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Quantitative evaluation of the effectiveness of Connaught freeze-dried BCG vaccine in mice and in guinea-pigs*

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Although the field trials carried out by the Medical Research Council of Great Britain demonstrated that BCG vaccination can confer a substantial degree of immunity against tuberculous infection, it does not follow that BCG substrains other than the one used for those trials will produce equally favourable results. In fact, there is increasing evidence that different BCG strains may differ widely in their protective potency. The experiments described here further confirm these differences. They also show how the determination of the minimum dose of a BCG vaccine capable of delaying the development of tuberculous infection in mice and in guinea-pigs can yield reproducible data that may help to characterize individual BCG strains.

The main purpose of these experiments was to determine the protective potency of Connaught freeze-dried BCG vaccine, lot 140, and to compare it with that of three other BCG vaccines. Marked differences were found with respect to the minimum protective dose for mice or guinea-pigs and the degree of immunity and tuberculin allergy produced in guinea-pigs as shown by the dose–response relationships recorded over a wide dosage range. The results suggest that the Connaught vaccine equals or surpasses the other vaccines in effectiveness.

Such tests require a reference BCG vaccine of high protective potency for both animals and man.

It is difficult to obtain reliable quantitative information on the effectiveness of a BCG vaccine in preventing tuberculosis in man. The testing of its ability to delay the development of experimental tuberculous infection in susceptible animals is therefore of great practical interest. By means of such protection tests—extensively used by previous workers, as reviewed by Swedberg (1), Conge & Dubos (2) and Jespersen (3)—we obtained useful information on the effectiveness of Connaught BCG vaccine.

The use of mice as test animals, in addition to guinea-pigs, was prompted by our observation (4) that vaccination with very small doses of the Connaught BCG strain, consisting of only a few viable units, significantly prolonged the life of white mice subsequently challenged with *Mycobacterium bovis*.

In subsequent experiments (unpublished data) we showed that, when Connaught freeze-dried BCG vaccine was tested simultaneously in mice and in guinea-pigs, using a wide dosage range, doses consisting of less than 20 viable units were capable of significantly extending the life of both species following challenge with virulent tubercle bacilli.

The purpose of the experiments described here was to assess the protective potency of Connaught freeze-dried BCG vaccine, lot 140 (referred to as " Connaught 140 " throughout the remainder of this paper), which is available as primary seed lot. The report is limited to tests in which this vaccine was compared with other BCG vaccines.

MATERIALS AND METHODS

BCG vaccines tested

The following freeze-dried BCG vaccines were obtained through the International Reference Centre for BCG Seed-Lots and Control of BCG Products, Copenhagen, Denmark: Japan 11012E, Japan 172,

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and London (Glaxo) F10. Japan 172—Japanese freeze-dried glutamate vaccine prepared from strain 172—was established in 1965 as the International Reference Preparation of BCG Vaccine by the WHO Expert Committee on Biological Standardization (5).

The three vaccines were tested along with Connaught 140, prepared at the Connaught Laboratories by Dr S. Landi. The Connaught BCG strain is derived from glycerinated bile-potato culture No. 340-4. Dr M. H. Brown obtained this strain in 1948 from Dr A. Frappier, Institute of Microbiology and Hygiene, University of Montreal, who in turn had obtained it in 1937 from the Pasteur Institute. The culture was maintained by periodic (14-day) passages on glycerinated potato medium, inserting after every fifth passage one passage on bile-potato medium.

The 4 freeze-dried BCG vaccines to be tested were reconstituted with sterile distilled water so as to contain 1 mg of BCG per millilitre. From these fresh suspensions serial dilutions were prepared in modified Tween-albumin medium[a] for protection tests and to determine—by means of colony counts on Löwenstein-Jensen medium—the number of viable units injected.

In addition to Connaught 140, the Connaught production strain, lot 146 (which will be referred to later in this paper as " Connaught 146 "), was tested. A " fresh harvest " suspension, containing 40 mg of BCG per millilitre, was made from the washed and finely ground surface pellicle harvested from Sauton's medium. Dilutions in modified Tween-albumin medium, corresponding to the dosage levels used for testing the freeze-dried vaccines, were prepared from the suspension.

Protection tests in animals[b]

Mice: Groups of 10 young female albino mice (Connaught breed), 4–6 weeks old, were vaccinated subcutaneously with BCG in doses ranging from 10^{-1} mg to 2×10^{-8} mg (moist weight). Twenty mice were left unvaccinated. After 5–6 weeks all mice were challenged by the intravenous route with 0.052 mg (moist weight) of the Ravenel strain of *M. bovis*, grown in Dubos Tween-albumin medium. According to colony counts this dose, contained in 0.4 ml, corresponded to 2.4×10^{6} viable units. The dates of all deaths were recorded and autopsies were performed to ascertain that death was due to tuberculous infection.

The minimum protective dose of a BCG vaccine was defined as the smallest dose that, under the conditions of the experiment, still prolonged life significantly beyond the time of death of the non-vaccinated control animals.

Guinea-pigs: Female albinos (Connaught breed) weighing 300–450 g, all Mantoux-negative to 250 TU of PPD (Connaught), were vaccinated with BCG intracutaneously, using the same dosage range as for mice. Groups of 20 animals were used for vaccination with the 10^{-1} mg dose and groups of 10 animals for the lower doses. About 20 guinea-pigs were left unvaccinated as a control group. A few days before challenge all guinea-pigs were Mantoux-tested. The vaccinated groups were tested with 100 TU of PPD. Guinea-pigs that gave a negative reading after 24 h were retested with 250 TU—the test dose used for the unvaccinated control animals.

For the purposes of this study, a positive tuberculin reaction was defined as an area of erythema more than 5 mm in diameter produced by the intracutaneous injection of 250 TU of PPD contained in 0.1 ml. In the case of BCG-vaccinated guinea-pigs that showed a positive reaction to 100 TU, it was assumed that 250 TU would also produce a positive reaction.

Twelve weeks after vaccination all guinea-pigs were challenged by a subcutaneous injection into the groin of 0.005 mg of *M. tuberculosis*, strain Johnston, grown in Tween-albumin medium. This dose corresponded to 0.34×10^{6} viable units. The Johnston strain and the maintenance of its virulence for guinea-pigs have been described (6).

The recording of deaths and autopsies as well as the definition of the minimum protective dose were essentially as stated for mice. However, in guinea-pigs, the determination of the conversion rate (the percentage of tuberculin-positive animals) prior to challenge was an integral part of the protection test.

The minimum allergenic dose of a BCG vaccine for guinea-pigs was defined as the smallest dose that still produced a positive tuberculin reaction in 50–100% of the animals.

[a] The composition of this medium is the same as that of Dubos Tween-albumin medium except that 1.35% of human serum albumin is substituted for the bovine albumin.

[b] Details on the survival of the animals vaccinated in this study, or left unvaccinated as a control, are given in three appendix tables. These tables, which include data on individual tuberculin tests, have been deposited in the WHO library and single copies may be obtained on request to: Chief Librarian, WHO, 1211 Geneva 27, Switzerland.

Table 1. Simultaneous vaccination of mice and guinea-pigs with graded doses of three freeze-dried BCG vaccines (experiments IA and IB)

BCG vaccine	Dose (mg)	Viable units (colony count)	Response: survival of mice (days)		Response: positivity of guinea-pigs to 250 TU of tuberculin	
			Geometric means ± SE	% increase over controls	No.	%
Connaught 146	10^{-1}	2 050 000	54.1 ± 5.80	79[c]	14/14	100
	2×10^{-5}	410	43.2 ± 6.37	43[d]	7/7	100
	2×10^{-6}	41	46.5 ± 5.66	54[c]	8/8	100
	2×10^{-7}	3–4	35.6 ± 4.27	18	5/9	55.5
	2×10^{-8}	0 or 1	27.4 ± 3.36	− 9	1/8	12.5
Connaught 140	10^{-1}	1 400 000	44.4 ± 4.89	47[d]	17/17	100
	2×10^{-5}	280	47.6 ± 7.05	58[c]	9/9	100
	2×10^{-6}	28	37.7 ± 7.05	25	10/10	100
	2×10^{-7}	0–3	43.1 ± 5.36	42[d]	6/8	75
	2×10^{-8}	0 or 1	28.0 ± 3.31	− 7	0/7	0
Japan 11012E	10^{-1}	4 500 000	36.0 ± 5.45	19	19/19	100
	2×10^{-5}	900	29.9 ± 3.69	− 1	0/8	0
	2×10^{-6}	71	28.3 ± 2.66	− 7	0/8	0
	2×10^{-7}	9	32.7 ± 3.81	8	0/7	0
	2×10^{-8}	0 or 1	27.6 ± 2.36	− 9	1/9	11.1
unvaccinated control group	—	—	30.2 ± 2.62	—	0/17	0

[a] Mice in groups of 10, with a control group of 20 animals.
[b] The guinea-pigs were Mantoux-tested 47 days after vaccination and remained unchallenged.
[c] This increase is significant ($P < 0.01$).
[d] This increase has some statistical significance ($0.01 < P < 0.06$).

RESULTS AND DISCUSSION

In experiments 1A and 1B the three vaccines listed in Table 1 were tested simultaneously in mice and in guinea-pigs. BCG doses that resulted in tuberculin positivity in more than 50% of guinea-pigs prolonged the life of mice beyond the survival of the unvaccinated control animals.

Connaught 140 extended the life of mice significantly in doses as low as 2×10^{-7} mg, corresponding to less than 10 viable units. Japan 11012E, even in a dose 100 times larger (2×10^{-5} mg) failed to prolong the life of mice and did not produce tuberculin allergy in guinea-pigs. Only the largest dose (10^{-1} mg) of this vaccine produced tuberculin allergy in guinea-pigs and a modest (19%) prolongation of life in mice.

For reasons unknown, Connaught 146, although effective in both species in a dose of 2×10^{-6} mg, did not fully measure up in effectiveness to Connaught 140 at a dosage level of 2×10^{-7} despite the fact that the latter contained fewer viable units.

In experiment 1B (in guinea-pigs), an intercurrent streptococcal infection produced some deaths, thereby reducing the initial number of animals per group from the original 10 or 20 to the numbers alive at the date of the tuberculin test (Table 1, column 6). This intercurrent infection precluded postvaccinal challenge with virulent tubercle bacilli and the subsequent determination of the survival time of the guinea-pigs.

It appeared desirable, therefore, to investigate further the protective potency of Connaught 140 in an experiment that permitted the determination of tuberculin conversion as well as the time of survival of guinea-pigs that had been infected with tubercle bacilli and had previously received graded doses of the vaccine, including in the test other known BCG vaccines for comparison.

The results of experiment 2 (Table 2) show that, for all 3 freeze-dried vaccines tested, the doses that resulted in a statistically significant prolongation of life were those that produced a high (80–100%) tuberculin conversion rate. Furthermore, the minimum

Table 2. Determination in guinea-pigs of minimum allergenic and minimum protective doses of three freeze-dried BCG vaccines (experiment 2)

BCG vaccine	Dose (mg)	Viable units (colony count)	Response: reaction to 100 TU of tuberculin — No. positive	%	mean diameter (mm) ± SE	survival (days) — means ± SE	% increase over controls
Japan 172	10^{-1}	5 000 000	12/12	100	20.9 ± 0.6	> 233 ± 14.0	> 85[c]
	2×10^{-5}	950	12/12	100	19.9 ± 0.4	190 ± 14.8	51[c]
	2×10^{-6}	95	9/11	82	14.6 ± 2.3	198 ± 10.3	57[c]
	2×10^{-7}	10	3/12	25	3.9 ± 2.0	147 ± 11.4	16.6[b]
	2×10^{-8}	0–2	0/11	0	0 ± 0.0	140 ± 14.5	11.1[b]
London F10	10^{-1}	6 250 000	12/12	100	18.5 ± 0.7	206 ± 11.4	63.5[c]
	2×10^{-5}	1 000	12/12	100	16.1 ± 0.6	189 ± 7.4	50[c]
	2×10^{-6}	100	12/12	100	15.5 ± 0.9	185 ± 7.1	46.8[c]
	2×10^{-7}	12	11/12	91.5	13.7 ± 1.3	199 ± 9.9	58[c]
	2×10^{-8}	0–3	4/12	33.3	4.3 ± 1.8	139 ± 15.9	10.3[b]
Connaught 140	10^{-1}	1 600 000	12/12	100	21.5 ± 0.9	229 ± 20.7	81.6[c]
	2×10^{-5}	320	12/12	100	20.7 ± 0.5	219 ± 15.2	73.8[c]
	2×10^{-6}	32	10/10	100	21.4 ± 0.8	210 ± 16.1	66.6[c]
	2×10^{-7}	3	12/12	100	18.8 ± 0.6	> 195 ± 16.5	> 54.8[c]
	2×10^{-8}	0 or 1	2/12	16.7	3.5 ± 2.3	142 ± 13.8	12.7[b]
unvaccinated control group	—	—	0/23	0	0	126 ± 9.9	—

[a] All guinea-pigs were Mantoux-tested 76 days after vaccination date, and challenged 6–8 days later with strain Johnston.

[b] This increase lacks statistical significance ($P > 0.05$).

[c] This increase is statistically highly significant ($P < 0.001$).

protective doses of Connaught 140 and London F10 were of the order of 2×10^{-7} (3 and 12 viable units, respectively), whereas the dose of Japan 172 required to produce a comparable extension of life was 10 times as high: 2×10^{-6}, corresponding to 95 viable units. The graphic recording of the survival time against the logarithm of the vaccine dose (Fig. 1) demonstrates visually the importance of including in the test the lowest BCG doses (2×10^{-6} to 2×10^{-8} mg), without which a difference in the minimum protective dose between Japan 172 and the other two vaccines could not have been detected.

The reduced immunogenicity of Japan 172, compared with Connaught 140, cannot be explained on the basis of any difference in the number of viable units injected, as the viable count of the former was in fact about 3 times as high as that of the latter (Table 2).

This example confirms that viable counts, which are useful for checking the stability of individual vaccines, cannot serve as a reliable measure of their protective potency. This particularly holds true when comparing vaccines from differing sources, as in the experiments described.

A comparison was made between the dose–response curves based on the mean survival times (Fig. 1) with the dose–response curves drawn for the corresponding mean sizes of the tuberculin reactions (Fig. 2). The two charts have the following features in common:

1. For each of the 3 vaccines the minimum protective dose (Fig. 1) coincides with the minimum allergenic dose (Fig. 2).

2. For the doses 2×10^{-6}, 2×10^{-5} and 10^{-1} mg of BCG, at which all vaccines gave statistically significant protection (Table 2), the dose–response lines for Connaught 140 run clearly above those of London F10, suggesting that the former has a higher protective and allergenic potency in guinea-pigs.

3. The lines representing Japan 172 follow an irregular intermediate course, the mean survival times and the mean sizes of the tuberculin reactions falling, as a rule, between the corresponding dose–response lines of London F10 and Connaught 140.

The accuracy of the determination of the minimum protective dose depends on the (10-fold) dosage intervals of the BCG dilution range, the number of

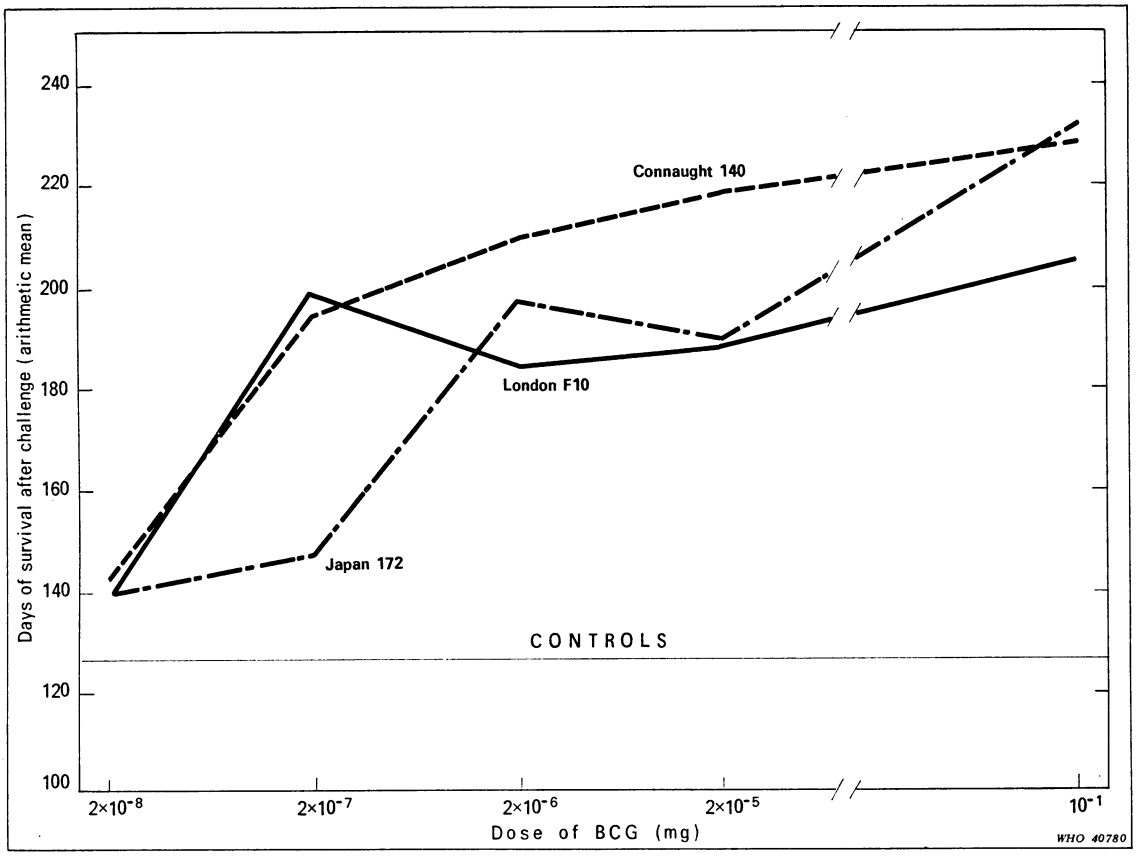

Fig. 1. Mean survival of challenged guinea-pigs as a function of the BCG dose (experiment 2).

test animals used per dose, and the degree of statistical significance ($P \leqslant 0.05$) required.

The observed close relationship between tuberculin allergy and immunity in guinea-pigs is essentially in agreement with observations made by Jespersen & Bentzon (7), who used for vaccinating guinea-pigs a dilution range of 4 ultrasonically treated BCG strains grown in Dubos medium. The results of experiment 2 of the present study show that the minimum allergenic doses, determined 11 weeks after vaccination, practically coincided with the minimum protective doses based on the survival data, although the 3 vaccines tested had not undergone ultrasonic treatment.

Jespersen & Bentzon (8) demonstrated that bank voles (red mice) are—like guinea-pigs—highly susceptible to infection with *M. bovis* and that even very small doses of BCG vaccine can induce partial immunity in these animals. Bank voles can therefore be used for testing BCG strains for their protective potency.

Although white mice of the Connaught breed are less susceptible to infection with *M. bovis*, requiring a higher challenge dose than bank voles, they are susceptible to the immunizing action of minute doses of BCG. These mice can therefore be used for determining the minimum protective dose of BCG vaccines. However, as stated by Jespersen (3), the white mice proved to be very sensitive to overdosage with the challenge strain, which makes the planning of successful experiments more difficult.

As test animals, guinea-pigs offer the advantage over mice and bank voles of providing quantitative information on both the immunogenic and the

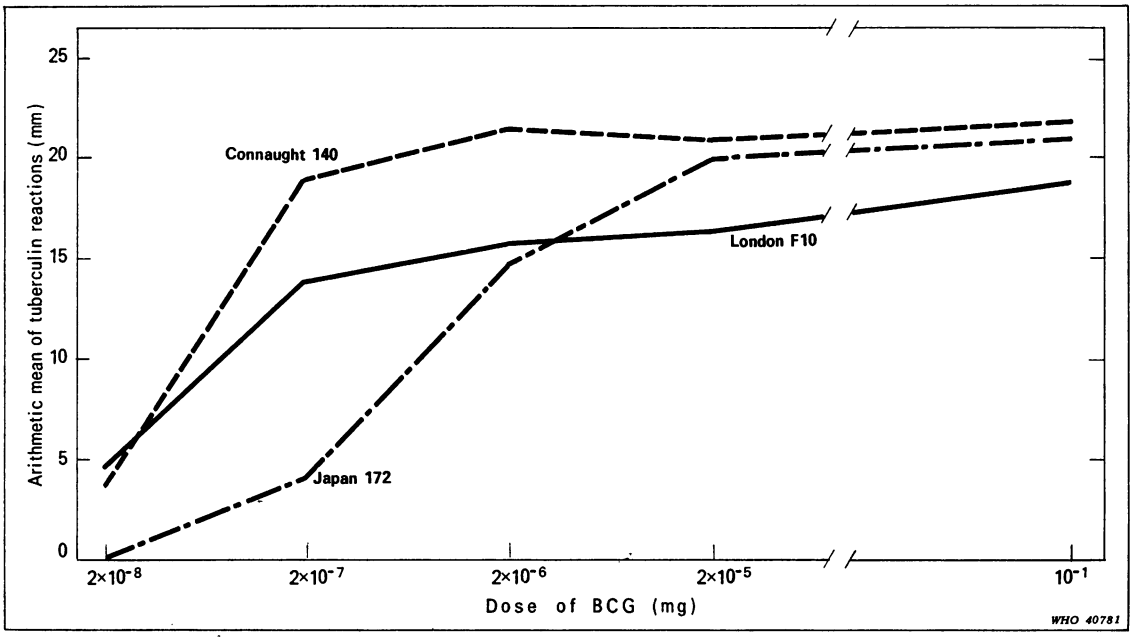

Fig. 2. Mean diameter of tuberculin reaction as a function of the BCG dose (experiment 2).

allergenic potency. Both sets of data are closely interrelated for a certain time following vaccination.

The observation, made in experiment 2, of a reduced level of allergy and immunity produced in guinea-pigs by Japan 172 and London F10, compared with Connaught 140, has its counterpart in a report by Ladefoged et al. (9) that the same 2 strains (172 and F10) proved definitely less effective in protection tests in bank voles than strains Copenhagen 1331 and Paris 1173 P2.

The experiments described were concluded before Japan 172 was established in 1965 as the International Reference Preparation of BCG Vaccine. In terms of the experimental conditions chosen for protection tests in guinea-pigs (experiment 2), a useful reference standard for animal tests could be expected to have a protective and allergenic potency corresponding to a minimum effective dose not larger than 2×10^{-7} mg, representing less than 20 viable units, and showing a high level of protective potency throughout a wide dosage range. Relative heat stability with regard to allergenicity in man, as described for freeze-dried Japanese glutamate vaccine (172) by Geser & Azuma (10), would be an additional asset if it were associated with a marked protective potency in experimental animals and in man.

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RÉSUMÉ

ÉVALUATION QUANTITATIVE DE L'EFFICACITÉ DU VACCIN BCG LYOPHILISÉ CONNAUGHT CHEZ LA SOURIS ET LE COBAYE

On a comparé des vaccins BCG de différentes origines quant à leur pouvoir de retarder l'apparition de la tuberculose expérimentale chez la souris et le cobaye: le vaccin Connaught 140 lyophilisé, le vaccin Japan 172 (préparation internationale de référence de vaccin BCG), le vaccin Japan 11012E et le vaccin London (Glaxo) F10. Les vaccinations ont été faites par voie sous-cutanée chez des souris blanches et par voie intracutanée chez des cobayes, en utilisant une large gamme de dosages. Les souris ont subi une inculation intraveineuse d'épreuve avec *Mycobacterium bovis* 5-6 semaines après la vaccination. Les cobayes ont subi un test de Mantoux (100 et 250 UT de PPD) avant l'inoculation d'épreuve pratiquée. après 12 semaines, avec une souche humaine virulente de *M. tuberculosis*.

La dose protectrice minimale de BCG a été définie comme la plus petite dose entraînant, dans les conditions de l'expérience, une prolongation notable de la vie par rapport à la survie des témoins non vaccinés. La dose allergisante minimale chez le cobaye a été définie comme la plus petite dose produisant encore une réaction tuberculinique positive à 250 UT de PPD chez 50-100% des animaux.

Au cours de la 1re expérience pratiquée sur des souris et des cobayes, une infection streptococcique intercurrente chez les cobayes n'a pas permis l'inoculation d'épreuve de bacilles tuberculeux virulents, le seul critère d'efficacité des vaccins, chez ces animaux, étant le taux de conversion tuberculinique. Le vaccin Connaught 140 a suscité un degré notable de protection chez la souris à la dose de 2×10^{-7} mg, qui est apparue aussi comme la dose allergisante minimale chez le cobaye. Par contre, une dose 100 fois plus forte (2×10^{-5} mg) de vaccin Japan 11012E n'a entraîné ni protection chez la souris ni conversion tuberculinique chez le cobaye.

Une 2e expérience, effectuée uniquement sur des cobayes, a permis d'attribuer au vaccin Connaught 140 une dose protectrice et allergisante minimale de 2×10^{-7} mg, du même ordre que la dose protectrice et allergisante minimale du vaccin London F10. Pour obtenir le même effet, on a dû utiliser une dose 10 fois supérieure (2×10^{-6} mg) du vaccin Japan 172.

L'étude des courbes dose-réponse a montré que dans la gamme de dosages de 2×10^{-6} à 10^{-1} mg de BCG les trois vaccins conféraient une protection statistiquement significative, mais que le vaccin Connaught 140 suscitait régulièrement des réponses nettement plus fortes que le vaccin London F10.

Les résultats des tests de protection chez la souris et le cobaye confirment l'existence de profondes différences entre divers vaccins BCG. Ils montrent la nécessité d'évaluer les préparations de BCG, par rapport à un vaccin de référence approprié, en vue de déterminer pour une gamme de dosages la plus étendue possible les doses protectrices et allergisantes minimales et le degré de protection conféré par chaque dosage.

REFERENCES

1. SWEDBERG, B. Studies in experimental tuberculosis. An investigation of some problems of immunity and resistance. Stockholm, Essette, 1951 (thesis).
2. CONGE, G. & DUBOS, R. J. *In*: Recherches sur le BCG. Paris, Flammarion, 1966, pp. 246-248.
3. JESPERSEN, A. The potency of BCG vaccines determined on animals. Copenhagen, 1971 (thesis).
4. SIEBENMANN, C. O. & BARBARA, C. *In*: Abstracts of papers, 16th International Tuberculosis Conference. Amsterdam, Excerpta Medica, 1961, p. 107 (International Congress Series, No. 41).
5. WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION. Eighteenth report. Geneva, 1966, pp. 13-14, 33-34, 38 (WHO Technical Report Series, No. 329).
6. SIEBENMANN, C. O. & BARBARA, C. *Amer. Rev. resp. Dis.*, **101**: 777-779 (1970).
7. JESPERSEN, A. & BENTZON, M. W. *Acta path. microbiol. scand.*, **71**: 114-131 (1967).
8. JESPERSEN, A. & BENTZON, M. W. *Acta tuberc. scand.*, **44**: 253-266, 276-289 (1964).
9. LADEFOGED, A. ET AL. *Bull. Wld Hlth Org.*, **43**: 71-90 (1970).
10. GESER, A. & AZUMA, Y. *Bull. Wld Hlth Org.*, **22**: 171-176 (1960).