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International Reference Preparations of Typhoid Vaccine

Potency Assay by the Active Mouse Protection Test with Three Different Routes of Immunization

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International collaborative laboratory studies on the International Reference Preparations of Typhoid Vaccine have so far failed to provide data on which international units for these vaccines can be based. Further assays carried out using the active mouse protection test, with immunization by the subcutaneous, intraperitoneal or intravenous route, confirmed the findings by some workers that the International Reference Preparation of Typhoid Vaccine (Acetone-Inactivated) (vaccine K) was more effective than the International Reference Preparation of Typhoid Vaccine (Heat-Phenol-Inactivated) (vaccine L), and indicated that intraperitoneal immunization was the most promising method. Vaccine K, together with the material extracted by the acetone in the preparation of the vaccine, had a significantly lower effectiveness (at the 5% probability level) only when intraperitoneal immunization was used. The reasons for the differences found between the various vaccines and routes of immunization are discussed at length.

It is suggested that challenge with a strain of Salmonella moscow *instead of the strain of* Salm. typhi *used until now gives a true infection and forms the basis of a reliable method for the potency assay of typhoid vaccines.*

The measurement of the protective activity of typhoid vaccines in terms of International Units of the International Reference Preparations of Typhoid Vaccine is not yet possible, owing to the inconclusive results of the international collaborative laboratory studies (Spaun & Uemura, 1964).

In this laboratory since 1959 we have been estimating the potency of typhoid vaccine by the active mouse protection test with a single immunization and subsequent challenge 2 weeks later. Our work for the above-mentioned international collaborative studies led us to the conclusion that the intraperitoneal route of immunization gives results which correspond to the findings obtained in humans—a conclusion that was not supported by the statistical analysis of the combined results of the international collaborative studies (Spaun & Uemura, 1964).

The International Reference Preparation of Typhoid Vaccine (Acetone-Inactivated) (referred to as vaccine K from now on, in conformity with previous usage; see Spaun & Uemura, 1964) was found by us to be more effective than the International Reference Preparation of Typhoid Vaccine (Heat-Phenol-Inactivated) (vaccine L); this finding was obtained by other laboratories in the international collaborative studies, using certain tests (Spaun & Uemura, 1964). Spaun (1964) suggested that the higher immunogenic effect of vaccine K might be connected with a lower toxicity owing to removal of toxic substances during the acetone treatment, and that this possibility could be investigated by assaying the potency of the K vaccine enriched with the material extracted by the acetone.

In the present studies, the potencies of vaccine K, vaccine L and vaccine K together with the acetone extract have been estimated by the active mouse protection test, with subcutaneous, intraperitoneal or intravenous immunization, in an attempt to elucidate the factors responsible for the differences

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between vaccines K and L. Since both vaccines were prepared from the same harvest of living *Salmonella typhi*, strain Ty-2, the differences must lie in the method of manufacture.

MATERIAL

We received several ampoules of freeze-dried acetone extract of vaccine K from the Statens Serum-institut, Copenhagen, Denmark. Each ampoule corresponded to 5 ml of the acetone supernatant of a dense vaccine suspension. When reconstituted with 5 ml of distilled water, 1 ml would be approximately equivalent to the extract from 50 ml of vaccine ready for use (10^9 organisms per ml).

In these experiments we checked the influence of the acetone supernatant used in a concentration 5 times higher than usual. Therefore, in order to obtain 50 ml of vaccine ready for use, we added 5 ml of acetone supernatant, which is equivalent to 250 ml of vaccine.

METHODS

White mice weighing from 13 g to 16 g were used for the vaccine assay. Batches of 10 mice were immunized subcutaneously, intraperitoneally or intravenously with a given dose of a given vaccine. The doses ranged from 1.28×10^4 to 4×10^7 cells in fivefold steps. The immunization was carried out only once. The interval between immunization and challenge was 15 days. The challenge doses of living *Salm. typhi* (Ty-2) suspended in saline were about 5 LD_{50}–10 LD_{50}.

The number of survivors was recorded on the third day. The statistical evaluation of results was carried out by probit regression analysis (Finney, 1952).

RESULTS

The results of these experiments are presented in the accompanying table. In general the acetone-inactivated vaccine K was more effective than the heat-phenol-inactivated vaccine L.

Our findings were in good agreement with the results of studies reported by Spaun (1964) on the influence of the route of immunization.

The 4 series of assays performed using the subcutaneous route of immunization gave consolidated potencies of vaccine L relative to that of vaccine K of 3.85, 1.67, 0.31 and 0.41. We have tested the homogeneity of these potencies by a χ^2 test and

POTENCIES OF VACCINE L AND VACCINE K + ACETONE SUPERNATANT, RELATIVE TO THAT OF VACCINE K ALONE, ESTIMATED BY THE ACTIVE MOUSE PROTECTION TEST AT DIFFERENT TIMES BETWEEN JANUARY 1961 AND JULY 1965 AND WITH DIFFERENT ROUTES OF IMMUNIZATION

Route of immunization	No. of tests	Period	Relative potency (and 95 % confidence limits) [a]	
			Vaccine L	Vaccine K + acetone supernatant
Subcutaneous	3	January 1961	3.85 [b] (2.56–5.88)	
	4	February-April 1963	1.67 (0.93–3.03)	
	3	December 1964–March 1965	0.31 [c] (0.099–0.97)	
	3	July 1965	0.41 [c] (0.17–0.99)	0.87 (0.38–1.98)
Intraperitoneal	3	Spring 1962	0.28 [d] (0.11–0.69)	
	4	February-April 1963	0.44 (0.10–1.92)	
	6	April-May 1964	0.18 [b] (0.098–0.33)	0.50 [c] (0.28–0.90)
	3	December 1964–March 1965	0.12 [b] (0.048–0.31)	
Intravenous	4	February–April 1963	0.17 [b] (0.08–0.36)	
	3	December 1964–March 1965	0.13 [b] (0.048–0.37)	
	3	July 1965	0.18 [b] (0.094–0.36)	0.70 (0.37–1.33)

[a] Vaccine K = 1.
[b] Significantly different from 1 at the 0.1 % probability level.
[c] Significantly different from 1 at the 5 % probability level.
[d] Significantly different from 1 at the 1 % probability level.

found $\chi^2=34.37$ (3 degrees of freedom). There is thus only a slight probability ($P<0.1\%$) that the different potencies of this series are mutually compatible. We have no explanation for this finding. The 4 consolidated potencies of vaccine L relative to vaccine K estimated by the intraperitoneal route of immunization—0.28, 0.44, 0.18 and 0.12—are mutually compatible according to the χ^2 test ($\chi^2=2.83$; 3 degrees of freedom). The mean potency for this series is 0.20 with 95% confidence limits of 0.13 and 0.30. The 3 consolidated potencies estimated by the intravenous route of immunization—0.17, 0.13, 0.18—were also mutually compatible ($\chi^2=0.26$; 2 degrees of freedom). The mean potency is 0.17 with 95% confidence limits of 0.11 and 0.26.

The potency of vaccine K with acetone supernatant relative to that of vaccine K alone was only found to be significantly different from unity when tested by the intraperitoneal route of immunization (relative potency of 0.5, significantly different from 1 at the 5% probability level).

DISCUSSION

As we have mentioned, we can offer no explanation for the discrepancies observed between the potencies estimated using subcutaneous immunization. Great efforts were made at the time of the collaborative studies to find an experimental reason for the outcome of our tests, but none could be detected. The possibility that the samples of vaccines K and L had been interchanged was ruled out with certainty. Two different scientific workers in two different laboratories of the vaccine department found vaccine L to be more potent than vaccine K when given subcutaneously, in both 1961 and 1963.

Our opinion that the subcutaneous route gives less consistent results is not only based on these findings. When we performed the assay using a single subcutaneous injection for immunization and the O strain of *Salm. typhi* for challenge the K vaccine was also significantly weaker than the L vaccine (Spaun & Uemura, 1964).

There is an indication (Pittman & Bohner, 1966) that in a type N sc assay (mucin challenge) vaccine K was slightly but significantly less potent than vaccine L (relative potency 0.78), while in a type P sc assay (saline challenge) the difference was more marked (0.54).

The results obtained in the laboratory of Dr Pittman in the USA and in our laboratory in the USSR using the intraperitoneal route of immunization were also nearly identical. Their potency value for vaccine K relative to vaccine L was 3.69 obtained with an N ip assay, while our value with a type P ip assay was 3.63.

At the same time it is known that Leslie, Wetterlow & Edsall [1] did not find good correlation between the results of the active mouse protection test using the intraperitoneal route of immunization and the findings obtained in human beings in the field trial in the USSR when vaccine L was compared with alcoholized vaccine. They suggested that the correlation between results obtained by the intraperitoneal route in the active mouse protection test on vaccines L and K and findings obtained in humans should be regarded as only coincidental, and that this route should be avoided.

We suppose that the reason for the influence of the route of immunization on the development of resistance in the animal is connected with (*a*) the different sensitivities of the peritoneum and subcutis to the harmful action of soluble antigens of microbial cells and of other toxic and sensitizing factors; (*b*) the different capacities of the peritoneum and subcutis to influence the development of immunity in the organism of animals when the antigen has no harmful action; in this case the peritoneum is more active than the subcutis; and (*c*) the qualities of different typhoid vaccines.

We think that the discrepancies between the results of the active mouse protection test using different routes of immunization and the findings in human beings are due to the fact that the nature and mechanism of immunity in mice and in human beings are quite different. Anti-endotoxic immunity, which plays the main role in the mechanism of mouse resistance, is less important for humans.

It is well known that typhoid does not generally produce true infection in mice, and that death is the result of severe intoxication after challenge with very large doses of microbial cells of *Salm. typhi*. Consequently, the results of the mouse protection tests and their correlation with findings in field trials will depend not only on the route of immunization but on the method of preparation of the vaccine. The different methods of treating live microbial cells do not render the antigens which are important in

[1] Unpublished document WHO/BS/66.861, obtainable, on request, from Biological Standardization, World Health Organization, Geneva, Switzerland.

the development of anti-endotoxic and anti-infectious immunity safe to the same degree. The former type of immunity is more important for mice and the latter for humans.

Each different method of treating the microbial suspensions has a different influence on the antigens of the typhoid vaccines. The washing of cells with acetone removes from the vaccine the substances which have sensitizing and toxic effects and are especially harmful when administered intraperitoneally. Therefore, mice give good results when they are immunized intraperitoneally by acetone-treated vaccine.

In acetone-treated vaccine, the antigen which is responsible for the development of anti-infectious resistance is not damaged and therefore it also provides high, stable immunity in humans.

Alcohol treatment causes damage to the antigen responsible for the development of anti-infectious immunity, but not to that responsible for the development of anti-endotoxic immunity. The alcohol-treated vaccine therefore protects mice very well against death caused by severe intoxication, but affords less effective protection against the development of typhoid in humans.

Treatment by heat and phenol damages the antigen important for the development of anti-endotoxic immunity (we think that this is the Vi antigen); the protection of mice by this vaccine is therefore less effective than that by acetone- and alcohol-treated vaccines. Intraperitoneal injection gives poor results because, apart from the reason mentioned, the microbial cells are not washed as carefully as in acetone- and alcohol-treated vaccines and the sensitizing factors of the heated vaccine damage the peritoneum, which is more sensitive than the subcutis. But the antigen in this vaccine responsible for resistance to infection (we suppose that this is the O antigen) remains intact or very little damaged. Therefore the heat-phenol-treated vaccine affords good immunity in humans—slightly less than, or equal to, that provided by acetone-treated vaccine and higher than that provided by alcoholized vaccine.

Thus, the acetone-treated vaccine provides the highest resistance in human beings, the heat-phenol-treated vaccine comes next, and the alcoholized vaccine is the weakest, while in mice the acetone- and alcohol-treated vaccines give the best protection, especially when injected intraperitoneally. The heat-phenol-heated vaccine is not so good for mice.

It is of interest to note that in other investigations by us on the reactivity of different types of typhoid vaccines using the Schwartzman phenomenon on rabbits, it had been shown that heat-phenol-treated vaccines have the strongest sensitizing action, especially those prepared using liquid media and aeration for the growth of microbial cells. This is connected with the presence of a great amount of soluble antigens in these vaccines, as shown in our experiments by haemagglutination-inhibition tests.

The alcoholized vaccines whose microbial cells had been grown in the same way (liquid media and aeration) gave much weaker Schwartzman reactions than the heat-phenol-treated vaccines. We think that the reason for this difference is the absence of heating and the repeated washing of cells with alcohol solutions (Melikova & Lesnjak, 1965; Lesnjak & Melikova, 1965; Melikova, Lesnjak & Kovalskaja, 1966).

There may be a difference between the alcohol-treated vaccine and the acetone-treated vaccine which could account for the discrepancies observed between the modest efficacy of the alcohol-treated vaccine in field trials on humans and the results of the active protection test in mice, particularly when the intraperitoneal route of immunization is used.

It should be noted that vaccines L and K also have a marked sensitizing action on rabbits, which is higher than that of alcohol-treated vaccines.

Active mouse protection tests in mice challenged with a typhoid strain which is able to produce a true infection would be expected to give more consistent and reliable results than those obtained hitherto.

The experiments performed in our laboratory by Lvovskaja (1963, 1965, 1966) showed that it was possible to obtain more objective data on the protective activity of different typhoid vaccines when immunized mice are challenged with a special strain of *Salm. moscow*, which is in group D of Kauffmann's classification. This strain gives a true infection in mice 5 or 6 days after injection, and the mice die in 2 to 3 weeks if they are not protected. The LD_{50} of this strain is nearly 30 microbial cells. Typhoid vaccine can give protection against the infection produced in this way.

The possibility of estimating the potency of different typhoid vaccines more objectively by the active mouse protection test using an appropriate route of immunization and challenge by *Salm. moscow* is being studied in our laboratory.

RÉSUMÉ

Les essais collectifs visant à définir à l'aide d'épreuves de laboratoire l'activité des préparations internationales de référence de vaccins antityphoïdiques n'ont pas donné de résultats concluants, et l'on n'est pas encore en mesure de définir une unité internationale de ces préparations.

Les recherches décrites ici ont fait appel au test de protection active de la souris: administration par voie sous-cutanée, intrapéritonéale ou intraveineuse d'une dose unique de vaccin, suivie 14 jours plus tard d'une inoculation d'épreuve de *Salmonella typhi* souche Ty-2 en soluté salin. Les résultats ont confirmé ceux d'observations antérieures et montré la supériorité du vaccin inactivé par l'acétone (vaccin K) sur le vaccin inactivé par la chaleur et le phénol (vaccin L). L'immunisation par voie intrapéritonéale est la méthode de choix.

On a émis antérieurement l'hypothèse que l'activité immunogène supérieure du vaccin K serait liée à une toxicité moindre résultant de l'élimination de substances toxiques lors du traitement par l'acétone. Des essais d'activité au moyen d'un vaccin K enrichi d'extraits acétoniques ont montré que l'efficacité de cette préparation n'est significativement inférieure à celle du vaccin K seul que si l'on recourt à la voie intrapéritonéale pour l'immunisation.

Les auteurs exposent leurs vues sur les raisons de ces différences d'activité entre vaccins et sur le rôle des modalités techniques de l'immunisation. Toute comparaison entre les résultats d'essais de laboratoire et les données recueillies sur le terrain doit tenir compte des particularités de la réponse immunitaire chez l'animal d'expérience d'une part et chez l'homme d'autre part.

De nouvelles recherches vont être entreprises en utilisant comme matériel d'épreuve *Salm. moscow*, qui détermine une infection vraie chez la souris, pour tenter de mettre au point une méthode d'évaluation plus objective.

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