Tuberculin sensitivity in guinea-pigs after vaccination with varying doses of BCG of 12 different strains *

A. LADEFOGED,[1] K. BUNCH-CHRISTENSEN,[2] & J. GULD [3]

In several previously reported studies a number of BCG strains, including those most widely used in vaccine production, were ranked according to their in vivo *activity in various experimental models in rodents and to the local and allergic response that they provoked in children. In this report, 12 strains are ranked in terms of tuberculin conversion in guinea-pigs according to the minimum sensitizing dose. For 10 of these strains, this minimum dose is very low, ranging from 5 to 50 culturable particles. Thus the traditional practice of tuberculin-testing guinea-pigs that have been vaccinated with a full human dose of BCG (of the order of one million culturable particles) has such a low discriminating power that it is useless as a routine test for currently used vaccines. The ranking obtained in this model was largely but not completely in accordance with similar rankings in other models. The Tokyo strain and, to some extent, the London strain ranked comparatively lower than they do in children. The pronounced lack of virulence of the Prague strain was confirmed.*

Guinea-pigs are widely used in the laboratory control of the biological activity of BCG vaccine, mostly because reactions to tuberculin in guinea-pigs are akin to those in man. But there is one great difference between guinea-pigs and human beings in their response to BCG inoculation. Whereas, in man, the tuberculin sensitivity is a function of the dose of BCG, as reported by Edwards et al. in 1953 (*6*), the tuberculin sensitivity and immunogenic effect in guinea-pigs are independent of the vaccine dose over a wide range, as reported by Jespersen in 1956 (*10*) and by Tolderlund et al. in 1960 (*15*). Large doses of BCG induce an earlier but not an ultimately stronger immunity than does even a remarkably small dose. It would seem that BCG is sufficiently virulent in guinea-pigs for it to multiply freely until the immunization has reached a maximum. A similar phenomenon was observed in experiments of protection in bank voles by Jerspersen in 1954 (*9*), and confirmed by Jespersen & Weis Bentzon in 1964 (*11*) and by Ladefoged et al. in 1970 (*12*). In comparing BCG strains expected to differ only slightly, it would seem justified to make a kind of titration, i.e. to vaccinate with decreasing doses of vaccine in order to estimate the lowest dose that induces tuberculin sensitivity and immunity, rather than to compare the effect of large doses of BCG.

We carried out a preliminary study along these lines (unpublished data, 1965) to compare 8 different BCG products, and showed the products to differ more clearly in terms of allergenic potency than of protective power. For some of the products, 10 culturable particles of BCG were enough to induce maximum tuberculin sensitivity. Around 50 culturable particles gave almost uniform protection for all products.

Based on these preliminary results and aiming at a characterization and ranking of BCG strains widely used in man, a number of further studies have been carried out in the WHO Collaborating Centre for BCG. As in our previously published studies of the effect of BCG in the hamster and the bank vole (*3*, *4*, *12*), liquid vaccines from the various strains were

* From the WHO Collaborating Centre for world-wide reference for BCG seed lots and for co-ordination of control of BCG products, BCG Department, Statens Seruminstitut, Copenhagen, Denmark.

[1] Research Assistant, BCG Department, Statens Seruminstitut.

[2] Chief, BCG Department, Statens Seruminstitut.

[3] Medical Officer, Tuberculosis, Division of Communicable Diseases, World Health Organization, 1211 Geneva 27, Switzerland.

prepared in this laboratory with a uniform technique aiming at uniform *in vitro* properties. A number of these vaccine batches have been used not only for the guinea-pig studies reported here but also for the investigations in man reported by Vallishayee et al. (*16*). This report, which is the last in a series, covers the results from 8 experiments in guinea-pigs, in which 12 different BCG strains were compared. The studies were undertaken in 1966–70. The guinea-pig investigations were planned to cover both the tuberculin sensitivity induced and the immunogenic power. However, while the results in terms of tuberculin sensitivity were very clear-cut, the results from the protection tests were much less precise.

METHOD AND MATERIALS

Design

Twelve BCG strains were examined during eight uniformly designed experiments. In experiments 1 and 2, four BCG strains were examined, in experiment 3, three BCG strains, and in each of the five remaining experiments, five BCG strains were involved. Each strain was examined in two or three experiments (Table 1).

For each BCG strain, doses ranging in fourfold steps from 1 to 1000 culturable particles, always in 0.1 ml (estimated from colony counts on solid medium) were inoculated into guinea-pigs by the intradermal route. Five to ten animals (equal numbers of males and females) were used for each dose, and 25–50 animals for each unvaccinated placebo group.

Table 1. Strains of BCG used in the eight experiments

Strain of BCG	Experiment							
	1	2	3	4	5	6	7	8
Copenhagen 1331	×	×	×	×	×	×	×	×
Former Danish strain	×	×						
Madras 809	×	×	×					
Prague 725	×	×	×					
Paris 1173 P2				×	×			
Moscow 3522				×	×			
Tokyo 172				×	×			
Y				×	×			
Rio de Janeiro (Moreau)						×	×	×
Gothenburg						×	×	×
London F 10						×	×	×
R						×	×	×

Twelve weeks after vaccination the vaccinated guinea-pigs and the unvaccinated group given placebo were tested with 10 TU of tuberculin RT 23 with Tween 80. The indurations were read 24 hours later by a specially trained nurse from the Danish Institute for Clinical Epidemiology, Copenhagen. That particular time for tuberculin-testing was chosen because, as shown by Tolderlund (*15*) with a 1/100 000 dilution of Danish vaccine (about 20 culturable particles), maximum allergy is obtained by the twelfth week.

As the studies were designed also to examine the protective effect of the vaccine, the guinea-pigs were challenged by the intraperitoneal route with virulent *Mycobacterium tuberculosis* one week later. Around 7–12 weeks after challenge, the animals were killed. Autopsy was performed, the spleen and omentum were weighed, and the degree of tuberculosis in the organs was determined on the basis of the macroscopic findings.

BCG strains

Strain 1331, which has been used in Copenhagen and Madras since 1966, was included in all eight experiments. The other strains were those routinely used in Prague (725), Paris (1173 P2), Moscow (3522), Tokyo (172), Rio de Janeiro (Moreau), Gothenburg, and London (Glaxo F10). The former Danish strain and the strain used in Madras (809) until 1966, as well as two experimental strains, designated Y and R, were also examined. Both the latter strains were obtained from the CIBA laboratories, Basle. Y is derived from the " Phipps " strain that was used by Aronson (*1*) in a controlled trial in American Indians, and R is derived from a single colony obtained from the Tokyo strain (H. Bloch, personal communication, 1965).

Vaccine preparations

All strains were grown as surface cultures on Sauton medium and harvested after 10–11 days. Liquid vaccine was prepared according to the routine method used in the BCG Department, Statens Seruminstitut, Copenhagen. The strains from Paris and Madras grew faster than the other strains did and showed pronounced pigmentation. The London strain grew more slowly than the others and the morphology of the surface culture was distinctly different from that of the other strains.

Table 2 shows the results of *in vitro* tests per-

Table 2. *In vitro* characteristics of BCG suspensions

Strain	Experiment No.	Opacity (suspension of 0.75 mg/cm³)	Oxygen uptake (μl per 120 mg per hour)	Germination rate (percentage at 24 h) [a]	Culturable particles (millions/mg)
Copenhagen 1331	1	0.34	118	75	21.2
	2	0.38	145	75	29.2
	3	0.32	171	75	19.0
	4	0.40	167	75-90	21.6
	5	0.34	150	75	11.4
	6	0.30	150	75-(90) [a]	12.8
	7	0.40	173	75	21.2
	8	0.36	166	75	15.5
Former Danish strain	1	0.42	149	75	28.3
	2	0.43	141	75	27.9
Madras 809	1	0.44	123	75-90	25.5
	2	0.34	138	75-90	23.9
	3	0.38	192	75-90	22.2
Prague 725	1	0.42	125	75-90	15.3
	2	0.43	129	50-75	17.6
	3	0.33	130	75	15.6
Paris 1173 P2	4	0.29	149	75-90	17.3
	5	0.37	110	75	12.9
Moscow 3522	4	0.39	208	75-90	34.6
	5	0.35	209	75	24.4
Tokyo 172	4	0.38	195	75	79.7
	5	0.35	194	75-90	73.2
Y	4	0.42	143	75	16.3
	5	0.38	134	75	15.3
Rio de Janeiro (Moreau)	6	0.32	183	75-90	17.4
	7	0.41	218	75-90	28.7
	8	0.41	155	75	22.9
Gothenburg	6	0.30	182	75	39.0
	7	0.40	166	75	48.0
	8	0.37	188	75	29.7
London F 10	6	0.32	90	75	5.0
	7	0.36	84	50	7.1
	8	0.36	83	50-75	6.2
R	6	0.33	190	75	25.6
	7	0.34	174	75	43.4
	8	0.40	161	75	25.3

[a] The result is considered to be closer to the figure not in parenthesis.

formed on the vaccines from the various strains. The strain from Tokyo yielded vaccines with a very high colony count and also the strain from Gothenburg showed a rather high colony count. The London strain yielded vaccines with a lower proportion of live organisms, as shown by the oxygen uptake, germination rate, and colony count. Otherwise, the vaccines from the various strains were much alike in a number of *in vitro* tests.

Animals

The guinea-pigs were 9–11 weeks old at the time of vaccination. The animals were, separately for each experiment and each sex, randomly allocated to the different strains and doses of BCG and to a placebo. Three guinea-pigs of the same sex were placed in each cage, and the cages were placed in the animal house in a formally randomized order.

Follow-up and analysis

Of the 3100 guinea-pigs included in the experiments, 2626 were vaccinated and 474 were given placebo only. Between vaccination and tuberculin-testing, 78 guinea-pigs died and have been excluded from the analysis: 62 in the vaccinated groups and 16 in the placebo groups. The tuberculin reactions for the individual animals are given in the Appendix tables [a].

RESULTS

The results are illustrated in Fig. 1–8 in terms of mean tuberculin reactions as a function of vaccine dose in nanograms of semidry weight. (Dosages in culturable particles can be derived from Table 2.) With very low dosages, around 0.5 ng (5–20 culturable particles), the strains are seen to differ. For dosages around 5 ng (50–200 culturable particles) the curves tend to level off, showing maximum reactions, except for the Prague strain (725), which shows only a slight increase in the tuberculin reactions even with a dose as high as 30–60 ng (around 500–1000 culturable particles).

The consistency of the experiments can be examined by comparing the graphs (Fig. 1–8). In experiments 1 and 2, dealing with the same 4 strains, and in experiment 3, in which 3 of these strains are included, the ranking of the strains is consistent, the Madras strain (809) being the strongest and the Prague strain (725) the weakest. Strain 1331 and the former Danish strain appear to be slightly weaker than Madras 809 and to an equal extent.

Of the 5 strains tested in experiments 4 and 5, those from Moscow, Paris, and Copenhagen seem to be almost equal in strength; the Tokyo strain is weaker, and strain Y the weakest.

In experiments 6, 7, and 8, the Rio de Janeiro, Gothenburg, and Copenhagen strains are the strongest. The Glaxo strain shows the weakest reactions and the R strain gives conflicting results. In two of the experiments—6 and 8—the curves for the R strain are similar to those obtained for the Tokyo strain in experiments 4 and 5. From experiment 7 it is obvious that a much larger dose (at least 8 times as large) of the R strain is needed for obtaining maximum reactions. Similar inconsistency in the R strain was observed in our experiments in the bank vole (*12*). There is no obvious explanation of the erratic behaviour of this strain.

Results in terms of protection

The protection tests gave much more variable results than the tuberculin tests. In some cases the animals were killed too early, and the tuberculous disease in the control animals was only slight. In other cases the animals were killed too late, and the disease was too severe. The strain used for challenge was apparently attenuated and the challenge dosage was not under sufficient control. The results (not shown), while very imprecise, did not contradict those reported here: guinea-pigs with increased tuberculin reactions after vaccination all showed some degree of protection compared with unvaccinated guinea-pigs.

In an examination of four different strains in the same model, Jespersen & Bentzon (*19*) found an association between tuberculin sensitivity and immunogenic potency, and concluded that the former can be used as an indication of the latter in this model.

DISCUSSION

The guinea-pig in quality control of BCG

The minimum sensitizing dose varied, among the strains examined, from 10 to 1000 culturable particles—even disregarding the weakest of the strains examined. Above this minimum dose, the response in guinea-pigs is dose-independent, in contrast to man, in whom (as already mentioned) the response to BCG is quantitative and dose-dependent. It is a

[a] The Appendix tables have been deposited in the WHO Library, and single copies may be obtained on request to: Chief Librarian, World Health Organization, 1211 Geneva 27, Switzerland.

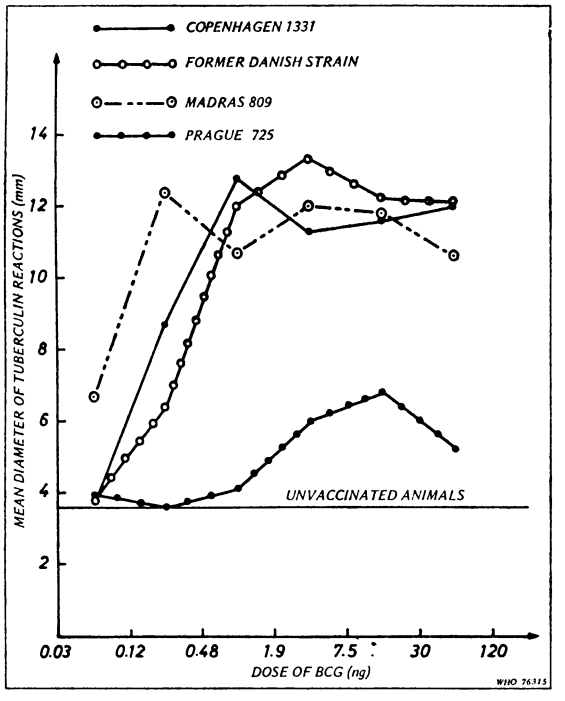

Fig. 1. Tuberculin reactions by strain and dose of BCG (experiment 1)

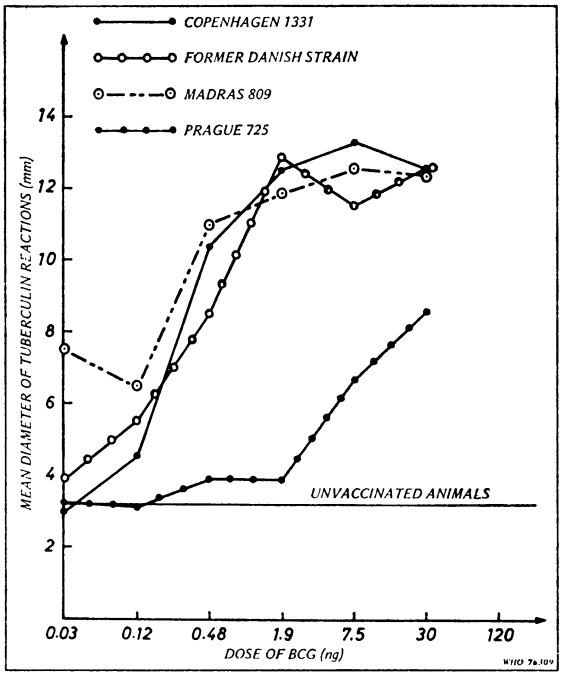

Fig. 2. Tuberculin reactions by strain and dose of BCG (experiment 2)

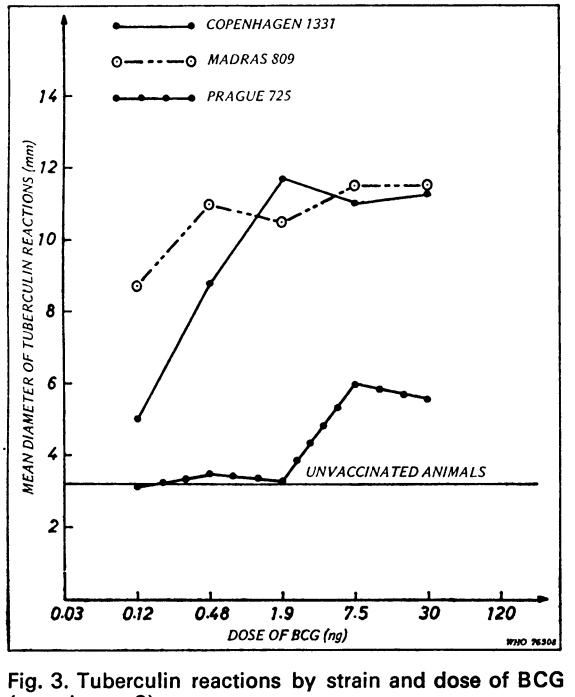

Fig. 3. Tuberculin reactions by strain and dose of BCG (experiment 3)

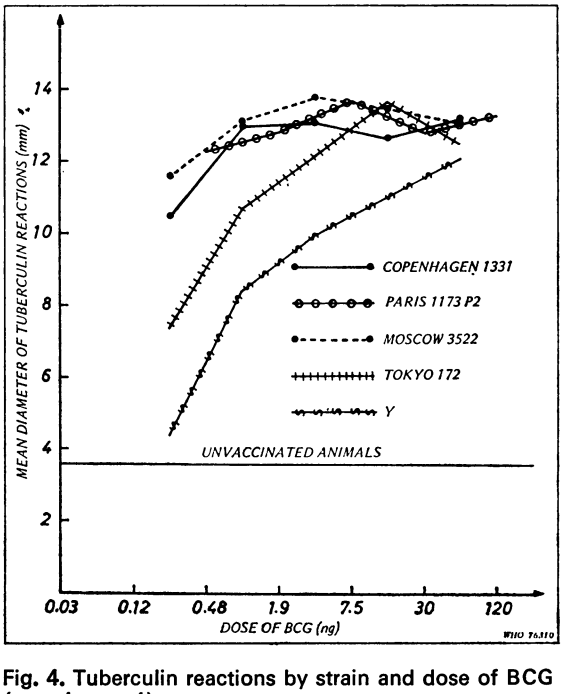

Fig. 4. Tuberculin reactions by strain and dose of BCG (experiment 4)

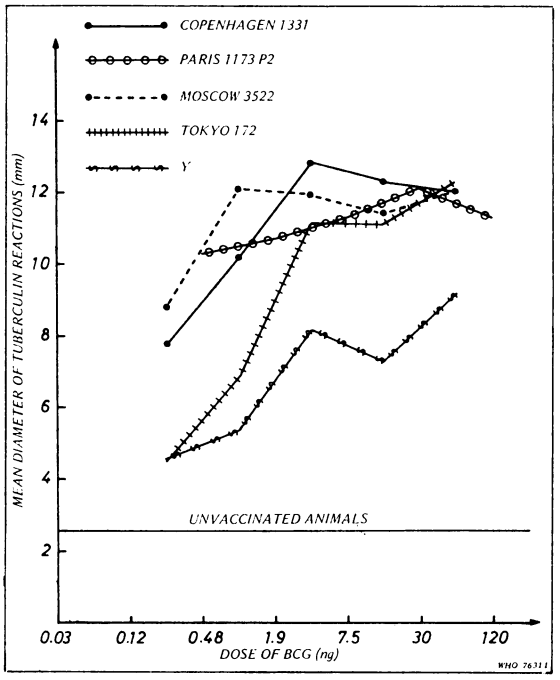

Fig. 5. Tuberculin reactions by strain and dose of BCG (experiment 5)

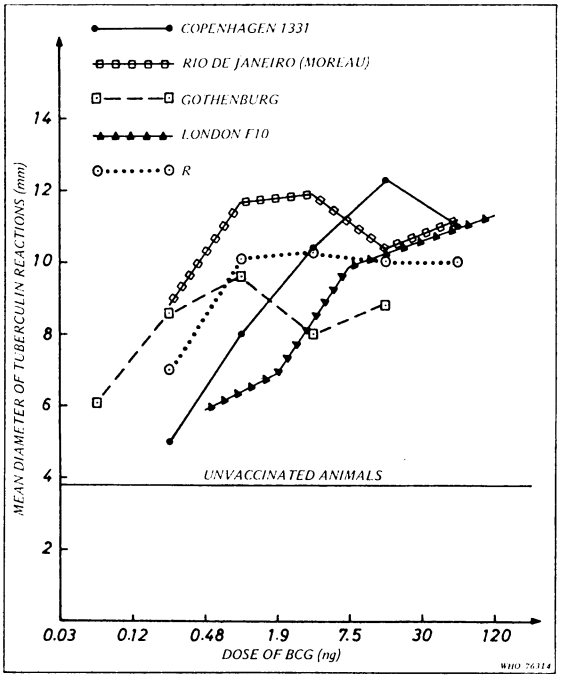

Fig. 6. Tuberculin reactions by strain and dose of BCG (experiment 6)

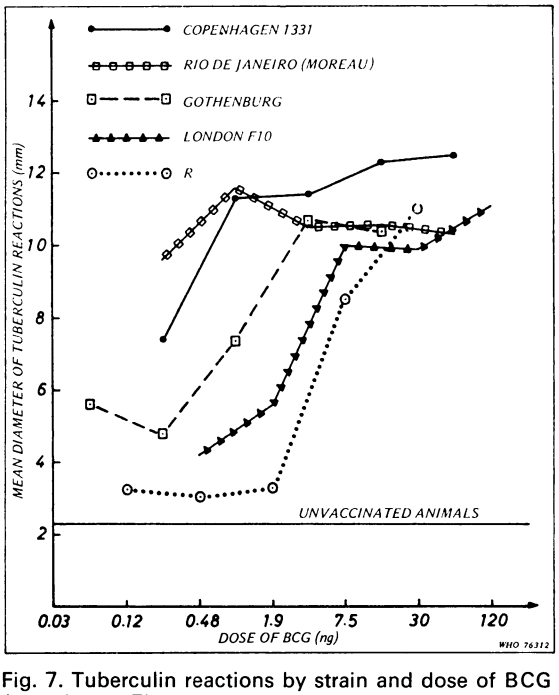

Fig. 7. Tuberculin reactions by strain and dose of BCG (experiment 7)

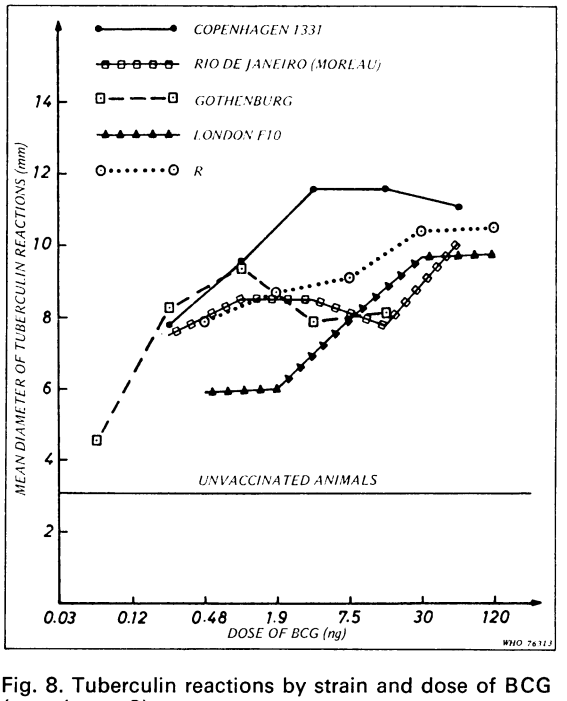

Fig. 8. Tuberculin reactions by strain and dose of BCG (experiment 8)

reasonable inference from the findings that, if the initial dose is sufficient to start multiplication at all, BCG multiplies freely in the guinea-pig (more so than in man) until the maximum immune effect is reached.

In many laboratories, a " test of skin reactivity in guinea-pigs " is carried out for every batch of vaccine, including the use of a human intradermal dose of BCG (*18*). In some laboratories, the same animals (or even animals given 50 human doses each, for the purpose of the safety test) are further tested with tuberculin after an interval so as to demonstrate the allergenic potency of the vaccine. It appears from the present results, as well as from earlier publications, that as a routine such a tuberculin test is meaningless because of the high BCG dosage. One could imagine instead a test of the " smallest effective allergenic dose ", but to carry out a test for every batch would in most cases be prohibitively expensive; for an otherwise well-examined strain it may indeed be merely a particularly expensive way of examining the viability of the batch. It is therefore proposed to use the model of the smallest effective dose only, as at present, for comparisons of strains—that is, seed-lots.

Ranking of strains

In 1956 Dubos (*5*) advanced the hypothesis that " invasiveness "—i.e., virulence as expressed by the ability of a BCG strain to multiply in the organs of the mouse—was associated with protective effect against virulent challenge. This hypothesis was confirmed in 1961 by Villis & Vandiviere (*17*) by a retrospective comparison of the effect of various strains in animal models, with controlled trials in human beings of three of these strains. A further discussion of this evidence was presented by Guld in 1971 (*8*).

On the basis of all the data from our experiments in four different models (see also *3*, *4*, *12*, *16*), it should now be possible to rank the strains according to *in vivo* activity, or virulence, in mammalian hosts. A strict ranking is not entirely possible because only one strain (1331) has been used consistently throughout: the choice of the other strains for the various experiments rather reflects the changing emphasis on the importance and promise held out by one or another strain—an emphasis that changed with time as experimental results accumulated, but also with developments in production practices. We have not attempted a strictly quantitative biometrical analysis, but should like to offer the following scoring and ranking, based on an inspection of the graphic presentations only.

	Hamsters	*Bank voles*	*Guinea-pigs*	*Children*	*Mean score*
Rio de Janeiro (Moreau)	5	5	4	5	4.8
Paris (1173 P2)	4	5	4	4	4.3
Copenhagen (1331)	4	4	4	4	4.0
Moscow	3	4	4	5	4.0
Gothenburg	4	5	3	3	3.8
Madras (former strain)	1	5	5	3	3.5
Copenhagen (former strain)	3	—	4	3	(3.3)
Tokyo	3	2	2	5	3.0
R	3	1–4	1–4	—	(2.7)
Y	2	4	1	3	2.5
London (Glaxo)	2	2	2	2	2.0
Prague	1	2	0	1	1.0

The mutual ranking of about half of these strains (those listed first), might very likely shift if yet another model were introduced, and that of strains close in ranking might even shift if the experiments were repeated.

For three of the more active strains, Paris, Copenhagen, and Moscow, there has been wide practical experience over a number of years. For the Moreau strain, scoring uniformly high in all models, there has been unfortunately only scanty experience in man with vaccines of high viability. The Paris and Copenhagen strains have given rise from time to time to complaints about complications (regional lymphadenitis)—nearly always in the new-born and associated with an unintentionally high dosage. While suppurative lymphadenitis after BCG is entirely benign and self-healing, a sudden increase in its frequency tends to upset both the public and the medical profession and thus may endanger the acceptability of a programme. On the other hand, its occurrence may be taken as yet another piece of evidence, in yet another " *in vivo* model ", that these are active, " invasive " strains in the sense meant by Dubos.

The Gothenburg strain has been used in the new-born and in infants in Sweden and Finland for many years, with an obvious protective effect, as reported by Bjartveit & Waaler (*2*). But in recent years there have also been reports of complicating osteomyelitis (G. Dahlström, unpublished observations, 1976) that is not entirely benign and needs treatment. Although rare, this complication is not negligible when vaccination of this age group is universal and infant tuberculosis is rare. Whether it is specific for the

Gothenburg strain is not known, since no other strain has been used as widely in the new-born in an area with well developed health services.

The strain formerly used in Copenhagen is of no particular interest compared with 1331, which was frozen as a seed-lot earlier. The strain formerly used in Madras, which scores low in hamsters and high in bank voles and guinea-pigs, is a striking example of disagreement between models.

That the London strain scores rather low throughout these studies is not an unambiguous finding. It was prepared for these experiments with a technique (surface growth) to which it is not adapted, and its viability was obviously lower (perhaps by 50%) than that which would have been obtained with the deep culture technique normally used for it. But even allowing for this the strain still appears to be rather low in rank. The Tokyo strain is perhaps more interesting. While second to no strain in inducing delayed hypersensitivity in children, it is definitely weak in bank voles and guinea-pigs. Actually, the response in guinea-pigs would have appeared even weaker had it been presented in terms of culturable particles and not moist weight. Whereas, for the Paris strain, the lowest dose used—approximately 7 culturable particles—gave an average tuberculin reaction size of 23 mm, it took approximately 70 culturable particles of the Tokyo strain to give an average of 18 mm.

The London and the Tokyo strains have both been widely used over the past decade, because 15 years ago they happened to be available as the first freeze-dried vaccines of high stability.

The Y and R strains are of historical interest only. The former is a laboratory strain derived from a vaccine that gave high protection in man 40 years ago, in Aronson's trial in American Indians (*1*), but there is no evidence that the strain has not become less active over the years. The R strain gave highly variable results. While derived from a single colony, it appears not to be genetically homogeneous.

The Prague strain is an interesting example of low allergenicity combined with low immune effect. A similar association was reported by Engbaek et al. (*7*) and by Mackaness (*13*).

Admittedly, the lack of complete agreement between different animal models raises the question of the validity of each model for man. However, taken together and as far as they agree, they can scarcely be completely disregarded in selecting a BCG strain for production.

No doubt better animal models are desirable. An elegant model has quite recently been proposed by Fok et al. (*14*), using respiratory challenge with so few viable units that not all lung lobes are involved in the primary infection. By counting the number of infected lobes after a suitable interval, the prevention of haematogenous spread to other lobes (which is the direct effect of BCG, also in man) can be directly observed. The use of this technique in an animal with approximately the same susceptibility as man to *M. tuberculosis* and to BCG (e.g., the white mouse and, if possible, also a non-rodent) would intuitively appear more valid than any other protection test in a particular animal and, it is to be hoped, also more relevant for man.

RÉSUMÉ

SENSIBILITÉ DES COBAYES À LA TUBERCULINE APRÈS VACCINATION PAR DIVERSES DOSES DE BCG PROVENANT DE 12 SOUCHES DIFFÉRENTES

Dans plusieurs études rapportées antérieurement, un certain nombre de souches de BCG, et notamment celles qui sont le plus couramment utilisées pour la production de vaccin, ont été classées en fonction de leur activité *in vivo* chez les rongeurs dans différents modèles expérimentaux, et en fonction de la réponse locale et allergique chez les enfants. Dans le présent travail, 12 souches sont classées sur la base du virage de la réaction tuberculinique chez le cobaye, selon la dose sensibilisante minimale.

La souche 1331, employée à Copenhague et à Madras depuis 1966, a figuré dans toutes les expériences. Les autres souches étaient celles qui sont couramment utilisées à Prague (725), à Paris (1173 P2), à Moscou (3522), à Tokyo (172), à Rio de Janeiro (Moreau), à Gothenbourg et à Londres (Glaxo F10). L'ancienne souche danoise et la souche utilisée à Madras (809) jusqu'en 1966 ont aussi été examinées ainsi que deux souches expérimentales désignées par Y et R, ces deux dernières provenant des laboratoires CIBA de Bâle.

Toutes les souches ont été cultivées en surface sur milieu de Sauton et récoltées au bout de 10 à 11 jours. Le vaccin liquide a été préparé selon la méthode habituellement utilisée au Département du BCG, Statens Seruminstitut, Copenhague. Les souches de Paris et de Madras ont poussé plus rapidement que les autres

et présentaient une pigmentation prononcée. La souche de Londres a poussé moins vite que les autres, et la morphologie de la culture superficielle était nettement différente de celle des autres souches.

Le tableau 2 montre les résultats d'un certain nombre d'épreuves *in vitro* effectuées sur les vaccins provenant des différentes souches.

Pour chaque souche de BCG, on a inoculé à des cobayes, par voie intradermique, des doses croissant de 4 fois en 4 fois, à partir de 1 à 1000 particules cultivables, sous le volume constant de 0,1 ml (les estimations étaient faites d'après les numérations des colonies sur milieu solide). On a utilisé 5 à 10 animaux (nombre égal de mâles et de femelles) pour chaque dose et 25 à 50 animaux pour chaque groupe non vacciné recevant un placebo. Douze semaines après la vaccination, les cobayes vaccinés et ceux qui avaient reçu le placebo ont été éprouvés par 10 unités de tuberculine RT 23 additionnée de Tween 80.

Les résultats des diverses expériences sont représentés dans les figures 1 à 8. Pour 10 des 12 souches étudiées, la dose minimale sensibilisante est extrêmement faible, allant de 5 à 50 particules cultivables. Ainsi, la méthode traditionnelle consistant à éprouver à la tuberculine les cobayes vaccinés avec la totalité d'une dose destinée à l'homme (qui est de l'ordre de 1 million de particules cultivables) a un pouvoir de discrimination tellement faible qu'elle est inutile en tant qu'épreuve de routine pour les vaccins utilisés actuellement.

Le classement obtenu avec ce modèle concordait en grande partie, mais pas totalement, avec des classements identiques établis avec d'autres modèles. Le rang de la souche de Tokyo, et, dans une certaine mesure, celui de la souche de Londres, ont été nettement plus bas d'après ce modèle que d'après leur effet chez l'enfant. L'avirulence prononcée de la souche de Prague a été confirmée.

REFERENCES

1. ARONSON, J. D. ET AL. *Archives of internal medicine*, **101**: 881 (1958).
2. BJARTVEIT, K. & WAALER, H. *Bulletin of the World Health Organization*, **33**: 289 (1965).
3. BUNCH-CHRISTENSEN, K. ET AL. *Bulletin of the World Health Organization*, **39**: 821 (1968).
4. BUNCH-CHRISTENSEN, K. ET AL. *Bulletin of the World Health Organization*, **43**: 65 (1970).
5. DUBOS, R. J. & PIERCE, C. H. *American review of tuberculosis and pulmonary diseases*, **74**: 699 (1956).
6. EDWARDS, L. B. ET AL. BCG vaccination. Geneva, World Health Organization, 1953, pp. 125-128 (Monograph series, No. 12).
7. ENGBÆK, H. C. ET AL. *Acta tuberculosea et pneumologica Scandinavica*, Suppl. LVI: 114-117 (1964).
8. GULD, J. *In*: Immunization in tuberculosis. Bethesda, U.S. Department of Health Education & Welfare, 1971, pp. 149-156 (DAEW publication No. (NIH) 72-68).
9. JESPERSEN, A. *Acta pathologica et microbiologica Scandinavica*, **35**: 396 (1954).
10. JESPERSEN, A. *Acta pathologica et microbiologica Scandinavica*, **38**: 203 (1956).
11. JESPERSEN, A. & BENTZON, M. W. *Acta tuberculosea et pneumologica Scandinavica*, **44**: 253, 276 (1964).
12. LADEFOGED, A. ET AL. *Bulletin of the World Health Organization*, **43**: 71 (1970).
13. MACKANESS, G. B. *American review of respiratory diseases*, **97**: 337-344 (1967).
14. FOK, J. S. ET AL. *Journal of infectious diseases*, **133**: 137-144 (1976).
15. TOLDERLUND, K. ET AL. *Bulletin of the World Health Organization*, **22**: 185 (1960).
16. VALLISHAYEE, R. S. ET AL. *Bulletin of the World Health Organization*, **51**: 489 (1974).
17. WILLIS, S. & VANDIVIERE, M. *American review of respiratory diseases*, **84**: 288 (1961).
18. WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION. Eighteenth report. Geneva, 1966, p. 42 WHO Technical report series, No. 329).
19. JESPERSEN, A. & BENZTON, M. W. *Acta pathologica et microbiologica scandinavica*, **71**: 114-131 (1967).