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Inactivated Poliovirus Vaccine and Test Development at Connaught Laboratories Ltd.

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Trivalent inactivated poliovirus vaccine (IPV) (MK) was made, purified, and inactivated according to the amended Rijks Instituut (The Netherlands) protocol from primary monkey kidney tissue grown on microcarrier cultures. Losses of the type 2 component due to adsorption to the glass ampule occurred with the purified vaccine preparation. This problem was solved by changing the diluent, and the vaccine was submitted for evaluation in clinical trials at Johns Hopkins (Baltimore, Md.). Phase 2 of the development was to standardize production of IPV from MRC-5 (human diploid) cells on microcarriers and otherwise follow the Rijks Instituut's method. Results of experimental trivalent vaccine production and testing showed that the number of effective doses harvested from MRC-5 cell cultures compared favorably with vaccine derived from monkey kidney. The yields could be further increased with stearyl tyrosine as adjuvant. Large-scale production using 200-liter fermenters is in progress. Poliovirus particles of various densities in cesium chloride can be found in any IPV preparation and give rise to different immunogenic responses. As shown in this paper, some of these virus fractions produce a low primary humoral antibody response but appear to be important for memory induction.

A program was introduced at Connaught Laboratories in 1980 to develop a large-scale production method for inactivated poliovirus vaccine (IPV) of increased potency. Although the method of the Rijks Instituut in The Netherlands [1] was adopted, the use of primary monkey kidney tissue has many obvious disadvantages and was replaced by serially subcultivated MRC-5 human diploid cells. Preliminary studies of the use of these cells were presented at the polio vaccine symposium in Bilthoven, The Netherlands, three years ago [2]. The physiologic growth conditions were further standardized and are now applied to produce trial vaccines. This paper describes the results obtained with an experimental trivalent IPV produced in this way, together with findings from the analysis of different antigenic components in the vaccine.

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Materials and Methods

Trial vaccine production. IPV (MK) had been prepared according to the formulation of the Rijks Instituut [1], and this vaccine had been filled into type 1 glass ampules, which require modification as described in the text. The protocol for the production of IPV from MRC-5 cells was as described earlier [2], and strict controls of pH 7.2 and 20% pO_2 during cell growth were applied. Medium CMRL-1969 was used with amino acid amplification towards the end of the growth cycle. The medium was supplemented with fetal bovine serum at the early passage levels and with 6% adult bovine serum at the production level. The cells were grown in 2.5 g of Cytodex beads (Pharmacia) per liter. For virus growth the medium was replaced with medium 199 containing Earle's salts.

Potency tests. Radioimmunoassays (RIA) were carried out with IgG derived from rabbit sera after immunization with purified D antigen. The specificity of the poliovirus type 1 IgG was further increased by adsorption with heat-treated antigen. The IgG was used for coating tubes and for the lactoperoxidase-iodinated reagent. The quantitative measurements were based on comparisons

Table 1. D-antigen content (by radioimmunoassay) of trivalent inactivated poliovirus vaccine (derived from monkey kidney) before and after filling into untreated or treated glass vials.

Inactivated poliovirus vaccine	No. of D-antigen units per dose of vaccine		
	Type 1	Type 2	Type 3
Glass vial untreated			
Bulk before filling	33	7	28
Filled vaccine	30	0.6	25
Glass vial treated			
Bulk before filling	45	7	30
Filled vaccine	40	8	28

with known standards in a parallel straight-line assay. Rat potency tests [3] were carried out with single dose (1 ml) injections in coronavirus-free Wistar rats. Antibody levels were determined 21 days later except in the adjuvant studies, where the test period was prolonged. To study priming effects, a two-dose schedule was followed. With 10 rats per sample, the antibody titers were expressed as $\log_2$ of the geometric means. Monkeys were used for the study of the adjuvant effect in a two-dose schedule. Groups of four were injected with 1 ml per monkey.

Adjuvant. Stearyl tyrosine HCl (U.S. patent no. 4258029, Canadian patent no. 1128773) was prepared and kindly submitted by Dr. S. Landi from our laboratories. The compound was supplied as a 1% saline suspension.

Table 2. Preparation of diploid-derived inactivated poliovirus vaccine (experimental).

Step	Poliovirus types		
	1	2	3
Harvests			
Volume (liters)	18	40	40
D-antigen units/ml	112	32	33
Prepurification/inactivation			
Volume of concentrate (ml)	173	270	255
D-antigen units/ml	8,023	1,554	1,789
Postpurification/inactivation			
Percent recovery	45	39	63
Final volume (ml)	2,250	3,200	2,600
Final D-antigen units/ml	200	31	111
Total D-antigen units	450,000	99,000	290,000
Total D antigen obtainable per lot	450,000	160,000	620,000

Table 3. Comparison of Salk diploid (MRC-5) vaccine with Rijks' standard (PU 78-02) in radioimmunoassay and rat potency tests with poliovirus Mahoney 1.

Vaccine	Adjuvant*	No. of D-antigen units per dose	Rat potency at geometric mean $\log_2$ days	
			21	63
Diploid	–	118	5.8	4.8
Diploid	–	55	5.0	4.7
Diploid	+	55	5.4	5.8
Diploid	–	34	4.6	3.9
Diploid	+	34	6.5	5.1
Diploid	–	14	2.1	2.6
Diploid	+	14	5.6	4.9
Rijks	– *Standard*	*48*	*4.1*	*4.7*
Rijks	+	48	6.0	5.8

NOTE. Abbreviations: + = present; – = absent.
* Stearyl tyrosine, 1 mg/ml (U.S. patent no. 4258029, Canadian patent no. 1138773).

Preparation of C, D, and DP fractions. Virus fluids were concentrated by ultrafiltration (Millipore-Pellicon), treated with 1,1,2-trichlorotrifluoroethane (Freon 113), and the pellet from the aqueous extract was layered on the CsCl gradient. After 22–24 hr of centrifugation at 150,000 *g*, the fractions were collected and dialyzed against reticulocyte standard salt buffer (RSB).

Results

Losses of poliovirus type 2 antigen after filling. Trivalent IPV (MK) was successfully prepared by the Rijks Instituut's method [1]. The vaccine concentrate was diluted for the final formulation in medium 199 and transferred into glass ampules. An immediate loss of the type 2-antigen component was noted. The antigen could be eluted from the ampule with diluent containing Tween 80 and human serum albumin, indicating that the loss was a result of adsorption to the glass. This problem was solved by changing the composition of the diluent. As shown in table 1, no further losses occurred after filling.

IPV produced from human diploid cells, MRC-5. Approximately three to four cell population doublings were consistently obtained in microcarrier cultures during the seven-day growth cycle. Experimental vaccine was produced from 20-liter cultures, but scaling up to 150-liter cultures has been successfully carried out with similar

Table 4. Comparison of Salk diploid (MRC-5) vaccine with Rijks' standard (PU 78-02) in radioimmunoassay and rat potency tests with poliovirus MEF type 2.

Vaccine	Adjuvant*	No. of D-antigen units per dose	Rat potency at geometric mean log_2 days	
			21	63
Diploid	–	4.4	7.4	4.5
Diploid	–	4.7	6.5	5.0
Diploid	+	4.7	7.5	4.9
Diploid	–	3.4	6.8	4.3
Diploid	+	3.4	8.0	5.4
Diploid	–	1.6	6.0	5.1
Diploid	+	1.6	6.6	5.3
Rijks	– *Standard*	*4.1*	*6.1*	*6.0*
Rijks	+	4.1	7.8	5.6

NOTE. Abbreviations: + = present; – = absent.
* Stearyl tyrosine, 1 mg/ml (U.S. patent no. 4258029, Canadian patent no. 1138773).

Table 5. Comparison of Salk diploid (MRC-5) vaccine with Rijks' standard (PU 78-02) in radioimmunoassay and rat potency tests with poliovirus Saukett type 3.

Vaccine	Adjuvant*	No. of D-antigen units per dose	Rat potency at geometric mean log_2 days	
			21	63
Diploid	–	24	4.1	4.9
Diploid	–	26	3.5	4.9
Diploid	+	26	7.6	6.4
Diploid	–	13	2.5	2.1
Diploid	+	13	6.1	5.6
Diploid	–	6	0.9	1.0
Diploid	+	6	5.6	5.9
Rijks	– *Standard*	*9*	*3.7*	*3.9*
Rijks	+	9	7.9	6.5

NOTE. Abbreviations: + = present; – = absent.
* Stearyl tyrosine, 1 mg/ml (U.S. patent no. 4258029, Canadian patent no. 1138773).

growth rates and final yields of $\geq 1 \times 10^6$ cells/ml. These cells have a tendency to clump the beads together, which in moderation does not interfere with virus propagation. An experimental vaccine was prepared from virus harvest fluids containing 112, 32, and 33 D-antigen units/ml for poliovirus types 1, 2, and 3, respectively, as shown in table 2. The harvest fluids were concentrated and were used for further processing. The percent recovery after processing and inactivation with formaldehyde and the total D-antigen yields are shown. The monovalent concentrates were diluted and pooled to prepare an IPV for testing in comparison with the Rijks' vaccine standard, PU 78-02.

The effect of the stearyl tyrosine adjuvant mixed with the vaccine was also studied. The D-antigen units/ml were determined by RIA and the vaccine was tested at various dilutions in rats for in vivo potency, as shown in table 3 for type 1 poliovirus. The results obtained with the Rijks' vaccine standard at log_2 antibody titers of 4.1 and 4.7, at 21 and 63 days after inoculation, were similar to the results obtained with the MRC-5-derived vaccine between 34 and 55 D-antigen units/ml. The adjuvant improved not only the antigenicity of the standard, but a threefold increase in immunogenicity was obtained with the MRC-5-derived vaccine. Similarly, as shown in tables 4 and 5, the immune response with types 2 and 3 poliovirus antigens could be increased. These results are summarized in table 6, which shows how many "effective doses," based on the comparison with the Rijks' vaccine standard, could be obtained from 20-liter lots of virus harvest fluids. The number of doses could be increased fourfold by the stearyl tyrosine adjuvant.

Adjuvant stearyl tyrosine. As shown in table 7, the adjuvant effect in line 4, with the vaccine diluted 1:4, is superior to that of the undiluted vaccine (line 1) and equal to that of the undiluted vaccine plus adjuvant (line 3).

D, C, and DP particles. IPV consists of different poliovirus particles, and the vaccine composition may differ under certain conditions, making it difficult to assess its potency in vitro. Three major fractions were isolated by CsCl gradient centrifugation from a type 1 concentrate: the empty capsids (C particles at 1.32 g/ml); full particles (D-antigenic at 1.34 g/ml); and dense par-

Table 6. Doses obtained from 20 liters of virus harvest fluids in the production of Salk diploid (MRC-5).

Poliovirus type	Stearyl tyrosine	No. of D-antigen units per dose	No. of doses per 20-liter lot*
1	–	40	11,250
	+	10	45,000
2	–	2	40,000
	+	1	80,000
3	–	32	9,688
	+	8	38,750

NOTE. Abbreviations: + = present; – = absent.
* Calculation of doses was adjusted to 20-liter harvest volumes.

Table 7. Effect of the adjuvant stearyl tyrosine on the immune response in monkeys after the second (14-day booster) injection.

CLL vaccine, lot 282	Adjuvant*	2 weeks on poliovirus type 1	2	3	16 weeks after second injection on poliovirus type 1	2	3
Undiluted	–	8.0	8.5	9.0	2.0	4.9	2.4
Diluted 1:4	–	4.5	0.0	5.0	0.0	0.0	0.0
Undiluted	+	7.3	9.1	7.5	3.6	6.1	4.5
Diluted 1:4	+	7.1	10.1	8.8	4.0	7.3	4.6

NOTE. Abbreviations: + = present; – = absent.

* Stearyl tyrosine (U.S. patent no. 4258029, Canadian patent no. 1138773).

ticles (DP at 1.42 g/ml). The D and DP have similar appearance and require CsCl for differentiation. As shown in table 8, at ~1 μg of protein/ml, the RIA indicates high D-antigen values, whereas 21 days after the first injection, only the D fraction induced an antibody response. Also included in this experiment is the heated D fraction, with reduced D-antigen value and no primary immune response. However, as shown by the booster response after injecting 10 D-antigen units of a purified D fraction, the C and DP fractions had actually primed the immune response in the test animals. Similar results were obtained when the second dose contained the same fractions, as shown in table 9.

Table 8. Primary dose response of D, DP, and C poliovirus particles and low-antigen dose secondary immune response (Mahoney type 1).

Primary inoculation	No. of D-antigen units	Antibody titer* at indicated no. of days after injection 21*	7†	63‡
DP	93	0.0	8.0	4.6
D	123	4.9	10.0	8.1
D, heated	4	0.0	6.5	2.4
C	103	0.0	8.4	3.5
RIV 78-02	49	4.5	7.7	7.6
No antigen	0	0.0	3.0	. . .

NOTE. DP, D, and C refer to types of poliovirus particles. C = empty capsids; D = full particles (D-antigenic); DP = dense particles.

* No. of days after primary injection.

† No. of days after booster injection (10 D-antigen units/ml) on day 42.

‡ Antibody titer in rats, $\log_2$ geometric mean.

Table 9. Primary and secondary immune response with type 1 poliovirus (Mahoney 1)-derived inactivated poliovirus fractions.

Inocula	Antibody titer* 21 days	Inocula†	Antibody titer* at 7 days	21 days
DP	0	DP	6.6	4.6
D	3.6	D	8.3	7.9
C	0	C	3.4	1.5
Diluent	0	DP	0	0
Diluent	0	D	2.1	0
Diluent	0	C	0	0

NOTE. DP, D, and C refer to types of poliovirus particles. C = empty capsids; D = full particles (D-antigenic); DP = dense particles.

* Antibody titer in rats (metabolic inhibition test), $\log_2$ geometric mean.

† Fractions from CsCl density centrifugation, 1.0 ± 0.5 μg of protein per dose.

Discussion

The more potent Rijks Instituut's IPV [4, 5] was introduced and made at Connaught according to the protocol of van Wezel and colleagues [1]. This vaccine is now being evaluated in the Johns Hopkins' (Baltimore, Md.) clinical trial. Furthermore, an experimental lot of IPV produced from MRC-5 cells has been described and shown to yield poliovirus antigens equivalent to those in the monkey kidney–derived vaccine.

A very significant increase in antigenicity, approximately fourfold, was obtained by adding the adjuvant stearyl tyrosine. This adjuvant so far has been used only in animal potency tests, as described here. However, it is biodegradable, and preliminary studies have indicated that it is also nontoxic. Sensitization or reactions at the injection sites do not occur.

Quantitative measurements of poliovirus immunogenic protein depend on the specificity of the reagents used in any in vitro test. Antibody specificity is more easily achieved for serotype and strain differentiation than for the immunogens. Differences in antigenicity and cross-reactivities in in vitro tests have been shown for the C, D, and DP fractions in various poliovirus preparations [6–10]. The specific antigenicities of these fractions are not stable, i.e., changes in pH and temperature may cause reversible antigenic conversions [11]. Poliovirus type 1 is antigenically the least stable component of IPV, and the immune response in the rat

potency test described here showed that the D-antigen determination by RIA could be misleading in predicting the primary dose response. However, a small booster dose resulted in a priming effect even with the so-called nonimmunogenic fractions. For the acceleration of antibody production, a second stimulus was required. Determination of vaccine composition may be useful in monitoring IPV production, but further studies are required to determine the importance of poliovirus antigens in various configurations for a long-lasting protection from the disease.

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