Yonsei Medical Journal
Vol. 19, No. 1, 1978

Studies on Exocellular Substances of *Mycobacterium ulcerans*. I. Preliminary Report on a Susceptible Animal to the Substances

Young Nam Lee and Joon Lew

Department of Microbiology, Yonsei University College of Medicine, Seoul, Korea

This is a report on attempts to search for a suitable experimental animal model which is sensitive to the exocellular substances of *M. ulcerans*, prior to study of biochemical and pathogenic natures of the substances.

Cells of *M. ulcerans* grown in a broth medium were harvested by filtration and washed with phosphate-buffered saline. The filtrate of culture supernatant was subjected to fractionation by addition of various amounts of ammonium sulfate. The washed cells and the preparations resulted from ammonium sulfate fractionation (ASF 45 and ASF 70) were inoculated by either foot-pad injection, subcutaneous or intradermal injection to the selected animal groups. Any skin response due to administration of the preparations; erythema, edema, pus formation, etc. was macroscopically observed along with time progression.

Among the animals employed, such as guinea pig, mouse, and rabbit, the rabbit was the only animal group showing strong skin response to the washed cells, ASF 45 and ASF 70. The heat-treated preparation of ASF 45 seemed to be inactive in elucidating skin response of rabbits. Dependence of skin response upon dose of the washed cells and the preparation of ASF 45 was also discussed.

Mycobacterium ulcerans infects man and elicits necrosis of the dermis and subcutaneous tissue. There were a few reports that this disease is more likely to be endemic in tropical areas, such as New Guinea, Malaysia (Pettit, *et al.*, 1966, Reid, 1967), Mexico (Lavalle Aguilar, *et al.*, 1953) and especially the countries of Central Africa (Connor and Lunn, 1966, Gray, *et al.*, 1967 and Bayley, 1971). One of clinical characteristics of the disease is chronic ulceration of skin with undermined edges. Microscopic examination of the necrotic lesion reveals clumps of acid-fast bacilli only in the central zone of the vast necrotic tissue. In other words, the necrotic tissue extends beyond the nidus of organism where acid-fast bacilli are confined to a relatively small area. This fact leads one to speculate that devitalization of the tissues may be due to the diffusible products of *M. ulcerans*, but the cause and mechanism of necrosis by the organism are not elucidated yet.

Although it became of interest to many

* Received August 31, 1978
* This study was supported by a Faculty Research Grant (1976-1977) of Yonsei University College of Medicine.

people to learn about the pathogenic mechanisms of this acid-fast bacilli in causing ulceration, there is not yet sufficient information even on the susceptible animals to the organism and/or exocellular substances of *M. ulcerans*. Thus, attempts to look for susceptible animal groups to the exocellular products of the organism were made prior to study of pathogenic mechanisms of the bacterium in elucidating necrosis and ulceration.

MATERIALS AND METHODS

Strains: *Mycobacterium ulcerans* 340 obtained from the Research Institute of Mycobacterium, Hiroshima University, Hiroshima, Japan and maintained at the authors' laboratory was used in the experiments.

Growth of bacteria: Colonies of *M. ulcerans* grown on Ogawa slant were transpanted into Dubos liquid medium and incubated at 33°C for two to four months with occasional aeration. Cells were harvested by centrifugation at 4,000 RPM for 30 minutes (International centrifuge-temperature controlled) at 4°C. Cells were washed three times with 10-ml of K-Na-phosphate buffered saline (PBS, 6.6 x 10^{-2} M phosphate, 0.85% Nacl, pH = 7.5) and resuspended in the same PBS to give 4 x 10^8 cells per ml.

Fractionation of culture supernatant with ammonium sulfate: The culture supernatant was fractionated by adding dried solid ammonium sulfate finally to give 45 and 70% (w/v) of the saturation concentrations, sucessively and the precipitates resulting from each addition were designated as ASF 45 and ASF 70, respectively. The ASF 45 and ASF 70 were dialyzed against 2 liters of glass distilled water for two days at 4°C in order to decrease ionic strength of the peparations.

Protein measurement: Protein was quantitated by the method of Lowry, *et al.* (Lowry, *et al.*, 1951). Commercial bovine serum albumin (Cohen fraction V, Sigma Chemicals, St. Louis, Mo.) was used to construct a standard curve relating protein concentration to absorbance at 540 μm.

Screening of animals susceptible to washed whole cells, ASF 45 and ASF 70: Mice, rabbits, and guinea pigs were chosen for the test. One-tenth ml of concentrated Dubos medium (5 mg of protein per ml), concentrated culture supernatant (1.25 mg of protein/ml), ASF 45 (1.5 mg protein/ml), ASF 70 (1.2 mg of protein/ml) and PBS washed whole cells (4 x 10^8 cells/ml) were injected subcutaneously into mice, and guinea pigs (two of each group and two sites for each animal, a total of four sites). The same amount of each preparation was given intradermally to rabbits. Foot-pad injection of each preparation was also made in mice.

Dose-dependence of rabbit skin response to ASF 45: Different amounts of ASF 45 preparation (2.2 mg of protein/ml) were injected intradermally into rabbit skin to test dose dependence. One portion of the ASF 45 preparation was heated in a 75°C water bath for 30 minutes an the heat treated preparation was also injected into the rabbit to see whether it still held activity inducing skin response. Sterile distilled water was adopted as a control.

Dose-dependence of rabbit skin response to the PBS washed *M. ulerans* cells: In order to observe the response of rabbit skin vs. number of the washed whole cells, cells harvested by membrane filtration were washed three times with phosphate buffered-saline and resuspended in the same buffer to give 4 x 10^8 cells/ml. One-, two- and three-tenths ml of this cell suspension were intradermally given to rabbits, with 0.3 ml of PBS injection at the control sites.

RESULTS

Among the animals employed for a susceptibility test to the PBS washed whole cells of *M. ulcerans* and the preparations of cell culture supernatant, only rabbit skin showed marked response to the washed cells, ASF 45 and ASF 70 as depicted in Table 1. Skin responses considered for apparent susceptibility included erythema, swelling, pus formation etc. during the early stage and erythema, scarring etc. at the progressed stage. Degree and intensity of skin responses induced by each preparation varied along with time progression. While erythema induced by administration of ASF 70 faded within five days, erythema caused by injection of ASF 45 was fairly intensive for the first week, then they became confined into a smaller area and lasted for more than several weeks as shown in Table 2 and Figure 1. When the washed cells were intradermally given to rabbits, there was erythemata in a small area for the first two weeks (ca. 0.5 cm in diameter), but the erythematous region extended further with time progression (maxium at 3-5 weeks, ca. 1.5 cm in diameter).

Table 1. Response of Animals to Preparations of *M. ulcerans* Culture

Preparation	Mouse		Guinea pig	Rabbit
	Foot-pad	Skin(S.C.)	Skin(S.C.)	Skin(I.D.)
Conc-medium (500 ug of protein)	–	–	–	–
Conc-culture suppernatant (125 ug of protein	–	–	–	–
Washed cell (4×10^7 cells)	–	–	–	0.5*
ASF 45 (150 ug of protein)	–	–	±	1.3
ASF 70 (120 ug of protein)	–	–	–	1.0

Records were made two days after injection of each preparations.
– : no response, ± : response in doubt.
* : diameter of erythema expressed in centimeters. The values are average of four sites.

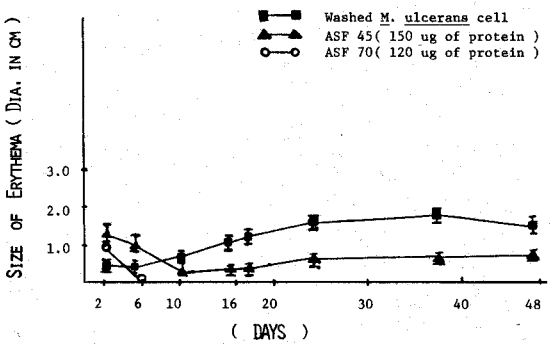

FIG. 1. SIZE OF ERYTHEMA INDUCED BY ASF 45, ASF 70 AND WASHED M. ULCERANS CELL WITH TIME PROGRESSION

Table 2. Duration of Skin Reponses of Rabbits to Preparations of *M. ulcerans* Culture

Preparation	Length of time	Skin response
Washed *M. ulcerans* cells (4×10^7)	> 50 days	erythema, edema, nodule
ASF 45 (150 ug of protein)	< 20 days	erythema, edema
	> 20 days	erythema, nodule
ASF 70 (120 ug of protein)	< 5 days	erythema

Erythrogenic activity of the preparation of ASF 45, upon injection in the rabbit, seemed to be lost by heating the preparation in a 75°C water bath for 30 minutes, as one may notice in Table 3. Both of erythematous area and persistance of the response seemed to be related to the amounts of ASF 45 (expressed in protein concentration), but not with a linear relationship (Table 3 and Table 4).

When the PBS washed *M. ulcerans* cells were given to rabbits, erythema induced by injection increased along with the number of cells (Table 5). There seemed to be a linear relationship between size of erythema and number of the washed cells injected as shown in Figure 2. Counts of acid-fast bacilli in the erythematous lesions of rabbit skin were made at three weeks

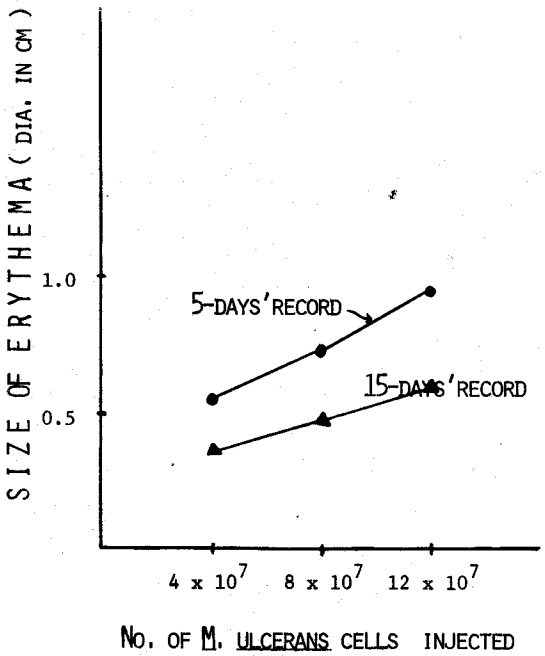

FIG. 2. DEPENDENCE OF ERYTHEMA SIZE TO NO. OF WASHED M. ULCERANS CELL

Table 3. Dose Response of Rabbit Skin to Preparation of ASF 45

Material	Size of erythema recorded days after injection		
	3 (days)	8 (days)	12 (days)
D-H_2O	–	–	–
Heat treated ASF 45 (160 ug of protein)	0.3*	–	–
ASF 45 (80 ug of protein)	0.5	0.5	red points
ASF 45 (160 ug of protein)	0.55	0.5	0.6
ASF 45 (220 ug of protein)	0.5	0.5	0.5
ASF 45 (360 ug of protein)	0.6	0.7	0.7

* Diameter of erythema in centimetres. The values are average of four sites.

Table 4. Duration of Skin Responses of Rabbits to Preparation of ASF 45

Material	Length of time (days)	Skin response
D-H_2O	0	–
Heat treated ASF 45 (160 ug of protein)	7	erythema, pus
ASF 45 (80 ug of protein)	19	erythema, pus, nodule
ASF 45 (160 ug of protein)	43	erythema, pus, nodule
ASF 45 (220 ug of protein)	43	erythema, pus, erupted, nodule
ASF 45 (360 ug of protein)	43	erythema, pus, erupted, nodule

Table 5. Dose Response of Rabbit Skin to *M. ulcerans* Cell

#of cells injected	Size of erythema recorded days after injection			
	3(days)	5(days)	9(days)	15(days)
PBS	–	–	–	–
4×10^7	0.55*	0.55	0.48	0.37
8×10^7	0.75	0.73	0.65	0.48
12×10^7	0.95	0.95	0.95	0.6

* Diameter of erythema expressed in centimetres. The values are average of four sites.

and six weeks of post-injection of the washed bacterial cells but there was no change in the number of bacteria compared with that of bacteria injected.

DISCUSSION

The species of *M. ulcerans* was isolated as a new pathogen for man by a group of Australians (MacCallum, *et al.*, 1948) 30 years ago, yet the cause of the disease and pathogenesis of the organism have not been well understood. The disease elicited by this bacterium was reported as endemic in tropical regions of Australia, Africa and Central America, (This infectious disease is better known as *"buruli ulcer"*, whose name is derived from the area in Central Africa where such disease is heavily endemic).

The lesions of *M. ulcerans* infection are characterized by extensive coagulation necrosis, but with detection of acid-fast bacilli only in the central zone of a vast necrotic area. On the basis of such clinical and microscopic observations, one may speculate that the features of *M. ulcerans* infection are the consequence of effect of diffusible exocellular products of the bacterium on tissues. Read's group (Read, *et al.*, 1974) reported that the culture filtrate and the viable organisms of *M. ulcerans* produced a progressive infection in mice and a cytopathogenic effect on itssue culture cells. They also claimed that focal necrosis and inflammawhich resemble those in naturally occuring infections in mice, resulted from either the culture filtrate or the viable cells of *M. ulcerans* on guinea pig skin, and the exocellular substance responsible for producing the focal necrosis and inflammation seemed to be non-dializable.

However, results of the present study were somewhat different from those of Read's group; that is, the mouse and guinea pig were not sensitive to either preparations of culture supernatant or the washed cells of *M. ulcerans*. In contrast, there was apparent response of rabbit skin to the preparations of culture supernatant as well as to the washed cells. The non-dializable substances in the culture supernantant, which is capable of inducing erythema and edema on rabbit skin, were precipitated with 45% of the saturation concentration of ammonium sulfate. The substances lost their activities by heating at 75°C for 30 minutes.

Intensity of skin response of the rabbit, resulting from injection of the washed cells, was apparently correlated to the number of cells given. In the cases, the affected area persisted for several weeks, but there was no indication of multiplication of the organisms inside the affected area. This fact suggested that the skin response seemed to result from gradual release of toxic substances from the bacterial cells upon their destruction inside the tissues, even though it is not certain whether or not *M. ulcerans* loses its viability in the dermis of the rabbit.

The chemical nature and other properties

of the exocellular substances need further investigation. A simple method quantitating activity of the substances *in vitro* should be designed in order to purify the substance with less effort.

Pathological studies of the lesions caused by the injection of either the exocellular substances of *M. ulcerans* or the washed cells should be extensively made to understand the pathogenic mechanisms.

Mycobacterium marimum is known as a causative agent of water-borne skin infection, i.e. "swimming pool granuloma". Both *M. ulcerans* and *M. marimum* favor a rather low temperature for better growth (31-33°C) and the clinical pictures of these infections look similar, but there are not many studies on the cause of infection by *M. marimum.* Therefore, it might be worth-while investigating the pathogenic nature of *M. marimum* with relation to that of *M. ulcerans.* It would also be interesting to see whether these two organisms are related to each other with respect to their immunological natures.

REFERENCES

Bayley AC: *Buruli ulcer in Ghana, Letter to the editor. Brit Med J 2: 401-402, 1971*

Connor DH, Lunn HF: *Mycobacterium ulcerans infection. (with comments on pathogenesis) Int J Leprosy 33: 698-709, 1965*

Gray HH, Kingma S, Kok SH: *Mycobacterial skin ulcers in Nigeria. Trans Roy Soc Trop Med Hyg 61: 712-714, 1967*

Lavalle Aguilar P. Marquez Iturribarria F, Milldebrook G: *Un caso de infeccion human por Mycobacterium ulcerans en el Hemisferio Occidental. Nota previa. J Int Leprosy 21: 469-476, 1953*

Lowry, OHN Rosebrough J, Farr AL, Randall RJ: *Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275, 1951*

MacCallum P, Tolhurst JC, Buckle G, Sissons HA: *A new mycobacterial infection in man. J Pathol 60: 93-122, 1948*

Pettit JHS, Marchette NJ, Reeves RJ: *Mycobacterium ulcerans infection. Brit J Dermatol 78: 187-197, 1966*

Read JK, Heggie CM, Meyers WM, Connor, DH: *Cytotoxic activity of Mycobacterium ulcerans. Infect Immunity 9: 1114-1122, 1974*

Reid IS : *Mycobacterium ulcerans infection: a report of 13 cases at the Port Moresby General Hospital, Papua. Med J Aust 1 : 427-431, 1967.*