STUDIES ON THE DEVELOPMENT OF NEW LEPTOSPIRAL VACCINES

Hiroshi Kida

CONTENTS

PREFACE

Leptospirosis is one of the most important zoonoses because of its wide distribution in man and domestic and wild animals (31). In Japan, it is estimated that a considerable number of cattle, swine, dogs and rats are infected with leptospires (35), and at least a hundred cases of human leptospirosis occur every year (8, 10, 19).

Vaccines prepared from killed leptospires have been used in many countries for control of human and animal leptospirosis. A vaccine prepared from viable, avirulent leptospires has been studied (27, 28), but has not been used practically.

For the preparation of the killed leptospiral vaccines, media containing whole serum (mostly rabbit serum) (4, 7, 15, 29) or bovine serum albumin (5, 6, 13, 22) have been widely used. In Japan, KORTHOF's medium containing whole rabbit serum (15) has been recommended for the preparation of the leptospiral vaccine for human use (17). However, some problems are evident. The occurrence of allergic side reactions induced by the presence, in the vaccine, of the serum proteins which were

derived from the culture medium was described by BABUDIERI (2) and MORSI and SHIBLEY (18). They claimed that all traces of serum must be removed from the vaccine in order for the vaccine to be safe. Also, it is difficult to procure large quantities of normal rabbit serum for cultivation of the organisms in vaccine production.

It has been desired, therefore, that a vaccine should be prepared from leptospires grown in a chemically defined medium.

The present author developed a polyvalent vaccine for human use prepared from leptospires grown in a chemically defined medium. Furthermore, fractionation of the components of leptospiral cells was carried out for the purpose of the development of a cell component vaccine which is more effective and less toxic than a whole cell vaccine. Established procedures could be applied directly to the vaccine preparation for animal as well as for human use.

The results derived from the experiments are to be described in the present thesis, which is composed of the following three parts.

I. Comparison of Vaccines Prepared from Leptospires Grown in a Serum Medium and in a Chemically Defined Medium

II. Morphological and Immunological Comparison of Leptospira Treated with Sodium Dodecyl Sulfate and Sodium Deoxycholate

III. Fractionation of Components of Leptospiral Cells Obtained after Treatment with Sodium Dodecyl Sulfate by Zonal Centrifugation, and Immunological and Morphological Analysis of the Fractions

PART I Comparison of Vaccines Prepared from Leptospires Grown in a Serum Medium and in a Chemically Defined Medium

INTRODUCTION

The purpose of the present part is to prepare a polyvalent vaccine from leptospires grown in SHENBERG's chemically defined medium (24). Since the strains of leptospire used for the preparation of the vaccine did not grow in the chemically defiend medium, the variants which could grow in the chemically defined medium were isolated first. Then, a polyvalent vaccine was prepared from the variants grown in the chemically defined medium (the chemically defined medium vaccine) and compared with another polyvalent vaccine prepared from the parents grown in KORTHOF's medium containing normal rabbit serum (15) (the serum medium vaccine). KORTHOF's medium has been recommended for preparation of the leptospiral vaccine by the Ministry of Health and Welfare (17).

MATERIALS AND METHODS

Strains: The strains of Leptospira interrogans

used were strain Shibaura belonging to serogroup Icterohaemorrhagiae, which was recently identified as serovar copenhageni (32), strain Akiyami A of serovar autumnalis, strain Akiyami B of serovar hebdomadis and strain Akiyami C of serovar australis. The strain Shibaura was virulent in guinea pigs and has been used as a challenge strain for a potency test in the national assay of leptospiral vaccine in Japan. All of these strains were provided by the WHO/FAO Leptospirosis Reference Laboratory, the National Institute of Health, Tokyo.

Variants: Four variant strains which could grow in the chemically defiend medium were obtained from 4 strains (parents) accordihg to the method described in a previous paper (14). Fifth to tenth subcultures of the variants in the chemically defined medium were used for vaccine preparation. Identity of the variant with the parent was examined by the microscopic agglutination test and immunodiffusion with hyper-immunized anti-parent rabbit serum. The preparation of antigen extracted with sodium dodecyl sulfate (SDS, Sigma) and immunodiffusion were done according to the method reported by

YANAGAWA et al. (34).

Media: KORTHOF's medium containing 10 % normal rabbit serum (15) (the serum medium) and SHENBERG's chemically defined medium (24) (the chemically defined medium) were used for cultivation of the parents and the variants, respectively. Normal rabbit serum was heated at 56°C for 30 minutes, sterilized by filtration through a SEITZ filter, and stored at -20°C.

Immune sera: Rabbits were hyper-immunized with the cells of 4 parent strains grown in 0.2 % tryptose phosphate broth (Difco) containing 10 % rabbit serum (4). The cells were centrifuged and suspended in a small amount of supernatant. A rabbit for each strain was injected with the cell suspension intravenously 8 times at intervals of 7 days with increasing amounts of the cells, which were collected from the cultures up to approximately 200 ml in total. Seven days after the last injection, rabbits were bled and the sera were stored at -20°C.

Preparations of vaccines: Two polyvalent vaccines were prepared; one was prepared from 4 parents grown in the serum medium, and the other was prepared from 4

variants grown in the chemically defined medium.

Each strain was first grown in test tubes containing 10 ml of medium (the parents were grown in the serum medium and the variants were grown in the chemically defined medium) for 6 days at 30°C. Test tubes containing 2 to 4 x 10^8 leptospires / ml were inoculated into 2 to 4 one-liter ROUX bottles, each containing 500 ml of the same medium. The final concentration of the inoculum was 1 to 2 x 10^6 leptospires / ml. The inoculated bottles were incubated for 6 days at 30°C, and those containing 2 to 4 x 10^8 leptospires / ml were used for the vaccine preparation. Leptospires were killed by adding formalin to the final 0.2 % concentration at 4°C for 7 days. The leptospires were then harvested by centrifugation at 10,000 g for 30 minutes and washed once with 0.01 M phosphate buffered saline (PBS, pH 7.0) in one-tenth volume of the culture. The leptospires were resuspended in PBS containing 0.5 % phenol in a concentration of 2 x 10^9 (strain Shibaura) or 1 x 10^9 (the other strains) leptospires / ml. Efficiency of killing by formalin was checked by inoculation into KORTHOF's medium and by injection into guinea pigs.

Each vaccine was prepared by mixing 4 strains according to the ratio given by the Minimum Requirements for Biological Products (17): 5×10^8 leptospires of strain Shibaura and 2.5×10^8 leptospires of strain Akiyami A, Akiyami B and Akiyami C. Two polyvalent vaccines were bottled in 12 ml vials, each containing 11 ml vaccine.

Tests for sterility of the vaccines: Representative vials of each vaccine were checked for sterility by inoculating them into thioglycolate broth (Wako Pure Chemical Ind., Osaka) and nutrient broth containing 2 % glucose. One half ml of each vaccine was inoculated into 20 test tubes containing 10 ml of each test medium. Ten tubes were incubated at 25°C and the remainings were done at 36°C for 14 days.

Tests for potency of the vaccines: The tests were according to the Minimum Requirements for Biological Products (17) as follows. Each of the 10 guinea pigs weighing 300 to 350 grams was vaccinated subcutaneously with 2 doses (1 ml each) at 7 day intervals. Fourteen days after the second vaccination, the animals were challenged intraperitoneally with 1 ml of leptospire

suspension, which was prepared according to the Minimum Requirements; the procedure was as follows. The liver of a moribund guinea pig with typical jaundice by infection with strain Shibaura was emulsified with PBS at a concentration of 10 %. The emulsion was centrifuged at 2,000 g for 30 minutes to remove cell debris, and the supernatant was diluted 1 : 10 with PBS and used for the challenge.

An additional group of three guinea pigs for each vaccine was vaccinated as stated above for the purpose of seroconversion and determination of titers of agglutinin. The animals were bled on the 14th day after the second vaccination. The sera were pooled by the groups and their titers were determined by microscopic agglutination test. The titer was expressed as a 50 % agglutination end point.

Determination of protein nitrogen content and optical density of the vaccines: The content of nitrogen precipitated by 5 % trichloroacetic acid was determined by means of micro-KJELDAHL analysis. The turbidity of vaccines was determined by a spectrophotometer (139 Hitachi UV-Vis, Tokyo) and expressed as a value of

optical density at a wave length of 650 nm.

Electron microscopy: Each vaccine was placed on a collodion- and carbon- coated grid of 150 mesh for 20 minutes to allow adhesion of leptospires and other substances to the support film; and excess amount of fluid was removed by blotting the grid with filter paper. The grid was then washed by placing one drop of distilled water, which was filtered twice through a Millipore filter (100 nm), and removing the water from the grid by blotting with filter paper. The grid was negatively stained with 2 % phosphotungstic acid at pH 6.0 filtered through a Millipore filter (100 nm). Specimens were examined in a JEM-100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo) at an accelerating voltage of 80 kV.

Precipitation test for detection of rabbit serum in the vaccine: Each vaccine was examined by the precipitation test with hyper-immunized anti-rabbit guinea pig serum for the detection of rabbit serum in the vaccine. Anti-rabbit serum guinea pig serum was prepared as follows. Normal rabbit serum was mixed with an equal volume of alminum hydroxide solution

(pH 7.0, alminum concentration was 1 mg / ml) and 0.5 ml of the mixture was injected 3 times into 3 guinea pigs intramuscularly at 7 day intervals. Seven days after the third injection the animals were bled. The sera were pooled and stored at -20°C. The pooled serum formed a precipitin band with the rabbit serum which was diluted at 1 : 256. The anti-rabbit guinea pig serum was mixed with an equal volume of 0.8 % agarose (Katayama Chemical Co., Osaka) and placed in the lower part of a precipitation tube. Each vaccine was layered on it and a precipitin band was examined 90 minutes later.

Tests for safety of the vaccines: Each vaccine was tested by injection into mice and guinea pigs. One half ml of each vaccine was injected intraperitoneally into 5 5-week-old mice (dd/Hikari/Takeda/SPF), and 5 ml of each vaccine were injected into 3 guinea pigs weighing approximately 350 grams. Animals inoculated with saline served as the control. They were weighed on 1, 2, 3, 4 and 7 days after injection and were observed clinically.

Tests for pyrogenicity to rabbits: The tests were

conducted in the same way as described in the Minimum Requirements for Biological Products (17). Rabbits weighing between 2,200 and 2,500 grams were used for the tests. One ml of the serum medium vaccine per 1,000 grams of the body weight of the rabbit was injected intravenously into two of the rabbits and 1 ml of the chemically defined medium vaccine per 1,000 grams of the body weight of the rabbit was injected intravenously into two other rabbits. A rabbit injected with saline served as the control. The rectal temperature of the rabbits was taked 60, 90, 120 and 180 minutes after injection. Pyrogenicity was expressed by the difference between the maximal temperature reached after injection and that before injection.

Tests for peripheral leukocytic response in mice: Each vaccine was tested by injection into mice. One half ml of each vaccine was injected intraperitoneally into 10 4-week-old mice (dd/Hikari/Takeda/SPF). The mice injected with saline served as the control. Two hours after injection, blood flowing out from a small cut made with a sharp razor blade on the tail vein was taken into a melangeur and diluted with TURK's solution.

The number of leukocytes was counted by using a THOMA's counting chamber. Leukocyte reducing activity was expressed by mean peripheral leukocyte count relative to the mean control count.

RESULTS

Antigenicity of Variants

The parent and the variant were agglutinated by the anti-parent rabbit serum to the same degree, as shown in Table 1.

No difference between the parent and the variant in the antigenicity of the SDS extracted antigen was found by immunodiffusion with the anti-parent rabbit serum (Fig. 1).

Potency of the Vaccines

The data of the potency tests are shown in Table 2. Both vaccines completely protected the guinea pigs from the challenge with the virulent strain. All of the control animals developed hemorrhage and jaundice and died within 7 days. The potency of both vaccines did not differ according to the limitations of the test procedures.

Table 1. Agglutinability of variants

Strain	Parent	Variant
Shibaura	32,000*	64,000
Akiyami A	16,000	16,000
Akiyami B	160,000	160,000
Akiyami C	8,000	8,000

* Reciprocal of the 50 % end point microscopic agglutination titers with anti-homologous parent rabbit serum.

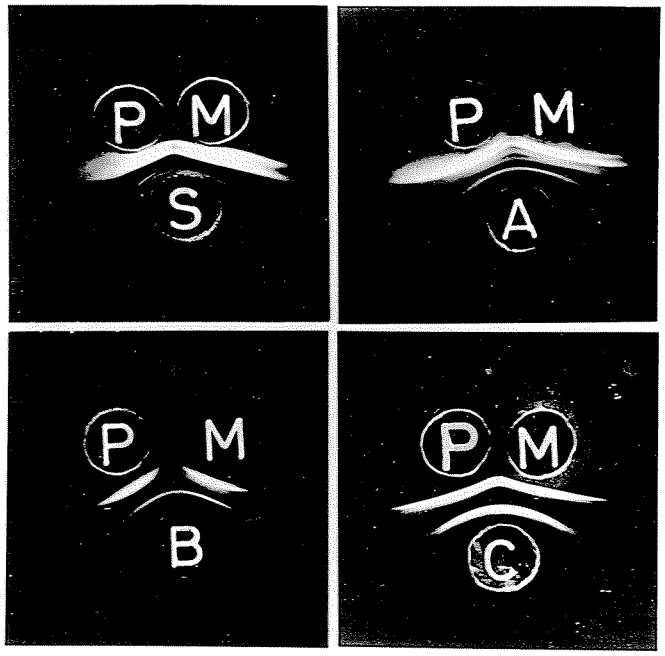

Fig. 1. Immunodiffusion of SDS extracted antigens of the parent and the variant with the anti-parent serum; (S) anti-strain Shibaura serum, (A) anti-strain Akiyami A serum, (B) anti-strain Akiyami B serum, (C) anti-strain Akiyami C serum, (P) antigen of the homologous parent strain, and (M) antigen of the homologous variant.

Table 2. Protective effect of the serum medium vaccine and the chemically defined medium vaccine against challenge with strain Shibaura

Vaccine	Preservation of vaccine*	Survivor rate**
Serum medium vaccine	3 weeks	10/10
Chemically defined medium vaccine	3 weeks	10/10
None		0/10***
Serum medium vaccine	1 year	10/10
Chemically defined medium vaccine	1 year	10/10
None		0/10

* Vaccines were preserved at 10°C.

** Numerator denotes the number of guinea pigs survived, while denominator indicates those challenged.

*** Control guinea pigs died 4 to 7 days after challenge. Hemorrhage and jaundice were found upon necropsy.

Agglutinin Titers of Vaccinated Guinea Pigs

Table 3 presents the data of the agglutinin titers of vaccinated guinea pigs. Seroconversion was obvious, and no marked difference of the titers was found between the sera of guinea pigs inoculated with the serum medium vaccine and the chemically defined medium vaccine.

Protein Nitrogen Content and Optical Density of the Vaccines

The data of the nitrogen content of the vaccines precipitated with trichloroacetic acid and their optical density at wave length of 650 nm are shown in Table 4. Both vaccines contained the same number of leptospires. The length of leptospiral cells did not differ greatly between the vaccines, as shown in Figs. 2 and 3. Obviously, the chemically defined medium vaccine contained a much smaller amount of protein and was much clearer than the serum medium vaccine.

Electron Microscopy

Electron micrographs of the serum medium vaccine and the chemically defined medium vaccine are shown in Figs. 2 and 3. A considerable number of impurities were observed in the serum medium vaccine. Aggregates

Table 3. Agglutinin titers in sera of vaccinated guinea pigs

Vaccinated with	Agglutinin titer with strains			
	Shibaura	Akiyami A	Akiyami B	Akiyami C
Serum medium vaccine	40*	20	40	80
Chemically defined medium vaccine	40	20	160	80
None	< 10	< 10	< 10	< 10

* Reciprocal of the 50 % end point microscopic agglutination titer in pooled serum of 3 guinea pigs, killed on 14th day after the second vaccination.

Table 4. Protein nitrogen content and optical density of the vaccines

Vaccine	Protein nitrogen* (ng/ml)	Optical density** (650 nm)
Serum medium vaccine	21.0	0.210
Chemically defined medium vaccine	7.5	0.078

* Determined by means of micro-KJELDAHL method.

** Optical density at a wave length of 650 nm.

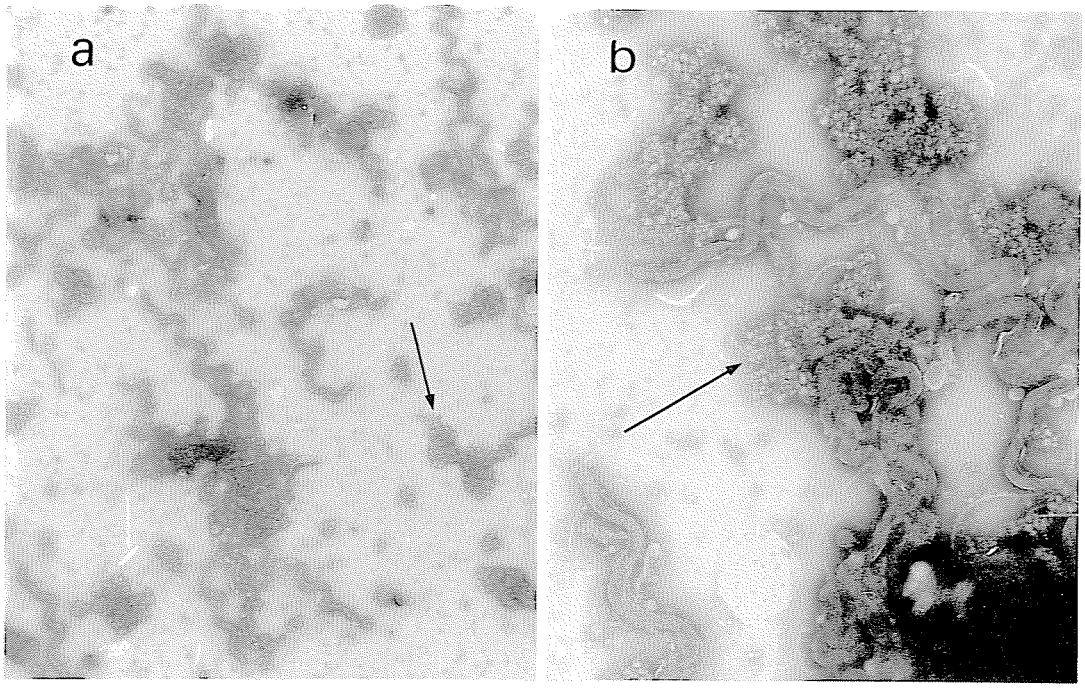

Fig. 2. Electron micrographs of the serum medium vaccine. (a) x 6,000 (b) x 20,000. The arrows indicate the impurity.

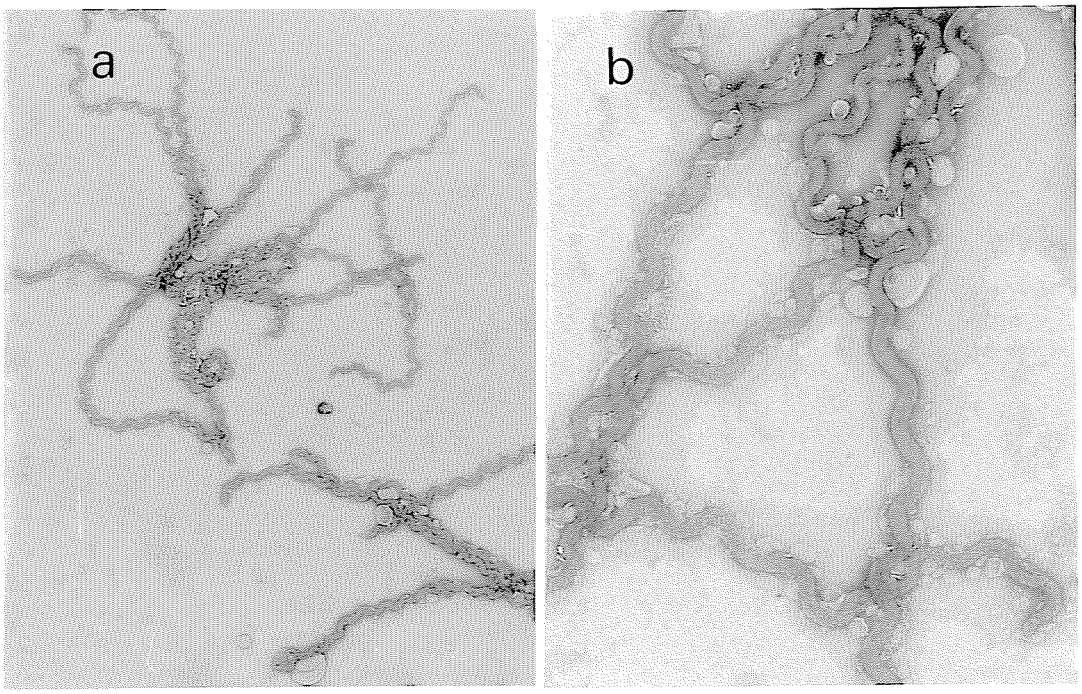

Fig. 3. Electron micrographs of the chemically defined medium vaccine; (a) x 6,000 (b) x 20,000. Impurity is not visible.

of small granules of various sizes, which seemed to be non-leptospiral components were attached to or free from leptospiral cells. Similar impurities were not observed in the chemically defined medium vaccine. Cells with loosened spirals were more frequent in the serum medium vaccine than in the chemically defined medium vaccine.

Precipitation Test for Detection of Rabbit Serum in the Vaccine

An attempt was made to detect rabbit serum in the serum medium vaccine by precipitation test with the anti-rabbit guinea pig serum. A precipitin band was formed by the serum medium vaccine, but not by the chemically defined medium vaccine (Fig. 4). It was clear that the serum medium vaccine contained rabbit serum. The supernatant of the serum medium vaccine gave no reaction, suggesting that rabbit serum was associated with the cell residue.

A leptospiral suspension which was prepared from the cells grown in the serum medium, washed three times with PBS, and suspended in PBS in one-tenth volume of the culture, still formed a precipitin band. Electron microscopically, the leptospiral suspension contained

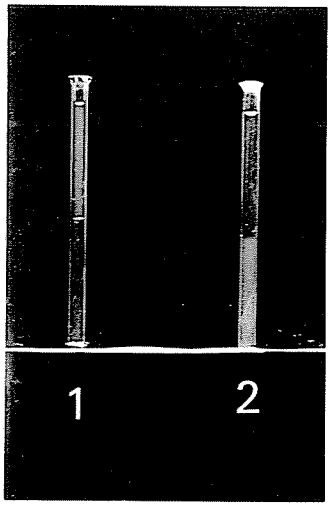

Fig. 4. Precipitation test of the serum meidum vaccine (1) and the chemically defined medium vaccine (2) with anti-rabbit guinea pig serum.

the impurities similar to those found in the serum medium vaccine.

It is presumed, therefore, that the procedure of washing did not remove rabbit serum completely from the leptospiral cells, or rabbit serum in the vaccine was in insoluble form, being aggregated with some medium components, or other unknown substances, and co-sedimented with leptospiral cells.

Safety of the Vaccines

No significant clinical signs were found in the mice and the guinea pigs injected with either the serum medium vaccine or the chemically defined medium vaccine. As shown in Fig. 5, the body weight of the animals injected with either vaccine decreased on the next day after injection. The control animals did not show any decrease of the body weight. The body weight of all the animals increased in 7 days after injection. The tests, therefore, showed each vaccine to be safe. Safety tests were done with the vaccines preserved for 1 year at 10°C. The results were essentially the same.

Pyrogenicity of the Vaccines

Pyrogenicity to rabbits was demonstrated in both

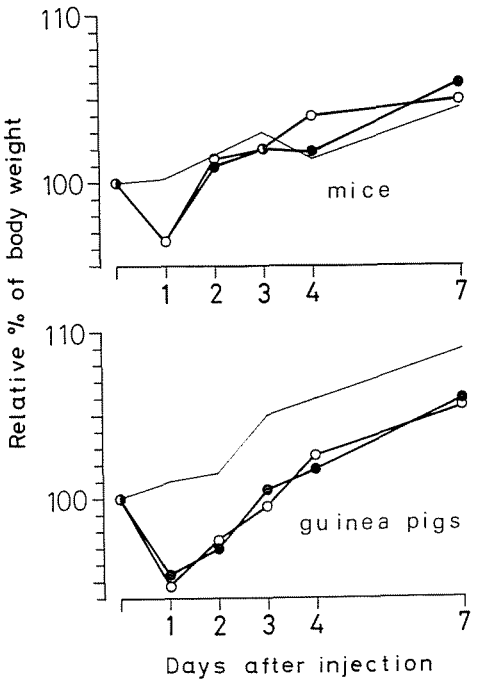

Fig. 5. Relative percentage of geometric mean of the body weight of the animals after injection with each vaccine.

The body weight of the animals injected with the serum medium vaccine (●——●), chemically defined medium vaccine (o——o) and saline (——).

vaccines. The difference between the maximal rectal temperature after injection and that before injection with the serum medium vaccine or the chemically defined medium vaccine was 1.2°C and 2.0°C or 1.4°C and 1.4°C, respectively. Temperature of the control rabbit injected with saline did not increase.

Leukocytic Response Caused by the Vaccines

It was found that both vaccines reduced the number of peripheral leukocytes of mice 2 hours after injection. The relative percentage of the number of the leukocytes of mice injected with the serum medium vaccine or the chemically defined medium vaccine to those injected with saline was 48 % and 56 %, respectively.

DISCUSSION

The serum medium vaccine contained three times more protein than the chemically defined medium vaccine, despite the fact that both vaccines contained the same number of leptospires and that there was no significant difference between the length of leptospiral cells in both vaccines. The serum medium vaccine, thus, contained a protein impurity which may have been derived from the

medium components, such as the rabbit serum and the peptone. Contamination of rabbit serum in the serum medium vaccine was detected by precipitation tests. The suspension of leptospires which were grown in the serum medium and washed three times with PBS still gave the precipitin reaction. The facts indicated that rabbit serum could not be removed by the procedure of washing, as far as the serum medium was used. Whether or not the rabbit serum was adhered to the surface of leptospiral cells needs further investigation.

The electron microscopical impurities were found in the serum medium vaccine, and in the suspension of leptospires which were grown in the serum medium and washed three times with PBS. Whether or not the impurities have any association with rabbit serum was not examined.

The risk of allergic side reactions exists when vaccines are prepared from leptospires grown in a medium containing rabbit serum or bovine serum albumin. SHENBERG and TORTEN (25) pointed out that the presence of even traces of serum proteins in a vaccine might lead to local and / or systemic allergic reactions.

MORSI and SHIBLEY (18) described that on using media containing serum in the preparation of leptospiral vaccines, serum must be removed to avoid anaphylactic reactions in vaccinated animals but soluble leptospiral antigens are also removed during the centrifugation processing of these vaccines. From these points of view, a vaccine prepared from leptospires grown in a chemically defined medium is preferable. Caution should be taken, however, before using a chemically defined medium for the preparation of a leptospiral vaccine because selective changes in antigenicity may occur in leptospires grown in this way (26). In the present experiment, identity of the antigenicity of the variants with their parent strains was carefully examined by the microscopic agglutination test and immunodiffusion.

Toxic reactions such as body weight decrease, febrile response and leukopenia were induced in animals equally by both vaccines. These reactions are known to be related to bacterial endotoxin (12, 16). Although it is unknown whether or not leptospires have endotoxin, these findings suggest that leptospires may also possess

endotoxin. The location of the endotoxin-like substance is unknown, but deprivation of the endotoxin-like activity from a chemically defined medium vaccine without diminishing its protective activity is a subject for future study.

An explanation for the greater loosening of spirals from cells in the serum medium vaccine than in the chemically defined medium vaccine is as follows. The protein content was so high in the leptospiral culture in the serum medium that the addition of 0.2 % formalin to kill the leptospires was ineffective in fixing them, while the leptospiral culture in the chemically defined medium contained much less protein; therefore, the formalin concentration was effective for fixation.

It has been reported that protective activity of leptospire is mainly located in enveloping sheath (1, 3, 30). Preparation of such a component vaccine from the cells grown in the chemically defined medium is promising.

BRIEF SUMMARY

Two types of leptospiral vaccines consisting of the organisms of four strains of L. interrogans (strain Shibaura of Icterohaemorrhagiae serogroup, strain Akiyami A of serovar autumnalis, strain Akiyami B of serovar hebdomadis and strain Akiyami C of serovar australis) were prepared; one type was prepared from these strains grown in KORTHOF's medium containing normal rabbit serum, and the other was made from variants grown in SHENBERG's chemically defined medium. The protein nitrogen was lower and electron microscopical impurities were fewer in the chemically defined medium vaccine than in the serum medium vaccine. The rabbit serum was detected by the precipitation test in the serum medium vaccine. The results of the potency test did not differ between the two vaccines. These results indicate that the chemically defined medium vaccine is preferable to the serum medium vaccine.

However, toxic reactions such as body weight decrease, febrile response and leukopenia were induced in animals equally by both vaccines. Deprivation of these toxic activities from a chemically defined medium

vaccine without diminishing its protective activity is a subject for future study.

PART II Morphological and Immunological Comparison of Leptospires Treated with Sodium Dodecyl Sulfate and Sodium Deoxycholate

INTRODUCTION

It was described in PART I that body weight decrease, febrile response and leukopenia were demonstrated in the animals injected with the chemically defined medium vaccine as well as the serum medium vaccine. These reactions are considered to be induced by leptospiral cells themselves. Isolation of (an) important component(s) for protection from other undesirable components may lead to a vaccine which is more effective and less toxic than whole cell vaccines.

It has been known that sodium dodecyl sulfate (SDS) or sodium deoxycholate (SDC) destroys and solubilizes the structural components of leptospiral cells (1, 20, 33, 34).

The present part deals with morphological and immunological investigations which were conducted for the purpose of bringing light to the question of which removes a larger amount of structural components from

leptospiral cells, SDS or SDC.

MATERIALS AND METHODS

Strains: Strains used were the same as described in PART I.

Media: Tryptose phosphate broth (0.2 %, Difco) containing 10 % normal rabbit serum (4) was used. SHENBERG's chemically defined medium (24) was used for the cultivation of the variant.

Preparation of SDS extract and cell residues, and SDC extract and cell residues: Cells of a variant strain Shibaura of Icterohaemorrhagiae serogroup grown in 400 ml of SHENBERG's medium were harvested by centrifugation at 10,000 g for 30 minutes and washed once with 0.01 M phosphate buffered saline (PBS, pH 7.2). The cells were resuspended in 20 ml or 6 ml of PBS and an equal volume of 2 % SDS or SDC (Wako Pure Chemical Ind., Osaka) was added and then treated at 45°C for 4 hours, followed by centrifugation at 12,000 g for 30 minutes. The supernatant was named as SDS or SDC extract and the sediment was named as SDS or SDC cell residues, respectively.

Electron microscopy: The specimens were negatively stained with 2 % phosphotungstic acid, or shadowed by chrome, or thin-sectioned and stained with lead (21) and 1 % uranyl acetate. They were examined in a JEM-100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo) at an accelerating voltage of 80 kV.

Immune sera: Immune sera used were the same as described in PART I.

Complement fixation (CF) test: CF tests were conducted by a microtiter technique (23). Titrations of antigenic activity were performed with the immune sera which were diluted at 1 : 100. The titer was expressed as a 50 % inhibition of hemolysis.

Indirect hemagglutination (IHA) test: The tests were carried out according to IMAMURA et al. (11). Titrations were performed with the immune sera which were diluted at 1 : 100.

Protection of guinea pigs: Each 1 ml of whole cells, SDS extract, SDS cell residues, SDC extract and SDC cell residues was injected into 5 guinea pigs subcutaneously. Two weeks later, those were challenged intraperitoneally with 1 ml of leptospire suspension which

was prepared according to the same method as described in PART I.

RESULTS

Morphology of Leptospiral Cells after SDS or SDC Treatment

In negatively stained (Fig. 6a), thin-sectioned (Fig. 6b) and shadowed (Fig. 6c) preparations, intact cells of strain Shibaura exhibit three major anatomical structures. These structures are an enveloping sheath, an axial filament and a protoplasmic cylinder.

After SDS treatment, cell residues consisted mostly of cell walls lacking cytoplasmic substances in negatively stained, thin-sectioned and shadowed preparations. These were concluded from their width and thickness. A small amount of fragments of enveloping sheath and other substances (Figs. 7a, 7b and 7c) were also found.

Cell residues were also examiend, after SDC treatment, and it was found that negatively stained, thin-sectioned and shadowed preparations consisted mostly of protoplasmic cylinders containing cytoplasmic

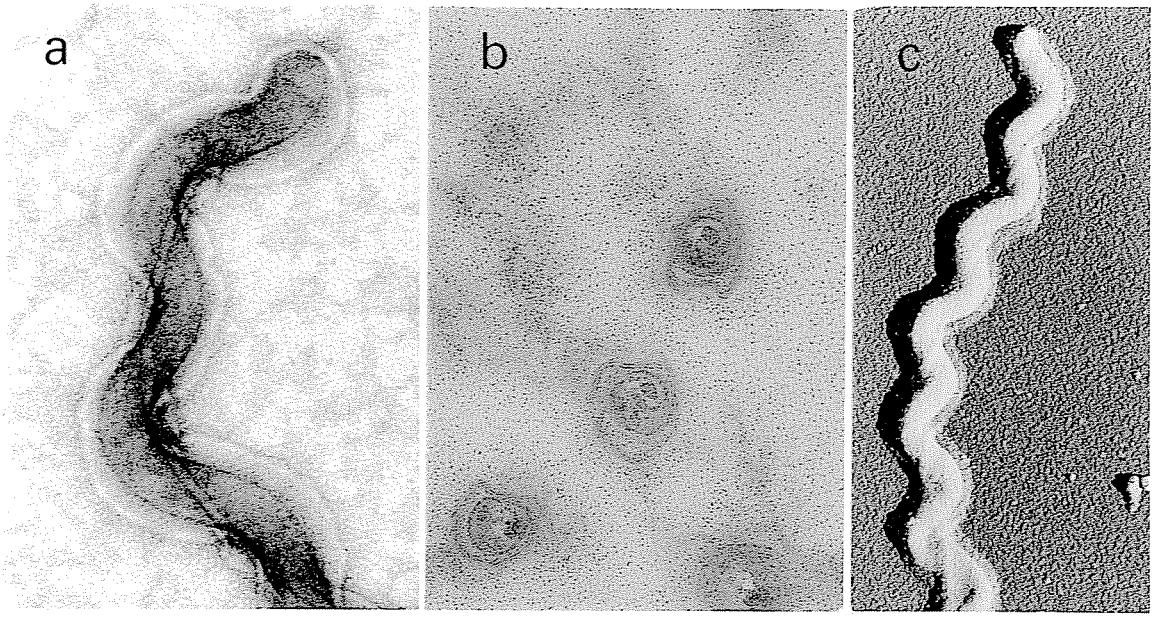

Fig. 6. Electron micrographs of the intact cells of strain Shibaura; (a) negatively stained with 2 % phosphotungstic acid x 60,000, (b) thin-sectioned and stained with lead and uranyl acetate x 60,000 and (c) shadowed by chrome x 30,000.

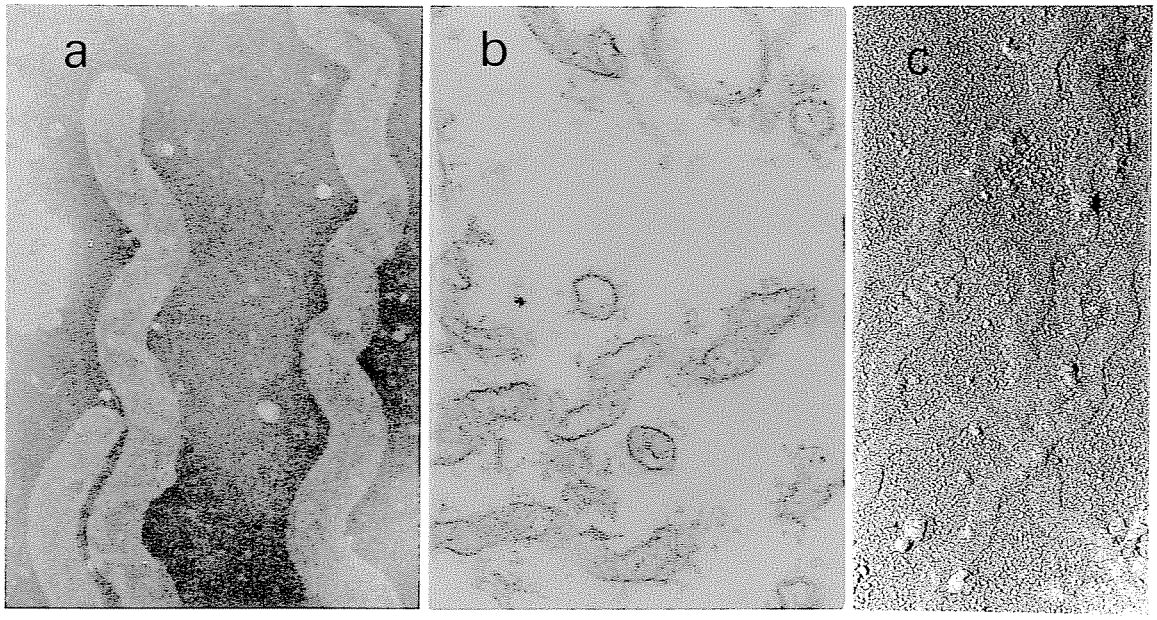

Fig. 7. Electron micrographs of the cells extracted with SDS; (a) negatively stained x 60,000, (b) sectioned x 60,000 and (c) shadowed x 30,000.

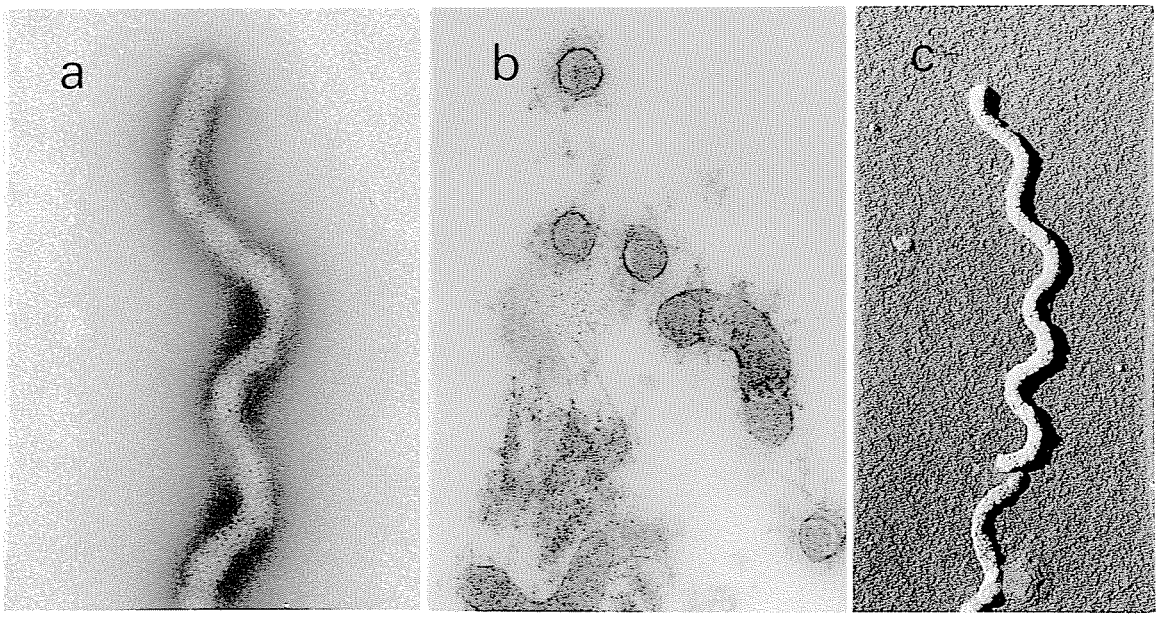

Fig. 8. Electron micrographs of the cells extracted with SDC; (a) negatively stained x 60,000, (b) sectioned x 60,000 and (c) shadowed x 30,000.

substances which were surrounded by cell walls, with a small amount of fragments of enveloping sheath and other substances (Figs. 8a, 8b and 8c). The shadowed preparations clearly showed that SDS cell residues were much thinner than SDC cell residues (Fig. 7c and Fig. 8c).

It was found, electron microscopically, therefore, that SDS removed a larger amount of components from the cells than did SDC.

Immunological Investigations of SDS Extract, SDS Cell Residues, SDC Extract and SDC Cell Residues

Antigenicity of whole cells, SDS extract, SDS cell residues, SDC extract and SDC cell residues was investigated serologically by CF and IHA tests using anti-whole cell sera. The data obtained are shown in Table 5. Whole cells reacted strongly with the homologous antiserum and less strongly with the heterologous antisera in the CF test, and reacted weakly with these antisera in the IHA test. SDS extract reacted strongly with the homologous and heterologous antisera in the IHA test, but reacted only with the homologous antisera in the CF test. Thus, SDS extract contained both

Table 5. Antigenic activities of whole cells, SDS extract, SDS cell residues, SDC extract and SDC cell residues of Ictero-haemorrhagiae strain Shibaura with hom-ologous and heterologous antisera

Preparations	Antisera	CF titer	IHA titer
Whole cells (before treating)	Shibaura	256	80
	Akiyami A	32	80
	Akiyami B	32	< 40
	Akiyami C	64	40
SDS extract	Shibaura	64	640
	Akiyami A	< 8	320
	Akiyami B	< 8	320
	Akiyami C	< 8	640
SDS cell residues	Shibaura	64	< 40
	Akiyami A	64	< 40
	Akiyami B	16	< 40
	Akiyami C	32	< 40
SDC extract	Shibaura	128	320
	Akiyami A	8	40
	Akiyami B	8	40
	Akiyami C	16	40
SDC cell residues	Shibaura	64	< 40
	Akiyami A	16	< 40
	Akiyami B	16	< 40
	Akiyami C	32	< 40

Titrations of activities were performed with the antisera diluted at 1 : 100. Concentration of each preparation was equivalent to 2×10^9 cells / ml.

genus-specific and serovar-specific antigens; the former was detected by the IHA test and the latter by the CF test. SDS cell residues reacted with the homologous and the heterologous antisera in the CF test but not in the IHA test. SDC extract reacted strongly with the homologous antiserum and reacted weakly with the heterologous antisera in the both CF and IHA tests. SDC cell residues reacted with the homologous and heterologous antisera in the CF test but not in the IHA test. This was the same as the reaction of SDS cell residues. Thus, SDS extract contained a larger amount of genus-specific antigen which was detected by the IHA test than SDC extract.

An additional experiment of treatment of strain Shibaura with SDS and SDC was made. Immunogenic activities of whole cells, SDS extract, SDS cell residues, SDC extract and SDC cell residues were investigated by the guinea pig protection test. Antigenic activities of these were also investigated by the CF and IHA tests with the homologous antiserum. The data obtained are shown in Table 6. SDS extract and SDC extract gave 80 % protection to guinea pigs. Whole cells, SDS cell

Table 6. Immunogenic and antigenic activities of whole cells, SDS extract, SDS cell residues, SDC extract and SDC cell residues of strain Shibaura with homologous antisera

Preparation	Guinea pig* protection	CF titer**	IHA titer**
Whole cells	5/5	1280	20
SDS extract	4/5	640	2560
SDS cell residues	5/5	640	< 20
SDC extract	4/5	640	320
SDC cell residues	5/5	640	< 20
control (PBS)	0/5	< 20	< 20

* Concentration of each preparation was equivalent to 10^9 cells /ml.

** Concentration of each preparation was equivalent to 10^{10} cells/ml.

residues and SDC cell residues gave complete protection to guinea pigs. Thus, no difference was shown between SDS extract and SDC extract by the guinea pig protection test. SDS extract, SDS cell residues, SDC extract and SDC cell residues all had the same degree of reaction with anti-whole cell serum in the CF test. On the other hand, SDS extract showed the strongest antigenic activity of all preparations in the IHA test. Thus, it was confirmed that SDS extract contained a larger amount of antigen than SDC extract.

DISCUSSION

From the findings of electron microscopic observation, it was shown that SDS removed a larger amount of components of leptospira than did SDC. It was shown serologically, furthermore, that SDS extract contained both genus-specific and serovar-specific antigens; the former was detected by the IHA test and the latter by the CF test. YANAGAWA et al. (34) also reported that SDS extract from leptospiral cells showed good antigenicity in immunodiffusion and contained both serovar- and genus-specific antigens.

SDS cell residues gave complete protection to guinea pigs. It was shown, electron microscopically, that SDS cell residues contained membrane fragments of enveloping sheath and other substances besides cell walls. The role of cell walls for protection, therefore, should be clarified with a further purified preparation.

In the following part, the isolation of the component(s) associated with protectivity from leptospriral cells will be described.

BRIEF SUMMARY

Extracts and cell residues obtained from L. interrogans serogroup Icterohaemorrhagiae strain Shibaura after treatment with SDS and SDC were compared morphologically and immunologically.

After SDS treatment, cell walls lacking cytoplasmic materials, a small amount of membrane fragments of enveloping sheaths and other substances were found, electron microscopically, in cell residues. Cytospasmic cylinders containing cytoplasmic materials which were surrounded by cell walls, a small amount of fragments of enveloping sheaths and other substances were found in cell residues after SDC treatment. SDS, thus, removed a larger amount of components of leptospire than did SDC.

Immunologically, SDS extract contained a larger amount of antigen which reacted with heterologous antisera in the IHA test than SDC extract. SDS extract, thus, contained both genus-specific and serovar-specific antigens; the former was detected by the IHA test and the latter by the CF test.

These findings suggest that SDS is more useful for fractionation of leptospiral components than SDC.

PART III Fractionation of Components of Leptospiral Cells Obtained after Treatment with Sodium Dodecyl Sulfate by Zonal Centrifugation, and Immunological and Morphological Analysis of the Fractions

INTRODUCTION

It was shown, electron microscopically, in PART II that SDS extract from leptospiral cells contained most of the components of the cells except cell walls. Immunological investigations showed that it was composed of serovar-specific and genus-specific antigens, and gave a significant protection to guinea pigs. Cell residues obtained after SDS treatment of cells gave a complete protection to guinea pigs. Since they contained not only cell walls but also membrane fragments of enveloping sheaths and other substances, the role of cell walls, as well as enveloping sheaths or the other structural components, for protection has still not been independently determined.

The purpose of the present study is to locate the protective activity and to clarify its correlation with

other immunological activities as well as to determine its morphological entity, and furthermore, to isolate the component(s) associated with protective activity from other substances.

At first, SDS extract obtained from strain Shibaura was fractionated by zonal centrifugation in sucrose density gradient, and the fractions were analysed immunologically and morphologically.

Secondly, further experiments were conducted in order to confirm the results obtained from the former experiments in detail and to subsequently clarify the immunological activity of cell walls. SDS treated cell suspension, which contained both SDS extract and cell residues, was fractionated by zonal centrifugation in a sucrose density gradient, and the fractions were analysed.

Additionally, a toxicity to mice in the fraction associated with protective activity, which was obtained from SDS treated cell suspension, was examined and compared with that in whole cells.

MATERIALS AND METHODS

Strains: Strains used were the same as described in PART I.

Media: Media used were the same as described in PART II.

Preparation of SDS extract and zonal centrifugation in sucrose density gradient: The technical procedures of SDS treatment were essentially the same as described in PART II. One hundred and twenty ml of SDS extract was prepared from 4,000 ml of the culture. The zonal centrifugation was performed at 40,000 rpm for 90 minutes (B-IV rotor, L-4 centrifuge, Spinco, Beckman). One hundred ml of the SDS extract obtained from approximately 7×10^9 cells per ml was applied on the top of a pre-formed gradient (1,200 ml of 7-40 % sucrose, in PBS, linear gradient underlaid with 225 ml of 60 % sucrose solution) and overlaid with 200 ml of PBS. After the centrifugation, 80 fractions of 20 ml each were collected.

Preparation of SDS treated cell suspension and zonal centrifugation in sucrose density gradient: One hundred and ten ml of cell suspension prepared from 10,000 ml of culture by the same methods as described

in PART II was added to an equal volume of 2 % SDS and treated at 45°C for 4 hours. Thus, 220 ml of the SDS treated cell suspension which was obtained from approximately 6 x 10^9 cells per ml was prepared. Two hundred ml of the SDS treated cell suspension was fractionated by zonal centrifugation in sucrose density gradient. The procedures of the zonal centrifugation were essentially the same as described above except for the following: linear gradient of 7-50 % sucrose was used, overlaid with 100 ml of PBS and the centrifugation was performed for 60 minutes.

Immune serum: Hyper-immunized anti-strain Shibaura rabbit serum which described in PART I was used.

CF test: The technical procedures were the same as described in PART II.

IHA test: The technical procedures were the same as described in PART II.

Immunodiffusion: The agar gel for immunodiffusion was prepared with distilled water at a concentration of 1 % agar (Difco).

Agglutinin production in mice: A half ml of each fraction was injected intraperitoneally into 10 or 5

4-week-old mice. Three weeks after the injection, the mice were bled, sera were pooled, and agglutinin titer was measured by microscopic agglutination test. The titer was expressed as a 50 % agglutination end point.

Protection of guinea pigs: The technical procedures were the same as described in PART II.

Electron microscopy: The specimens were negatively stained and examined according to the procedures described in PART I.

Test for body weight decrease of mice: A half ml of the fraction associated with protection obtained from zonal centrifugation of the SDS treated cell suspension was injected intraperitoneally into 5 5-week-old mice (dd/Hirari/Takeda/SPF). Mice inoculated with whole cells (6×10^8 cells/ml) prepared from 0.2 % formalin-killed leptospires and with saline served as the control. They were weighed 1, 2, 3 and 7 days after the injection.

RESULTS

1. Fractionation of SDS Extract by Zonal Centrifugation

Zonal Centrifugation Profile of SDS Extract

Zonal centrifugation profile of SDS extract in sucrose density gradient is shown in Fig. 9. Two main peaks of antigenic activities which were in accord with the peaks of optical density at the wave length of 280 nm were found. The first peak exhibited strong IHA and weak CF activities, while the second peak exhibited strong CF and no IHA activities.

Immunogenic Activities of the Fractions of SDS Extract

Immunogenic activities of the fractions are shown in Table 7. Agglutinin production was high in mice inoculated with the second peak but not so in those inoculated with the first peak. Fraction 67 (second peak) produced agglutinin in mice at the titer of 1 : 64, while fraction 13 (first peak) produced agglutinin in mice at the titer of 1 : 8. Thus, agglutinin production in mice was found to be related to CF activity.

Protection of guinea pigs was high in the second peak; 100 % protection was given with each of the fractions 65, 66 and 67. However, the first peak showed only a partial protection; zero, 40 and 20 % protection with fractions 12, 13 and 14, respectively (Table 7).

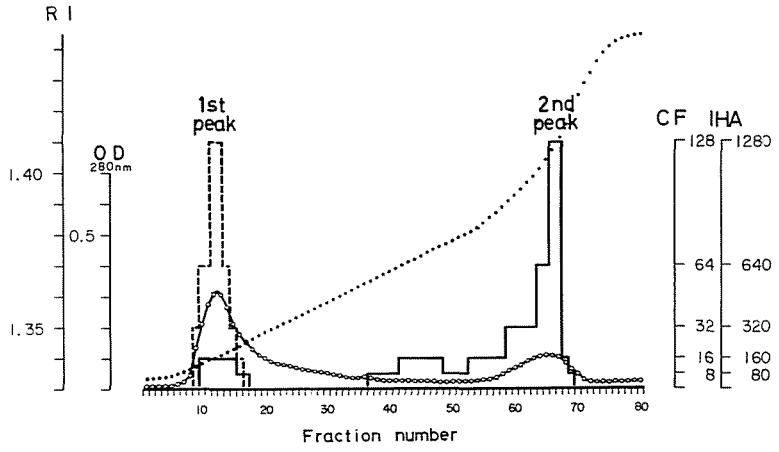

Fig. 9. Zonal centrifugation profile of SDS extract in sucrose density gradient.

— CF titer, --- IHA titer, ··· Refractive index (RI), o Optical density at the wave length of 280 nm (CD_{280} nm).

Table 7. Immunogenic activities of the fractions

Fraction number	CF	IHA	Agglutinin titer produced in mice	Guinea pig protection	Morphology****
	16*	1280*	4**	0/5***	
13	16	1280	8	2/5	soluble
14	16	640	4	1/5	
30	< 4	< 40	< 4	0/5	
42	16	< 40	4	NT	
43	16	< 40	8	3/5	
44	16	< 40	4	NT	
65	64	< 40	16	5/5	
66	128	< 40	32	5/5	
67	128	< 40	64	5/5	enveloping sheaths
SDS extract before fractionation	128	1280	64	5/5	

* Reciprocal of the complement fixation (CF), indirect hemagglutination (IHA) titers reacted with anti-Icterohaemorrhagiae strain Shibaura serum.

** Reciprocal of the titer of agglutinin produced in mice.

*** Number of animals surviving 14 days after challenge/number of animals challenged.

All of 5 control guinea pigs died 4 to 6 days after challenge. Hemorrhage and jaundice were found upon necropsy.
NT Not tested.

**** Electron micrographs are shown in Fig. 11.

In immunodiffusion, as shown in Fig. 10, SDS extract and the first peak (fraction 13) similarly produced 5 precipitin lines while the second peak (fraction 67) produced no line.

Morphology of the Fractions of SDS Extract

Electron microscopically, large amount of membrane fragments and a small amount of helical structures were seen in the SDS extract (Fig. 11a) and the second peak (fraction 67) (Fig. 11c), while no obvious structural components were seen in the first peak (fraction 13) (Fig. 11b).

2. Fractionation of SDS Treated Cell Suspension by Zonal Centrifugation

Zonal Centrifugation Profile of SDS Treated Cell Suspension, and Morphology and Immunogenic Activities of the Fractions

Zonal centrifugation profile of the SDS treated cell suspension in sucrose density gradient is shown in Fig. 12. Three main peaks of optical density at the wave length of 280 nm were found. CF activity was high in the second peak (fraction 46).

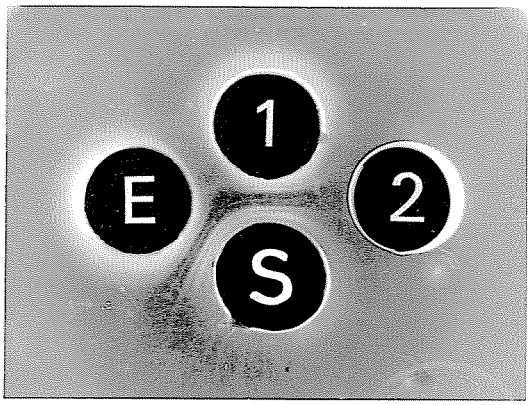

Fig. 10. Immunodiffusion of anti-Icterohaemorrhagiae strain Shibaura serum (S) with the following antigens: SDS extract (E), fraction 13 (1) and fraction 67 (2).

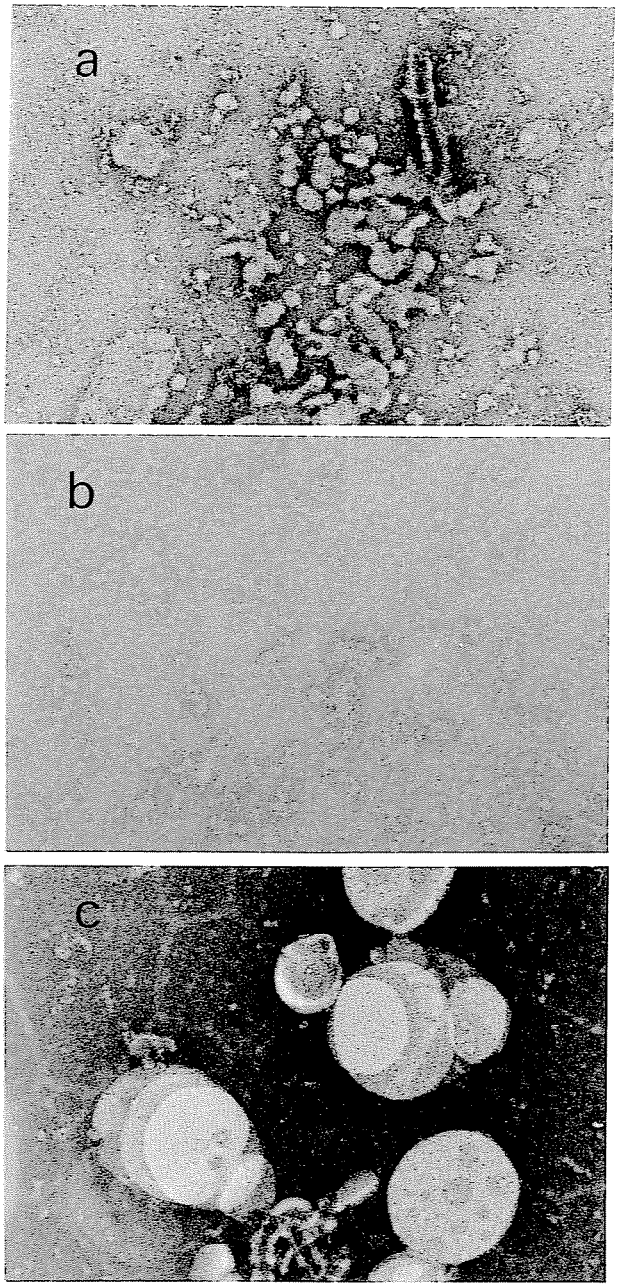

Fig. 11. Electron micrographs of (a) SDS extract, (b) fraction 13, and (c) fraction 67. Negatively stained. x 60,000.

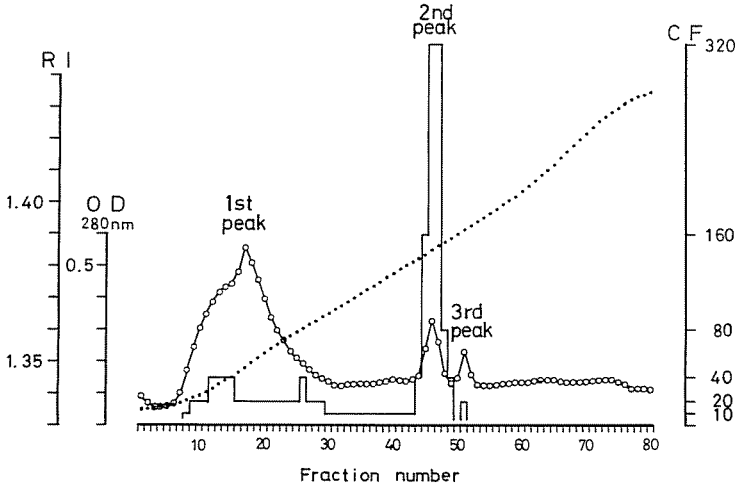

Fig. 12. Zonal centrifugation profile of SDS treated cell suspension in sucrose density gradient.

— CF titer, ··· Refractive index (RI), o Optical density at the wave length of 280 nm (OD_{280} nm).

Electron microscopically, the second peak (fraction 46) contained a large amount of membrane fragments of enveloping sheaths (enveloping sheath fraction) (Fig. 13a) and the third peak (fraction 51) contained cell walls (cell wall fraction) (Fig. 13b), while no obvious structural components were seen in the first peak (fraction 17, soluble fraction).

Immunogenic activities of the fractions are shown in Table 8. The second peak (fraction 46, enveloping sheath fraction) gave complete protection to guinea pigs even when it was diluted at 1 : 6. The first peak (fraction 17, soluble fraction) diluted at 1 : 2.5 showed only a partial protection of 40 %. The third peak (fraction 51, cell wall fraction) showed no protection. Thus, protective activity in the guinea pig existed mostly in the enveloping sheath fraction. This finding confirmed the results obtained from the investigation of SDS extract, which were described above. Agglutinin production was high in the mice inoculated with the enveloping sheath fraction, but not so high in those inoculated with the soluble fraction. The mice inoculated with the cell wall fraction did not produce

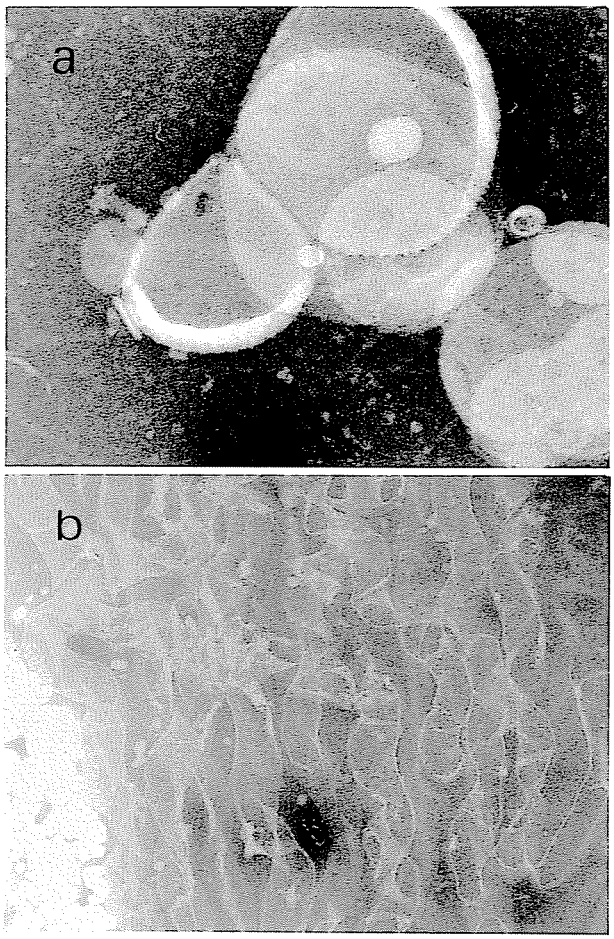

Fig. 13. Electron micrographs of (a) fraction 46 (enveloping sheath fraction) and (b) fraction 51 (cell wall fraction). Negatively stained. x 60,000.

Table 8. Immunogenic activities of the fractions of SDS treated cell suspension

Fraction number	Morphology	OD_{280} nm	Dilution 1 :	Cell number $\times 10^8$/ml equivalent	Guinea pig protection	Agglutinin titer produced in mice	CF titer
46	enveloping sheath	0.33	1	(16.5)*	NT	512***	320****
			1.5	(11)	5/5**	512	
			3	(5.5)	5/5	256	
			6	(2.75)	5/5	128	
51	cell wall	0.24	1	(12 .)	0/5	< 8	20
			2	(6)	0/5	< 8	
17	soluble	0.55	1	(27.5)	NT	16	20
			2.5	(11)	2/5	32	
			5	(5.5)	NT	< 8	
			10	(2.75)	NT	< 8	
Suspension before fractionation		1.20	1	60	NT	256	160
			5	12	4/5	64	
			10	6	3/5	32	
Whole cells		0.30	1	6	4/5	NT	64
			2	3	3/5	NT	
			4	1.5	3/5	NT	

* Parenthesis shows conversion from OD_{280} nm of SDS treated cell suspension before fractionation.

** No. of animals surviving 14 days after challenge/No. of animals challenged. All of 5 control animals died 4 to 5 days after challenge. Hemorrhage and jaundice were found upon necropsy.
NT Not tested.

*** Reciprocal of the titer of agglutinin produced in mice.

**** Reciprocal of the complement fixation (CF) titer reacted with anti-strain Shibaura serum which was diluted at 1 : 100.

detectable agglutinin.

Absence of Body Weight Decreasing Toxicity in the Enveloping Sheath Fraction

As shown in Fig. 14, no decrease of body weight was found in the mice injected with 0.5 ml of the enveloping sheath fraction (fraction 46, the second peak in Fig. 12). Despite the fact that this fraction gave complete protection to guinea pigs even when it was diluted at 1 : 6 (Table 8), a toxicity was not detected. On the other hand, whole cells which were prepared from leptospires killed with formalin and contained 6×10^8 cells per ml caused a decrease of the body weight of mice the day after the injection. Despite the fact that whole cells gave 80 % protection to guinea pigs (Table 8), a toxicity was thus demonstrated.

DISCUSSION

The immunological and morphological analysis of the fractions of SDS extract and SDS treated cell suspension of leptospires obtained by rate zonal centrifugation in sucrose density gradient indicated that protective activity was associated with agglutinin-

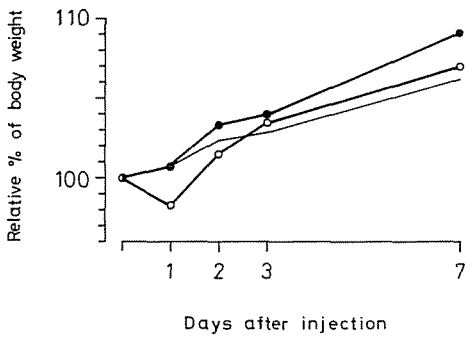

Fig. 14. Relative percentage of geometric mean of the body weight of the mice after injection with the enveloping sheath fraction, whole cells and saline. The body weight of the mice injected with the enveloping sheath fraction (fraction 46) (●——●), whole cells (o——o) and saline (——).

producing and CF activities and was related to the membrane fragments of the enveloping sheath.

Membrane fragments found in the second peak in both zonal centrifugation profiles were similar to those reported by NAUMAN et al. (20), who also identified these structures as the enveloping sheath. A small amount of the helical structures, which was the helical outer coat of the axial filament named by NAUMAN et al. (20), was also seen electron microscopically in the second peak. However, the helical outer coat of the axial filament was considered to be too small in amount to exhibit its own biological activities.

The protective activity of the enveloping sheath has been shown by AURAN et al. (1), GLOSSER et al. (9), BEY et al. (3) and TAKASHIMA and YANAGAWA (30). YANAGAWA and FAINE (33) reported that the enveloping sheath of leptospire was the structural component responsible for agglutination by antibody.

The absence of body weight decreasing toxicity in the mice injected with at least the 6 times concentration, which gave complete protection to guinea pigs in the enveloping sheath fraction, should be noted. Whole

cells, on the contrary, decreased the body weight of the mice injected even at the concentration which gave incomplete protection to guinea pigs (80 %, Table 8).

Cell walls were found electron microscopically in the third peak in the zonal centrifugation profile of SDS treated cell suspension. This fraction gave no protection to guinea pigs and showed no detectable agglutinin producing activity and weak CF activity. It is concluded, therefore, that cell walls are not important for protection. Immunological activities of SDS cell residues shown in PART II (Table 6) must have been exhibited not by cell walls but by the other component(s) such as the membrane fragments of enveloping sheath which were observed electron microscopically (Fig. 7). These were considered to be separated from cell walls by zonal centrifugation.

Leptospiral vaccines of whole cells induce toxic reactions in experimental animals as described in PART I and the present part. The method presented here should be useful as a method of preparing a cell component vaccine which is more effective and less toxic than whole cell vaccines.

BRIEF SUMMARY

Sodium dodecyl sulfate (SDS) extract and SDS treated cell suspension of L. interrogans serogroup Icterohaemorrhagiae strain Shibaura was fractionated by rate zonal centrifugation in sucrose density gradient, and the fractions were analysed immunologically and morphologically. The following three main fractions were obtained.

The fraction (enveloping sheath fraction), which contained mainly the membrane fragments of the enveloping sheath, gave complete protection to guinea pigs. It showed activities of high production of agglutinin in mice, strong complement fixation (CF) and no indirect hemagglutination (IHA).

The fraction (cell wall fraction), which contained cell walls, gave no protection to guinea pigs. It showed no detectable agglutinin producing activity in mice and low CF activity.

The fraction (soluble fraction), which contained no recognizable structural components, gave only a partial protection to guinea pigs. It showed activities of low production of agglutinin in mice, weak CF

and strong IHA.

The protective activity of the leptospiral cell was thus intimately associated with the activities of agglutinin production and CF, as well as with the membranous structures of the enveloping sheath and not with cell wall.

The enveloping sheath fraction did not induce a toxic reaction of body weight decrease in mice.

The method presented here should be useful as a method of preparing a cell component vaccine which is more effective and less toxic than whole cell vaccines.

CONCLUSION

In order to avoid the allergic side reactions caused by a vaccine prepared from leptospires grown in a medium containing serum, a polyvalent vaccine was prepared from leptospires grown in a chemically defined medium and compared with the serum medium vaccine now being used.

The protein nitrogen was lower and electron microscopic impurities were fewer in the chemically defined medium vaccine than in the serum medium vaccine. The rabbit serum was detected by the precipitation test in the serum medium vaccine. The results of the potency test did not differ between the two vaccines. These findings indicate that the chemically defined medium vaccine is preferable to the serum medium vaccine.

However, toxic reactions such as body weight decrease, febrile response and leukopenia were equally induced in animals by both vaccines. These findings suggest that side reactions which are caused by leptospiral cells themselves may occur even when the chemically defined medium vaccine is used. Therefore, components which play an important role in protection

and are less toxic than a whole cell vaccine were isolated.

Prior to the isolation of the components, leptospires were disrupted by treatment with either sodium dodecyl sulfate (SDS) or sodium deoxycholate (SDC) and the effect of the disruption was examined morphologically and immunologically. It was found that SDS solubilized a larger amount of components of leptospires than did SDC.

Therefore, SDS-treated leptospires were fractionated by rate zonal centrifugation in sucrose density gradient, and the fractions were analysed immunologically and morphologically. The enveloping sheath fraction, which contained mainly the membrane fragments of the enveloping sheath, gave complete protection to guinea pigs and showed activities of high production of agglutinin in mice, strong complement fixation (CF) and no indirect hemagglutination (IHA). The cell wall fraction, which contained cell walls, gave no protection to guinea pigs and showed no detectable agglutinin producing activity in mice and low CF activity. The soluble fraction, which contained no recognizable structural

components, gave only a partial protection to guinea pigs and showed activities of low production of agglutinin in mice, weak CF and strong IHA. The protective activity of the leptospiral cell was thus intimately associated with the membranous structures of the enveloping sheath as well as with the activities of agglutinin production and CF, and not with the cell wall.

Furthermore, the enveloping sheath fraction did not induce a toxic reaction of body weight decrease in mice.

These findings indicate that the enveloping sheath fraction is avilable for a vaccine which is more effective and less toxic than whole cell vaccines.

It is concluded, from the present results, that the serum medium vaccine now being used had better be replaced by a cell component vaccine prepared from leptospires grown in a chemically defined medium.

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