

ARTIFICIAL IMMUNIZATION OF RABBITS*† AGAINST SYPHILIS

I. EFFECT OF INCREASING DOSES OF TREPONEMES GIVEN BY THE INTRAMUSCULAR ROUTE

BY

M. METZGER AND W. SMOGÓR

From the Department of Medical Microbiology, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

The results reported in a previous paper (Metzger, Michalska, Podwińska, and Smogór, 1969) have clearly shown that the immunogenic properties of *Treponema pallidum* are related to their protein component. A high degree of immunity to challenge inoculation by virulent treponemes was obtained in rabbits that had been injected with killed treponemes with the protein antigen preserved, as judged by their high agglutinability in a serum that contained antibody to this component only. Two vaccine preparations proved very efficient when given intravenously to rabbits:

(1) Treponemes maintained without any additions at 4°C. for from 7 to 10 days before administration.

(2) Treponemes stored for the same time but with penicillin added (4 µg./ml.).

The main purpose of the present study was to check the efficiency of these preparations when administered intramuscularly and to investigate the effect of treponeme dose on the development of resistance.

Material and Methods

The virulent Nichols strain of *T. pallidum* was used throughout.

Adult Danish albino rabbits weighing about 3 kg., with non-reactive VDRL and TPI tests, were used for the study; they were housed in individual cages out of doors.

Vaccines

Three vaccines prepared from killed *T. pallida* were given as immunizing agents:

(1) Treponemes suspended in phosphate-buffered saline of pH 7.4 and stored at 4°C. for from 7 to 10 days.

(2) Treponemes suspended in phosphate-buffered saline of pH 7.4 with penicillin† added (1 µg./ml.); stored at 37°C. for 24 hours and at 4°C. for 6 to 9 days.

(3) Treponemes suspended in phosphate-buffered saline of pH 7.4 with penicillin added (4 µg./ml.); stored at 37°C. for 24 hours.

Non-infectiousness of the vaccines was ascertained by the following experiments:

(1) Each new batch of treponeme suspensions was tested for virulence by intracutaneous injection of 0.1 ml. containing 29 million organisms into each of four sites on the shaven back of a rabbit, and by injection of 0.5 ml. containing 145 million organisms into each testis of the same rabbit. None of the rabbits developed lesions during a 4-month observation period, and lymph node transfers from these rabbits to other rabbits were negative. All the animals remained VDRL and TPI negative.

(2) 4 or 5 weeks after the completion of the immunization procedure, the left popliteal lymph node of each animal was removed and emulsified in 1 ml. 10 per cent. normal rabbit serum-saline; one drop of the emulsion was inspected by darkfield microscopy for the presence of treponemes, and the rest was injected into one testis of a normal rabbit; the second testis was³ utilized for lymph node transfer from another rabbit. Microscopic examination of the testes performed one month later did not show the presence of treponemes in any of the testicles.

Immunization

Only female rabbits were used. They were injected intramuscularly with various vaccines over a period of 7 weeks. Each rabbit received 1 ml. inoculum into the gluteal muscles of each side alternately four times a week for 3 weeks, and 2 ml. inoculum four times a week for an additional 4 weeks. The total dose of treponemes

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‡The penicillin used was crystalline G (potassium salt) produced by Polfa (Poland).

for different groups of animals was approximately 12,000, 6,000, and 3,000 million organisms.

Infectivity Test

5 weeks after completion of the immunization procedure, an intradermal challenge of 300,000 virulent *T. pallida*, suspended in 0.1 ml. 10 per cent. normal rabbit serum-saline, was made at each of four sites of the shaven back of each animal. The rabbits were inspected daily, and the time at which syphilitic lesions appeared at the challenge sites was noted. After 4 months, the rabbits were killed by an injection of air into the marginal ear vein; three to four lymph glands were then excised from the rabbits which had failed to develop lesions and emulsified in 1 ml. 10 per cent. normal rabbit serum-saline. One drop of the emulsion was examined microscopically for the presence of treponemes, and the rest was injected into one testis of a normal rabbit; the second testis was utilized for lymph node transfer from another rabbit. The infection was allowed to develop for one month. After that time, the rabbits were killed, and the testes were removed and examined by darkfield microscopy for the presence of treponemes.

Serological Tests

VDRL Slide Flocculation Test This was carried out as described in the "1959 Manual of Serologic Tests for Syphilis".

TPI Test This was performed by the procedure of Nelson and Diesendruck (1951) with the following modifications: the basal medium contained double strength sodium thioglycolate, and bovine serum albumin was replaced by gelatin to a final concentration of 100 mg. per cent.

Results

Clinical Response

As can be seen from the data presented in Table I and summarized in Table II, all three vaccines given intramuscularly conferred a state of immunity, which was demonstrated either by absence of lesions at the challenge sites, associated with non-infectiousness or infectiousness of the lymph glands, or by reduced number and delayed incubation period of lesions as compared to those in controls.

TABLE I
RESULTS OF INFECTIVITY TESTS ON RABBITS INJECTED INTRAMUSCULARLY WITH INCREASING NUMBERS OF VARIOUSLY TREATED KILLED TREPONEMES

Vaccine No.	Type of Vaccine	Total Dose (million)	Results of Infectivity Test			
			No. of rabbits with lesions/ No. inoculated	No. of positive challenge sites/ No. of sites	Mean Incubation Period (days)	Number of rabbits without lesions but with positive lymph node transfer/ No. examined
1	Treponemes stored at 4°C. for from 7 to 10 days	12,000	3/10	4/40	41	2/6
		6,000	3/9	6/36	37	3/6
		3,000	6/8	16/32	22	1/2
2	Treponemes stored with penicillin (1 µg./ml.) at 37°C. for 24 hrs and at 4°C. for 6 to 9 days	12,000	3/8	4/32	41	2/5
		6,000	5/8	9/32	34	1/3
		3,000	7/8	24/32	25	0/1
3	Treponemes stored with penicillin (4 µg./ml.) at 37°C. for 24 hours	6,000	5/9	8/36	25	2/4
No vaccine			8/8	31/32	18	

TABLE II
RESULTS OF IMMUNIZATION

Vaccine No.	Total Dose (million)	Total No. of Rabbits Vaccinated	Reaction to Challenge		
			Immune	Asymptomatic (lymph node transfer positive)	Symptomatic (Lesions at Challenge Sites)
1	12,000 6,000 3,000	10 9 8	5	2	3
			3	3	3
			1	1	6
2	12,000 6,000 3,000	8 8 8	3	2	3
			2	1	5
			1	0	7
3	6,000	9	2	2	5

The total number of treponemes injected during the period of immunization had a marked effect on the immune response of the animals; rabbits that had been given 3,000 million treponemes showed markedly less resistance to infection than those given 6,000 or 12,000 million organisms.

Of the three vaccines used in this study, the best immunizing effect at each dosage level was that of Vaccine 1, which contained only treponemes stored at 4°C. for from 7 to 10 days.

Vaccines 2 and 3 containing treponemes with added penicillin were equally effective in protecting rabbits against challenge inoculation but the degree of immunity was lower than that conferred by Vaccine 1, as indicated by the number of animals that developed lesions and the number of positive challenge sites.

Serological Response

Qualitative TPI and quantitative VDRL tests were performed on the sera of all rabbits before and after immunization, before the challenge inoculation, and 3 or 4 months thereafter. The results are recorded in Tables III and IV where the rabbits are grouped on the basis of their reactions to challenge irrespective of the type of vaccine used.

Table III shows that none of the vaccinated animals developed immobilisins after immunization, but taken 3 and 4 months after challenge their sera exhibited a different reactivity in the TPI test. The sera of those rabbits that showed a complete

resistance to challenge (the "immune" group) remained TPI-negative, while most of the sera of symptomatically infected animals became TPI-positive. Although all asymptotically infected rabbits were found to be TPI-negative after the infection had lasted 3 months, a tendency towards TPI-positivity was clearly indicated by the results of tests performed 4 months after inoculation.

The sera from almost all rabbits showed a Wassermann antibody response after immunization (Table IV). The titres ranged from "undiluted" to a dilution of 1:16, declining slightly during the 5-week rest-period before challenge. The sera of asymptotically and symptomatically infected rabbits examined 3 and 4 months after challenge showed significantly higher titres to VDRL antigen than those from the "immune" group. It will also be noted that there was a pronounced tendency towards VDRL-negativity in the "immune" group.

Discussion

These results show that it is possible to induce in rabbits a high degree of resistance to challenge inoculation by large numbers of virulent *T. pallida* by intramuscular injections of killed treponemes having their protein component preserved. The results previously reported (Metzger, Michalska, Podwińska, and Smogór, 1969) have thus been corroborated and extended.

The vaccine preparations used in this study have

TABLE III
TPI ANTIBODY RESPONSE

Sera of Rabbits showing Reaction to Challenge	Results of TPI Test*	Number of sera showing TPI Reactivity				
		Before Immunization	After Immunization	Before Infection	After Infection	
		7-wk interval	5-wk interval		3 mths	4 mths
Immune	Negative	17	17	17	17	17
	Doubtful	0	0	0	0	0
	Positive	0	0	0	0	0
Asymptomatic	Negative	11	11	11	11	3
	Doubtful	0	0	0	0	5
	Positive	0	0	0	0	3
Symptomatic	Negative	32	30	31	9	4
	Doubtful	0	2	1	1	2
	Positive	0	0	0	22	26
Controls	Negative			8	0	0
	Doubtful			0	1	0
	Positive			0	7	8

*Percentage of treponemes immobilized: <20 = negative <50 ≥ 20 = doubtful >50 = positive

TABLE IV
WASSERMANN ANTIBODY RESPONSE

Sera of Rabbits showing Reaction to Challenge	Result of VDRL Test	Number of sera showing VDRL reactivity			
		Before Immunization	After Immunization	Before Infection	After Infection
		7-wk interval	5-wk interval		3 mths 4 mths
Immune	Negative	17	0	2	0 2
	Positive undil.	0	5	10	15 15
	dil. 1:2	0	3	2	2 0
	1:4	0	7	3	0 0
	1:8	0	2	0	0 0
Asymptomatic	Negative	11	1	3	1 0
	Positive undil.	0	7	7	7 6
	dil. 1:2	0	1	1	2 3
	1:4	0	1	0	0 1
	1:8	0	1	0	1 1
Symptomatic	Negative	32	3	5	0 0
	Positive undil.	0	15	18	13 14
	dil. 1:2	0	6	5	7 6
	1:4	0	3	4	1 6
	1:8	0	4	0	8 2
	1:16	0	1	0	2 2
	1:32	0	0	0	1 2
	Negative			8	0 0
Controls	Positive undil.			0	4 3
	dil. 1:2			0	1 3
	1:4			0	1 0
	1:8			0	1 1
	1:16			0	1 0
	1:32			0	0 1

already been found effective in rabbits when injected intravenously (Metzger and others, 1969). To make a fair comparison of the immunizing effects of intravenous and intramuscular administration, experimental conditions in this study were kept essentially the same as in the previous one.

Vaccine 1, containing treponemes stored in phosphate-buffered saline of pH 7.4, without any additions, at 4°C. for from 7 to 10 days before injection, proved the best immunizing agent in both studies. The minimum 7-day storage period was accepted as ensuring that none of the organisms remained alive in the suspension. The maximum storage period was 10 days because treponemes maintained for longer than this quickly lost their protein component (Metzger and Podwińska, 1967). Previous results (Metzger and others, 1969) have shown that treponemes stored for 20 days were unable to protect rabbits against challenge. For this reason, a new treponeme suspension was prepared every week, and portions of it were injected on four successive days, *i.e.* on the 7th, 8th, 9th, and 10th days of storage.

Vaccines 2 and 3 contained treponemes stored with added penicillin. Although Vaccine 2 was effective when given intravenously, penicillin was

found slightly to impair the immunogenic properties of the treponemes (Metzger and others, 1969). The concentration of penicillin was therefore decreased to 1 µg./ml.

Vaccine 3 was a modification of Vaccine 2, in which the treponemes were maintained at 37°C. for only 24 hours before injection and a higher concentration of penicillin (4 µg./ml.) was added to ensure the death of the organisms. It was previously found (Metzger and others, 1969) that treponemes treated with this much penicillin were unable to infect rabbits only 6 hrs after contact with the antibiotic.

The present results show that the total dose of treponemes injected during the course of immunization influences the immune response. The best immunizing effect was obtained in rabbits given the highest dose, *i.e.* 12,000 million organisms, and the effect was less in those given 6,000 million. Rabbits that had received 3,000 million treponemes showed a significantly lower degree of resistance to challenge.

Every step of the immunization procedure used in this study was arbitrarily established. Different experimental conditions may give more complete protection, and this is now being studied in this laboratory.

None of the vaccinated animals developed TPI

antibody after immunization. Rabbits of the "immune" group remained TPI-negative during a 4-month observation period, whilst those which developed symptomatic or asymptomatic infection tended to show TPI-positivity. This was further evidence that no infection developed after inoculation with virulent *T. pallidum* in the "immune" group. Although almost all the rabbits were found to be positive to VDRL antigen after immunization, the situation after infection was similar to that of the TPI response.

These results confirm the conclusions of Magnuson, Thompson, and McLeod (1951), McLeod and Magnuson (1953), Miller, Fazzan, and Whang (1963), and Metzger and others (1969) that circulating antibodies, particularly those involved in the TPI test, play either a very limited role or none in the development of immunity against syphilitic infection.

Summary

Groups of rabbits were immunized over a period of 7 weeks with intramuscular injections of three vaccines prepared from killed treponemes:

(1) Treponemes suspended in phosphate-buffered saline of pH 7.4 and stored at 4°C. for from 7 to 10 days.

(2) Treponemes suspended in phosphate-buffered saline of pH 7.4 with penicillin added (1 µg./ml.) and stored at 37°C. for 24 hours and at 4°C. for 6 to 9 days.

(3) Treponemes suspended in phosphate-buffered saline of pH 7.4 with addition of penicillin (4 µg./ml.) and stored at 37°C. for 24 hours.

The total doses of treponemes injected in different groups of animals were approximately 12,000, 6,000 and 3,000 million.

It was ascertained by appropriate control experiments that the treponeme suspensions were non-infectious.

An intradermal challenge inoculation was made 5 weeks after completing the immunization procedure.

The three vaccine preparations given intramuscularly conferred a degree of immunity on rabbits which was shown by:

(1) The absence of lesions at the challenge sites, associated with noninfectiousness or infectiousness of the lymph glands.

(2) A reduced number and delayed incubation period of lesions as compared with those in controls.

The total number of treponemes injected had a marked effect on the immune response. None of the vaccinated rabbits developed TPI antibody after immunization.

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Immunsation artificielle des lapins contre la syphilis

I. Effet des doses croissantes de tréponèmes inoculés par voie intramusculaire

SOMMAIRE

Des groupes de lapins furent immunisés pendant 7 semaines par des injections intramusculaires de 3 vaccins préparés à partir de tréponèmes tués:

(1) Tréponèmes suspendus en eau physiologique tampon phosphate de pH 7,4 et conservés à 4° C. pendant 7 à 10 jours.

(2) Tréponèmes suspendus en eau physiologique tampon phosphate de pH 7,4 avec addition de pénicilline (1/µg./ml.) et conservés à 37°C. pendant 24 heures puis à 4°C. pendant 6 à 9 jours.

(3) Tréponèmes suspendus en eau physiologique tampon phosphate de pH 7,4 avec addition de pénicilline (4/µg./ml.) et conservés à 37°C. pendant 24 heures.

Les doses totales de tréponèmes injectées pour les différents groupes d'animaux furent approximativement de 12.000, 6.000, et 3.000 millions.

On s'était assuré par des contrôles appropriés que les suspensions tréponémiques étaient avirulentes. Cinq semaines après la fin du dispositif d'immunsation on pratiqua une inoculation intra dermique d'épreuve.

Les préparations des 3 vaccins donnés par voie intramusculaire ont conféré un degré d'immunité qui fut démontré de la manière suivante:

(1) L'absence de lésions aux points des inoculations d'épreuve accompagnée ou non de la virulence des ganglions lymphatiques.

(2) La réduction du nombre et l'augmentation de la durée d'incubation des lésions en comparaison avec ces mêmes éléments chez les témoins. Le nombre total des tréponèmes inoculés a un effet important sur la réponse immunitaire.

Il ne fut trouvé d'anticorps immobilisants (TPI) chez aucun des lapins vaccinés.