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A RICKETTSIA-LIKE ORGANISM FROM *IXODES URIAE* TICKS COLLECTED ON THE KERGUELEN ISLANDS (FRENCH SUBANTARCTIC TERRITORIES)

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Summary. - A rickettsia-like microorganism was isolated in suckling mice from *Ixodes uriae* ticks collected from penguins breeding on Mayes Island, Kerguelen Archipelago, French Subantarctic Territories. At isolation, this agent mimicked a tick-borne arbovirus. Finally, electron microscopy studies of infected suckling mouse livers showed the presence of inclusions filled with pleomorphic microorganism in the cytoplasm of some hepatocytes, sometimes dividing by binary fission and thus of obviously non-viral nature. No firm serological relationship was demonstrated with *Chlamydia psittaci, C. trachomatis, C. pneumoniae, Coxiella burnetii, Cowdria ruminentium, Ehrlichia canis, E. phagocytophila, E. risticii* or the WSU/1044 agent. The exact taxonomic position of the "Mayes" agent remains to be clarified.

Key words: rickettsia-like organism; Mayes agent; Ixodes uriae; penguins; Kerguelen Islands

Introduction

The Kerguelen Islands are located in the South-Western Indian Ocean (49° S - 70° E) and are equivalent in surface area to Corsica in Europe. Many seabird species have their beeding sites there and their breeding status has recently been reviewed (Weimerskirch *et al.*, 1989). Two endemic tick species parasitize seabirds on Kerguelen Islands, the circumpolar and bipolar *Ixodes uriae* White 1852 and the more geographically restricted *Ixodes kerguelensis* André and Colas-Belcour 1942 (Zumpt, 1952; Wilson, 1970). During demographic studies on seabirds performed in 1990, two of us (O.C. and F.G.) collected a number of *I. uriae* from Rockhopper and Macaroni penguins, cormorants and albatrosses.

When two strains of an apparently identical agent, highly pathogenic for suckling mice (sm), were isolated from pooled *I. uriae* they were considered as probably viral in origin. Previously, at leat 31 tick-borne arboviruses had been isolated world-wide from this tick species (Chastel, 1988).

Table 1. Isolation from Ixodes uriae ticks on Kerguelen Islands in 1990

Date	Localization	Eggs	N	♀	Hosts	Isolate
Feb. 19	Mayes	–	4*	6	Rockhopper Penguin, chick *(Eudyptes chrysocome)*	T 2810
Feb. 19	Mayes	–	2	7*	Macaroni Penguin, immature *(E. chrysolophus)*	T 2813
Feb. 26	Port-aux-Français	–	–	2	Kerguelen Cormorant, chick *(Phalacrocorax verrucosus)*	–
Mar. 20	Mayes	> 100	–	4	*E. chrysocome*, adult	–
Apr. 10	Canyon des sourcils noirs	> 100	–	2	Black-browed Albatross, chick *(Diomedea melanophris)*	–
Apr. 10–11	Canyon des sourcils noirs	> 400	–	33	*E. chrysolophus*, adults	–
Total		> 600	6	54		2

* positive pool

The characterization of this agent proved subsequently that it was not a virus but an intracellular rickettsia-like organism.

Materials and Methods

Tick collection. I. uriae were collected between February and April 1990 from (1) one Rockhopper penguin (*Eudyptes chrysocome)* and one Macaroni penguin (*E. chrysolophus*) on Mayes Island, "Golfe du Morbihan", (2) Kerguelen cormorants (*Phalacrocorax verrucosus*) near "Port-aux-Français", the head-quarters of the Kerguelen administrative district, and (3) Black-browed Albatrosses (*Diomedea melanophris*) on "Canyon des sourcils noirs, Presqu'île Jeanne d'Arc" (Table 1). Ticks were sent alive by sea and/or by air to the Virus Laboratory in Brest. During their transport (minimal delay: two months and a week), some females layed hundreds of eggs that were later also used for virus isolation attempts, along with nymphs and females *I. uriae* (Table 1).

Isolation procedures. Ticks were pooled according to host and developmental stage and processed by classical methods for virus isolation. They were then inoculated intracerebrally (i.c.) into sm and in Vero cells. Isolates were studied and characterized by methods described by Chastel *et al.* (1981). In particular, sensitivity to ether, pH 3.0 and heating at 60 °C for 1 hr was determined.

Histological methods. Sections of whole infected sm or their brains were stained using haematoxylin and eosin.

Electron microscopy. Infected sm brains or livers were processed as previously described (Chastel *et al.*, 1981) and ultrathin sections were examined and photographed using a Zeiss EM 9 S electron microscope at 60 kV.

Antigen and antibody production. Haemagglutination or complement fixation (CF) antigens were prepared by sucrose-acetone extraction of infected sm brains (Clarke and Casals, 1958). However, as this classical method failed to yield any suitable antigen from our isolates (T 2810 and T 2813, see below) we tried to extract antigens from infected sm livers because this method was known to be more suitable for certain arboviruses, such as group C Bunyaviridae (Clarke and Casals, 1958). Antigens were obtained for both isolates when infected sm livers were ground in sterile PBS and clarified at 3 000 rpm for 20 min at + 4 °C. The supernatants were then collected, mixed with an equal volume of Freon 113, agitated from time to time during three days at + 4 °C and finally centrifuged at 10 000 rpm for 1 hr at + 4 °C. The clear supernatants were then used as antigens for CF tests. Immune ascitic fluids (IAFs) were prepared against the two isolates in adult mice using infected sm livers as antigens. IAFs were used in CF tests to identify the isolates and to localize them by immunofluorescence assays in infected sm liver replicas or liver cells following trypsination. After adaption of the two isolates, infected MacCoy cells (Flow Laboratories) provided an excellent immunofluorescent assay for further antigenic analysis.

Serological identification. Antigens to the two isolates were screened by CF using a number of polyvalent or monospecific antisera or ascitic fluids available in the Brest Virus Laboratory, covering a large number of arboviruses, arenaviruses or other viruses. In addition a further lot of antibodies prepared by the Pasteur Institute in Dakar (Digoutte *et al.*, 1991), against many African mosquito-borne arboviruses were included in CF tests. When the non-viral nature of the isolates was suspected, CF tests and immunofluorescent assays were performed on infected MacCoy cells using antibody to *Chlamydia psittaci, C. trachomatis, Coxiella burnetii, Cowdria ruminentium, Ehrlichia phagocytophila, E. canis, E. risticii* and the WSU/1044 agent isolated from an aborted bovine foetus (Dilbeck *et al.*, 1990). IAFs prepared against T 2810 and T 2813 were also tested by CF and immunofluorescent assays using *C. psittaci, C. trachomatis* and *C. pneumoniae* as antigens.

Results

Isolation

Two strains of an apparently identical agent were isolated in sm but not in

Table 2. Sensitivity of isolates to ether, acidic pH and heat

Isolate	Initial titer (log ID_{50}/0.02 ml)	Titer after exposure (log 10 ID_{50}/0.02 ml)		
		Ether	pH 3.0	60 °C (for 1 hr)
T 2810	5.36	$< 10^2$	1.62	$< 10^2$
T 2813	3.75	$< 10^2$	$< 10^2$	$< 10^2$

Vero cells, from *I. uriae* pools Brest/Ar. T 2810 and Brest/Ar. T 2813, both from Mayes Island. These isolates originated from 2 engorged nymphs collected on one *E. chrysocome* (T 2810) and 4 engorged females collected on one *E. chrysolophus* (T 2813). This later bird was an immature stray within the Mayes's Rockhopper roockerie.

On initial passage, inoculated sm were paralysed or found dead 3 to 9 days p.i. During the second sm passage, the incubation time was reduced to 4–5 days, and then remained unchanged during further passages. Strain T 2813 was successfully reisolated three months after the original isolation but not the strain T 2810.

Biological properties

At the 3rd passage, both strains killed 100 % of sm inoculated by either ic or ip route. Adult mice were resistant to inoculation by both routes. Both strains passed through a 200 nm membrane (Acrodisc Gelman). As seen in Table 2, both strains were sensitive to ether, pH 3.0 and 60 °C for one hour. After passages in sm, both strains adapted readily to Vero cells at 37 °C and to MacCoy cells at 30 °C and 37 °C.

Antigenic properties

Using chick erythrocytes, haemagglutination was not demonstrated for either

Table 3. Cross CF tests between isolates T 2810 and T 2813

Isolates	Immune ascitic fluids	
	T 2810	T 2813
T 2810	128/32*	512/128
T 2813	256/64	512/128

* Reciprocal of antibody titer/reciprocal of antigen titer

T 2810 or T 2813. No CF antigen was obtained after sucrose-acetone extraction of infected sm brains. On the contrary, potent CF antigens with a titer of 32–128 were obtained after sucrose-acetone or PBS/freon extractions of infected sm livers. IAFs with a immunofluorescent titer of 2048 were achieved when adult mice were immunized using infected sm livers as antigens. As determined by cross CF tests, the two strains proved to be identical (Table 3).

Both T 2810 and T 2813 antigens were then screened by CF tests with all available polyvalent, group and type virus antibodies. These tests failed to demonstrate any antigenic relationship with any arboviruses, arenaviruses, paramyxoviruses, orthomyxoviruses or other viruses described in the "International Catalogue of Arboviruses" (Karabatsos, 1985). It was thus suspected that our isolates were not tick-borne arboviruses or, if so, highly unconventional ones.

Pathology and electron microscopy studies

By light microscopy, we found no consistent histological alterations in cerebral cortex, cerebellar cortex or midbrain of infected sm. On the contrary, lesions were prominent in the bulb and the spinal cord showing marked neuronal degeneration, oedema, glial infiltration and capillary damage. In the liver of infected sm, alterations were limited to small foci of hepatocyte necrosis and pronounced diffuse congestion.

By electron microscopy, lesions were also found in spinal cord including focal neuronal necrosis, diffuse oedema and marked endothelitis. No clear viral structure was identified. In infected sm livers, inclusions were found in the cytoplasm of hepatocytes. These cells harboured enveloped pleomorphic particles, some of which were observed to be dividing, thus confirming their non-viral nature (Fig. 1, 2 and 3). They appeared rickettsia- or chlamydia-like in morphology. Immunofluorescent assays applied to both infected sm liver replicas or trypsinized liver cells demonstrated the inclusions were specific for the T 2813 isolate.

Antigenic characterization of the agent

Because *Chlamydia psittaci* has been isolated from seabirds in the Northern hemisphere (Hoagen and Mauer, 1938; Mykytowycz *et al.*, 1965) or mites parasitizing seabirds (Terkikh *et al.*, 1961) and since serosurveys have evidenced antibodies to *C. psittaci* and *Coxiella burnetii* in seabirds (Flint, V. B., personal communication) our attention was first focused on these pathogens.

Accordingly, IAF T 2813 was tested by immunofluorescent assay (Bio Mérieux and Biosys) and CF (Hoescht - Behring) for chlamydia and coxiella antibodies. IAF showed low titer antibody to *C. psittaci* (1:32), and negative results for *C. burnetii* (< 1:4), *C. trachomatis* (< 1:16) and *C. pneumoniae* (< 1:16). A polyclonal *C. psittaci* antibody (kindly supplied by Prof. J. Orfila, Chlamydia Reference Centre, Amiens) failed to react (< 1:8) in CF test against a potent T 2813 liver antigen (homologous titer, 1:512).

Fluorescein conjugated monoclonal antibodies to *C. psittaci* and *C. tracho*

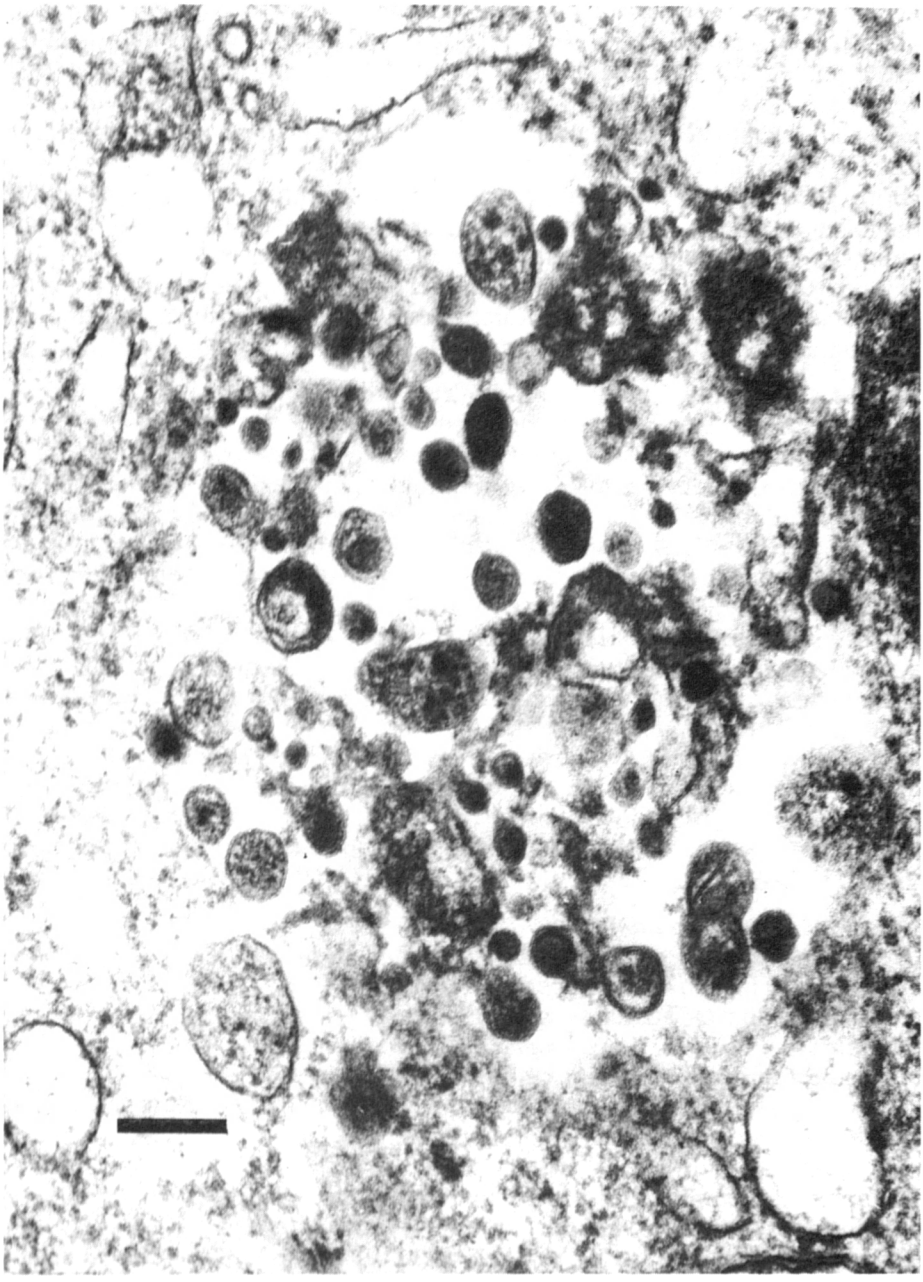

Fig. 1
For legend see page 19

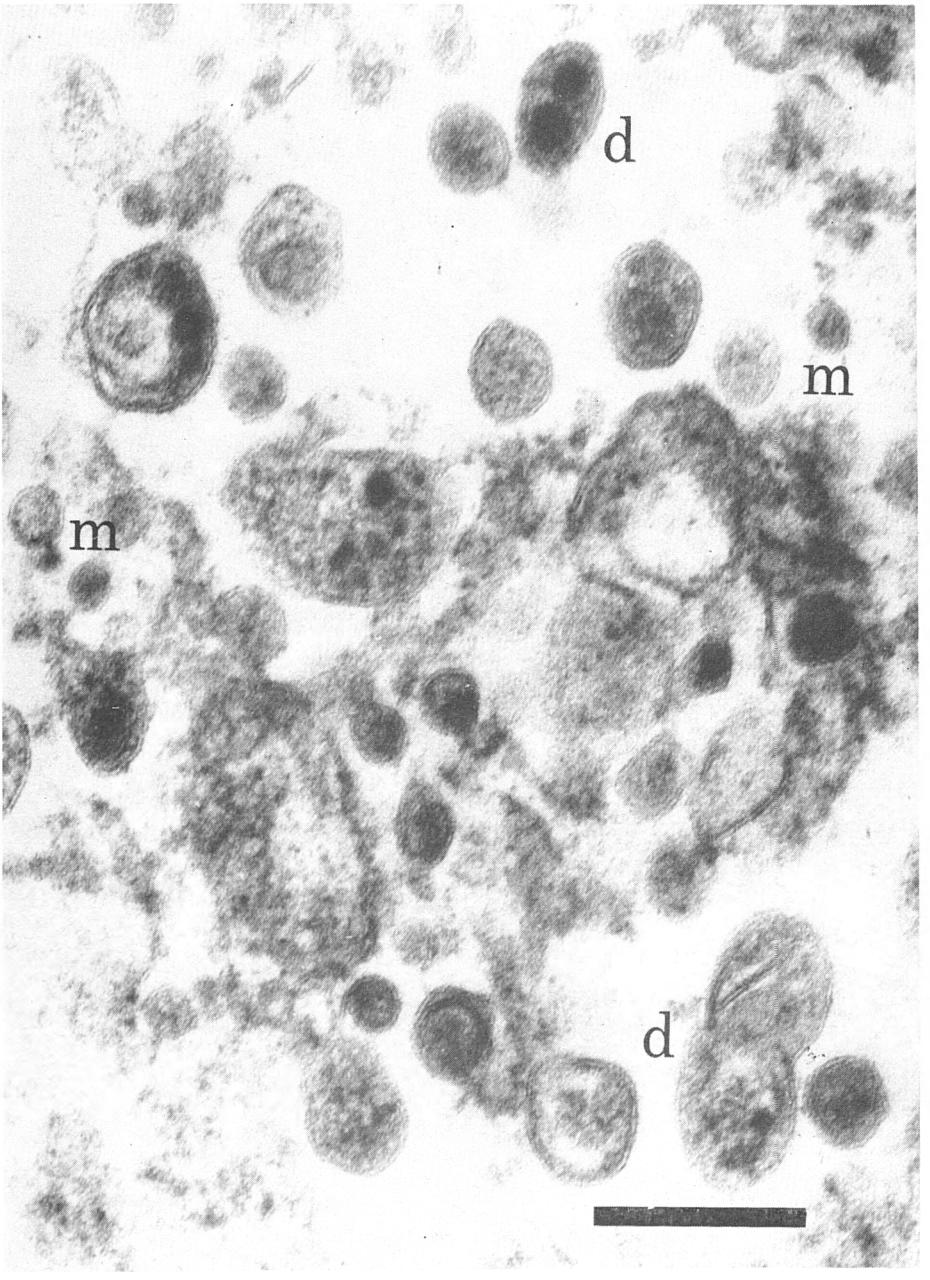

Fig. 2
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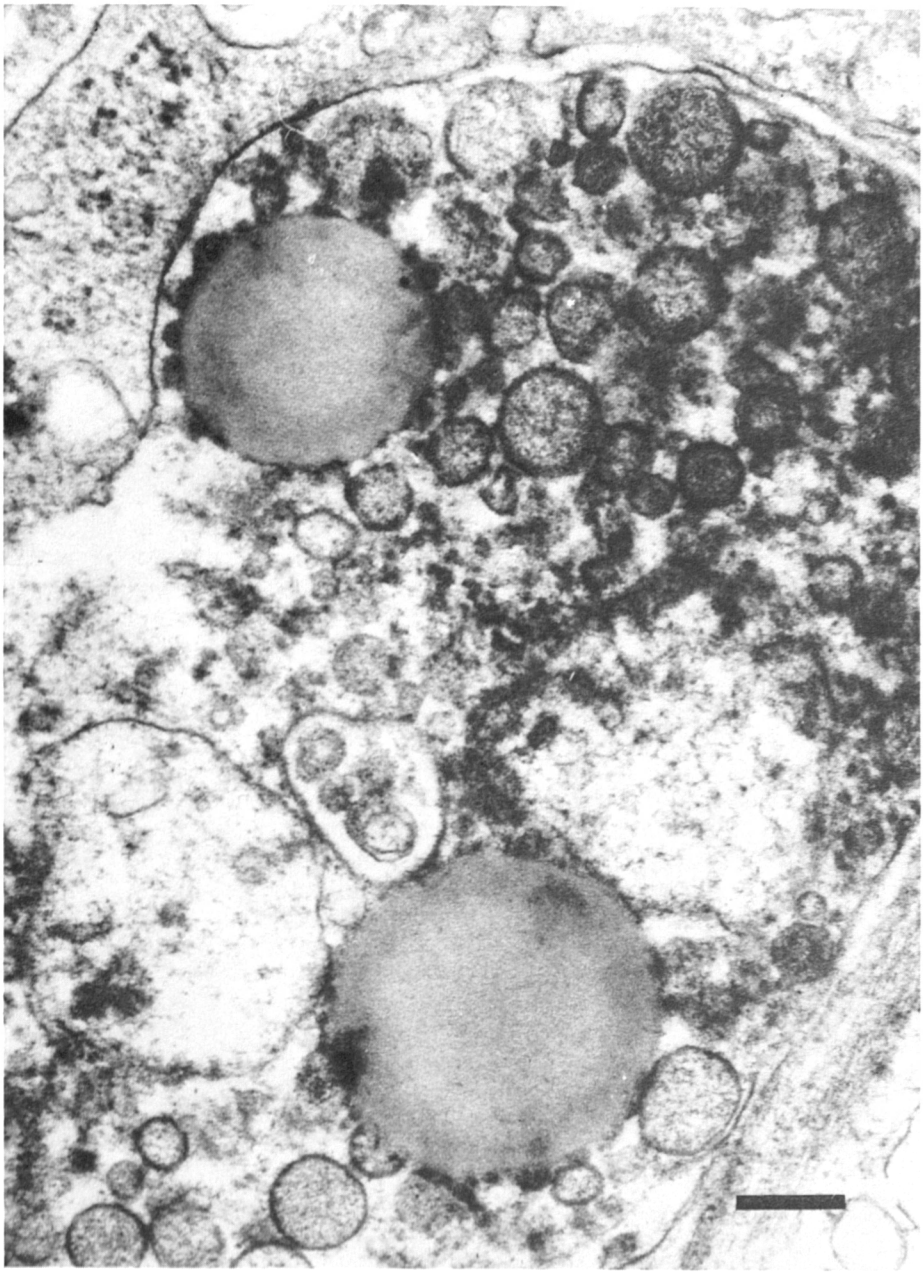

Fig. 3
For legend see page 19

matis (culterested Chlamydia Ortho Kit) did not react in direct immunofluorescent assays with both T 2810 and T 2813 infected MacCoy cells nor did polyclonal antibodies to *C. burnetii, Cowdria ruminentium, Ehrlichia phagocytophila, E. risticii, E. canis* and the WSU/1044 agent in indirect immunofluorescent assays.

Discussion

From our serological and ultrastructural data, it seems clear that isolates T 2810 and T 2813 (provisionally named "Mayes agent") did not correspond to a virus, but to a rickettsia-like microorganism. However, the exact taxonomic position of this agent among Rickettsiales or Chlamydiales remains to be clarified.

The marked pleomorphism of the agent is probably indicative of a complex multiplication cycle which is characteristic of Chlamydiales, including elements of differents shape (70 to 450 nm), variable electron density and occasionally binary fission (Fig. 2). The smaller electron dense elements may represent miniature forms as those of *C. psittaci* according to the definition of Tanami and Yamada (1973) and the larger and more transluscent forms may be equivalent to reticulate bodies (Fig. 3). However, a positive one-way immunofluorescent reaction with *C. psittaci* antigen is insufficient to classify definitely the "Mayes agent" in the Chlamydiales order. In addition, a number of Rickettsiales belonging to genera *Wolbachia* and *Rickettsiella* have been found in ticks, mites and insects. No antibodies were available against these organisms (W. Bugdorfer, personal communication).

Thus, more morphological and biological studies are needed, particularly in cell cultures, before a complete identification can be achieved. On the other hand, it seems important for arbovirologists, and particularly those working on tick-borne arboviruses to be aware of the possibility of isolating such an agent from ticks. It is, therefore, proposed that the "Mayes agent" should be systematically included in screening procedures of unclassified or "new" viruses.

Fig. 1

T 2813 infected mouse liver

Large inclusion in the cytoplasm of a hepatocyte. Note the pleomorphism of the agent. Bar = 0.25 μm.

Fig. 2

T 2813 infected mouse liver

Pleomorphic enveloped elements, sometimes dividing (d) by bineary fission. Some elements are of "miniature" type (m). Bar = 0.25 μm.

Fig. 3

T 2813 infected mouse liver

Another cytoplasmic inclusion. Enveloped elements mimicking the so-called "reticulate" bodies. Bar = 0.25 μm.

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