Bull. Org. mond. Santé } 1950, 3, 33-42
Bull. World Hlth Org. }

CASEIN HYDROLYSATE CHOLERA VACCINE [a]

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Manuscript received in March 1950

It is customary to prepare prophylactic vaccines against bacterial infections from growths of the organisms on agar. In 1940 the Cholera Advisory Committee of the Indian Research Fund Association (now the Indian Council of Medical Research) recommended that "the [cholera] vaccine should consist of a suspension of the vibrios obtained by washing off the growth from a 24-hour agar culture with 0.85 per cent saline solution" (Taylor [10]). This recommendation was in accordance with the practice of the laboratories preparing cholera vaccines in large quantities. No one has ever given any reason why agar cultures exclusively should be employed for the purpose, but it is likely that the preference for growths on agar to growths in a liquid medium must be due to the anxiety of workers to obtain their suspensions of the organisms as free from extraneous proteins as possible.

The acute shortage of the supplies of agar in India brought about by the outbreak of war with Japan forced us to investigate the possibility of preparing a cholera vaccine in a liquid culture-medium. We were quite used to preparing another vaccine, the Haffkine plague vaccine, in a liquid medium. It was merely a matter of finding a suitable liquid medium for the growth of cholera vibrio. In Mueller & Johnson's [5] acid hydrolysate of casein as modified by us[9] we found an excellent medium. It is entirely free of proteins, is easy and cheap to make, and gives rich growth of cholera vibrio. Our very first vaccines showed a very high protective power as measured by our biological assay method, using the mouse as test animal.[8] We have produced this vaccine in quantity since early 1945, and 40 million ml of it have been used in India alone. We give below the method of preparation.

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[a] A preliminary note was published in *Curr. Sci.* 1944, **13**, 230

Method of Preparation

Strains of Vibrio cholerae

A number of freshly isolated strains of *V. cholerae* having the following characters are selected and preserved by drying from the frozen state :

(1) Serological characters of O Group I (Gardner & Venkatraman[3]) subtypes Inaba and Ogawa : should agglutinate to titre with standard monospecific high-titre sera ;

(2) Typical smooth, translucent colony appearance ;

(3) Stable in salt solution ;

(4) Producing acid from mannose and saccharose but not from arabinose ;

(5) Non-haemolytic ;

(6) Virulence : Approximately 1,000 organisms of the strain suspended in 0.5 ml of 5.0% mucin suspension given intraperitoneally to white Swiss mice—of the strain of the Yellow Fever Laboratories of the International Health Division of the Rockefeller Foundation, New York, or any other strain equally susceptible—about five weeks old and weighing 18-21 g should kill not less than five animals out of the ten infected.

The method we employ for the preservation of the strains, by freeze-drying, and for their regeneration is described in another paper. [8]

Medium

Acid hydrolysate of casein is dispensed in 500-ml quantities in 3-litre, modified Haffkine flasks [b] (see fig. 1) and sterilized. The flasks are not plugged with cotton wool but are covered with a stainless-steel cap, $2\,^{3}/_{16}$ inches in diameter and $2\,^{1}/_{4}$ inches in height. This cap, without a cotton-wool plug, is adequate to keep the medium sterile and has the added advantage that there are no cotton fibres to fall into and spoil the medium ; furthermore, it saves the recurring cost of the cotton wool.

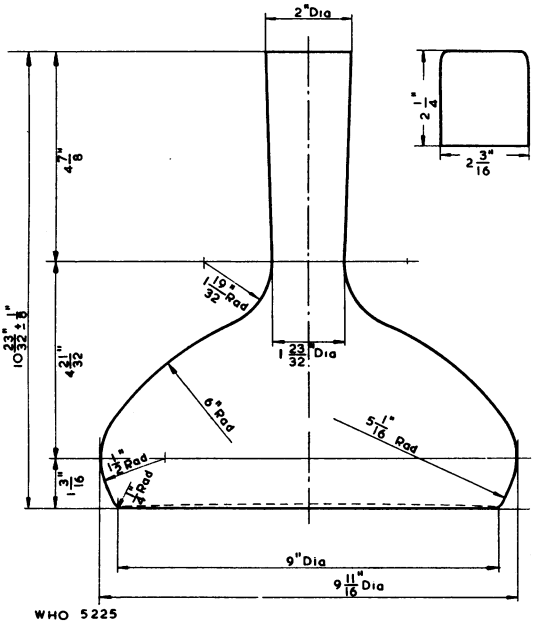
Inoculum

One freeze-dried culture of each of the two subtypes, Ogawa and Inaba, is regenerated, and from each of these a number of agar slopes are plated and incubated for 24 hours at 37°C. The slopes are stored at 4°C and are used up in a week.

A smooth, translucent, typical colony from a slope is picked up and emulsified in 3 ml of peptone water and the emulsion inoculated into a Pasteur balloon containing about 500 ml of acid hydrolysate of casein.

b The original Haffkine culture flask was a 4-litre flask designed to prepare plague vaccine. The widest part of the flask was reached only if it contained 2 litres of the medium. One of us (Sokhey [7]) has shown in another paper that in the case of plague bacillus the growth is roughly proportional to the surface area of the medium and not to its quantity. To prepare a potent plague vaccine he found it necessary to put 1 litre of the medium in this flask. To get the best results he modified the flask so that the medium reached the widest part of the flask when 1 litre of it was dispensed into the flask. We have used this modified flask for the preparation of the cholera vaccine also, but dispense only 500 ml of the medium in each flask. By actual experimentation we found that larger quantities than this gave poorer vaccines.

FIG. 1. 3-LITRE MODIFIED HAFFKINE CULTURE FLASK [c]



The balloon is incubated at 37°C for about six hours, and a sample is taken for testing purity. The balloon is returned to the incubator for 24 hours. Two sets of balloons are prepared in this way, one set with the subtype Ogawa and the other with the subtype Inaba. These balloons are then used for seeding culture flasks containing 500 ml of the medium. About 5 ml of the inoculum are added to each flask.

Temperature and duration of incubation

The cultures are incubated at 37°C for three days.

Killing the growth

At the end of the period of incubation, flasks are individually tested for purity. For this purpose a small sample of the culture is removed, and, immediately after this is done, 4 ml of formalin 1/10 are added to each flask to make a strength of 0.08%. The flasks are again incubated at 37°C for three days. Each flask is now tested for sterility.

Preservation of the vaccine

Immediately after a sample for testing sterility has been taken from each flask, 15 ml of a solution of phenyl mercuric nitrate containing 50 mg per 100 ml are added to each flask.

[c] This diagram is reproduced by courtesy of James A. Jobling & Co., Ltd., Sunderland, England.

Making a divalent vaccine

Up to this stage we have two monovalent vaccines, Ogawa and Inaba. These are now mixed in equal quantities and ampouled. Each batch of ampoules is tested for sterility in accordance with the regulations in force (British Therapeutic Substances Act, 1925).

Divalent vaccine. It will be noted that we prepare a divalent vaccine ; the reason is that there is little cross-protection between the two subtypes, Ogawa and Inaba, with which we are concerned in India. In another paper [8] we have given results of some cross-protection experiments with experimental vaccines. In this paper we give results of the assay of some mass produced vaccines in actual use (tables I and II), which bring out the same point. Vaccines used to be made from single subtype strains

TABLE I. ASSAY OF SEVEN OF OUR DIVALENT ROUTINE VACCINES *

Vaccine batch no.	Challenge strains	Mouse protective dose (ml)
320	569B (Inaba) 1 (Ogawa)	0.00022 0.00021
2211	569B (Inaba) 1 (Ogawa)	0.00017 0.00019
15672	569B (Inaba) 41 (Ogawa)	0.00028 0.00016
7159	569B (Inaba) 41 (Ogawa)	0.00017 0.0001
8229	569B (Inaba) 41 (Ogawa)	0.00038 0.00006
20412	569B (Inaba) 41 (Ogawa)	0.00018 0.00037
2981	569B (Inaba) 41 (Ogawa)	0.00025 0.00005

*** For making these vaccines both the subtypes were seeded together into the same flask ; they are not mixtures of two separately grown cultures.**

TABLE II. ASSAY OF SOME MASS-PRODUCED VACCINES IN ACTUAL USE AND OF BRF VACCINE

Vaccine batch no.	Challenge strains	Mouse protective dose (ml)
C121/44-8A	569B (Inaba) 1 (Ogawa)	0.0012 0.036
467/L	569B (Inaba) 1 (Ogawa)	0.0027 0.0004
165845	569B (Inaba) 1 (Ogawa)	0.0038 —
BRF direct cholera vaccine	569B (Inaba) 1 (Ogawa)	0.004 0.06

in the belief that there was a high degree of cross-protection between the two subtypes. This belief was almost universal when we took up this study, and Ranta & Dolman [6] actually produced experimental results in support of this thesis. We have dealt with their work elsewhere.[8]

Virulence of the Strains

As we have explained elsewhere, we began our work on cholera vaccine with the development of our assay method. We had to select strains lethal to the mouse for our challenge dose, and naturally made our first experimental vaccines from the same strains. When we compared the protective power of our experimental vaccines with that of the vaccines in use at the time, we found our vaccines had 5 to 120 times greater protective power (see page 36). We then obtained the strains from which some of these vaccines had been prepared and found that they were not fully virulent to the mouse, in the manner previously described (page 34, point (6). We give in table III the results of measurement of the virulence of the strains and the protective power of the vaccines made from them. These results show that the protective power is linked with the virulence of the strains used.

TABLE III. VIRULENCE TESTS OF STRAINS AND ASSAY OF VACCINES MADE FROM THEM

Vaccine batch no.	Strain from which vaccine is made	Virulence of strain		Protective value of the vaccine	
		Number of organisms per mouse	Proportion of mice killed *	Challenge strains	Mouse protective dose (ml)
75 (C)	569 B (Inaba)	1,630	5/6	569 B (Inaba)	0.000028
		16,300	6/6		
61 (E)	1 (Ogawa)	1,240	4/6	1 (Ogawa)	0.000035
		12,400	6/6		
75 (F)	3 (Inaba)	1,080,000	0/10	569 B (Inaba)	0.000074
		10,800,000	1/10		
75 (G)	169 (Ogawa)	920,000	0/10	1 (Ogawa)	0.14
		9,200,000	3/10		

* The first figure represents the number of mice killed and the second the number of mice injected.

Protective Power of the Vaccine

It will be seen from our paper on biological assay [8] that we do not standardize our vaccine by a count of the bacterial content and consequently do not specify it on the label. The evidence we have presented

shows that the bacterial count by itself gives no indication of the protective power of the vaccine. The mouse protective dose is the criterion we employ, but this measurement requires the use of a large number of mice for every batch of vaccine. We have, however, found from our experience over a period of five years that, if the various steps of manufacture are rigidly standardized, the protective power of different batches of vaccine lies between reasonably narrow limits ; and we now measure the protective power of only one batch of vaccine every month. The results of the last seven tests are given in table I. We wish to emphasize here two points for particular attention :

(1) The crop yield of each batch of casein hydrolysate must be measured and the medium used for the preparation of a batch of vaccine only if it passes the test. We have given the results of tests of 103 batches of the medium in another paper.[9] During a period of 18 months, since we started using a standard brand of casein, 103 batches of the hydrolysate were tested, and, of them, 101 batches proved to be suitable for the production of a vaccine of a constant strength, showing that our method of preparing casein hydrolysate can be depended upon to yield a reasonably constant product. In spite of this fact, we insist on determining the crop yield of each batch of the medium before we use it for the production of the vaccine.

(2) The constancy of the virulence of the strains used as the inoculum must be checked.

Protective Power of Cholera Vaccines Used in India in 1945 and of BRF Direct Cholera Vaccine

When we had worked out our method of biological assay and had made some experimental casein hydrolysate vaccines, we were interested in knowing how the protective power of our vaccines compared with that of the mass-produced vaccines in use in India at that time. For this purpose we obtained a commercial sample and perfectly fresh supplies from two of the main public-health laboratories which then met the major part of the demand of the whole of India.

In this test was also included another vaccine, Biochemical Research Foundation (BRF) direct cholera vaccine (Jennings & Linton [4]). In 1945, a colleague had drawn our attention to it and had sent us a reprint of the paper describing the vaccine. The authors had obtained a vaccine with a turbidity equal to 10,000 p.p.m. of silica. They had injected the vaccine subcutaneously in 0.1- and 0.2-ml doses in human volunteers without any objectionable results, and observed that :

> the serum of vaccinated individuals was found to compare favourably in mouse protection tests with that obtained from individuals vaccinated with the present commercial products. We feel that there may be more actual protection against the disease

provided by this preparation than is apparent from such tests, since the use of entire culture results in the products of growth being included in the vaccine. If the unknown toxic agent which is responsible for the actual symptoms of cholera should prove to be antigenic, the BRF vaccine might be expected to prove a more satisfactory prophylactic than the usual washed suspension of bacillary bodies.

They suggested that

such vaccine may be used in 0.1-ml doses to produce immunity equivalent to that obtained by present methods as regards mouse protection units.

The sample of BRF vaccine tested was obtained from the Director of the Biochemical Research Foundation of the Franklin Institute, Newark, Delaware.

Results of the tests are given in table II. All the vaccines showed poor protective power. Vaccine C121/44-8A is evidently a monovalent vaccine made from subtype strain Inaba, as it shows little protection against Ogawa. It has 1/40th of the protective power of our monovalent Inaba vaccine 75C (tables III and IV). The BRF direct cholera vaccine is evidently also a monovalent Inaba vaccine and so is vaccine No. 165845. Both these vaccines have about 1/120th of the protective power of our Inaba vaccine 75C. Vaccine 467/L, on the other hand, is a bivalent vaccine and has for the Inaba fraction a protective power 1/27th, and for Ogawa fraction 1/5th, of the protective power of our bivalent vaccine No. 22, described in another paper.[8]

TABLE IV. ASSAY OF VACCINES FROM THE SAME VIRULENT STRAINS GROWN IN DIFFERENT MEDIA

Strain from which vaccine is made	Challenge strains	Mouse protective dose of vaccine (ml)	
		Agar growths *	Casein hydrolysate
569B (Inaba)	569B (Inaba)	0.00022	0.000028
1 (Ogawa)	1 (Ogawa)	0.00023	0.000035

* The agar-grown vaccine contained 8,000 million organisms per ml.

The low protective power of these vaccines may be a possible explanation of the not very high estimation in which cholera vaccines have been held as a prophylactic measure by some health authorities in India. The notes prepared by the British Army in India for the guidance of its medical officers (*Field Service Hygiene Notes, India*, 1945) express the view that the immunity "conferred by inoculation, if indeed any such immunity is conferred, lasts, it is thought, barely for three months". Even as regards these vaccines with a low protective power this view has been corrected by Adiseshan et al.[1] and Chandra Sekar.[2] Their field inquiry showed that cholera inoculation in severe epidemics lowered the attack rate by one-half to four-fifths, though the case fatality-rate showed no significant

difference between the inoculated and uninoculated populations. They found that the immunity lasts for at least 6 months, and may last as long as 12 months. However, it would be interesting to see, if suitable field trials could be conducted, whether our much more potent vaccine, at least as estimated by the mouse-protection test, would give a higher degree of immunity and immunize for a longer period.

Vaccines Made from Agar Growths

Some of the vaccines in use in India at that time which were made from agar growths, containing 8,000 million organisms per ml, showed poor protective power when assayed by our method (table II). We found, as shown above, that at least one reason for their low protective power was the low virulence of the strains from which they had been made. Did the agar growth itself give lower protection power ? To clear up this issue we prepared vaccines from agar growths of virulent strains and compared them with casein hydrolysate vaccines made from the same strains. The results are given in table IV. The agar vaccines showed 1/6th to 1/9th of the protective power of the casein hydrolysate vaccines made from the same strains. This raised the issue whether the casein hydrolysate vaccines did not have a higher bacterial content than 8,000 million organisms per ml. An actual count in a Petroff-Hauser Chamber, however, showed it to be only 3,000 million organisms. Though this count may not represent the total bacterial content because of the factor of lysis, yet it does draw attention to the possibility of metabolites of the cholera vibrio playing a role in immunizing mice.

Conclusions

1. A method is described for the manufacture of cholera vaccine in a specially prepared casein hydrolysate medium.
2. It is shown that the virulence of the strains to white Swiss mice determines the protective power of the vaccine as measured by the mouse-protection test.
3. The two subtypes, Ogawa and Inaba, were found to give little cross-protection. Therefore divalent vaccines must be prepared for general use.
4. It was found that the casein hydrolysate vaccine had a much higher protective power than the agar-grown cholera vaccines available in India at the time of its introduction, largely because of the use of avirulent strains.
5. Even when virulent strains were used for the preparation of agar-grown vaccines, these were found to have 1/6th to 1/9th of the protective power of casein hydrolysate vaccines made from the same strains.

SUMMARY

Until now it has been common practice to prepare cholera vaccine from cultures on agar, probably with the idea of avoiding the introduction of extraneous proteins. The shortage of agar in India during the war forced the authors to investigate the possibility of preparing cholera vaccine in a liquid medium. Mueller & Johnson's acid hydrolysate of casein as modified by the authors was tried and found to be a suitable medium—inexpensive and free of proteins—for the manufacture of cholera vaccines.

The preparation of monovalent vaccines of each of the two subtypes, Ogawa and Inaba, is described in detail. By mixing the two monovalent vaccines, a divalent vaccine is obtained. Experience has shown a need for a divalent vaccine since, contrary to the opinion generally held, the cross-protection afforded by vaccines of the two above-mentioned subtypes is, in reality, very little. A table summarizes the results of investigation on this point.

An experimental study of vaccines prepared with acid hydrolysate of casein has shown that the protective power of a strain is linked with its virulence.

The potency of the vaccine obtained by this new method is not defined by the number of organisms it contains, but by the result of a biological assay on the mouse as devised by the authors. If the various processes for the preparation of the vaccine are rigidly standardized, its protective power is fairly constant. However, it is recommended that the crop yield of each batch of the medium be ascertained to ensure that it meets the requirements. The constancy of the virulence of the strains used should also be checked.

The casein hydrolysate vaccine has a much higher protective power than both the cholera vaccines generally used in India in 1945 and the BRF (Biochemical Research Foundation) direct cholera vaccine. The weak protective power of the vaccines in general use is probably one of the reasons for certain doubts regarding

RÉSUMÉ

Jusqu'à présent, il a été d'usage courant de préparer le vaccin anticholérique à partir de cultures sur agar, sans doute en vue d'éviter l'introduction de protéines étrangères. Le manque d'agar dans l'Inde durant la guerre a conduit les auteurs à envisager la possibilité de préparer un vaccin anticholérique sur milieu liquide. L'hydrolysat acide de caséine, selon la formule de Mueller & Johnson modifiée, s'est révélé un milieu approprié à la préparation des vaccins anticholériques, qui présentait l'avantage d'être exempt de protéines et peu coûteux.

La préparation des vaccins monovalents Ogawa et Inaba est décrite en détail. Par mélange des deux vaccins monovalents, on obtient un vaccin bivalent, dont l'expérience a montré la nécessité. En effet, contrairement à l'opinion généralement admise, la protection croisée conférée par les vaccins des deux sous-types précités est très faible. Un tableau résume les résultats des recherches sur ce point.

L'étude expérimentale des vaccins préparés sur l'hydrolysat acide de caséine a montré que le pouvoir protecteur d'une souche est lié à sa virulence.

L'activité du vaccin obtenu avec cette nouvelle méthode n'est pas définie par le nombre d'organismes qu'il contient, mais par le résultat d'une épreuve biologique sur la souris, mise au point par les auteurs. Si les divers processus de préparation du vaccin sont rigoureusement standardisés, le pouvoir protecteur de ce dernier est assez constant. Toutefois, il est recommandé de mesurer pour chaque lot de caséine le rendement des cultures, afin de s'assurer qu'il satisfait aux conditions requises. Il importe également de vérifier la constance de la virulence des souches utilisées.

Le vaccin sur hydrolysat de caséine possède un pouvoir protecteur plus grand que celui des vaccins anticholériques utilisés couramment dans l'Inde en 1945, et que celui du vaccin BRF (Biochemical Research Foundation). Le faible pouvoir protecteur des vaccins d'usage courant constitue peut-être une des raisons pour

prophylactic cholera vaccines held by some health authorities in India. These doubts are not shared by other workers, however, whose investigations in the field have shown that during severe epidemics attack rates were decreased from 50% to 80% as a result of vaccination, although there was very little difference in the case fatality-rates in vaccinated and non-vaccinated groups. If suitable field-trials could be conducted, it would be interesting to determine whether the vaccine which is most effective in the mouse test also gives immunity of a higher degree and for a longer period in man.

Vaccines containing 8,000 million organisms per ml, prepared on agar cultures, gave a protective power which was only one-sixth or one-ninth of that given by vaccine prepared from the same strain, in casein hydrolysate, which had a vibrio content of 3,000 million organisms per ml. This last figure may not represent the total bacterial content, on account of the lysis factor ; it suggests, however, the possibility that metabolites of the cholera vibrio may play a role in the immunization of mice.

lesquelles les vaccins prophylactiques anticholériques ont été peu appréciés par certaines autorités sanitaires de l'Inde. Plusieurs auteurs, cependant, ne partagent pas les mêmes vues. Des enquêtes ont montré que, lors de graves épidémies, le nombre de cas a diminué de 50% à 80% à la suite de la vaccination, bien qu'il n'y ait guère eu de différence entre les taux de léthalité observés dans les groupes vaccinés et dans les groupes non vaccinés. Il serait intéressant de se rendre compte par des essais pratiques si le vaccin qui apparaît le plus actif dans l'épreuve sur la souris confère également à l'homme un plus haut degré d'immunité, et pour une plus longue période.

Les vaccins contenant 8.000 millions d'organismes par millilitre, préparés à partir de cultures sur agar, ont présenté un pouvoir protecteur qui n'était que le sixième ou le neuvième de celui des vaccins, provenant des mêmes souches, préparés sur l'hydrolysat de caséine, et dont la teneur était de 3.000 millions de vibrions par millilitre. Ce dernier chiffre ne représente peut-être pas la teneur totale en bacilles, en raison du phénomène de lyse ; il suggère cependant l'idée que des produits du métabolisme du vibrion cholérique pourraient jouer un rôle dans l'immunisation de la souris.

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