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Methenamine Silver Stain Impregnates Amyloid-related Components of Senile Plaques in the Alzheimer Brains as Clearly as β protein Immunostaining

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SUMMARY: We developed a new type of methenamine silver (MS) stain for senile plaques (SP) on paraffin-embedded tissue sections. This method is a modification of both Gomori's methenamine silver nitrate, and Jone's periodic acid-methenamine silver (PAM) stains. We omitted chemical pre-oxidation by either periodic acid in the PAM stain or chromic acid in Gomori's stain, and determined the best condition of silver solution for SP. This resulted in selective staining of SP and amyloid angiopathy, because capillary basement membranes, corpora amylacea, macrophage granules, and nerve cell pigments remained unstained. The argyrophilia of SP was not affected by pretreatment with aldehyde blocking reagents. In comparison with β protein immunostain, our MS stain showed almost the same staining pattern as that of the β protein immunostain. Moreover, the argyrophilia of SP was selectively abolished by formic acid pretreatment, which has been shown to destroy β-pleated sheet structure of amyloid, suggesting that our MS stain impregnates amyloid-related components of SP. Our MS stain is a new, rapid, easy, and reliable method, and sensitive enough for routine or screening studies of the SP.

INTRODUCTION

The formation of senile plaques (SP) is one of the hallmarks of

Alzheimer-type dementia (ATD) including senile dementia of the Alzheimer type (SDAT) and presenile Alzheimer's disease, and the extent of SP formation has been known to correlate with intellectual decline in the disease (1,2). Abundant SP formation is confined to ATD and Down's syndrome, whereas Alzheimer's neurofibrillary tangles (ANT) are present in a wide range of diseases (3). Moreover, Terry et al. (3) have reported that a considerable minority of the SDAT lacked neocortical ANT. Therefore, the sensitive detection of SP is necessary for the pathological diagnosis of ATD, especially in the SDAT, where proper diagnosis depends on knowing the extent of SP formation (4).

Recently, the periodic acid-methenamine silver (PAM) stain has been shown to demonstrate SP sensitively in the brains of the ATD patients (5,6). In the present study we show a new type of methenamine silver (MS) stain. This method is more specific to the amyloid of SP than the PAM stain, and results in a stain similar to that of the β protein immunostain. Moreover, our MS stain is rapid, easy, and sensitive.

MATERIALS AND METHODS

We examined formalin-fixed paraffin-embedded tissue blocks of brains from five ATD patients (four SDAT and a Alzheimer's disease), aged between 62 and 83 years old and five nondemented aged controls.

To determine the best condition for labeling SP, deparaffinized sections were stained with various conditions as follows: 1) Working silver solution: a. various concentrations of methenamine of 3, 6, and 12%; b. various amounts of 5% borax of 0, 2, 4, 6, 8 and 12ml added to 100ml of silver solution. 2) Various exposure times from 90 to 180 minutes. The recommended procedure of new MS stain is shown in Table 1.

Prior to MS staining, some sections were immersed in a molar aniline hydrochloride or 5% aqueous phenylhydrazine hydrochloride solutions for 24 hours in order to block aldehyde groups in the section. Other sections were pretreated with 99% formic acid for 5 minutes and processed for the MS stain.

Serial 6 μm-thick sections were cut from the formalin-fixed and paraffin-embedded tissue blocks. Deparaffinized sections were processed for new MS and PAM (7) stains (Table 2), and immunohistochemistry for β protein (8). For β protein immunostain, sections were pretreated with 86

or 99% formic acid for 5 minutes (9), incubated with rabbit antiserum to β protein (8) (diluted in 1:2,000 in 2% normal goat serum), and followed by the avidin-biotin peroxidase complex method (Vector Lab., USA). Some sections were used for Gomori's methenamine silver nitrate stain (9).

RESULTS

The specificity of the MS stain for amyloid depended on the concentration of methenamine and amount of borax. The most specific stain was obtained at the condition of 6% methenamine and 2ml of 5% borax to 100 ml of silver solution (Fig. 1a). However, this low dosage of borax made the silver solution unstable, and resulted in some early deposition of small silver granules. Background staining increased with each increase in the amount of added borax. Therefore, we determined the best condition to be 6% methenamine and 6ml of borax (Table 2).

We carefully observed serial sections stained by our MS (without pretreatment), PAM (pretreated with periodic acid) or β protein stains, and compared the same SP in different slides (Fig. 2 and 3). Each plaque demonstrated by our MS stain was clearly labeled by the β protein immunostain. In addition to SP, the PAM stain labeled basement membranes of the blood vessels, corpora amylacea, and cytoplasmic granules of the macrophages and neurons which were positive for periodic acid-Shiff (PAS) reaction (Fig. 2c and 4). Occasionally, blackened macrophages were hardly distinguishable from the amyloid core of SP in the PAM sections (Fig. 4). Our MS stain did not label these PAS-positive components. Thus, the SP were selectively demonstrated by our MS stain(Fig. 2 and 3). Amyloid angiopathy was also clearly verified in MS and β protein preparations (Fig. 3a,b), although the MS stain impregnated the nuclei of endothelial cells. The PAM stain blackened both normal and amyloidotic vessels (Fig. 3c). Normal axons and degenerated neurites of SP were not labeled by any of these methods. ANT were occasionally impregnated (Fig. 1b), especially by over-staining, but not consistently.

Toning in gold chloride solution markedly reduced the background stain, making it useful for long period-fixed materials, which showed reduced argyrophilia of SP and high background staining.

In five brains from mentally-normal aged people, our MS stain impregnated SP demonstrated by the β protein immunostain.

Gomori's original method (pretreatment with chromic acid) faintly impregnated SP with a high background stain.

Aldehyde blocking reagents did not block the argyrophilic reaction of SP, showing that the argyrophilia of SP did not depend on the aldehyde groups. A formic acid pretreatment completely abolished the argyrophilia of SP in MS preparations (Fig. 5).

DISCUSSION

In 1946, Gomori (9) reported his methenamine silver nitrate stain for mucin and glycogen. The mode of this staining action is, in part, probably based upon the hydrolysis and oxidation of polysaccharides to create new aldehyde groups (10). The silver of methenamine-silver complexes is then reduced to metal particles by these aldehydes upon warming. In 1953, Jones (11) applied PAM stain for kidney, where periodic acid was used as pretreatment instead of the chromic acid of Gomori's original method, and he reported the actual technique in 1957 (7). The PAM stain blackens the tissue elements which are positive to the PAS reaction. Blackened macrophages, which appeared in the center of SP, resembled the amyloid core of SP and made it difficult to classify the SP. To obtain a clearer staining of SP than achievable using the PAM stain, we tried omitting the periodic acid pretreatment. Resulting MS stain showed that SP were argyrophilic even when chemical pre-oxidation was omitted, and that PAS-positive materials lost their argyrophilia. Moreover, aldehyde blocking reagents caused minimal effects on argyrophilia of SP in our MS preparations. The SP may contain intrinsic reducing substances.

We have already reported that most argyrophilia of SP disappears when sections are treated with formic acid prior to PAM or modified Bielschowsky stains, but ANT and altered neurites of SP remain argyrophilic after formic acid pretreatment in modified Bielschowsky preparations (6). Here, we demonstrated that our MS stain did not impregnate SP after pretreatment with formic acid, which destroys β-pleated sheet structure of amyloid, abolishes Congophilia of amyloid, and highly enhances the βprotein immunostain (12). Therefore, we suggest that the argyrophilia of SP in our MS preparations depends on amyloid-related substances, but not on neuritic components.

The SP were generally classified into three types: primitive, classic

and compact plaques (13). Recently, we demonstrated a new type of SP, diffuse plaques, which were labeled by the β protein immunostain with formic acid pretreatment, modified Bielschowsky stain and PAM stain as an ill-defined area of fine fibrillar material (6,8). Diffuse plaques primarily consist of SP in SDAT brains (6). The diffuse plaques were not detected by the Bodian stain because of lacking altered neurites. Neither Congo red nor PAS stains can demonstrate diffuse plaques (8). Bielschowsky preparations ordinarily tend to suppress diffuse plaques, but these plaques appear most distinctly when the Bielschowsky method is unsuccessful (14). The diffuse plaques were clearly labeled by the von Braunmuhl technique (15) and King's silver stain for amyloid (14), although these methods generally work best for frozen sections (Table 2). In this study we demonstrated that our MS stain is sensitive enough to detect any type of SP including diffuse plaques on paraffin sections in ATD, Down's syndrome, and mentally-normal aged brains. Recent autopsy criteria for the diagnosis of ATD depends on the age of the patient, and the extent (location and density) of SP and ANT formation (4). This criteria demands quantitative measurement of SP. Our MS stain is useful for this purpose because of its clear and sensitive stain of SP.

Compared to the β protein immunostain, our MS stain has many advantages: simplicity, speed, cost, and avoidance of any specific antiserum. Therefore, the MS stain is appropriate for routine and screening studies for SP.

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Table 1. Recommended procedure of methenamine silver stain

1) Hydrated sections are washed well (3 changes) in distilled water (DW).
2) Place in methenamine silver solution until sections become light tan in color, checking before the finish. This takes about 2 hours at 56 °C incubation.
3) Wash in DW (3 changes).
4) Place in 4% neutral formalin reducing solution for 5 minutes. If sections are over-stained and showed a deep tan-back color, immerse in 0.2% gold chloride toning solution for 10 minutes instead of the reducing solution.
5) Wash in DW (3 changes).
6) Place in 5% sodium thiosulfate solution for 5 minutes.
7) Wash in running tap water for 5 minutes.
8) Dehydrate in graded alcohol line, and mount.

Methenamine silver working solution: Add 5 ml of 5% silver nitrate solution to 100 ml of 6% methenamine solution, mix well until complete clearness, then add 6ml of 5% borax, and mix well. Prepare just before use.

Table 2: Comparison of silver impregnation methods

Report Name	Year	Sectioning	Oxidation as pretreatment	Silver solution Amino-group	Silver solution Alkali	Temperature of exposure	Original purpose
Gomori	1946	Paraffin	Chromic acid, next, Na_2SO_3	Methenamine 1.5 %	Borax* 2-4 ml	37-45°C	Mucin and glycogen
Jones	1957	Paraffin	Periodic acid	Methenamine 3 %	Borax 12 ml	70°C	Glomerular basement membrane of kidney
Yamaguchi et al.	1989	Paraffin	Not treated	Methenamine 6 %	Borax 6 ml	56°C	Amyloid-related components of senile plaques
King	1948	Frozen	Not treated	Ammonia (+Pyridine)	Na_2CO_3	45°C	Amyloid

*Amount of 5% borax added to 100 ml of working silver solution.

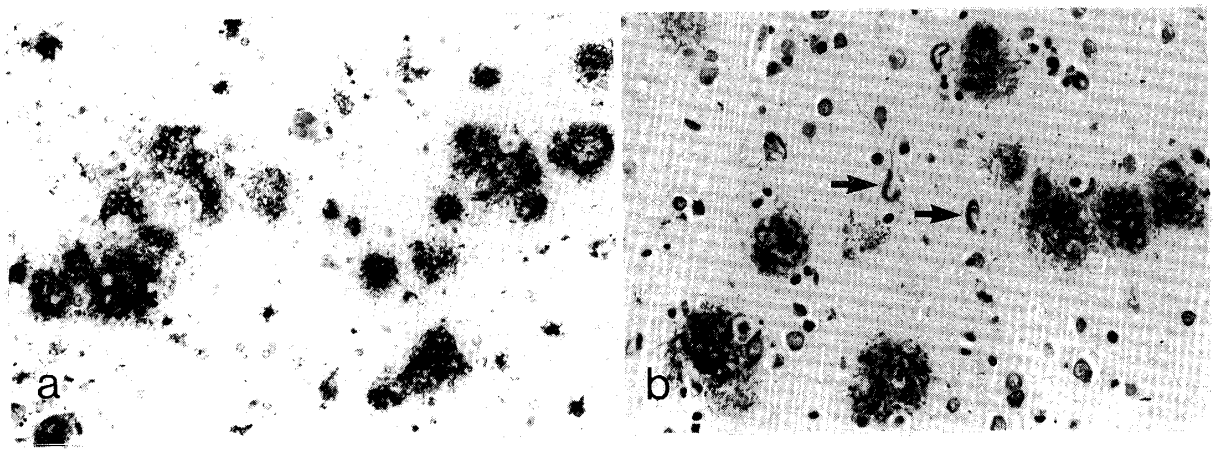

Fig. 1: Variations of MS stain. a: the most selective stain of SP was obtained at the condition of 6% methenamine and 2 ml of 5% borax added to 100 ml of silver solution without toning. Frontal cortex, SDAT, X260. b: ANT (arrows) were labeled in the section which was over-stained in silver solution with 3% methenamine and 12 ml of borax, and then toned in gold chloride solution. Frontal cortex, Alzheimer's disease, X260.

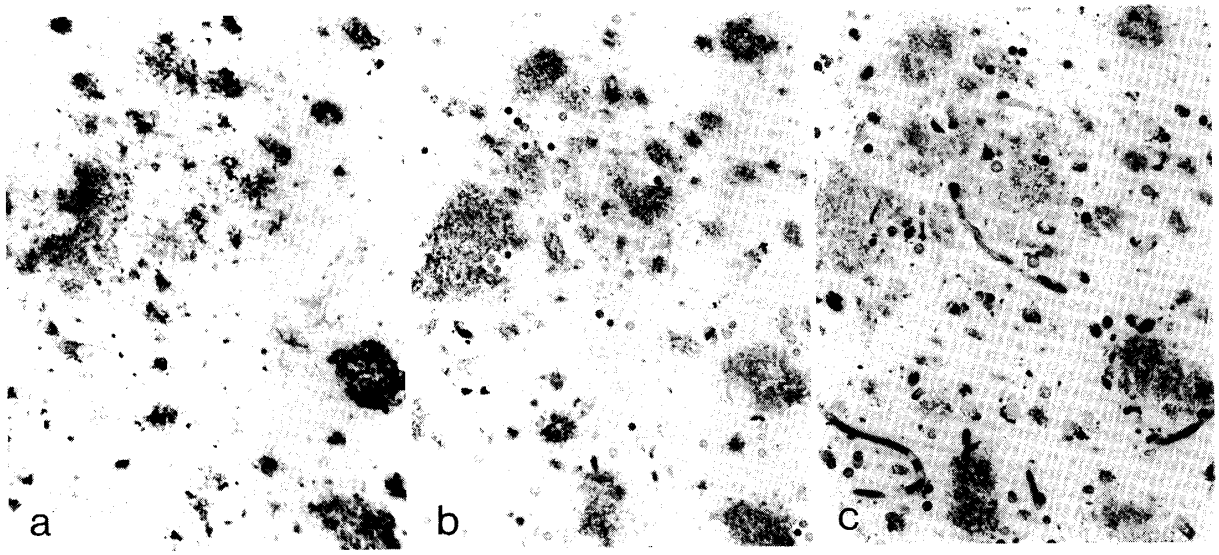

Fig. 2: Serial sections stained with β protein immunostain (a), new MS stain (b), and PAM stain (c), showing numerous SP. The MS stain is more specific to the amyloid of SP than the PAM stain, and results in a stain similar to that of the β protein staining. Frontal cortex, SDAT, X280.

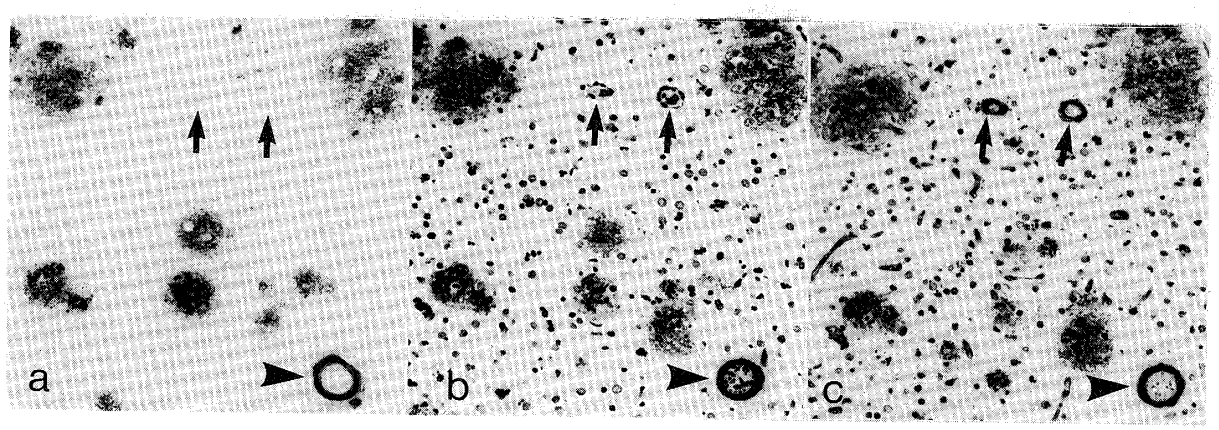

Fig. 3: Serial sections stained with β protein (a), MS (b), and PAM stains (c). Vessels with amyloid angiopathy (arrow heads) and without (arrows), were verified in β protein and MS, but not in PAM preparations. Frontal cortex, Alzheimer's disease, X140.

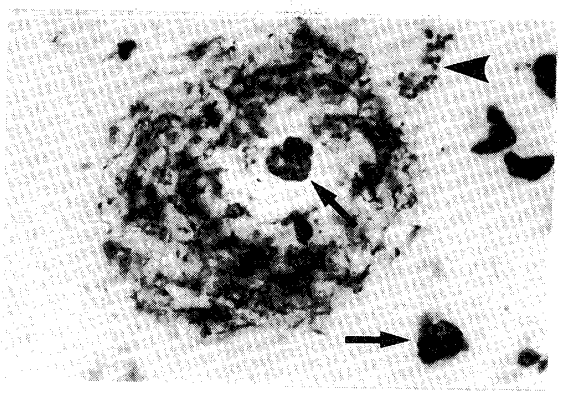

Fig. 4: The PAM stain impregnated cytoplasmic granules of macrophages (arrows). The macrophage resembles amyloid core of SP when it appears in the center of SP. Arrow head indicates the neuronal cell pigments. Frontal cortex, SDAT, X1,000.

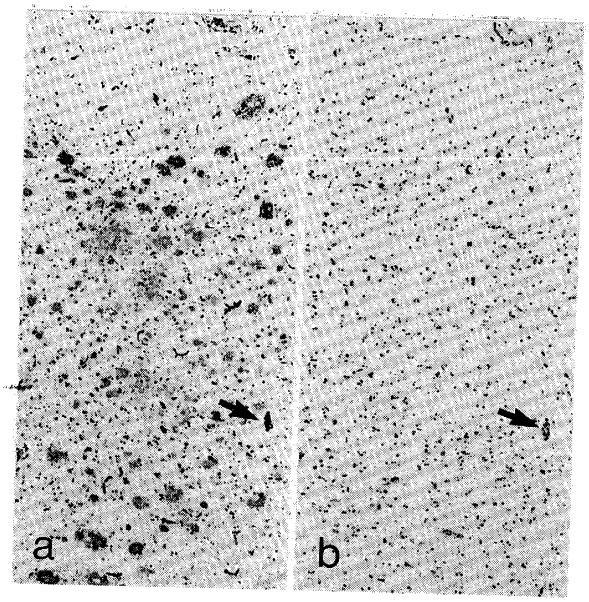

Fig. 5: The effect of formic acid pretreatment on the MS stain. Senile plaques in the non-treated section (a) disappeared with pretreatment (b). Stain of nuclei and erythrocytes (arrows) were resistant to formic acid. Frontal cortex, SDAT, X60.