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FREEZE-DRIED B.C.G. VACCINE FROM AN ISONIAZID-RESISTANT STRAIN

A LABORATORY INVESTIGATION

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The use of a B.C.G. vaccine made from an isoniazid-resistant strain for vaccinating tuberculosis contacts while they are receiving prophylactic treatment with isoniazid would have obvious advantages. Such a vaccine has been proposed by Canetti (1956). With the availability of more precise methods of preparing stable freeze-dried B.C.G. vaccine (Ungar *et al.*, 1956; Muggleton, 1960) a comparison of B.C.G. from isoniazid-resistant and isoniazid-sensitive strains has been undertaken. This paper describes the preparation of the freeze-dried vaccines and their properties as determined in the laboratory.

Experimental

Preparation of the Isoniazid-resistant B.C.G. Culture

The strain of B.C.G. used in our laboratories was derived from the Copenhagen substrain in 1954. It is maintained in freeze-dried master cultures, which are recovered, as required, on Löwenstein-Jensen egg medium. These cultures were used to inoculate tubes of Dubos's liquid medium; these, after nine days' incubation at 37° C., in turn provided inocula for a series of tubes of the same medium containing falling concentrations (double dilutions of 100 µg./ml., 50 µg./ml., 25 µg./ml., etc.) of isoniazid. After nine days' incubation at 37° C. the tubes were examined for growth of the B.C.G. The culture containing the highest concentration of isoniazid showing visible growth was used as inoculum for a further set of tubes, and the process was repeated until 14 transfers in isoniazid had been made.

The B.C.G. strain was originally sensitive to 0.05 µg. of isoniazid per ml. Resistance development took place

in slow steps up to 25 µg./ml.; at this point it appeared to stop, and further serial transfers failed to increase the resistance. Freeze-dried master cultures of the resistant substrain were then prepared.

At this point the substrain developed a tendency to grow poorly in Dubos's medium—a phenomenon that was later associated with its inability to produce catalase. Sauton's medium would not support its growth at all, although it grew freely on Löwenstein-Jensen egg medium, on glycerol/potato slopes, and on blood agar.

Tests for catalase on washed centrifuged cells from Dubos's cultures were negative, whereas the control parent isoniazid-sensitive strain gave a positive result.

Morphologically the resistant and sensitive cultures were identical in size, shape, and staining characteristics. The differentiation of living and dead cells from older cultures could be made with both cultures by the neotetrazolium reduction technique of Greenberg *et al.* (1958) or by the differential staining technique of Lack (1953).

The virulence of the resistant substrain to guinea-pigs was compared with that of the parent sensitive strain. A group of six 250-g. male albino guinea-pigs was used for each, and each animal received the equivalent of 5 mg. of moist weight of centrifuged organisms from a 9-day-old Dubos culture. The animals were observed for 10 weeks, during which they each gained more than 220 g. in weight. They were then killed; post-mortem examination did not show any progressive disease in any of the animals. Both the resistant substrain and its sensitive parent produced an abscess 5 mm. in diameter at the site of injection, with some enlargement of regional lymph nodes.

Preparation of Freeze-dried Vaccine

The isoniazid-resistant culture was used to prepare three batches of ampoules of freeze-dried vaccine by the method we have described previously (Ungar *et al.*, 1956). Since the resistant culture would not grow in the Sauton medium usually employed for preparing vaccine from the sensitive parent strain, two of the batches (192 and 331) were prepared from cells grown for 12 days in Dubos's medium. The cells were collected in the centrifuge, washed once in saline to free them from traces of serum albumin from the culture medium, and used to prepare the vaccine. The third batch (390) was grown in a modified medium, based on Sauton's formula, in which the glycerol was replaced by sodium glutamate, glutamine, and a purified casein hydrolysate. Two batches (258 and 389) of vaccine prepared from the parent isoniazid-sensitive strain were used for control purposes.

The residual moisture contents of the three resistant and two sensitive batches were measured by the vapour-pressure method (Beckett, 1954) and found to fall between 0.9 and 1.1% residual moisture.

Viability Counts

The viabilities of the batches were assessed by a modification of the technique of Miles *et al.* (1938) on Petri dishes, with an oleic acid-albumin medium containing 10% human blood. The colonies were counted under a stereobinocular microscope after 28 days' incubation at 37° C. Sometimes, and unpredictably, the isoniazid-resistant culture would grow poorly and give low counts in one set of tests, a phenomenon that seemed to be associated with the lack of catalase formation by the substrain.

The viability counts of the batches of vaccine one to two months after preparation are shown in Table I. The batches were stored at 4° C. in a refrigerator, and on further testing over a period of 12 months were found to retain their full viability during their use in laboratory and clinical tests.

Safety and Efficiency Tests of the Freeze-dried Vaccines on Laboratory Animals

Jensen Tests.—The sizes of the local lesions produced in guinea-pigs by intradermal injection of the resistant and sensitive cultures were studied by Jensen's (1946) method to determine their degrees of virulence. All the batches of vaccine listed above were tested; the results from batches 390 (resistant) and 389 (sensitive) were typical of the others and are shown in Table II. It was evident that vaccines from resistant and sensitive strains produced similar local lesions at equivalent viability levels and that the resistant strain should there-

fore be safe for preparing vaccines to be used in human patients.

Tuberculin Conversion in Guinea-pigs with and without Isoniazid Treatment.—Groups of six male albino guinea-pigs, weighing approximately 250 g., were inoculated by injection intradermally in the depilated flank with 0.1-ml. dilutions (1/10, 1/100, 1/1,000, and 1/10,000) of the two vaccines. Two sets of animals were inoculated with each dilution of each vaccine; one set was treated once daily with 20 mg. of isoniazid per kg., and the second untreated set served as controls. The isoniazid was given in 1 ml. of water by stomach-tube on week-days and in the drinking-water at week-ends; the doses were continued throughout the experiment for 42 days. After 14, 28, and 42 days all of the animals were tuberculin-tested (Mantoux test) with 10 T.U. of P.P.D. The reactions were recorded after 24 hours by measuring with callipers the diameter of the oedema. The results relating to batches 389 and 390 are summarized in Table III. It is evident that, after treatment with the vaccine from the isoniazid-sensitive substrain, isoniazid treatment inhibited tuberculin conversion in the guinea-pigs, particularly in those vaccinated with low doses. At the highest dose level (0.1 ml. of 1/10 dilution) sufficient B.C.G. was introduced to induce tuberculin conversion, albeit of a low degree, despite the isoniazid treatment.

In contrast, the vaccine from the isoniazid-resistant substrain at all dose levels tested (down to 0.1 ml. of 1/10,000 dilution) induced the tuberculin conversion of guinea-pigs whether treated with isoniazid or not.

Discussion

The preparation of an isoniazid-resistant B.C.G. culture by "training" an isoniazid-sensitive parent can fairly readily be accomplished. A significant feature of this culture is that it loses the ability to produce catalase—a property often observed in strains of isoniazid-resistant *Mycobacterium tuberculosis* var. *hominis*. The avirulent character of the trained resistant substrain is, however, clearly maintained, since it causes neither progressive disease when injected intramuscularly in large amounts into guinea-pigs nor excessive local reactions on intradermal injection in the same species.

TABLE I.—Viability Counts of Batches of B.C.G. Vaccine Prepared from Isoniazid-resistant and Sensitive Cultures

Batch No.	Sensitivity to Isoniazid	Viable Cell Count (per ml. of Reconstituted Vaccine)
192	Resistant*	8.85×10^8
331	"	4.90×10^8
390	"	6.3×10^8
258	Sensitive	8.0×10^8
389	"	6.25×10^8

* The sensitivities to isoniazid of the cultures recovered from the three batches of freeze-dried vaccine were tested. All were found to grow in 25 µg. of isoniazid per ml. of Dubos's medium.

TABLE II.—Jensen Tests of B.C.G. Vaccine from Isoniazid-resistant (Batch 390) and Isoniazid-sensitive (Batch 389) Substrains

Vaccine	Dilution of Vaccine	Diameter (mm.) of Intradermal Lesion at 21 Days	
		Average (of 6)	Range
Isoniazid-resistant (batch 390)	Undiluted	8.25	7-9
	1/10	4.75	4-5
	1/100	3.50	2-4
	1/1,000	2.00	0-3
Isoniazid-sensitive (batch 389)	Undiluted	7.25	6-8
	1/10	4.25	4-5
	1/100	4.25	4-5
	1/1,000	2.00	0-5

TABLE III.—Tuberculin Reactions in Guinea-pigs after Vaccination with B.C.G. Vaccines from Isoniazid-resistant and Isoniazid-sensitive Substrains

Vaccine	Isoniazid Treatment	Dilution* of Vaccine	Tuberculin Reaction (mm. Oedema)					
			14 Days		28 Days		42 Days	
			Average	Range	Average	Range	Average	Range
Isoniazid-resistant (batch 390)	None	1/10	13.3	12-14	14.8	14-17	14.8	14-16
		1/100	10.7	9-13	13.8	12-15	14.1	12-16
		1/1,000	4.5	0-10	11.7	8-14	13.5	12-14
		1/10,000	2.3	0-6	9.8	4-13	11.4	9-14
Isoniazid-sensitive (batch 389)	None	1/10	11.5	7-13	13.8	11-16	14.4	13-16
		1/100	8.5	5-11	14.0	12-16	12.3	10-13
		1/1,000	1.0	0-3	13.3	11-15	13.8	13-15
		1/10,000	0	0	12.3	10-14	13.2	12-15
Unvaccinated control	—	—	0	0	0	0	0	0
Isoniazid-resistant (batch 390)	20 mg./kg. daily orally	1/10	9.6	6-12	12.7	10-15	12.6	11-15
		1/100	9.4	7-11	13.6	13-15	13.6	11-15
		1/1,000	4.7	0-12	14.7	12-17	13.7	12-16
		1/10,000	2.5	0-9	12.5	12-15	13.0	10-14
Isoniazid-sensitive (batch 389)	20 mg./kg. daily orally	1/10	0	0	8.3	6-9	11.5	10-12
		1/100	1.0	0-6	2.3	0-8	6.3	4-9
		1/1,000	0	0	0	0	0	0
		1/10,000	0	0	0	0	0	0
Unvaccinated control	—	—	0	0	0	0	0	0

* Dilutions prepared in Sauton's medium with 1/4,000 Triton WR1339 to prevent clumping on dilution.

There is as yet no proof that we have produced a stable substrain in which the characteristic isoniazid-resistance will be retained on repeated subcultivation. This property would be important if large-scale production of B.C.G. vaccine from the resistant culture were contemplated. The difficulty has, for the present, been avoided by the use of freeze-dried master cultures.

In guinea-pigs, tuberculin conversion after vaccination with the sensitive parent strain is strongly inhibited by concurrent isoniazid treatment—a finding that is consistent with the clinical observation that isoniazid treatment inhibits tuberculin conversion in children vaccinated with B.C.G. The vaccine from the isoniazid-resistant substrain was, however, fully effective in producing a positive tuberculin reaction with concurrent isoniazid therapy. It is therefore assumed that freeze-dried vaccine prepared from the isoniazid-resistant cultures should be satisfactory for the vaccination of tuberculin contacts while they are on prophylactic doses of isoniazid, and trials on children and adults with such a vaccine are described by Gaisford and Griffiths (1961).

Summary

An isoniazid-resistant substrain of B.C.G. has been produced by repeated subcultivation in the presence of isoniazid.

The resistant culture was unaltered in virulence, morphology, and staining properties, but its growth characteristics in some culture media were altered owing to its having become catalase-negative.

Batches of freeze-dried vaccine have been prepared from the substrain and a comparison made with batches from the isoniazid-sensitive parent strain.

Guinea-pigs, treated orally with isoniazid at 20 mg./kg./day, when vaccinated with even small doses of the isoniazid-resistant substrain became tuberculin-positive. With a control isoniazid-sensitive B.C.G. substrain, tuberculin conversion was inhibited by the isoniazid treatment.

The use of vaccine made from isoniazid-resistant B.C.G. in human tuberculosis contacts receiving prophylactic doses of isoniazid is suggested.

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A FREEZE-DRIED VACCINE FROM ISONIAZID-RESISTANT B.C.G.

A CLINICAL INVESTIGATION

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The use of B.C.G. in the protection of contacts is well established and reaches its acme in the vaccination of newborn infants of tuberculous mothers or of infants born into tuberculous households. Unfortunately, in both these situations a period of segregation is necessary after vaccination until Mantoux conversion has occurred, whereafter protection may be assumed, and during this time, usually three to four weeks, the infants have to be nursed away from the risk of exposure to tuberculosis and from the risks of nosocomial infection. The abolition of this segregation period would be a major advance, and there are two possible ways in which it could be effected; the first, already tried successfully by Dormer and his colleagues (1959), of giving isoniazid from birth: the second, originally suggested by Canetti (1956), by giving an isoniazid-resistant vaccine at birth and concurrently administering isoniazid orally until the post-B.C.G. tuberculin test becomes positive, whereafter it may be discontinued. The obvious advantages of the latter proposal prompted us to undertake trials with such a vaccine. The preparation of the vaccine and its properties are described by Ungar *et al.*, (1961).

Preliminary trials in a few adults showed that the antigenicity of the first isoniazid-resistant vaccine (batch 192) was comparable with that of the ordinary freeze-dried vaccines used routinely in our B.C.G. clinic. The viable count of this batch was 8,800,000 per ampoule and the contents of each ampoule were dissolved in 2 ml. of saline. Each intradermal injection of 0.1 ml. therefore consisted of approximately 440,000 viable organisms. Alternate cases were given 650,000 viable organisms of an ordinary routine batch intradermally for comparison. The local reactions were similar—a fairly uniform 4–6 mm. papule—and the mean of the Mantoux response to 10 T.U. after three weeks was 12 mm. induration in each group.

Investigations were then undertaken in newborn infants: (1) to assess the effect of giving isoniazid at the same time as an ordinary freeze-dried vaccine, the treatment being continued for three to four weeks; (2) to assess the effect of giving isoniazid at the same time as the isoniazid-resistant vaccine; (3) to compare the resistant vaccine with an ordinary routine freeze-dried vaccine without giving isoniazid; and (4) to determine the degree to which post-vaccination conversion was maintained with the resistant preparation.

For these purposes three batches of freeze-dried vaccine were used: two were isoniazid-resistant-batch 192 (concentration 8,800,000 viable organisms per ampoule), which was prepared in March, 1958, and batch 331, prepared in December, 1959, with a concentration of 4,900,000 viable organisms per

During the year 1959–60 the East African Institute for Medical Research continued its work on body composition, energy expenditure, analysis of milk of African mothers, nutritional ophthalmology, filariasis, and schistosomiasis. (*East African Institute for Medical Research Report*, 1959–60.)