

螢光物質－腐植酸與烏腳病之相關研究

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民國六十四年作者分析嘉南沿海烏腳病流行區居民飲用之井水，經紫外線照射後會發出藍綠色螢光，作者將之稱為「螢光物質」，並懷疑其與烏腳病有關。於是進一步分析其成分，發現其主要成份為腐植酸 (humic acid)。

腐植酸是一種深棕色，具有螢光的有機酸，分子結構並沒有一定，分子量介於數千至數十萬之間，但基本上是酚酸 (phenolic acid) 的聚合體。

將烏腳病流行區—臺南縣鹽水鎮舊營里之井水樣品經過濃縮、萃取及膠體過濾 (gel filtration) 所得之腐植酸，以動物實驗模式來說明腐植酸與烏腳病的關係。

以每日 5 毫克腐植酸／20 克體重的劑量，腹腔注射的方式，注射入 16 隻 Balb／c 小白鼠體內。在 22-32 天的連續注射中，有 8 隻小白鼠分別發生腳趾跛行、關節腫大及浮腫、潰爛等症狀，其中有一隻小白鼠之尾部全部變黑又壞疽，另一隻小白鼠之腳掌轉變為黑色。所有實驗小白鼠均未死亡，發病率達 50%。

關鍵詞：螢光物質，腐植酸，烏腳病

Introduction

“Blackfoot disease” is a folk term for an endemic peripheral vascular disease being prevalent along the southwestern coast of Taiwan[1]. The initial symptom of this disease is usually the insidious onset of numbness or coldness of one or more extremities. It usually progresses, resulting in an area of ulceration with subsequent gangrenous changes giving, as the name suggests, the characteristic black discoloration.

of dry gangrene, especially in the feet. The clinical symptoms, signs, and the course are similar to those of Buerger's disease[1]. Pathologically, the patients show vascular changes comparable with either thromboangiitis obliterans or arteriosclerosis obliterans [7]. The first cases were reported in 1954[3], and there were more than 1,800 cases reported by 1985[4]. Arsenic [1,2,4-9] and the fluorescent compounds [9,1] are generally believed to be the two main possible etiologic factors found in the well water that the inhabitants used to drink. However, there was no direct evidence to support these assumptions before this study.

The fluorescence noted from the drinking water in the endemic area of Blackfoot disease was first noticed in 1975[11]. Under ultraviolet light irradiation, the water showed a bluish-green fluorescence [11]. Later, the crystallized fluorescent compounds isolated from the well water were identified as humic

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substances[12].

After injection into the yolk sack of 8-day old chicken embryos, the fluorescent compounds induced stretching back and trembling of the feet, bowed neck, sparse plumage on the neck and the wings, and even death of the chicken[13]. They showed more cytotoxicity on normal cultured cells than to continuous cell lines[14], and enhanced the incorporation of protein and nucleic acid into growing HeLa cell[15]. Moreover, their effects such as inhibition of histamine-induced capillary permeability, degeneration at the capillary endothelial cells in vivo, and mutagenesis were also reported[9]. Our previous intention to induce peripheral vasculopathy in rats did though succeed in one case, 8 of the 10 experimental animals died during the experimental course[16]. The purpose of this study is thus to report that, under a suitable condition, fluorescent compounds in artesian well water can induce mice's Blackfoot disease, and this study may contribute to further understanding of the pathogenesis of Black-foot disease.

Materials and Methods

Isolation of fluorescent compounds:

The well water from the endemic area of Blackfoot disease was first filtered through a filter paper and then concentrated under low pressure until it dried. 12N HCL was added. The mixture then was left standing until no more bubble appeared. The mixture was condensed once more to dry the powder under low pressure. The powder of fluorescent compounds was extracted with methanol. The white precipitate resulting from this was discarded. The remaining brown methanol extracting solution was then condensed to the least volume. Distilled water was added, and the whole solution passed through a Sephadex G-25 column (48.5 × 2.0 cm). After washing with distilled water, three groups of fluorescence compounds were then obtained[17]. Group 1, which had the heaviest molecular weight and a yellow

color, obtained 170 mg per liter from the original water and used as material for injection (Table 1, Figure 1).

Animal experiments:

20 male Balb/c mice weighing approx. 20 g each were purchased from the Animal Center of Medical College of the National Taiwan University. They were fed with Rodent Laboratory Chow (5001, Ralston Purina Company, USA) and put in separate cages. Water and food were freely accessible.

The control group consisted of 4 mice receiving one intraperitoneal injection of 0.2 ml of distilled water every day. The remaining 16 mice, making up the experimental group, were daily injected with 0.2 mg of fluorescent compounds per one gram of body weight dissolved in a total of 0.2 ml of distilled water. The pH value of this solution was adjusted to 7.8. The mice were closely examined and then sacrificed 32 days after the first injection of fluorescent compounds. An autopsy was carried out on mice No. 7, 14, and the extremities and tails were decalcified in D-Calcifier (Lerner Lab, USA) for another 12 hours. The tissues then were embedded in paraffin, sectioned (6 μ) and stained with hematoxylin and eosin (H&E). A light microscopic examination was conducted thereafter.

Results

Immediately after injection, the mice shrieked and showed intermittent tremors and then laid still. Thirst was often noticed. One or two hours later, all mice recovered to their normal appearance.

After 14 to 26 (mean=22, S.D.=2.5) days of injection, 8 mice of the experimental group showed apparent symptoms of the disease: intermittent claudication was first noticed (14 to 23 days) followed by the edema of the extremities (20 to 25 days), and by ulceration and gangrene of the affected limbs (20 to 27 days). A spontaneous amputation of a limb also had to be carried out on one mouse (Table

2). Among these mice, No.7 developed an intermittent claudication, edema, ulceration, gangrene, and finally black discoloration of the right lower limb (Figure 2). Ulceration, gangrene and black discoloration were observed on the tail of mouse No.20 (Figure 3).

All the mice of the control group were quite normal. None of the mice in those two groups died before the sacrifice. The incidence rate was 50% (8/16).

The body weight of the control group increased steadily, whereas the experimental group slightly lost body weight during the first several days, and then regained their weight steadily. However, when

clinical symptoms appeared, their weight decreased suddenly, and they lost even more weight than before the injection.

The microscopic examination of the affected limbs and tail revealed local necrosis of the skin, in which bacteria clumps and various degrees of neutrophil and monocyte infiltration were also found. Congestion and microthrombi occurred in the vessels of the dermis. There was neutrophil infiltration between the muscle bundles and between the muscle fibers (Figure 4,5,6) as well. Various degrees of congestion were observed in the brain, heart, lung, liver, and in the kidney. There were however no significant changes in other organs.

Table 1. Isolation of Fluorescent Compounds from Well Water

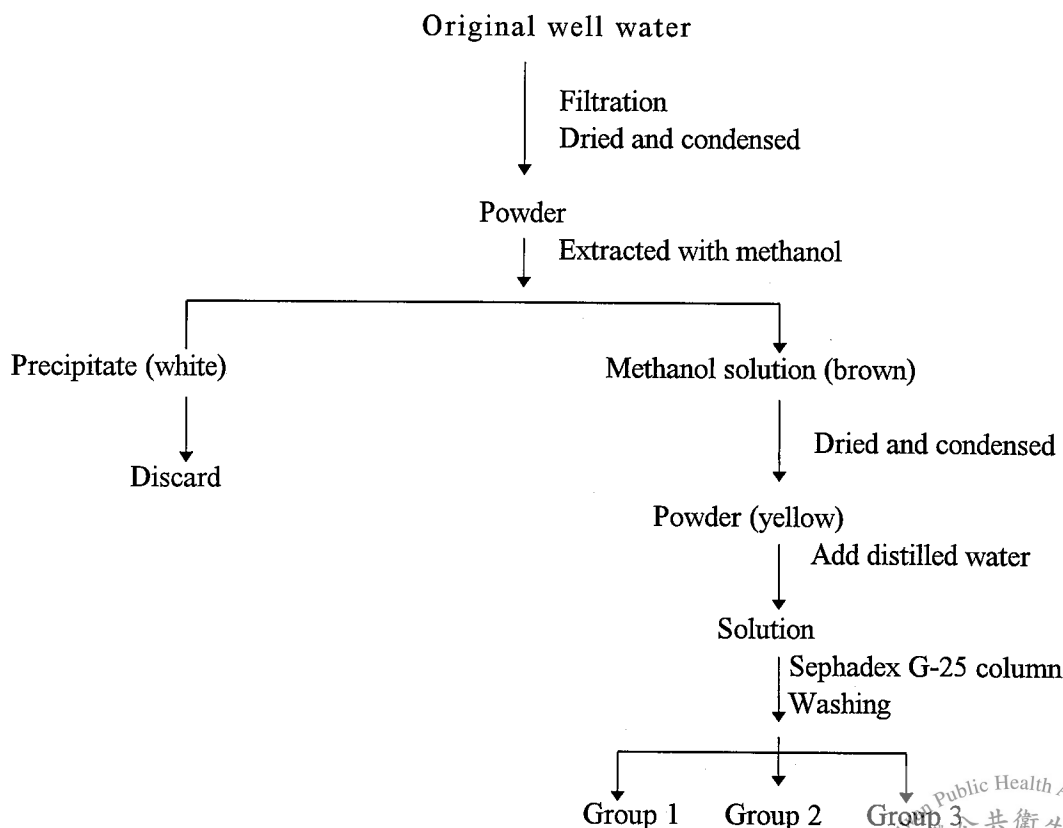
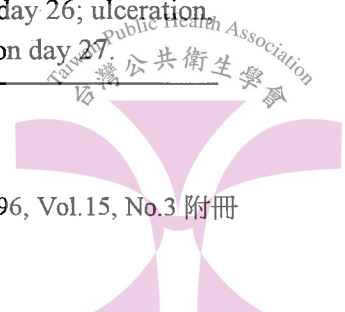


Table 2. Signs of the Experimental Mice

No.	Date (day) signs appeared	Signs
5	23	Intermittent claudication of left lower limb.
6		No apparent signs
7	14	Right lower limb, claudication began on day 14, edema and ulceration began on day 25, gangrene began on day 28, black discoloration on day 31.
8		No apparent signs
9		No apparent signs
10		No apparent signs
11	23	Digital joint swelling of both lower limbs
12	20	Edema and ulceration of left upper limb
13		No apparent signs
14	23	Edema and ulceration, right lower limb on day 23, left lower limb on day 25.
15		No apparent signs
16		No apparent signs
17	22	Loss of right upper limb
18	22	Claudication , edema, and ulceration of left lower limb
19		No apparent signs
20	26	Cyanotic changes in the tail began on day 26; ulceration, dry gangrene and black discoloration on day 27.



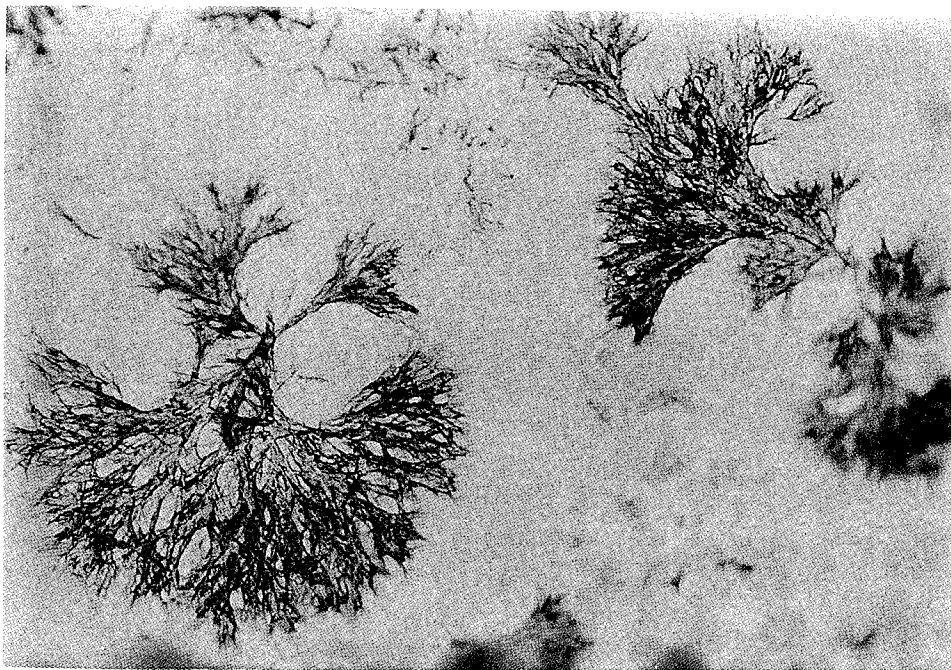


Figure 1. Crystal of 1st group of fluorescent compounds (x100).

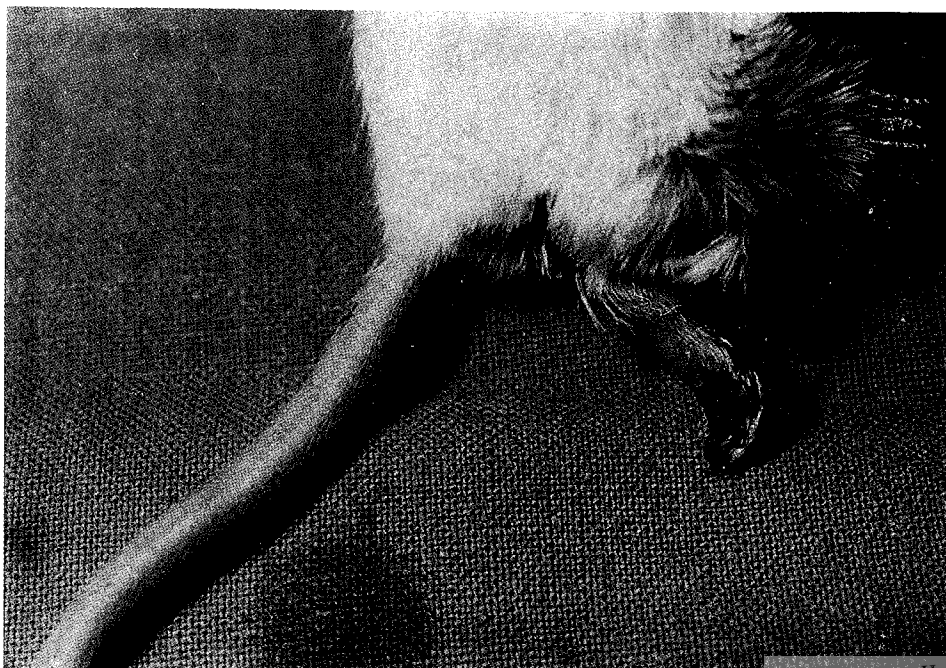


Figure 2. Edema, ulceration, gangrene, and black discoloration in the right lower limb of mouse No. 7.

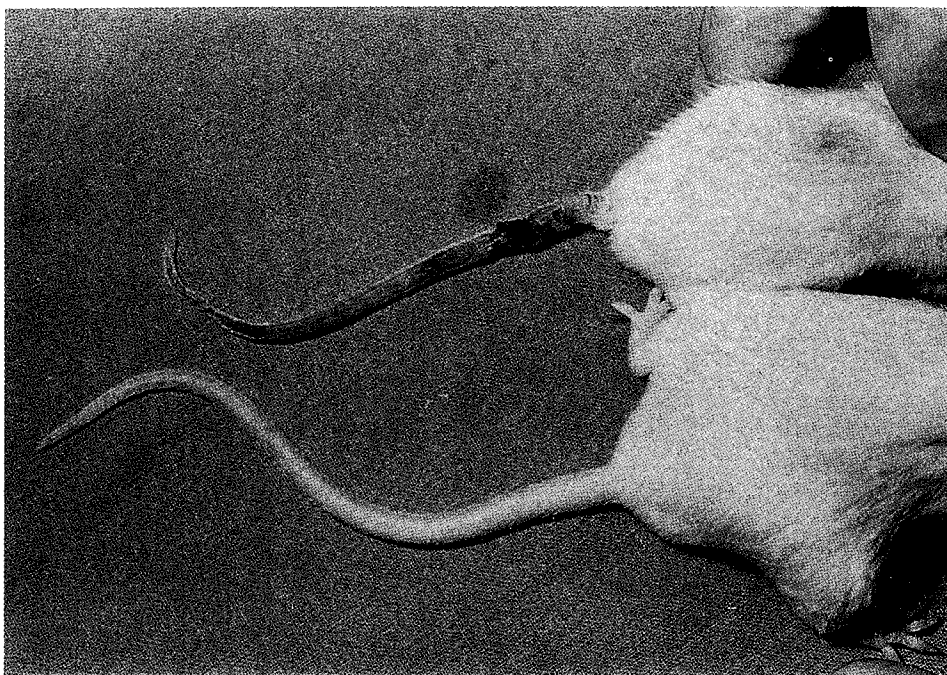


Figure 3. Ulceration, gangrene, and black discoloration in the tail of mouse No.20 (top) in comparison with a mouse of the control group (bottom).

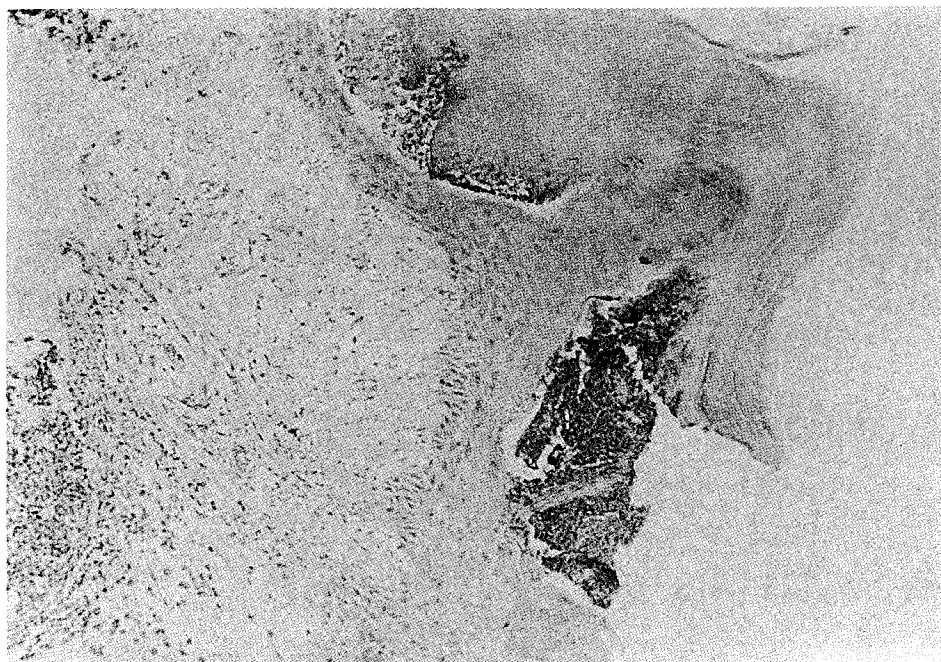


Figure 4. Ulcerated foot of mouse No.7. Local necrosis of skin with bacterial clumps and neutrophil and monocyte infiltration was found (x100).



Figure 5. Ulcerated tail of mouse No.20. Congestion and microthrombi were found in the vessels (x100).

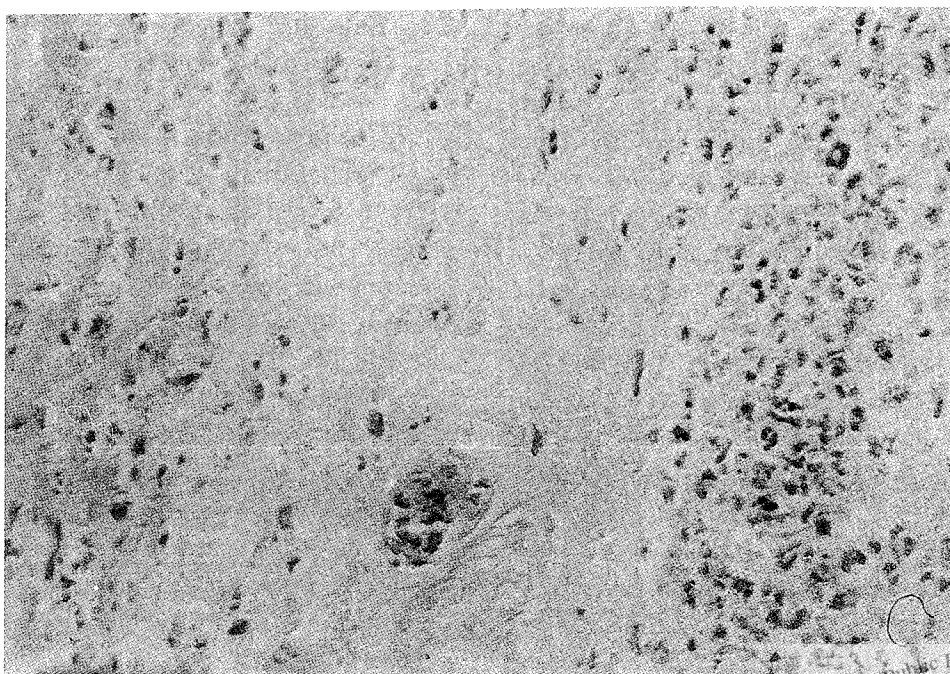


Figure 6. Ulcerated foot of mouse No. 15. Neutrophil infiltration was found within the muscles (x100).

Discussion

The Blackfoot disease is an endemic peripheral vascular disease found among the inhabitants living along the southwestern coast of Taiwan. The largest series of clinical investigations which covered 1,623 patients showed that, although the clinical onset is usually insidious, it may be quite sudden as well, and it usually starts with numbness (75.1 %) or coldness (57.0 %) in one or more extremities, usually in the feet. An intermittent claudication is also a common initial symptom about which approx. 42.2 % of the patients complained. The lesser common initial symptoms include cyanotic change in the feet (15.3 %), a burning sensation in the soles (15.0 %), pale feet (14.9 %), weakness of extremities (10.2 %), and itching in the plantar surface of the feet (5.3 %)[18]. Furthermore, rest pain of shooting nature interfering with sleep and causing anorexia occurred. After a certain period of time, which varied from several days up to 2 years, however usually within 6 months, gangrene develops with lancinating, gnawing or burning pain, and gives the affected limbs the characteristic black discoloration [1]. An edema was noticed in 46.7 % of the patients during the course [19]. 68.8 % of the victims eventually underwent either spontaneous or surgical amputation. 167 cases reported spontaneous amputation before 1959. After that, all the amputations were performed surgically. Among those 167 cases, 20.4 % had to undergo a spontaneous amputation within one month after gangrene began, and 58.7 % within 4 months. The majority (83.3 %) of the spontaneous amputations had to be conducted within 6 months after gangrene developed, and all of them occurred within 2 years[18].

Since both numbness and coldness are subjective sensations and cannot be told by mice, it is reasonable to say that the first symptom we observed in the mice was mainly intermittent claudication, which was the most common initial symptom of Blackfoot disease, with the exception of numbness

and coldness. Edema, a common symptom of Blackfoot disease in an early stage, also occurred in 5 (31.3 %) of the experimental mice. Ulceration and gangrene, which are both important diagnostic criteria for the Blackfoot disease, were observed in 5 of the experimental mice as well. The most striking characteristic signs of Blackfoot disease -- black discoloration (2/16) and spontaneous amputation (1/116) of the affected extremities -- were also successfully induced. Both the spreading from one limb to another, as seen in mouse No.144, and the onset of signs in more than one limbs, as observed in mouse No.11, might also be observed in patients suffering from Blackfoot disease[1]. A sudden onset without any preceding sign as occurred in mouse No.17 was observed in approximately 13.9 % of the cases of Blackfoot disease[18] as well. Therefore, both the clinical symptoms and the course of the disease of the mice induced by injecting fluorescent compounds during this study were quite comparable with those of Blackfoot disease.

Clinically, the Blackfoot disease is more similar to thromboangiitis obliterans than any other peripheral vascular disease. However, in pathological terms, it can be divided into 2 distinctly different reaction groups: the thromboangiitis obliterans group and the arteriosclerosis obliterans group. The pathological changes of the vessels in Blackfoot disease are quite characteristic, and yet complicated. In the thromboangiitis obliterans group, the basic initial change is fibrinoid degeneration of the intimal connective tissue of either arteries or veins, with or without inflammatory cell reaction. If this occurs in large vessels and not so extensive as to cause secondary thrombosis, it will result in an intimal collar fibrosis, usually described as intimal proliferation. If it occurs in small vessels, thrombotic occlusion may ensue immediately. The fibrinoid degeneration may sometimes extend to the whole wall and be associated with a marked inflammatory reaction. This is particularly common in arterioles, precapillaries, and in venules. Such fibrinoid degen-

eration may occur repeatedly, resulting in collar fibrosis of the intima, cushion-like intimal thickening, or even in an organized occluding lesion. The proliferation and the activation of vascular endothelium are the second most important finding in this group which can hardly be seen in pure arteriosclerosis obliterans. The occluding lesion in this group is highly cellular, not only with fibroblasts and endothelial cells of newly formed capillaries, but also with lymphocytes and some mononuclear or hemosiderin-laden macrophages. Diffuse infiltration of inflammatory cells throughout the whole vessels wall is another characteristic finding in this group. Such vascular changes are seen involving small arteries in skeletal muscles and are never seen in ordinary thromboangiitis obliterans. In this arteriosclerosis obliterans group, the occluding lesions are variables; from a red or mixed thrombus to complete occlusion, marked intima sclerosis was shown. The most common intima change is fibrous thickening. Atheroma formation and calcification can be found, and simple necrosis in deeper layers sometimes occurs. Although fibrinoid degeneration of intima connective tissue has never been found in some cases. Medical calcification of occluded arteries is found in many cases of this group, and so is the bone formation of the occluded arteries. Atrophy, fibrosis and vascularization are also common in the subject. Periarterial fibrosis is not uncommon in this group, though not as marked as in the thromboangiitis obliterans group [7].

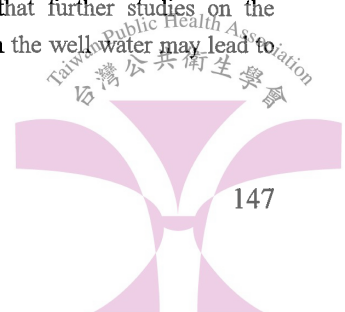
Concerning other organs, a general congestion which was found in the experimental animals was also found in 2 of the only 3 reported autopsy cases, at either moderate or marked levels. Therefore, from a pathological point of view, the findings in the experimental mice are comparable with those found in humans suffering from Blackfoot disease.

The general applied diagnostic criteria for Blackfoot disease are (a) objective signs of ischemia, such as absence or diminution of arterial pulsation, paleness or cyanotic changes of the affected

extremities, and various ischemic changes of the skins, (b) subjective symptoms of ischemia, such as numbness or coldness of the affected extremities, intermittent claudication, resting pain, and ischemic neurological changes, (c) local ulceration or gangrene [18]. The symptoms induced in the mice during this experiment are fulfilling those criteria. Furthermore, the pathological findings are comparable with those found in humans suffering from Blackfoot disease. We suggest that the fluorescent compounds in the well water can induce Blackfoot disease.

Although arsenic content in the well water was thought to be the causative factor of the Blackfoot disease for many years, all the attempts to induce Blackfoot disease in animals have failed.

The intramuscular injection of concentrated well water to the legs of rats had successfully induced ulceration, gangrene, and spontaneous amputation similar to those seen in Blackfoot disease, and ulceration and gangrene could also be induced by injection of arsenic at pH 12, but not at pH 8.5. However, the similar ulceration and gangrene could be induced by injecting NaOH or KOH solution at pH 12 as well. However, the characteristic spontaneous amputation due to Blackfoot disease could not be induced by the induction of arsenic or by the injection of alkaline solution[20]. Thus, the ulceration and gangrene induced during these studies might have been contributed to the alkaline solution, not to the arsenic content. However, the pH value of the injected solution in our experiment was 7.0 throughout the course, so we can rule out the effect of the pH condition on the results. Furthermore, since there was no pathological report in that study, we cannot be sure whether the induced disease is comparable with the Blackfoot disease found in humans. We therefore believe that the experimental model conducted on animals, which we have developed, is the most suitable for the study of the Blackfoot disease, and that further studies on the fluorescent compounds in the well water may lead to



the understanding of the pathogenesis of this special disease, or even other vascular disease.

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