

Original Article

Immunohistochemical Detection of Aflatoxin in Lesions of Aflatoxin-producing *Aspergillus flavus* Infection

Takeshi Mori¹, Hiroshi Abe², Mitsutaka Yoshida³, Yutaka Tsukune¹, Yuriko Yahata¹, Tomoiku Takaku¹, Jun Ando¹, Miki Ando¹, Shinichi Torii⁴, and Makoto Sasaki¹

¹ Division of Hematology, Department of Internal Medicine, Juntendo University Graduate School of Medicine

² Department of Host Defense and Bio-chemical Research, Juntendo University Graduate School of Medicine

³ Laboratory of Morphology and Image Analysis, Research Support Center, Juntendo University Graduate School of Medicine

⁴ Laboratory of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Science, the University of Tokyo, and Vermilion Therapeutics Inc

ABSTRACT

Aflatoxin produced by *Aspergillus flavus* is known to be strongly related to liver injury (hepatocellular carcinoma) and immune system damage involving leukocytes. This toxin suppresses both the cell-mediated immune system and macrophage function, and decreases the production of complement and interferon molecules.

Purpose: To evaluate the presence of aflatoxin in infectious lesions as well as how the toxin is taken up by leukocytes.

Method: Pathological specimens from a patient who died from aspergillosis caused by aflatoxin-producing *A. flavus* were used. Anti-aflatoxin B₁ antibody was reacted with paraffin-embedded lesion specimens from the heart, kidney, and thyroid gland of the patient and observed microscopically.

Result: Positive reactions were detected in fungal elements and leukocytes (neutrophils and macrophages) in inflammatory lesions.

Conclusion: Within the patient's body, *A. flavus* likely produced aflatoxin, which then was taken up by neutrophils and macrophages.

These results suggest that leukocyte function and the immune mechanism are locally suppressed by aflatoxin.

Key words : aflatoxin, *Aspergillus flavus* infection, immunohistochemical detection, pathological finding

Introduction

Several studies have evaluated hepatic toxicity following oral intake of plant products, such as peanuts, or after consuming meat or dairy products from animals that have consumed feed contaminated with aflatoxin.

Numerous reports have described the role of aflatoxin in hepatic cancer as well as its effect on the immune system.

Infection with an aflatoxin-producing strain of *Aspergillus flavus* is very rare, particularly in Japan. We previously reported the only clinical case of aflatoxin-producing *A. flavus* infection lesions.

Pathological studies have been conducted to determine the localization of aflatoxin. However, it remains unclear how

leukocyte function is disrupted by aflatoxin intoxication.

In this study, we examined the location of aflatoxin in the body using pathological specimens from a patient who died from aflatoxin-producing *A. flavus* infection^{1,2)}.

Methods

Pathological specimens (formalin-fixed and paraffin-embedded sections) of the heart, kidney, and thyroid gland of a previously reported case^{1,2)} were used.

Immunohistochemistry using anti-aflatoxin B₁ antibody was performed on paraffin sections, and the localization of aflatoxin in the focus was evaluated.

The aflatoxin B₁ Mouse Monoclonal Antibody (Clone 6A10) [Novus Biologicals, Littleton, CO, USA] was used.

Address for correspondence: Takeshi Mori, MD

Division of Hematology, Department of Internal Medicine, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

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E-mail: mtlemonade753@yahoo.co.jp

Hematoxylin-eosin (HE) staining, periodic acid-Schiff (PAS) staining, and immunohistochemical staining using the anti-aflatoxin B₁ antibody (6A10) were performed, and the results were compared with those obtained from other specimens. A negative control sample was also examined.

Immunohistochemistry was performed as follows:

1. Paraffin sections at thickness of 4 µm were dried and dewaxed in xylene.
2. The specimens were placed in 0.3% hydrogen peroxidase in 100% methanol to quench endogenous peroxidase for 20 min.
3. The specimens were incubated with 10% normal goat serum to prevent nonspecific binding of antibody to tissues.
4. The specimens were incubated with 1:100 solution of the primary antibody (anti-aflatoxin B₁) in PBS overnight at 4°C.
5. The specimens were incubated with the secondary antibody (peroxidase-conjugated goat anti-mouse IgG-antibody (Fab')) for 30 min at room temperature. Histofine Simple Stain MAX-PO (M) (Nichirei Bio-sciences, Inc., Tokyo, Japan) was added and reacted for 30 min at room temperature and then washed with PBS.
6. For colorimetric detection, the sections were reacted with 0.05% hydrogen peroxide, and then 3,3-diaminobenzidine solution was added for incubation at room temperature for 30 min. The samples were washed with PBS followed by washing with water.
7. Specimens were stained with hematoxylin to allow visualization of the nuclei and were observed by light microscopy.

As control (negative contrast), a mouse normal serum antibody rather than primary antibody was reacted using the same procedure and conditions. A positive reaction was considered as the appearance of color in the specimen compared with that in the control.

Results

In the HE and PAS staining, numerous fungal elements were detected in cardiac muscles and blood vessels with inflammatory cell infiltration (Fig. 1a, b, Fig. 2a, b).

In kidney specimens, fungal elements and leukocytes that had phagocytosed the fungus were observed in the blood vessels and interstitial lesions in inflammatory lesions, similar to those observed in the heart specimens. Fungal elements were not detected in the glomerulus or tubules (Fig. 1c, d, Fig. 2c, d).

Fungal elements and leukocytes attached to fungal elements were observed in the follicle, follicle walls, and interstitial inflammatory tissues of the thyroid gland (Fig. 1e, f, Fig. 2e, f).

In the staining for the negative control samples, numerous fungal elements were observed in the cardiac muscle, blood

vessels, and vessel walls with inflammatory lesions. In addition, the cytoplasm of fungal elements and that of leukocytes adhered to fungal elements were stained as bluish purple in some places (Fig. 3).

Fungal elements were stained by immunohistochemistry. In each lesion of the heart (Fig. 4), kidney (Fig. 5), and thyroid gland (Fig. 6), the fungal elements were stained as tea brown, indicating a positive antibody reaction. A negative antibody staining result was indicated by a bluish purple color. The areas that were stained bluish purple and tea brown indicated that aflatoxin was unequally distributed in elongated fungal elements.

Scattered leukocytes in the lesions and leukocytes attached to fungal elements were mixed together, showing a positive reaction as tea brown around a negative reaction as bluish purple.

In both large and small follicles, and interstitial tissues of the thyroid gland, positive reactions were detected in the fungal elements and in the leukocytes (neutrophilic leukocytes and macrophages) close to and distant from the fungal elements.

Discussion

A. flavus, *Aspergillus parasiticus*, and *Aspergillus nomius*, among others, are generally known to produce aflatoxins B₁, B₂, and G₁. Aflatoxins M₁ and M₂ are respectively hydroxylated metabolites of aflatoxins B₁ and B₂. Aflatoxin B₁ is the most toxic, whereas aflatoxins M₁ and M₂ are considered less toxic³⁾.

Numerous studies have reported the production and metabolic mechanism of these proteins^{4, 5)}.

In addition to aflatoxin M₁, aflatoxins B₁ and B₂ were previously detected in lesions in our case^{1, 2)}.

Aflatoxin is thought to suppress the cellular immune response, reduce the function of macrophages, decrease the production of complement and interferon, and reduce the function of reticuloendothelial cells, thereby increasing the susceptibility to infectious diseases⁶⁻¹⁸⁾.

In addition, aflatoxin reduces enzymatic activity in metabolism, prevents sugar metabolism, suppresses the production of phosphoric acid and free fatty acid, decreases the synthesis of triglycerides, cholesterol, and esters, and inhibits DNA, RNA, and protein synthesis¹⁵⁾.

Detection of aspergillosis in lesions caused by aflatoxin-producing strains of *Aspergillus* spp. is extremely rare. Additionally, one case of keratitis due to a mycotoxin-producing *Aspergillus pseudotamarii*¹⁹⁾ is the only reported case, excluding our case.

Not all fungal elements produced aflatoxin, and the toxin appeared to remain localized at its site of production.

The staining patterns of leukocytes in lesions showed that

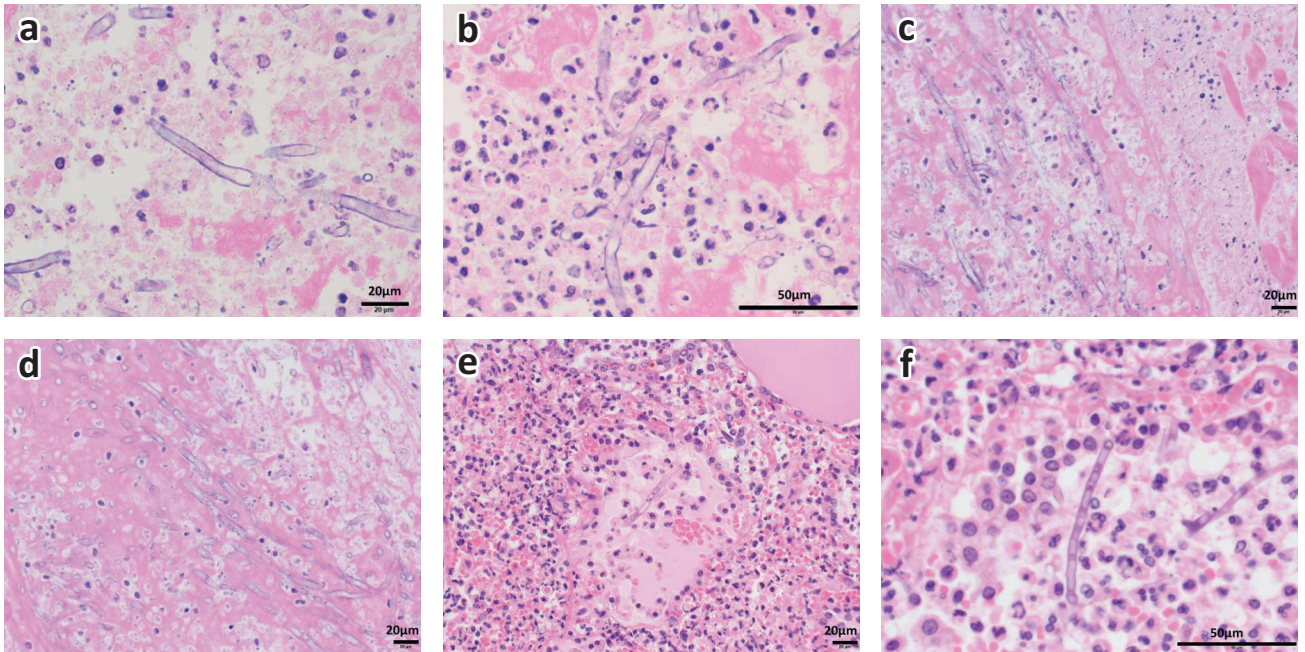


Fig.1. Pathological findings by HE staining.
Fungal elements in lesions of the heart (a, b), kidney (c, d), and thyroid gland (e, f).

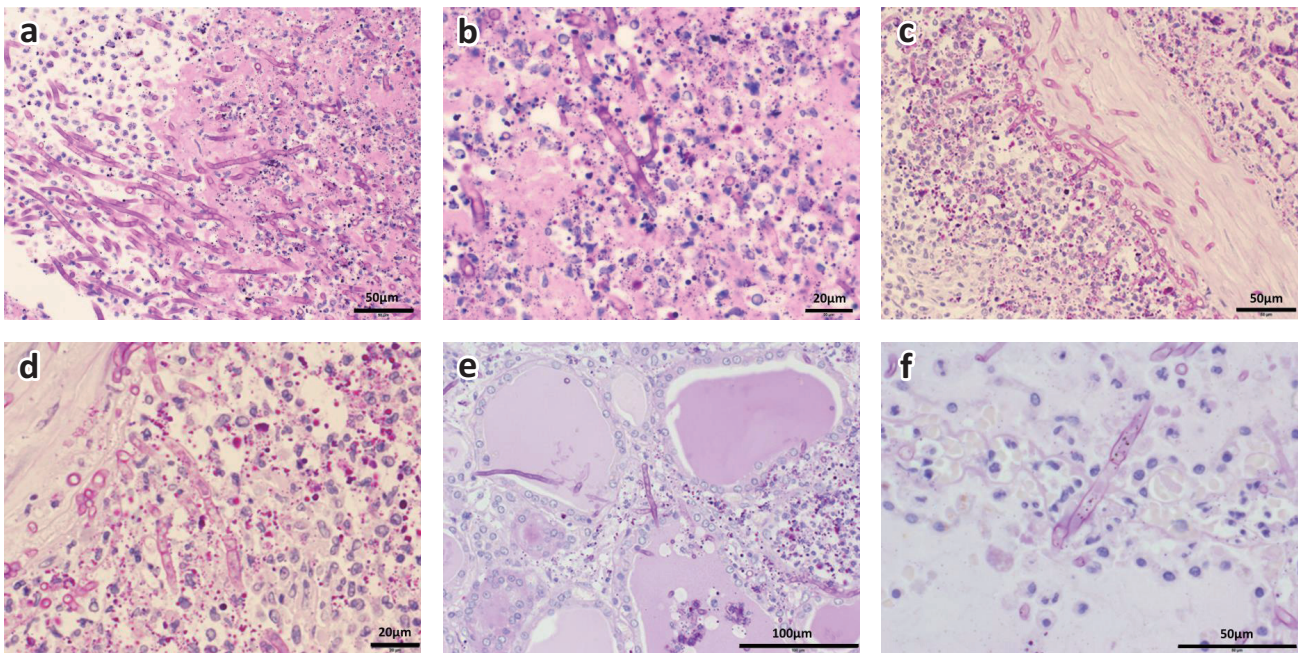


Fig.2. Pathological findings by PAS staining.
Fungal elements in lesions of the heart (a, b), kidney (c, d), and thyroid gland (e, f).

the localization of aflatoxin differs among individual leukocytes.

Aflatoxin M₁ was detected in lung samples in our reported case, indicating that aflatoxin B₁ is metabolized to aflatoxin M₁ inside the patient's body^{1, 2}.

Neutrophilic leukocytes and macrophages in the inflammatory focus were stained as tea brown, indicating that aflatoxin

was taken up into these cells, where the pathogen exerted its effects.

Our reported case with acute myelogenous leukemia was complicated with aspergillosis, which was treated with antifungal therapy mainly using amphotericin B, and the lesions of *Aspergillus* infection subsided. Bone marrow transplantation (BMT) was therefore performed. After the

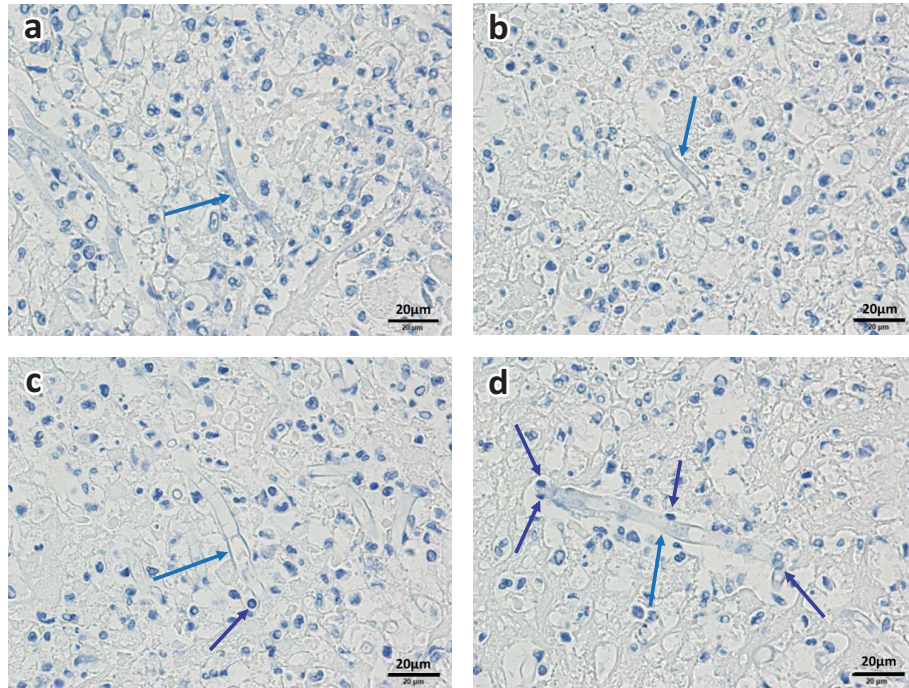


Fig.3. Pathological findings in heart lesions by control staining.

Antibody-negative fungal elements (a-d, arrow) and antibody-negative leukocytes adhered to fungal elements (c-d, deep blue arrow).

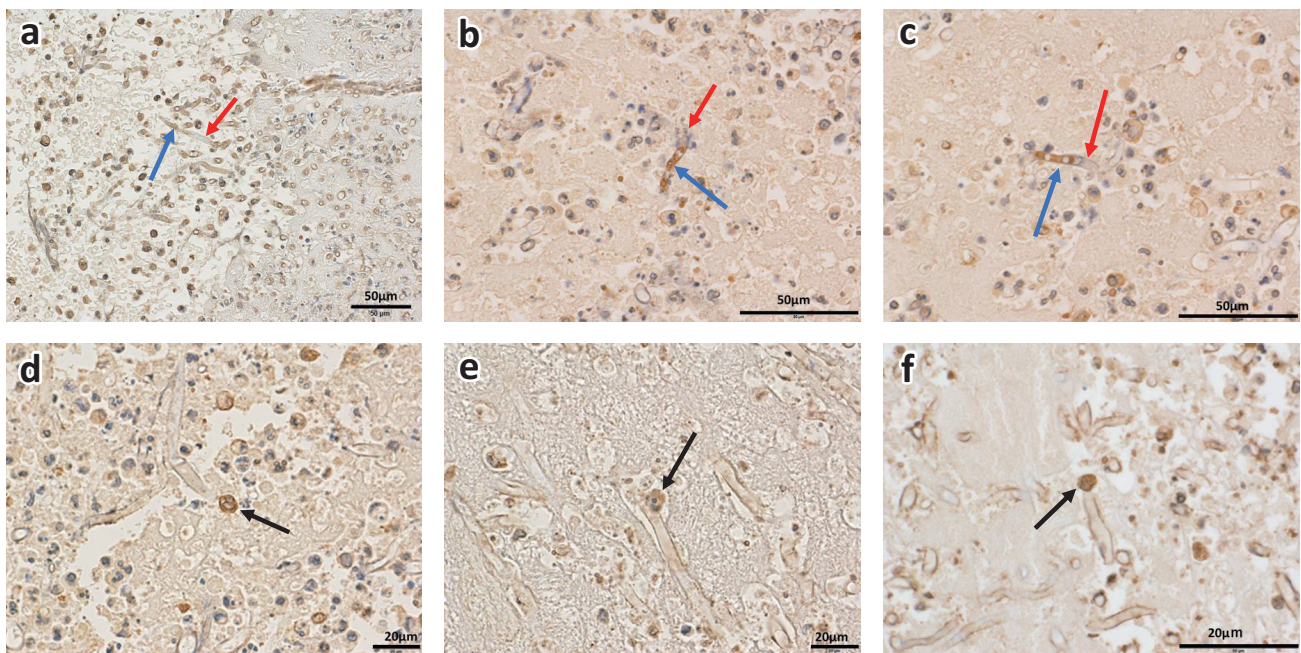


Fig.4. Pathological findings in the heart by immunohistochemical staining.

Extended hyphae with antibody-positive (a-c, blue arrow) and antibody-negative regions (a-c, red arrow). Antibody-positive leukocytes in surroundings of hyphae (d-f, black arrow).

BMT, the *Aspergillus* infection relapsed, and thus the antifungal therapy was performed again. Although bone marrow examination showed complete remission after BMT, the aspergillosis persisted. This indicates that the function of macrophages may have been greatly reduced.

This study demonstrated that *A. flavus* produced aflatoxin, which was released into the surrounding area to be taken up by leukocytes and affect leukocyte function. This is the first study to suggest that leukocyte function is disrupted in the lesions of patients with aflatoxin-producing *A. flavus* infection.

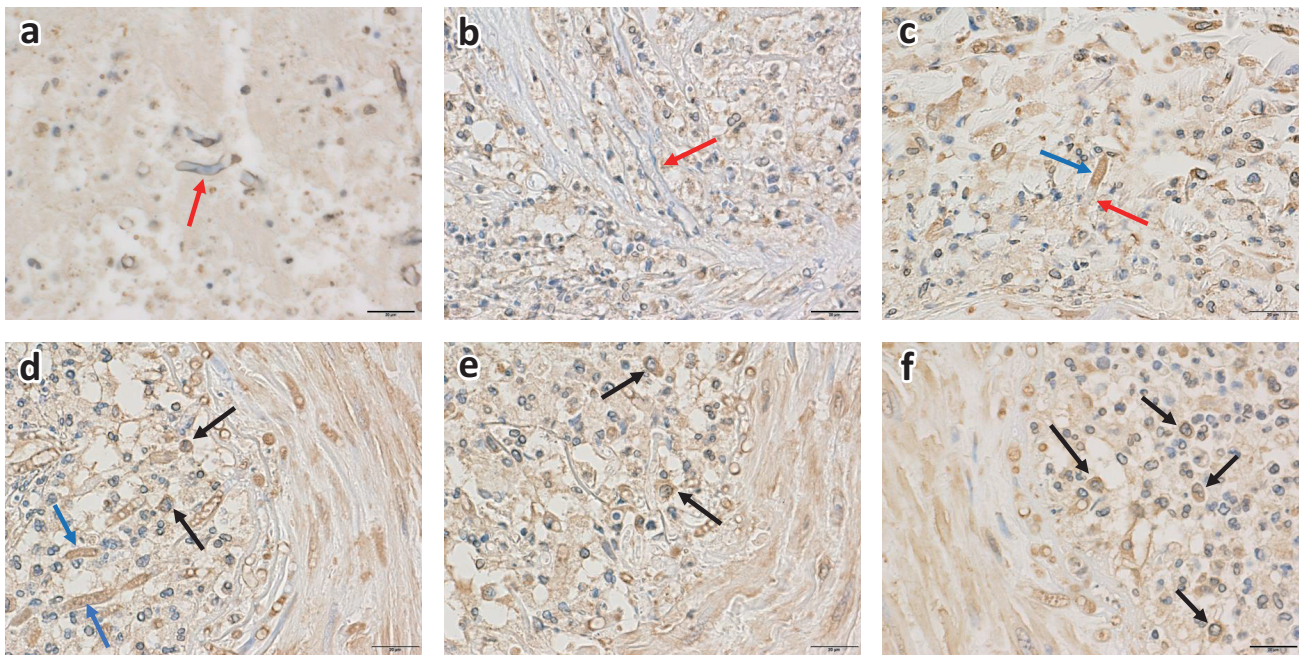


Fig.5. Pathological findings in kidney by immunohistochemical staining.

Similar findings as in Fig.4.

Extended hyphae with antibody-positive (c, d, blue arrow) and antibody-negative regions (a-c, red arrow). Antibody-positive leukocytes in surroundings of hyphae (d-f, black arrow).

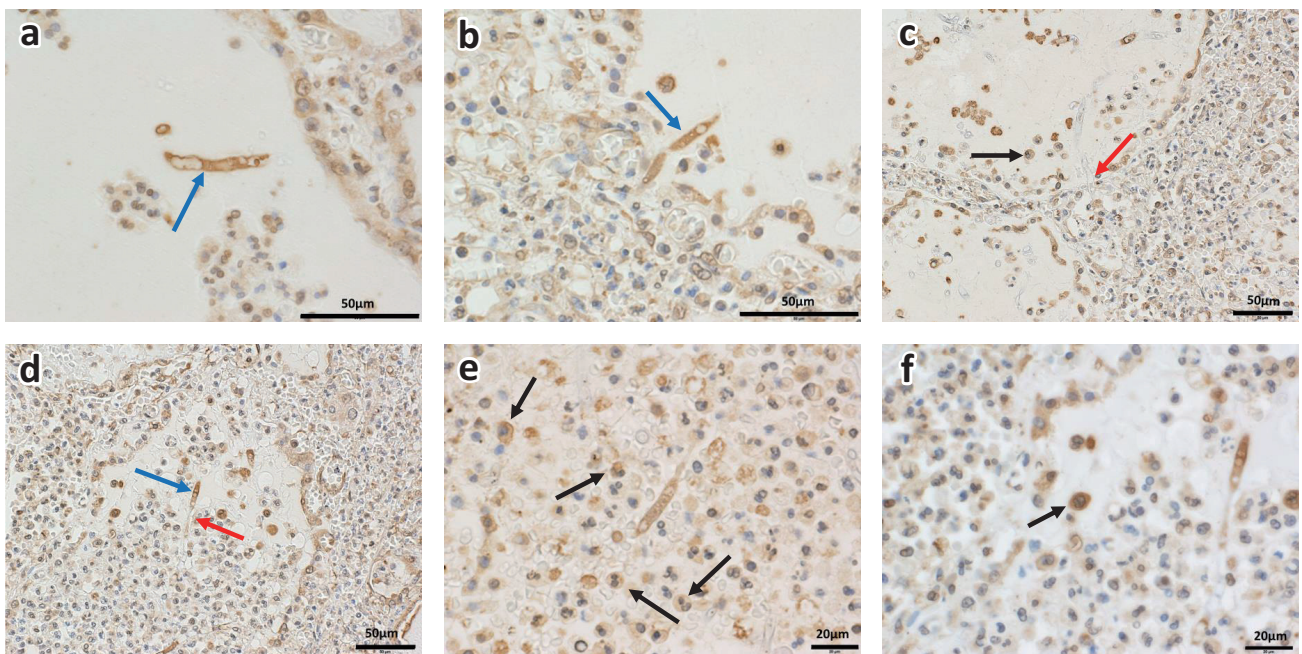


Fig.6. Pathological findings in thyroid gland by immunohistochemical staining.

Similar findings as in Fig.4 and 5.

Extended hyphae with antibody-positive (a, b, d, blue arrow) and antibody-negative regions (c, d, red arrow). Antibody-positive leukocytes in surroundings of hyphae (c, e, f, black arrow).

Conclusion

Aflatoxin was observed in the fungal elements and leukocytes. These results show that aflatoxin was produced

and released by *A. flavus* in the patient's lesion. The aflatoxin was then taken up by leukocytes where it disturbed their function.

Moreover, the metabolism of aflatoxin M₁ occurred *in vivo* in the disease focus in this case.

When considering the treatment and control of aspergillosis caused by aflatoxin-producing strains of *A. flavus*, underlying diseases must also be considered because they have serious effects on immune deficiency. Aflatoxin can reduce immune function in patients. Therefore, future studies should focus on developing strategies to improve the prognosis of immunocompromised patients with aspergillosis.

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Conflicts of interest

None.

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