INTERNATIONAL JOURNAL OF LEPROSY Volume 37, Number 2
Printed in U.S.A.

Acid-Fast Organisms in the Skin of Man and the Human Fetus[1, 2]

Tatsuo Mori, Yoshiharu Kishi, Seiji Innami and Shinji Nishimura[3]

Nishimura *et al.* ([8, 9]) found acid-fast organisms in the subcutaneous connective tissue of a high percentage of healthy mice which had not been in contact with known murine leprosy infected rodents. These acid-fast organisms were almost impossible to cultivate. Strains of murine leprosy-like bacilli, which proliferate well in a second generation of mice, were present among these. Later investigations ([7]) have shown that numerous acid-fast organisms are also present in the dermal tissues of guinea-pigs, rabbits and monkeys. The distribution of acid-fast organisms in the mouse was also determined in an attempt to elucidate the pathway of infection ([7]), but no constant pattern was found. Sushida *et al.* ([11, 12]) reported that acid-fast organisms are often found in fetuses of healthy as well as murine leprosy infected mice. On the basis of these studies it seemed reasonable to expect similar findings in man. A search was made of the skin of human subjects and human fetal material, unrelated to leprosy, for the presence of acid-fast organisms.

MATERIALS AND METHODS

Skin tissue material. Samples of skin were obtained at surgery and at legal and routine autopsies. The autopsy material was obtained within two days of death. The material obtained at surgery was mostly from patients with various forms of tumor; material obtained at legal autopsy was mostly from cases of accidental death from trauma or poisoning and that from routine autopsies was mostly from cerebral hemorrhage and heart failure cases and included some cases of old age. In collecting the material from the autopsy cases, the epidermis was cleansed well and care taken to avoid contamination as much as possible.

Fetal material. Fetuses, obtained by induced abortion, were collected from hospitals. They were refrigerated over night and used within one day after abortion to avoid as much contamination as possible.

Amnionic fluid. Forewaters were collected aseptically using sterile vacuum aspiration tubes.

All the material was placed in sterile petri dishes and stored in the deep freezer.

Precautions against contamination. All instruments and equipment employed were maintained exclusively for this study so as to avoid contamination. All glassware was washed well after use, placed in potassium chromate solution for two days and then washed with water before being used again. The chloroform, ether, water and acetic acid were redistilled before use. In staining for bacilli, carbol fuchsin and decolorizing 1 per cent HCl-alcohol were poured dropwise on the glass slide. Staining solution and decolorizing 1 per cent HCl-alcohol were discarded each time so as to avoid contamination.

Alkali-treated smear method. A small amount of material was placed in a mortar, and macerated with 1N NaOH. A thin smear was then made on a glass slide, dried, and the alkali washed away by gentle shaking in water and then dried again. Fixation was by heat and staining was with the Ziehl-Neelsen method.

Bacillary concentration technic. It being difficult to find bacteria in smears from skin and fetal material and microscopic examination of the alkali-treated smear requiring

[1] Received for publication 6 September 1968.
[2] This study was made possible in part by a grant from World Health Organization.
[3] T. Mori, M.D., Y. Kishi, M.D. and S. Innami, M.D., Staff members, Department of Leprology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; S. Nishimura, M.D., D.V.M., Professor of College of Biomedical Technology, Osaka University, Instructor in Department of Leprology, Research Institute for Microbial Diseases, Osaka University.

TABLE 1. *Detection of acid-fast bacillus by alkali-smear method.*

No.	Age (yrs.)	Sex	Disease	Presence of bacilli[a]	Type of specimen
1.	21	female	ovarian cancer	(−)	(S)
2.	22	male	dislocation of shoulder	(+)	(S)
3.	23	male	death from a fall	(−)	(A)
4.	25	female	abdominal hernia	(−)	(S)
5.	27	female	breast tumor	(+)	(S)
6.	28	male	heart rupture	(−)	(A)
7.	28	female	uterine myoma	(−)	(S)
8.	29	female	sarcoma	(−)	(S)
9.	31	male	gastric ulcer	(−)	(S)
10.	32	female	breast cancer	(−)	(S)
11.	32	female	acute death	(−)	(A)
12.	35	female	breast cancer	(−)	(S)
13.	35	female	breast tumor	(+)	(S)
14.	36	female	breast cancer	(−)	(S)
15.	37	female	mastitis	(−)	(S)
16.	37	female	breast tumor	(+)	(S)
17.	38	female	breast cancer	(−)	(S)
18.	38	female	chorio-epithelioma malignum	(−)	(S)
19.	39	female	spinal caries	(−)	(S)
20.	39	male	neurinoma of plexus brachialis	(−)	(S)
21.	40	male	atheroma of buttock	(−)	(S)
22.	40	female	breast tumor	(−)	(S)
23.	40	female	sarcoid	(−)	(S)
24.	40	female	breast tumor	(−)	(S)
25.	40	male	atheroma	(−)	(S)
26.	42	male	apoplexy	(+)	(A)
27.	43	female	breast tumor	(−)	(S)
28.	43	female	breast cancer	(−)	(S)
29.	46	female	breast cancer	(−)	(S)
30.	47	male	submaxillary tumor	(−)	(S)
31.	47	female	breast cancer	(−)	(S)
32.	49	female	breast abscess	(−)	(S)
33.	49	female	cervical tumor	(−)	(S)
34.	50	female	breast cancer	(−)	(S)
35.	50	male	gastric ulcer	(−)	(S)
36.	50	female	gastric ulcer	(−)	(S)
37.	51	female	axillary tumor	(−)	(S)
38.	51	female	breast cancer	(−)	(S)
39.	52	female	breast cancer	(−)	(S)
40.	52	female	breast cancer	(−)	(S)
41.	52	female	mammary abscess	(−)	(S)
42.	53	male	gastric cancer	(−)	(S)
43.	53	female	breast cancer	(+)[b]	(S)

TABLE 1 *Continued*

No.	Age (yrs.)	Sex	Disease	Presence of bacilli[a]	Type of specimen
44.	53	male	sarcoma	(−)	(S)
45.	53	female	breast cancer	(−)	(S)
46.	55	female	uterine cancer	(++)[c]	(S)
47.	56	male	abdominal tumor	(−)	(S)
48.	56	female	breast cancer	(−)	(S)
49.	56	female	breast cancer	(−)	(S)
50.	58	female	inguinal hernia	(−)	(S)
51.	59	male	mandibular cancer	(−)	(S)
52.	60	male	apoplexy	(−)	(A)
53.	60	female	uterine cancer	(−)	(S)
54.	60	male	gastric cancer	(−)	(S)
55.	61	male	Hodgkin's disease	(−)	(S)
56.	63	female	gastric cancer	(+)	(S)
57.	63	female	breast cancer	(−)	(S)
58.	64	female	breast cancer	(+)	(S)
59.	64	female	aneurysma	(+)	(A)
60.	64	male	hanging	(−)	(A)
61.	71	male	sarcoma	(−)	(S)
62.	78	female	old age	(−)	(A)

[a] Present in 10 (16%); absent in 52.
[b] No. 43 (+) = Specimen of serial section.
[c] No. 46 (++) = Specimen of antigen.
(S) = Surgical biopsy.
(A) = Specimen from autopsy.

much time, a bacteria concentration technic was utilized. By combining the Kar *et al.* method([5]) with that of Dharmendra and Mukherjee ([1]), a Kar-Dharmendra method was developed. The material (0.5 gm. of adult human skin, or 2 gm. of fetal skin together with hands and feet in two or three month fetuses) was cut into small pieces with a pair of scissors. Two per cent acetic acid was added (4.5 ml. for adult skin, 1 ml. for fetal skin) to break down the tissues which were each placed in a Waring blender and mixed for two minutes. Twenty ml. of chloroform was added and mixing in the Waring blender continued for one minute. In the case of adult skin, the chloroform layer was transferred to an Erlenmeyer flask by decantation while with fetal skin, the chloroform layer was separated by centrifugation at 2,000 rpm for five minutes and the chloroform layer aspirated with a syringe and placed in an Erlenmeyer flask. This treatment with chloroform was repeated five times. The chloroform washings of each specimen were then pooled and placed overnight in the refrigerator. Floating tissue fragments were allowed to adhere to the wall of the flask and removed by carefully wiping away with clean gauze. The chloroform solution was placed in an evaporating flask and the chloroform evaporated under reduced pressure. The residue was suspended in ethyl-ether and centrifuged at 2,000 rpm for five minutes after which the supernatant was discarded. The sediment was suspended in one drop of distilled water, spotted on a slide glass heated on a constant temperature plate. In some cases, this spotting was repeated 2-3 times for greater concentration. After evaportation and fixation by heat, the specimens were stained by the Ziehl-Neelsen method, taking care not to lose bacteria.

With the amnionic fluid, a small portion

TABLE 2. *Detection of acid-fast bacillus by Kar-Dharmendra method.*

No.	Age (yrs.)	Sex	Disease	Presence of bacilli[a]	Type of specimen
1.	19	male	neurofibromatosis	(−)	(S)
2.	19	female	pancreas tumor	(−)	(S)
3.	20	male	gastric cancer	(−)	(S)
4.	29	male	gastric cancer	(−)	(S)
5.	29	female	intestinal adhesion	(−)	(S)
6.	30	male	tongue cancer	(−)	(S)
7.	31	male	tumor of parotid region	(−)	(S)
8.	32	female	breast cancer	(−)	(S)
9.	32	male	chronic pancreatitis	(+)	(S)
10.	34	male	gastric cancer	(−)	(S)
11.	35	female	breast cancer	(−)	(S)
12.	38	female	breast cancer	(−)	(S)
13.	38	female	gastric cancer	(+)	(S)
14.	38	male	gastric ulcer	(+)	(S)
15.	39	female	bile stone	(−)	(S)
16.	39	male	tongue cancer	(−)	(S)
17.	41	male	duodenal ulcer	(−)	(S)
18.	41	female	breast cancer	(−)	(S)
19.	42	female	breast cancer	(−)	(S)
20.	43	male	gastric ulcer	(−)	(S)
21.	43	male	gastric cancer	(−)	(S)
22.	44	male	gastric ulcer	(−)	(S)
23.	45	female	femoral sarcoma	(+)	(S)
24.	46	male	gastric ulcer	(−)	(S)
25.	47	female	breast tumor	(−)	(S)
26.	47	male	gastric cancer	(−)	(S)
27.	50	male	retroperitoneal tumor	(−)	(S)
28.	51	male	gastric ulcer	(−)	(S)
29.	51	male	gastric cancer	(−)	(S)
30.	52	male	gastric cancer	(+)	(S)
31.	52	male	gastric cancer	(−)	(S)
32.	53	male	gastric ulcer	(+)	(S)
33.	53	female	breast cancer	(−)	(S)
34.	54	female	gastric cancer	(−)	(S)
35.	54	male	gastric ulcer	(−)	(S)
36.	54	female	gastric cancer	(−)	(S)
37.	56	male	gastric cancer	(−)	(S)
38.	57	male	sarcoma in shoulder	(+)	(S)
39.	58	male	pancreas cancer	(−)	(S)
40.	58	male	gastric ulcer	(−)	(S)
41.	59	female	gastric ulcer	(−)	(S)
42.	59	male	gastric ulcer	(−)	(S)
43.	63	male	gastric cancer	(−)	(S)

TABLE 2. *Continued*

No.	Age (yrs.)	Sex	Disease	Presence of bacilli[a]	Type of specimen
44.	63	female	gastric cancer	(−)	(S)
45.	63	male	gastric cancer	(−)	(S)
46.	64	male	gastric cancer	(−)	(S)
47.	64	male	rectal cancer	(−)	(S)
48.	64	female	breast cancer	(−)	(S)
49.	64	male	gastric ulcer	(+)	(S)
50.	66	female	gastric cancer	(−)	(S)
51.	68	male	gastric ulcer	(+)	(S)
52.	69	male	gastric cancer	(−)	(S)

[a] Present in 9 (17%); absent in 43.
(S) = Surgical biopsy.
(A) = Specimen from autopsy.

was retained for cultivation and the major part was immediately combined with chloroform, mixed in a Waring blender and treated as above.

Method of cultivation. Bacteria positive material was inoculated on egg medium (mono-potassium phosphate, sodium glutamate, potato extract, glycerine, whole egg), neutral egg medium and acid egg medium. Since the surgical material was considered to be uncontaminated, a part of the tissue sample was homogenized in physiologic saline and inoculated on neutral egg medium. Contaminated material was treated with 4 per cent NaOH and inoculated on acid egg medium. The cultures were incubated at 33°C and 37°C for two months. The Dubos medium supplemented with mouse liver extract and Jucho mycobacterial species bacilli heat extract was used as bacterial proliferation media. The material was treated with 4 per cent NaOH, neutralized with 1 normal HCl until the neutral red indicator became orange, cultivated in the proliferation medium for two weeks and then inoculated on egg medium.

Preparation of tissue specimen and method of staining. Since stainability of the leprosy bacillus declines when paraffin is removed with xylol, the method of Ishihara and Hagihara([4]) using toluene was followed. Staining was with a modified Koch-Ehrlich method([6]). The specimen was

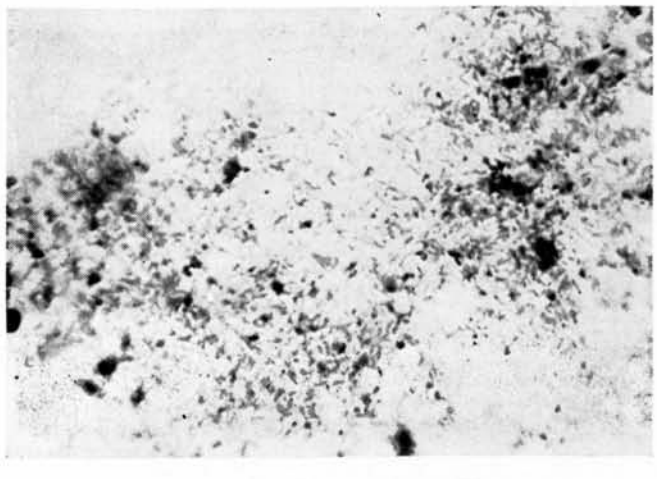

FIG. 1. Acid-fast bacilli obtained by the Kar-Dharmendra method from case No. 43. Ziehl-Neelsen stain. Magnification: X1,200.

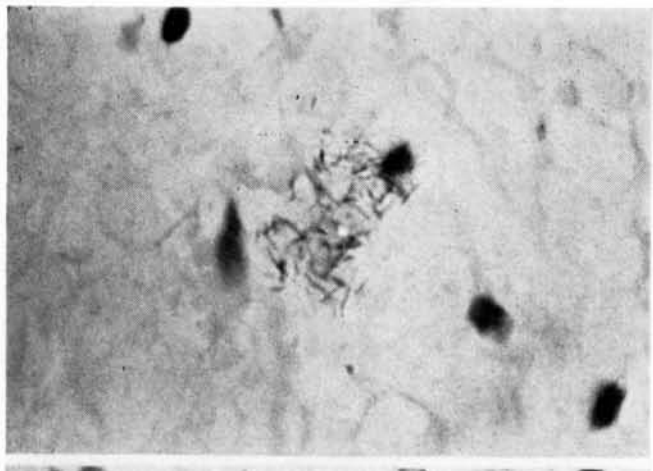

FIG. 2. Acid-fast bacilli present in the subpapillary layer of the skin from case No. 43. Aniline-fuchsin-hematoxylin stain. Magnification: X1,200.

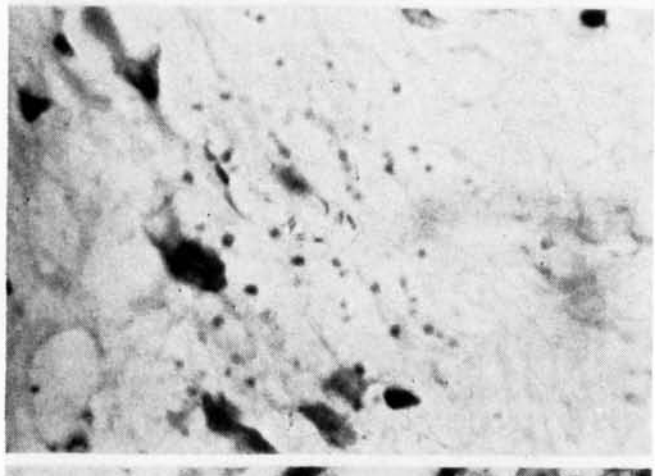

FIG. 3. Acid-fast bacilli present in the dermal layer of the skin from case No. 43. Analine-fuchsin-hematoxylin stain. Magnification: X1,200.

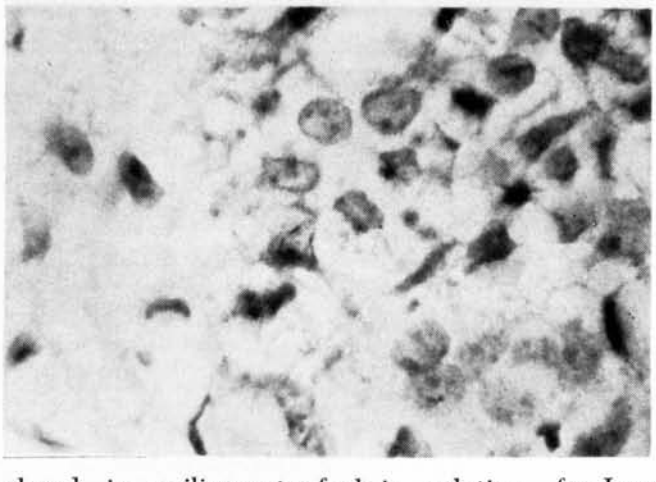

FIG. 4. Same as Figure 3. Bacilli are seen in the center of the photograph.

placed in aniline-water-fuchsin solution and stained for 30 minutes at 50°C, decolorized with 1 per cent HCl-alcohol and stained with hematoxylin as the counter stain.

Standard lepromin. Standard lepromin was obtained from the National Institute for Leprosy Research (Tokyo) and had a bacterial count of 1.3×10^8/ml.

Blank test. In order to check for contamination, commercially available products of chloroform and ether, redistilled chloroform and ether and acetic-acid-water solutions were treated in the same way as the tissue samples and examined for bacilli.

TABLE 3. *Lepromin reaction compared with reaction to antigen of isolated bacilli from human skin.*

Antigen / Subjects	Isolated bacilli No. 46		*M. leprae* lepromin	
	24 hr. mm.	48 hr. mm.	24 hr. mm.	48 hr. mm.
No. 46 adult isolated acid-fast bacilli	0	0	0	0
Lepromatous leprosy	0	0	0	0
Borderline leprosy	0	0	4 × 4	0
Tuberculoid leprosy	0	0	30 × 30	30 × 30
Tuberculoid leprosy	4 × 3	0	15 × 20	8 × 10
Healthy adult (BCG−)	11 × 10	8 × 10	7 × 8	4 × 5
Healthy adult (BCG+)	10 × 11	11 × 13	15 × 13	15 × 19

Numbhr of bacilli, 5×10^6/ml.; inoculum, 0.1 ml.

RESULTS

With the alkali smear method, acid-fast organisms were found in 10 of 62 cases (16%), unrelated to age, sex and disease as shown in Table 1. Examination by the Kar-Dharmendra method yielded nine positive determinations out of 52 (17%) as detailed in Table 2. The organisms seen were bacilli of varying lengths which stained solidly with almost no signs of degeneration. As with the alkali smear method, there was no relationship to age, sex or disease. All positive material was inoculated on medium for acid-fast organism but not a single case of bacillary proliferation was found.

Case No. 43 in Table 1 was a 53 year-old patient with breast cancer organisms. Acid-fast organism had been found in large numbers in the skin which was obtained by surgical operation. Figure 1 shows bacilli collected from the skin of case No. 43 by Kar-Dharmendra method. Serial sections of the skin were examined and a bacterial aggregate was observed in the cutis in three preparations of successive sections (Figs. 2, 3, 4). Figure 2 shows the bacterial aggregate below the Malphigian layer and there was no associated inflammatory response.

Five grams of abdominal skin from case No. 46, obtained at surgical biopsy of a 55 year-old patient with uterine cancer, was examined by the Kar-Dharmendra method and 5 x 10^6 organisms were obtained (Fig. 5). These were suspended in one ml. of 0.5 per cent carbol-physiologic saline, sterilized

TABLE 4. *Detection of acid-fast bacilli in human fetuses by Kar-Dharmendra method.*

	Months							
	2	3	4	5	6	7	8	Total
Positive	3	1	5	1	1	3	1	15
Negative	0	17	23	10	10	5	2	67
Total	3	18	28	11	11	8	3	82

Positive = 18%.

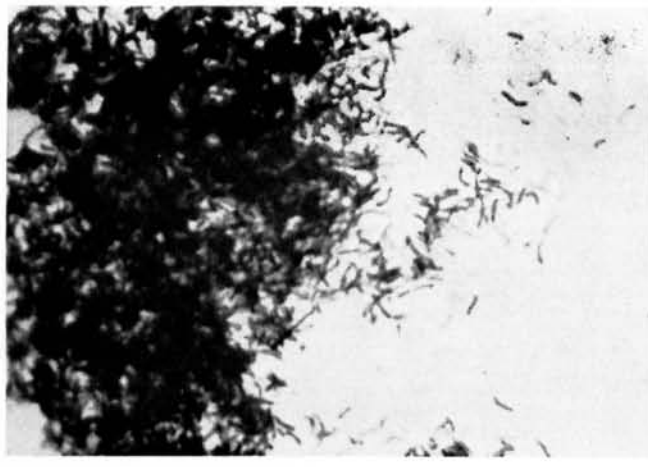

FIG. 5. Acid-fast bacilli collected by Kar-Dharmendra method from case No. 46. Ziehl-Neelsen stain. Magnification: X1,200.

and antigen No. 46 prepared. Standard lepromin, diluted to the same bacterial count was utilized as a control. The comparative lepromin reaction was tested in the patient from which the organism was isolated (No. 46), and also in two cases of lepromatous leprosy, two cases of tuberculoid leprosy and two healthy subjects. Table 3 presents the results. Though the number of cases is small, the fact that the skin reactions to the two antigens do not coincide in the tuberculoid leprosy patient, suggests that the organism isolated from this patient differs from the leprosy bacillus which is not cultivatable by the methods employed. The finding that both the tuberculoid leprosy cases were negative and the two healthy subjects positive to antigen No. 46 is interesting.

Table 4 shows the results of examination by the Kar-Dharmendra method in aborted fetal material. Acid-fast organisms were found in 15 of 82 cases (18%). Possible contamination during passage through the birth canal could not be avoided, but no bacterial growth was found on egg medium such as employed for cultivation of tubercle bacilli. One specimen consisted of a fetus completely enclosed in the amnionic membrane and no contamination was possible. Nevertheless, acid-fast organisms were isolated from this material. Acid-fast organisms were isolated from aseptically collected forewaters in one case out of six, but the number of specimens was so small as to be suggestive only. From these findings it is evident that human fetuses are significantly contaminated by unidentified acid-fast bacilli.

DISCUSSION

Figueredo and Desai[2] state that six to 60 acid-fast organisms were found in 31 out of 48 specimens of skin from non-symptomatic contacts of human leprosy patients by bacterial collection with the chloroform-acetone method. They suggested that these were healthy carriers of human leprosy bacilli. From our findings in non-contacts in Japan where leprosy is relatively infrequent (incidence 1 in 1,000,000), it is concluded that the acid-fast organisms isolated in the present study did not originate through contact with human leprosy patients. If the organisms were due to contamination by acid-fast organisms normally present in nature, growth should take place in culture medium for acid-fast bacillus but not one case of bacterial proliferation was noted. Approximately 18 per cent of the fetal material and 16 per cent of adult material examined showed acid-fast organisms.

The reactions to No. 46 antigen differed from that to the Dharmendra antigen. Therefore it is concluded that the acid-fast organism found in this patient is an unknown bacillus which is not presently cultivatable and that differs from the leprosy bacillus. If the invasion of such acid-fast organisms occurs at the fetal stage, then the acid-fast organisms may induce a state of immunologic tolerance according to the immunologic theory of Medawar and Burnet[3, 10]. The absence of inflammatory cell infiltrates around the bacterial locus and the differing reactions to antigen No. 46 can perhaps be explained by the immunologic tolerance phenomenon.

SUMMARY

Acid-fast organisms were found in 10 of 62 samples (16%) of adult human skin by the alkaline smear technic.

With the Kar-Dharmendra bacterial concentration method, acid-fast organism was observed in 9 of 52 samples of adult human skin (17%), and in 15 of 82 specimens (18%) of two to three month old human fetal material obtained from abortions.

All attempts to cultivate the organism on culture medium for acid-fast organisms were unsuccessful.

Serial sections were prepared from tissue of one surgical biopsy (No. 43) showing a large number of organisms, and bacterial aggregates were found in three preparations from these successive tissue sections.

From 5 gm. of skin tissue of one surgical biopsy (case No. 46) which showed numerous organisms, 5 x 10^6 bacilli were collected by the Kar-Dharmendra method and from these an antigen was prepared. The dermal reaction to this antigen was examined in comparison with 5 x 10^6/ml. diluted standard lepromin. The results that both the tuberculoid leprosy cases tested were negative and two healthy subjects positive to this antigen suggested that the isolated organism was not *M. leprae*.

The findings are discussed in the light of Burnet's theory of immunologic tolerance.

RESUMEN

Se encontraron organismos, acido-resistentes en 10 de 62 muestras (16%) en la piel de adultos humanos mediante la técnica del frotis alcalino.

Por medio del método de concentración bacteriana de Kar-Dharmendra organismos ácido-resistentes se observaron en 9 de 52 muestras de la piel de adultos humanos (17%), y en 15 de 82 muestras (18%) obtenidas en material fetal humano de dos a tres meses de edad, obtenido por abortos.

Todos los ensayos para cultivar los organismos en medios de cultivos para organismos acido-resistentes fueron infructuosos.

Se prepararon secciones seriadas de tejidos de una biopsia quirúrgica (No. 43) mostrando un gran número de organismos y bacterias; estos fueron encontrados en tres preparaciones de estas sucesivas secciones de tejidos.

De 5 gm. de tejido de piel de una biopsia quirúrgica (Caso No. 46) la cual mostró numerosos organismos, 5 x 10^6 bacilos, por el metodo de Kar-Dharmendra, fueron reunidos y de esto se preparó un antígeno. La reacción dérmica de este antígeno se examino comparándola con una lepromina standard diluída 5 x 10^6/ml. Los resultados que ambos casos examinados de lepra tuberculoide fueron negativos y dos individuos sanos positivos a este antígeno, sugieren que el organismo aislado no era *M. leprae*.

Estos hechos se discuten a la luz de la teoría de Burnet de la tolerancia inmunológica.

RÉSUMÉ

Par la technique des frottis alcalins on a trouvé des organismes acido-résistants dans 10 échantillons de peau d'homme adulte sur 62 (16%).

Par la méthode de concentration bactérienne de Kar-Dharmendra, on a observé des organismes acido-résistants dans 9 énchantillons de peau d'homme adulte sur 52 (17%), et dans 15 énchantillons sur 82 (18%), de matériel humain foetal âgé de 2 à 3 mois et obtenu à la suite d'avortements.

Tous les essais menés en vue de cultiver cet organisme sur des milieux de culture, afin de mettre en évidence des organismes acido-résistants, ont échoué.

Des coupes en série ont été préparées à partir du tissu d'une biopsie chirurgicale (Case No. 43), montrant un grand nombre d'organismes; des agglomérats bactériens ont été observés dans 3 préparations provenant de ces coupes successives de tissu.

A partir de 5 gm. de tissu cutané, provenant d'une biopsie chirurgicale (Case No. 46) qui montrait des organismes en grand nombre, 5 x 10^6 bacilles ont été recueillis par la méthode de Kar-Dharmendra; un antigène a été préparé à partir de ces bacilles. La réaction dermique à cet antigéne a été comparée avec celle obtenue par une lépromine standard diluée contenant 5 x 10^6 bacilles par ml. Les cas de lèpre tuberculoïde qui ont été soumis à cet antigéne ont présenté des réactions négatives, alors que 2 individus en bonne santé se sont révélés positifs à cet antigène; ces résultats suggèrent que l'organisme isolé n'est pas *M. leprae*.

Ces observations sont discutées à la lumière de la théorie de Burnet sur la tolérance immunologique.

Acknowledgments. We wish to thank Professor Shiba, Drs. Sugawa and Taguchi and their colleagues in the Research Institute for Microbial Diseases, Osaka University; Professor Matsukura in the Department of Forensic Medicine, Osaka University Medical School; Professor Kammbe in the Department of Pathology, Osaka City University and Drs. Okimoto and Hasegawa in the Hospital of Obstetrics and Gynecology, who so kindly made the samples of tissue available.

REFERENCES

1. Dharmendra and Mukherjee, N. Simple method of concentration of leprosy bacilli from "closed cases of leprosy." Leprosy in India **24** (1952) 169-177.
2. Figueredo, N. and Desai, S. D. A new method for the detection of leprosy bacilli in neural cases and contacts. Indian J. Med. Sci. **6** (1952) 296-301. (*Abstract in* Internat. J. Leprosy **21** (1953) 128.)
3. Hasek, M., Lengerova, A. and Hraba, T. Transplantation immunity and tolerance. Adv. Immunol. **1** (1961) 1-66.
4. Ishihara, S. and Hagihara, S. Study on the procedure for demonstrating lepra bacilli in paraffin sections. La Lepro **23** (1954) 143-146. (*Abstract in* Internat. J. Leprosy **23** (1955) 107.)
5. Kar, S., Elliston, E. P. and Taylor, C. E. Field method for concentrating *M. leprae* in skin biopsy specimen. Internat. J. Leprosy **32** (1964) 18-23.
6. Klimmer, M. Koch-Ehrlich method "Technik und Methodik der Bakteriologie und Serologie." (1923) 155.
7. Mori, T., Kohsaka, K., Kishi, Y., Kamei, M. and Nishimura, S. Distribution of acid-fast bacilli in the skin, extremities and internal organs of various experimental animals. La Lepro **35** (1966) 27-32. (*Abstract in* Internat. J. Leprosy **34** (1966) 452).
8. Nishimura, S. Transmission of *M. leprae* to mice and identification of increased bacilli in injected animals. La Lepro **32** (1963) 1-13. (*Abstract in* Internat. J. Leprosy **31** (1963) 377.)
9. Nishimura, S., Kawaguchi, Y., Kohsaka, K. and Mori, T. Contamination of healthy mice with murine leprosy-like acid-fast bacillus. La Lepro **33** (1964) 245-256. (*Abstract in* Internat. J. Leprosy **33** (1965) (58.)
10. Smith, R. Immunological tolerance of nonliving antigens. Adv. Immunol. **1** (1961) 67-129.
11. Sushida, K. and Hirano, N. Investigation for acid-fast bacilli in normal mice with special reference to normal pregnant mice and their fetus. La Lepro **35** (1966) 179-186.
12. Sushida, K., Hirano, N. and Nakano, H. Placental infection in the fetus of pregnant murine leprous mice. La Lepro **35** (1966) 237-245. (*Abstract in* Internat J. Leprosy **35** (1967) 416.)