The Use of Improved Silver Impregnating Staining Method in the Differential Diagnosis of Tuberculoid Leprosy

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The histopathological picture of tuberculoid leprosy is epithelioid cell granuloma. However there are many skin diseases such as sarcoidosis, granuloma annulare, granuloma multiforme, secondary syphilis, leishmaniasis, some deep fungal diseases and other mycobacterioses which are manifested as epithelioid cell granuloma, too (1, 2). The key to differential diagnosis is the involvement of cutaneous nerves and the presence of acid-fast bacilli (AFB). Therefore it is most important to identify cutaneous nerves, then to confirm the correlation among nerves, infiltration and AFB (1, 2, 3). Some authors utilized silver impregnating methods for the demonstration of axons of nerve fibers as an aid in the differential diagnosis of leprosy. Unfortunately, most silver impregnating methods used for paraffin sections of skin biopsies were not so stable for practical use (4). The immunostaining with anti S-100 antibody has also been used for the same purpose (2, 3). Recently, K. Kawatsu being good at medical photography adopted some chemicals, which are usually used as components of emulsion or developer, and thus established his silver impregnating method (5, 6). This paper presents a study of ten cases, who had been firmly diagnosed as borderline tuberculoid leprosy in the National Institute for Leprosy Research, Tokyo, Japan, to compare Kawatsu’s silver impregnating method with S-100 protein ABC technique for the detection of cutaneous nerves in the differential diagnosis of tuberculoid leprosy.

MATERIALS and METHODS

Paraffin blocks of 10% formalin-fixed skin biopsies taken from ten active borderline tuberculoid leprosy cases were retrieved from the files of the National Institute for Leprosy Research, Japan. The blocks were cut into 5 μm thick serial sections, then stained with hematoxylineosin (H & E) for routine histopathological examination, by Kawatsu’s method and S-100 protein ABC technique for the demonstration of cutaneous nerves.

Kawatsu’s staining.

1. Deparaffinize sections in xylene and hydrate through graded alcohols to water.
2. Bring sections to distilled water.
3. Stain with freshly prepared gelatin silver solution at 60°C for 16 to 24 hrs, changing the solution once.

The gelatin silver solution was prepared as follows:

- distilled water 90ml
- 5 % gelatin (BBL, DIFCO, SIGMA type III) 10ml
- 1 % silver nitrate 1ml
- 1 % borax 1ml

4. Place in reducing solution (hydroquinone 1 g, sodium sulfite 2 g, add distilled water to 100ml) at 30 to 35°C for 10 min.
5. Wash in three changes of distilled water for 10 min.
6. Tone in 0.5-1% gold chloride solution for 20 min.
7. Wash in three changes of distilled water for 10 min.
8. Place in 2% oxalic acid for 10 min.
9. Wash in three changes of distilled water for 10 min.
10. Fix in 5% sodium thiosulfate solution for 3 min.
11. Rinse in tap water for 5 to 10 min.
12. Stain with nuclear fast red for 5 min.
13. Rinse in tap water.
14. Counterstain with 0.01% methyl blue (Merck, art. 16316) in saturated picric acid aqueous solution for 5 min.
15. Dehydrate rapidly in absolute alcohol, clear in terpineol-xylene (1:1), followed by xylene and mount.

The staining scheme is: axons, black; nuclei, red to light brown; connective tissue, light blue to green; muscles, yellow to light green.

S-100 protein ABC technique:
1. Deparaffinize and hydrate through xylene, graded alcohols to water.
2. Rinse in distilled water.
3. Incubate in 0.3% H₂O₂ in methanol for 30 min.
4. Wash in PBS (pH 7.4) for 20 min, three changes.
5. Incubate with blocking serum for 20 min.
6. Blot excess serum from sections.

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K = Kawatsu's silver impregnating method
S = S-100 protein ABC technique
7. Incubate with anti S-100 protein antibody (Dako), at a dilution of 1:1000, for 30 min.
8. Wash in PBS for 10 min, three changes.
9. Incubate with diluted biotinylated secondary antibody solution for 30 min.
10. Wash in PBS for 10 min, three changes.
11. Incubate with VECTASTAIN ELITE ABC reagent for 30 min.
12. Wash in PBS for 10 min, three changes.
13. Incubate in DAB solution for 5 min.
14. Wash in tap water for 5 min.
15. Counterstain with hematoxylin, clear and mount.

The staining scheme is:
Schwann cells, melanocytes, Langerhans' cells and eccrine glands are brown.

RESULTS

Examples stained by Kawatsu's method and S-100 protein ABC technique are shown in Fig. 1-4. Results of neurohistopathology are given in the table. Severe infiltration and destruction in the peripheral nerve were seen in all 10 cases. The neurohistopathological changes observed were as follows: nerve endings around skin appendages disappeared in 5 cases; no unaffected nerve bundles were shown in 4 cases; nerve bundles involved in non-specific inflammation in 3 cases; nerves invaded by epithelioid cell granulomas were seen in all 10 cases; nerve bundles with fibrosis and/or hyalination were suspected in 2 cases.

DISCUSSION

The results obtained from unaffected nerve bundles, nerve bundles involved in non-specific inflammation or granulomatous infiltration were almost the same by Kawatsu's method or S-100 protein ABC technique.

The minimal remnants were beyond recognition in H&E staining sections. By S-100 protein ABC technique, these were shown in 5 cases and suspected in 2 cases because in case of weak positive cases, the minimal remnants were indistinguishable from the processes of Langerhans' cell (3, 8). However by Kawatsu's method, the minimal remnants were detected in 9 cases, including the suspected cases, by the appearance of black axon in the background of blue endoneurium. Additionally, some completely fibrosed and/or hyalinated nerve segments, whose S-100 protein and axon staining were all negative, were stained in bright blue that looks different from normal connective tissue in green.

As for nerve endings, when stained by Kawatsu's method, the nerve endings were showed in 5 cases around hair follicles while stained by S-100 protein ABC technique, seen only in 2 cases, one was around hair follicle, another was surrounding sweat glands. Demyelination is an early change of nerve fibers caused by M. leprae (7). Consequently, some nude axons, which are similar to nerve endings, still exist after being demyelinated. This kind of axons with degeneration in varying degrees can be demonstrated by silver impregnating staining, even though there is no possibility of showing by S-100 protein ABC technique.

Besides, as discussed above, Kawatsu's method is a cost-effective method compared with S-100 protein ABC technique. Therefore its usefulness for the diagnosis of tuberculoid leprosy will be, not only in a research centre but also in peripheral laboratories directly serving the field work of the leprosy control programmes, especially in developing countries.
Fig. 1.
a large number of nerve endings around hair follicle. Kawatsu's method; ×1000.

Fig. 2.
axons inside an epithelioid cell granuloma. Kawatsu's method; ×1000.

Fig. 3.
an axons within an epithelioid cell granuloma. Kawatsu's method; ×1000.
SUMMARY

A comparative study on the usefulness of Kawatsu's silver impregnating staining method compared with S-100 protein ABC technique for the differential diagnosis of tuberculoid leprosy was carried out. The results of neurohistological examination obtained from both methods were almost the same. It can be said that Kawatsu's method is useful and cost-effective, and thus suitable for practical use in developing countries.
REFERENCES


類結核ららいの鑑別診断における改良鍍銀染色法の有用性

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キーワード: 改良鍍銀法, ららい, 神経組織病理学

類結核ららいの鑑別診断における, 川津の鍍銀法と, ABC法によるS-100蛋白質の免疫組織染色法の有用性を比較検討した。その結果, 皮膚生検標本中の神経の組織学的検索において, 両法の結果はほぼ同じであった。この結果からわれわれは, 川津法は, 類結核らいの鑑別診断のために有用であり, 費用便益の面でも開発途上国での実用に適した方法であると結論した。