The wandering treponeme

*A possible source of error in fluorescent antibody staining of spiral forms in body fluids**

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Current techniques for both direct and indirect fluorescent antibody (FA) staining of treponemes in body fluids require that, at some time during the procedures, excess reagents be washed from smears of experimental specimens and control treponemal antigen (Venereal Disease Research Laboratory, 1967; Israel, 1969). Smith and Israel (1968) have mentioned the possibility that, if control antigen smears are washed in the same bath with experimental smears, treponemes may wash off control slides and float onto slides containing experimental specimens. More recently, Chandler and Cannefax (1969) have reported that nearly 75 per cent. of treponemes present on antigen slides may be lost during the direct FA procedure. This study examines the possibility that the organisms lost from control slides during staining and washing may adhere to other slides present in the wash bath.

Material and methods

ANTIGEN SMEARS

Treponema pallidum, Nichols strain, used as antigen for the fluorescent treponemal antibody absorption (FTA-ABS) test, was prepared by re-suspending the lyophilized organisms in sterile distilled water. Using a standard bacteriological loop, one loopful of the treponemal suspension was placed within the two pre-etched circles of alcohol-cleaned glass slides and allowed to dry in air. The slides were then fixed in acetone at room temperature for 10 minutes, air dried, and stored at -20°C . (U.S. Department of Health, 1969). When prepared in this manner, the antigen slides contained approximately 25 organisms per $540 \times$ microscopic field, distributed evenly within each pre-etched 14-mm. circle.

STAINING TECHNIQUE

Experimental slides

Six treponemal antigen smears were stained at one time. Two alcohol-cleaned blank glass slides, containing two pre-etched circles, were carried throughout the procedure to test the hypothesis of antigen migration from one slide to

another. The direct FA technique was performed by layering fluorescein isothiocyanate (FITC)-labelled anti-*T. pallidum* globulin, rabbit origin (Mothershed and Bullard, 1968), onto the six antigen smears. The conjugate had been diluted 1:40 in phosphate buffered saline (PBS), pH 7.2, containing 2 per cent. Tween 80 (U.S. Department of Health, 1969) and was spread evenly onto the dried antigen suspension within each etched circle with a wooden applicator stick. Care was taken not to allow the stick to touch the surface of the antigen smear. The six antigen slides overlaid with conjugate and the two blank glass slides were incubated for 30 minutes in a moist chamber at room temperature. After staining, excess reagent was removed by rotating each antigen slide 90° and tapping its edge against dry bibulous paper. The eight slides were then placed in the carrier of a glass staining dish with one empty glass slide alternating between two stained treponemal smears. The slides were soaked twice in PBS for 5 minutes and once in distilled water for 5 minutes (Israel, 1969). Approximately 200 ml. wash fluid were required in each wash to cover the slides completely. At the beginning of each wash, the slide carrier was agitated gently five times. After soaking, the eight slides were dried in air and stored at -20°C .

Before microscopic examination, frozen slides were allowed to thaw, and a small drop of mounting medium, prepared with 9 parts glycerine to 1 part PBS, pH 7.2, was applied to each etched circle. Care was taken to prevent contamination of the mounting medium with treponemes from the antigen smears. Disposable glass Pasteur pipettes were used to apply the mounting medium to the slides. The pipette never touched the glass slides, and after one pipette had been used to apply the medium it was never reintroduced into the medium. Cover slips were then affixed.

Control slides

FITC-labelled anti-*T. pallidum* globulin was layered evenly within the pre-etched circles of six alcohol-cleaned glass slides. No treponemal antigen had been smeared onto these slides. These six slides and two empty glass slides were incubated in a moist chamber for 30 minutes and placed in a slide carrier, as had been the antigen smears and experimental blank slides described previously. The slides were similarly soaked twice in PBS and once in distilled water. The six slides on which the conjugate had been layered were then discarded and the two empty

Received for publication July 22, 1971.

*Trade names are used for identification only and do not represent an endorsement by the Public Health Service or the U.S. Department of Health, Education, and Welfare.

slides were dried in air and stored at -20°C . These empty slides served as controls for spiral forms which might be present in the conjugate, PBS, distilled water, mounting medium, and on the glass itself.

MICROSCOPICAL EXAMINATION

Observations were made under oil at $540\times$ magnification with a Leitz ultraviolet SM microscope using a darkfield condenser, an HBO 200 mercury lamp, a BG-12 3-mm. thick excitation filter, an OG-1 barrier filter, and a 15-watt tungsten lamp. All observations were made by one observer using the same microscope. Every microscopic field within every etched circle in the experimental and control slides was examined for the presence of fluorescing spiral forms resembling *T. pallidum*. Morphological observations were confirmed with tungsten illumination. The observed microscopic field was 0.21 mm. in diameter. Since the inner diameter of each etched circle was 14 mm., the area examined within one circle contained approximately 4,500 microscopic fields. One stained treponemal antigen smear was examined with each slide as a control for the intensity of fluorescence of known *T. pallidum*.

Results

Ten previously blank experimental slides were examined. Brightly fluorescing spiral forms, morphologically indistinguishable from *T. pallidum* seen on the stained antigen smears, were found within the etched circles of eight of the ten slides. The number of fluorescing spiral forms varied greatly from slide to slide. In some only one or two scattered forms were present, and in others clumps as well as numerous single forms were seen. Although only the area within the etched circles was carefully examined for the presence of spiral organisms, rapid scanning over the remainder of some of the glass slides demonstrated scattered fluorescing forms outside the borders of the 14-mm. circles. No spiral forms seen by tungsten illumination failed to fluoresce when examined by ultraviolet light.

Twenty control slides were examined. No spiral organisms were found after thorough search.

Discussion

Brightly fluorescing spiral organisms, appearing identical to treponemal test antigen, were found on blank glass slides soaked in the same solution as stained treponemal antigen smears. No such forms were found on control slides using identical conjugate, buffer, distilled water, and mounted with the same media. Thus, stained *T. pallidum* organisms washed off the antigen smears, floated in the wash solution, and adhered to previously blank glass slides.

According to the calculations of Chandler and Cannefax (1969), approximately 75 per cent. of stained treponemal antigen washes off the smear during the direct staining procedure. Using a rough estimate,

approximately 2.25×10^5 *T. pallida* are present in the two etched circles of each antigen slide (25 organisms/field $\times$ 4,500 microscopic fields/etched circle $\times$ 2 etched circles/glass slide), or 13.5×10^5 organisms for the six antigen slides in the wash bath. If roughly 75 per cent. of these organisms wash off, approximately 1×10^6 organisms would be floating in the wash fluid at some time or another during the three washing periods. Each of the three baths requires 200 ml. of either PBS or distilled water. Therefore, if treponemes float off equally well during each wash, the concentration of organisms in the wash fluid is 1×10^6 organisms dispersed in 600 ml. total volume of wash fluid, or 1,600 treponemes/ml. wash fluid. However, since many organisms probably adhere to both sides of the blank glass slides, and re-adhere to the original antigen slides as well, the concentration of organisms floating free in the bath at any one time is likely to be lower than this approximate figure. Darkfield examination of the wash bath fluid revealed no treponemes. This is not surprising, as data from our laboratory (Clark, 1971) indicate that, at such a low concentration, organisms are quite difficult to find in fluid.

In view of these findings, it is obvious that smears of stained experimental material suspected of containing treponemes and smears of stained control treponemal organisms must be washed in separate dishes. Indeed, slides containing material from different experimental samples should be washed separately. Even though there may be very few spiral forms, if any, present in stained experimental smears, all chances that a spiral form in one specimen may migrate through the wash solution to another specimen must be eliminated. Furthermore, all washing dishes should be scrupulously cleaned before each batch of smears is washed to avoid the possibility that spiral forms attached to the walls of the dish during a previous washing, may float off and adhere to the slides being washed. Care must also be taken that the mounting medium is free of contamination by treponemes. Although the direct FA technique was used exclusively in this experiment, similar precautions should be used when an indirect FA staining method (Israel, 1969) is performed, if reagents are washed from smears in a similar manner.

The implications of this experiment, using a direct FA technique to search for spiral organisms, cannot be applied to laboratory procedures designed to detect the presence of antibodies directed toward *T. pallidum*, such as the FTA-ABS test on serum. The washing procedure used in the present experiment, consisting of three 5-minute washes, is different from that used in the FTA-ABS test, in which slides are rinsed in running wash solution before and after two

5-minute washes (U.S. Department of Health, 1969). Furthermore, in the FTA-ABS test, so many stained organisms are already present on each slide that the migration of a few organisms between slides would not affect the interpretation of overall fluorescence. It is only when few organisms are present on the entire slide, as when fluids are examined for the presence of treponemes, that an organism floating from one slide to another may be falsely interpreted as being highly significant.

Summary

Fluorescing *T. pallida* were found on previously blank glass slides which were soaked in the same bath as stained antigen smears of *T. pallidum*. No fluorescing spiral forms were found on control slides washed in the presence of FITC-labelled anti-*T. pallidum* globulin. Therefore the stained *T. pallida* floated in the wash bath from the antigen smear to the blank slides. During FA staining, experimental and control smears should be washed separately to avoid contamination of experimental material with *T. pallidum* organisms.

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Le tréponème errant: une cause d'erreur possible dans la coloration par l'anticorps fluorescent de formes spiralées dans les humeurs de l'organisme

SOMMAIRE

Des *T. pallida* fluorescents furent trouvés sur des lames de verre antérieurement 'vides' qui avaient été trempées dans un même bain que des lames portant de l'antigène *T. pallidum* marqué. Il ne fut pas trouvé de formes spiralées fluorescentes sur des lames témoins lavées en présence de globulines anti-*T. pallidum* marquées par l'isothiocyanate de fluorescéine. Ainsi, des *T. pallida* marqués flottaient dans le bain de lavage, passant de lames chargées d'anticorps aux lames propres. Lors de la coloration par l'anticorps fluorescent, les prélèvements expérimentaux et témoins doivent être lavés séparément pour empêcher la contamination du matériel expérimental par des *T. pallida* errants.