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Morphology of *Borrelia burgdorferi*: Structural Patterns of Cultured Borreliae in Relation to Staining Methods

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The microscopic recognition of *Borrelia burgdorferi* in biologic fluids and tissues is difficult and challenging because of low numbers of organisms occurring as single isolated spirochetes, the apparent lack of colony formation in tissues, and differing lengths and structural morphologies. To identify the most common morphologic forms, we studied numerous cultures by a variety of microscopic techniques. Culture suspensions of *B. burgdorferi* were stained by several different histochemical procedures (Gram, Wright, Wright-Giemsa, Giemsa, and polychromes), fluorochromes (thioflavin-T, acridine orange, and rhodamine), silver impregnation techniques (Warthin-Starry, modified Dieterle, modified microwave Dieterle, and Bosma-Steiner) and immunocytochemical methods with different polyclonal and monoclonal antibodies. Additionally, borrelia culture suspensions were embedded in agar and also injected into skin biopsies and processed for histologic study. The different staining properties were found to be influenced by fixation methods and the type of antibody in immunocytochemical stains. This study provides evidence for marked cytomorphic variations in individual spirochetes. Variations detected by these staining procedures provide a basis for future study of tissue sections and for how borreliae can be expected to appear in tissue sections.

Borrelia burgdorferi, the spirochetal agent that causes Lyme borreliosis, has been isolated in culture from ticks and organs and body fluids of humans and different animals (3, 4, 9, 15–17, 22). The ability of *B. burgdorferi* to be cultured in Barbour-Stoenner Kelly (BSK) medium (5, 16) or agar containing solid culture medium (13) sharply distinguishes it from *Treponema pallidum*. Morphologically, *B. burgdorferi* is an irregularly coiled spirochete, 10 to 40 μm in length and 0.2 to 0.3 μm in diameter, that is found in multiple in vitro forms as nonuniformly coiled, twisted, or intertwined over one another and is often found in aggregated forms. Colony formation has been observed in both fluid medium and semisolid plates (5, 13). It has also been shown that changing the culture conditions can alter the spirochete morphology. These spirochetes tend to lose coiled morphology and become unusually elongate in older cultures (6). Moreover, pH alterations cause dissociation of the outer envelope from the axial cylinder and can lead to the formation of outer membrane blebs (6).

The possibilities of visualizing *B. burgdorferi* by staining methods are numerous, but not all staining methods can readily be applied to tissue sections. We report herein our experiences with many staining techniques performed on culture suspensions of *B. burgdorferi*, with emphasis on the spectrum of spirochetal morphologies.

MATERIALS AND METHODS

Strains, culture methods, and preparation of borreliae. *B. burgdorferi* B31 (ATCC 35210), FM323 (Wisconsin), BAPA (Pennsylvania), MM (Minnesota Mouse; R. Johnson, University of Minnesota, Minneapolis), SON and DN (California; C. Moody, Yale University, New Haven, Conn.), H-1, H-2 (skin isolate; C. Poitschek, Vienna, Austria), W (cerebrospinal fluid isolate; G. Stanek, Vienna, Austria) were cultivated at 33°C in BSK-II medium (5) and passaged no more frequently than one time per week or longer if the pH indication was stable. Strains FM323 and BAPA have recently been obtained by one of us (P.H.D.): FM323 was isolated from a white-footed mouse (*Peromyscus leucopus*) found on the military reservation of Fort McCoy, Wis., while BAPA was obtained from an *Ixodes dammini* nymph derived from Bryn Athyn, Pa., collected by Amy Jones. FM323 has protein bands nearly identical to those of B31 by polyacrylamide gel electrophoresis, while BAPA has an attenuated 32-kDa band (outer surface protein B) compared with that of B31 (data not shown).

Culture pellets were prepared as follows. Seven-day BSK-II culture aliquots were washed twice with phosphate-buffered saline (PBS), centrifuged, and resuspended in PBS. Drops of the resuspensions were either placed on slides containing wells or prepared on standard ringed slides in order to direct the cell pellet to a confined space. Slides were air dried or acetone fixed and stored at −20°C until stained. Slides destined for cytochemical staining were usually air dried, while those to be used for immunocytochemical methods were cold acetone fixed for 10 min, air dried, and stored at −20°C until stained.

In addition to air-dried and acetone-fixed spirochete pellets, those destined for immunoperoxidase staining were also fixed in 100% methanol, 7.5% buffered formaldehyde, or 4% paraformaldehyde for a range of 5 to 10 min in order to determine the optimal fixative. *T. pallidum* Nichols (Bio Merieux) (Formalin and acetone fixed) was used as a control for the staining exercises. Fixation was achieved by use of coplin jars.

Agar embedding. Borrelia culture suspensions were centrifuged at 2,000 U/liter for 10 min, and 1 ml of the pellet was fixed in 7 ml of 7.5% buffered Formalin and again centrifuged at 1,000 U/liter. Warm liquid agar (20 g/liter; Bacto-Agar [Difco]) was then added to the pellet, pulled up in a plastic syringe, and allowed to solidify by cooling. The solidified agar blocks were then fixed in Formalin and embedded in paraffin by the method of Chaplin et al. (10). These agar

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preparations were handled as if they were tissue blocks during the embedding procedure, processed, and stained just as in a routine histologic preparation. This technique allowed us to determine the influence that histologic preparations have on the cytomorphology of *B. burgdorferi*.

Borrelia-injected skin. In order to study the cytomorphology of *B. burgdorferi* in relation to dermal collagen, spirochete-infected skin was prepared as follows: 0.5 ml of unfixed, centrifuged borrelia cultures containing 10^6 cells per ml were injected into a freshly obtained biopsy of normal human skin obtained from the resection margins of a nevus from a patient in the Dermatology Clinic. The normal skin components were Formalin fixed immediately, embedded in paraffin, and processed for routine histologic examination. India ink was added to the culture suspension to facilitate localization of the injected borreliae.

Culture pellet staining methods. (i) Universally used peripheral blood cytochemical aniline dye stains. Gram, Wright, Wright-Giemsa, Giemsa, May-Grünwald, and Hemacolor (EM Diagnostic Systems, Inc., Gibbstown, N.J.) stains were used as described by Sheehan and Hrapchak (18). Hemacolor preparations consisted of methanol fixation, followed by a brief treatment in phosphate-buffered eosin and suspension in thiazine solution, according to the manufacturer (EM Diagnostic).

In the Wright stain procedure, slides of fixed spirochete pellets were stained as for routine peripheral blood methods as follows. The slides were covered with Wright stain (EM Diagnostic) for 1 min, followed by the addition of 2 volumes (twice the amount of Wright stain used to cover an individual slide) of dilute sodium phosphate buffer (0.3 g of dibasic plus 0.7 g of monobasic sodium phosphate in 100 ml of distilled water) until a surface metallic sheen was visible for at least 3 min, rinsed in distilled water, mounted with permount, and coverslipped.

The May-Grünwald stain is a modification of the Giemsa stain (both of which are members of the Romanowsky group containing thiazine eosinates) and has the potential of yielding a metachromatic hue to certain parasite structures, optimizing microscopic visibility. The procedure can be done in two ways. The first method, while requiring some extra steps, has the advantage of achieving good metachromasia. Jenner stock solution was prepared by dissolving 1 g of Jenner stain powder in 400 ml of absolute methanol. Thirty milliliters of Jenner stock was then combined in our procedure with 30 ml of double distilled water (DW) just prior to staining. Slides were hydrated in DW, dehydrated in two changes of absolute methanol, stained in Jenner working solution as described above for 6 min and then in Giemsa stain for 45 min, rinsed copiously in DW, immersed in 1% acetic acid for color differentiation, rinsed again in DW, dehydrated with a series of graded alcohols, cleared in xylene, mounted with permount, and coverslipped. The second method utilizes May-Grünwald desiccated powder in one step (Roboz Surgical Instrument Co., Washington, D.C.).

(ii) Fluorochromes. Thioflavin-T (19), acridine orange (7, 20), and auramine-rhodamine (21) desiccated fluorochrome powders were all obtained from Roboz.

In the thioflavin-T procedure, slides were fixed in 10% neutral buffered Formalin, hydrated with a series of graded alcohols to DW, and placed in Mayer hematoxylin for 4 min. This was followed by 2 min of tap water washing, and the slides were overlaid with 1% aqueous thioflavin-T for 3 min and three changes of DW washes. Color differentiation was achieved by treatment in 1% aqueous acetic acid for 15 min. Slides were then incubated in PBS (pH 6.0) for a full minute, coverslipped in an aqueous immunomount (Shandon), and examined with an Olympus fluorescence microscope with a UG2 exciter filter along with a colorless barrier filter to search for faint yellow spirochetes.

In the acridine orange procedure, spirochete-pelleted slides were fixed in ether-alcohol for 30 min (in place of Formalin which does not work well with this fluorochrome). Slides were next hydrated with a series of graded alcohols and finally DW, dipped for 6 s in 1% acetic acid, rinsed twice in DW, and flooded with 0.1% acridine orange in PBS at pH 6.0 for 3 min. Washing was for 1 min in the pH 6.0 PBS (an acid pH is required throughout the staining procedure in order to optimize DNA fluorescence). Slides were coverslipped in 0.1 M PBS and examined with the fluorescence microscope.

Formalin-fixed slides were used for the auramine-rhodamine fluorochrome procedure. They were rinsed in DW and immersed in auramine *O*-rhodamine B solution in a coplin jar for a full hour in a 60°C oven. Slides were next differentiated in 0.5% acid alcohol (995 ml of absolute alcohol with 5 ml of concentrated HCl) until the pellet appeared colorless. Slides were rinsed with running tap water and then DW and were oxidized in 0.5% potassium permanganate for 2 min. Slides were well rinsed in DW, dehydrated with a series of graded alcohols, cleared in xylene, mounted with permount, coverslipped, and examined by fluorescence microscopy with a UG2 exciter filter and a colorless UV barrier filter.

(iii) Silver impregnation methods. The Warthin-Starry (23), modified Dieterle (12), and Bosma-Steiner (11) methods were performed as previously described. The microwave Dieterle method was performed as follows. Reagents were prepared as previously described (12) with regard to the uranium nitrate solution, preparation of the developer solution, and the silver nitrate solution. All manipulations of the microwave Dieterle method were done under a ventilated hood. The procedure was the same as that in the Bosma-Steiner and modified Dieterle methods with the following additional changes as follows. Slides to be stained were placed in a plastic coplin jar which was half-filled with 1% silver nitrate solution. Microwaving was performed for 60 s, after which the slides were allowed to incubate in the hot silver nitrate solution for an additional 5 min. Copious rinsing in DW then followed, and the slides were then dipped in the developer solution that had been preheated in the microwave for 30 s. Slides were left in the hot developer for 3 to 4 min until a rich yellow color with a faint tan hue appeared. Slides were dipped in 95% alcohol, dehydrated with a series of graded alcohols, cleared in xylene, and mounted with permount. Care was taken to secure the lids tightly on the coplin jars in order to prevent pressure loss during heating.

(iv) Immunocytochemical methods. The monoclonal antibodies used throughout were supernatants derived from murine hybridoma fusions immunized against antigens and treated as described below.

Indirect immunofluorescence was performed as follows. A 1:500 dilution (1% bovine serum albumin in PBS) of murine monoclonal antibody H-5332 (Alan Barbour, University of Texas, San Antonio) was placed on acetone-dried slides that were rehydrated in PBS, incubated for 30 min, washed in PBS for 5 min, incubated with goat anti-mouse secondary antibody conjugated with fluorescein isothiocyanate at a 1:50 dilution, and examined with a fluorescence microscope.

Immunoperoxidase trials employed fixed borrelia cultures

(B31, H-1, H-2, and W), which were studied following immunocytochemical staining with the following multiple antibodies: polyclonal human antiserum from a patient with high antibody titers against *B. burgdorferi*, polyclonal rabbit antiserum against *B. burgdorferi* B31 and W (kindly provided by G. Stanek, Hygiene Institute, Vienna, Austria), and anti-*B. burgdorferi* murine monoclonal antibodies (hybridoma supernatants; H9724, H604, and H6TS directed against a 41-kDa protein [flagellin]; H3TS and H5332 directed against the 31-kDa outer surface protein Osp A; H63, H68, H6831, and H614 directed against the 34-kDa Osp B; and H10022 directed against a 38-kDa protein and H9E11 directed against a 20-kDa protein of *Borrelia hermsii* [kindly provided by A. Barbour]). The sources of the murine monoclonal antibodies and their protein concentrations were described in detail previously (for a review, see reference 1).

Additional antibodies were used in the following immunocytochemical tests: (i) the peroxidase-antiperoxidase (PAP) method with rabbit antiserum (Dako anti-rabbit immunoglobulin G from swine [1:20; PAP, 1:50] or the Tissu Gnost R Universal kit with anti-rabbit PAP-P 20004 [Merck]); and (ii) the avidin-biotin complex (ABC) method for visualizing spirochetes with the human polyclonal antibody (2) and mouse monoclonal antibodies with biotinylated sheep anti-mouse immunoglobulin G (1:200; Amersham), avidin-biotin (1:300), Tissu Gnost R antimouse ABA-M 20001 (Merck), ABC kit (Vectastain), or PK 6102 (Elite). (iii) The phosphatase-antiphosphatase technique (Dako APAAP kit systems with universal mouse K670 [Dakopatts] or the vector alkaline phosphatase substrate kit SK-5100) was also employed for staining borreliae with mouse monoclonal antibodies.

TABLE 1. Histo- and cytochemical and fluorochrome stain reactions with *B. burgdorferi* strains

Stain	Staining of strain[a]:					
	B31	FM323	PABA	SON	DN	MM
Wright	3	3	2	3	2	3
Giemsa	2	2	3	2	2	2
May-Grünwald	2	3	3	3	2	3
Gram	1*	0	0	1*	0	0
Hemacolor	3	3	3	2	3	3
Thioflavin-T	1	0	2	1	1	2
Acridine orange	2	2	2	2	1	2
Auramine-rhodamine	1	1	0	0	0	1

[a] Staining was scored as follows: 0, no staining; 1, incomplete, faint staining; 1*, very faint gram-negative reaction (see text); 2, complete, faint staining; and 3, good staining.

RESULTS

In general, all the borrelia strains tested gave similar results by using the cytochemical and fluorochrome methods as follows.

(i) Reactions from the cytochemical aniline dye stains. The Hemacolor peripheral blood stains adequately demonstrated spirochetes, with a superior quality of staining reactions, as evidenced by the sharpness of individual spirochetal outlines. Gram stain attempts were generally unsuccessful. There was an incomplete, faint apparent gram-negative reaction with two strains (Table 1). This reaction was regarded as insufficient evidence that this spirochete is gram negative. Wright-Giemsa and Wright stains gave adequate

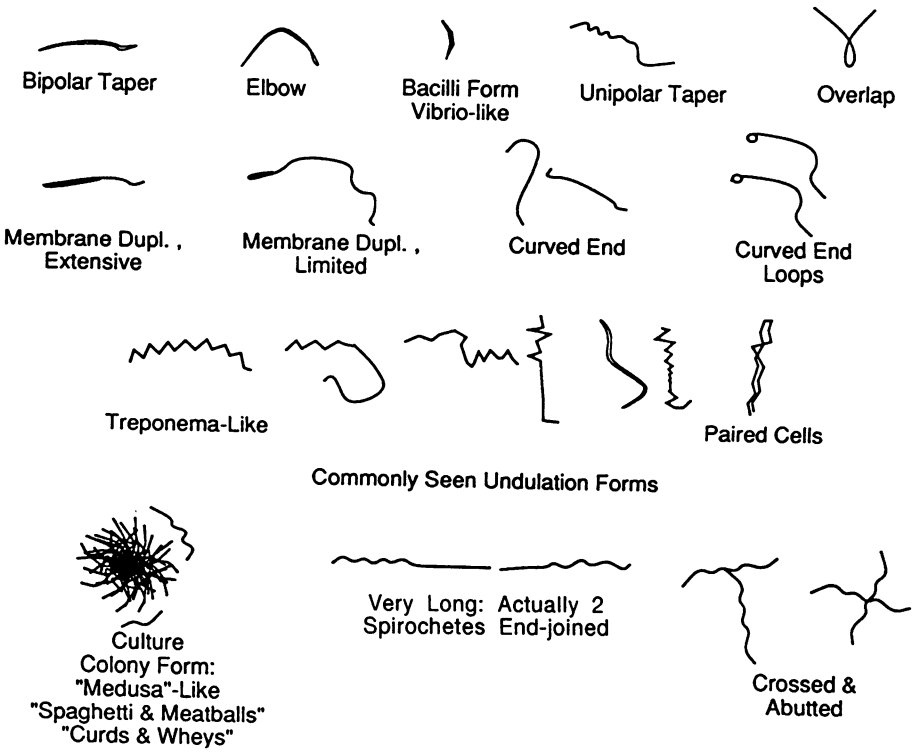

FIG. 1. Common morphologies of *B. burgdorferi* B31.

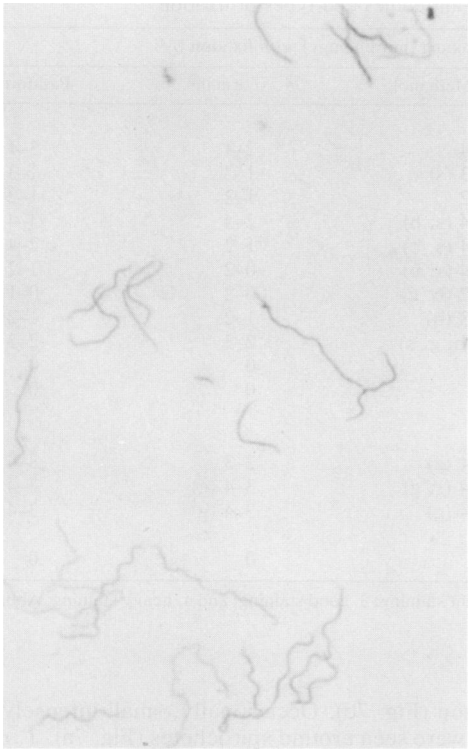

FIG. 2. Several classic forms of *B. burgdorferi* B31 are seen in this photomicrograph, including uniform undulations, irregular undulations, tapered ends, and no undulations of the tapered spirochete in the approximate center. Overlap forms are also present. Eosin-thiazine stain was used. Magnification, ×1,000.

results, yielding similar blue-pink color reactions comparable to those obtained with Giemsa. May-Grünwald stain occasionally yielded a clearly metachromatic blue-green color, but the reaction seemed to vary from batch to batch with some difficulty in consistency. Eosin-thiazine (Hemacolor) gave a violet-blue-to-light-violet reaction, but the cytomorphology of an individual spirochete was sharp in outline and reproducible from batch to batch. Details of spirochete cytomorphology are shown in Fig. 1 through 3.

(ii) Fluorochrome reactions. Fluorescence of spirochetes occurred with the fluorochromes, with acridine orange yielding the strongest reaction (a faint green color [Table 1]). The rhodamine-auramine reaction was essentially negative. Fluorescence reactions were of very short duration, with a given spirochete showing rapidly (within seconds) extinguishing fluorescence. Details of spirochete morphology could not be adequately evaluated by any of the fluorochrome dyes because of the phenomenon of rapidly extinguishing fluorescence.

(iii) Silver stains. The Warthin-Starry, modified Dieterle, and Bosma-Steiner silver stains all stained the spirochetes adequately, allowing for the study of cytomorphologic details of all borrelia strains (Fig. 4). Reagents in the modified Dieterle and Bosma-Steiner stains are essentially the same, but the Bosma-Steiner stain incorporates a prestaining enzyme incubation (amylase) which is reported to be advantageous in delineating greater numbers of spirochetes in tissue sections (12). Spirochete structural details are equivalent when patterns yielded by silver stains and those yielded by the rapid eosin-thiazine stain (Hemacolor) are compared.

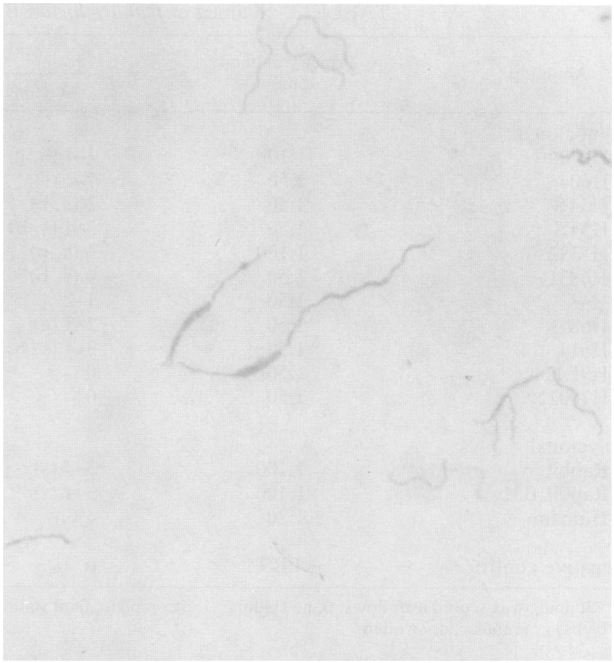

FIG. 3. *B. burgdorferi* B31 in the center shows apparent membrane thickening, which highlights the polymorphism of this spirochete. Eosin-thiazine stain was used. Magnification, ×1,000.

Generally, silver-stained borreliae appeared more straight than the immunohistochemically stained organisms.

(iv) Immunocytochemistry. Immunofluorescence yielded good results in demonstrating spirochetes, but as in all such procedures, the fluorescence reaction was readily extinguished, requiring use of photomicrographs to conduct morphologic studies. All immunofluorescence studies were carried out with *B. burgdorferi* B31. Immunoperoxidase results are shown in Tables 2 and 3. There were variations in intensity and in the delineation of morphology depending on the fixative used prior to the antibody incubation. The intensity of immunoperoxidase staining was scored as fol-

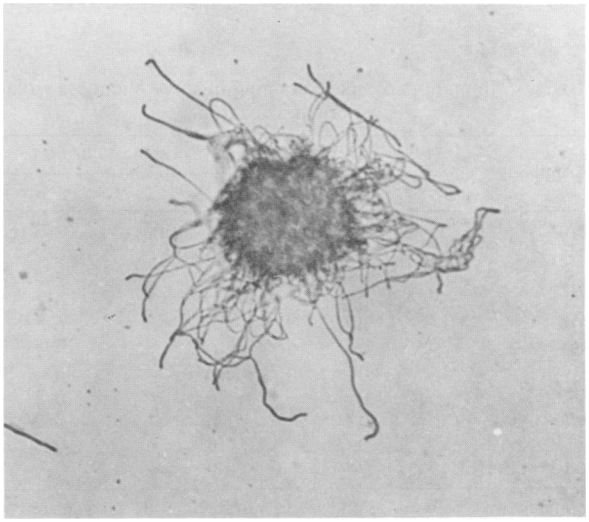

FIG. 4. *B. burgdorferi* B31. Dieterle stain was used. Magnification, ×1,000.

TABLE 2. Staining of *B. burgdorferi* B31 with different antibodies and types of fixation

Antibody	Primary antibody dilution	Staining (morphology) with fixation by[a]:			
		Acetone	Methanol	Formalin	Paraformaldehyde
Monoclonal					
H9724	1:100	3–4 (b, g)	3–4	2–4	3–4
H604	1:50	1–2 (s)	2–3 (s)	1–2	1–3
H6TS	1:50	2 (s, b)	1–2	1–2	1–2
H3TS	1:50	3–4 (s, b)	3–4 (s, b)	2–4	3–4
H5332	1:100	4 (s, b)	3–4 (s, b)	1–2	2–4
H6831	1:50	4 (s, b)	3–4 (s, b)	0–2	1–2
H63	1:50	1–2	1–2 (b, g)	0–2	0–1
H68	1:50	2–3 (g)	2–3 (b)	1–2	1–2
H614	1:50	3–4 (s, b, g)	3 (b, g, s)	2–3	2–3
H9E11	1:50	0	0	0	0
H10022	1:50	0	0	0	0
Polyclonal					
Rabbit W	1:100	3–4 (s)	3–4 (g)	2–3	3–4
Rabbit B31	1:100	3–4 (s)	3–4 (s, g)	3–4 (g)	3–4 (g, s)
Human	1:20	4 (s)	3–4 (g)	3–4 (g)	3–4
Negative control	PBS	0	0	0	0

[a] Staining was scored as follows: 0, no staining; 1, incomplete, faint staining; 2, complete, faint staining; 3, good staining; and 4, heavy staining. Abbreviations: b, blebs; g, granular; s, swollen.

lows: 0, no staining, 1, incomplete, faint staining; 2, complete, faint staining; 3, good staining; and 4, heavy staining. Generally, acetone-fixed borreliae appeared more swollen, with outer membrane blebs appearing more prominent (Fig. 5a) than those of methanol- or Formalin-fixed preparations (Fig. 5b). The antibodies generally gave a higher staining score in the acetone-fixed pellets (Table 2).

Methanol-fixed borreliae yielded a decreased staining intensity, while the Formalin- and paraformaldehyde-fixed suspensions resulted in a still fainter staining reaction. However, retention of spirochetes on the slides was superior in the Formalin- and paraformaldehyde-fixed slides than with either acetone or methanol, where sometimes fewer borreliae remained on the slides.

Polyclonal human antiserum gave a very strong stain reaction with prominent membrane surface blebs (Fig. 6), while murine monoclonal antibody reactions stained less strongly (Fig. 7a) and often gave a granular reaction in comparison (Fig. 7b). Occasionally, small intensely stained granules were seen around spirochetes (Fig. 7a). *T. pallidum* Nichols strain spirochetes reacted with the anti-*B. burgdorferi* polyclonal human and rabbit antibodies but did not stain

TABLE 3. Immunoperoxidase staining of three Viennese isolates by Formalin fixation

Antibody	Staining (morphology) of[a]:		
	H-2	H-1	W
H9274	2–3 (g)	2–3 (g)	2–3 (g, s)
H604	0–2 (g)	0	0–2
H6TS	0–2 (g)	0	2–4
H3TS	0	0	0
H5332	0	0	0
H6831	0	0	0–1
H63	0	0	0
H68	0	0	0–2
H614	0	0	0–2
H9E11	0	0	0
Negative control	0	0	0

[a] Staining was scored as follows: 0, no staining; 1, incomplete, faint staining; 2, complete, faint staining; 3, good staining; and 4, heavy staining. H-1, H-2, and W are described in the text. Abbreviations: g, granular; s, swollen.

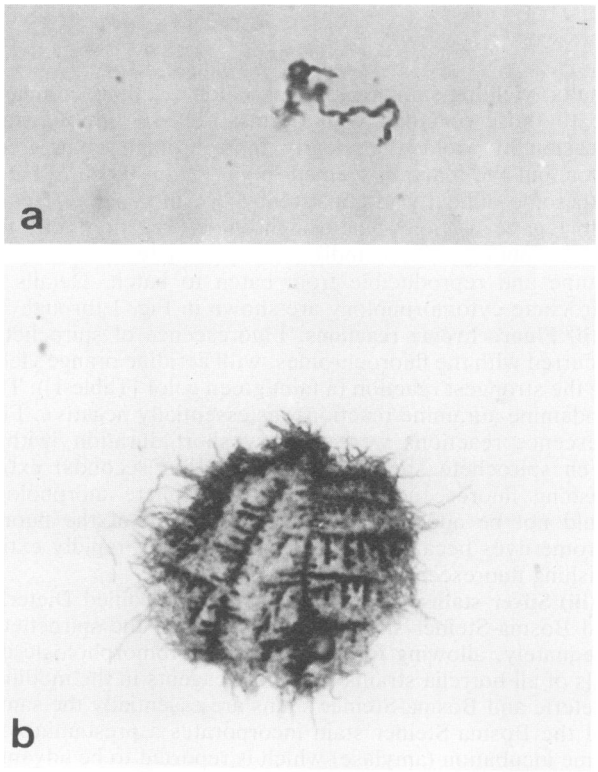

FIG. 5. (a) *B. burgdorferi* B31. Acetone fixation and H604 antibody were used. (b) *B. burgdorferi* B31. Formalin fixation was used. Crystallike deposits of H9724 antibody can be seen. Magnification, ×1,000.

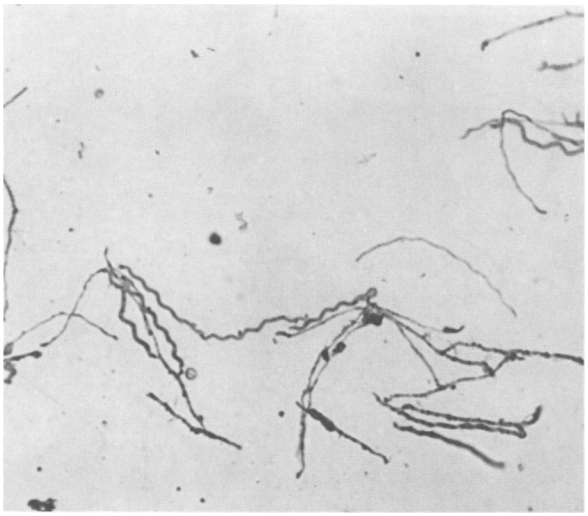

FIG. 6. *B. burgdorferi* B31. Formalin fixation and anti-human antibody were used. Magnification, ×1,000.

with any of the borrelia-specific murine monoclonal antibodies. Some of the Formalin-fixed treponemes, however, showed a diminution of the usual tight, uniformly spiralled cork-screw configuration so classic with silver stains and were replaced by a more curved and undulated morphology as in borreliae (Fig. 8). However, this phenomenon did not occur in the acetone-fixed treponema preparations. The H-1, H-2, and W isolates were stained by the H9724 antibody only, and isolate W was additionally stained by the H6TS

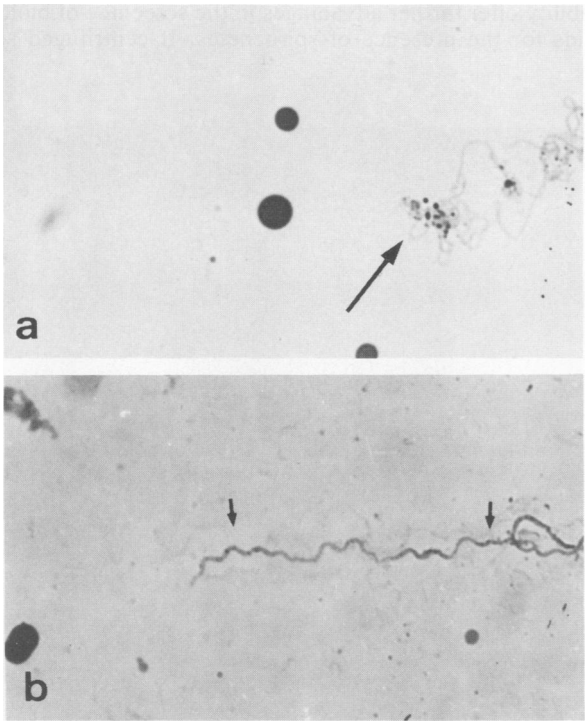

FIG. 7. (a) *B. burgdorferi* B31. Formalin fixation and H5332 antibody were used. Released granules between spirochetes can be seen. (b) *B. burgdorferi* H-1. Formalin fixation was used. Granular staining with H9724 antibody can be seen. Magnification, ×1,000.

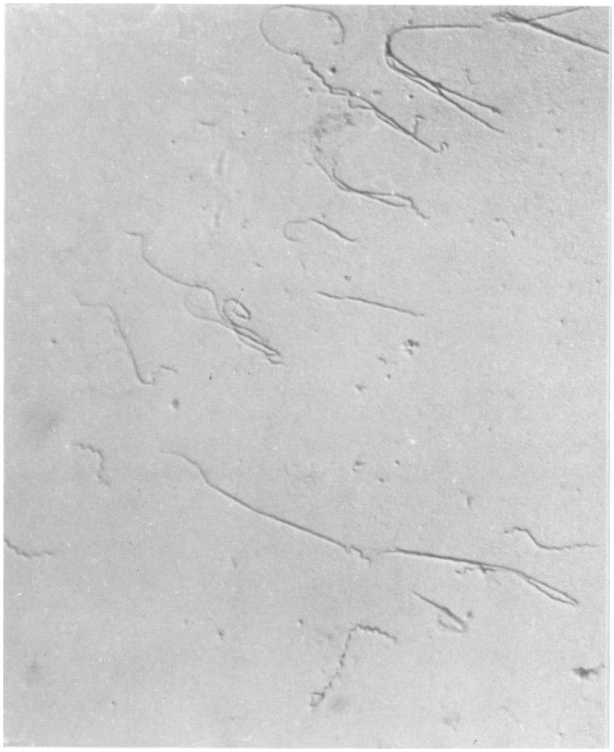

FIG. 8. *T. pallidum* Nichols. Formalin fixation and anti-rabbit W antibody were used. Magnification, ×1,000.

antibody (Table 3). None of the spirochete preparations reacted with anti-*B. hermsii* monoclonal antibodies (H9E11 and H10022) in this study.

Agar-embedded borreliae showed prominent staining with both the human and rabbit antisera and the H9724 anti-flagellin antibody, whereas they were only partly stained by the H5332 anti-Osp A antibody. Many spirochetes aggregated into clumped structures, producing large tangles of spirochetes (Fig. 9). Although they were demonstrable immunocytochemically, it was not possible to stain agar-embedded borreliae by silver stains.

The borrelia-injected skin control samples (simulating cutaneous borrelia infection) demonstrated a low number of spirochetes. On occasion, we experienced difficulty in detecting individual spirochetes against collagen filters that had diffuse background staining (Fig. 10). Another problem was the occasional presence of linear granules between collagen fibers in stain trials with the monoclonal antibodies.

Labeling the spirochetes with India ink prior to skin injection successfully allowed their detection, but they were seen scattered in different areas of the dermis, indicating rapidly moving dispersion. By this technique, staining of the injected dermal spirochetes was comparable to that of the agar-embedded organisms (Fig. 11). In the skin preparation, the uniquely clumped spirochetal aggregate bodies were also demonstrated just as in the agar-embedded preparations (Fig. 9 and 10).

DISCUSSION

This study compared many staining methods that can be used to visualize the morphology of *B. burgdorferi* grown in cultures. Borreliae were detected with both the histochemi-

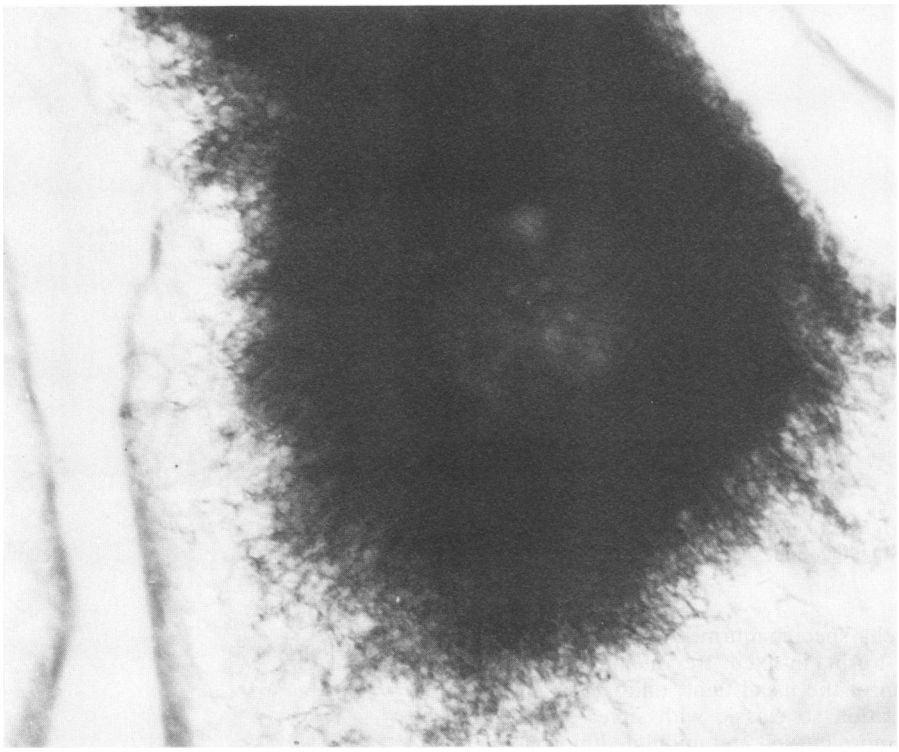

FIG. 9. *B. burgdorferi* B31. Agar embedding of clumped, aggregated borreliae can be seen. H9724 antibody was used. Magnification, ×1,000.

cal and fluorochrome methods. However, while these stains strongly suggest *B. burgdorferi*, monoclonal antibody reactivity would be optimal for specificity. Some of these methods, particularly the peripheral blood aniline dye stains, are suboptimal for routine use in tissue sections because of heavy background staining which makes borreliae indiscernible from the surrounding tissue.

The aniline dye stains, however, have potential for use in screening biologic fluids such as synovial, pericardial, pleural, ascitic, cerebrospinal, and urine. The ease of reagent preparation, their low cost, and their relative chemical stability offer further advantages in the screening of biologic fluids for the presence of spirochetes. If centrifuged sedi-

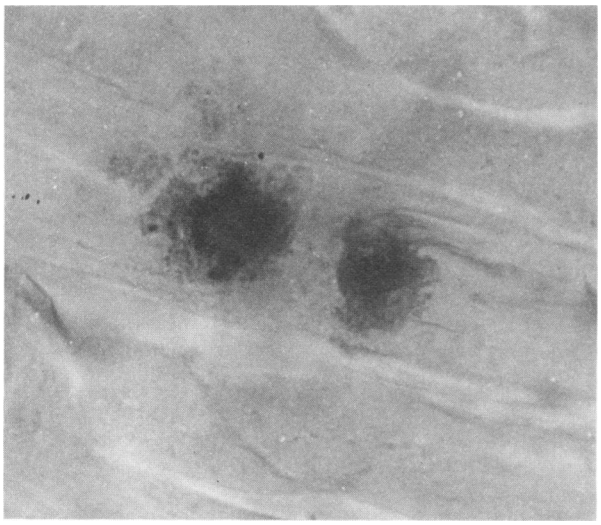

FIG. 10. *B. burgdorferi* B31-injected skin. Anti-rabbit W antibody was used. Magnification, ×630.

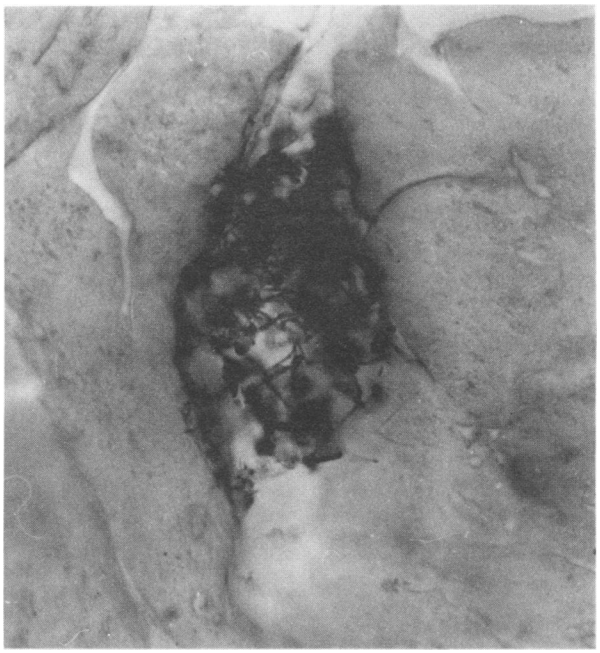

FIG. 11. *B. burgdorferi* B31-injected skin. Anti-human antibody was used. Magnification, ×1,000.

ment demonstrates likely structures in an initial laboratory screening, the sediment can be further examined with monoclonal antibodies such as H5332 or H3TS for confirmation, since these monoclonal antibodies react with a borrelia-specific 31-kDa protein antigen. The aniline dye stains were comparable in stain results, but occasionally the May-Grünwald stain produced sharper details of the spirochete. Wright stain offers the advantage of being a simple procedure. The eosin-thiazine reaction (Hemacolor) also was simple to use, but the reagents are more costly. The fluorochromes offered no advantage because their weak fluorescence was readily extinguished. Auramine-rhodamine fluorescence is more durable and is useful for screening for the tuberculosis bacterium, but our results were disappointing with this reagent.

Silver impregnations, especially the modified Dieterle and Bosma-Steiner techniques, do allow a very intense staining of borreliae. Moreover, in the silver stains single borreliae show better definition than the large, clumped, agglutinated forms, which were better outlined by the immunoperoxidase method. Aggregated borrelia forms yielded crystallike antibody deposits in some immunohistochemical antibody reactions, possibly reflecting an artifact (Fig. 5b). Antibody binding or fixation may be influencing this effect. We also found immunostain variability (incomplete staining or granular staining), depending on which monoclonal antibody was used (Fig. 7b). The strongest staining in the immunoperoxidase trials was obtained with the polyclonal human antiserum, with which outer membrane blebs were also seen very distinctly (Fig. 6 and 10).

Immunoperoxidase stains showed further variation in the thicknesses of the individual spirochetes. Some were thick and irregularly swollen (Fig. 5a), while other borreliae were thin and delicate (Fig. 5b and 7). Thus, structural features of *B. burgdorferi* varied with immunostaining conditions, such as the fixative used, whether the antibody was monoclonal or polyclonal, and what the target antigen was (e.g., flagellin or one of the membrane surface proteins). The age of the cultures also influenced spirochete cytomorphology, because younger cultures (e.g., 6 to 9 days) exhibited variably and irregularly coiled organisms, whereas older cultures demonstrated much longer, straighter single spirochetes with fewer coils. So-called filamentous forms were detected following treatment of the culture tubes and flasks with ultrasound (Fig. 12).

Acetone fixation seems to preserve the antigen receptors better than methanol or Formalin fixation, and the borreliae appeared thicker in the acetone-fixed preparations (Fig. 5a). We were especially interested in elucidating the cytomorphology of Formalin-fixed cultures with monoclonal antibodies to define the structural polymorphism under these conditions as a frame of reference for identifying similar structures in tissues. Formalin fixation is and has historically been a common, universal fixative for human tissues, and building a library of the variations in *B. burgdorferi* cytomorphology was one of our major aims. Additionally, we wanted to provide a framework for a better approach to the identification of putative spirochetal structures in tissue stromas. Moreover, if proven to be sensitive in labeling such tissue structures, anti-Osp A monoclonal antibody H5332 would render confidence that the spirochete was quite likely *B. burgdorferi*. In this study, H5332 antibody gave very weak staining, especially of H-1, H-2, and W (Viennese isolates), which may be due to variable amounts of Osp A protein in some European borrelia strains (Table 3) (24). These strains were strongly stained by only the H9724 antibody. Of the isolates from Vienna, only strain W reacted with another flagellar antibody (H6TS). The heterogeneous staining might reflect clonal polymorphism of borrelia cultures (8).

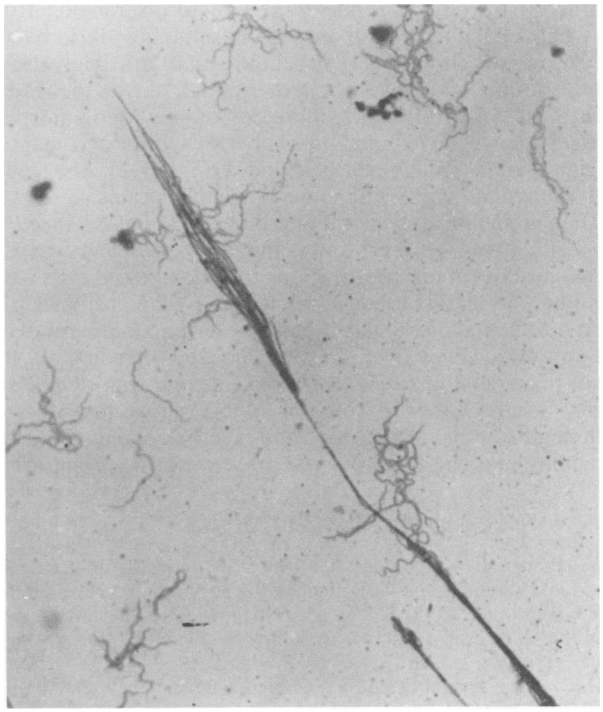

FIG. 12. *B. burgdorferi* B31. Formalin fixation and H63 antibody were used. Filamentous borreliae can be seen. Magnification, ×1,000.

Immunohistochemical stains of agar-embedded borreliae demonstrated clumps or bodies of agglutinated spirochetal structures (Fig. 9). These bodies were also present in the borrelia-injected skin preparations from dermal connective tissue (Fig. 10), the significance of which is not known.

Staining intensities and configurations varied depending on the antibody and fixative methods used (Table 2). Polyclonal immune sera on acetone-fixed spirochetes yielded the strongest staining reactions and demonstrated surface membrane blebs. As expected, monoclonal antibodies, which react with fewer antigenic determinants, resulted in more specific but weaker staining. This raises questions about the potential use of monoclonal antibodies in screening tissue sections for *B. burgdorferi*. *T. pallidum* Nichols was stained by the human and the two rabbit polyclonal antisera but not by the monoclonal antibodies, which should favor the use of monoclonal antibodies in tissue sections to exclude the syphilis spirochete. The fact that human polyclonal hyperimmune borrelia antiserum also stained *T. pallidum* Nichols (the substrate for the fluorescence treponema antibody test) is consistent with recent serologic findings that patients with antibodies against *B. burgdorferi* may also have a positive fluorescence treponema antibody test (14).

The cytomorphologic features of *B. burgdorferi* show marked polymorphism, a fact that makes its detection in tissue or biologic fluid samples challenging to the inexperienced microscopist (Fig. 1). *T. pallidum*, in contrast, is recognizable in tissues by the presence of relatively numerous spirochetes (compared with *B. burgdorferi* infections), having more or less regular corkscrew spirals. Structural cytopolymorphism, as seen in BSK-II cultures, seems to be

characteristic of *B. burgdorferi*, with forms ranging from very short bacilluslike curved cells resembling vibrio bacteria to remarkably long spirochetes that at times are a manifestation of two spirochetes joined "head to head." Figure 1 is a diagram of the more common cytopolymorphic forms that we have encountered. The laboratory-adapted original isolate from Shelter Island, N.Y. (B31), is exceedingly long compared with some European strains.

The significance of membrane blebs in some *B. burgdorferi* cells awaits further study, but their presence was detected in some of our preparations. Formalin-fixed cells were less likely to manifest blebs, while blebs were easily seen in acetone-fixed slides and in some of the immunoperoxidase preparations. They were also visualized in some of the eosin-thiazine-stained slides. Eosin-thiazine was clearly superior to the other peripheral blood stains tested, was technically simple to perform, and may be advantageous for screening biologic fluids for the presence of *B. burgdorferi*.

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