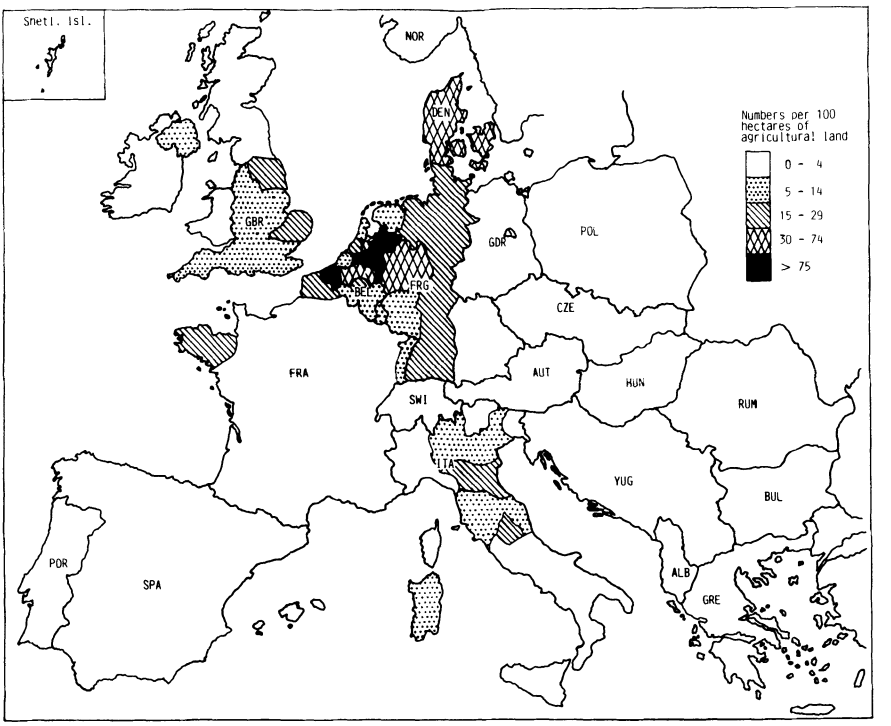

FIGURE 3. Density of the pig population (breeding sows) within the Member States of the EEC in December 1981. (From: EEC livestock holdings and structures, May 1984, European Booklet 84/1, Meat and Livestock Commission, Queensway House, Bletchley, Engl.)

Many people, even veterinarians, have taken it for granted that CSF would fade away or could easily be eliminated when modern pig production systems were introduced. Unfortunately it is not so. One reason is that many holdings are not run as 'protected herds', there is no control of the health status of

animals before they are integrated, many animals are bought on markets or from dealers without health guarantees, and many holdings are still of the traditional type, where cleaning and disinfection is difficult to perform. In many modern, intensive production systems it is difficult to control and prevent infectious diseases from spreading to the whole herd, as they are not subdivided into smaller units.

Hygienic and prophylactic measures are often difficult to perform in many herds, where swill feeding is practiced, and others where the disposal of manure and urine in the form of slurry is a permanent risk of recycling of contaminants.

It may be concluded that any systems for control and eradication of CSF can only give results if the farmers are ready to accept that the structure of their pig production is changed and that certain limitations are followed in the movement of pigs between herds. Even the most perfect public legislation on CSF control and eradication will give no favourable results unless the producers are involved in it and ready to co-operate with the veterinary services.

4. PUBLIC CONTROL OF CSF

The responsibility for control and eradication of CSF cannot be left up to the owner or the farmers' organizations. Too many combined and extensive actions are needed to eliminate the infection when an outbreak appears and to stop further spread. This is why all countries have established national CSF regulations according to which the veterinary services take action when an outbreak is discovered. Historically the European countries have used different types of public control systems for CSF: Some have very strict rules for import of live pigs and fresh pig meat, others have used more or less systematic prophylactic vaccinations. In case of outbreaks some have been very rigorous in stamping out all pigs in the infected herds, others have limited the measures to destruction of the sick pigs and vaccination of the rest of the herd.

5. THE LEGISLATION OF THE EEC ON CSF

It is evident that in the EEC, where free trade is one of the important principles, such differences will produce problems. This is why Community legislation was adopted in order to co-ordinate the national measures to control CSF; the following texts deal with CSF:

a. Council Directive 80/217/EEC introducing Community measures for the control of classical swine fever (2) amended by Council Directive 80/1101/EEC and 84/645/EEC (3,4)
b. Council Directive 80/1095/EEC laying down conditions designed to render and keep the territory of the Community free from classical swine fever (5)
c. Council Decision 80/1096/EEC introducing Community financial measures for the eradication of classical swine fever (6)
d. Council Decision 81/859/EEC concerning the designation and function of a liaison laboratory for classical swine fever (7)
e. Some of the already existing directives were announced in order to establish rules for CSF in relation to trade, namely:
   - Council Directive 64/432/EEC on health requirements which must be fulfilled by live bovine animals and swine intended for intra-Community trade amended by Council directive 80/1098/EEC (8)
   - Council Directive 72/461/EEC on animal health requirements which must be fulfilled by animals from which meat is obtained, amended by Council Directive 80/1099/EEC (9)
   - Council Directive 80/215/EEC on animal health requirements which must be fulfilled by meat products intended for intra-Community trade amended by Council Directive 80/1100/EEC (10).

In establishing common rules for trade in pigs, the Community had to take into account that the CSF situation was different in the different Member States. It was, therefore, necessary to permit those Member States who were free from CSF

to maintain certain restrictions for imports of live pigs and fresh meat, until all had reached the same health level.

6. LEGAL MEASURES AGAINST CSF IN CASE OF EMERGENCY SITUATIONS

It was decided to introduce common rules for the emergency situation in relation to outbreaks (Directive 80/217) (2). In this way it was hoped that CSF would be controlled and eradicated more efficiently whenever it appeared, which would eventually lead to the halt of further spread of CSF from the first outbreaks, and elimination of epizootics before they got started. The directive, therefore, contains all the minimum criteria to follow in case of CSF outbreaks, and whenever infections are found in a pig herd. In order to support the Member State in these actions it was decided to make Community financial support available (6) when the infected herds were eliminated correctly.

In this Directive all the principles are given which must be followed when an outbreak occurs.

a. Presence of, and suspicion of presence of CSF are compulsorily and immediately notifiable to the competent authority.
b. In case of suspicion, the official veterinarian must immediately set in motion official means of investigation. The holding is placed under official surveillance, the pigs listed and isolated. No pigs may enter or leave the holding. After 15 days a slaughter permit may be issued. Furthermore rules are given to avoid possible spread of infection. They may be lifted only when the suspicion is officially ruled out.
c. In case of official confirmation of CSF the competent authority prescribes that all pigs must be slaughtered without delay under official supervision, the carcases of the pigs destroyed, including carcases of dead pigs. All measures are taken to avoid any spread of CSF virus including cleaning and disinfection of buildings, vehicles, equipment, substances and waste likely to be contaminated; all following the instructions of the official veteri-

narian.

d. Reintroduction of pigs to the holding may not take place until at least 15 days after completion of cleaning and disinfection; disinfectants and their use must be officially approved by the competent authority, and cleaning and disinfection carried out under official supervision, according to instructions of the official veterinarian.

e. The epizootiologic enquiry shall be carried out to identify the sources of infection, other herds which may have become infected and possible spread by movement of persons, vehicles, etc.

f. Member States may authorize slaughter of pigs which are in the infected holding, but are not infected or suspected of being infected, if they are brought to a special establishment for slaughter and if the meat undergoes heat treatment to ensure that CSF virus is destroyed (FC3), all under veterinary supervision.

g. If holdings are organized in such a way that production units provide completely separate facilities for housing, keeping and feeding so that virus cannot spread from one production unit to another, then pigs from non-infected units may be finished for slaughter.

h. A protection zone with a minimum of 3 km shall be established around the infected holding. Movement of pigs is prohibited. However, pigs may be moved directly to a slaughter house under official supervision and after examination of all pigs on the holding by an official veterinarian. Itinerant boar service is prohibited. Fairs, markets, shows or other gatherings of pigs are prohibited. Collection and distribution of pigs by dealers is forbidden. The duration of the measures of the protection zone is 30 days after destruction of infected pigs, and completion of cleaning and disinfection.

i. For welfare reasons it may be permitted by the competent authority to move fattening pigs to another holding within the protection zone or within 20 km of that zone. Breeding

pigs may also be moved between two holdings within the same protection zone.

k. Sampling and laboratory investigation of material to detect CSF shall be carried out according to common approved methods, which are described in Annex I to Directive 80/277/EEC (2). It is regarded as an important part of the control system in the EEC that the diagnostic procedures are harmonized. The Directive, therefore, includes technical specifications which function as guidelines, standards and minimum criteria for the diagnostic procedure. In each Member State a national laboratory is responsible for co-ordination with the individual diagnostic laboratories of the Member State. It shall provide them with diagnostic reagents, control the quality of reagents used, arrange comparative tests periodically, and shall hold isolates of CSF virus from field cases.

l. The diagnostic procedures include the following methods:
   - Demonstration of antigen in organ tissues by the use of the direct immunofluorescence technique.
   - Virus isolation and identification in cell cultures (PK15).
   - Detection of antibody in blood by the plaque reduction test (PRT), neutralization index test (NI test) or virus neutralization and immunofluroescence test (NIFT).

m. Differential diagnosis between antibodies induced by CSF or BVD virus is made by parallel end-point titrations of sera.

n. A liaison laboratory has been designated by the Council (7) in order to co-ordinate the diagnostic activities of the Member States. It holds cell cultures, virus strains, test sera, conjugates and other reference material in order to standardize the test and reagents used by the Member States. It maintains a collection of CSF virus isolates. It organizes comparative tests. It distributes information about new diagnostic methods. This activity has proven its value in the common diagnostic system of the Member States.

o. Measures to control the disease may be supplemented by vaccinating pigs threatened by field virus in an area

where outbreaks occur. When it is decided to carry out an area vaccination all pigs in the area shall be vaccinated as soon as possible; there is a stand-still during the campaign. Vaccinated pigs may leave the vaccination area 3 months after completion of the vaccination operation if they are vaccinated at least 15 days beforehand, and if they go directly to slaughter or - on special authorization - to a fattening herd, from where they go directly to slaughter.

p. Herds of very great genetic value may be exempted from the systematic vaccination campaign.

q. If a CSF epizootic is exceptionally serious and tends to spread, the Member State shall declare a demarcated area "a high health risk area". If this is not done, a Commission recommendation (SVC-procedure) may be taken to establish such "a high health risk area". If the Member State does not apply this recommendation the Community financial procedure is suspended. Vaccinated pigs are marked permanently. Vaccinated sows of breeding age may leave the vaccinated area only to be taken to a slaughterhouse. Use of specific immune-serum or sero-vaccination is prohibited. The commission, through the Standing Veterinary Committee (SVC), decides about the requirements relating to CSF vaccine.

r. Swill is regarded as a dangerous material in relation to spread of CSF. Swill from means of international transport (ships, aircraft, vehicles) must be collected and destroyed under official supervision. National swill must be heated to ensure destruction of CSF virus, and for feeding fattening pigs only. Such pigs can only leave the holding to go for slaughter. Swill collection and treatment must be organized in such a way that the risk of spread of virus is eliminated.

7. THE EEC PROGRAMME FOR ERADICATION OF CSF

In 1980 the Council adopted a Directive in order to accelerate the national campaigns to eliminate CSF. The CSF

situation appeared favourable. The prevalence of CSF was the lowest ever seen, only 122 outbreaks registered in 1979 and about 40 up to November 1980. The Member States had reduced the routine vaccination against CSF to a minimum, and it was believed that subclinical infections were rare and could be found by systematic screening for antibody-carriers in the breeding herds. It was the plan that the herds could be cleared by slaughter of a relatively limited number of reactors.

In Directive 80/1095/EEC the formal basis for the establishment of national plans was given. The principles were the following:

- Systematic slaughter of all pigs in herds where outbreaks occured.
- Epidemiologic investigations wherever clinical and subclinical cases of CSF were discovered followed by serological examination in all suspect herds.
- Stop of the routine vaccination programmes within the first two years of the eradication period.

The Member States who were not yet declared officially free of CSF had to produce a national plan for CSF eradication. While four Member States, ie. Ireland, United Kingdom, Denmark and Luxembourg were declared officially free from CSF by a Commission decision of May 15, 1981, national plans were approved by the Commission from January 1, 1982 for Belgium and the Federal Republic of Germany, for the Netherlands, March 1, 1982 and France, June 1, 1982; Italy and Greece from 1983.

The Community financial support according to the Council Directive 80/1096/EEC (6) is available for the eradication of infected animals.

8. THE CSF EPIZOOTIC IN BELGIUM, THE NETHERLANDS AND THE FEDERAL REPUBLIC OF GERMANY

In 1981/82 a CSF epizootic started in West Flanders from where it spread into southern Netherlands during 1983 and 1984 and continued into Germany (see Figs. 1 and 4) where it became especially widespread in the regions of Münster and Weser-Ems. In spite of vaccination it spread into still wider areas of the

Netherlands and to many areas of North, Mid and South Germany. In Belgium and the Netherlands the main reason for spreading was trade in live pigs. In Germany feeding with swill was often declared as the cause of the initial outbreaks in an area, while trade in live pigs appeared to be the most common cause of spread later on.

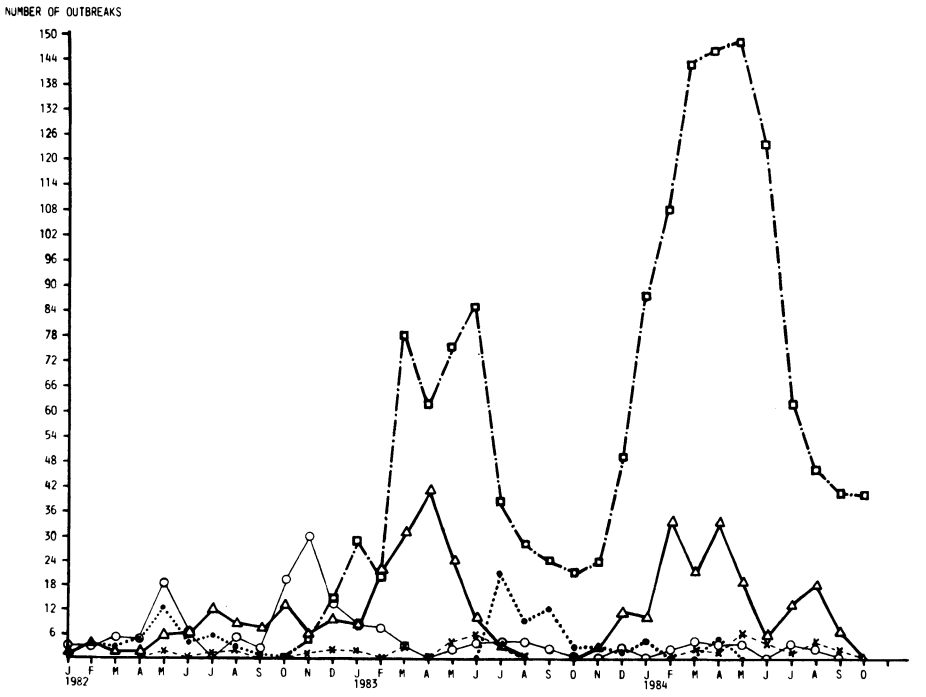

FIGURE 4. The CSF epizootic in Belgium, the Netherlands, Federal Republic of Germany, Italy and France 1981-85. Number of outbreaks per month.

There seemed to be serious obstacles in applying the measures to eliminate the disease quickly in some areas. This is why area vaccination has been started in these countries.

During the same period the national CSF eradication plan has been applied in France with good results. Vaccination has not been necessary. Some outbreaks have been notified, which in

many cases can be related to imports from infected Member States. Greece and Italy have started their national plans but hesitate to stop vaccination until the epizootic is under control.

9. CONCLUDING REMARKS

The CSF situation in Europe during the recent years has clearly illustrated how difficult it is to control and eradicate CSF in a group of countries where the pig production is intensive and is still being even more intensified. In some areas of western Europe the pig population has more than doubled over the last 10 years. Although the public legislation in some of the countries has been reinforced, veterinary control extended and modern vaccines used, it has not been possible to stop the epizootic and to continue with plans to eliminate the CSF infection from the herds in order to obtain official freedom of CSF. The development of co-ordination between the national services in the legislative and technical form of the EEC and the co-operation with the international organizations of FAO and OIE has been very useful.

But it is now clear that further progress depends on a closer co-operation between the pig producers and their organizations on one side and the veterinary authorities on the other. The aim of such a co-operation would be to change the pig production systems in order to make them less sensitive to epizootics and reduce the risks of extensive spread of infections. In order to achieve this aim some elements need consideration:

- reduced density of the pig population in some areas;
- maximum size of pig holdings;
- separation between individual holdings and, within larger holdings, separation of production units to avoid immediate and direct contact infection;
- movement of pigs directly from one holding to another avoiding markets, etc.;
- better co-ordination when an epizootic makes restrictions to trade and production necessary.

It is important to modernize traditional pig holdings and to improve the animal health situation in general in pig herds. Such a programme should be fortified by strategic serlogical screening procedures in order to detect inapparent virus reservoirs in the midst of a pig population which is otherwise CSF-free. This is especially true for the production systems where pigs are kept under semi-wild conditions.

The veterinary services should be especially organized to deal with CSF, in accordance with the recommendations of the FAO/EEC Expert Consultation in 1976 (11,12). Special importance is given here to the training of field and laboratory staff, to the application of measures to avoid all sorts of spread of virus, and to have adequate diagnostic methods available. Surveillance systems in order to discover and notify cases suspect of being CSF should be developed and notification to other countries and to the OIE should be an important part of a general warning system. The Council Directives 82/894/EEC and 84/80/EEC (13,14) have been established in order to make it possible for the Member States of the EEC to organize common actions in case of CSF emergency. It should be stressed that this system is an internal co-ordination system for the EEC, and that it in no way interferes with the disease reporting system of the OIE. This system is supported and used fully by the Member States of the EEC, who are aware of the value of information on the CSF situation in the rest of Europe and in other parts of the world, when the conditions for trade in live pigs and pig products are laid down.

Of special importance is the close co-operation between neighbouring countries, both in the emergency situation, when the first outbreaks are discovered and eradication measures organized, and later on, when preventive vaccination campaigns are organized. This may be especially needed in Europe, and the initiatives of FAO in this respect should be fully supported.

This survey of the principles and problems related to control of CSF has been built up on the basis of observations made in the West European countries, who are members of the

EEC. It is my conviction that the general principles which are followed in the formal and technical functions of the EEC systems for control of CSF can be taken over by other countries. But it is also important to stress that any control programme must be adapted individually to the conditions of the pig production in the country and region concerned. It is this practical adaptation which will determine the result, and it depends on the skill and energy of the veterinary staff and the awareness of the producers and managing staff whether the elimination of the CSF may be finally achieved.

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12

THE COMPARATIVE BIOLOGY OF CLASSICAL SWINE FEVER

J.W. HARKNESS AND P.L. ROEDER

1. INTRODUCTION

All three viruses which together form the pestivirus genus are pathogens of significance for farm animals. In this chapter, some of the characteristics of infection with ruminant pestiviruses will be considered in relation to classical swine fever/hog cholera, which we will refer to as hog cholera (HC), and the similarities and differences between the disease syndromes they cause discussed. Attempts will be made to identify important gaps in current knowledge and their implications for the practical control of disease in cattle, sheep and pigs. In the available space it will be impossible to provide a comprehensive survey of the extensive literature on pestiviruses; the aim is to present a current perspective on a rapidly changing field of knowledge which is of concern to all those interested in HC.

Hog cholera was apparently first reported in southern Ohio, USA, in the early 1830's. By contrast the disease conditions in ruminants caused by the other two pestiviruses were recognised relatively recently. The first of these, bovine virus diarrhoea (BVD) was described in 1946 as an infectious and highly contagious disease of cattle characterised clinically by high fever, nasal discharge, diarrhoea and coughing (1). Case mortality rates were low but morbidity was high, and the condition was transmitted experimentally with relative ease (2). A similar condition reported by Ramsay and Chivers (3) occurred mainly in young cattle, and clinical signs and lesions were considerably more severe. Morbidity rates were low but case fatality rates were high and extensive ulceration of the gastrointestinal tract was present at post-mortem. Attempts to transmit this disease by experimental inoculation failed, and the authors concluded that it was a new condition, which they termed mucosal disease (MD). A number of virus isolates from various

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outbreaks of these two clinically different diseases were characterised in the laboratory (4,5). Some isolates induced cytopathic changes in cell culture (10) while others were resolutely non-cytopathic. Further studies showed that these agents formed an immunologically related group (6,7,8,9). Latterly, the virus responsible for both BVD and MD has been referred to by most authors as BVD virus (BVDV).

The third member of the genus is the virus which causes Border disease (BD) of sheep (BDV). BD was first described in 1959 by Hughes and his co-workers (11) after studies among sheep flocks in the region along the border between England and Wales. The disease occurs when the ewe encounters the virus during pregnancy; it is typified by abortion and stillbirth, and by the appearance of lambs with an abnormally hairy birthcoat, body tremors and a markedly slow rate of growth. The cardinal lesion in affected lambs is hypomyelinogenesis of the central nervous system, which is associated with the presence of abnormal glial cells and significant neurochemical changes. The viral nature of the aetiological agent was demonstrated using antibiotic-treated cell-free filtrates of suspensions of tissues from affected lambs (12) and its immunological relationship with BVDV was discovered soon afterwards (13,14). Further investigation showed that there was also a relationship with HC virus (15,16,17). Serological relationships between pestiviruses and the other non-arthropod-borne togaviruses, rubella virus, equine arteritis virus and lactic dehydrogenase virus, have not been demonstrated (18).

The first evidence of the antigenic relationship between HCV and BVDV came in reports (19,20) of a cross-reaction in the agar gel precipitin test, subsequently confirmed by other workers (21,22). Cross-reactions were also observed in complement fixation and immunofluorescence tests (23,24) and neutralisation assays (25,26,27). Cross-neutralisation between pestiviruses has been repeatedly demonstrated, but it is clear that there are different degrees of antigenic relatedness and cross-reactions may be strong, weak or non-existent (27,28,29). Antigenic relationships between these ruminant pestiviruses and HCV are generally much more distant than between the isolates from cattle and sheep. Some studies of these cross reactions are difficult to evaluate. Early workers did not

always appreciate the complications introduced by frequent contamination of cell cultures and fetal calf serum with BVDV, the existence of BVDV infection in pigs, or the necessity for strict control over the methods used in production and selection of antisera. Neutralisation tests are the main basis on which serological reactions to the viruses are differentiated and because of the occasional need to distinguish between BVDV and HCV infections in pigs in the course of the control of HC, the relationships are of considerable importance. As new information emerges, the picture becomes more complex. Thus, although Corthier and co-workers (27) concluded that HCV strains form a relatively compact antigenic grouping by comparison with BVDV, the degree of heterologous neutralisation is dependant upon the BVDV strain involved (28,30). Further complications are introduced by a recent report from Japan (31) of the isolation of virus strains from pigs which appeared more closely related to BVDV than to HCV in neutralisation tests but which caused HC in experimentally inoculated pigs. These findings bring into question the validity of the criteria currently used to differentiate the viruses and emphasises that it is difficult to discuss the application of control measures for HC without reference to the ruminant pestiviruses.

The pestiviruses, together with other non-arthropod-borne togaviruses including rubella and equine arteritis viruses, are capable of crossing the placenta and invading the fetus. For ruminant pestiviruses this is an event of central importance in their biology.

2. PRENATAL INFECTION

It has long been recognised that when pregnant sheep, cattle and pigs are infected with pestiviruses, transplacental infection occurs readily. In sheep it has been demonstrated that such transmission occurs within seven days (32,33) and investigations of naturally occurring infections indicate similarly rapid transmission to the fetus in cattle (34,35). Fetal death with mummification or abortion occurs commonly after pestivirus infection in all three species of domestic livestock (36,37,38,39,40,41,42). However, infections of the fetus which persist throughout gestation and postnatal life are of major importance in the epidemiology of pestivirus-associated diseases,

particularly as these infections are not always accompanied by readily appreciable clinical evidence of fetopathic effects.

Viruses which induce persistent infections in their host are often non-cytopathic in cell cultures (43) and this is also common among pestiviruses. The expression of cytopathic effects, usually after 'in vitro' manipulation, has been described in many reports in the case of BVDV (44,45,305) and more rarely for BD (46,47,48,49) and HC (50,51,52,53) viruses, but isolates obtained from newborn animals surviving intrauterine infection and from asymptomatic persistently-infected individuals have generally been non-lytic (34,41,54,55,56,57), even when the viruses used to infect the dams were lytic in culture (39,58,59). Thus, it seems that the fetus destined to become a persistently-infected individual rapidly assumes a mutually tolerant relationship with the virus involving lack of cytopathogenicity and reduced pathogenicity on the part of the virus (60), and the failure of the fetus to mount the immune response which would otherwise eliminate the virus (54,55,56,57,59,61,62,63,64,65,66).

The role played by the development of immune responses in the fetus is of crucial importance in understanding the pathogenesis of fetal pestivirus infections. Somewhat surprisingly, there is more information concerning the sheep fetus than the other economically more important species and this data will be considered in more detail before assessing the information available from cattle and pigs. Clear indications of fundamental mechanisms emerge when all the available information is correlated. The terms BVDV and BDV are used here to indicate virus isolates from cattle and sheep respectively although there is currently no sound basis upon which BVDV may be distinguished from BDV.

2.1. Response of the ovine fetus to pestivirus infection

The ability to mount an immune response to BD virus, as assessed by detection of serum neutralising (SN) antibody in fetal sera and presuckling sera from newborn lambs, has been demonstrated in the progeny of ewes inoculated from as early as 52 days of gestation (56). The proportion of fetuses capable of responding increases as gestation progresses (67, 68, 69, 70). The work of Parsonson et al (33) and

Snowdon et al (71) shows that a similar responsiveness occurs in the ovine fetus at between 59 and 87 days of gestation. In no case where antibody was detected was it possible to isolate virus from the fetus or lamb. Congenital persistent infections have resulted from vertical transmission in persistently-infected ewes (58,72,73), and from infections of normal susceptible ewes at between 19 and 72 days of gestation, but never later (41,56,60,69,73,74), and were associated with the absence of SN antibody at birth. Assuming that, as already indicated, it requires approximately seven days for the virus inoculated into the ewe to gain access to the fetus, it appears that the ability to control infection and subsequently produce SN antibody is acquired by the ovine fetus at between approximately 60 and 80 days of gestation; the exact timing and rate of development of immune competence probably differs between individuals and is influenced by genotypic and perhaps phenotypic factors.

When correlated with the times of developing fetal immune responses, the published accounts of the abnormalities caused by fetal infection, which are primarily neuropathological, show that the maturing immune response modulates and may fleetingly enhance the direct fetopathic effects of the virus. Typical BD results from infections of the fetus earlier than 60 days of gestation. The lesions observed in the newborn, which result from deviant differentiation of the organs and tissues undergoing rapid development at this stage in fetal life (75), are always accompanied by persistent infection with the virus. In marked contrast, necrotising inflammatory lesions, or their residua, termed "alternative " pathology by Barlow (76), and consisting of dysplastic and severe cavitating lesions in the CNS, have all been associated with maternal infections occurring at 54 to 71 days of gestation (41,77,76) and therefore fetal infections occurring at approximately 61 to 78 days of fetal life, when the fetal immune response is at an early stage of development. These lambs, which show hydranencephaly and cerebellar dysplasia, lack the stigmata of classical BD (77); they have eliminated infection by birth and the majority possess pre-colostral antibody (78). The acute encephalomyelitis described by Clarke and Osburn (79) in fetuses infected experimentally at 66 to 91 days of gestation may be the basic

pathogenetic mechanism, and the variability in the extent of the lesion could be a function of changes in the availability or susceptibility of target cells; more mature cells perhaps provide a less favourable substrate for viral replication. It has not been shown that the virulence of the virus for particular target cells changes during the course of infection of the fetus, and the fact that neural elements in the newborn lamb are still susceptible to damage when virus is introduced intracerebrally (80) suggests that changes of this kind do not occur. It is relevant to note that the presence of a defective interfering genome attenuates the cell killing effects of vesicular stomatitis virus in mature, but not immature, murine neurones in vitro (81), but it must be stressed that there is no evidence at the present time for a defective interfering genome in pestiviruses (82). Increasing effectiveness of the maturing immune response, perhaps in association with a decreasing availability of target cells, therefore appears to offer the best explanation at present.

The occurrence of hydranencephaly and cerebellar dysplasia after infection at the earliest stages of immune responsiveness to the virus suggests that the exact timing of infection in relation to that responsiveness is a critical factor, and the data indicate that the pathogenetic mechanism is operative for only a very short period in any individual fetus. A cellular inflammatory response mediated by large mononuclear cells, first seen in the ovine fetus at 63 days of gestation, is the earliest fetal defence mechanism and it can be excessive and of a different nature from that observed post-natally (83,84,85). It is therefore possible that the observed damage is a function of immunological immaturity.

Less severe cavitating lesions in the CNS, ranging from gelatinous softening in the cerebral and cerebellar white matter through malacic lesions to frank cavitation, occur in lambs with other lesions characteristic of BD (77,86). These lambs are persistently infected and available assays detect no evidence of an immune response (73,86). Thus development of the leukoencephalomalacic lesion is more likely to be virally mediated than immune mediated, but may not be a direct viral effect since the white matter in which the lesion is found does not become differentiated in the fetal brain until later in

gestation than the time of infection. Virus strain variation alone could account for observations of severe effects on oligodendroglial differentiation and function, and extensive myelin breakdown which was more severe than that observed in typical BD (87).

It is perhaps more likely that the severity of CNS pathology is influenced by the interaction of virus strain differences with host genotype. It has also been claimed that the immune status of the dam in respect of previous infections with antigenically different pestiviruses modifies fetal pathogenesis (75). Virus variation clearly exerts an effect on the extent of fetal damage in all three pestivirus infections. One bovine isolate was shown to replicate to a higher level in the placentome and caused more severe placental pathology than other strains studied (71), and it was suggested that this might explain the occurrence of the leukoencephalomalacic lesion (69). Different strains of BD virus may also elicit variations in the clinical features of the disease (86). Congenital tremor in calves has been reported several times but appears relatively rare in nature (35,39,88,89), and not all strains of HC virus cause congenital tremor in piglets (90,91,92).

From approximately 80 days of gestation the ovine fetus is able to mount an apparently mature immune response, resulting in the elimination of infection and the production of SN antibody before birth (41,56,69). Lambs infected after 80 days of gestation show minimal pathology; however, a nodular periarteritis has been described after fetal infection at this stage (93).

2.2 BVD virus infections of the bovine fetus

The changes in the resulting fetal pathology which occur with the onset of immunological competence is also apparent from studies in cattle (35), although interpretation of many reports is made difficult by the lack of adequate virological data and the tendency to describe all the different pathological manifestations as though they were the result of a single process.

An intact zona pellucida affords a degree of protection to both the bovine (98) and ovine (99) embryos in vitro. Although infection introduced directly into the uterus before implantation may prevent

fertilisation in cattle (100) or adversely affect the development of pre-implantation embryos (101) and result in reduced conception rates (102), this appears to be less important where infection is acquired naturally or by parenteral inoculation. In sheep (67,103,104,105), cattle (102) and possibly pigs (106), the embryo appears to be protected from infection until after implantation.

Infections persisting to term have been established by direct inoculation of the bovine fetus from as early as 45 days and as late as 125 days gestation (57). No evidence has been found to support statements that lambs and calves born persistently-infected after early intra-uterine infection can eliminate virus post-natally (128,129). Numerous records show that the fetuses of cows inoculated after 90 days of gestation can eliminate infection and produce neutralising antibody before birth (39,94,95,96,97). Fetal death and premature birth are common adjuncts to fetal pestivirus infection in all species, but their pathogenesis is poorly understood. Diagnostic experience in cattle and sheep indicates that there are two forms with differing pathogeneses. In the first, an autolysed fetus is expelled within five to six weeks of infection in early and mid-gestation; infectious virus is not detectable but antibody to BVDV is sometimes present. Fetal death could be due to the direct effects of viral replication in fetal tissues, compounded by placentitis and consequent interference with placental function (107). However, the presence of antibody in some cases suggests that mortality may also be the result of severe immune-mediated tissue damage. The report that BVDV antigen and specific antibody but not infectious virus were detectable in fetuses with severe inflammatory pathology three weeks after direct inoculation at 120-165 days of gestation is supportive of this hypothesis (108).

The second form is markedly different; when fresh fetuses in a good state of preservation are expelled in late gestation it is frequently possible to isolate virus. The pathological lesions and the distribution of viral antigen in these fetuses is characteristic of persistent infections established in early gestation. Expulsion may be a result of precocious adrenocortical development resulting in raised plasma corticoid levels (109).

In cattle it has been reported that lesions of hypomyelinogenesis,

associated with the presence of abnormal glia, and hydrocephalus accompany congenital and persistent infection, but the majority of affected calves lack macroscopic and microscopic lesions (35,39,88,110,111). As in sheep, infection at the onset of the fetal immune response is associated with the development of cavitating cerebral and degenerative cerebellar lesions (39,42), cataracts and retinal degeneration. These abnormalities occur in calves when maternal infection is established at between approximately 100 and 150 days of gestation. Published evidence suggests a correlation between the presence of these lesions, elimination of virus, and the presence of pre-colostral antibody (94,96,113,114,115,112). Sequential studies of fetuses carried by cows infected at 150 days of gestation clearly indicate that acute inflammation first seen 17 days after inoculation of the dam, which results in ocular and cerebellar lesions, resolves to leave atrophic abnormalities (114,115).

After infection in late gestation, the virus is eliminated and precolostral antibody is found if sufficient time elapses between infection and birth (116,117). As in sheep, these infections are associated in cattle with minimal pathology.

Naturally occurring fetopathy in cattle has been studied less thoroughly, but degenerative cerebellar lesions have been associated with the presence of pre-colostral antibody (118,119). Reports of the presence of both virus and pre-colostral antibody in cases of naturally occurring hydranencephaly must be mentioned but are impossible to reconcile with our concept of the pathogenesis (120,121).

Thus, it appears that the dysplastic lesions encountered after infection of the bovine fetus are associated temporally with, and conceivably induced by, the early immune response of the fetus; the onset of immune competence appears to change the pathogenesis and contribute to the development of lesions (35). Viral strain differences appear to influence both the severity and the nature of the lesions. By comparison, we have already noted that some but not all strains of HCV produce congenital tremor in pigs after maternal infections initiated at between 10 and 72 days of gestation (64,90,91,92,122,123).

2.3. Prenatal infections in pigs

The fetal lesions described for sheep and cattle have their counterparts in pigs but it is more difficult to establish from published studies when immune responses to HCV first appear. In swine, the gestational length is relatively short and the neonate is immunologically immature in comparison with the ruminants (124). Consequently, congenital infections are not always persistent infections established by exposure prior to the development of fetal immune competence. Elimination of infection can occur after birth (125) but it is not known whether this indicates termination of persistent infection acquired in early fetal life, which occurs in rubella in man (126), or delayed elimination of virus acquired after the onset of immune competence. The latter explanation is plausible since it has been shown that not all fetuses in a litter become infected with porcine parvovirus simultaneously, and that spread between fetuses may occur subsequently. This has the effect of broadening the spectrum of congenital abnormalities seen within the litter at birth, even when the sow has been infected at a stage when her fetuses are not yet immunologically competent.

Infection of the sow at up to 100 days of gestation can undoubtedly result in congenital infection in her piglets (130) but persistent infections have been associated mainly with infection of the sow at 40 to 69 days (125,130) or earlier (64,90). The period in gestation from 50 to 60 days has been infrequently studied. Occasionally, persistent infections occur after infections as late as 90 days (125,131). Precolostral antibody and elimination of virus infection has been observed in a small proportion of newborn piglets from sows infected as early as 72 days of gestation (64,132). Thus in pigs, the transition period from immune non-responsiveness to immune responsiveness to HCV appears to occur when sows are infected at between approximately 70 and 90 days of gestation.

In the congenital tremor syndrome, reduced cerebellar size, hypomyelinogenesis, micro-ophthalmia and pulmonary hypoplasia have resulted from maternal infections occurring at between 10 and 72 days of gestation (64,90,122,123,123,133). In contrast, inflammatory and degenerative cerebral changes have been observed following infection at

between 65 and 67 days and non-suppurative CNS vasculitis following infection at between 85 and 109 days (132,134).

There are many similarities between the factors influencing the fetal response to pestivirus infection in pigs and ruminants and in the variable manifestations of these responses. There may also be important fundamental differences. Unlike the ruminant pestiviruses some strains of HCV appear to be able to modify the immune response of the fetal and neonatal pig and induce a state of "tolerance" and consequent persistent virus infection, after the onset of immune competence (135,136). This might reflect biological differences between the porcine and ruminant pestiviruses and their interplay with differences in the timing of the maturation of the immune response in pigs and ruminants. The generation of persistent infections after the normal time of onset of immune competence in the fetus has not been observed in ruminants. Two reports of prolonged infection in calves (137,138) suggest a condition which resembles chronic HC (139,140,141) more closely than persistent infection. Chronic HC is considered to be distinctly different from persistent infections induced from fetal infections (91). However, it must be recognised that few strains of ruminant pestivirus have been examined in this way and only 6 of 135 HCV isolates had this potential (136).

2.4. Cross-infections between species

It is not surprising in view of the close phylogenetic relationships between the species that there is much evidence that pestivirus isolates from cattle and sheep are reciprocally fetopathic (33,71,142,143,144,145,146). In addition, BDV produces BD in goats both experimentally (147,148) and naturally (149).

Although HCV virus replicates after inoculation by parenteral routes and induces the production of serum antibody (150) there is no evidence that it has any fetopathic potential in ruminants. In the only study known to the authors (O.Papadopoulos, G.Koptopoulos, M.Artiopou, C.Richardson, P.L. Roeder and J.T. Done - unpublished) pregnant ewes inoculated in early gestation with the 'Weybridge' congenital tremor strain of HCV all seroconverted, but suffered no fetal loss and abnormalities were not detected by morphometric study of

the newborn lambs.

After inoculation with cell cultured material or contact exposure to persistently-infected sheep and cattle, pigs become actively infected with both BDV (56) and BVDV (151,152). Serological evidence indicates that infections with ruminant pestiviruses are common in pigs in many countries (89,152,257) yet the fetopathogenicity of such infections appears to be very limited. Inoculation of pregnant sows at 34 days of gestation produced equivocal results (153). There was some evidence of cerebellar hypoplasia but only one piglet was clearly abnormal having a meningocoel associated with severe cerebellar dysgenesis. Liveborn piglets had light and dark striping along their bodies, caused not by pigmentation but by the reflective or refractive properties of abnormal hair fibres. The abnormality was likened to the pigmented striping found in the young of wild progenitors of the domestic pig (154). The lighter stripes, and to a lesser extent the darker stripes contained larger medullated fibres than samples from control pigs and the results of hair fibre analysis were much like those obtained in studies of experimentally produced BD in sheep. A limited unspecified virological examination failed to demonstrate virus in a pool of spleen tissue from some piglets.

More convincing evidence that transplacental infection is possible in sows infected with BVDV came from a series of experiments conducted in the USA. The finding that anti-pestivirus antibody was present in hysterectomy derived piglets in higher titre to BVDV than to HCV (155) suggested that transplacental infection had occurred and subsequently virus was isolated from a sow and three of her hysterectomy-derived piglets in the same herd (156). These porcine isolates produced severe, and even fatal, disease when inoculated into calves. Transplacental infection with porcine "BVD" viruses was confirmed (157) and in this later study evidence was obtained that BVDV isolates derived from cattle also caused death of the porcine fetus. The most interesting report of such an occurrence is also the most recent (158). The authors investigated reports of increases in piglet mortality, mainly during the second week of life, and of sows returning to service. The syndrome in affected piglets comprised locomotor disturbance, ill-thrift, palpebral oedema, a high mortality rate and,

at necropsy, haemorrhagic lesions resembling those of HC. The problem arose from the use in early gestation of one batch of modified live Aujeszky's disease vaccine which contained a pestivirus contaminant. The syndrome was reproduced experimentally and the serological responses to HCV were found to be inconstant, of low titre and slow to develop, quite unlike those usually seen in HC. Affected piglets were persistently-infected, but although horizontal transmission of virus to in-contacts occurred readily, it lacked pathogenicity for the pig post-natally. The investigation showed that BD virus had been introduced into the vaccine by the lamb kidney cell substrate. This is the first record of an incident of this kind in pigs, but disastrous results have followed the use of sheep pox (159) and orf (160) vaccines contaminated with pestiviruses of ovine or bovine origin. It is probable that other incidents have gone unreported and there is a need to examine more strains of ruminant pestivirus for their fetopathic potential in swine.

No comprehensive review of BVDV fetopathogenicity exists but the reader is referred to excellent reviews of togavirus (128) and BVDV (129) congenital infections and to a comprehensive BD monograph (161). Undoubtedly the interpretations we have presented are partly speculative, but they are supported by a wealth of observational and experimental data. A clearer understanding of the pathogenesis of fetal pestivirus infections should result in better recognition of naturally occurring disease, which is certainly under-diagnosed at present. Persistent infections generated 'in utero' are of great importance in the epidemiology and pathogenesis of pestivirus-associated diseases in postnatal life.

3. THE LEGACY OF INTRA-UTERINE INFECTION

3.1. Clinical and pathological sequelae and their relation to immune responses.

There is no evidence that the calf or lamb, born with circulating antibody and with destructive lesions, harbours infectious virus. Yet in sheep the antibody response to the virus has been shown to persist long after birth. Antibody titres in the affected newborn rise progressively for months, attaining levels much higher than those

encountered after postnatal infection (33,56,73,95) before falling slowly to normal levels. The serological picture thus suggests that viral antigen persists and continues to be presented to the immune system. Support for this interpretation comes from evidence that BDV antigen persisted for at least six months after birth (70) in association with lesions of nodular periarteritis observed in lambs infected with BDV between 90 days of gestation and the first week of life (93).

The clinical signs in lambs with severe intracranial malformations closely resemble those of calves surviving naturally occurring Akabane virus infection (73,162) and are consistent with the presence of central vestibular syndrome (163). Perinatal mortality is high unless lambs are cared for individually; survivors maintained in housed conditions show a depressed growth rate (159, P.L.Roeder, unpublished). Similarly infection of the bovine fetus soon after the onset of immune responsiveness can be associated with post-natal stunting of the calf (35,97). Superficially these individuals may appear normal at birth but close examination often reveals locomotor dysfunction caused by CNS damage (42). The long term prognosis for animals which have responded immunologically to infection in utero is unknown in terms of their viability and weight gain.

Many immediate losses are directly attributable to severe congenital defects which make it difficult for the weak newborn animal to obtain milk and seek shelter. The variable pathology reported makes it difficult to unravel the direct cause of such losses, which are probably multifactorial. Mortality is also high in persistently-infected neonates (56,74,91,97,125,135). Many deaths are undoubtedly due to the superimposition of other infections on the underlying dysfunction caused by the pestivirus. Pigs which die in the first few weeks of life frequently exhibit haemorrhages in the skin and internal organs reminiscent of acute postnatal HC (64,125). In calves, an acute fatal diarrhoeic episode may occur and a necrotising enteritis has been described (57,111); pneumonia and joint pathology have also been recorded (57,97). Weaning appears to be a particularly traumatic period with many deaths at that time (57,164,165) and in lambs subsequent development of a chronic and ultimately fatal illness has

been described in which abnormal susceptibility to parasitic infestation was implicated (166). Growth retardation is a common adjunct to persistent infection in all three species, varying in extent from the barely recognisable to dramatic stunting (34,55,75,125,131,163,167,168). The mechanism of growth retardation is not established, but by analogy with rubella (169) a growth hormone deficiency may be involved, in addition to direct effects of the virus on cell growth; cells of the pituitary gland are commonly infected (170).

It must be stressed, since it is of major epidemiological significance, that although persistent infection is frequency disclosed by overt clinical signs, a significant proportion of animals are clinically normal and their subsequent development may be unremarkable (56,57,88,91,97,171,172). Such persistently-infected cattle have been detected commonly in field surveys (55,65,66,173) and their prevalence in the adult cattle population in Denmark has been stimated to be as high as 0.9 per cent (173). Clinically affected calves and lambs may recover (74,88). In contrast, persistently-infected pigs are often clinically normal at birth and for some weeks or months afterwards; however, all such individuals studied to date have eventually developed a syndrome of anorexia, ill-thrift, depression, conjunctivitis, dermatitis, locomotor disturbance and diarrhoea, terminating fatally before one year of age (91,125,135,167).

Characteristically, animals persistently-infected with pestivirus are born seronegative and after the loss of colostral antibody usually remain seronegative (39,56,63,97). The acquisition of maternal antibody via colostrum has little effect on the persistence of infection or viral excretion (56,73,91,97) but it is possible that the uptake of antibody and intact sensitised lymphocytes from the dam could play a role in neonatal mortality (174). Passively acquired antibody is lost much more rapidly in persistently-infected pigs than in controls (63,123,125) and the antibody half-life in lambs is reduced (56) to about four days compared with 19 days in unaffected lambs (73). The high concentration of free virus in serum makes it likely that antibody is removed by the formation of immune complexes but attempts to demonstrate infectious complexes in the sera of

persistently-infected piglets (63) have rarely been successful.

The majority of persistently-infected animals appear to be immunologically tolerant to the virus they carry, but there are indications that in a significant proportion of animals tolerance may be neither absolute nor permanent (129). Although lacking neutralising antibody, persistently-infected pigs have been shown to develop low level antibody which is detectable by indirect immunofluorescence and precipitation reactions (62,63). Studies in persistently-infected sheep and cattle have largely been limited to the attempted detection of neutralising antibody. The production of antibody in apparently tolerant animals is indicated by the presence of viral antigen-antibody immune complex deposition in the glomeruli of cattle affected by mucosal disease (176). In addition, since it has been convincingly demonstrated that persistently-infected cattle unresponsive to the virus they carry retain the ability to produce antibody to an antigenically different strain of virus (65,66,172), it is possible that in studying groups of affected lambs assembled from different sources the neutralising antibody demontrated may have been a response to cross-infection with one or more antigenic variants of virus. Other observations show that persistently-infected sheep can develop low titre antibody transiently and intermittently, and some undergo frank seroconversion after many months of apparent tolerance (56,58,72,73). In one study in sheep (73) superinfection was considered to be an improbable event, and failure to isolate virus from clotted blood after the appearance of neutralising antibody suggested that the antibody neutralised the persisting virus. In no case did the development of antibody in a persistently-infected animal result in the elimination of infection.

The most dramatic manifestation of pestivirus infection in cattle is that of MD and there is now little doubt that persistent infection resulting from intra-uterine infection early in gestation is a necessary prerequisite for its development. This concept has evolved over many years (34,65,66,176,177,178) but the factors which precipitate the acute disease episode are still unclear. Post-vaccinal reactions caused much concern in the USA (182) and the similarity between naturally-occurring MD and the acute disease episode sometimes

provoked by the vaccination of persistently-infected cattle (65,66,179) has suggested a common pathogenesis.

3.2. The significance of viral cytopathogenicity

The superinfection hypothesis has been forcefully expounded by one group (181) who reported an association between the isolation of cytopathic BVDV and MD. It was suggested that cytopathogenic strains of virus have significantly different biological properties and that MD ensues when cattle persistently-infected with non-cytopathic virus are superinfected with a cytopathic strain. Fatal MD occurred within three weeks of challenge in two cattle which had previously been persistently-infected but asymptomatic. Concurrent work in the USA yielded very similar results in five out of six superinfected cattle (175). The hypothesis has been criticised on the grounds that it does not adequately explain the observed epidemiology of the condition (182,183) but it is clear that the presence of a cytopathic pestivirus is associated with late-onset disease in persistently-infected sheep (59) and cattle (181,184). The analogous situation has not been reported in pigs.

Cytopathology is the result of a complex interaction between virus and cultured cells and perhaps not always a reliable marker for 'in vivo' virulence. There is therefore a need to identify genotypic markers which have a consistent relationship with 'in vivo' virulence. Biochemical analysis showed that cells infected with cytopathic BVD virus contained a virus-coded polypeptide with a molecular weight of about 87,000 daltons which was not present in cells infected with non-cytopathic BVDV derived from the same MD-affected animal (185). A similar observation was made while studying the differences between cytopathic and non-cytopathic BDV strains (186), and recent studies using monoclonal antibodies which reacted only with cytopathogenic strains of BVDV also indicated significant differences in the viral polypeptides (306). The importance of these observations has yet to be determined. It is unlikely that the pathology of MD results directly from cell damage caused by infection with cytopathic virus since infection with cytopathic strains produces few clinical signs and minimal pathology in normal susceptible cattle, but it is possible that

the immune responses to viral antigens might precipitate disease. A complex interplay between antigenically differing but otherwise closely related strains of virus (184) may thus be of central importance in the pathogenesis of MD. The "mucosal disease-like syndrome", consisting of diarrhoea, respiratory distress and death, evoked in persistently-infected sheep by superinfecting virus (179) may not be truly analogous since the observed lesions were essentially inflammatory and lymphoproliferative. No signs of illness occurred in other lambs superinfected with homologous or heterologous virus isolates (129).

In our studies the occurrence of MD among persistently-infected cattle has frequently followed a pattern in which an index case in a single animal precedes by up to one month the development of clinical MD in other members of the group. The index case might follow the occurrence of a mutation in the noncytopathic virus causing the persistent infection, altering the mutually tolerant relationship established in utero and initiating the mechanism which results in MD. It is to be expected that the mutant virus would spread rapidly to other animals in contact; the outcome in other persistently-infected cattle may then depend on the precise form of their tolerance to the virus. Viral mutation is a chance event and could occur at any time, and this partly explains the observation that MD occurs at any age in post-natal life. The probability of explosive outbreaks of MD clearly increases in large groups of persistently-infected animals. Viral mutation is now included in a modified superinfection hypothesis (184). Whether in addition, it is possible that other factors may upset the balance between virus and host, breaking the tolerant state and inducing MD, has yet to be determined.

The chronic disease syndrome observed in pigs persistently-infected with HCV seems different from MD. One persistently-infected pig superinfected with the virulent Brescia strain of HCV survived for even longer than susceptible uninfected pigs similarly challenged (125). However, the non-cytopathic nature of the superinfecting virus may be important to the interpretation of this result.

3.3. Immune responses in persistently-infected animals

High post-natal mortality rates and evidence of increased susceptibility to intercurrent disease have fuelled much speculation on the subject of "immunosuppression" in persistently-infected animals. Few studies have been made of immune responsiveness in such animals before the onset of terminal disease, which may be the cause, rather than be the effect of, immune dysfunction. For example severe immunosuppression might be expected to result from the lymphocyte necrosis present in persistently-infected pigs terminally, and is contributed to or caused by adrenal hyperplasia (187). The reduced mitogen responsiveness of lymphocytes in cattle affected by MD has been attributed to a non-specific antistimulatory effect of autologous serum (65) although a similar result has been reported for purified lymphocytes (188). A low proportion of lymphocytes bearing surface immunoglobulin has also been reported in affected cattle (189).

Van Oirschot's studies of persistently-infected pigs (62) showed little abnormality in these responses of lymphocytes to mitogen stimulation or in antibody response to porcine parvovirus among clinically normal persistently-infected pigs. The mean antibody titres elicited by the inoculation of sheep erythrocytes were "even slightly higher than those of the control". As in MD-affected cattle, mitogen responsiveness declined as late onset disease progressed (62,63).

Evidence of immunological abnormality has been found in lambs exposed to infection 'in utero', although it was clear that "immunosuppression" was too superficial a concept to explain the observations (73). Lambs showed a reduced delayed type hypersensitivity response after BCG vaccination and yet generated enhanced antibody responses to several antigens. The abnormal responses were less marked in older animals and if control sheep of the same age had not been available it is likely that the conclusions drawn would have been different. Studies of this kind include only animals which survive the neonatal period and they are almost certainly the least severely damaged. More severe immunological dysfunctions probably leads to early death.

It has been implied that immunological abnormalities are a function of persistent infection, but in this study (73) both

persistently-infected lambs and lambs with cavitating CNS lesions and an immune response to the virus exhibited similar dysfunctions. The indications are that persistent infection may not itself be responsible for the altered immune responsiveness. A major abnormality seen in both classes of sheep is damage to neural elements in the CNS. This might be expected to result in the release of oligodendroglial and/or myelin antigens and could link the neural damage to the observed immunological modulation. Antisera raised against fetal human brain tissue marks about 20 per cent of human T cells (190) and cultured ovine oligodendrocytes share at least one antigen or receptor site with human T suppressor cells (191). No such relationship exists with cultured human, calf and rat oligodendrocytes (192). It is conceivable that neural damage associated with fetal CNS pathology might release neural antigens capable of stimulating an immune response which is cross-reactive with sub-sets of T lymphocytes, thus modulating their activity. Depressed peripheral blood lymphocyte counts and T lymphocyte counts observed in BD lambs are consistent with this hypothesis (73). A relationship of this kind between oligodendrocyte antigen and the modulation of T-suppressor cell function by lymphocytotoxic antibody may be important in the pathogenesis of human multiple sclerosis (192). Brain antigens showing common determinants with other lymphoid elements have been described (192). There is also support for this hypothesis from studies of antimyelin antibodies, which are a consistent finding in BD lambs and persist for some months after birth (193).

There has been no report of correlation between the presence of antimyelin antibody in lambs and the lesions associated with the maturing immune response, but one report (194) concerns the detection of antibodies to myelin basic protein in the sera of two lambs affected by "hairy shaker disease" (BD). One of the lambs had a rising neutralising antibody titre to BVDV when tested at 10 and 20 weeks of age and may be representative of the immune response-associated form of BD.

In the absence of active oligodendroglial breakdown the cross-reactive antibody response might be expected to wane, resulting in the amelioration of immune dysfunction. In turn this could explain

why, apart from MD, the high mortality in congenitally-affected sheep and cattle occurs early in life. Calves and lambs which outlive the critical early period have little evidence of immune dysfunction and often survive for years (56,110). Variation in the intensity of immunomodulation could result from differences in the severity of neural damage, itself partly a function of viral strain characteristics. HCV may induce more severe immunological damage than ruminant pestiviruses and thus appears to be less well adapted to its host. The raised immunoglobulin level demonstrated in pigs exposed to intra-uterine infection does not represent anti-viral antibody; its specificity has not been determined (197). Persistent infections may enhance immune dysfunction since pestiviruses have an affinity for macrophages and antigen handling cells (73,123,125,197,198,199,200) and a reduced neutrophil function may contribute to increased susceptibility to intercurrent infections (73).

Thus, tolerance to pestivirus and deviant immunological function may be two completely separate phenomena, tolerance arising from the presence of virus antigen in the lymphocyte differentiation environment at a critical time in ontogeny. Immune dysfunction may be an autoimmune disease arising as a side effect of neural damage.

Despite all the phenomena which mitigate against the survival of persistently-infected sheep and cattle, a significant number survive to breeding age and vertical transmission results when these animals are bred (34,55,58,72,110). Clinical evidence of the damage resulting from deviant differentiation after maternal infection early in gestation may be evident at birth (58,72,73); mortality can be high in the neonatal period (55) and MD may supervene later (34). Surviving lambs and calves perpetuate the virus both vertically and horizontally into the next generations (72,88).

4. ACUTE POSTNATAL INFECTIONS

The characteristics of BVD infection of the bovine fetus described in the preceding sections necessitate the adoption of a radically different view of the biology of the virus, incompatible with some of the ideas expressed in earlier texts. It is, however, based upon recent work in several laboratories throughout the world and supported

by both experimental and field studies. The revised concept explains the association of the virus with two clinically different diseases, one of which (MD) is highly fatal and the other which is relatively benign (BVD). They also explain the past failure of many investigators to reproduce MD experimentally, even with unpassaged material from outbreaks in which mortality rates were high (202,203,204). Putative variations in virus strain virulence were thought to account for these findings; the missing element in these studies was persistent infection which, as we have indicated above, is an essential prerequisite for development of MD (34,171,205).

4.1. Clinical responses to acute infection

One major difficulty that this new perspective on pestivirus infections brings is that many early studies of the field disease in cattle undoubtedly described herds in which both MD and BVD were present, and in retrospect it is not easy to establish the severity of the clinical condition associated with the acute postnatal infection, which we refer to as BVD. The earliest reports contain descriptions of disease in dairy cows in which infection was followed by an acute diarrhoeic episode, profound (though temporary) drop in milk yield, pyrexia and depression (1,206). It seems probable that these observations are a true reflection of the pathogenicity of the virus in postnatal life but in the light of the recent findings there is a need to re-evaluate its significance as a primary pathogen and whether it causes disease of economic significance. Such information can best be gathered through cohort studies on infected farms.

As a rule, the clinical picture seen after experimental BVDV infections is much milder than that described for field disease. Typically, after a 3-5 day incubation period experimental calves show a pyrexia of up to 41C, which may last only 24-48 hours but can be diphasic in character. Leucopoenia is common and may precede the febrile response by several hours. It is accompanied by variable levels of depression, anorexia and nasal discharge, and a viraemia which may be detectable from about day 3 and persist up to about day 15 post inoculation; the illness is of short duration and recovery is generally complete within 12 days. Respiratory symptoms, diarrhoea and

erosive lesions of the oral cavity are relatively uncommon, but more severe reactions of this type are recorded and relapsing viraemia and virus persistence in lung and lymphoid tissue for up to three months or more has been claimed for two American BVDV isolates (137,138). It is possible, therefore, that BVDV strains may exist which have some of the attributes of those HCV strains which are not rapidly fatal and are able to produce chronic infections persisting for long periods of time (136). Studies on this scale and with this number of field isolates have not been undertaken with BVDV. Route of infection, age of experimental animal and passage history of virus inoculum may also be important in determining the severity of the clinical reaction (207). However, it is difficult to escape the conclusion that, in general, postnatal infection causes a comparatively trivial illness. The discrepancy between the high prevalence of serum antibody in many cattle populations (44,208,209,210,211,212,213) and the relative lack of clinical disease attributable to the virus supports this view, suggesting that the great majority of field infections are undetected.

4.2. Immune function during acute infection

In spite of this, infection with BVDV may be of considerable significance in postnatal life. Of particular importance in this connection is the rapidly accumulating body of data which shows that acute infections with the virus causes temporary immunosuppression of the host, and although much of this information is derived from 'in vitro' laboratory studies its practical importance is becoming increasingly evident. The virus has an affinity for cells of the immune system and an increasing amount of evidence indicates that cell-mediated immunity plays a central role in recovery from viral infections. The virus is known to suppress interferon production in cell cultures (214), and to depress immunoglobulin synthesis by bovine spleen cells in vitro (215). Monocyte chemotaxis is impaired (216) which is of importance since the accumulation of macrophages at local sites of inflammation is especially relevant to defence against infectious agents (217). The response of peripheral lymphocytes to various mitogens is also reduced (218,219,220). The ability of the virus to replicate in cultures of lymphocytes and macrophages prepared

from peripheral blood has been demonstrated, (222,223) and may be important in explaining hyporesponsiveness to mitogens. Response to T-lymphocyte stimulation is depressed in peripheral blood lymphocytes collected within one week of exposure of cattle to BVDV (224), and may be explained by the observation that the virus causes a decrease in the absolute numbers of circulating B and T lymphocytes lasting for about seven days (225,303). Some vaccinal strains of virus are undoubtedly capable of suppression of lymphocyte function (221).

Another effect observed after BVD infection is a defect in polymorphonuclear (PMN) leukocyte function compounded by a marked decrease in the number of circulating PMN (226). This state may both facilitate infection with the virus and predispose the animal to secondary viral and bacterial infection, since bovine PMN are known to be one of the most active cell types mediating antibody-dependant cell-mediated cytotoxicity against, for example, herpesvirus infected cells (227).

Few comparable studies have been conducted with HCV. Van Oirschott and others (228) showed that a low virulence strain depressed the response of peripheral blood lymphocytes to B cell mitogens, and absolute numbers of circulating B cells were reduced. Later it was found that low virulence HCV did not inhibit antibody responses to lipopolysaccharide or sheep erythrocytes (229,230), and a transient hyporesponsiveness to T cell mitogens together with a significant increase in B cells in lymph nodes was observed. These results apparently differ substantially from those for BVDV, although the procedures adopted were not directly comparable.

4.3. Role of pestiviruses in other disease complexes

Further indications of depression of normal host defences by BVDV were obtained by Reggiardo and Kaeberle (224) who demonstrated interference with clearance of endogenous bacteria from the bloodstream. In another study, clearance of IBR virus from the lungs of experimentally infected calves was reduced, allowing spread of virus to many tissues (231). Recently, concurrent BVDV infection was shown to significantly increase virus shedding in vaccinated calves

challenged with field strain IBR virus (232). These and other observations are of particular relevance to bovine respiratory disease (BRD).

There is evidence that BVDV may be important in BRD. Under experimental conditions the virus by itself produces only mild respiratory tract lesions characterised by interstitial pneumonia (233,234,235). Using the Oregon C24V strain of BVDV which had been passaged 28 times in tissue culture, Lopez and others (236) found no effect on the capacity of the bovine lung to clear inhaled P. haemolytica at 3, 5 or 7 days after infection. The results were of interest since in laboratory animals decreased bacterial clearance rates are correlated with susceptibility to pneumonias (237,238). However, Potgieter and co-workers showed that superinfection with P. haemolytica 5 days after exposure to a low passage field isolate of BVDV produced a severe fibrinopurulent bronchopneumonia and pleuritis involving 40-75% of the lung (239). This work has since been repeated and extended (240) with two field strains of virus, one of which was cytopathic and the other non-cytopathic. The cytopathic strain induced more severe disease providing further evidence of the significance of biological variation among BVDV strains.

Experimental studies of this kind do not provide information about the importance of the virus in BRD in the field. Circumstantial evidence has been used to implicate BVDV in this context (241,242,243,244), but there are surprisingly few published investigations into the association between infection with the virus and the occurrence of BRD. In one outstanding study (245), however, a significant relationship was established between infection and field disease. There was no clear temporal association, but the result was obtained in spite of the use of sampling methods and laboratory techniques that were less than optimally sensitive. Thus, while the relative importance of this agent in the BRD complex is not firmly established, the immunosuppressive effects of the virus and demonstrated ability to potentiate respiratory tract disease experimentally indicate that further attention must be paid to this aspect of its biology. In addition, BVDV may be expected to enhance the pathogenicity of organisms not involved in BRD, either through its

general immunosuppresssive effects or by facilitating spread. This facet is largely unexplored.

4.4. Post-natal BD virus infections in sheep

The consequences for sheep of primary infection with BD virus in postnatal life have received relatively little attention. Before the first description of BD, Huck (202) transmitted an agent from cattle affected with MD to sheep, and passaged it in sheep by transference of blood. Pyrexia was observed at 5-8 days after inoculation, associated with inappetance and diarrhoea. By contrast, tissue cultured BVDV produced no clinical signs (67,145,246) in experimentally infected sheep, though Ward (145) recorded febrile responses in ewes given a non-cytopathic strain of BVDV. Similarly, sheep inoculated with spleen tissue from a steer with MD were pyrexic at 2-4 days after exposure (67). Pestivirus isolates from sheep with typical BD have produced conflicting results. In the earliest experiments (67) neither pyrexia nor leucopaenia were observed, though animals had a brief viraemia and virus was recovered from kidneys, thymus and lymph nodes of ewes killed at 15 days post-infection. Most later workers found no clinical abnormality in ewes or lambs (12,32,76,104,247,248) but a few record pyrexia with or without neutropaenia (103,249,250). The only reports of pathological lesions following infection are those concerning placentitis (107) and a nodular periarteritis (93) or non-suppurative encephalitis (80) in young lambs infected by the intracerebral route. The clinically mild nature of the infection was confirmed in recent experiments in which sheep were infected by contact with persistently-infected lambs from three different flocks, by parenteral and intranasal inoculation of two tissue cultured BD virus isolates, and by intracerebral inoculation of a third (73,80). A transient low grade pyrexia and transient leucopaenia was accompanied to a variable extent by slight ocular and nasal discharge, anorexia, depression, tachypnoea and the passage of altered faeces. Nasal shedding of virus was minimal, helping to explain the low rates of transmission observed on several occasions (37,251,252), and both neutralising and precipitating antibodies were late to appear. The clinical reaction, virus shedding patterns, leucopaenia and kinetics of antibody responses

resembled those of calves infected with isolates of BVDV (207,221,224,226,253). Acute postnatal infections with pestivirus in sheep must therefore be regarded as relatively benign.

Acute infections in early life are likely to occur more frequently with the adoption of more intensive husbandry practices, and concurrent infection with BDV and other agents could become economically important. Some information about the effects of acute BDV infection on immune function in sheep is emerging. Recent experiments (73) showed that the lymphocytopaenia following BDV infection is accompanied by minimal changes in the numbers of circulating 'B' (SmIg-positive) lymphocytes but a marked depression in the number of 'T' (peanut agglutinin-positive) lymphocytes and the parallel with results for BVDV in cattle (225) is evident. A fall in T cell populations can be expected to compromise many immune functions, particularly cell-mediated functions. Delayed-type hypersensitivity responses were also depressed, indicating a profound effect on cell-mediated immune function. In addition, in these experiments the random migration potential of peripheral blood leucocytes was reduced, an effect which would interfere with participation by these cells in immune function by reducing their ability to respond to chemo-attractants and gain access to extra-vascular sites. In concurrent infections with Chlamydia psittaci, in the control of which cell-mediated immune mechanisms are probably of vital importance, disease was very much more severe than with either agent alone (73). These results require verification, but suggest that postnatal infection may play an unrecognised role in sheep disease through these mechanisms.

Experimental postnatal infections of sheep with BDV are followed by seroconversion, sometimes within three weeks but more usually at between three and five weeks later (13,14,67,254). Once established the humoral antibody can persist for many months or years (255,256) but in the absence of reinfection declines progressively, and can become undetectable within three years (73).

4.5. Acute BVD virus infections in pigs

BVD virus isolations from pigs under field conditions have been reported on a number of occasions. Fernelius (156) isolated a

non-cytopathic BVD virus from a sow and her litter on a farm where pigs were kept in close contact with cattle, and used it to produce disease experimentally in neonatal calves. Another report records BVD virus isolation from naturally-infected swine (152), and more recently isolations have been made from pigs in the Netherlands (257). The frequency of BVDV detection in pigs was extremely low in the latter case, amounting to only 12 isolates in 9 years from material from over 21,000 farms. These are remarkably rare encounters in view of apparently widespread antibody to this virus in some pig populations. Some of the evidence for BVDV infection in pigs in USA was reviewed by Carbrey and others (258) who recorded two specific-pathogen free pigs born in the laboratory which had precolostral BVDV antibody, suggesting prenatal infection. Also observed were pigs in close contact with BVD vaccinated cattle which had antibody to the virus, and BVDV antibody in pigs fed slaughterhouse offal. Experimentally, pigs placed in contact with cattle with MD developed neutralising antibody to BVDV, and resisted challenge with virulent HCV (259).

The pathogenicity of BVDV for pigs requires further study, but there is no published evidence suggesting a constant association between infection and clinical disease on farms with antibody. Many workers have infected pigs experimentally and infection has almost always been clinically silent (155,258,260,262,262), although in one study febrile reactions were recorded in some pigs exposed to the NADL strain of BVDV (263). Evidence of viral replication in the form of detectable viraemia was observed in a proportion of pigs only. In another study virus of bovine origin could not be re-isolated in porcine cell culture and was thought to replicate poorly in the live pig (264). Carbrey and others (258) found indications of a relationship between reproductive problems and BVDV infection in pigs, but experimental infection of the pregnant sow (157) produced transplacental infection in only one of twenty animals, although the virus strains used had been passaged several times in porcine cell cultures. If this result reflects the situation in the field, BVDV is unlikely to be a pathogen of economic significance for the pig industry.

Cross-protection against HCV produced by BVD vaccines is apparently strain dependant and therefore unreliable (25,260,261,262)

and the practice of using live BVD vaccines to protect pigs has been discontinued in most countries.

It is conceivable that there are variants of BVDV with differing biological characteristics, one of which is the ability to infect pigs with ease. Few of these infections result in clinical disease, but continual cycling of BVDV in pig populations could enhance pathogenicity resulting in significant losses. The elucidation of the importance of BVDV in pigs is partly dependant upon the availability of more precise methods, such as monoclonal antibodies (306,307,308,312), for the characterisation of virus strains, and their application as epidemiological tools.

In summary, it may be concluded that the pestiviruses of ruminants do not usually cause significant economic losses following postnatal infection, and are not thought to be pathogenic for pigs. Present indications are, however, that they do cause profound, if transient, immunosuppression in both cattle and sheep which may potentiate the development of concurrent infections with other agents. Low virulence strains of HCV may have similar effects on the pig, though the mechanisms are possibly different. The incidence of variants of the ruminant viruses which do produce clinical disease is unknown, but probably low. This contrasts with HC as it occurs in many countries, though not with HC observed in some parts of Europe in recent years (260).

5. EPIDEMIOLOGY AND CONTROL

5.1. Routes of Transmission

As we have already seen, congenital persistent pestivirus infections of farm animals are characterised by generalised infection of the host. Cattle, sheep and pigs can show a lifelong viraemia, and virus is present in many tissues, especially in epithelial and endothelial cells and cells of the reticulo-endothelial system. In consequence, unlike the 'carrier' animals of diseases such as salmonellosis or pseudorabies, they shed substantial amounts of virus continually. Virus can be isolated regularly from oral and nasal secretions and urine and, particularly under intensive management systems, the weight of infection accumulating in the environment can be

great. In-contact non-immune animals become infected easily and the rate of spread is often very high. In addition to horizontal spread of virus to in-contacts, vertical transmission takes place through spread to the fetuses of persistently-infected females. The live bovine and ovine offspring of these animals may themselves be persistently-infected, thereby perpetuating the virus in the next generation. How frequently this occurs is unknown: it has not been recorded among pigs congenitally infected with low virulence strains of HCV, but among cattle and sheep with BVDV or BDV it is well documented (72,110,183). Meyling's observations that about 1% of apparently normal cattle going to slaughter in Denmark were persistently-infected with BVDV (173) suggests that enough calves born persistently-infected survive to breeding age to make this mechanism important in the perpetuation of infection. Apparently healthy persistently-infected individuals are undoubtedly of major significance in transmission of all three pestivirus-induced diseases, and their removal from the population may be expected to have an important effect on the incidence of new infections on affected farms.

Transmission rates after acute postnatal infections are poorly defined, but a number of observations suggest that for BVD and BD viruses rates of spread are comparatively slow. BD virus rarely transmits to contacts from acutely infected sheep presumably reflecting the minimal amounts of virus shed in excretions and secretions (37,67,103,251,252), and paddocks grazed by ewes and their BD affected progeny and isolation pens which had housed infected lambs did not constitute a risk for susceptible sheep introduced later (38). It might be expected that under intensive housing conditions transmission of virus would occur more frequently, but even among cattle which are housed or yarded, spread of infection can be slow (302,303,309,312). This contrasts with the behaviour of virulent strains of HCV, which spread rapidly, but parallels observations of low virulence strains (167). The implication may be that viral biotype is as important as husbandry systems in determining transmission rates. A knowledge of the rate at which pestiviruses spread in particular circumstances is important in helping to predict the extent and duration of disease outbreaks in the field and appropriate mathematical models can help to

identify key factors in disease control. They may also indicate under what conditions acute infections are able to sustain themselves in the absence of persistently-infected animals.

5.2. Viral stability

The stability of BVDV and BDV in the environment, and in meat and meat products has received much less attention than other aspects of their biology. For HC virus, considerably more work has been completed and although further studies are required, several important questions have been answered, particularly with regard to survival in foodstuffs, a route of transmission of HCV known to be of major importance in some countries. It is exceedingly improbable that the food chain plays any important part in maintaining infection cycles in ruminants. It would be important to know, however, if BVDV survives in meats or meat products and is a significant source of infection for pigs fed kitchen or abattoir waste. Tissues from persistently-infected cattle would be a potent source of virus, and Carbrey and others (258) have already documented one incident in which pigs may have become infected by this means. It is of interest that serological reactions in pigs to BVDV occur at relatively low prevalences in countries where waste food is heat-treated before it is fed to pigs. Excretion and survival of virus in milk may also be important. Whether persistently infected cattle excrete virus in milk is unknown, but if this is the case virus might survive in whey fed to pigs.

More relevant to intra-species spread of infection is the persistence of BVDV in manure and slurry, and in water supplies, in buildings and in the face of disinfectants and disinfection procedures. The data available at present relate principally to laboratory experiments concerned with virus inactivation by various physical and chemical agents (265) and do not answer questions relevant to disease control. The possibility of airborne spread must also be considered. There is no indication that this is a significant route of transmission at present; the influence of relative humidity and temperature on virus survival would need to be measured in order to assess its importance.

5.3. Serological evidence of virus distribution

Primary postnatal infection with BVDV in cattle results in the production of serum neutralising antibody, which may be detectable as early as sixteen days after experimental infection, but more usually does not appear until three to four weeks and may occasionally be delayed even longer. The presence of neutralising antibody correlates well with immunity to challenge (266), but antibody has not been shown to be the basis of that immunity. Data from Kahrs (212) indicate that antibody levels persist for many years, but the interpretation of these results has been challenged (267) and the persistence of antibody requires further study. Except in the first six months of life when colostral antibody may be present (212,268), the presence of neutralising antibody thus reflects exposure to the virus at some unknown time in the past. In cattle populations, the prevalence of antibody is often in excess of 60% in adult animals (44,208,209,210,211,212,213). In sheep, antibody prevalence is much more variable (49,246,274,275,276,277,278,279,280) and may have a patterned geographical distribution, perhaps reflecting differences in husbandry practices. In countries where HC has been eradicated, the prevalence of antibodies to BVDV in pigs is often very low, in spite of a high incidence of BVD in cattle. Conversely, in some areas where HC is widespread, BVDV antibodies in pigs are also widespread (257,276). The explanation for this contrast may lie with the differences in the amount of contact between pigs and other species, the characteristics of the circulating virus strains, the use of BVDV-contaminated porcine vaccines or, as already indicated the control measures over the feeding of waste food to pigs. The first and last factors should be examined in seroepidemiological studies, and the second by investigations to determine whether rates of transmission of BVDV among pigs are sufficiently high to sustain the virus in herds of various sizes. Control over vaccine quality is a matter for national regulatory authorities.

Whatever their origin, antibodies to BVDV in pigs are of importance in the control of HC. Experimental evidence (25,260,261,262) suggests that such antibody could suppress clinical HC, making it more difficult to detect newly infected herds rapidly.

Attempts to differentiate HC infection from BVD infection on the basis of serological examinations alone are sometimes necessary and place a considerable additional burden on laboratories. Monoclonal antibody panels may help in the identification of viral isolates, but distinguishing between the infections on the basis of serological reactions to the two agents can be difficult and time consuming. Experiment suggests that primary infection with BVDV followed by super infection with HCV could give false negative results. False positive results might also arise, when HCV-like strains of BVDV are used for differential serology (28,277). As a corollary the importance of the choice of test antigen in serosurveillance studies is apparent, and the need for more discriminating serological methods self-evident.

5.4. Pestivirus reservoirs in other species

Other species are probably involved in the epidemiology of ruminant pestiviruses. Human and equine sera do not carry antibodies (213,278) but there is a wealth of serological evidence, gathered on a worldwide basis, that the majority of free-living or captive ungulate species harbour, or are occasionally infected by, one or more antigenically-related pestiviruses (284,285,286,287). Since there is no means by which serological responses to ovine and bovine pestiviruses may be differentiated, the origin of these antibodies is unknown. Hamblin and Hedger (279) considered that some free-living antelope species might be reservoirs of infection. Few attempts have been made to isolate pestivirus from such animals, but isolates have been obtained from various species of deer in Hungary (283), Germany (284,285), New Zealand (286) and Scotland (287), sometimes associated with fatal enteritis and pneumonia, or fetal death. The contribution that reservoirs of infection of this kind might make to infection cycles in farm livestock should therefore be noted.

Variable, usually modest, seroprevalence rates have been reported in goats from many regions of the world, including Africa (274,288), the USA (289,290), Australia (44) and Europe (149,255). Experimentally, BDV infects goats eliciting an antibody response and producing in the offspring of the susceptible pregnant dam many of the clinical and pathological features characteristic of BD (12,147),

except for the absence of interfascicular lipid droplets in the CNS (148). Loken observed a natural outbreak of BD in a goat herd in Norway (149) and in another study a non-cytopathic pestivirus was isolated from the lungs of a kid which died at 4 months of age (291).

Inter-species spread of bovine and ovine pestiviruses generally appears to take place readily. Calves and piglets placed in contact with persistently-infected BD lambs rapidly underwent subclinical infection and seroconverted to BVDV (56,58) and so did most of a group of ten pregnant heifers experimentally exposed at about 50 days gestation to a tissue suspension prepared from BD lambs (146). Nine of these later aborted or failed to calve and three aborted fetuses showed severe growth retardation and cavitation and oedema of the cerebral white matter with no evidence of a macrophage reaction. One also showed cerebellar abnormalities, including Purkinje cell necrosis, rarefaction of the internal granular layer and oedema of the white matter core. The same tissue suspension given to pregnant sows at the 34th day of gestation produced equivocal evidence of fetopathogenicity (153). Two cell cultured BD virus strains given to bacon weight pigs failed to evoke clinical disease, but seroconversion was observed and one strain protected against challenge with virulent HCV (48). The significance of this experimental evidence is that the potential clearly exists for spread to the other species from sheep.

The facility with which the bovine pestiviruses cross species barriers has already been discussed and the complexity of the epidemiology of the ruminant pestivirus infections of cattle, sheep and pigs now becomes apparent. There is no evidence that HCV infects ruminants under natural conditions, though these species have been shown to be susceptible experimentally (150,310). The increasing evidence of biological variability among HCV strains (31) suggests the possibility of spread to cattle or sheep from pigs on rare occasions. Modern farming practices make it unlikely that this is of significance in the control of HC but the current dogma concerning inter-species transmission in this direction may need re-examination.

5.5. Potential spread through breeding activities

Embryo transfer is a potential route of spread of pestiviruses

little considered until recently. At the time of writing there is no published information on the ability of HC to transmit by this means. For BVDV, a single report indicates that the virus is not taken up by pre-implantation embryos (292), but doubts concerning the methodology employed led the authors to express the need for confirmatory observations. BVDV given by the intrauterine route had a significant effect on conception rates in seronegative cattle but not in seropositive cattle, or seronegative cattle infected by intranasal and oral routes (102) and the conclusion was that the virus is unlikely to be a major cause of repeat breeding. In a later study, the results of two separate experiments showed that the presence of BVDV in the uterus at the time of breeding interfered with fertilisation (100). Shedding of virus in semen has been reported on a number of occasions and could interfere with conception rates in seronegative cows and heifers where natural service is used. Natural exposure of seropositive cows by venereal contact with a bull whose semen contained the virus did not result in the birth of calves with evidence of prenatal infection, although an average of 2.3. services were required for conception (55). Poor semen quality is a notable finding, but is not directly attributable to the persistent infection of the bull with the virus (311). Intrauterine antibody may be important in neutralising the virus in these circumstances; it has been suggested that in sheep BD virus may persist in the uterus after insemination with infected semen, even in the presence of circulating antibody (12). This is an experimental result which requires further investigation.

It seems important to ensure by appropriate laboratory screening that bulls acting as AI donors are not persistently-infected with BVDV, though many such individuals are probably rejected on the basis of poor performance and fertility records. Adverse effects may be important for those pedigree breeders and beef herds using natural service and whose selection of the breeding male is based on evaluation of non-performance related characteristics. The importance of the persistently-infected breeding male may be adduced for sheep flocks. In contrast, the survival of congenitally infected piglets to breeding age has not been demonstrated and, in any case, the intensive nature of pig production is likely to highlight suboptimal breeding performance

at an early stage.

5.6. Control considerations

The epidemiology of all three pestivirus infections is complex, but there are sufficient similarities to suggest that all the viruses had a common origin in evolutionary history. The indications for control of the ruminant pestivirus diseases are, however, rather different from those for HC control. Present information suggests that most of the economically important losses in cattle (and sheep) occur as a direct result of transplacental infection, and that the majority of postnatal infections are of comparatively little consequence. An exception may be concurrent infection with pneumopathogenic organisms, but the size of the contribution such interactions make to the overall problem of BRD is not accurately known. The role of BVDV in calf pneumonia will have to be re-examined but it appears that the majority of identifiable losses are associated with fetal death, congenital malformations, neonatal losses, poor growth rates and the late sequel to fetal infection which we have referred to as mucosal disease. The high incidence, ease of transmission, frequent inapparent infection and presence of non-bovine hosts make eradication of ruminant pestiviruses an unreasonable objective and for these reasons any national control program is likely to be difficult to operate and of uncertain gain to the livestock industry as a whole. At the local level, the requirement to control the disease depends primarily on the results of cost-benefit analyses, and these vary greatly with the circumstances of the individual farm. Closed cattle herds containing valuable pedigree stock of unknown immune status are most vulnerable to the introduction of infection. On the other hand, many herds are partially immune, suffering only sporadic losses. The disease problem then becomes difficult to identify and costs are hard to establish. The decision to intervene with control measures may not be easy to justify in every case.

Mention has been made of the key role that persistently-infected animals play in the spread of infection. Where control measures are considered appropriate, identification of these individuals is an important preliminary to further action. Methods designed for this

purpose have been available for some years (34,173). Once identified, persistently-infected cattle can be removed from the breeding programme and kept isolated from pregnant stock. Further measures which have the aim of boosting levels of immunity in female cattle before breeding may then be expected to break the cycle of infection and prevent future losses following fetal infection.

The use of vaccines against these viruses is problematical. Live vaccines have been widely deployed in some countries and several types of adverse reaction are recorded (180,222,293). Vaccination in early pregnancy may result in fetal infection with vaccine virus, causing all the effects attendant upon infection with field virus (97,117) and is to be avoided at all costs. Vaccination in the last two months of gestation has been recommended (294) but the consequences of this practice are not well studied. The fetus is growing rapidly at this age and the growth check which could occur in utero as a result of infection with vaccine virus may be a serious disadvantage to the newborn calf. The safest and most efficacious age at which breeding cattle can be vaccinated is therefore between six months of age, after colostral antibody has disappeared and before the time of first service. Similar recommendations might apply for sheep flocks.

Live BVD vaccines also present not inconsiderable hazards for feedlot cattle. Martin and others (295) found in the course of a large epidemiological study in Canada, that the use of such vaccine was significantly associated with increased mortality rates in beef calves. Some of these losses may result from the potentiation of intercurrent infections by vaccine virus (221). In other cases losses are probably the result of vaccination of persistently-infected cattle, a procedure which appears to precipitate the development of MD in a proportion of cases (180). For the breeding herd operating a planned control program, this represents a problem largely confined to a single generation of animals, avoided by pretesting stock at risk. In feedlots it may be virtually unavoidable. In the past, vaccine testing procedures have not generally included assessments of protection for the fetus and safety for persistently-infected individuals, both of which must now be considered.

The disadvantages of live vaccines have led to the development of

inactivated vaccines (296,297,298,299,303) which avoid these adverse reactions and are claimed to be sufficiently immunogenic to provide protection. Much more work is required on this aspect. In addition to the difficulties identified above, the extent of cross-protection between BVDV strains remains unclear. Castrucci and others (300) after a study of three strains of BVDV concluded that there was a reciprocal protective response to BVDV strains which were serologically quite different. Unfortunately, experiments of this kind conducted in 4-6 months-old calves reveal little about the ability of challenge virus to cross the placenta and infect the fetus. The experimental evidence concerning the efficacy of post-exposure immunity in providing protection against subsequent challenge with pestivirus in sheep is conflicting (12,38,249,256,277). The role which reinfection with an antigenically different virus strain might play in field vaccine performance is thus far from clear. The position is complicated by the suggestion that host genotype, immune status of dam and viral biotype interact to vary the pathogenesis of the disease (77,301).

Vaccines undoubtedly have a place in the control of ruminant pestivirus diseases, as they do in the control of HC in certain circumstances. At the present time, the safety and efficacy of existing BVD vaccines requires review. Some of the available vaccines may be satisfactory in their present form, but the improved knowledge of the epidemiology of pestivirus infections in cattle and sheep has added to the list of essential vaccine characteristics both the ability to stimulate immunity in the dam which protects the fetus, and avoidance of the adverse reactions discussed above. It is imperative that the many questions surrounding vaccines be resolved and that studies with inactivated vaccines should be continued. The scope for a new generation of vaccines, developed using the techniques of modern biotechnology, is large, and offers the real prospect of markedly more efficient control of the pestivirus diseases of domesticated ruminants.

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