BACTERIAL CHARACTERISATION AND PATHOGENICITY

Fine structural characterisation of a Rickettsia-like organism in human platelets from patients with symptoms of ehrlichiosis

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Since 1982, Ehrlichia phyls infection has been diagnosed in canines from Venezuela by the use of buffy coat smears. In 1992, ehrlichia-like bodies were observed in platelets from a severely ill girl by light microscopy. The patient was seropositive to E. chaffeensis by the indirect fluorescent antibody test (IFAT). Tetracycline was administered and the patient recovered. More than 400 cases with such intra-platelet organisms have been studied at this laboratory over the past 6 years, and all the patients had a good response to the treatment. To determine whether the organisms in human blood platelets were truly platelet ehrlichiae, IFAT and transmission electron microscopy (TEM) studies were undertaken in four patients. Light microscopic examination of blood samples revealed the dense organism inside platelets, and a great reactivity of the blood cells. Sera from the four patients were seronegative against E. chaffeensis and E. phyls antigens. Three of four samples contained the intra-platelet organisms when examined by TEM. Electron microscopy showed platelets with vacuoles containing pleomorphic organisms. These organisms had a thickened membrane, an electron-translucent inner area and an electron-dense granular component in the periphery. An abundant electron-dense material was observed surrounding them. The ultrastructure of such micro-organisms has not been reported previously. Based on the similarity of many of their characteristics with rickettsiae, we suggest that the micro-organisms found in the present study might belong to the family Rickettsiaceae.

Introduction

Ehrlichiosis is a disease caused by intracellular bacteria belonging to the genus Ehrlichia of the family Rickettsiaceae and affects various animals and man.

Ehrlichia canis, which causes canine ehrlichiosis, was the first ehrlichial organism to be discovered; it was recognised by Donatien and Lestoquard in Algeria [1]. Later, the disease was described for the first time in the Americas [2]. E. canis is known to be transmitted, trans-stadially but not transovarially, by the brown dog tick, Rhipicephalus sanguineus, leading to the conclusion that canids, but not ticks, are reservoirs [3, 4]. Ewing et al. found a granulocytic ehrlichia in dogs [5] which was called E. equi until 1972, when it was demonstrated as new species, E. ewingii [6]. Harvey et al. [7] described ehrlichias in canine platelets, in the USA. A few years later, those organisms were named E. phyls [8] and the disease that this species caused in dogs was called infectious cyclic thrombocytopenia [9].

In Venezuela, all the above mentioned ehrlichias have been detected in dogs since 1982 [10]. Arraga-Alvarado et al. [11] prepared an E. phyls antigen from platelet-rich plasma from an acutely infected dog from Maracaibo in Venezuela; the male patient contained 97% of infected platelets and reacted with serum samples from infected dogs, but not serum samples from man. A transmission electron microscopy (TEM) study was initiated to observe the ultrastructure of infected platelets from dogs in Maracaibo [12].

The first case of human ehrlichiosis in the USA was reported in 1987 [13]; the organism observed in a human blood smear was morphologically and serologically similar to E. canis. Later, Dawson et al.

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reported the isolation in cell culture of *E. chaffeensis*, a new species obtained from a preparation with ehrlichiosis [14]. In 1991, the same species was detected in human blood by PCR [15]. The susceptibility of dogs to infection with *E. chaffeensis* was demonstrated by Dawson *et al.* [16] and it was found that dogs are reservoirs for *E. chaffeensis* in urban areas [17].

In Venezuela, the first case of human ehrlichiosis was reported by Arraga-Alvarado *et al.* [11], in an extremely sick 17-month old girl; some inclusions resembling ehrlichial morulae were observed inside leucocyte cells in a buffy coat smear. Acute and convalescent serum samples were analysed by the indirect fluorescent antibody test (IFAT) at the Centers for Disease Control (CDC) in Atlanta, USA by B. Anderson; anti-*E.chaffeensis* antibodies were demonstrated. This patient also had *E. platys*-like organisms in blood platelets.

No information about the ultrastructure of organisms inside human blood platelets in patients with ehrlichiosis has been reported thus far. The purpose of the present study was to determine whether the organisms observed inside blood platelets by light microscopy were truly platelet ehrlichiae. To achieve this, fine ultrastructural examination of the organisms by conventional TEM and a comparison with *E. platys* in canines from Maracaibo were undertaken.

**Materials and methods**

Since 1992, blood samples from patients with symptoms of ehrlichiosis [11, 13, 18–20] have been sent by physicians from different Zulia State Hospitals and Clinics to the Unit of Clinical Research, Faculty of Veterinary Sciences, University of Zulia. These samples were submitted to detect the presence of ehrlichial organisms (by buffy coat smear) or their antibodies (by IFAT). Four individuals in whom the organisms in blood platelets were most evident on light microscopy were selected from the above group of patients. The Declaration of Helsinki for research in humans was taken into account.

Paired serum samples from the patients were tested by IFAT with two antigens: *E. chaffeensis* (provided by J. Dawson, CDC, Atlanta GA, USA) and *E. platys* (obtained from this laboratory).

Blood samples of healthy individuals, matched by age and sex with the patients, were also taken and simultaneously processed as follows. Blood samples from patients and controls were collected into EDTA-coated tubes. Buffy coat smears were prepared using the microhaematocrit method [21] to concentrate platelets and leucocytes. Smears were stained with Dip Quick Stain (Jorgensen Laboratories, Loveland, CO, USA) for examination of platelet organisms by light microscopy.

To demonstrate the ultrastructure of the intra-platelet organisms, the remaining blood samples were centrifuged at 103 g for 10 min and the platelet-rich plasma (PRP) was separated into centrifuge measuring tubes. This PRP was fixed by gentle addition of glutaraldehyde 0.1% solution, pH 7.3, buffered with sodium cacodylate, to give a final concentration of 0.05%. The suspension of platelets was incubated at 37°C for 10 min and then centrifuged at 1315 g for 10 min at room temperature. The supernate was discarded and the sediment was overlayered on a glutaraldehyde 3% solution (pH 7.3 buffered as above) and held for 72 h. This platelet pellet was post-fixed in buffered osmium tetroxide 1% for 1 h. After block-staining in uranyl acetate 3%, platelets were dehydrated through ascending grades of ethanol and embedded in a low viscosity epoxy resin. Ultra-thin sections were cut with a Sorvall MT-2B ultramicrotome, collected on 300-mesh copper grids, stained with lead citrate and examined with a Hitachi H-7000 electron microscope at 80 kV.

The size of the intracellular structures was determined with a light scale 10× lupe and an ultrastructure size calculator.

**Results**

Between Jan. 1994 and May 1998, this laboratory studied 339 human patients with ehrlichia-like organisms demonstrated in blood platelets by light microscopy. Of these, 92 were diagnosed in 1994, 100 in 1995, 82 in 1996, 35 in 1997 and 21 in the first four months of 1998. Since July 1994, the IFAT with *E. chaffeensis* antigen has also been used. Only seven of the patients gave positive results for anti-*E. chaffeensis* antibody. None of the human patients studied gave positive results for anti-*E. platys* antibody.

More than 95% of the human patients who had this platelet organism had severe illness; only a few cases had mild illness. All the patients recovered after treatment with doxycycline.

Examination of the blood samples from the patients by light microscopy revealed great reactivity of the blood cells: monocytes and platelets with numerous vacuoles, macro-platelets or giant platelets, atypical lymphocytes and immature lymphocytes (Fig. 1). Platelet phagocytosis was visualised in one of the patients and erythrophagocytosis in another (Fig. 2). The platelet granulomere was not so evident, using Dip Quick Stain as with Giemsa’s, thus permitting the observation of any organism inside the platelet.

Most of the platelet organisms were observed as dense bodies with deep purple colour and variable size,
whereas the platelet cytoplasm showed a pale pink colouring (Fig. 3). Occasionally, bodies with a variable intensity of staining were noted. Some of the organisms were so large that they occupied a great part of the platelet cytoplasm. Even though buffy coat smears were used, very few intracellular organisms were observed, indicating that bacteraemia was low.

Sera from the four patients studied were tested by IFAT and were negative with both antigens. Three of four patient samples were shown to contain the organisms when examined by TEM, despite the low number of intra-platelet organisms observed by light microscopy. Electron microscopy revealed aggregates of platelets, which contained vacuoles containing pleomorphic organisms. These organisms were ultrastructurally similar to the *Ehrlichia* species observed in canine platelets when they were examined at low magnifications (Fig. 4).

Some patients showed an increase in the number of peripheral vacuoles inside platelets (Fig. 5a, b). Only one vacuole containing the organisms was observed in each platelet. The vacuole occupied an extensive area of the platelet, and the number of organisms per vacuole varied from 1 to 14 (Fig. 6a). Most of the organisms had an electron-translucent inner zone and an electron-dense granular component in the periphery (Fig. 6b). Most of the organisms were round, but some were elongated. They ranged from 0.1 to 0.8 μm in maximum diameter. Organisms with lobe-shaped structures were observed. All the organisms showed a thickened membrane, but a well-defined double membrane was not observed (Fig. 7a). Fine strands in the central region of the organisms were visible (Fig. 7b). The platelets that contained a considerable number of organisms showed a clear cytoplasm and scarce granulomere (Fig. 8a). Normal platelets from the same sample had an electron-dense cytoplasm and abundant granulomere. An abundant electron-dense material
Fig. 5 (a) Buffy coat smear from a patient with symptoms of ehrlichiosis showing a giant platelet (arrow) with peripheral vacuolisation. (b) Electron micrograph of large vacuoles (arrow) in the peripheral cytoplasm of the platelet.

surrounding most of the organisms was also observed (Fig. 8b), but other organisms were seen inside a clear vacuole.

Discussion

*E. platys* is the only recognised *Ehrlichia* species that infects blood platelets [7, 20, 22]. Organisms resembling *E. platys* have been observed inside human platelets at this laboratory [11]. Similar organisms have also been observed in other animals, such as cats, horses, cows, sheep and goats, by the use of light microscopy and buffy coat smears (Arraga-Alvarado et al., unpublished observations). In experimental infections with *E. platys*, patterns of bacteraemia and cyclic thrombocytopenia take place every 2 weeks [23]. Similar patterns were not to be detected in human patients. A daily evaluation of the patients would have been necessary at the onset of clinical signs to obtain this information.

Because of the morphological similarity, by light microscopy, between the organisms inside human platelets studied here and *E. platys* reported by other researchers [7, 8, 10, 23, 24], IFAT tests with two antigens (*E. chaffeensis* and *E. platys*) were performed. None of 339 patient serum samples gave positive results with *E. platys*; only seven of them were positive with *E. chaffeensis*. Therefore, organisms present in most of the patients might correspond to another species. It is assumed that a mixed infection was present in the seven patients who gave positive results for *E. chaffeensis*.

By the time the patients were seen at this laboratory, considerable time had elapsed since the onset of clinical signs. Therefore, most of them showed severe illness at the time their sample was taken. At this time, there was no established diagnosis and the patients had received different kinds of antibiotic treatment without improvement. Tetracycline was not used. The fact that the patients recovered after doxycycline treatment [25, 26] indicated that the causative agent was probably a rickettsia, because tetracycline has been documented...
to be a broad-spectrum antibiotic with activity against many micro-organisms – particularly rickettsiae, chlamydiae and ehrlichiae [22, 27].

The reactivity of blood cells (lymphocytes, monocytes and platelets) has been reported when ehrlichiae are present in animals and man [9, 10, 12, 13]. The evidence of leucocytes phagocytosing platelets and erythrocytes demonstrated the immunological activity present in some of the patients [9, 22].

In buffy coat smears, a scanty number of human platelets with organisms inside was observed. In contrast, when the same technique was used on canine samples, a large number of infected platelets was observed in 60–70% of naturally infected cases (unpublished observations). The individual organisms inside the morulae of the human platelets were so tightly packed that they could hardly be distinguished by light microscopy. They were similar to the densely packed morulae described for E. canis [22, 28], and to most of the E. platys morulae described in the literature [7, 8, 10, 23, 24]. In this study, loosely packed morulae as described in canines [28] were not observed.

As the organisms described here were inside human platelets, ultra-thin sections of E. platys obtained from canines in Maracaibo were examined first. Then, a comparison with organisms inside human platelets was performed to determine similarities or differences between platelet organisms from man and dogs. The electron density of platelet organisms was similar to that of the host cell cytoplasmic background. This low contrast made it difficult to find the organisms in infected cells at low magnification by electron microscopy, as Rikihisa reported in a study of ehrlichiae [22]. The organisms inside vacuoles in human platelets were not so electron-dense as in E. canis and other ehrlichiae. Sells et al. [29] and Perez et al. [30] have reported that the matrix of these inclusions was filled with filamentous ground substance. In the present study, the inclusions or organisms were not filled with such ground substance; only thread-like material was seen in them.

The platelets with numerous organisms had a clear cytoplasm and scarce granulomere; we believe that the host cells (platelets) had degenerated due to infection, as described by Wright et al. [31], who studied the
 ultrastructure of the Wolbachia pipientis, a rickettsialike microorganism, in the ovaries of mosquito Culex pipiens. The electron-translucent inner zone and electron-dense granular component in the periphery of organisms were similar to those in ehrlichial organisms within feline mononuclear cells [27]. The granular component might be clusters of ribosomes as described in E. equi and E. canis [22, 29, 32, 33]. Fine strands in the central region of the organisms have been reported previously in ehrlichial species as fine fibrils of DNA [7, 22, 29, 32–34]. The lobe-shaped protuberances observed in these organisms could correspond to some stage of binary division.

In the E. platys study of dogs from Maracaibo, the double membrane, characteristic of genus Ehrlichia, was rarely observed surrounding the whole organism. Moreover, in some organisms only a thickened membrane was observed. At low magnification, the organisms from human patients were similar, but not identical to those ehrlichial organisms observed in canine platelets [7, 8, 12, 22–24]. This fact might lead to the suggestion that the studied organisms were closely related to the genus Ehrlichia. Nevertheless, at high magnification, some differences between the organisms from man and canines were detected. In canine organisms, a well-defined double membrane was evident and the intravacuolar space was clear, whereas in organisms from man, the membrane was thickened and the intravacuolar space was electron-dense. In human platelets, the dense material in the vacuole that contained the organisms was similar to that described by Anderson et al. [34]. They identified degenerate Rickettsia rickettsii in dense vacuoles in the host cell cytoplasm, and these vacuoles were reported to be digestive organelles. The organisms observed in the present study were not degenerate; therefore, it cannot be assumed that the vacuole was a digestive organelle. Other authors have reported a fibrillar matrix in the morula of some Ehrlichia species [29, 35]. In the large vacuoles of E. risticii, similar material is either present in small quantities or absent completely [32]. It is not clear whether or not the morula of the platelet-parasitising ehrlichia, E. platys, contains this fibrillar matrix [35].

The membrane of different species within the genus Ehrlichia has been studied by several researchers. For example, Sells et al. [29] studied strains of E. equi, which consisted of individual organisms bounded by two distinct membranes. This was confirmed by other authors in different ehrlichial species [14, 20, 22, 24, 33, 35–37]. Members of the genus Ehrlichia are surrounded by a thin bi-molecular leaflet of outer and inner membranes and, unlike the genus Rickettsia, ehrlichial organisms show no thickening of the outer membrane [22]. The reproduction of ehrlichial organisms takes place inside host cell phagosomes, whereas rickettsial organisms replicate freely within the host-cell cytoplasm [38]. Based on these differences between Ehrlichia and Rickettsia spp. it was difficult to determine the identity of these organisms in human platelets; they were incorporated into a vacuole, as occurs in Ehrlichia and they showed a thickened membrane as does the genus Rickettsia. Final confirmation of genus and species will require sequencing of the 16S rRNA gene.

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References
