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The histology of "taches noires" of boutonneuse fever and demonstration of *Rickettsia conorii* in them by immunofluorescence

Mario R. Montenegro[1], Serafino Mansueto[2], Barbara C. Hegarty[1], and David H. Walker[1]

[1] Department of Pathology, University of North Carolina, Chapel Hill, N.C., 27514, USA
[2] Cattedra di Clinica delle Malattie Tropicali e Sub-tropicali, Piazza delle Cliniche, 2, 90127 Palermo, Italy

Summary. The recent increase in the incidence of boutonneuse fever in Italy provided the opportunity to study the pathology of six "taches noires," the lesions at the site of tick bite. The center of the lesion has either an ulcer or an area of necrosis of the epidermis and superficial dermis; in some cases the epidermis is intact. The alterations are mainly in the dermis and subcutaneous tissues where the small vessels show endothelial swelling and intramural and perivascular oedema and inflammation with macrophages, lymphocytes and smaller numbers of plasma cells, PMNs and eosinophils. In a few small arteries and fewer veins there are either nonocclusive mural or occlusive thrombi; there is no spatial or quantitative correlation between thrombosis and necrosis. We propose that cutaneous necrosis results from severe injury to many small vessels. Rickettsiae which had not been previously observed in "taches noires" were demonstrated in blood vessels by immunofluorescence, a finding that may be used as a means for early aetiological diagnosis of the disease. The "tache noire" is an excellent human model for localized rickettsial injury.

Key words: Tache noire – Boutonneuse fever – Immunofluorescence – Rickettsia

The information on the histology of the inoculation site in boutonneuse fever is limited to a few sketchy descriptions made in the early 1930's by Olmer [12] and Combiesco [3] in men and to more extensive descriptions made in experimental animals by Baltazard [2], Giroud [6] and Hass and Pinkerton [8]. *Rickettsia conorii*, the agent of this spotted fever, has never been observed in these lesions [4, 8, 16].

The recent increase in the incidence of the disease in Italy [15] gave us the opportunity of obtaining biopsies of taches noires (TN) from Sicilian

Offprint requests to: M.R. Montenegro, Caixa Postal 213, 18600 Botucatu SP, Brasil

patients. In this paper we will describe the histology of the lesions as well as the demonstration in them of rickettsiae by immunofluorescence. The possibility of identifying the agent in TN may be useful to establish the diagnosis of boutonneuse fever in its early phases even before the development of the characteristic rash.

Materials and methods

Biopsies were taken in Sicily by one of us (SM) after obtaining the patients' consent. In 5 patients the TN was biopsied; a sixth patient had biopsies of the TN and the rash, and in a seventh patient only the rash was biopsied.

The specimens were cut in half and fixed in formalin; one half was processed in the University degli Studi di Palermo and the other half was mailed to the Department of Pathology of the University of North Carolina at Chapel Hill. The formalin-fixed specimens were embedded in paraffin, cut at 4 μm, and stained by HE, Masson trichrome, PAS, Brown Hopps, PTAH, reticulum and elastic stains; other sections were deparaffinized and trypsinized for immunofluorescence identification of the agent.

The clinical records of the patients were reviewed and the serological results tabulated.

Direct immunofluorescence identification of rickettsiae in tissues: Sections of the skin were processed according to the technique proposed by Huang [10] as used by Walker and Cain [18] with slight modifications. Formalin fixed, paraffin embedded sections were affixed to slides with Le Page Bond Fast Resin glue (Le Page, Ltd., Montreal, Canada) to avoid loss of the sections, incubated in an oven at 60° C for 1 h, deparaffinized in three changes of xylene for 10 min each, and rehydrated through serial changes of ethanol in concentrations of 100%, 95%, 70%, 50%, 35% and distilled water. The slides were then incubated for 4 h in 0.1% trypsin (Grand Island Biological Co., Grand Island, NY, USA) with 0.1% $CaCl_2$; pH 7.8 at 37° C. After incubation slides were washed in distilled water, washed for 30 min in phosphate buffered saline (PBS), and allowed to react for 30 min with fluorescein isothiocyanate (FITC) conjugated globulin fraction of rabbit antiserum to *Rickettsia rickettsii* (Center for Disease Control, Atlanta, GA.), washed for 30 min in PBS, rinsed in distilled water, mounted in a solution of glycerol in PBS, and examined by ultraviolet light microscopy with use of barrier and exciter filters appropriate for fluorescein.

Indirect immunofluorescent test for anti-*R. conorii* antibodies in patients was performed according to the method of Philip, et al. [13]. The antigen was *R. conorii*, strain 7, (obtained from the American Type Culture Collection) cultivated in our laboratory. Antigens were applied to slides containing 12 wells, dried and fixed in acetone at room temperature. Serial twofold dilutions of serum in PBS were placed in the wells, incubated at 37° C for 30 min, washed in PBS for 20 min, and dried; anti-human gamma-globulin FITC conjugate 1:80 (DAKO, Accurate Scientific Co., Westbury, NY) was added to the wells, incubated for 30 min at 37° C, washed in PBS, mounted in buffered glycerol, and examined by ultraviolet microscopy with the use of FITC barrier and exciter filters. The titer was considered the highest dilution of the serum conferring definite fluorescence to rickettsiae.

Results

Table 1 gives the age, time at which the biopsies were obtained, immunofluorescent antibody titers, and presence of rickettsiae in the eschars of the six patients.

In the majority of the cases the biopsies were performed less than 10 days after the initiation of fever. In patient 2, the eschar was the only manifestation of the disease; no fever or rash were observed, but rickettsiae were still present in very small numbers in the eschar that was 23 days old. In this patient, as in the others, the clinical diagnosis was confirmed by specific serology.

Table 1. Age, Time of Biopsy, Antibody Titers and Presence of Rickettsiae in "Tache Noire" of Six Patients with Boutonneuse Fever

Patient	Age	Day of Biopsy[a]	Antibody Titers to *R. conorii*	Presence of Rickettsiae in Eschar[b]
1	51	11	1:40	+
2	60	23[c]	1:640	+
3	24	7	1:80	+
4	64	5	1:320	+
5	33	8	1:1,280	+
6	32	?	1:80	Absent

[a] Days after first day of fever
[b] By immunofluorescence
[c] Days after tick bite; patient did not develop fever or rash

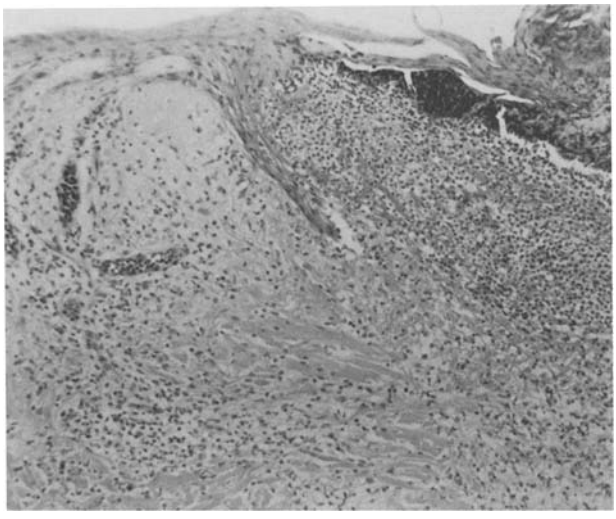

Fig. 1. Case 1. True eschar with necrotic epidermis and superficial dermis separated from normal tissues by band of suppuration. Perivascular oedema and infiltration by macrophages in the better preserved dermis. H and E, ×150

Histopathology

In four of the cases there was necrosis of the epidermis without ulceration. The areas of necrosis were small. In one case there was a true eschar, i.e., ischemic necrosis of the superficial dermis and epidermis delineated from the deeper dermis by a band of suppuration (Fig. 1). In three cases there was only a small, 2–3 mm long, shallow ulceration; the epidermis of the margins was necrotic, and the bottom of the ulcers, at the level of the papillary dermis, was the site of intense acute inflammation. In case 5, in which the biopsy was taken on the sixth day after the onset of fever, the epidermis was intact, covering a band of intense odema of the papillary

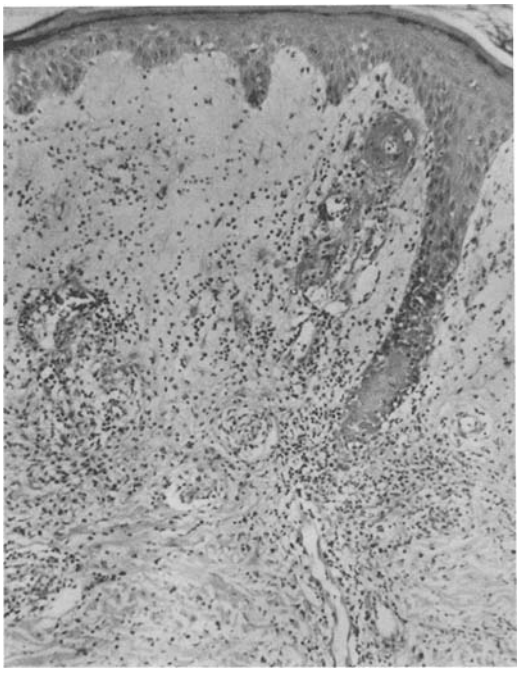

Fig. 2. Case 5. Epidermis is intact; oedema of papillary dermis with scattered interstitial macrophages and red blood cells. Fibrin in the wall of small artery. Endothelial swelling, oedema and infiltration of perivascular tissues in deeper dermis. H and E, ×150

dermis where the vessels were dilated and congested with extravasation of red blood cells into the edematous tissues (Fig. 2).

In all the cases there was an intense focal inflammatory reaction extending from the papillary and reticular dermis to the subcutaneous tissues. Everywhere the inflammatory foci were associated with blood vessles. Small arteries, veins and capillaries were lined by swollen endothelial cells, and their walls and the perivascular tissues were separated by oedema and inflammatory cells (Figs. 1, 2, 3). In the majority of the foci, macrophages and lymphocytes were the dominant cells, but in some of the foci PMNs and plasma cells were also present; in patient 2 eosinophils were prominent. PMNs were more frequently observed in the vascular lumens or adhering to the swollen endothelial cells; some were seen within the walls of small and large vessels. As a rule they were few and focal; however, in the vicinity of the ulcers they were numerous. Macrophages were also seen in the vessel walls, but most of them, accompanied by lymphocytes, were accumulated around the vessels forming perivascular cuffs (Fig. 3). The swollen endothelial cells bulged into the lumen and in some cases appeared to reduce it markedly (Fig. 4). Rare mitoses were observed in endothelial cells. Seldom was the inflammatory reaction of the vessel wall so intense as to destroy the external elastic membranes (Fig. 5). Only exceptionally was there fibrinoid necrosis of the vessel wall (Fig. 1) with or without mural thrombosis. There was no correlation between these rare, partially occluded, thrombosed vessels and necrosis of the epidermis.

The connective tissue of the dermis away from the perivascular inflammatory cuffs was oedematous and its small vessels lined by swollen endothe-

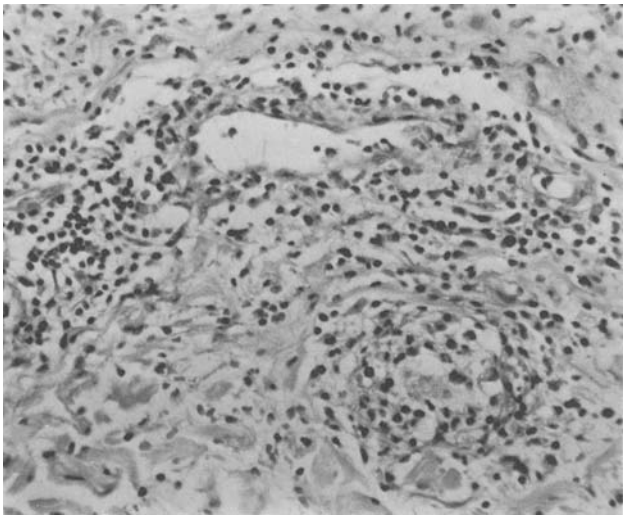

Fig. 3. Case 3. Vessels in reticular dermis show endothelial swelling and infiltration of walls and perivascular tissues by macrophages and lymphocytes. There is perivascular oedema. H and E, ×375

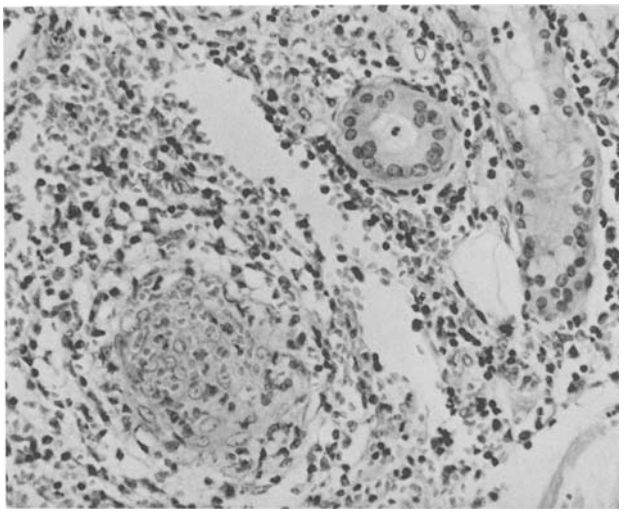

Fig. 4. Case 4. Vessel with occlusion of lumen by mass of macrophages and swollen endothelial cells. There is perivascular oedema and infiltration by macrophages and lymphocytes and extravasation of red blood cells. H and E, ×375

lial cells; a few isolated macrophages and lymphocytes were scattered among the collagen fibers. As a result of these features the whole dermis appeared to be diffusely hypercellular and oedematous. The perivascular foci of inflammation were more evident in the vicinity of hair follicles and sweat glands.

In two of the six biopsies the site of the tick bite could be recognized histologically. In one, the lesion was more recent and consisted of a thin,

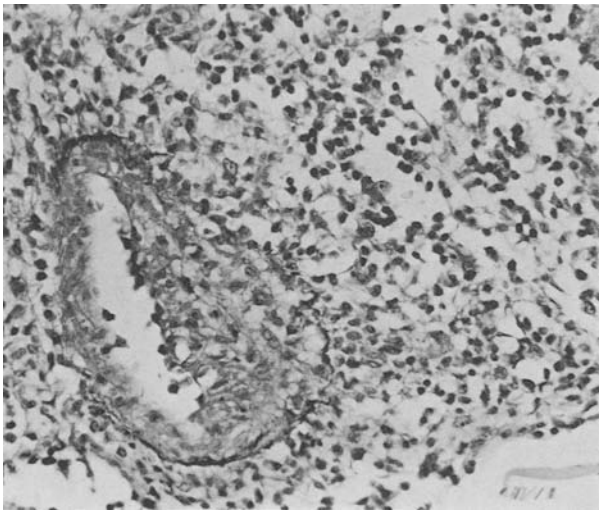

Fig. 5. Case 4. Artery with partial interruption of elastic membrane by inflammatory reaction; perivascular oedema and infiltration by macrophages and lymphocytes. Elastic stain, ×375

wedge-shaped area of acute inflammation that crossed the dermis perpendicularly reaching the superficial subcutaneous tissues. The area was densely packed with the same inflammatory cells described above with PMNs being much more numerous; close to the ulcerated epidermis there was necrosis of the papillary dermis. The other was from patient 2, without fever or rash. In the area of the tick bite there was an irregular mass of necrotic tissue containing nuclear debris partially covered by regenerated acanthotic epidermis and partially surrounded by granulation tissue containing numerous eosinophils, plasma cells, and a few giant cells. Away from this area and extending into the underlying subcutaneous tissue there was the typical perivascular reaction as described for the other cases; in this instance, however, the quantity of plasma cells and eosinophils was much greater. The perivascular reaction extended laterally well beyond the necrotic area.

In the biopsies of the rash the epidermis was normal, the dermis oedematous and its dilated vessels lined by swollen endothelial cells and surrounded by a few macrophages and lymphocytes. There was spilling of red blood cells into the interstitial tissues. The alterations were similar to those in the eschars but milder. They were more prominent in the papillary dermis but involved the whole thickness of the skin including the subcutaneous tissue.

Identification of rickettsiae in the eschars

Coccobacillary organisms were observed in all but one of the eschars examined. They were present in small numbers forming clusters on the internal aspect of the small vessels of the reticular dermis (Fig. 6) in a location that in HE preparations appeared to be occupied by swollen endothelial cells. They were seldomly observed in the perivascular inflammatory foci;

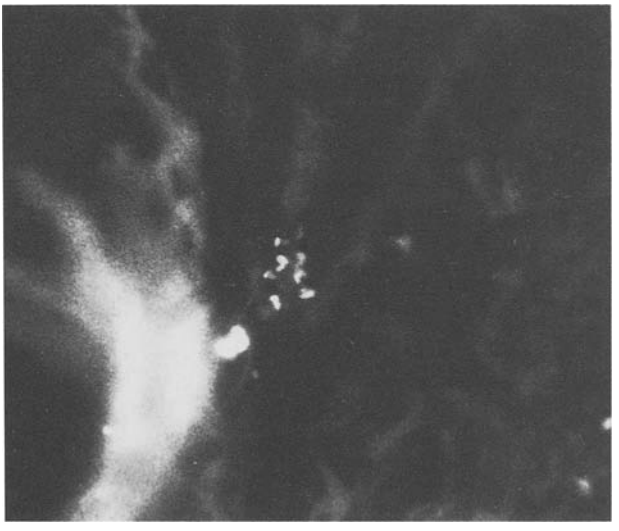

Fig. 6. Cluster of rickettsiae in wall of vessel in reticular dermis. Direct immunofluorescence with anti-spotted fever group rickettsia rabbit globulin conjugate. × 590

in some foci there was granular fluorescence but only very rarely identifiable organisms. Similar organisms were observed in one of the biopsies of the rash (patient 4).

Discussion

Boutonneuse fever is a mild but incapacitating disease; in debilitated persons it may be severe [14] and there are references to an epidemic in Israel [7] in which the disease had clinical manifestations similar to Rocky Mountain spotted fever (RMSF). The available methods for its diagnosis are serological, implying that there are no means to establish an early aetiological diagnosis. The identification of the agent in the TN, often the earliest manifestation of boutonneuse fever, would permit such early diagnosis, at least in the cases in which the TN is present.

Hebert, et al. [9] have shown that fluorescent conjugated anti-*R. rickettsii* rabbit serum reacts with *R. conorii* and *R. akari*. The titers are highest for *R. rickettsii* but also quite high for *R. conorii*, being lower for *R. akari*. The authors concluded that the anti-*R. rickettsii* conjugate is a specific spotted fever group immunofluorescent reagent. Our results applying the method in human tissues further suggest the utility of this previously standardized rabbit anti-*R- rickettsii* conjugate in the early diagnosis of boutonneuse fever.

Indeed, coccobacillary organisms with the morphological characteristics of rickettsiae were identified in all but one of the TN studied. The fact that they were scarce is probably related to the timing of the biopsies; they were performed at least 5 days after the initiation of fever at a time when specific host defenses were certainly effective; in some of them IF antibodies were already present in high titer in the serum.

The location of the fluorescent coccobacillary organisms in the small vessels of the reticular dermis away from the perivascular inflammatory reaction may also be explained by the time at which the specimens were obtained. Since the early works on typhus it is known that rickettsiae appear to grow by continuity from one endothelial cell to the other; Wolbach [21] described this propogation along the intima of the small vessels as typical. Walker [20], studying skin biopsies in RMSF, has also observed this tendency of the rickettsiae to occur in contiguous endothelial cells of blood vessels anastomosing in the center of the maculopapule of the rash. The absence of intact microorganisms in the area of more intense inflammatory reaction is probably the consequence of their destruction by the local inflammatory process that had been active for several days when the biopsies were performed.

The fact that fluorescent organisms were not observed in the rash of a patient is also related to the timing of the biopsy since in her case the biopsy of the rash was obtained 12 days after the initiation of fever and 6 days after therapy with tetracycline.

The early descriptions of the histology of TN were based on biopsies taken late in the disease when the lesions were in advanced stages of involution [2]. Furthermore, the better descriptions of Olmer [12] and Combiesco [3] made in the early 1930's are very succinct. Since then, to our knowledge, no other publications have dealt with this subject. The lesions of the inoculation site of other rickettsioses, including RMSF, have been thoroughly described [1, 4, 19, 21] and the alterations observed in our 6 patients are very similar. The type and distribution of the inflammatory reaction and the prominence of endothelial alterations, including the presence of mitoses, are the predominant pathological features of the rickettsioses. A few alterations, however, deserve comment: There was no correlation between vascular thrombosis and presence of necrosis of the skin, an observation suggesting that necrosis, where it occurs, is the result of diffuse microvascular involvement and not, necessarily, due to occlusion of an artery. The vascular involvement in the TN is diffuse in an extensive, localized area of the skin, and the entire vasculature of the reticular dermis is affected, an observation that was recently emphasized by Scaffidi and Ferrigno [16] in relation to the rash of BF.

Another remarkable histological observation was the fact that in one TN there was no necrosis or ulceration of the epidermis, the lesions being entirely intradermal. The histology in this case corresponded to what one would see grossly as a "tache noire", literally, a black spot and not an "eschar," a word used to describe lesions of necrosis of the skin covered by a crust (as seen in burns or as a consequence of the action of corrosives) [5, 17]. This fact has not been emphasized in the literature, but is known to occur in scrub typhus where only a few of the inoculation site lesions develop into true eschars [11]. It has also been mentioned in boutonneuse fever both in the past [8] and very recently [16]. Combiesco [3], describing 34 cases of boutonneuse fever in 1932, does not refer to necrosis and ulceration in any of the TN of his patients. Furthermore, he inoculated a few

persons with blood of patients with the disease; these persons had inoculation site lesions that did not develop into eschars; in his words, the lesions were "... papule d'une couleur plus fonceè." Hass and Pinkerton [8] described the TN as "indurated, hyperaemic, painless, and occasionally ulcerous papules." The picture selected by Zdrodovski and Golinevich [22] to illustrate the primary skin lesion in "Marseilles fever" does not appear to be ulcerated, and the same can be said of Fig. 4 of Scaffidi and Ferrigno [16]. In summary, there is ample evidence to conclude that the "tache noire" of boutonneuse fever does not always correspond to a true "eschar."

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