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TECHNICAL CONSIDERATIONS OF CHOLERA VACCINES AND SOME RESULTS OF CHOLERA – EL TOR MASS IMMUNIZATION (*)

by

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Among the bacterial vaccines used for prophylactic measures, Cholera vaccine does not give a complete protection or a satisfactory mass vaccinations result compared with other biologics used for the prevention of other human infectious diseases. This relative failure is partly due to the fact that cholera used to be, for many centuries, a problem in a limited part of Asia and from this asian focus it used to spread to other countries. In other words, cholera has never been a serious disease in those countries where it could be studied by all means and some specially potent vaccines could be developped. The lack of a potent cholera vaccine may also be attributed to the complex antigens inside the vibrio cells.

Therefore we can not show among the existing cholera vaccines one which could be accepted as an ideal prophylactic. It is also shown that repeated injections of cholera vaccine will not always increase the level of immunity. On the other hand natural infection is followed by a resistance which usually fades after 6 months and one may be reinfected during subsequent outbreaks of cholera.

The serological reactions are by no means parallel to the development of immunity and on many occasions while there is a complete protection against cholera the serum antibody level remains negative. The above mentioned remarks illustrate the difficulties we face when inducing cholera immunity on a nation wide basis.

Minimum requirement of cholera-vaccine

The main goal of cholera vaccination is to administer an effective vaccine which causes no, or only slight side effects which are acceptable to the public.

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Therefore a good vaccine should satisfy the following requirements.

1) The vaccine should be effective so that two injections develop a protection for 9 to 12 months.

2) The vaccine should not cause a local or general reaction not acceptable to the public.

3) The protective value of the vaccine should not change by keeping it for several days outside of cold storage. This is very important during mass vaccination in remote areas of tropical regions.

4) The majority of the population of a town or a city receiving only one injection should develop sufficient immunity, because people are often reluctant to receive a second and third injection of cholera vaccine. These conditions can be only obtained with killed vaccines.

Immunological evaluation of cholera vaccine

Although the first cholera vaccine was prepared and used 70 years ago, its effectiveness in man is not directly shown by ingestions of virulent vibrios. The effectiveness of cholera vaccine should be demonstrated by Field trials on a statistical basis. From data published during the last 30 years one can assume that there has been a definite protection among vaccinated people in comparison with non vaccinated controls. The duration of immunity will be about six months if 60 to 70 per cent of a population is immunized with a potent vaccine.

The laboratory evaluation of cholera vaccine

The existing laboratory methods, such as precipitation or agglutination tests to evaluate the presence of potent antigens in the vaccine are not accepted as a procedure to measure the protective value of the cholera vaccine.

Protection against experimental infection was first studied by Pfeiffer (1894) in his early work on intraperitoneal cholera in the guinea pig. The guinea pig has been used by a number of later workers but because of irregularities in the percentage of protected animals in different laboratories and the high cost of a great number of animals it is not suited to routine titration of the protection conferred by immunization.

Dutta & Habbu (1955) referring to the early work of Metchinkoff (1894) demonstrated a consistent infection which can be induced in baby rabbits by intraintestinal infection of the vibrio. The striking similarity of experimental rabbit infection and human cholera is mentioned by these authors and by Phillips (1963).

Baby rabbits have not been used for the routine protective value control of cholera vaccine.

The mouse protection test first suggested by Griffitts (1942-1944) and modified later on by Habbu (1950) and Sokhey and Habbu (1950) was proposed by the WHO study group on requirement for cholera vaccine (1959). This test, accepted by most manufacturers of cholera vaccine is normally done according to the procedure outlined by Pittman & Feeley (1962, 1963) as follows: mice in three groups of 16 are given one intraperitoneal injection of three graded doses of vaccine, respectively, and two weeks later are challenged, intraperitoneally with approximately 1.000 LD50 of culture suspended in 5% hog gastric mucin. A monotype reference vaccine should be included in each experiment. The potency is expressed relative to the reference control i.e., the ED50 of the reference is divided by ED-50 of the vaccine.

The types of cholera vaccine

The cholera vaccines can be divided as suggested by Delpy (1963) into 2 main types.

Type 1 — The agar grown and killed vibrios, the first prototype of this vaccine was prepared by Kolle (1896).

Type 2 — Vibrios are grown in a liquid medium and are killed by heat or by chemical reagents. Sokhey & Habbu (1948) were among the first to suggest the use of this type of vaccine.

In bothe types there are variations based on the mode of preparation. These variations can be summarized in three groups. (Delpy 1963)

a) Vaccine is prepared by a suspension of vibrios killed immediately after harvest by heat or chemical reagents without waiting for lysis of the vibrio cells and release of protective antigens.

Group 2 — In this group of vaccines the suspension of vibrios are submitted to a certain degree of lysis before the addition of chemicals normally used for killing the vibris.

Group 3 — Similar to group 2 but in order to enhance immunity, some adjuvants are added to the vaccine.

It is worth mentioning that the vaccines prepared in liquid media are not usually recommended for mass vaccinations.

The agar grown and heat killed vaccines have been widely used in different countries during the last two decades but because of a possible denaturation of

heat labile immunogenic cholera antigens the agar grown and phenol killed cholera vaccines have been used since 1964 as suggested by WHO cholera vaccine Experts. The addition of 0.5% phenol to a suspension of vibrios grown on agar and submitted to a partiel lysis will keep the immunogenic quality of vaccine when tested by mouse protection tests. Formaldehyde is also used to kill the vibrios but experimental results were less satisfactory. Merthiolate (0.01%) may also be added as a preservative to the vaccine.

Combined vaccines prepared by mixing cholera antigen with typhoid-paratyphoid vaccine or with tetanus toxoid have been used with good results. The addition of mineral or oil adjuvants to the cholera vaccine has improved the immunogenic response of laboratory animals.

Finally some investigators have studied the possibility of using a live attenuated cholera vaccine.

Experimental results of cholera immunization

Experimental studies were done with heat killed agar grown cholera vaccine containing four billions of organisms per ml of each sero type ogawa and inaba. The protective value of different fractions isolated from vibrio cells have also been studied. Aswami et Rao (1952) found that the immunogenic fraction of vibrio cells were passed into the aqueous portion of the vaccine after lysis, and this fraction can be precipitated by Ethylene glycocolle. Pustulova (1963) isolated this fraction by precipitation with ammonium sulfate. Bhatia et al (1964) and Watanabe et al (1965) isolated a lipopolysaccharide from the cholera vibrio. This fraction showed a remarkable protective value in mice. Sodhi et al (1961) by adding Freund adjuvant to cholera vaccine induced immunity in rabbits. Husain (1962) after killing vibrios grown in casein-glucose broth with phenyl mercuric nitrate concentrated the suspension by repeated dialysis. This antigen induced in mice an immunity which lasted for 9 months.

Joo et al (1964) by adding typhoid-paratyphoid, tetanus and mineral adjuvent to cholera vaccine induced an excellent immunity in volunteers but the local and general reactions were severes. Mirchamsy & Taslimi (1966) were able to kill a suspension of 160 billions/ml of cholera - EL Tor vibrios by seven rapid cycles of freezing and thawing. This suspension was diluted to 8 billions of vibrios/ml and then after a further 1:5000 dilution, and detoxification with a trace of Formaldehyde, 0.5 ml volume injected into mice induced a solid immunity.

The utilization of classical cholera vaccine for protection against outbreaks of cholera EL Tor was first regarded as a doubtful procedure. Mukerjee & Ray

(1962) suggested the cholera vaccine should not be used to prevention of EL Tor infection. Vella (1963) found that mice immunized with cholera vaccine resist challenge with EL Tor vibrios but EL Tor vaccine alone cannot induce satisfactory protection against classical cholera. A number of differences between the two vibrios have been described: Protein composition, heat and chloroform inactivation of agglutinability, hemolytic activity, haemolysin testing and bacteriophage typing are different. But the classical cholera and EL Tor vibrios have common characteristics. They have common O group antigens and belong to the Heiberg fermentation group I and finally Pittman and Feeley by using mouse protection tests have found that with classical cholera vaccine, protection against EL Tor vibrios is as good as that against cholera vibrios. This finding supports the use of cholera vaccine for immunization against EL Tor cholera. It is worth mentioning here the experimental live vaccines. Panse (1964) immunized rabbits with living vibrios and found a definite superiority of the vaccine to killed vaccines. Oral vaccines were used by Murkerjee (1964) who believes that an avirulent vibrio can induce a solid immunity in man; but the stability and lack of mutation of such a modified vibrio as proposed by Mukerjee must first be guaranteed. The oral use of killed vaccine can also induce immunity. Freter et al (1963, 1965) vaccinated a group of 9 volunteer by the oral route on seven consecutive days using a killed vaccine. Seven out of nine persons had adequate amounts of coproantibody.

The chemical composition of cholera EL Tor vibrios has been studied by several workers-Landsteiner and Levine (1927) first isolated a carbohydarte fraction from the cholera cell.

The relationship between Proteins, Lipopolysaccharides and Enzymes of cholera vibrios and its protective value has been investigated by Linton et al (1934) Shrivastava & Misra (1960), Shrivastava (1964). Vatanabe (1965) and many others.

In their interesting reports Shrivastava et al (1959, 1960, 1961) studied the antigenic mosaic of a limited number of ogawa, Inaba and Rough strains derived from them and NAG strains fermenting mannose and sucrose. They showed that the vibrio cells have at least 7 antigens of which alpha antigen is heat stable, lipopolysaccharide in nature and is located in the cell wall. This antigen is probably responsible for the serological activity of the intact cells. The other antigens are heat labile and are located in the cytoplasm. The same authors have further observed a basic similarity in the antigens present in the cytoplasm of ogawa, Inaba, Rough and the NAG strains tested. The main difference would appear to be in the alpha antigen.

Human mass immunization

Between the two world wars the effectiveness of agar grown and heat killed cholera vaccine was studied by many workers. Among these workers Murty in Japan, Zabolotny in Russia, Savan in Balkan and many others in India, China, Burma and other countries have indicated interesting results. The index of susceptibility to cholera among vaccinated and controls in Burma is recorded as 1 to 15 and in Keshmir 1 to 41. Unfortunately these results are obtained by comparing limited numbers of vaccinated persons with a very large number of non vaccinated people. From the statistical point of view these results are doubtful especially when we consider the social and technical conditions of field trials under which the mentioned statistics were prepared. In a more carefull study when the number and the social conditions of the immunized and control groups were relatively similar the percentage of protection induced by cholera vaccine was much lower.

The field trials for mass immunization with cholera vaccine were effectively started at the end of the second world war. In this note we will give data of a field trial during an outbreak of cholera and another field trial during an outbreak of cholera EL Tor.

The first field trial is described by Kant in 1944 in the Bahar State of India.

The purpose of study was to evaluate the protective value of a single dose of cholera vaccine. The vaccine made at the Haffkine Institute was an agar grown, heat killed and phenol preserved antigen. Each ml of the vaccine contained four billions of ogawa and four billions of Inaba serotypes of classical cholera vibrios.

The vaccine was used in 49 villages each time when the first case of cholera was reported. Among the total population of 52.806 persons 30.683 persons received one injection of the vaccine, (1 ml for adults and 0.25 to 0.5 ml for children according to their age). The results recorded by kant are as follows: From the total population under survey 1.716 persons were infected and 820 persons died. Among the infected persons there were 257 and among the death cases only 89 persons from immunized group. From this experiment one can assume that with one injection of heat killed cholera vaccine (which seems to be immunologically a poor prophylactic) the immunized people are 3 to 4 times more resistant than the non-immunized subjects.

The second report belongs to the outbreak of cholera EL Tor in the Philippines in 1964 where the most important field trial to our knowledge was conduced. This report is published in detail in the 1965 WHO Bulltin. Although the types of

vaccine were not selected properly and the random groups of the population surveyed were not statistically quite significant, there are interesting data which we will discuss here briefly. The study was conducted under the auspices of WHO with the technical assistance of the Japanese Health autorities. Four types of vaccines were used as follows:

1) Agar grown, heat killed and phenol preserved classical cholera vaccine.
2) Agar grown, heat killed and phenol preserved EL Tor vaccine.
3) Standard cholera vaccine with oil adjuvant.
4) Typhoid vaccine (control).

The cholera and EL Tor vaccines contained four billion of ogawa and four billion of inaba vibrio serotypes. One ml was injected into the adulte and 0,25 to 0,5 ml into the children. The oil adjuvant was a mixture of mannide monooleate and petrol-ether marketed under the trade name of "Arlacel A" was added to the standard cholera vaccine.

584.000 persons were injected with one dose of one of these prophylactics. The close medical survey continued first for 26 weeks and then until 9 months. 325 cases of cholera EL Tor reported among the vaccinated groups as shown in table 1.

TABLE 1

Vaccine	Number immunized	Number of cases	Attack rate per 100.000
Cholera	146.000	87	59,6
EL Tor	146.000	68	46.6
Oil-adjuvant	146.000	52	35.6
Control	146.000	118	80.8
TOTAL	584.000	325	55.7

In the first survey a protection of 26% by cholera vaccine, 42% by EL Tor vaccine and 56% by oil-adjuvant cholera vaccine was recorded four and half months after vaccination. After six months these figures altered as follows:

While no immunity was observed among subjects vaccinated with cholera vaccine, 26% of persons immunized with EL Tor vaccine and 66% of those vaccinated with one injection of cholera vaccine with oil adjuvant were still protected. At the end of nine months when no protection was noticed among vaccinated subjects with cholera or EL Tor vaccines, 50% of the people immunized with cholera-adjuvant vaccine were still protected.

The oil adjuvant vaccine seems to be the best prophylactic, but unfortunately this vaccine provoked a severe local reaction in 96% of the immunized subjects followed by oedema and long lasting tumors. The biopsy of the tumors has not showed any malignancy, but because of the pain and the ulceration the vaccine could not be recommended for general use. As we have mentioned before the vaccines used in this field trial were the heat killed antigens which seem to be the poorest antigens. The new phenol killed and phenol preserved vaccines used recently in Iran and other Middle-East countries are more potent and may induce a higher percentage of immunity.

Production of cholera EL Tor vaccine in Iran

In August 1965 when the production of large amounts of chorela EL Tor vaccine was requested from the Razi and Pasteur Institute, the technical Committee of the Ministry of Health and the Experts of the mentioned Institutes decided to incorporate both bioypes of EL Tor vibrios in the cholera vaccine. Referring to the results obtained in the Philippines it was decided to produce vaccines containing 8 billions of vibrios per cc. Two billions of each of the four serotypes ogawa and inaba, classical cholera and EL Tor were mixed. Two injections of 1 ml at 4 weeks intervals and one injection of 1 ml, 6 months later were recommended. The suspension of vibrios was obtained on agar media. After a certain lysis, vibrios were killed with 0.5% phenol. The innocuity of each batch was tested in guinea pigs and mice. The potency test was done in mice according to the technique proposed by Pittman and Feeley (1963). The protective value of the vaccines controlled by mouse protection tests was done by the manufacturers. The comparative results of a batch of our vaccine tested by Pittman & Feeley in US Public Health Service, Bacterial control laboratory, Bethesda, Md. U.S.A. are indicated in table 2.

TABLE 2

Results of potency tests on Razi Institute Cholera Vaccine-Ogawa challenge (NIH 41)

Vaccine	Test Date 2—4—66 D50 ml x 10—4	Rel. pot./ml	2—18—66 ED50 ml x 10—4	Rel. pot./ml	Geometric mean ED50 ml x 10—4	Rel. pot./ml	Rel. pot. per single human dose	
Razi Institute mixte vaccine lot No 64	0.840	(50 — 196) *	3.02	1.60 (72 — 139)	1.40	1.16	2.05	2.05
U.S. Ogawa, Reference	2.54	(72 — 139)	1.0	2.24 (70 — 144)	1.5	2.38	1.0	

* ( ) = limits for 1 S.D. expressed in percent.

TABLE 2

Results of potency tests on Razi Institute cholera vaccine - Inaba challenge (NIH 35 A3)

Vaccine	Test Date 2—10—66 ED50 ml x 10^{-4}	Rel. pot./ml	2—24—66 ED50 ml x 10^{-4}	Rel. pot./ml	Geometric mean ED50 ml x 10^{-4}	Rel. pot./ml	Rel. pot. per single human dose	
Razi Institute mixte vaccine lot No 64	0.616 (63 — 160) *	4.85	0.648 (70 — 144)	4.06	0.632	4.45	4.45	
U.S. Inaba Reference	2.99 (63 — 160)		2,63 (77 — 131)	1.00	2.81	1.00	—	

* ( ) = limits for 1 S.D. expressed in percent.

From the data presented in table 2 we can assume that the ogawa fraction of the Iranian Vaccine was two times more potent than the US ogawa Reference Vaccine and that the inaba fraction showed more than four times the protection induced with the US inaba Reference Vaccine.

Summary

The existing types of cholera vaccines are described. During the last outbreak of cholera EL Tor in 1965 the agar grown, phenol killed cholera-EL Tor vaccine containing eight billion vibrios/ml of the four ogawa and inaba serotypes of classical cholera and EL Tor has been used in Iran. Some of the important field trials of cholera vaccine are discussed.

The experimental protective value of the Iranian cholera EL Tor vaccine is confirmed.

REFERENCES

1) Aswami, S.N. & Rao, S.S. (1962) - Indian J. Microbiol., 2, 51, 55.
2) Bhatia, A.L. & al. (1964) - Indian J. Med. Res. 52, 831.
3) Burrows, W. & al. (1947) - J. Infect. Dis., 81, 157.
4) Cvjetanovic, B. (1965) - Proc. of cholera Res. Symposium Honolulu.

5) Delpy, L.P. (1963) - Bull. Org. Mond. Santé, **28**, 369.
6) Freter, R. & Gangarosa, E.J. (1963) - J. Immunol., **91**, 724.
7) Freter, R., Mondal, A., Shrivastava, O.L. & Sunderman, F.W. (1965) - Am. J. Trop. Med., **14**, 83.
8) Felsenfeld (1966) - Bull. Org. Mon. Santé, **34**, 161.
9) Gallut, J. (1960) - Ann. Inst. Pasteur, **99**, 28.
10) Gosh, S.N. & Mukherjee, S. (1960 - Ann. Biochm. Exp. Med., **20**, 31.
11) Griffitts, J.J. (1942) - Publ. Hlth. Rep. (Wash.), **57**, 707.
12) Griffitts, J.J. (1944) - Publ. Hlth. Rep. (Wash.), **59**, 1347.
13) Husain, M.M.S. (1962) - J. Pak. Med. Ass., **12**, 543.
14) Joo, I. & al. (1964) - Z. Immun. Allergieforsch, **127**, 230.
15) Kant, L. (1951) - J. Trop. Med., **54**, 223.
16) Landsteiner, K. & Levine, P. 1927) - J. Exp. Med., **46**, 213.
17) Linton, R.W., Mitra, B.N. & Shrivastava, D.L. (1933) - Ind. J. Med. Res. **21**, pages 749, 91, 379, 385.
18) Linton, R.W. & Mitra, B.N. (1934) - Ind. Med. Res., **22**, 295.
19) Mirchamsy, H. & Taslimi, H. (1966) - Unpublished data.
20) Misra, S.B. & Shrivastava, D.L. (1960) - Ind. J. Med. Res., **48**, 683.
21) Morgan, F.H. & al. (1960) - Am. J. Hyg., **70**, 250.
22) Mukerjee, S. (1964) - Brit. Med. J., **2**, 546.
23) Pittman, M. & Feeley, J.C. (1963) - Bull. Org. Mond. Santé, **28**, 379.
24) Pustulova, M.L. (1963) - Z. Mikrobiol. (Mosk.), **40**, 136 cited by Felsenfeld (Bull. WHO, 1966).
35) Ranta, L.E. & Mc Creery, P.M. (195)) - Canad. J. Med. Sci., **31**, 339.
26) Shrivastava, D.L. (1964) - Ind. J. Med. Res., **52**, 817.
27) Sodhi, K. & al. (1961) - Indian J. Med. Res., **49**, 388.
28) Sokhey, S.S. & Habbu, M.K. (1950) - Bull. Org. Mond. Santé, **3**, 33,43,47.
29) Vella, E.E. (1963) - Brit. Med. J., **1**, 1203.
30) Watanabe, Y. & Verwey, W.F. (1966) - Bull. Org. Mond. Santé, **32**, 809.