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Preparation of Purified Suspensions of *Mycobacterium leprae* and *Mycobacterium lepraemurium* from Biopsy Material[1]

Laszlo Kato[2]

Calf-lymph-smallpox vaccines have been successfully purified by Dunlap ([1]) using enzymatic digestion with α-chymotrypsin; subtilisin and collagenase. The raw smallpox vaccine contained particulate debris derived from the lymph and dermis, thus resembling the impurities present in the homogenized granulomata of human and rat leprosy biopsy material. The principles applied in the method of Dunlap were used for the isolation of human and rat leprosy bacilli from the diseased tissues. Modification of the method of Dunlap permitted a simple and easily reproducible technic to prepare purified suspension of leprosy bacilli.

MATERIALS AND METHODS

Subcutaneous human lepromas were removed aseptically from untreated lepromatous leprosy or reactivated lepromatous leprosy cases. The tissue was placed in sterile saline solution adjusted to pH 3.5 with acetic acid and then lowered to pH 3 with HCl. Without refrigeration, this tissue was shipped and reached our laboratory within three days. The Hawaiian strain of rat leprosy was maintained on Wiersing rats by transmitting the disease subcutaneously at three month intervals.

α-Chymotrypsin, subtilisin and collagenase (CSC) (Nutritional Biochemicals Corp.) were dissolved at a concentration of 100 μg/ml in phosphate buffered saline (PBS, pH 7.2) containing 5 mg% $CaCl_2$ and sterilized by filtration. The biopsy material was homogenized in the enzyme solution in a Wortex four-blade blender, three times for ten seconds at 12,000 rpm at room temperature. The tissue, enzyme-solution ratio was 1:10 for the rat lepromas and 1:50 for the human lepromas. The homogenized granuloma was filtered through a sterile nylon filter. The filtrate was immediately centrifuged at room temperature at 4,500 rpm for seven minutes. The sediment was resuspended in the same enzyme solution and centrifuged at the same speed for seven minutes. Up to this point, the homogenate was exposed to enzymatic digestion for not less than 30, and not more than 45 minutes. The sediment was washed and centrifuged (seven minutes at 4500 rpm) successively with 50 ml of each of the following solutions for the sediment obtained from one gram human leproma or five grams of rat leprotic tissue.

1. PBS, acidified with acetic acid to pH 3.0 (to remove acid-soluble collagens and acid-soluble peptides).
2. Distilled water (to water soluble fragments of digested macromolecules).
3. 0.85% saline solution.
4. PBS adjusted to 8.4 with KOH (to remove the alkaline soluble collagens, peptides and alkali-homogenized fat or fatty material floating on the surface).
5. PBS pH 6.0, then resuspended in physiological saline.

Aliquots of the homogenized, purified suspensions were placed on siliconized slides, to which the bacilli adhere rapidly, by means of a tuberculin syringe. Fixation for two minutes at 80°C on hot plate was followed by treatment in ten percent periodic acid in water at 60°C for ten minutes. Staining for acid-fastness was performed at room temperature. The preparation was examined microscopically for purity and the percentage of solidly stained acid-fast rods were counted on 200 bacilli. Smears were also examined with phase contrast and dark field microscopy, to detect tissue debris and fibers.

In another series of experiments, lepromas were digested with trypsin (1:250 Gen. Biochem., 100 μg/ml) alone, followed

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[2] L. Kato, M.D., Head Department of Experimental Pathology, Institute of Microbiology and Hygiene, Montreal University, Canada.

by washings and in experimental conditions shown in Tables 1 and 2.

The wet weights of the sediments were obtained following centrifugation at 1600 rpm for 40 minutes. Salts were removed by washing twice in deionized water. Bacilli were counted using the microspot technic ([2]).

RESULTS

Crude homogenates of human or murine lepromas suspended in PBS contain large amounts of tissue debris which obscure the microscopic examination of the bacilli in stained preparations. With repeated washings in PBS, the tissue debris was only partially removed. While trypsin digestion and consecutive washings were effective in breaking up the debris, there still remained a large amount of blue stained components, dust-like particles and many partially acid-fast bacilli. When the homogenized lepromas were digested with the triple enzyme solution (CSC) for 45 minutes at 20°C, followed by washing with PBS five times, the solid/non-solid acid-fast ratio was not improved as compared to the crude homogenized leproma. Similarly, this treatment did not remove the small dust-like particles visible with phase contrast microscopy. When the lepromas were submitted to the triple enzyme treatment and consecutive washings in PBS at differing pH, distilled water and saline solution, the final fraction consisted mainly of acid-fast material characteristic of mycobacteria with only traces of blood stained elements. For both the human and the rat leprosy bacilli, the ratio of solid to non-solid rods was considerably improved. Microscopic examination of the purified suspension by contrast technics confirmed the findings of the stained preparations. The impurities consisted of few dust-like particles and short fibers, probably collagen.

Table 1 shows the percentage of solid acid-fast murine leprosy bacilli in the crude suspension of the leproma and in batches submitted to different washings and enzyme treatment plus washings. Each fraction was injected subcutaneously into separate groups of ten rats on the day of removal of the leproma and after 14 days refrigeration at +4°C. Each rat was injected subcutaneously with 5 x 10^5 bacilli in 0.5 ml saline in the scapular region. Table 1 shows that the procedure permitted not only improved recovery of the percentage of solidly stained acid-fast rods, but that the infectivity was well preserved during the 14 days refrigeration period. When the purified, 14 day old bacilli were injected into rats, lepromas developed in two months in seven to ten animals. No palpable tumors developed in any of the rats injected with the crude suspension of the 14 day old preparation. The largest lepromas developed after four months with the purified *M. lepraemurium* suspension. Table 2 shows that the percentage of solidly stained, acid-fast rods increased considerably in the trypsin digested and washed preparations of human as well as rat leprosy bacilli. In both cases, the quality of the suspension was considerably higher when purified with the triple enzyme technic. With both human and rat leprosy bacilli and with both purification methods, the loss of bacilli during the procedures can be attributed to the elimination of the non-acid-fast and non-solidly staining acid-fast elements.

DISCUSSION

Both *M. leprae* and *M. lepraemurium* are non-cultivable pathogenic microorganisms. Experimental evidence indicates that the lack of success in its cultivation is partly due to the failure of energy utilization or assimilation of added exogenous substrates. Non-specific antibacterial substances and immune entities, present in the crude or partially purified bacillary suspensions prepared from the lepromas, act as growth inhibitors transferred into culture media. The use of the triple-enzyme solution and consecutive washing with different aqueous solvents permit the elimination of most of the undesired impurities and the removal of most of the non-solidly stained acid-fast rods. The fact that the purified murine leprosy suspensions produced accelerated granuloma formation when reinjected into rats, suggests that the triple enzyme treatment removed or destroyed most, if not all, the inhibitors. The yield of the purified bacilli was in the 10^6 to 10^7 range per gram of leproma but the wet weight of the densely packed, purified sediment was un-

TABLE 1. *Acid-fastness and infectivity of variously treated* M. lepramurium.

Preparation of homogenized murine leproma for rat transfer	Transfer after refrigeration, days	% solid acid-fast before transfer	Development of lepromas; average in 10 rats/group	
			Palpable at 2 months	Mean weight gm in 4 months
Suspended in PBS	0 14	24 16	2/10 0/10	6.5 ± 2.49 1.5 ± 0.65
Washed in PBS 5 times	0 14	32 18	0/10 0/10	12.5 ± 5.33 2.1 ± 1.56
Trypsin 1 h. 40°C washed in PBS 5 times	0 14	20 14	4/10 0/10	8.2 ± 3.06 2.5 ± 1.37
Trypsin 45′ 20°C washed in PBS 5 times	0 14	42 24	5/10 0/10	10.9 ± 4.51 0.8 ± 0.61
CSC, 45′ 20°C washed in PBS 5 times	0 14	38 32	6/10 0/10	16.7 ± 4.7 1.6 ± 0.96
CSC, 45′ 20°C. Washed in PBS pH 3.0 H_2O, saline, PBS pH 8.4, PBS pH 6.0, saline.	0 14	78 60	9/10 7/10	18.4 ± 7.53 18.1 ± 5.98

TABLE 2. *Comparative bacillary yields.*

Preparation of the leproma	HUMAN			RAT		
	Yield mg/gm sediment/ leproma wet weight	Solid AF %	Bacilli/ gm tissue	Yield mg/gm sediment/ leproma	Solid AF %	Bacilli/ gm tissue
Homogenized in PBS filtered on nylon		16	1.6×10^7		24	2.3×10^8
Trypsin digested, 45′ 20°C washed in PBS 5 times	56	42	1.04×10^7	80	42	1.6×10^8
CSC digested, washed in PBS, pH 3.0, H_2O, saline, PBS, pH 8.4, PBS, pH 6.0, saline	18	64	9.4×10^6	32	78	1.1×10^8

usually high. Since the microscopic examinations showed but relatively small amounts of impurities, the high weight of the wet paste can be considered as a result of high water binding capacity of the paste.

SUMMARY

The non-cultivable human and rat leprosy bacilli were separated from lepromas by enzymatic digestion of tissue debris and macromolecules followed by a series of washings. The homogenized biopsy material was exposed for 45 minutes to chymotrypsin, subtilisin and collagenase at room temperature. Solubilized debris and degraded macromolecules were removed by washings with phosphate buffered saline solution (PBS) adjusted to pH 3.0, with distilled water, with 0.85% saline solution, and with PBS at pH 8.4, and at pH 6.0 and with 0.85% saline solution successively. Phase contrast and light microscopic examination revealed that the final suspension was composed mostly of high quality acid-fast rods and only small amounts of tissue debris, subcellular particles and fibers. Most of the non-solidly stained or partially acid-fast bacilli were removed. The enzymatic digestion and the process of purification did not reduce the infectivity of *Mycobacterium lepraemurium*.

RESUMEN

Los bacilos no cultivables de lepra humana y murina se separaron de los lepromas mediante digestión enzimática de los restos de tejido y de las macromoléculas, seguida de una serie de lavados. El material de biopsia homgeneizado se expuso durante 45 minutos a quimotripsina, subtilisina y colagenasa, a temperatura ambiente. Los restos solubilizados y las macromoléculas degradadas se separaron por medio de lavados con solución salina fisiológica tamponada con fosfato (SFF) ajustada a un pH de 3,0, con agua destilada, con solución salina al 0,85% y con SFF a un pH de 8,4 y a un pH de 6,0 y luego con solución salina al 0,85% sucesivamente. El examen microscópico con contraste de fases y con microscopio de luz mostró que la suspensión final estaba compuesta en su mayor parte de bacilos ácido-resistentes de alta calidad y que contenía solamente pequeñas cantidades de restos de tejido, partículas subcelulares y fibras. La mayor parte de los bacilos no-sólidos o que eran parcialmente ácido-resistentes fueron removidos. La digestión enzimática y el proceso de purificación no redujeron la infectividad del *Mycobacterium lepraemurium*.

RÉSUMÉ

Des bacilles de la lèpre humaine et de la lèpre du rat, non cultivables, ont été recueillis de lépromes par digestion enzymatique des débris tissulaires et des macromolécules, suivie d'une série de lavages. Le matériel homogénéisé de la biopsie a été mis au contact de chymotrypsine, de subtilisine et de collagénase, durant 45 minutes à la température ambiante. Les débris solubilisés et les macromolécules dégradées ont été séparés par des lavages dans une solution physiologique tamponnée au phosphate (PBS) ajustée à pH 3,0, dans de l'eau distillée, dans une solution physiologique à 0,85 pour cent et dans la solution physiologique tamponnée (PBS) à pH 8,4, puis à pH 6,0, et enfin dans une solution physiologique à 0,85 pour cent, successivement. Les examens au microscope optique et au microscope à contraste de phase ont montré que la suspension finale était composée surtout de bâtonnets acido-résistants de haute qualité, accompagnés d'une petite quantité seulement de débris tissulaires, de particules sub-cellulaires et de fibres. La plupart des bacilles colorés de façon non uniforme ou partiellement acido-résistants avaient été ôtés. La digestion enzymatique et la procédé de purification ici décrit ne réduisent pas le pouvoir infectant de *Mycobacterium lepraemurium*.

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