Attachment 1. (5) 62

Date 10 May 1983

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator ANSARUDDIN AHMED Trainee Investigator (if any) _____

Application No. 83-018 Supporting Agency (if Non-ICDDR,B) _____

Title of Study Modulation of murine antibody responses to V. cholerae by enteric immunization.

Project status:
(✓) 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:
   (a) Ill subjects Yes No
   (b) Non-ill subjects Yes No
   (c) Minors or persons under guardianship Yes No
2. Does the study involve:
   (a) Physical risks to the subjects Yes No
   (b) Social Risks Yes No
   (c) Psychological risks to subjects Yes No
   (d) Discomfort to subjects Yes No
   (e) Invasion of privacy Yes No
   (f) Disclosure of information damaging to subject or others Yes No
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) Yes No
   (b) Use of fetal tissue or abortus Yes No
   (c) Use of organs or body fluids Yes No
4. Are subjects clearly informed about:
   (a) Nature and purposes of study Yes No
   (b) Procedures to be followed including alternatives used Yes No
   (c) Physical risks Yes No
   (d) Sensitive questions Yes No
   (e) Benefits to be derived Yes No
   (f) Right to refuse to participate or to withdraw from study Yes No
   (g) Confidential handling of data Yes No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes No
5. Will signed consent form be required:
   (a) From subjects Yes No
   (b) From parent or guardian (if subjects are minors) Yes No
6. Will precautions be taken to protect anonymity of subjects Yes No
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)
   NA 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)
   NA Informed consent form for subjects
   NA Informed consent form for parent or guardian
   NA Procedure for maintaining confidentiality
   NA 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.

Note: This protocol is exclusively restricted to animal (mice) experimentations.

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

A. Ahmed.
Principal Investigator

Trainee

83-018

15/5/83

SECTION I - RESEARCH PROTOCOL

1. Title: Modulation of a parenterally induced IgA/IgG antibody response to Vibrio cholerae in mice by the adoptive transfer of Peyer's patch lymphocytes from enterally immunized donors

   Short Title: Modulation of murine antibody responses to V. cholerae by enteric immunization

2. Principal Investigator: Dr. Ansaruddin Ahmed

   Co-Investigator: Dr. K.A. Abdullah Al Mahamud

3. Starting Date: October 1, 1983

4. Completion Date: September 30, 1984

5. Total Direct Cost: US$15,078

6. Programme Head of the Science (Host Defence) Working Group: Dr. Thomas C. Butler

This protocol has been approved by the Host Defence Working Group

Signature of the Programme Head: _____

Date: _____

7.7. Summary

This study will test the hypothesis that the ingestion of Vibrio cholerae generates in the gut-associated lymphoid tissue (Peyer's patches), suppressor or helper cells or both. This can be shown in mice, by the adoptive transfer of Peyer's patch lymphocytes (PPL) from donor mice immunized enterally, to modulate a parenterally induced specific antibody response of of syngeneic recipients by a concurrent IgG suppression and IgA enhancement. These two immune events have previously been shown to occur separately in different animals fed with various antigens but not a non-invasive enteric pathogen like V. cholerae.

In the methods, PPL from enterically immunized donor mice, followed in close succession by V. cholerae, will be adoptively transferred intravenously into test receipients two times at an interval of a fortnight. The control groups of mice will receive PPL from normal donors in the same experimental design. On days 4 and 6 after the challenging (second) injection, spleens from the test and control groups will be compared for numbers of specific antibody secreting cells (Jerne PFC assay) of the IgG (2a+2b) and IgA immunoglobulin classes to assess any significant suppression or enhancement. Spleen lymphocytes from enterically immunized donors, in place of PPL, will also be used in similar cell-transfer experiments to get evidence that the suppressor cells reach the site of examination i.e., the spleen and thus, the splenocytes may be expected to transfer non-IgA antibody suppression.

It may be emphasized, despite a deceptive academic aura ~~area~~ around the experiments, that the expected results will have a significant impact on the basic knowledge on antibody modulation by the enteral route; and will promote additional confidence, from the point of view of antibacterial immunity, on the experimental basis of oral vaccination previously derived from the extensive work of N.F. Pierce and his associates using cholera toxin or toxid as antigens on rat and dog models.

Human subjects will not be used in this research project and the experiments will be fully restricted in the mouse model.

8. Reviews:

a. Research Involving Human Subject: _____

b. Research Review Committee : _____

c. Director: _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION:

1. Objectives:

This study addresses the question whether specific IgG antibody response to V. cholerae in mice, — using adoptive transfer of Peyer's patch lymphocytes (PPL) from orally immunized donors and Jerne antibody-forming cell (PFC) assay of recipient spleens — is suppressed with a concurrent enhancement of IgA leading to a 'local kind' of antibody response having a high IgA/IgG ratio. Similar findings, using the same experimental design, were obtained with the IgM response in a previous study (Ahmed Ansaruddin et al, 1983.).

2. Background:

The presentation of an antigen in the gut of an individual may lead to a suppression of the systemic antibody responses and delayed hypersensitivity on a subsequent parenteral challenge. This is known as Sulzberger-Chase phenomenon and has been well established with several assays and antigens but not with a living non-invasive intestinal pathogen. The mechanism of this phenomenon, however, is not yet well determined though the key role of suppressor Peyer's patch lymphocytes (PPL) is gaining acceptance (Mattingly and Waksman, 1978; Ngan and Kind, 1978). Peyer's patches (PP) also have been shown to be the major source of antigen-sensitive IgA-committed precursor cells that are distributed by 'homing' in gut lamina properia (LP); (Craig and Cebra, 1971; Husband and Gowans, 1978; and Guygrand, Gricelli and Vassalli, 1974).

Antibody production in mice challenged with an antigen (V. cholerae) by various routes was studied earlier by enumeration of antibody-secreting cells (PFC assay) in spleen (Ahmed Ansaruddin et al, 1982, manuscript in preparation). Repeated intragastric (i.g.) priming followed by an intravenous (i.v.) challenge created a peak response (between 3 to 6 days) showing high average IgA/IgM ratio (1.06/1). An i.g. challenge of similar i.g.-primed mice led to a response of a lower magnitude but of very high average IgA/IgM ratio (16.4/1). However, i.v. priming followed by i.v. challenge yielded quite low average IgA/IgM ratios (1/4.1 to 1/13.4 by various dose regimens) allowing a demarkation between local and systemic stimulations. All results of IgA/IgM ratio in antibody responses created primarily by local stimulation(s) lay above the value of 0.5. This category of responses could arbitrarily be defined to be of 'local kind'. In addition, it has also been shown that presentation of antigen in the gut can lead to the IgA synthesis at distant sites such as colostral, salivary and lachrymal secretions in man (Mesteckey et al, 1978) and salivary secretations in the rhesus monkey (Challacombe and Lehner, 1980), without any significant humoral response.

B. SPECIFIC AIM

The aim of this investigation is to test the hypothesis that local presentation of a noninvasive gram-negative enteric pathogen (V. cholerae) in the gut leads to the emergence in the PP of supressor or helper cells or both, which are responsible for the modulation of the antibody responses ultimately into the 'local kind' i.e., possessing a high IgA/IgG ratio. It is postulated that this happens by a concurrent occurrence of IgG (and IgM)* suppression and IgA enhancement.

*IgM response was studied earlier as mentioned in the 'background'

C. METHODS:

1. Antigen

A streptomycin-resistant Classical Inaba strain of V. cholerae, selected for the property of adherence, designated 569B, SR, Beads VII will be used as the bacterial antigen for immunizing mice. A phenol-water extracted, purified and alkali-treated lipopolysaccharide (LPS) soluble antigen from the same vibrio strain will be used for sensitizing the indicator sheep erythrocytes in the Jerne PFC assay of mouse spleens.

2. Antibodies

a. Affinity-purified antimouse IgG (L/γ chain): for coating plastic surface in the separation of T and B cells from the PPL of orally immunized donors.

b. FITC coupled antimouse gamma-globulin: for monitoring the purity of T&B cell separation by UV microscopic identification of fluorescent sm Ig-positive B cells.

c. Antimouse IgG 2a and IgG 2b (pooled): for enumeration of antibody-forming cells (PFC) of IgG type.

All the above antibodies will be either proprietary immunochemical reagents or procured from research institutions. Monoclonal antibodies, if available, will always be prefered.

3. Animals

The inbred Swiss albino mice of ICDDR,B animal house will be used. During a few pilot experiments this stock of mice was found to be suffering from helminthic (Cestodes & Nematodes) and protozoal infestation (Giardia muris). To get fully healthy mice we need to renovate the animal house

to be rodent and verminth-free and start breeding a fresh non-infected stock. This will incur considerable expenditure. As ICDDR,B is undergoing economic constraint, the present stock, while being treated continually and concurrently with anthelmintics and metronidazole, will be used in the experiment. It is expected that experiments on the mice taken as controls will be scientifically valid because they will be picked up from the same drug-treated stock; though they may not be delcared perfectly normal in the strictest definition of norm.

4. Lymphocyte preparation and separation of PPL into enriched T&B subpopulations

Lymphocytes from surgically removed and washed PPL of donor mice will be enzymatically isolated by digestion with collaqenase (10 units/ml) in Hank's balanced salt solution (HBSS) containing fetal calf serum (FCS,20%) followed by washings in HBSS-5%FCS. Lymphocytes from spleens will be obtained by mechanical desaggregation in HBSS-5%FCS with the help of a glass tissue grinder. The lymphocytes will be counted for viability by Trypan blue exclusion technique. Percentage of true lymphocytes, when required, will be verified by a stained smear made with the help of a cytocentrifuge.

The mixed population of PPL single cell preparation will be partitioned into enriched T and B subpopulations using antimouse IgG (L/γ) antibody-coated flat plastic surface of a tissue culture flask by the method of Mage et al (1977). The B cells will adhere to the plastic surface by their surface membrane immunoglobulin (smIg) receptors, allowing the nonadherent T cells to be harvested. The B cells, in turn, will subsequently be recovered from the surface by xylocaine-treatment. As a result, both the purified T and B cells from the same donor group of

mice will be ready for adoptive transfer into two groups of syngenic recipients in the same sitting. The purity of the enriched subpopulations will be determined by immunofluorescence, counting the percentage of smIg-positive fluorescent cells using direct-staining with fluorescein isothiocyanate (FITC) conjugated anti-mouse gamma-globulin and examining under the UV microscope in epi-illumination.

5. Jerne PFC assay:

A standard thin layer modification of Jerne technique will be used for the enumeration of Ig class specific antibody-forming plasma cells. A volume of 0.1 ml single-cell suspension of spleen at appropriate dilution, 0.05 ml of 30% indicator sheep erythrocytes and 1 ml of 0.7% agarose in RPMI 1640-10% FCS cell culture medium at 48°C will be mixed and spread immediately on a small (60 mm-diameter) sterile plastic petri dish. Enumerations of IgG and IgA indirect plaques will be done by facilitating with the corresponding anti-Igs after the elimination of IgM direct plaques with the treatment of concanavalin A (Con A). The conventional method of computing IgG/IgA plaques by subtracting direct plaque counts from indirect plaque counts will not be used; because most anti-Ig sera used to develop indirect plaques exert variable but significant degree of inhibitory effect on the IgM plaques.

6. Experimental design:

Live PPL will be isolated from donor mice, previously fed three times at weakly intervals with 5×10^{10} V. cholerae per feed on day 4 or 5 after their last feed. About 5×10^{6} viable morphologic PPL followed in close sucession by 1×10^{8} V. cholerae will be administered intravenously

twice in syngenic recipients with an interval of two weeks in between. The control experiments will have identical design in which primed PPL will be replaced by PPL from normal conventionalized mice using comparable numbers of viable cells.

Another set of cell-transfer experiments, similar in design as above with primed PPL, will be performed using spleen lymphocytes form orally immunized donor mice for the test and spleen lymphocytes from normal mice for the control. (This portion has been included later according to the suggestion given by the external reviewer Prof. Nathaniel F. Pierce of The Baltimore City Hospitals, Baltimore, Maryland 21224, USA). This will allow us to know whether spleen cells from enterically immunized mice also transfer non-IgA antibody suppression. If so, there would be more direct evidence that the suppressor cells normally reach the site of examination i.e., the Spleen, a central lymphoid organ and thus may be expected to be exerting an effect there.

The numbers of IgA and combined IgG 2a and IgG 2b PFC/10^7 viable lymphocytes in the recipient spleens will be enumerated by the Jerne PFC assay on day 4 and 6 (peak antibody period) after the second injection (the booster). The significant Ig class-specific suppression, enhancement or no change in the test groups of mice will be ascertained by statistical comparisons with the results obtained in the control group in which unprimed mixed PPL/spleen cells will have been used.

Two more test experiments will be performed with identical designs in which about 3 x 10^6 purified T or B cells instead of mixed PPI population from vibrio-fed donors will be used' but the results of the same control group comprising the use of unprimed mixed PPL will be accepted as reference for the assessment of significant IgG suppression and IgA enhancement.

7. Statistics

The comparison of the independent samples in the test and control (6-10 mice) will be done by nonparametric Mann-Whitney U (rank-sum) test.

D. SIGNIFICANCE

Parenteral vaccination have been the main-stay of immunization against cholera for nearly a century. But its futility in the form of injection of either the somatic (killed V. cholerae/purified LPS) or the toxin (adjuvanted toxoid) antigen have been proved earlier by scientists working at the ICDDR,B. All these parenteral vaccines evoked high level of serum antibody but none of them could give lasting protection.

Data on the age-related acquisition of immunity in the population of endemic areas and studies on volunteers undergoing induced cholera suggest convincingly that presentation of the living antigen in the gut is the main factor to be correlated with significant lasting protection against the disease. Consequently, successful immunization against cholera in future is envisaged to be the oral administration of an attenuated living vibrio having optimum mucosal colonizing character leading to stimulation of gut- associated lymphoid tissue (mainly Peyer's patches), local synthesis of IgA antibody and its active secretion to the mucosal surface.

Till date, the basic characteristics of the local antibody responses are much less understood than those of the systemic one; especially the questions like (i) what is the best procedure of stimulating a mucosal response? and (ii) how the overall immune response of an individual is modulated by gut presentation of an antigen? Pierce's group formed the experimental basis (in rats and dogs) of oral immunization of cholera by answering the first question.

(Pierce and Gowans, 1975; Pierce, Sack and Sircar, 1977; Pierce, 1978). The results of these experiments strongly favoured oral stimulation as the most likely means to evoke optimum immunity to cholera. My earlier work on this question, at the University of Adelaide, fully coroborated this finding of Pierce and his associates. The present project will try to answer a part of the second question.

There has been sufficient body ofdata to support seperately the two gut-related immune events e.g., (i) systemic unresponsiveness for non-IgA antibody synthesis by prior feeding of antigen and (ii) production of secretory IgA antibodies by antigen feeding; but few experiments were done to prove whether these two events occur concurrently in animals exposed to the gut with a living non-invasive gram negative organism. Only Challacombe and Tomasi (1980) demonstrated in mice the concurrency ofthe above two immune events using nonliving particulate (formalin-killed Streptococcus mutans) and soluble protein (Ovalbumin) antigens. Using similar experimental design and assay system as mentioned, a concurrency of adoptively transfered IgM suppression and IgA enhancement in mice through the transfer of PPL from V. cholerae-fed donors, has been shown by my earlier work. The question whether a similar concurrency ofIgG suppression andIgA enhancement is also demonstrable in similar experiments, — will be answered by this research protocol.

The current knowledge in the nature of mucosal immunity of mammalian gut is far from comprehensive. We need to increase the vista of basic knowledge in this field so that we can put our future immunization programmes on sound scientific grounds. Though this study apparently looks to be of academic interest, its results are expected to have significant implications towards achieving the above goal.

E. FACILITIES REQUIRED:

1. Laboratory space for immunologic work:already provided in room 107 (assigned for HDWG work, to be shared with Dr. M. Struelens.)

2. Animal resources. Pending ideal construction/renovation of verminth and rodent-proof animal house, one room is to be altered to minimize protozoal and helminthic infestation of mice.

A small special expenditure will be incurred for continual treatment of mice with anthelmentics and metronidazole concurrently with the experiments.

3. Major items of equipment :

i. Clinical Microscope - one provided in the room (for M. Struelens), will be used.

ii. Epi-illumination assembly to an UV microscope - one already ordered under DTWG, will be used.

iii. A cytocentrifuge - for examination of smears of various cell preparation - has to be ordered.

iii. 37^{o} and 56^{o}C water baths -
iv. Table-top clinical centrifuge with swingout head and buckets

] were present in the room from before as HDWG property

v. 37^{o} C Incubator - one ordered by M. Struelens will be shared.

4. Specialized requirement:

i. Chemicals: EDTA, EGTA, Dithiothrietol, Mercapto ethenol, Iodoacetamide, Lidocaine brand Xylocaine injectable solution, Agarose powder, Trypan Blue, common laboratory reagents etc.

ii. Biobedical reagents: Tissue culture dehydrated medium - RPMI-1640, Foetal bovine serum, Bovine serum albumin dehydrated

guinea pig complement, Heparin, Collagenase (Clostridiopeptidase A; Sigma), concanavalin A, Brain heart infusion, dehydrated culture medium etc..

iii. Immunochemical reagents: Rabbit antimouse secretory IgA, preferably raised against monoclonal antigen, Rabbit affinity-purified anti-mouse IgG(L/γ chain), FITC conjugated goat (or rabbit) anti-mouse gamma-globulin, Rabbit or goat antimouse IgG 2a and IgG2b.

iv. Other important items: Nickel-steel wire gauze (100-and 300-mesh) Plastic tissue culture flask ('Corning' glass No.25100); Conical graduated centrifuge tubes (15ml); Hemocytometer counting chambers, improved Neubauer rulling;Sterile petri dish (60 mm diameter); "Optivisor" - binocular visual aid - 5x and 10x magnification; Plastic serological test tubes, disposable, 5 ml; Interval timer; Stopwatch; tissue grinders etc....

F. COLLABORATIVE ARRANGEMENTS

Contact will be maintained with the laboratories of Professor D. Rowley and Professor N.F. Pierce regarding obtaining good quality immunochemical reagents and theoretical and technological advices, if necessary.

ABSTRACT SUMMARY

This study will address the question whether the ingestion of Vibrio cholerae generates in the gut-associated lymphoid tissue (Peyer's patches), suppressor or helper lymphocytes or both. This will involved transfer of Peyer's patch lymphocytes from orally immunized donor subjects into test recipients for observing the modulation in their immune responses against V. cholerae. Obviously, invasive techniques will have to be used in the methodology and thus, human subjects cannot be used in any part of the experiments.

As all the experiments will be solely restricted to the mouse model, the question of furnishing informations listed under 1 through 8 in "Information to include in abstract summary" does not arise at all.

References

1. Ahmed, Ansaruddin, et al. Modulation of antibody responses to Vibrio Cholerae in mice by adoptive transfer of Peyer's patch lymphocytes from orally immunized donors. Sent for publication in the Aust. J. Exp. Biol. Med. (1983)

2. Ahmed, Ansaruddin. Characterization ofanti-bacterial antibody responses to Vibrio cholerae in mice by various routes of immunization using Jerne PFC assay (1983) in preparation.

3. Challacombe, S.J. and Tomasi, Jr, T.B. Systemic tolerance and secretory immunity after oral immunization J. Exp. Med. 152:1459 (1980)

4. Craig, S.W. and Cebra, J.J. Peyer's patches: an enriched source of precursors for Ig-producing immunocytes in the rabbit. J. Exp. Med., 134:188 (1971)

5. Guy-Grand, D. et al. The gut-associated lymphoid system: nature and properties of the large dividing cells. Europ. J. Immunol., 4:435 (1974)

6. Husband, A.J. and Gowans, J.L. The origin and antigen dependent distribution of IgA-containing cells in the intestine. J. Exp. Med. 148:1146 (1978)

7. Mage, M.G. et al. Mouse lymphocytes with or without surface immunoglobulin: Preparative scale seperation on polystyrene tissue culture dishes coated with specifically purified anti-immunoglobulin. J. Immunol. Meth.15:47 (1977)

8. Mattingly, J.A. and Waksman, B.H. Immunologic suppression after oral administration of antigen. J. Immunol. 121:1878 (1978)

9. Mestecky, J. et al. Selective induction of an immune response in human external secretions by ingestion of bacterial antigen. J. Clin. Investigation, 61:731 (1978)

10. Ngan, J. and Kind, L.S. Suppressor T cells for IgE and IgG in Peyer's patches of mice made tolerant by the oral administration of ovalbumin. J. Immunol, 120:861 (1978)

11. Pierce, N.F. The role of antigen form and function in the primary and secendary intestinal immune responses to cholera toxoid and toxin in rats. J. Exp. Med. 148:195 (1978)

12. Pierce, N.F. and Gowans, J.L. Cellular kinetics of intestinal immune response to cholera toxoid in rats. J. Exp. Med. 42:1550 (1975)

13. Pierce, N.F. et al. Immunity to experimental cholera. III. Enhanced duration of protection after sequential parenteral-oral administration of toxoid to dogs. J. Infect. Dis. 135:888 (1977)

SECTION III - DETAILED BUDGET

1. Personnel Services:

Name	Designation	% Effort	Project requirement (approximate) Taka	Dollars
Dr. A. Ahmed	Asso.Scientist(PI)	50%	76,500	-
Dr. K.A. Al-Mahmud	Asso.Scinetist(CI)	10%	12,300	-
Mr. P.K. Neogi or a substitute of equal position	Sr. Research Off	50%	36,000	-
Mr. Shahjahan Mia or a substitute	Lab. Technician	100%	24,000	-
Mr. Mohiuddin or or a substitute	Lab. Attendent	25%	4,225	-

2. Major equipment

One cytocentrifuge - 2,000

3. Supplies & Materials:

a. Special requirement

Chemicals 643.00

EDTA (1 lb: $50); EGTA (100 gm: $63)
Dithiothrietol (5gm: $58); Mercaptoethanol (500gm: $17);
Iodoacetamide (25gm: $33); Lidocaine ampoule, 100mg xylocaine in 5ml (2 dozens: $75); Agarose powder (100G: $100); Trypan blue (25gm: $14)

Common laboratory reagents e.g., Sodium chloride, potassium chloride, Magnesium-chloride and sulphate, calcium chloride, phosphates (mono & dibasic), glucose, indicators etc. ($250)

Biomedical reagents 810.00

Tissue culture dehydrated medium, RPMI 1640 (2x10x1L:$40);
Foetal bovine serum, frozen, 100ml vial (1½ dozen: $180);
Bovine serum albumin, 50gm bottle (6 bottles: $20); Collagenase, type IA Sigma (100mgx5: $60); Concanavalin A, type IV (100x5: $60);
Dehydrated G.P. complement (50x5ml: $250); Brain-heart infusion, dehydrated culture medium (1 lbx5: $200)

Detailed Budget	Taka	Dollar
Immunochemical reagents		900.00

Rabbit antimouse secretory IgA (2mlx2: $160); Rabbit antimouse IgG (L/γchain), an Fab_2 fraction, affinity purified (2mlx2: $160); FITC congugated goat (or rabbit) antimouse gamma globulin (2ml: $100); Rabbit(or goat) antimouse IgG 2a and IgG2b or IgG(2a+b), (2mlx2x2: $320); goat antimouse IgM (μ chain specific or FC specific) (2ml: $80); Goat antimouse Igs (IEP) (2mlx$80)

Other important items		823.00

Nickel-steel wire gauze, 100-and 300-mesh ($40); Plastic tissue cultuer flask, Corning 'No 25100 (one case 500: $145); Sterile petri dish, 60mm diameter, (500x5: $230); Plastic serological test tube, disposable, 5ml capacity (2000x2: $115); Hemocytometer counting chamber, improved Neubauer rulling (1: $42); Counting chamber, Fuchs-Rosenthal, improved Neubauer rulling (1: $43); Optivisor' binocular visual aid, 5x and 10x magnification (2: $60) Interval timer (one: $35); Stop watch one: $45); Tissue grinders, size 13x66mm (2: $48), 13x100 Corning (4: $150); graduated centrifuge tube (pyrex), lifetime red, 17x120mm, 15ml (12x2: $100).

b.	Standard laboratory glass & plastic wares		1,000.00
4.	Cost of animals and special animal handling/care		
	50 mice per set of test and control experiments x 10 sets @ Tk.8 per mice	Tk. 4,000	167.00
	Handling/care charge - per set of experiment with 50 mice, experiment period per mice 2 or 3 weeks - @ Tk.2 mice/day per mice/day: 1,820x10 sets	Tk.18,200	758.00
5.	Stationary	Tk. 720	30.00
6.	Printing and reproduction	4,800	200.00

Note: Most of the items shown under special requirement will have to be ordered in excess to the exact requirement because of getting best advantage of the interlock between quanitity & price as well as the limitations of minimum packages. These are expected to last for a couple of similar experiments in future.

BUDGET SUMMARY

		Taka	US $
1.	Personnel Services	153,025	6,376
2.	Major equipment	-	2,000
3.	Supplies and Materials	-	4,176
4.	Animals plus special handling and care	22,200	925
5.	Stationery	720	30
6.	Printing and Reproduction	4,800	200
	Grand Total :	US $13,707	
	Incremental Cost 10% :	1,371	
		US $15,078	

(Conversion rate US$1 = Taka-24)