VIRUS AETIOLOGY OF BEHÇET'S SYNDROME*

BY

ALY MORTADA

Faculty of Medicine, Cairo University, Egypt

AND

IMAM Z. E. IMAM

Virus Research Centre, Agouza, Ministry of Public Health, Cairo, Egypt

BEHÇET (1937) described a muco-cutaneous-ocular syndrome characterized by recurrent uveitis and ulcers of the oral mucous membrane and external genitalia. Sezer (1953) reported the isolation of the causal virus from the subretinal fluid of affected eyes and later isolated the virus from the blood and urine of affected patients in the viraemia stage (Sezer, 1954). Behçet (1939) and Ruggs-Gunn (1947) noted intracellular inclusion bodies in smears from buccal and genital aphthous ulcers, and Kozousek and Jandasek (1956) and Nakagawa and Shingu (1958) isolated the virus from the ocular fluids and genital aphthous ulcers of patients with Behçet's disease.

Attempts to isolate the virus from the aqueous humour of diseased eyes (Adamantiadis, 1953; Latte, 1953), from cutaneous lesions (Hugonnier, 1941; Curth, 1946; Franceschetti, Jadassoku, and Paillard, 1953), and from buccal aphthae (Jebejian and Kalfayan, 1945; Cavara, 1950) were not successful, and various workers considered the disease to originate from tuberculosis, syphilis, allergy, or septic foci. Curth (1954) considered the syndrome to be a type of collagen disease. Most text-books of ophthalmology (e.g. Woods, 1961) suggest that Behçet's disease is probably of virus aetiology, but unfortunately this virus is not available in many laboratories so that serological tests against it cannot be done.

Case Reports

In the two cases to be reported the family history was not significant. The spleen, liver, and salivary glands were not enlarged. The chest and abdomen were normal. There was no clinical or laboratory evidence of tuberculosis, syphilis, sarcoidosis, brucellosis, toxoplasmosis, parasitic infection, dysentery, gout, gonorrhoea, diabetes, leprosy, allergy, or neurological disease. A blood count showed the total leucocytes to be within normal limits. The differential count showed a slight shift to the left. There was mild monocytosis. The blood pressure was 120/90. The urine was free of albumen and sugar. The blood Wassermann reaction and tuberculin skin test were negative. X rays of the chest and long and short bones, and also of the teeth, nasal sinuses, ears, tonsils, urinary tract, gall bladder, appendix, prostate, and posterior urethra showed no abnormality.

Case 1, a 30-year-old footballer, had had recurrent bilateral uveitis causing diminution of vision for 2 years. His father, mother, and two brothers, who were living with him in the same house, had perfect health. For football training the players were accustomed to wear each other's jerseys and shorts. The disease had begun 7 years before with a month of high fever, since which time scrotal ulcers had recurred every 2 months. For the last 4 years the patient had also had recurrent ulcers of the oral mucous membrane.

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The scrotal ulcer (Fig. 1) appeared first as a dark papule which ulcerated in 2 to 3 days. The ulcer was painful and oval in shape (1×0.5 cm.), with a clear-cut edge and sloughing base. The appearance of the ulcer was preceded by 2 to 3 days ($38^{\circ}\text{C}.$) fever, headache, and malaise. During the ulcerative stage the inguinal lymph glands on the affected side were enlarged. The ulcer remained for 10 days and healed in 5 days.

The buccal ulcer (Fig. 2) was small (0.5×0.25 mm.), shallow, and painful, with a necrotic base and a clean-cut edge surrounded by a reddish circle. The submaxillary glands on the affected side were enlarged during the ulcerative stage.

FIG. 1.—Case 1, showing typical scrotal ulcer in a 30-year-old man.

FIG. 2.—Case 1, showing buccal mucous membrane ulcer in a 30-year-old man.

The right eye showed posterior synechiae, a clear lens, and a cloudy vitreous, but no keratic precipitates. The fundus could not be seen. The visual acuity was 1/60 and the ocular tension normal.

The left eye showed posterior synechiae, with a pale optic disc and attenuated retinal vessels. The visual acuity was 6/36 with no refractive error and normal ocular tension.

Case 2, a 20-year-old male, complained of bilateral diminution of vision (right eye 1/60 and left eye 2/60). He had recurrent bilateral uveitis and scrotal and oral ulcers similar to those in the first case. The recurrent scrotal ulcers had left many scars (Fig. 3).

Recurrent axillary ulcers (Fig. 4) resembled the scrotal ulcers.

FIG. 3.—Case 2, showing two typical scrotal ulcers and scars of healed ulcers in a 20-year-old man.

FIG. 4.—Case 2, showing axillary ulcer in a 20-year-old man.

Laboratory Investigations

The edges of the scrotal, axillary, and buccal ulcers all showed similar histopathological appearances. There was dense subepithelial chronic, non-specific, mononuclear cellular infiltration (Fig. 5), with residual polymorphonuclear cells from the acute stage. Venules, capillaries, and arterioles showed pan-vasculitis (Fig. 6). Most of these small vessels showed endothelial proliferation obliterating the lumina (Fig. 7), and many were obliterated by thrombus formations mostly in the stage of organization (Fig. 8).

FIG. 5.—Scrotal ulcer edge, showing subepithelial chronic non-specific mononuclear cellular infiltration and marked pan-vasculitis. $\times 90$.

FIG. 6.—Scrotal ulcer edge, showing pan-vasculitis. $\times 405$.

FIG. 7.—Scrotal ulcer edge, showing endothelial proliferation obliterating the vascular lumina. $\times 405$.

FIG. 8.—Scrotal ulcer edge, showing organizing thrombus occluding the vascular lumen. $\times 405$.

Examination of the cerebro-spinal fluid during the feverish stage of ulceration showed a clear appearance, protein 35 mg., per cent., sugar 40 mg. per cent., chloride 740 mg. per cent., and 2 lymphocytes per c.mm. Gram-stained films showed no organisms. Zeil-Neilsen stain showed no tubercle bacilli. Giemsa stain showed no inclusion bodies. Cultures on chorio-allantoic membranes of developing chick embryos gave a negative results for viruses.

Scrotal, buccal, and axillary ulcer scrapings when stained with Giemsa stain showed small, round, pink, intranuclear inclusion bodies in the epithelial cells (Fig. 9).

FIG. 9.—Scrotal ulcer scraping, showing epithelial intranuclear inclusion bodies. Giemsa. $\times 1,200$.

The affected nuclei were faintly stained. Several cells in the early stages of multi-nucleate cell formation were present. The cytoplasm of these cells is not seen, having been destroyed during the preparation of the slide.

Fig. 10 shows intranuclear inclusions in epithelial cells of the edge of the scrotal ulcer.

FIG. 10.—Epithelial cells at edge of scrotal ulcer, showing intranuclear inclusion bodies surrounded by clear zone. Giemsa. $\times 1,200$.

In the first case, during a fresh attack of uveitis, while the patient's temperature was 37.7°C , a hypopyon of 2 days' duration was seen in the right eye. This hypopyon, which was 3 mm. in height and whitish-yellow in colour, was withdrawn by an Amsler's needle. Smears of the hypopyon were Gram-negative, but Giemsa stain showed both leucocytes

and epithelial cells with intranuclear inclusion bodies (Figs 11 and 12), having the same appearance, size, and staining properties as those in the smears from the buccal or the scrotal ulcers. Culture of the hypopyon on blood agar was negative.

Fig. 11 $\times 1,200$.

FIGS 11 and 12.—Hypopyon of 2 days' duration. Smear showing both epithelial and leucocytic intranuclear inclusion bodies. Giemsa.

FIG. 12.— $\times 1,800$.

Virus Isolation

The virus was isolated from the blood and from scrapings of the scrotal and buccal ulcers, as well as from the hypopyon fluid.

The blood was taken from one patient in the feverish stage and was allowed to clot. The clot was ground in broth and 10 per cent. emulsion was prepared and tested for sterility.

The ulcer scrapings were ground in broth and penicillin in a concentration of 500 units per ml. and streptomycin 200 mg. per ml. was added. The prepared fluid was tested for sterility and then used in the virus isolation experiments.

The hypopyon fluid was similarly prepared.

Chorio-allantoic Membrane

0.2 ml. of each of the prepared fluids (blood, ulcer scrapings, and hypopyon) was inoculated on to the chorio-allantoic membrane of ten 10-day old chick embryos. After 48 hrs' incubation at 37°C., the membranes were harvested and examined. Small whitish plaques each of about 1 mm. diameter were seen on the membranes (Figs 13 and 14, opposite), but no plaques appeared on ten control eggs inoculated with normal saline.

Smears made from these plaques (Fig. 15, opposite) showed inclusion bodies exactly like those seen in smears from the hypopyon and ulcer scrapings.

A 10 per cent. suspension of the harvested membranes was prepared in broth and the infected emulsions were tested for sterility by culture on agar, blood agar, and

FIGS 13 and 14.—Chorio-allantoic membrane infected with blood taken during viraemia stage, showing small whitish plaques each of about 1 mm. in diameter that developed after 48 hrs. (Fig. 14 reproduced from kodachrome negative).

FIG. 15.—Chorio-allantoic membrane, showing epithelial cells infected with Behçet's disease virus. Smear showing intranuclear inclusions of varying sizes. Each inclusion is surrounded by a halo of clear zone. Giemsa. $\times 1,200$.

sodium thioglycollate medium as well as Sabaro medium and pleuropneumonia-like organisms agar medium. All these cultures gave a negative result, the emulsions being completely sterile.

A second passage was made on the chorio-allantoic membranes of 10-day-old chick embryos. After 48 hrs' incubation at 37°C. the same whitish plaques were seen.

A 10 per cent. suspension of the infected chorio-allantoic membrane was prepared and passed through a Seitz filter (E. K. S11). This filtrate was inoculated on the chorio-allantoic membrane of ten more 10-day-old chick embryos.

Small whitish plaques appeared on the chorio-allantoic membranes after 48 hrs' incubation at 37°C., and smears from these plaques showed the same inclusion bodies as those in the smears from the hypopyon and ulcer scrapings. After inoculation with saline the control membranes remained normal.

Five passages were carried out on the chorio-allantoic membrane for each of the four isolates.

Mouse Brain

The filtrates described above were inoculated intracerebrally into 3-week-old white Swiss mice, six mice for each of the four isolates. These mice died 4 to 5 days after inoculation, but a control group inoculated with saline remained alive (Table I).

TABLE I
RESULTS OF INTRACEREBRAL INOCULATION OF 0.3 ML. PRIMARY VIRUS ISOLATES INTO THE BRAINS OF 3-WEEK-OLD MICE

Material from	No. of Mice Inoculated	No. of Deaths after 48 hrs
Blood	6	5/6
Hypopyon	6	4/6
Scrotal ulcer	6	5/5
Buccal ulcer	6	3/4
Saline	6	0/5

The results of a second passage are shown in Table II. Again there were no deaths in the control group.

TABLE II
RESULTS OF SECOND PASSAGE OF ISOLATES IN 3-WEEK-OLD MICE BY INTRACEREBRAL INOCULATION OF 0.03 ML. SUSPENSION

Passage 1 Material	No. of Mice Inoculated	No. of Deaths after 48 hrs
Blood	6	6/6
Hypopyon	6	4/4
Scrotal ulcer	6	5/5
Buccal ulcer	6	6/6
Saline	6	0/6

The results of titration of the agents in adult mice are shown in Table III.

TABLE III
RESULTS OF TITRATION OF ISOLATES IN 3-WEEK-OLD MICE

Virus Dilution	No. of Mice Inoculated	No. of Deaths after 48 hrs
10 ⁻¹	6	6/6
10 ⁻²	6	6/6
10 ⁻³	6	2/6
10 ⁻⁴	6	1/6
10 ⁻⁵	6	0/6
10 ⁻⁶	6	0/6

L.D.₅₀ 10^{-2.9}

The titre of the virus causing the death of half the animals after mouse brain inoculation was always about 10^{-2.9} to 10^{-3.0} for all agents isolated.

Rabbit Serum

An immune serum was prepared in rabbits against each isolate by subcutaneous inoculation with a 50 per cent. mixture of the concentrated 20 per cent. suspension of the agent together with an adjuvant. Each rabbit was inoculated with 1 ml. on four occasions at weekly intervals. The serum was collected one week after the last injection.

Neutralization Tests**Mice (Table IV)**

Serial dilutions of the virus, from 10^{-1} (which from previous titration was taken as 100 L.D₅₀) to 10^{-4} , and concentrated immune sera were used. The immune rabbit and the normal rabbit sera were inactivated at 56°C. for 30 min. before testing.

The same test was repeated for each isolate with homologous and heterologous serum. A log. neutralization index of about 1.5 to 1.9 was obtained in each experiment. On using convalescent human serum, a log. neutralization index of 2.9 was obtained

TABLE IV
NEUTRALIZATION TEST IN MICE WITH NORMAL AND IMMUNE RABBIT SERUM AND
NORMAL AND CONVALESCENT HUMAN SERUM

Serum		Final Virus Dilution				Log. Neutralization Index
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	
Rabbit	Normal	6/6*	2/6	1/6	0/6	1.9
	Immune	0/6	0/6	0/6	0/6	
Human	Normal	6/6	3/6	1/6	0/6	2.9
	Convalescent	0/6	0/6	0/6	0/6	

Chorio-allantoic Membrane (Table V)

Plaques were found only in a virus dilution of 10^{-1} and were very rare in a dilution of 10^{-2} . Equal amounts of 20 per cent. virus concentration and concentrated immune rabbit serum were mixed together and incubated for 1 hr at 37°C. A similar mixture was made with normal rabbit serum.

Each virus-serum mixture was inoculated onto the chorio-allantoic membranes of ten 10-day-old chick embryos and incubated at 37°C for 48 hrs. When the harvested membranes were examined, all those inoculated with the immune serum-virus mixture were normal and showed no plaques, whereas the controls inoculated with the normal serum-virus mixture showed the same plaques as were seen before.

TABLE V
NEUTRALIZATION TEST IN CHORIO-ALLANTOIC MEMBRANE OF 10-DAY-OLD CHICK
EMBRYOS WITH 0.2 ML. NORMAL AND IMMUNE RABBIT SERUM

20 per cent. Virus Concentration		No. of eggs	Result
Rabbit Serum	Normal	10	Plaques
	Immune	10	No plaques

Complement Fixation.—Antigen was prepared from the infected chorio-allantoic membranes, and the complement-fixation test carried out on four sera from patients with Behçet's disease and on normal sera. All the normal sera gave a negative result. The results from the Behçet patients' sera being as follows:

Patient No.	Complement-Fixation Titre
1	1/32
2	1/16
3	1/32
4	1/8

Immune animal sera also gave positive complement-fixation titres ranging from 1:16 to 1:64 when tested against the prepared antigen. All sera were negative when tested against an antigen prepared from normal chorio-allantoic membrane.

Discussion

The cases of Behçet's syndrome from which many workers have failed to isolate the causal virus were perhaps atypical, or the ulcer scrapings were not taken during the early stages of ulceration, or the patient's blood or hypopyon were not examined during the early feverish stage.

It was interesting to note that, although both patients were living with relatives, none of the latter was affected by the disease. In the first case the patient used the football kit of other players and the garments were probably infected. In both cases the onset of the syndrome was preceded by a long spell of fever which may be related to the time of infection. The recurrent axillary ulcers in Case 2 showed the importance of friction in the development of the ulcers typical of Behçet's syndrome.

Smears from the hypopyon and from buccal and scrotal ulcer scrapings stained with Giemsa stain and also tissue cultures showed that the essential pathological feature of Behçet's disease is the recurrent virus infection of the epithelial cells. The edges of the scrotal, axillary, and buccal ulcers clearly showed recurrent patchy panvasculitis and vascular thrombosis giving rise to vascular obliteration. It seems that the virus lies dormant in the epithelial cells of the external genitalia, skin, and buccal mucous membrane and in the neuro-epithelial cells of the eye. When the virus becomes active it provokes a marked secondary vascular reaction in the underlying tissue.

The vascular reaction in the uvea and retina, which was thought to be the essential ocular lesion of Behçet's disease by Touraine (1944), France, Buchanan, Wilson, and Sheldon (1951), Curth (1954), and other workers, is secondary to the virus infection of the neuro-epithelial cells of the iris, ciliary body, and retina.

It seems that virus-infected leucocytes in the scrotal skin, buccal ulcers, and eye lesions transfer the virus to the blood, giving rise to the feverish viraemia stage. In a few days, however, antibodies develop so that the virus becomes inactive again, and the skin and buccal ulcers heal quickly. The residual ocular damage is comparatively severe because of the much greater delicacy of the structure of the eye.

Summary

(1) This is the first account of the isolation of virus from cases of Behçet's disease to be reported from Egypt, and provides strong evidence of the virus aetiology of this condition. The virus was isolated from a hypopyon in the anterior chamber, from scrapings from buccal and external genital ulcers, and from the patient's blood during the febrile stage of the disease.

(2) Intranuclear inclusion bodies were seen in epithelial cells in the hypopyon and in scrapings from the buccal and genital ulcers.

(3) The essential ocular pathology of Behçet's disease is caused by virus infection of the neuro-epithelium of the iris, ciliary body, and retina, provoking secondary vasculitis of the uvea and retina and producing the characteristic signs of uveitis and retinitis.

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