in roller tube tissue culture‖ at the same time they initially were implanted in rats. After the first generation of growth in these animals (and often after 2 or 3 generations of transfer), some of the harvested embryonic material was again placed in tissue culture. Without exception, much better *in vitro* growth was obtained from the rat grown tissue than the original material. The possible explanation for this finding will be discussed elsewhere(5).

Summary. Human embryonic tissues particularly lung, skin, cartilage, and intestine have been propagated in x-irradiated rats or nonirradiated hamsters both treated with cortisone. The method of growing these tissues is simple and so uniformly reproducible that it offers a good source of human embryonic material for experimental use and study.

‖ All tissue cultures were done in the laboratory of Dr. Alice Moore of The Sloan-Kettering Institute.

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Preparation, Characterization, and Antigenicity of a Saline Soluble Fraction From Shigella Types. (21179)

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Several investigators have extracted and characterized the somatic antigen of *Shigella sonnei* and the *Sh. paradysenteriae* (Flexner) group(1,2). The complete antigen appears to be a complex of polysaccharide, phospholipid, and protein. The polysaccharide of the antigenic complex determines specificity and it can elicit antibodies in man and the mouse, but not in the rabbit. The protein fraction appears to be the most toxic component of the complex. Numerous attempts have been made to reduce the toxicity of the complete antigen(3) but decreased antigenicity usually accompanies reduced toxicity.

Vaccines made of whole cells have been used in prophylactic immunization against bacillary dysentery. Most preparatory methods(4,5), and most extraction methods for somatic antigens, include the washing of cells with saline solution, an act that may cause a significant loss of antigen from the cells. The successful extraction of Vi antigen from *Escherichia coli* with isotonic saline has recently been reported (6,7). A similar method was used here for the extraction of shigellae. This report concerns the preparation, preliminary analyses, and some immunogenic properties of saline soluble antigens from 3 *Shigella* types.

Materials and methods. The cultures used in this study were *Sh. sonnei,* strain (Ch); *Sh. paradysenteriae, Flexner III,* strain (Be); and *Sh. paradysenteriae, Flexner II,* strains (Km) and (PR).* The organisms were grown in a chemically defined medium (Table I). The (Km) strain of Flexner II required 17 g of dibasic phosphate and 5 g of glucose per liter of this medium. Cultures were incubated with shaking for 24 hours at 37°C in liter quantities. The cultures were centrifuged and the packed cells resuspended in cold acetone and dried on filter paper. One liter of the chemically defined medium yielded approximately 2 g of dry cells.

* Obtained through the courtesy of Dr. M. L. Cooper, The Children's Hospital Research Foundation, Cincinnati, O.

TABLE I. A Chemically Defined Medium for Cultivation of Shigella Species.

L-glutamic acid	3.4 g
L-aspartic acid	2.8
L-lysine	.5
DL-valine	.4
DL-serine	.4
L-leucine	.25
L-histidine	.2
DL-threonine	.16
DL-methionine	.13
DL-isoleucine	.1
L-arginine	.05
L-cysteine	.05
L-proline	.05
L-tyrosine	.03
DL-phenylalanine	.015
Glycine	1.0
$K_2HPO_4 \cdot 3\ H_2O$	12.25
NaCl	8.0
Nicotinamide	5.0 mg
$MgSO_4 \cdot 7\ H_2O$	50.0 "
Glucose	10.0 g
Distilled water	1000.0 ml

(Adjusted to pH 7.3-7.4 with 10% NaOH.)
Glucose added after sterilization.

Preparation of extract. One gram of acetone dried cells was added to 100 ml of sterile 0.9% saline previously adjusted to pH 4.0 with N/1 HCl. This cell suspension was shaken at 37°C for 30 minutes and then centrifuged at 3000 rpm for one hour and the supernatant fluid removed. If the fluid was not clear at this point it was recentrifuged at 10,000 rpm for 30 minutes. The clear supernatant was placed in cellulose casing (Visking) and dialyzed overnight in running tap water. Then the extract was filtered through a Seitz filter and stored in sterile vaccine vials at 4°C. Extracts were thus prepared from cells of *Sh. sonnei* (Ch), Flexner III (Be), and Flexner II (Km). A polyvalent extract was prepared by mixing 10 ml of each extract and bringing the volume to 100 ml with saline.

Mouse protective potency tests. The antigenicity of the extracts was determined by an active immunization mouse protective potency test based upon a constant immunizing dose and a graded challenge. Albino Swiss-Webster mice† of both sexes weighing between 15 to 18 g were used in all tests. Mice were given an immunizing dose of 0.1 ml of the respective extract or a dilution thereof by the intraperitoneal route. Seven days later the mice were challenged with graded doses of the homologous or heterologous strain of *Shigella*. The challenge culture was removed from a semisolid brain heart infusion stock and given at least five 12-hour subcultivations in trypticase soy broth (BBL), supplemented with 5 μg/ml nicotinamide and 7 mg/ml KH_2PO_4. The last 12-hour subculture for challenge was diluted to a standard turbidity reading equal to approximately 2 billion/ml viable cells. This suspension was diluted 1:5 and serial 10-fold dilutions made thereafter. A portion of each dilution was mixed with an equal quantity of sterile 6% hog gastric mucin.‡ Plate counts were made on 2 appropriate dilutions before the addition of mucin to determine the number of organisms in the LD_{50} dose. Each mouse received a challenge dose of 1.0 ml intraperitoneally. The mice were observed for 5 days. The 50% end points were computed according to the Reed and Muench method(8). The protective index was obtained by subtracting the reciprocal of the LD_{50} dilution of culture for immunized mice from the reciprocal of the LD_{50} dilution of culture for nonvaccinated mice. The index gives the logarithm of the number of LD_{50} doses that the mice resisted.

Toxicity tests. Early mortality in mice was noted at the time of immunization. Normal temperature and weight curves on guinea pigs were kept for 3 successive days after which graded amounts of the extracts were given intraperitoneally to each of 2 animals weighing between 350 to 500 g. Temperatures were recorded every 4 hours for the first 24 hours and then daily thereafter for one week. The weights were recorded daily for one week.

Chemical characterization. Preliminary chemical analyses of extracts included determination of nitrogen by the micro-Kjeldahl method(9), carbohydrates by either the Molisch or Dische method(9), and chromatographic tests for proteins, amino acids, simple sugars, and polysaccharides(10). n-Butanol-acetic acid-water (4:1:5, v/v) and buffered phenol solvents followed by n-butanol-ninhydrin spray was used to determine proteins and amino acids. n-Butanol-acetic acid-

† Obtained from Hamilton Laboratory Animals, Inc., Cincinnati, O.

‡ Type 1701-W obtained from Wilson Laboratories, Chicago, Ill.

TABLE II. Homologous and Heterologous Immunity Tests with Saline Extract from *Shigella paradysenteriae* (Flexner II) (Km).

Vaccination extract	Challenge culture	Challenge dilution $1 \times 10^{-}$: 1	2	3	4	5	6	7	8	9	10	LD_{50} $1 \times 10^{-}$	Protective index
Flexner II (Km)													
Undiluted	Flexner II (Km)	5/7*	2/7	1/6	0/7	0/6						1.6	6.1
1: 10	"	4/7	1/7	1/6	0/7	1/7						1.4	6.3
1: 50	"	6/6	5/6	0/6	2/6	0/6						2.6	5.1
1:100	"	4/6	6/6	3/6	2/6	1/6						3.1	4.6
	" control					5/5	4/5	4/5	2/5	0/5	1/5	7.7†	—
Undiluted	Flexner III (Be)				5/5	5/5	3/5	3/5	3/5			7.4	1.7
	" control					4/5	4/5	5/5	5/5	4/5	0/5	9.1†	—
"	Sonnei (Ch)					6/6	5/6	6/6	4/6	6/6		>9.0	<.4
	" control					4/5	5/5	5/5	5/5	4/5	1/5	9.4†	—
"	Flexner II (PR)		5/6	3/6	1/6	1/6	1/6					3.3	6.0
	" control					5/5	5/5	3/5	4/5	4/5	2/5	9.3†	

* Numerator denotes No. of deaths; denominator, total mice tested.
† No. of organisms in LD_{50} dose: Flexner II (Km), 9; Flexner III, 1; Sonnei, 1; Flexner II (PR), 1.

water (4:1:5) followed by alkaline permanganate or m-phenylenediamine spray was used for carbohydrate qualitative tests.

Results. The results of mouse protective potency tests on animals immunized with the 3 saline fractions are presented in Tables II to IV. The Flexner II extract induced protection in excess of one million LD_{50} doses against the homologous organism and against one million LD_{50} doses of the (PR) strain of Flexner II (Table II). There was little or no immunity against heterologous challenge. Essentially similar results were obtained with the Flexner III and Sonnei extracts. Following vaccination with the Flexner III extract (Table III) mice resisted between 7 and 8 logs of the homologous strain but only 2.6 logs and 0.7 log of the heterologous Flexner II and Sonnei strains, respectively. Following vaccination with the Sonnei extract (Table IV) mice resisted considerable amounts of the homologous strain but only 1.6 logs of the heterologous Flexner II and 3.9 logs of the heterologous Flexner III. The results also indicate that the extracts may be diluted as much as 100-fold with little loss in homologous antigenicity.

In Table V are shown the results after vaccination with the polyvalent extract. Mice developed good immunity against all 3 types of *Shigella* with protective indices of 6 logs or more against each organism.

Toxicity studies in mice with the undiluted extracts revealed the following mortality:

TABLE III. Homologous and Heterologous Immunity Tests with Saline Extract from *Shigella paradysenteriae* (Flexner III) (Be).

Vaccination extract	Challenge culture	Challenge dilution $1 \times 10^{-}$: 1	2	3	4	5	6	7	8	9	10	LD_{50} $1 \times 10^{-}$	Protective index
Flexner III (Be)													
Undiluted	Flexner III (Be)	2/5*	1/5	0/5	0/5	0/6						1.0	7.8
1: 10	"	0/6	1/6	1/5	0/5	1/5						<.7	>8.1
1: 50	"	2/5	1/6	1/6	0/6	2/6						1.5	7.3
1:100	"	1/5	1/5	0/6	1/5	0/5						<.9	>7.9
	" control					5/5	5/5	5/5	5/5	2/5	0/5	8.8†	—
Undiluted	Flexner II (Km)		4/5	5/5	4/5	4/5	4/5	4/4				7.0	2.6
	" control					5/5	5/5	5/5	5/5	5/5	1/5	9.6†	—
"	Sonnei (Ch)					4/5	4/5	5/5	4/5	4/5	1/5	9.1	.7
	" control					5/5	5/5	5/5	5/5	5/5	2/5	9.8†	—

* Numerator denotes No. of deaths; denominator, total mice tested.
† No. of organisms in LD_{50} dose: Flexner III control, 1; Flexner II control, 1; Sonnei control, 1.

TABLE IV. Homologous and Heterologous Immunity Tests with Saline Extract from *Shigella sonnei* (Ch).

Vaccination extract	Challenge culture	Challenge dilution $1 \times 10^{-}$:										LD_{50}	Protective index
		1	2	3	4	5	6	7	8	9	10	$1 \times 10^{-}$	
Sonnei (Ch)													
Undiluted	Sonnei (Ch)	3/7*	3/7	0/7	1/5	1/6						1.6	7.9
1: 10	"	7/7	1/7	1/7	1/7	0/7						1.7	7.8
1: 50	"	6/6	1/6	0/6	2/6	1/6						1.9	7.6
1:100	"	4/6	5/6	3/6	0/6	1/6						2.7	6.8
	" control					5/5	5/5	4/5	5/5	4/5	2/5	9.5†	—
Undiluted	Flexner II (Km)					3/6	0/6	1/6	1/5	1/5	0/5	5.4	1.6
	" control					5/5	2/5	3/5	1/5	1/5	0/5	7.0†	—
"	Flexner III (Be)			4/5	1/5	3/5	3/5	2/5				5.1	3.9
	" control					5/5	5/5	5/5	4/5	3/5	0/5	9.0†	—

* Numerator denotes No. of deaths; denominator, total mice tested.

† No. of organisms in LD_{50} dose: Sonnei control, 1; Flexner II (Km) control, 14; Flexner III control, 1.

Flexner II, 4.6%; Flexner III, 19%; and Sonnei, 5.2%. Diluting the extracts 1:10 removed the toxic components of the Flexner II and Sonnei extract but still gave a 10% mortality with the Flexner III extract. No deaths were observed when any one of the extracts was diluted 1:50 or above. The number of mice tested ranged from 30 to 100 for each dilution. A close correlation was observed when the extracts were given to guinea pigs. Doses of 1.0 ml or 5.0 ml of the Flexner II and Sonnei extract caused only a slight febrile reaction and some transient weight loss while 5.0 ml of Flexner III extract was lethal to 2 guinea pigs in 8 hours. The polyvalent vaccine composed of 1:10 dilutions of each of the 3 extracts was lethal to only 9/421 (2.1%) of mice immunized. No untoward reactions were observed in guinea pigs receiving 5.0 ml of the polyvalent extract.

The extracts were found to contain only small amounts of nitrogen. Dialysis reduced the nitrogen content of the extracts approximately one-half. Final nitrogen values of the extracts (mg % N) were: Flexner II, 19.2; Flexner III, 11.2; and Sonnei, 11.6. The polyvalent extract contained 3.02 mg N/100 ml. There was no chromatographic evidence of protein or free amino acids. Concentration of 5.0 ml amounts to 0.2 ml did not give chromatographic evidence of free amino acids. The extracts gave positive Molisch tests. The Dische quantitative carbohydrate test has been performed on the Flexner II extract. Small amounts of carbohydrate (16 mg/100 ml extract) were found. Chromatographic analysis failed to reveal any free simple sugars.

Discussion. The results presented demonstrate a significant immune response in mice following injection of a saline soluble fraction from *Shigella* cells. The heterologous protection afforded by the 3 saline extracts is low but homologous protection exceeds a million LD_{50} doses in all instances. The polyvalent extract is reduced in toxicity but still gives significant cross protection against the 3 strains of *Shigella*. With the use of female mice, where rapid weight increases do not occur over a week's time, protective indices of 100 million LD_{50} doses have been obtained. It is felt that the toxicity of the polyvalent extract resides in the Flexner III extract and that mouse mortality could be further reduced by increasing the dilution of Flexner III extract.

The extraction procedure is rapid and subtle and does not seem to remove a great deal of the cell wall material or diffusible cell constituents. The extract appears to contain a nondialyzable polysaccharide consisting of possibly an amino sugar. Slein and Schnell (11) in recent studies on a polysaccharide isolated from Flexner III have identified an acetylated amino sugar (D-glucosamine), rhamnose, glucose, and phosphorus. Further chemical studies are in progress to identify and purify the active fraction. In addition,

TABLE V. Summary of Immunity Tests with Polyvalent Saline Extract of Flexner II, III, and Sonnei. (Final dilution of 1:10 for each extract.)

Challenge culture	Challenge dilution $1 \times 10^{-}$										LD_{50}	Protective index
	1	2	3	4	5	6	7	8	9	10	$1 \times 10^{-}$	
Flexner II (Km)	6/6*	4/6	1/6	1/6	0/6	1/6					2.6	6.0
" control					5/5	5/5	5/5	5/5	0/5	1/5	8.6†	—
" (PR)	4/5	0/5	3/5	1/5	1/5						1.9	6.8
" control					5/5	2/5	5/5	5/5	2/5	2/5	8.7†	—
Flexner III (Be)	4/5	0/5	1/5	0/5	1/5	1/5					1.7	6.8
" control					5/5	5/5	4/5	4/5	1/5	1/5	8.5†	—
Sonnei (Ch)	3/5	2/5	3/5	2/5	0/5	0/5					2.5	6.9
" control					5/5	5/5	5/5	4/5	5/5	0/5	9.4†	—

* Numerator denotes No. of deaths; denominator, total mice tested.
† No. of organisms in LD_{50} dose: Flexner II (Km) control, 5; Flexner II (PR) control, 5; Flexner III control, 5; Sonnei control, 1.

further work is in progress to determine the antigenicity of the extracts in rabbits and to investigate the activity of the polyvalent extract when given by other immunizing routes to the mouse.

Summary. A procedure for the extraction of a saline soluble fraction from *Shigella* species is described. Each of the 3 extracts protects mice against massive challenge with the homologous strain of *Shigella*. Heterologous protection is of low order or insignificant. A 1:10 dilution of the extracts reduces the toxicity while retaining the essential protective properties. A polyvalent extract has been prepared containing a 1:10 dilution of each of the 3 extracts. Preliminary chemical characterization indicates a nondialyzable carbohydrate-containing compound with small amounts of nitrogen present.

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