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Pleural effusion disease agent as passenger of Treponema pallidum suspensions from rabbits

Survey of laboratories

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SUMMARY Material from rabbits used for the propagation of *Treponema pallidum* in 12 selected laboratories was examined for a viral passenger agent of the treponemal suspensions. An agent which causes clinical or subclinical pleural effusion disease (PED) in rabbits and which is serologically identical or related to the PED agent isolated in Copenhagen was found as a contaminant in treponemal suspensions in laboratories in Europe, the USA, and Japan. The experience gained in the Scandinavian treponematoses laboratories suggests that strains of *T pallidum* contaminated with the PED agent can be purified by passage through hamsters.

Introduction

Pleural effusion disease (PED) is an intercurrent infection among rabbits used for the propagation of pathogenic treponemes. The infection was originally recognised as an intercurrent mortality problem in Scandinavian laboratories using Nichols pathogenic strain for the *T pallidum* immobilisation (TPI) test.[1-3]

The same disease appears to have emerged independently under similar circumstances in France,[4] and there is some evidence to suggest that a subclinical form of the disease occurs in the USA.[5] At present, however, PED appears to be confined to treponematoses laboratories.

The aetiological agent of PED is considered to be a virus which is transmitted as a passenger of the treponemal suspension, but as yet it has not been demonstrated by culture, electron microscopy, or by a specific serological technique.[6] Experiments in rabbits show that the agent can be demonstrated regularly in the circulating blood for 2-30 days after infection. The pathogenicity for rabbits can be varied by manipulation from fatal to subclinical disease. After infection the PED agent multiplies rapidly in the host, reaching titres of about 10^5 rabbit-infective doses per ml of blood within a couple of days. After 3-4 weeks protective antibodies become demonstrable.[8]

PED is considered to be a "new" rabbit disease, characterised clinically by fever, lymphocytopenia, leucocytosis, anaemia, iridocyclitis, anorexia, and death. The most conspicuous finding at necropsy is large amounts (up to 50 ml) of plasma-like fluid in the pleural cavities, hence the name pleural effusion disease.[8] The histopathological findings are minimal. Fatal infections are characterised by reduction of the splenic white pulp and focal degeneration of the thymus and lymph nodes. Later in the infection there is hyperplasia of the splenic white pulp and an increased amount of interstitial follicular lymphoid tissue in the lungs.[9 10]

Various methods have been proposed to alleviate the problem of intercurrent rabbit mortality.[4 11 12] Passage of the rabbit testicular suspension of treponemes through hamsters and back to rabbits appears to remove the PED agent from the treponemes,[11] and this principle has been applied as a practical solution in some of the Scandinavian laboratories.

The widespread use of treponemes propagated in rabbits for various serological tests and for experimental purposes prompted the present investigation for the PED agent in rabbit material from selected treponematoses laboratories. The investigation also provided an opportunity to study the Scandinavian experience with hamster passages of contaminated Nichols strains.

Materials and methods

PARTICIPATING LABORATORIES

Fifteen laboratories from 11 countries were asked to supply material. The laboratories were selected on

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TABLE 1 *Survey of treponematoses laboratories from which paired rabbit sera were received**

Laboratory		*T pallidum strain used*		*History of rabbit mortality*	*Hamster passages of treponemal strain*	*Paired sera*	
No	*Location*	*Designation*	*Received from*			*Date*	*No of rabbits*
Category (a)							
1	Copenhagen	Nichols	London (1972)	+	+	4/4/1979	4
2	Stockholm	Nichols	Copenhagen (1954)	+	+	20/12/1978	6
3	Olso	Nichols	Copenhagen (1969)	+	0	17/10/1978	4
4	Helsinki	Nichols	Copenhagen (1973)	+	+	27/3/1979	4
5	Paris	Nichols	Baltimore (1950)	+	0	8/12/1978	5
6	Utrecht	Nichols	Copenhagen (1975)	+	0	1/11/1978	4
	Utrecht	Utrecht	Local strain (isolated 1967)	+	0	1/11/1978	4
Category (b)							
7	Wrocław	Nichols	Copenhagen (1958)	0	0	9/5/1979	4
8	Tokyo	Nichols	Copenhagen (1967)	0	0	21/7/1979	5
Category (c)							
9	Los Angeles	Nichols	Baltimore (1950)	0	0	26/3/1979	2
10	London	Nichols	London (1952†)	0	0	15/12/1978	4
Category (d)							
11	Atlanta	Nichols	Chapel Hill NC (1961)	0	0	6/2/1979	4
12	Minneapolis	Nichols	Atlanta (1973)	0	0	19/3 and 31/7/1979	4

*Information from laboratories were given in personal communications as follows: No 1, Jørgensen BB (1970-79); No 2, Hederstedt B (1974-79); No 3, Erichsen S, Loe KG (1974-79); No 4, Aho K (1971-79); No 5, Vaisman A, Paris A (1978); No 6, Kruijt BC, Menke HE (1978); No 7, Metzger M (1979); No 8, Sugahara T, Nakagawa M (1979); No 9, Miller JN (1979); No 10, Wilkinson AE (1978); No 11, Clark JW (1979); and No 12, Johnson RC (1979).
†From C W Chacko, St Mary's Hospital Medical School.
+ Positive, 0 negative

the basis of (a) a history of present or past excessive rabbit mortality; (b) having received a Nichols strain from the Copenhagen laboratory without subsequent increased rabbit mortality; (c) having supplied the Copenhagen laboratory with a Nichols strain; and (d) having no mortality problem or exchange of strains with the Copenhagen laboratory.

One laboratory in each of categories (a) and (d) did not respond within the time limit, and one in category (c) was unwilling to supply any material for examination. Table I gives a survey of the participants, which included three of the four WHO collaborating centres dealing with research in treponematoses.

SERUM SAMPLES

Each laboratory was asked to send frozen paired sera from 2-4 rabbits obtained before and 48 hours after intratesticular infection with treponemes. Freeze dried sera were received from two laboratories (Nos 8 and 12), and from another (No 11) two of the four postinfection sera were not obtained until 96 hours after infection. One preinoculation specimen was lost during shipment (No 2). On arrival all sera were stored at −70°C in aliquots of 0·5 ml.

Information was obtained on the origin of the *T pallidum* strain currently used, the total dose of treponemes given to the rabbits, and whether or not the strain in question had been maintained in animal species other than the rabbit. The Scandinavian laboratories, including the Finnish laboratory, supplied more detailed information.

The choice of paired rabbit sera had several objectives. Examination of the normal rabbit serum before inoculation with treponemes served to demonstrate the presence or absence of the PED agent. Examination of the 48-hour postinoculation serum was used to demonstrate the presence or absence of the PED agent in the treponemal suspension given to the rabbit. The primary reason for examining the 48-hour postinfection rabbit serum rather than the treponemal suspension—which is routinely prepared in the second week after treponemal infection—was the enrichment effect secured by giving the PED agent a chance to be in the logarithmic phase of growth.[6]

DEMONSTRATION OF THE PED AGENT

Animals

Conventional albino rabbits originating from the closed colony at Statens Seruminstitut (Ssc:CPH) are considered to be free from PED infection.[8] Observations to the contrary have never been made since the discovery of PED in the early 1960s. Male rabbits weighing 1900-2900 g were used for all inoculations. Before use, these animals had been employed once for pyrogen testing of protein fractions of human blood.

Method of examination

A 0·2-ml quantity of each serum specimen mixed with 0·8 ml PBS (pH 7·0) was inoculated subcutaneously into one rabbit. The rabbit was then observed for fever, iridocyclitis, loss of weight, and death for a period of 10 days. Twenty days after the end of the observation period, all surviving rabbits were challenged by subcutaneous inoculation of 10^3 rabbit-infective doses of the virulent Copenhagen PED agent[6] and observed for another 10 days in the same manner as before. Animals that died were examined as described previously.[8] In five cases the test was repeated either because the rabbit died intercurrently (two animals) or because the necropsy findings did not exclude convincingly a cause of death other than the PED infection (three animals).

Fever together with iridocyclitis or death with characteristic necropsy findings of PED or both after the inoculation were considered as evidence of the presence of the PED agent in the specimens. Challenge 30 days after the serum inoculation served to demonstrate the presence or absence of immunity to the Copenhagen PED agent.

Results

EXAMINATION OF RABBIT SERA BEFORE TREPONEMAL INFECTION

The results of rabbit inoculations with 53 serum specimens from 12 laboratories and the results of the subsequent challenge of these animals with the PED agent are shown in table II.

Only one out of the 53 specimens gave a positive reaction in the rabbit test (table II). This specimen is considered to have been contaminated inadvertently with a postinfection serum from the same laboratory (No 3). Titration of content of rabbit-infective doses in the suspect pair of sera and determination of interferon concentration in all eight specimens (kindly performed by Dr Sv Haahr, University of Aarhus) support this contention.

When the inoculated animals were challenged with the Copenhagen PED agent, all except the above-mentioned rabbit proved to be susceptible to infection and developed clinical signs or showed necropsy findings typical of PED or both. This indicates that none of the rabbits used by the laboratories was harbouring the PED agent before infection with treponemes.

EXAMINATION OF RABBIT SERA AFTER TREPONEMAL INFECTION

The 54 postinoculation sera originated from rabbits

TABLE II *Results of rabbit inoculation with "normal" serum followed by challenge with PED agent 30 days later*

Preinoculation sera			
Laboratory	*No of rabbits inoculated*	*With clinical evidence of PED*	*With clinical protection after challenge*
Category (a)			
1	4	0/4	0/4 (3 died)
2	5	0/5	0/5 (5 died)
3	4	1/4	1/4 (2 died)
4	4	0/4	0/4 (2 died)
5	5	0/5	0/5 (3 died)
6*	4	0/4	0/4 (3 died)
	4	0/4	0/4 (2 died)
Category (b)			
7	4	0/4	0/4 (3 died)
8	5	0/5	0/5 (3 died)
Category (c)			
9	2	0/2	0/2 (2 died)
10†	4	0/4	0/4 (4 died)
Category (d)			
11	4	0/4	0/4 (1 died)
12	4	0/4	0/4 (1 died)

*Represented by two strains of *T pallidum*.
†Two of the four specimens from rabbits given cortisone.

which had each received an intratesticular total dose of $1{\cdot}6\times10^6$ to 2×10^8 treponemes. In seven laboratories the inocula were treponemal suspensions prepared on two to four different dates. In each of the remaining five laboratories the rabbits were probably inoculated with the same suspension.

Table III shows that a positive clinical reaction for PED infection occurred in rabbits inoculated with material from six laboratories (Nos 2, 3, 5, 6, 7, and 8). With the exception of laboratory No 2, surviving animals from this group challenged with the PED agent all proved to be protected, thus indicating that immunity to the Copenhagen PED agent had developed.

Rabbits inoculated with serum specimens from laboratory No 12 showed no clinical signs of disease except for transient fever in two of the four animals. Nevertheless, all these four rabbits were completely protected when challenged, thus indicating the presence in the specimens of an agent of low virulence conferring immunity to PED infection.

A problematic response to inoculation and challenge was observed with the specimens from laboratory No 2. Clinical signs of PED were observed in three of the six animals, but in these three animals the onset of iridocyclitis was atypically delayed. On challenge, one of the six animals developed clinical signs of PED and two of the other five animals became febrile.

No clinical evidence of PED infection could be demonstrated in any of the specimens from five laboratories (Nos 1, 4, 9, 10, and 11), and on challenge all rabbits were found to be susceptible to the Copenhagen PED agent.

REMOVAL OF THE PED AGENT FROM CONTAMINATED NICHOLS STRAINS BY PASSAGES IN HAMSTERS

In the 1960s, the four Scandinavian TPI laboratories experienced high mortality among rabbits inoculated with treponemes. The Copenhagen and Stockholm laboratories carried out passages of their Nichols strains in hamsters to remove the agent causing death among the rabbits while the Oslo laboratory made no such change. The Helsinki laboratory received hamster-passaged Nichols strains from Copenhagen.

The date of initiation of hamster passages, the number of passages, the date of the resumption of the passages in rabbits, and the results of the present examination for the PED agent are shown in table IV.

None of the initial passages of the Nichols strains in hamsters was successful in any of the three laboratories. Admittedly, the rabbit mortality was reduced immediately, but intercurrent deaths typical of PED occurred within one to two years. After the latest passage in hamsters, it would appear that the Helsinki and Copenhagen laboratories have been free from PED contamination for more than five and two years respectively. In the same periods the annual mortality in these two laboratories has been close to zero.

The Stockholm laboratory apparently harbours an

TABLE III *Results of rabbit inoculation with postinoculation serum followed by challenge with PED agent 30 days later*

Postinoculation sera			
Laboratory	*No of rabbits inoculated*	*With clinical evidence of PED*	*With clinical protection after challenge*
Category (a)			
1	4	0/4	0/4 (1 died)
2	6	3/6	5/6
3	4	3/4 (2 died)	2/2
4	4	0/4	0/4 (2 died)
5	5	4/5 (3 died)	2/2
6*	4	3/4 (2 died)	2/2
	4	4/4 (1 died)	3/3
Category (b)			
7	4	3/4	4/4
8	5	4/5	5/5
Category (c)			
9	2	0/2	0/2 (2 died)
10†	4	0/4	0/4 (3 died)
Category (d)			
11	4	0/4	0/4
12	4	0/4	4/4

*Represented by two strains of *T pallidum*.
†Two of the four specimens from rabbits given cortisone.

agent in spite of the hamster passages, but the mortality has been less than 1% during the last three years.

The PED agent still exists in the Oslo laboratory, and the annual mortality during the last three years has varied from 4 to 12%.

It would appear from the results that passages of the Nichols strains through hamsters and back to rabbits were not convincingly successful in removing the contaminating agents in Stockholm and Copenhagen. As yet there are no explanations for these failures, but from the experience in Copenhagen it seems that recontamination may also occur in the laboratory when contaminated and non-contaminated treponemal suspensions for rabbit inoculation are prepared in the same room.

Discussion

Four types of reactions could be differentiated in the rabbit test used. A negative reaction, observed with the preinoculation specimens from all laboratories and also with the postinoculation specimens from five laboratories, shows that neither clinical nor immunological evidence of the PED agent could be demonstrated. The question as to whether previously acquired antibodies against the PED agent were present in these negative specimens cannot be shown by the rabbit test, since undoubtedly such antibodies in the small inoculum used no longer exerted a protective effect at the time of challenge.

A strong positive reaction was observed with the postinoculation specimens from five laboratories (Nos 3, 5, 6, 7, and 8). Inoculation of these specimens resulted in clinical signs or death typical of PED infection or both. The result of challenge shows that the causative agents were serologically identical or closely related to the PED agent isolated in Copenhagen. It is reasonable to conclude that the agents demonstrated after inoculation with treponemes were also present in the suspension of treponemes given to the rabbits, since no evidence of PED infection could be demonstrated in the normal sera obtained before the treponemal inoculation.

Apparently the more pathogenic PED strains were isolated from laboratories which had had high rabbit mortality (Nos 3, 5, and 6), and the less pathogenic strains from laboratories with no history of mortality (Nos 7 and 8). Such a difference in virulence is in accordance with experimental observations on the PED infection.[8]

From this point of view, the third type of reaction is not surprising. The only evidence of PED infection demonstrated in material from one laboratory (No 12) was the development of immunity, thus suggesting a subclinical infection with the PED agent. This strain with a low virulence is now being passed serially in rabbits to be observed for increase of virulence.

The fourth type of reaction was encountered with the postinfection specimens from the Stockholm laboratory (No 2). The provisional interpretation of this reaction is that the agent demonstrated is related to but not identical with the PED agent.

It would appear that the repeated passages in hamsters did not remove this agent from the Nichols strain in Stockholm (table IV). Gudjónsson and Skog,[3] reporting in 1970 on the contamination of the Swedish Nichols strain, mention that the findings in the intercurrent disease in Stockholm were similar to

TABLE IV *Results of hamster passages of contaminated Nichols strains in Scandinavian TPI laboratories*

Laboratory	*Hamster passages*		*Transfer back to passages in rabbits*	*Examination for PED*	
	Onset	*No*		*Date*	*Result*
Copenhagen	Apr 1970	5	Sep 1970		
	Sep 1970	1	Oct 1970		
	Nov 1972	2	Jan 1973*		
	Aug 1976	2	Oct 1976	Apr 1979	0
Helsinki	Apr 1970	5†	Sep 1970‡		
	Nov 1972	2†	Jan 1973§	Mar 1979	0
Stockholm	Aug 1970	1	Nov 1970		
	May 1971	3	Oct 1971		
	Mar 1975	2	Aug 1975		
	Dec 1975	2	May 1976	Dec 1978	+?
Olso				Oct 1978	+

*Rabbit passages temporarily interrupted by storage of treponemes in liquid nitrogen.
†Hamster passages carried out in Copenhagen.
‡Helsinki received twentieth rabbit passage from Copenhagen in April 1971.
§Helsinki received eighth rabbit passage from Copenhagen in October 1973.
+ Positive, 0 negative

those observed in Copenhagen.[1] Therefore, our observations raise the question as to whether the so-called "Stockholm agent" is identical with the agent examined in the present rabbit test.

It should be remembered that the Stockholm agent was also studied at the International Treponematoses Laboratory in Baltimore. A challenge experiment made there caused Gudjónsson *et al*[5 12] to suggest that the local Nichols strain in Baltimore might be contaminated by an agent similar to the Stockholm agent.

The present isolation of an agent from the Minneapolis laboratory conferring immunity to challenge with the PED agent makes this suggestion more credible.

Furthermore, in this survey, the isolate from Minneapolis may represent the most recently acquired contamination of treponemes, provided that the Atlanta Nichols strain was also free from the PED agent in 1973 when the strain was received in Minneapolis.

It has been suggested that the PED agent may be present subclinically for 10 or 20 years in rabbit passages of the Nichols strain.[8] This assumption is supported by the demonstration of the PED agent in material from two laboratories (Nos 7 and 8) which received the Nichols strain from Copenhagen three years before and six years after the recognition of rising intercurrent rabbit mortality in Copenhagen in 1961.

The importance of the PED agent as a cause of rabbit mortality has been reported previously.[1 8] This survey shows that the PED agent may also exist as a silent or unrecognised contaminant in other laboratories which maintain the Nichols strain in rabbits.

The significance of the subclinical contamination of rabbit-passaged strains of *T pallidum* is not known. It should be pointed out that a concomitant PED infection may possibly interfere not only with yield of treponemes from rabbit testes but also with other host reactions, which may lead to hazards in interpretation of experimental results.

Addendum

A recent article by Small *et al*[13] describes the manifestations of rabbits infected with the Stockholm agent, brought to Baltimore in 1970 from Stockholm.[5] The clinical and histopathological observations by these authors are essentially the same as previously described for the PED agent.[8-10] This is in agreement with the notion that the agent present in Stockholm in 1978 is no longer identical with the Stockholm agent.

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