Bull. Org. mond. Santé
Bull. Wld Hlth Org. } 1970, **43**, 431-437

Studies on Vaccination Against Bacillary Dysentery

5. Studies in *Erythrocebus patas* *

DAVID M. MEL,[1] BRANKO CVJETANOVIĆ[2] & OSCAR FELSENFELD[3]

In earlier studies in man it has been demonstrated that streptomycin-dependent shigellae, if given orally in 5 sufficiently large doses, confer a very high type-specific protection against bacillary dysentery.

In the present study, 2 groups of Erythrocebus patas *monkeys were immunized with live streptomycin-dependent* Shigella flexneri *2a, and a third group was not vaccinated. One of the vaccinated groups was given streptomycin with each dose of the live vaccine. The animals that received streptomycin with the vaccine were shedding these organisms in their faeces for a significantly longer period than the animals that did not receive streptomycin. A week after the last dose of vaccine, the animals were challenged with virulent* Sh. flexneri *2a organisms. All the control animals developed diarrhoea and excreted challenge organisms for an average of 9 days. None of the 9 immunized animals showed pathological changes nor symptoms of dysentery. On average, they excreted challenge organisms for only 2.3 days.*

Immunological tests confirmed the immunity of the vaccinated animals.

This study shows that 3 doses of live oral streptomycin-dependent Shigella *vaccine given together with streptomycin are at least as effective as immunization with 5 doses of the same vaccine without streptomycin. Oral application of streptomycin, therefore, seems to have had an enhancing effect on the immunizing property of live oral streptomycin-dependent enteric vaccines.*

In earlier studies it was shown (Mel et al., 1965a, 1965b, 1965c, 1968) that live oral vaccine freshly prepared from streptomycin-dependent (SD) strains of shigellae confers, to men aged 20–21 years, an 84%–100% protection against infection with homologous *Shigella flexneri* serotypes. In other controlled field trials (Mel et al., 1965a, 1965b, 1965c) similar results were obtained with lyophilized vaccines of the same type. Protection could be achieved by administering 5 doses of the vaccine orally at 2-day intervals, or by 4 doses given at 3-day intervals. The lengths of the intervals and the number of doses were designed according to the period of shedding of the vaccine organisms, which was usually about 3 days. An extension of the excretion period of the vaccinal organisms could be accomplished by the administration of streptomycin simultaneously with the vaccine (Mel et al., 1968). Consequently we hoped to achieve an efficient protection also by administering only 3 doses of the vaccine, if given together with small doses of streptomycin, at more extended intervals.

The results of the present study conducted on *Erythrocebus patas* seem to justify the conduct of trials on man with only 3 doses of the vaccine administered together with streptomycin.

* This study was carried out at the Delta Primate Research Center, Covington, La., USA, and was supported by grants from the World Health Organization and the US Army Medical Research and Development Command.

[1] Military Medical Academy, Belgrade, Yugoslavia.

[2] Chief Medical Officer, Bacterial Diseases, Division of Communicable Diseases, World Health Organization, Geneva, Switzerland.

[3] Chief, Division of Communicable Diseases, Tulane University, Delta Primate Research Center, Covington, La., USA.

MATERIALS AND METHODS

Vaccine

The vaccine was freshly prepared from live SD *Sh. flexneri* 2a organisms which require for their growth 0.4 mg of streptomycin per ml of medium, as described earlier (Mel et al., 1965a, 1965b).

Experimental animals

Apparently healthy *Erythrocebus patas* monkeys of 6.2 kg average weight (range 4 kg–10 kg) were used: they were divided into 3 groups. One group, which consisted of 6 animals, average weight 5.8 kg (4 kg–6.6 kg), received 5 doses of the live SD oral vaccine. The second group was composed of 3 animals with an average weight of 5.9 kg (5.5 kg–6.2 kg): these animals received 3 doses of the same vaccine but with streptomycin given simultaneously. The third group of 4 animals, average weight 6.9 kg (5 kg–10 kg), served as control. All three groups were caged in the same room, and were otherwise fed and treated identically.

On 3 successive days, both before vaccination and before challenge, stool specimens of all animals were examined for the possible presence of *Shigella* organisms.

Vaccination

The first group of monkeys was vaccinated with 5 doses, given at 4-day intervals, the individual doses containing 40×10^9, 50×10^9, 50×10^9, 60×10^9 and 60×10^9 live organisms of SD *Sh. flexneri* 2a. The second group of 3 animals was immunized with 3 doses consisting of 50×10^9, 50×10^9 and 60×10^9 of live organisms of SD *Sh. flexneri* 2a, respectively, together with 40 mg streptomycin with the first, 50 mg with the second, and 60 mg with the third dose of the vaccine. The streptomycin was mixed with the vaccine immediately prior to its administration.

After the animals had been tranquillized with 2.5 mg per kg body-weight of phencyclidine, given intramuscularly, 2 g of sodium bicarbonate in 20 ml water were introduced in the stomach through a nasal catheter: 5 minutes later the indicated number of vaccine organisms in 20 ml of physiological saline were introduced through the same catheter.

Challenge

All monkeys were challenged 26 days after the first vaccine dose was given, with 60×10^9 virulent organisms of *Sh. flexneri* 2a strain 2457 T (obtained from Dr Samuel B. Formal, of the Walter Reed Army Institute of Research, Washington, D.C., USA). The challenge was administered through a nasal catheter after the monkeys had been tranquillized.

Stool examination

Fresh stool specimens were collected daily from all animals under study, before vaccination and again several times after vaccination, and plated to meat-extract agar plates containing 0.4 mg/ml streptomycin to determine the length of time during which the vaccine strain was shed in the faeces. Each plate was examined under the stereoscopic microscope, using oblique illumination. Colonies suspected of being those of shigellae were identified serologically by the slide agglutination test. Stools were plated daily also on *Salmonella-Shigella* (SS) agar to reveal any *in vivo* reversion of the vaccine SD strain to streptomycin-independence.

Stool specimens were also collected every day after challenge to determine the length of shedding of the challenge strain and they were plated to both streptomycin-free and streptomycin-containing meat extract and SS agar.

All monkeys were inspected daily and signs of illness recorded. The animals were weighed both before and at the time of vaccination, as well as after the challenge.

Immunological procedures

The animals were bled before vaccination and on days 26, 32, 36, 40, 48, and 63 after the first dose of vaccine.

The immunoglobulins IgG, IgA and IgM in the sera were determined by the single-radial-diffusion method of Mancini et al. (1965) using rabbit anti-*patas* IgG, IgA and IgM sera prepared and tested for purity and specificity in this laboratory.

The agglutinin titres were determined by the indirect haemagglutination method of Haltalin et al. (1966), using extracts of both SD and non-dependent *Sh. flexneri* 2a.

The bactericidal titres were performed according to Muschel et al. (1955, 1966) with brain-heart-infusion agar as the growth medium, phosphate-buffered saline as the diluent, and 4-h incubation; the ensuing opacity was read at a wave-length of 550 nm. The results were recorded as the maximal dilution giving a reduction in opacity to half that of the control without serum.

The opsonic indices of the sera were estimated by mixing 0.2 ml of a suspension containing 1.2×10^8–1.5×10^8 organisms per ml of shigellae with 0.2 ml of the tested serum diluted serially in phosphate-saline with 0.003 M $MgCl_2$, pH 7.2, and 0.3 ml of blood of a "negative" *patas* monkey. After 30 min at 37°C, slides were made, stained with Giemsa stain and the engulfed organisms were counted. The serum dilution giving 50% phagocytotic capability was considered as the end-point.

TABLE 1

DURATION OF SHEDDING OF THE VACCINE ORGANISMS AFTER ADMINISTRATION OF VACCINE

Vaccine group	Animal no.	Immunization period (days) [a]																					Total	
		Dose ↓ 1st					↓ 2nd				↓ 3rd				↓ 4th					↓ 5th				No. of days positive per animal	Percentage of days positive for all animals
		Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22		
Without streptomycin	1		+	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−			+	−	−	−	5	18
	2		−	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−			+	−	−	−	4	
	3		−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−			−	−	−	−	2	
With streptomycin		Dose ↓ 1st													↓ 2nd						↓ 3rd					
	4		+	+	+	−									+	+	−	−			+	+	+	−	8	63
	5		+	+	+	−									+	+	−	−			+	+	−	−	7	
	6		+	+	−	−									+	+	−	−			+	+	+	−	7	
	7		+	+	+	+									+	+	−	−			+	+	−	−	8	
	8		+	+	−	−									+	+	−	−			+	+	−	−	6	
	9		+	+	+	−									+	+	+	−			+	+	+	−	9	

[a] + = positive bacteriological finding of vaccine organisms in the stools in the four consecutive days immediately following immunization.

Stool specimens were taken before vaccination and 5, 13, 21, 32, 40, 63 and 76 days after the first dose of vaccine. Fresh stool specimens were added to weighed centrifuge tubes each containing a measured quantity of phosphate-buffered saline; after the specimen had been emulsified, it was pressed through a Millipore syringe with a filter of average pore size 0.5 nm. The specimens were stored at −20°C until tested.

The immunoglobulins IgG, IgA and IgM in stools were determined using the Preer method (1956) and the antisera employed in the estimation of immunoglobulins in the sera.

All other tests were carried out after 10-fold concentration of the stool extracts in Viscose bags with polyvinyl pyrrolidon.

The agglutination and bactericidal titres of the stools were determined as for the serum.

The antitoxin determinations were carried out in monolayer tissue cultures of human embryonic kidney cells, using Eagle's minimal essential medium with 20% calf serum, without rotation, and inoculation with serial dilutions of the examined materials. Then 10 minimal cytopathic effect (CPE) units of the supernate of sterile sonicated virulent streptomycin-independent *Sh. flexneri* 2a were added. The rules of standard tissue culture techniques were followed for 3 days, and the CPE was recorded daily. The 50% effective dose was calculated and reported as the antitoxin titre of the tested material.

All tests were carried out in duplicate.

RESULTS AND DISCUSSION

The data in Table 1 show that, considering the 4-day periods after vaccination, the proportion of days during which the monkeys shed the vaccine strain was significantly higher (45 days out of 72 days = 63%) in the group treated with 3 doses of vaccine plus streptomycin than in animals that received 5 doses of the same vaccine but without streptomycin (11 days out of 60 days = 18%). The difference between the two proportions is significant ($P<0.0001$).

These findings show the favourable effect of the administration of streptomycin together with the vaccine. This is consistent with our observation with SD *Salmonella typhi* vaccine that streptomycin extends the period of shedding of the immunizing strain and that this is apparently a parameter of the immunizing efficiency of live oral enteric vaccines (Cvjetanović, Mel & Felsenfeld, 1970).

Table 2 demonstrates that the length of shedding of the challenge strain, considered to be an index of the severity of the infection (Mel et al., 1965a, 1965b, 1965c), was in fact significantly shorter in vaccinated animals than in the controls. The same data also show that 3 doses of vaccine, administered together with streptomycin, were at least as effective in reducing the period of excretion of the challenge strain as 5 doses of vaccine without streptomycin.

After the challenge, all 4 control animals developed diarrhoea, 2 of them with mucus and

TABLE 2

DURATION OF SHEDDING OF CHALLENGE STRAIN BY VACCINATED MONKEYS AND CONTROLS

Vaccine group	No. of monkeys	No. of shedding days after challenge [a]		No. of monkeys shedding the challenge strain [b]	
		Individual animals	Total no. of shedding days / total no. of days of observation	for <5 days	for >7 days
Controls	4	8, 9, 9, 10	36/48 [c] (75%)	0	4
5 doses without streptomycin	3	2, 2, 4	8/36 (22%)	9	0
3 doses with streptomycin	6	1, 2, 2, 2, 2, 4	13/72 (18%)		
			21/108 [c] (19%)		

[a] Each animal was observed for 12 days.

[b] Significance of difference between the proportions compared according to Diem ($P = 0.02$).

[c] $\chi^2 = 43$ ($P < 0.0001$).

1 also with blood in the stools. The mean duration of the *Shigella* excretion was 9 days. On the other hand, none of the 9 vaccinated animals showed pathological changes in the stools after challenge, and the average length of shedding dysentery bacilli was 2.3 days.

Table 3 shows that both groups of animals, those vaccinated with 5 doses without streptomycin, and those receiving 3 doses of vaccine with streptomycin, gave comparable results in the biochemical tests conducted on the extracts of their stool specimens.

Immunochemical examinations were conducted on serum specimens obtained from the animals before vaccination, on day 26 after vaccination (but before the challenge) and several times after challenge. The results are shown in Table 4. There was an increase in serum IgG and IgM levels after the challenge, principally in the unprotected control animals. Agglutinin titres remained elevated for a longer time in the latter group, as well as in the monkeys that received streptomycin. Both these groups also displayed higher bactericidal titres, and they remained high for a longer time. The antitoxin levels did not show a clear pattern but were elevated for 2 months in all animals. The opsonic index followed a similar course. These immunological findings give the impression that simultaneous admi-

TABLE 3

RESULTS OF IMUNOCHEMICAL EXAMINATION OF STOOL SPECIMENS [a]

Test	Group of animals [b]	Before vaccination	No. of days after 1st vaccination [c]						
			5	13	21	32	40	63	72
IgG	C	0	0	0	0	0.9	0.1	—	0.2
	VO	0	0.5	0.7	0.7	1.4	0.7	0.3	0.2
	VS	0	0.4	0.5	0.9	1.3	0.5	0	0
IgA	C	0	0	0.8	0	2.5	0	—	0.1
	VO	0	1.5	1.9	2.6	5.2	0.5	0	0
	VS	0	1.1	1.6	3.1	3.5	0.8	0	0
IgM	C	0	0	0	0	0	0	—	0
	VO	0	0	0	0	0	0	0	0
	VS	0	0	0	0	0	0	0	0
Agglutinin titre	C	0	0	3.3	0	2.5	0	0	0
	VO	0	13.3	31.6	176.0	213.3	13.3	0	0
	VS	0	3.3	11.7	146.0	290.0	18.3	0	0
Bactericidal titre	C	0	0	0	0	10.0	0	6.6	10.0
	VO	0	6.6	30.0	86.6	186.0	10.0	6.6	0
	VS	0	6.0	28.3	144.0	352.0	44.0	10.0	6.6
Antitoxin titre	C	0	0	0	0	5.0	0	6.6	15.0
	VO	0	0	6.6	73.3	80.0	6.6	0	0
	VS	0	0	6.6	84.0	90.0	26.6	6.6	3.3

[a] Mean values in groups of animals.

[b] C = Controls; VO = Vaccine without streptomycin on days 1, 5, 9, 13 and 19; VS = Vaccine with streptomycin administered on days 1, 13 and 19.

[c] All monkeys were challenged on day 26.

TABLE 4
RESULTS OF IMUNOCHEMICAL EXAMINATION OF BLOOD SPECIMENS [a]

Test	Group of animals [b]	Before vaccination	No. of days after 1st vaccination					
			26 [c]	32	36	40	48	63
IgG	C	1027	1027	1137	1190	1077	1067	1085
	VO	1000	941	940	975	1000	1006	980
	VS	1005	980	1035	916	1000	1039	1008
IgA	C	302	295	347	360	285	264	267
	VO	313	286	246	256	255	245	218
	VS	296	248	316	352	291	278	280
IgM	C	124	130	180	208	147	177	177
	VO	95	112	118	126	126	135	133
	VS	104	100	145	149	156	166	148
Agglutinin titre	C	0	5	30	95	40	50	35
	VO	0	0	16	46	33	0	6
	VS	0	13	28	67	41	10	20
Bactericidal titre	C	0	0	5	75	90	95	35
	VO	0	0	23	33	40	13	20
	VS	0	3	16	65	46	30	30
Antitoxin titre	C	0	0	5	30	70	50	25
	VO	0	7	10	33	40	6	13
	VS	0	10	26	60	50	7	20
Opsonic titre	C	0	0	0	5	20	35	45
	VO	0	53	73	106	80	53	66
	VS	0	13	104	93	103	70	56

[a] Mean values in groups of animals.
[b] C = controls; VO = vaccine without streptomycin; vaccine with streptomycin.
[c] All animals were challenged on day 26, after the collection of the blood samples.

nistration of streptomycin and vaccine is accompanied by changes that quantitatively resemble those accompanying natural infection.

The possible mechanisms of the enhancing effect of streptomycin both on the period of shedding of the vaccine strain and on the immunogenicity of the live oral vaccine seem to merit further study.

RÉSUMÉ

ÉTUDES SUR LA VACCINATION CONTRE LA DYSENTERIE BACILLAIRE: 5. ÉTUDES CHEZ *ERYTHROCEBUS PATAS*

De précédentes recherches ont montré que l'administration de 4 à 5 doses d'un vaccin buccal vivant préparé à partir de souches de *Shigella* streptomycinodépendantes conférait à l'homme un taux de protection de 84-100% contre l'infection homologue. Au cours de l'expérimentation actuelle, menée chez le singe *Erythrocebus patas*,

on a étudié la possibilité d'obtenir une immunisation aussi efficace par 3 prises du vaccin, plus espacées, grâce à l'administration concomitante de streptomycine.

Deux groupes de singes ont été immunisés à l'aide d'un vaccin vivant *Sh. flexneri* 2a (souche streptomycinodépendante): 3 animaux ont reçu, à 4 jours d'intervalle, 5 doses de la préparation contenant 40×10^9 à 60×10^9 organismes; 6 autres ont reçu en 19 jours 3 doses (50×10^9 à 60×10^9 organismes) et en outre, lors de chaque prise de vaccin, 1 dose de streptomycine (40, 50 et 60 mg). Un groupe témoin de 4 singes a été constitué.

Les singes traités par l'association vaccin-streptomycine ont éliminé la souche vaccinale dans les selles pendant une période notablement plus longue (durée globale: 45 jours) que les singes traités par le vaccin seul (durée globale: 11 jours). Vingt-six jours après la prise de la première dose de vaccin, tous les animaux ont subi une infection d'épreuve par 60×10^9 organismes d'une souche *Sh. flexneri* 2a virulente. Les animaux témoins ont présenté de la diarrhée et ont excrété la souche d'épreuve pendant 9 jours en moyenne. Aucun des animaux vaccinés n'a présenté de symptômes morbides et, chez eux, l'élimination de la souche virulente ne s'est pas prolongée au-delà de 2,3 jours en moyenne. L'examen de sérums couplés a montré que la réponse immunitaire des singes traités à la fois par le vaccin et la streptomycine rappelait celle suscitée par une infection naturelle.

Les auteurs concluent que l'immunisation par 3 doses de la préparation vaccinale additionnée de streptomycine est au moins aussi efficace que la vacination par 5 doses du même vaccin sans antibiotique. L'administration de streptomycine par voie buccale paraît renforcer l'action immunisante des vaccins buccaux vivants préparés à partir de souches streptomycinodépendantes.

REFERENCES

Cvjetanović, B., Mel, D. M. & Felsenfeld, O. (1970) *Bull. Wld Hlth Org.*, **42**, 499

Haltalin, K. C. et al. (1966) *J. Immunol.*, **87**, 517

Mancini, C. et al. (1965) *Immunochemistry*, **2**, 235

Mel, D. M., Arsic, B. L., Mikolić, B. D. & Radovanović, M. L. (1968) *Bull. Wld Hlth Org.*, **39**, 375

Mel, D. M., Papo, R. G., Terzin, A. L. & Vukšić, L. (1965a) *Bull. Wld Hlth Org.*, **32**, 637

Mel, D. M., Terzin, A. L., & Vukšić, L. (1965b) *Bull. Wld Hlth Org.*, **32**, 633

Mel, D. M., Terzin, A. L. & Vukšić, L. (1965c) *Bull. Wld Hlth Org.* **32**, 647

Muschel, L. H. & Jackson, J. E. (1966) *J. Bact.*, **91**, 1399

Muschel, L. H. & Treffers, H. P. (1965) *J. Immunol.*, **76**, 1

Preer, J. R., Jr (1966) *J. Immunol.*, **77**, 52