Attachment 1. Date 30.1.1981

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr. M.Subba Rao Trainee Investigator (if any) _____

Application No. 80-004 (P) Supporting Agency (if Non-ICDDR,B) _____

Title of Study Investigations on the Bio-logical activities of Lipopolysaccharides ~~Cholera Toxin/Toxoid available in the~~ routinely manufactured Anti cholera Vaccines

Project status:
( x ) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

Circle the appropriate answer to each of the following (If Not Applicable write NA).

1. Source of Population: N.A
   (a) Ill subjects ~~Yes No~~
   (b) Non-ill subjects Yes No
   (c) Minors or persons under guardianship NA ~~Yes No~~
2. Does the study involve: NA
   (a) Physical risks to the subjects N.A ~~Yes No~~
   (b) Social Risks ~~Yes No~~
   (c) Psychological risks to subjects N.A ~~Yes No~~ N.A
   (d) Discomfort to subjects ~~Yes No~~ N.A
   (e) Invasion of privacy Yes No N.A
   (f) Disclosure of information damaging to subject or others ~~Yes No~~ N.A
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) N.A ~~Yes No~~
   (b) Use of fetal tissue or abortus N.A ~~Yes No~~
   (c) Use of organs or body fluids N.A ~~Yes No~~
4. Are subjects clearly informed about:
   (a) Nature and purposes of study N.A ~~Yes No~~
   (b) Procedures to be followed including alternatives used N.A ~~Yes No~~
   (c) Physical risks ~~Yes No~~ N.A
   (d) Sensitive questions ~~Yes~~ No N.A
   (e) Benefits to be derived ~~Yes No~~ N.A
   (f) Right to refuse to participate or to withdraw from study ~~Yes No~~ N.A
   (g) Confidential handling of data ~~Yes No~~ N.A
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure ~~Yes No~~ N.A
5. Will signed consent form be required:
   (a) From subjects ~~Yes No~~ N.A
   (b) From parent or guardian (if subjects are minors) Yes ~~No~~ N.A
6. Will precautions be taken to protect anonymity of subjects ~~Yes No~~ N.A
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ___ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary.
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

We agree to obtain approval of the Ethical Review Committee for any changes involving the rights and welfare of subjects before making such change.

~~Principal~~ Investigator 30/1/81 Trainee

80-004(P)
rec'd 2/2/81

## SECTION I - RESEARCH PROTOCOL

1. Title: Investigations on the Biological activities of Lipopolysaccharides and Cholera Toxin/Toxoid available in the routinely manufactured Anti-cholera Vaccines

2. Principal Investigator: Dr. M. Subba Rao, M.D. (WHO Fellow)
   Co Investigator:

3. Starting Date: 1st January 1981

4. Completion Date: 1st April 1981
   (Provisional)

5. Final Completion Date: 31 December 1981

6. Total Direct Costs: US $ 2,200

7. Availability of Fund: Combined Pathogenesis & Therapy Working Group and Host Defense Working Group.

8. Scientific Programme Head:

   This protocol has been approved by the _____ Working Group

   Signature of Scientific Programme Head: _____

   Date: _____

9. Abstract:

Although there has been nearly a century of experience with anti-cholera vaccine there is no sound evidence of its effectiveness as a immunological protective agents in endimic areas. Despite the impressive progress in our understanding of the mode of toxin, and antibacterial antibodies (against lipopolysaccharide LPS), it has not been possible to translate this new knowledge into an effective immunizing agent for man.

In this study it is proposed to identify the importance of exoenterotoxin or choleragen (CT) by the ELISA and $GM_1$ ELISA, which may be one of the main polypeptide extra-cellular metabolite, released during the growth of V. cholera on the conventional media such as Douglas Agar or papain digest Agar media which are routinely used in the large scale manufacture of anti cholera vaccine.

By studying the biological activities of LPS which are usually released in varying proportion into the surrounding saline media and cholera toxin or toxoid for a period of 18 months it is expected that useful results may be made available to introduce suitable and practicable changes in the technological procedure of manufacture of anti cholera vaccines.

## SECTION II - RESEARCH PROTOCOL

### A. INTRODUCTION

The current concepts regarding the development of combined prophylaxis of whole cell vaccine coupled with natural toxoid the choleragenoid, developed by Swedish group of workers have made it possible to give a fresh opportunity to look into the matters of technological procedures and their efficacy on biological extracellular products such as enterotoxin (LT produce during the course of the growth of V. cholera in the time-honoured Douglas Agar media or papain digest media which are incubated usually for 18 hours at 37°C.

These extra cellular metabolites may have some protective role in the immunity of cholera. Of all these, the exoenterotoxin or choleragen (CT) is the most important one. During the course of various stages of manufacture this polypeptide is subjected to various physical and chemical agents such as heat (60°C for one hour), 1% phenol, 1% merthiolate or phenylmercuricnitrate which may denature the extotoxin and thus render biologically inactive or may preserve its immunological activity.

#### Objective

The immediate objective of the present study on "Investigations on the biological activities of Lipopolysaccharides and Cholera Toxin/Toxoid available in the routinely manufactured Anti-cholera Vaccines", is the identification of exoenterotoxin (CT) in the extracellular metabolites

that are usually elaborated in the exponential growth phase of the V. cholera in culture filtrates and also in the media like Douglas Broth Agar and papain digest agar media commonly employed in the large scale manufacture of anti-cholera vaccine.

It is the objective of the present study to delineate the effects of various physical and chemical agents such as phenol, merthiolate, as described above, not only on the choleragen (CT) but also on the LPS which is partly or fully released into the surrounding medium.

It is also proposed in the study to utilize passive immune haemolysis cholera may reveal the true nature of the immunogenicity of toxoid and LPS. Further, the investigations may be carried out to study the biological activities of LPS and entero toxin (CT) and toxoid for a period of 18 months in order to fix the expiry date, of vaccine which is now arbitrarily done.

Objective of the second phase of work (September to December 1981)

While the above activities are going on to study the biological reactivity of toxoid and LPS, it is proposed to implement ELISA technique to verify the actual quantities of enterotoxin (CT) that are present in the supernatants of cholera harvested materials, and to verify the biological activity which may be preserved with the various physical and chemical agents. The toxin present in the supernatant may separately be concentrated by ultra-filtration so that a suitable dose of cholera toxoid, 30 ug per ml (Germanier et al 1977) amy be added to the whole

cell vaccine to confirm the synergistic effect of combination of the whole cell cholera vaccine with highly purified cholera toxin by suitable experiments. A separate protocol is being submitted for approval in the month of April 1981, since the details of the procedures and costs involved are to be worked out.

Background

There is a great variation in the method of inactivating the harvest of V. cholera which are routinely followed in the individual laboratories. Heat killing, with phenol 0.05% preservation (Institute of Preventive Medicine, Hyderabad), treatment with formalin and preservation with phenyl mercuricnitrate, (Haffkine Institute Bombay), phenol killing with 1% phenol and phenol preservation 0.05%, (C.R.I. Kausali, King Institute, Madras), inactivation with merthiolate (2% solution) and merthiolate preservation (Budapast Vaccine Institute) are some of the methods employed in dealing with the inactivation of vibrios. These procedures are apparently harmless and have been widely accepted for a long time without any reservation. According to the observations in the field trials and laboratory tests (Joo et al 1969, 1970 and Feely 1970), it was observed that none of the commonly used killing and preserving agents is superior to any other.

This may be true with lipopolysaccharides (LPS) which are usually resistant to such chemical treatment. But it is not known which one would be better when we are dealing with enterotoxin (CT). Usually the extracellular metabolites are spinned off and thrown out and not included in the final whole cell vaccine, as practised in States Bacteriological Laboratories, Sweden. Further it is possible the strain variation of V. cholera in their toxigenic propensities as reported by Craig (1978), may be responsible for poor antitoxic antibody responses when injected in experimented animals (Feely and Roberts 1969).

569B strain which is a classical Inaba one, as a vaccine candidate strain may be useful in the process of large scale manufacture of cholera vaccine as it can cross protect against Ogawa infections as well as elaborate useful cholera toxoin which can be toxoided by a mild heat i.e., 56°C for one hour (Mekalanos et al 1977) or by any other suitable method such as merthiolate treatment. The detoxification procedures and resultant toxoid formation are designed to be checked by rabbit skin permeability test (Craig 1965) and the ELISA tests. The biological activities of such toxoid will be confirmed by modified passive immune haemolysis techniques.

Thus the present investigations are being designed to investigate the scientific basis of usefulness of toxoids which may enhance the protective value of whole cell vaccines. (Svennerholm and Holmgren 1976).

The Nature of Protective Immunity in Cholera

There has been nearly a century of experience with anticholera inoculation as a control measure. However until 1963 there was no sound evidence that inoculation prevented the occurrence of the disease although several observations, particularly in military settings strongly suggested that it did. Greenwood, commented upon the impressive experience of the Greek Army during the second Balkan War 1930. Greenwood wrote that the data that 4 per 1000 in vaccinated suffered from cholera, as against 18 per 1000 in unvaccinated, were not complete enough to satisfy the epidemiologist but he could not discover no material fallacy in the study.

Studies in the endemic settings during the present pandemic have shown that certain Vaccines do prevent diarrhoeal diseases associated with vibrios, but it was found that the protection was very shortlived and of a limited value (Feely and Gangarosa 1980). The apparent discrepancies in the immunological profiles observed during the various cholera vaccine field trial may be possibly explained on the basis of the toxoid content in the vaccine which may be biologically active in varying degrees depending on the procedures of inclusion of supernatant extracelluler metabolites during the course of the manufacture of vaccines. However, the following conclusions are drawn from the point of nature of protective immunity.

1. Protection is typically only modest 50-70% and of short duration (3-6 months).
2. Inapparent infections are not prevented.
3. Analysis of the difference between 1 or 2 doses of El tor vaccine versus a single double strength dose was made in Philippines showed no discernable effectiveness (Philippines Cholera Committee 1968).
4. Monovalent vaccine made from Inaba serotype protects against Ogawa serotype but not vice versa. (Philippines Cholera Committee 1973a).
5. Biotype of vaccine production strains whether classical or El tor is irrelevant. (Pitman and Feely 1963).
6. The possible persistent immunity with an oil adjuvant vaccine was most interesting but unfortunately this vaccine caused an unacceptable incidence of local side reactions. Further studies with a aluminium

hydroxide-adjuvant containing vaccines were however carried out at Calcutta. (Pal 1977) (Azurin et al 1967).

7. The monovalent Inaba vaccine, 2 annual injections averaged 85% protection over the 3 year foolow-up period. (Mosley 1973).
8. Ogawa vaccine failed to protect against classical Inaba infection while significant protection was seen in 0-4 and 5-14 year age groups with Inaba vaccine and Inaba fraction. (Mosley 1973).
9. The mouse protection assay which bears no apparent resemblance to cholera in man, was a more reliable predictor of vaccine effeciency than the human antibody response. (Feely and Pitman 1962), (Watanabe 1974).
10. Children less than 15 years of age paradoxically had the highest antitoxin levels and the lowest vibriocidal titres and suffered the highest incidence of cholera (Benenson et al 1968).
11. In endemic areas the rise of vibriocidal titres with age shows a close correlation with the fall of cholera case rate with age. (Mosley et al 1968 a,b) as the latter falls by about 50% with a doubling of the vibriocidal titres. (Mosley et al 1969).

Correlation of mouse protection tests with the recent research findings.

After analysing the protective antibody levels and their possible immune mechanism in various animal models studied by different workers, (Dogs - Pierce et al 1977), (Rabbits - Holmgren et al 1977) (Hamsters - Basu et al 1964) (Chinchillas - Blackman 1974), (Rats - Aziz et al 1968) (Mice - Basilski et a l 1977), (Chaicurupa et al 1974), (Iwamaga et 1971)

(Ujiiye et al 1968), (1970), (Fujita and Finkelstein 1972). (Lange and Holmgren 1978), Stefan Lange (1979) concludes that there was no correlation between the resistance to cholera toxin challenge and the serum levels of anti-toxic anti-bodies indicating that the intestinal protective immunity was exclusively due to a local immune response. Somehow due to various reasons, probably due to species differences in immunological response a significant protective immunity is obtained much more easily by enteral than by parenteral immunization in mice. There seems to be influx of protective anti-toxin antibody in the gut from the serum which is seen in dogs. This difference may explain the findings of Feely and Pitman (1962), Mosley et al (1972) and the observation made by Feely and Gangarosa (1980) that "surprisingly the mouse protection assay which bears no apparent resemblance to cholera in man was a more reliable predictor of vaccine efficacy than the human antibody response." Since IgA with its secretory piece 'J' form, is usually resistant to the intestinal enzymatic activity, these protective antibodies against toxin as well as LPS, may have an important role in contributing the "protective nature" of antibodies against V. cholera. The subcutaneous injection of cholera vaccine which is reported to be capable of acting as a booster to release IgA antibodies in experimental animals deserves special attention, in designing the modified mouse model for testing the potency of anticholera vaccines at a future date. Thus, this significant finding has a bearing on the standardization of cholera vaccine manufacture.

Rationale

There are several reasons which justify the presented investigations of the LPS and cholera toxin:

a. Synergism between antibacterial and antitoxic antibodies is recently demonstrated in animals by Svennerholm and Holmgren (1976).

b. There is no standard procedure of the inclusion of the supernatants of the harvested vibrios which are usually not included and are thrown out as inconsequential elements because of lack of specific knowledge of the immunological activity and potentiation of enterotoxin (CT) and other cellular metabolites.

c. By identifying the immunological activity of CT and consequent inclusion of suitable dose of 30 ug of choleragen (Cholera toxoid) will improve the protective value of cholera vaccine.

d. Suitable expiry date and its potency in the mouse model, i.e., per. oral immunization followed by subcutaneous injections may lead to better immunogenic cholera vaccine.

B. SPECIFIC AIMS

1. To extimate the presence of enterotoxin and their derivatives such as toxoid (choleragenoid), in the culture filtrates of vibrio cholera which are being incorporated in the course of the procedures adopted for large manufacture of anti-cholera vaccine.

2. To implement ELISA (Enzyme Linked Immunoabsorbent Assay) for the sensitive detection of enterotoxin (choleragen), choleragenoid or toxoid and LPS liberated during the course of manufacturing vaccines which employed different kinds of media varying from laboratory to laboratory.
3. To study qualitative and quantitative biological activities of LPS and toxoid and also to estimate that the degradation or preservation of such activities over a period of 18 months.

## C. MATERIALS AND METHODS

1. ELISA techniques, are useful for accurate sensitive quantitative estimation methods for both somatic and enterotoxin. By comparing the inhibition using known concentration of pure LPS and CT or choleragenoid the amount of test antigen is readily quantified as indicated by Holmgren J. Svennerholm (1973 and 1978), Engvall and Perlmann (1972).

2. In $GM_1$ ELISA method Holmgren (1973 and 1978) ganglioside is attached to polysterene tubes by incubation of the tubes with 1.5 uMGM, ganglioside at room temparature, overnight. After washing 3 times with phosphate buffered saline (PBS - 0.15 M), pH 7.2, and unoccupied binding sites on the plastic surface are blocked by incubation with 1% serum albumin (Foetal calf serum). The tubes are then incubated for 2 hours at room temperature with serial dilutions of the test samples containing unknown quantities of cholera toxin or toxoid in 0.5% serum albumin. Non bound material is removed by rinsing

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the tubes with PBS supplemented with 0.05% tween 20. Tween 20 is used to avoid non specific absorption. (Engvall and Perlmann 1972). The enterotoxin bound to the $GM_1$ sorbent is thereafter demonstrated by sequential incubation of the tubes with the (a) human or rabbit anti enterotoxin serum, (in PBS containing 0.05% tween 20 and 0.02% sodium azide) for 30 minutes incubation. (b) Anti rabbit immunoglobin, IgG or IgA or combined coupled two alkaline Phosphatese at an enzyme concentration of 0.2 ug/ml, (c) Nitrophenyl phosphate substrate (Engvall and Perlmann 1972).

Usually 0,2 ml volumes of all reactants except the substrate (1 ml) i.e., p. nitrophenyl phosphate (NPP, sigma) in concentratione on 1 mg/ml NPP and $10^{-3}$ M $Mgel_2$ in 0,97 M.D.E.A. buffer are added.

After suitable incubation for either 60' or 100's, depending on the system used the enzyme hydrolyses the bound phosphate and liberates yellow p. nitrophenyl which is measured in a spectrophotometer at 400 nm. The increase in absorbance with the time is linear up to approximately one. After suitable incubation time at room temparature the enzyme reaction in individual tubes was stopped by addition of 0.1 N. NaOH. The results are plotted on logarithmic scales and interpreted.

3. Passive Immune Haemolysis

There is a possibility of the enterotoxin and its derivatives such as toxoid may be bound to the bacterial content or LPS released in

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the surrounding medium or saline during the course of large scale manufacture of cholera vaccine. To examine this possibility the whole cell vaccines with the supposed toxoid elements are proposed to be injected in Rabbits subcutaneously without adjuants in duplicate 0.5 ml portions above and left posterior legs thrice with a 2-week interval. A pair of control rabbits are to be injected with PBS. Five to seven days after the booster injection serum samples are to be drawn and to be tested for the presence of antitoxic antibodies on the basis of ELISA method as well as modified passive immune haemolysis (PIH).

It is proposed to carry out these controlled studies on LPS on toxoid in the course of next three months to establish the biological activities in vitro after satisfying with the immunogenicity of the agents in rabbit studies. The immunogenic materials can be studied in vitro either by inhibation test of by $GM_1$ ELISA every 15 days so that at least 5 reliable readings may be made available as base line data.

It is further proposed to study the same biological activities after 9 months, 12 months and 16 months on the lines indicated so that the expiry date of the routine anti cholera vaccine may be fixed on sound scientific data.

Materials

a. Antigens:

1. 569 B strain is grown in the usual media commonly employed (Douglas Broth Agar) in the large scale manufacture. The growth after 6 hours are harvested along with extra cellular metabolites. The materials are subdivided into three parts - a) whole cell vaccien along with natural extra cellular metabolites adjusted to contain $8 \times 10^9$ organisms freshly harvested which equivalent to 10 International units standard supplied by (WHO) when diluted with equal amount of PBS. b) the supernatant which is collected after spinning in a cold centrifuge at 12000 rpm for 20's to which sodium azide is added - 20 ug/ml. c) The deposit is treated with phenol (1%) after suitable digestion to contain $8 \times 19^9$ ml organisms per ml at stated above, and finally preserved with 0.5% phenol (A.R.).

2. Similar preparation can be made with heat as a killing agent and the cells are injected without centrifugation so that the enterotoxin if present is also easily converted into toxoid during the heating stage.

3. Supernatant is treated separately with 0.5 ml of 10% merthiolate and combined later on with merthiolate killed vibrio cholera so that the final concentration may be about 1:10,000.

b. Antisera:

Purified antisera against LPS and Antitoxic antisera prepared from rabbits are made available through the courtesy of Professor Holmgren.

c. Conjugates:

Antirabbit gamma globulin G. conjugate with alkaline phosphates is made available through the courtesy of Professor Holmgren.

D. SIGNIFICANCE

The present concept of the immunogenic ability of whole cell vaccine is that it is unable to produce antitoxic antibodies since the extra cellular metabolites are usually removed. The media* may be able to produce C.T. during the incubation period when 569B is used as seed strains. If it is found feasible that the present methods are suitable for the development of C.T. and consequesnt toxoid formation determined by ELISA techniques, the enhancement can be easily achieved by suitably altering the techniques commonly used in the large scale manufacture of cholera vaccines.

This study may lay sound foundation in fixing usual expiry date of vaccines, the period of which may vary under different ambient temparatures prevalent in tropical countries.

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* Douglas Agar medium, Papain Digest agar medium are commonly used for large scale manufacture of cholera vaccines.

E. FACILITIES REQUIRED

1. Office space : None
2. Laboratory space : None
3. Hospital resources : None
4. Animal resources : 24-30 rabbits weighing 2 kg, mice.20, goats 2
5. Logistic support : None
6. Statistical support : Needed
7. Major item of equipment : None
8. All other minor equipment are available in the Host Defence Working Group of laboratories.
9. Collaborative arrangemens with Biochemistry Branch are required. for comparative studies as a semimicro spectrophotometer is needed.

F. COLLABORATIVE ARRANGEMENT

1. Preparation of 569B vaccine in the adjoining public health laboratories for the Director Dr. M.A. Latif has kindly consented to extend facilities.
2. Highly purified antitoxoid antisera and anti-LPS antisera of rabbit are required in 1 ml quantitites each in the near future. It may be imported if necessary.

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## ABSTRACT SUMMARY

All the available recent research activities point that a combined whole cell vaccinewith the cholera toxoid (choleragenoid) is desirable in view of the synergistic activities recently demonstrated, in animals. (Svennerholm 1975 and Svennerholm and Holmgren 1976). This study aims to demonstrate the presence of cholera toxin and its possible conversion to toxoid during the preparation of large scale manufacture of cholera vaccine. The study also aims at the ultimate effect of physical and chemical agents on LPS and CT naturally produced during the course of the growth of V. cholera in Douglas Broth Agar medium. Studying the qualitative and quantitative biological activities of LPS and cholera toxoid over a period of 12-18 months by ELISA techniques it would be possible to establish the expiry date of cholera vaccines and modify the techniques on sound scientific data and knowledge.

## SECTION III - BUDGET

### A. DETAILED BUDGET

| 1. Personnel Services | Position | % Time Used | Annual Salary | Actual Requirement | Remarks |
|---|---|---|---|---|---|
| Dr. M.S. Rao | - | - | - | - | 7 mths. |
| Shamsul Huda | Lab.Ofr(Gr.II) | 10% | 42,000 | 4,400 | 7 mths. |
| G. Mondal | Res. Techn. | 10% | 30,000 | 1,750 | 7 mths. |
| | Lab. Techn. | 80% | 24,000 | 9,470 | 7 mths. |

2. Supplies and Materials

Media, reagents, ELISA plates, Chemicals Biologicals, Phosphocellulose Resins 10,000

3. Equipment - None

4. Patients Hospitalization ; None

5. Outpatients Care - None

6. ICDDR,B Transport - None

7. Travel and Transporation of Persons - None

8. Transportation of Things and Materials ; 2,000

9. Rent, Communications etc. - None

10. Printing and Reproduction of Reprints ; 2,500

11. Other Contractual Services : None

12. Construction and Renovation : None

13-Animal Requirements : Rabbits - 60, Mice - 100, Goats - 4, Guinea Pigs - 20

6,180

Total: Tk.36,300

Total US$ 2,200

## BUDGET SUMMARY

| Category | Dollars |
|---|---|
| 1. Personnel | 947 |
| 2. Supplies | 606 |
| 3. Equipment | - |
| 4. Hospitalization | - |
| 5. Outpatients | - |
| 6. ICDDR,B Transport | - |
| 7. Travel Persons | - |
| 8. Transportation Things | 122 |
| 9. Rent/Communication | - |
| 10. Printing & Reproduction | 152 |
| 11. Other Contracts | - |
| 12. Construction and Renovation | - |
| 13. Animal Requirements | 375 |
| Total: | 2,200 |

(Conversion rate US$ 1 = Taka 16.5)