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Pleural Effusion Disease in Rabbits. Properties of the Aetiological Agent

By

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With 1 Figure

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Summary

The size and heat sensitivity of Pleural effusion disease (PED) agent or virus (PEDV) propagated in rabbits were examined. The infectious particles were estimated to be between 25 and 50 nm by filtration. Residual infectivity of infectious serum was 0.1 per cent after heating at 56° C for 4 hours.

PEDV and the Stockholm agent appeared identical concerning pathogenic and immunogenic properties by infection experiments and protection tests in rabbits. Two of the three PEDV isolates were less pathogenic but appeared immunogenically identical to PEDV. The third isolate, obtained from the laboratory, which several years previously had supplied material for demonstration of the Stockholm agent, differed from PEDV in pathogenic and immunogenic properties.

Serological examinations of paired rabbit sera did not indicate any antigenic relationship between PEDV and representative members of the two mammalian coronavirus antigenic groups. It is concluded that the aetiological agent of PED is a virus not belonging to the coronaviridae.

Introduction

Pleural effusion disease (PED) agent is found as a passenger in rabbit testicular suspensions of *Treponema pallidum* and causes subclinical to fatal infections in rabbits (1, 6, 17, 22). PED has been reported as a new disease (1) and another name proposed for it is rabbit infectious cardiomyopathia (19). The disease has been known since the 1960s (4, 9), but as yet the agent or virus (PEDV) has not been convincingly demonstrated by tissue culture or electron microscopy. A

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remarkable feature of PED is the long persisting viraemia (1, 3); nevertheless, the infection induces clinical protection and there is also evidence indicating that protective IgG antibodies are produced as a result of the infection (3).

During a survey in 1978/79, isolates were obtained from several laboratories. All these isolates induced clinical protection to challenge with PEDV, except one (SBL) from Stockholm which induced only partial protection (2).

SMALL *et al.* (19) have suggested that the Stockholm agent, which was brought to the Johns Hopkins University School of Medicine, Baltimore in 1970 (5), might be a coronavirus antigenically related to human coronaviruses (HCVs) 229E and OC43. This concept was supported by OSTERHAUS *et al.* (18) who observed coronavirus-like particles in rabbits infected with PEDV. However, in this study we show that PEDV appear to be smaller and more heat resistant than coronaviruses, and that PEDV and related isolates are antigenically unrelated to representative mammalian coronaviruses by enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

PEDV Isolates

Stock PEDV consisted of pooled rabbit sera obtained 48 hours after subcutaneous inoculation with pleural fluid or infectious serum. A low virulent variant of PEDV from the blood of a rabbit six months after infection was also used (3). Stock of this variant was infectious serum from an 11th serial rabbit passage, obtained 72 hours after inoculation. The Stockholm agent was kindly supplied in 1980 by Dr. J. D. Small, NIH, Bethesda, U.S.A. as freeze dried infectious serum 73—015. Three other isolates used were from different laboratories and designated SBL, Paris I and Minneapolis (Table 1). Stocks of the Stockholm agent and the three isolates were from the first or second rabbit passage, prepared in the same manner as for PEDV. All stocks were stored at —70° C.

Table 1. *History of isolates from* Treponema pallidum *infected rabbits and titres of prepared virus stocks*

Designation of isolate	Date isolated	Origin	Titre of virus stock
PEDV	22/10/70	Copenhagen	10^6
PEDV, low virulent	27/ 8/80	Copenhagen	10^2
Stockholm agent[a] (73-015)		Stockholm	10^6
SBL	20/12/78	Stockholm	10^4
Paris I	8/12/78	Paris	10^5
Minneapolis	19/ 3/79	Minneapolis	10^4

[a] Brought to Baltimore in 1970 as a contaminant of rabbit testicular suspension of Nichols pathogenic *T. pallidum*

PEDV Titration

The number of rabbit-infectious doses per ml of the virus stocks was determined by making 10-fold dilutions in PBS (pH 7.0) using one to four rabbits per dilution. The term rabbit-infectious dose refers to a dose capable of producing typical clinical responses, i.e. fever together with uveitis or death with characteristic necropsy findings and/or clinical protection to challenge with PEDV 30 days after inoculation. An exception to this procedure was the titration of SBL, where the homologous stock virus was used for challenge.

Coronaviruses and ELISA Procedure

Representative members of the two mammalian coronavirus antigenic groups were used. These were HCVs 229E (7) and CV Paris (20), the latter being closely related to HCV OC43 (8). All mammalian coronaviruses, apart from one or two possible exceptions that have not been fully characterised, are antigenically related by ELISA to one or the other of these strains (8, 13, 21, 23).

HCV 229E was grown in MRC continuous cells (14) and CV Paris was grown in HRT 18 cells (8). The cells were frozen and thawed once and the resulting suspensions were clarified at $2000 \times g$ for 30 minutes. The preparations of HCV 229E and CV Paris contained between 10^7 and 10^8 particles per ml, as determined by electron microscopy.

The ELISA was based on methods described previously for detecting HCV antibodies in rabbit (10) and human (11, 15) sera.

Filtration

Samples of PEDV stock were diluted 1:10 in PBS (pH 7.0) and passed serially through Millipore filter membranes graduated in porosity from 800 to 25 nm. Samples of filtrates were taken for titration in rabbits after passage through 220, 100, 50 and 25 nm filters. Similar experiments were done with HCV 229E and polio virus, type 3, strain Saukett. The infectivity of the HCV 229E samples were determined by fluorescent focus assay (16). The Saukett strain was grown and titrated in primary monkey kidney cells. The culture was concentrated and purified, but was diluted 1:100. (The Enterovirus Department at Statens Seruminstitut supplied the polio virus and performed the infectivity titrations).

Inactivation by Heat

Two ml cryotubes containing PEDV stock, undiluted and diluted 1:100 in PBS (pH 7.4), were submerged in a stirred water bath at 37° and 56° C. At selected intervals thereafter the tubes were removed and the contents immediately titrated in rabbits using chilled PBS diluent.

Rabbits and Sera

Albino rabbits, aged 3—5 months, from the closed colony at Statens Seruminstitut (Ssc: CPH), were used. The majority of these animals had been used once for pyrogen testing of protein fractions of human blood. All inoculations were made subcutaneously.

For serological examination paired sera were obtained before and 30 days after proven infections of groups of four rabbits with PEDV, Stockholm agent, SBL, Paris I, or Minneapolis. The sera were stored at —20° C until examined.

Infection Experiments

Uniform groups of rabbits were infected with the same dose of the various isolates.

The Stockholm agent was passaged serially in rabbits at intervals of 3—10 days and 30 days as described for PEDV (1). The results of the PEDV passages are shown in Table 3 for comparison. Passages of the three PEDV isolates were done in the same way but only at intervals of 3—10 days. The inoculum for the first passage was the original material while 1 ml of blood or pleural fluid was used for the subsequent passages. The low virulent variant of PEDV was also passed serially in rabbits, with an interval of 7 days between passages. A 0.2 ml amount of serum mixed with 0.8 ml of PBS was used as the inoculum. Several rabbits were challenged with PEDV 30 days after inoculation and observed for another 10 days for clinical signs of PED.

Active Immunization and Challenge

Groups of rabbits were inoculated with diluted stock of the low virulent variant of PEDV. Thirty days after inoculation each group was challenged with diluted stock virus of PEDV, Stockholm agent, SBL, Paris I, or Minneapolis. After challenge the animals were observed for 10—12 days for clinical signs of PED.

Similarly, groups of rabbits were inoculated with SBL, Paris I, and Minneapolis and challenged with PEDV, Stockholm agent, SBL, and Paris I.

Results

Estimation of Size of PEDV

Infectious serum diluted 1:10 and with a titre of 10^5 passed the 100 nm filter without loss in infectivity. The virus also passed through 50 nm membrane, but with a 10 to 100-fold loss in titre. No detectable infectivity passed 25 nm filter. Infectious HCV229E particles did not pass the 100 nm filter, and polio virus particles passed the 50 and 25 nm filters with a 100-fold loss in infectivity. These findings indicate that PEDV particles are between 25 and 50 nm, smaller than coronavirus particles and larger than polio virus particles.

Inactivation of PEDV by Heat

The rate of inactivation of undiluted and diluted infectious serum at 56° C was about the same and the calculated half life of infectivity of the two preparations was 0.12 hours (Fig. 1). There was a 1 to 10-fold loss in infectivity of the diluted preparation after 2 days at 37° C, but after 5 days residual infectivity was 0.01 per cent. Stock virus kept at 4—5° C for 5 months showed no loss of infectivity.

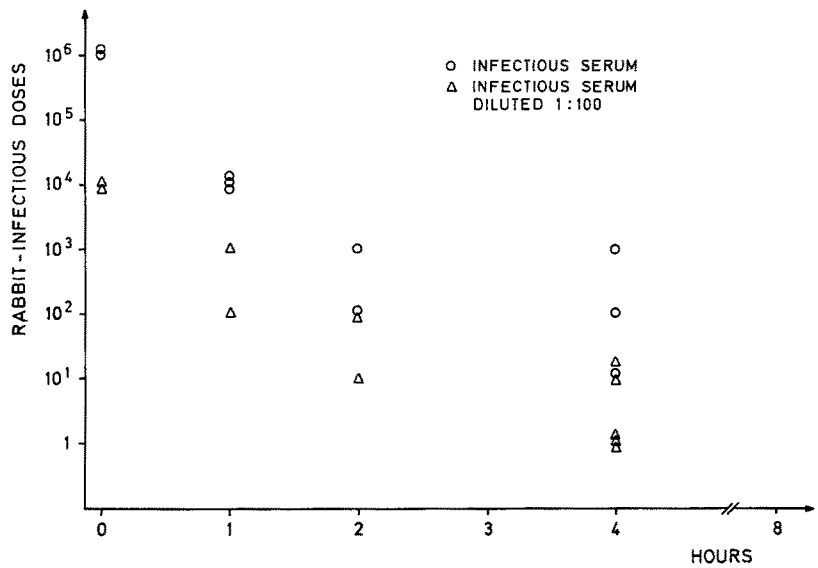

Fig. 1. Inactivation of rabbit propagated PEDV at 56° C

Pathogenicity of Isolates in Rabbits

The pathogenic properties of the isolates were compared directly (Table 2) and by passage in rabbits to simulate the "natural" infection, i.e. a concomitant infection of rabbits used for propagation of pathogenic treponemes (Table 3).

Both the Stockholm agent and PEDV produced high mortality with almost the same incidence of fever and uveitis among the survivors. When passed at an

Table 2. *Comparison of rabbit pathogenicity of isolates*

Isolate	Inoculum[a]	Number of rabbits	Died	Survivors[b] showing fever	Survivors[b] showing uveitis
PEDV	10	8	5	3	3
PEDV, low virulent	10	16	0	3	0
Stockholm agent	10	8	4	4	4
SBL	10	8	0	3	2
Paris I	10	8	0	8	5
Minneapolis	10	8	0	2	0

[a] Number of rabbit-infectious doses
[b] All survivors failing to show fever or uveitis were protected when challenged with PEDV, except the SBL-infected animals

interval of 30 days, there was no mortality and the clinical signs of PED were reduced. This indicates that the two isolates are similar in their pathogenesis and persistence in blood.

Minneapolis and the low virulent variant of PEDV produced a febrile response, but rarely detectable uveitis. The clinical response by the SBL or Paris I was intermediate between these responses. A distinguishing feature of the SBL-infection was the delayed onset of uveitis, i.e. 8th—11th day post-inoculation as compared with 3rd—6th days for the other isolates.

As measured by clinical response only PEDV appeared to increase in virulence during passage.

Table 3. *Comparison of PEDV with other isolates as measured by clinical response during serial passages of isolates and results of challenge with PEDV*

Isolate	Number of rabbit passages	Interval between passages (days)	Mortality (per cent)	Number of survivors	Percentage of survivors showing fever	Percentage of survivors showing uveitis	Result of challenge with PEDV[a]
PEDV	170	3—10	92 (54)	78	87	100	n.d.[b]
PEDV	12	30	0	12	33	42	12/12
PEDV, low virulent	54	7	0	54	56	2	47/47
Stockholm agent	20	3—10	9 (45)	11	91	91	5/5
Stockholm agent	10	30	0	10	50	20	9/9
SBL	48	3—10	1 (2)	47	81	19	4/7
Paris I	21	3—10	0	21	67	62	9/9
Minneapolis	48	3—10	0	48	63	4	10/11

[a] Challenge inoculum: 10^4 rabbit- infectious doses. Numerator equals number of protected and denominator number of rabbits challenged
[b] Not done

When rabbits were challenged with PEDV 30 days after infection, they were nearly always clinically protected, the only exception being the SBL-infected animals.

Protection Tests

PEDV and the Stockholm agent, which both caused high mortality in rabbits, were not used for active immunization. Minneapolis was used for challenge in only one experiment since this isolate does not cause typical PED (Table 4).

The immunizing infections of the various groups of rabbits produced the same clinical syndromes as observed in the infection experiments. When challenged all the rabbits were protected except for the SBL-infected rabbits challenged with PEDV or the Stockholm agent. The majority of animals in these two groups developed PED symptoms, although none died. The SBL-infected rabbits challenged with Paris I did not produce PED symptoms, but several showed a transient ephemeral fever. Challenge with SBL did not cause clinical signs of PED.

These results together with those of the infection experiments demonstrate a similarity in immunogenicity between PEDV and the Stockholm agent, and between Paris I and Minneapolis. The immunogenicity of SBL is evidently different from PEDV and the Stockholm agent.

Table 4. *Results of cross-protection tests between the isolates*

Immunizing isolate (inoculum)	Challenge isolate (inoculum)[a]				
	PEDV	Stockholm agent	SBL	Paris I	Minneapolis
	10^4—10^5	10^4—10^5	10^2—10^3	10^3—10^4	10^2—10^3
PEDV, low virulent (10^1—2×10^1)	10/10[b]	10/10	12/12	12/12	12/12
SBL (10^2)	3/8	1/8	10/10	8/8	n.d.[c]
Paris I (10^4)	5/5	5/5	12/12	4/4	n.d.
Minneapolis (10^2)	4/4	4/4	12/12	4/4	n.d.

[a] Inoculum expressed as number of rabbit-infectious doses, given subcutaneously
[b] Numerator equals number of protected rabbits and denominator number of challenged rabbits
[c] Not done

Examination of Antibody Rises to Coronaviruses by ELISA

HCV 229 E and CV Paris were used as antigens in ELISA to detect antibody rises to coronaviruses in paired sera from 20 rabbits infected with the 5 isolates. A ratio of 2 or more in the absorbance values obtained with postinoculation serum compared to preinoculation serum at the same serum dilution was considered to represent a significant antibody rise. Using this criterium no antibody rises to HCV 229 E were detected in any of the paired sera, whereas only one paired sera from an SBL-infected rabbit showed an antibody rise to CV Paris, i.e. it had a ratio of 2.2. The same results were obtained when the same or other pairs of sera were

examined by a neutralisation test with HCV 229 E (kindly performed by Dr. Sylvia E. Reed, Central Public Health Laboratory, London, England) and in a CF test with HCV OC43 antigen (kindly performed by Dr. T. Hovi, University of Helsinki, Finland).

Discussion

It has been suggested that the Stockholm agent and PEDV are similar on the basis of history and pathology (1, 5, 22), but they have never been compared directly. This study shows that they are very similar if not identical. The results also indicate that the less pathogenic Paris I and Minneapolis are similar to PEDV, and that SBL is probably closely related to PEDV, but differs in its pathogenic and immunogenic properties. This agrees with the observations at isolation, although Paris I did not cause mortality (2).

SBL was isolated in 1978 from treponema-infected rabbits from the laboratory, that had supplied the rabbit material from which the Stockholm agent was isolated in 1970 (2). Attempts in this laboratory to remove the treponemes from the Stockholm agent were made on several occasions. This was done by passaging contaminated treponemal suspensions through hamsters, which are not susceptible to PEDV, but which allow the multiplication of treponemes (2, 6). These efforts appeared to be successful since intercurrent rabbit mortality fell to less than one per cent after 1976. However, the isolation of SBL two years later suggests this was a failure and that the reduction in intercurrent mortality was due to a mutational change in the Stockholm agent.

The apparent stability in the virulence of the isolates during the 20 to 54 rabbit passages may well be due to the number of passages. PEDV had 170 rabbit passages over a 3-year period and during this time the annual mortality increased from 39 to 68 per cent.

SMALL *et al.* (19) and OSTERHAUS *et al.* (18) suggested a role for coronaviruses in PED, while the latter and LAPIERRE *et al.* (12) also reported the association of coronavirus-like agents with intestinal infections in rabbits. Both SMALL *et al.* and OSTERHAUS *et al.* detected coronavirus-like particles in infectious serum. However, in our hands no coronavirus-like particles were detected by electron microscopy in rabbits infected with PEDV (FENNESTAD *et al.*, unpublished results), and by filtration infectious PEDV particles were clearly smaller than infectious HCV 229 E particles. The heat stability of PEDV, which is in agreement with SMALL *et al.* (19), was greater than that reported for coronaviruses (21, 23).

SMALL *et al.* used a CF assay to show that antigen(s) in infectious serum displayed a cross-reactivity with HCVs 229 E and OC43 and that hyperimmune sera from convalescent rabbits contained antibodies to HCV 229 E. These results are surprising as these HCVs have been shown to be antigenically unrelated (13, 15, 23). Our ELISAs show that PEDV does not generally produce antibodies related to HCV 229 E and CV Paris. One of 20 rabbits showed a positive reaction to CV Paris, but this is probably not due to infection with PEDV, as normal rabbit sera contains antibodies to both CV Paris and HCV OC43, but not HCV 229 E, indicating the existence of a possible rabbit coronavirus antigenically related to CV Paris and HCV OC43 (MACNAUGHTON, unpublished results).

Both Small *et al.* (19) and Osterhaus *et al.* (18) left open the possibility that the cause of PED might be a virus different from coronavirus. Our findings agree with this interpretation.

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