THE NATURE OF BANDS IN
PARASITIZED BOVINE ERYTHROCYTES

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ABSTRACT

Anaplasma marginale is the etiological agent of a hemolytic disease of cattle, known as anaplasmosis. The organism appears as a marginal inclusion in parasitized erythrocytes, but certain isolates also have bands associated with the inclusion. Inclusions and associated bands in parasitized erythrocytes in the liver and peripheral circulation were studied by light microscope cytochemistry and electron microscopy. Bands were comet- and dumbbell-shaped by light microscopy and were stained by techniques used to demonstrate protein and fibrin. The same forms, as well as other shapes, were seen in infected erythrocytes which were sectioned and examined by electron microscopy. Bands had longitudinal and transverse periodicity. They did not appear to have a crystalline structure. Their appearance was collated with that of bovine fibrin. Bands were well differentiated in erythrocytes that were extensively hemolyzed by natural or artificial means, but poorly differentiated in mildly hemolyzed erythrocytes. Hemolysis methods appeared to influence the morphology of bands and their demonstration.

Anaplasmosis, a hemolytic malady of bovines, was first recognized as a specific disease in 1895 (18). The acute stage of the disease is readily diagnosed by the presence of marginal inclusions, 0.4 to 0.8 μ in diameter, in infected erythrocytes stained by Romanovsky stains. The nature of the etiological agent, Anaplasma marginale, has not been resolved, but most workers classify the organism as either protozoan or rickettsia. However Bergey’s Manual classifies it as a rickettsia (3). The first electron microscope study (5) of the organism indicated that marginal inclusions, as seen by light microscopy, were composed of several small bodies. More recently, marginal inclusions have been described as consisting of 1 to 8 initial bodies (subunits), surrounded by a mass of undifferentiated matrix (15). Initial bodies were round or oval, 300 to 400 μ in diameter, and enclosed by a double membrane. Isolates from Mexico, California, and Oregon have been reported to differ from other strains by having projections that extend from the inclusion. Such projections have been observed in lysed, infected erythrocytes observed by phase-contrast microscopy or specific staining techniques (2, 6). The nature of projections is not known although specialized methods have been employed for their study, including fluorescein-labeled antibody, acridine orange staining, and electron microscopy of shadowed preparations. According to most reports, these structures are not visible by Giemsa staining. The projections have been described as an integral part of the marginal inclusion (12), as a flagellate (6), as an artifact (17), and as an unknown parasite (10).

The nature of projections, hereafter called bands, present in bovine erythrocytes parasitized by a strain of A. marginale is described in this paper. Interpretations were based on the results of light microscopy of blood smears and the electron microscopy of thin sections and surface preparations of normal and parasitized erythrocytes.
MATERIALS AND METHODS

Acute cases of anaplasmosis were produced in 6 month old splenectomized dairy calves, using an Oregon strain of *A. marginale* as the source of inoculum. When at least 60 per cent of the erythrocytes were parasitized, as determined by the examination of Giemsa-stained blood smears, blood was collected in sterile tubes containing disodium ethylenediaminetetraacetate dihydrate (EDTA). This infected blood was used for all light and electron microscope studies.

Unfixed, heat-fixed, and alcohol-fixed blood smears of infected blood were stained by Giemsa, Goodpasture's stain in pH 7.2 Sorenson's buffer, new methylene blue (16), and Noland's stain (1).

Blood containing EDTA was lysed in a test tube in one of three ways. First, 4 drops of blood were treated with 10 ml of 0.1 per cent acetic acid. In the second method, 5 drops of blood were added to 10 ml of 10 per cent neutral formalin, agitated for 5 minutes, and centrifuged. Five ml of 0.25 per cent aqueous ammonium acetate was added to the centrifuged sediment and the suspension shaken vigorously. The resultant sediment was resuspended in fresh ammonium acetate. In the third method, water was used for hemolysis. Red cell suspensions from each type of lysate were smeared on glass slides and stained with new methylene blue, Noland's stain, mercury-bromphenol blue (MBP) for protein, Perls' reaction for iron, and phosphotungstic acid hematoxylin stain for fibrin. In addition, erythrocyte suspensions from each type of lysate were placed on copper grids, shadowed with chromium, and examined by electron microscopy.

Infected erythrocytes were treated in various ways prior to being embedded in Araldite (11) for thin sectioning and examination with a Philips EM 200 microscope. Acetic acid-lysed erythrocytes were fixed for 1 hour in OsO₄ (4). Blood in EDTA was fixed in 10 per cent neutral formalin for 2 hours, or OsO₄ for 1 hour.

Liver biopsies were obtained from infected calves which had 60 per cent infected red cells. Small pieces of hepatic tissue were fixed in OsO₄ for 2 hours prior to being embedded in Araldite and sectioned for electron microscopy. Thin sections on grids were stained with lead citrate (14), aqueous uranyl acetate, aqueous phosphotungstic acid, or a combination of the last two compounds prior to being examined by electron microscopy. Thin sections of clotted blood from a normal bovine were also observed with the electron microscope. Electron diffraction patterns of infected erythrocytes in thin sections of liver were obtained using the diffraction lens on the Philips EM 200 microscope.

RESULTS

Light Microscopy

Marginal inclusions without observable bands were present in Giemsa-stained blood smears. Marginal inclusions with elongated comet- and dumbbell-shaped bands were apparent in unfixed smears stained with Goodpasture's, Noland's, and new methylene blue stains. Bands were not observed by staining methods in which smears were first fixed with methanol. No reaction for iron was seen in smears stained with Perls' stain; however, in the phosphotungstic acid hematoxylin stain the inclusion stained blue and the bands stained a yellow-pink color. There was particularly good differentiation of inclusions and bands in Noland's stain after non-clotted blood had been treated with formalin and ammonium acetate (Fig. 1). Bands were associated with about

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**Figure 1** Micrograph of parasitized erythrocytes in a blood smear. Notice inclusions (C) with comet-shaped bands (P). Dumbbell shapes (D) are also seen. Noland's stain. X 1500.

**Figure 2** The inclusion (C) appears to be swollen in this erythrocyte lysed with water. A remnant of the band (P) persists. X 15,000.

**Figure 3** The inclusion appears to be normal in size and is composed of subunits (U). The band (P) is altered. The electron-opaque inclusion casts a more prominent shadow (arrow) than the band. Acetic acid-lysed erythrocyte, shadowed with chromium. X 15,000.

**Figure 4** The inclusion (C) appears to be well preserved, and the well differentiated band (arrow) has the form of a cylinder. Formalin-fixed, ammonium acetate–lysed, chromium-shadowed. X 15,000.
70 per cent of the inclusions, but only the bands stained like protein in the MBP stain. Bands were generally closely associated with the marginal inclusion, but sometimes a space separated the two structures. Inclusions, but not bands, were seen in stained smears prepared from erythrocytes lysed with water or acetic acid.

**Electron Microscopy: Surface Studies**

Blood lysed in water or acetic acid, dropped on a grid, and shadowed did not display well differentiated bands. Inclusions appeared swollen in erythrocytes lysed by water (Fig. 2), but not swollen in acetic acid-lysed preparations. However, remnants of bands were seen in erythrocytes treated by both of these techniques (Fig. 3). Formalin-fixed and ammonium acetate-lysed erythrocytes had excellent detail. In this method in which shadowing was employed, bands appeared as elongated cylinders (Fig. 4) which cast a less conspicuous shadow than inclusions. When two or more inclusions were present in the same erythrocyte, they were connected by a band which produced a dumbbell appearance (Fig. 5).

**Studies of Thin Sections**

Inclusions, but not bands, were observed in erythrocytes hemolyzed in 0.1 per cent acetic acid, fixed in OsO₄, embedded, and sectioned at a thickness of about 300 Å. Nor were bands observed when blood was fixed in neutral formalin, embedded, and sectioned.

Blood fixed in OsO₄ contained well preserved inclusions and several manifestations of well differentiated bands. Bands were elongated, straight, and had a rigid appearance. Sometimes they were forked at the free extremity (Fig. 6), but generally they were widest where they were closely related to the inclusion (Fig. 7). Bands had periodicity. They were seen as a pattern of longitudinal and transverse striations (Fig. 15). The latter were subdivided into two major striae consisting of alternating electron-opaque and electron-translucent striae. The translucent striae were about 125 to 135 Å wide. The opaque striae were 500 to 510 Å wide and contained fine translucent intrabands (Fig. 15). The longitudinal units were slender and electron-opaque. One extremity of the band appeared to adhere but not attach to the limiting membrane of the inclusion. The band was not limited by a membrane, and its periphery seemed to blend irregularly into the surrounding medium of the parasitized erythrocyte.

Good micrographs of bands in parasitized erythrocytes were also obtained from OsO₄-fixed liver preparations. Banding was seen in naturally hemolyzed erythrocytes in the sinusoids, being particularly evident in extensively hemolyzed cells and poorly defined in slightly lysed cells (Fig. 8). In some erythrocytes, longitudinal units were better developed than transverse striae (Fig. 18). Apparently, neither tissue fixation nor processing was responsible for erythrocytic bands or hemolysis, because well differentiated non-parasitized erythrocytes, leukocytes, and macrophages were found in the same sinusoids in which lysed, parasitized erythrocytes were photographed (Fig. 10).

In most sections, bands were undivided, comet-shaped, and in close contact with one face of the limiting membrane of an inclusion body. However, sometimes two or even three bands were associated with a single inclusion (Figs. 11 and 12). In other instances, bands were completely unassociated with inclusions (Fig. 13). Sometimes bands appeared to be cut in cross-section or diagonally with respect to the median axis (Fig. 14).

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**Figure 5** Dumbbell-shaped structures were formed when a band (P) was associated with two inclusions (C). Formalin-fixed, ammonium acetate-lysed erythrocyte, chromium-shadowed. Compare with Fig. 1 (D). \( \times 15,000 \).

**Figure 6** Some bands (arrow) in erythrocytes were forked at their free extremity. Uranyl acetate and phosphotungstic acid stained. This and the following micrographs represent sections of parasitized erythrocytes. \( \times 15,000 \).

**Figure 7** Thin section of a parasitized erythrocyte. Notice prominent transverse striations (arrows) in the band and its close relationship to the inclusion (C) which contains two subunits (U). \( \times 40,000 \).
**Figure 8** The band (P) is indistinct in this slightly hemolyzed erythrocyte in a liver sinusoid. The inclusion (C) is distinct. × 30,000.

**Figure 9** A band (arrow) is associated with two inclusions. × 25,000.

**Figure 10** A well preserved monocyte (N) and a lysed erythrocyte containing a banded inclusion (arrow) are present in a sinusoid. × 6000.
FIGURES 11 and 12  Sometimes two ($P_1$) or even three bands ($P_2$) were associated with a single inclusion ($C$). $\times$ 30,000.

FIGURE 13  In this almost completely hemolyzed erythrocyte in the liver, a well preserved band ($P$) is unassociated with the degenerated inclusions ($C$). $\times$ 30,000.

FIGURE 14  The lysed erythrocyte in a liver section contains a degenerated inclusion ($C$) and bands cut transversely ($X$) and diagonally ($D$). $\times$ 30,000.
FIGURE 15 When a band (P) was associated with two inclusions (C), dumbbell forms were generally seen. The opaque striae contain delicate intrabands (arrows). × 45,000.
FIGURE 16. An irregular shaped band (P) is related to two inclusions (C). The erythrocyte (E) is extensively hemolyzed. × 33,000.

FIGURE 17. The band (P) extends beyond one of the inclusions (C). × 75,000.

FIGURE 18. Longitudinal units converge (arrows) and resemble an imperfect crystal. Selected area diffraction indicated that the band was not crystalline. × 80,000.

FIGURE 19. Section of normal bovine fibrin. Notice gross resemblance between fibrin and bands present in erythrocytes parasitized with A. marginale, (Fig. 17). × 75,000.
Bands associated with two inclusions usually were dumbbell-shaped (Figs. 9 and 15), but sometimes they were seen in other shapes. Occasionally they were wider than the diameter of the inclusion (Fig. 16), or extended beyond the marginal body (Fig. 17). Bands had a stronger affinity for phosphotungstic acid than for uranyl acetate or lead citrate. Some of the bands had patterns similar to an imperfect crystal, since some adjacent longitudinal units converged (Fig. 18). Selected area diffraction patterns of the bands and the surrounding liver area, devoid of erythrocytes, gave an unchanged pattern. It appeared, therefore, that the bands were not crystalline in nature.

**DISCUSSION**

There are numerous references reporting the presence of projections in close association with the inclusion of *A. marginale*. With one exception (7), these were observed when certain strains of *Anaplasma* were stained by special stains involving hemolysis. Projections were described by light microscopy as tails, comets, rings, loops, and dumbbells. Electron microscope studies have been limited to surface studies, and projections have been described as sac- or tubular-like structures. Similar appearing structures have been observed in the gut and excreta of ticks fed on anaplasmosis-infected calves (1).

The nature of the projections (described as bands in this paper) has not been established, except that reports indicate that they do not have an affinity for acridine orange, as does the marginal inclusion (10).

Although hemolysis is apparently necessary for demonstration of bands in erythrocytes parasitized by certain isolates of *A. marginale*, it has been shown in this paper and elsewhere (13) that water lysis is not effective for demonstration of the entirety of bands. Acetic acid is likewise a poor hemolyzing agent for demonstrating complete bands.

It has been suggested that bands associated with *A. marginale* inclusions might be artifacts produced by staining and hemolytic techniques (17). However, our observations indicated that bands were present in erythrocytes hemolyzed by disease conditions (Fig. 12), or in cells almost normal in hemoglobin content (Fig. 8). In the latter, bands appeared to be masked by cellular hemoglobin because both had about the same electron opacity. This regular structure, as far as we know first demonstrated in this study, also suggests that the bands are not artifacts. Bands were pleomorphic in size and shape and were not limited by a membrane. Thus, they are not believed to be a part of the inclusion. Instead, it appeared from this study that bands might have been formed from organization of erythrocytic material since the irregular peripheries of bands blended into the surrounding medium of the erythrocytes. Bands, stained like protein by light microscopy (mercury–bromphenol blue stain) and by electron microscopy (phosphotungstic acid), in being striated, resembled bovine fibrin (Fig. 19) and fibrin demonstrated in published electron micrographs of fibrin from other species (8, 9). However, fibrin in our studies had an axial periodicity of 220 Å, while the bands had an axial periodicity of 500 Å. It has been postulated previously that bands are composed of erythrocytic substances drawn to the marginal inclusion (2). Apparently bands are antigenic because in fluorescein-labeled antibody studies (10), both bands and inclusions fluoresced. These data appear to substantiate the theory that bands result from organization of proteinaceous materials in parasitized bovine erythrocytes.

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