Effect of Passive Immunization with Mouse Anti-BCG 64 kDa Protein on Ehrlich Ascites Carcinoma Cells

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Submitted by
Maisha Mosharrat Chowdhury
Student ID: 15276001
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Biotechnology Program
Department of Mathematics and Natural Sciences
BRAC University
Dhaka, Bangladesh

Dedicated to

My Respected Mentor

DECLARATION

I hereby declare that the research work embodying the results reported in this thesis entitled "Effect of Passive Immunization with Mouse Anti-BCG 64 kDa Protein on Ehrlich Ascites Carcinoma Cells" submitted by the undersigned has been carried out under the supervision of Chowdhury Rafiqul Ahsan, PhD, Professor, Department of Microbiology, University of Dhaka and Professor Naiyyum Choudhury, former Coordinator of Biotechnology and Microbiology programs, BRAC University. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

Candidate:

_____

(Maisha Mosharrat Chowdhury)

Certified:

(Professor Naiyyum Choudhury)

Supervisor

Former Coordinator, Biotechnology and Microbiology Program
Department of Mathematics and Natural Sciences
BRAC University

Chairman, Bangladesh Atomic Energy Regulatory Authority

(Chowdhury Rafiqul Ahsan, PhD)

Supervisor

Professor
Department of Microbiology
University of Dhaka

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(Maisha Mosharrat Chowdhury)

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Abstract

Cancer is one of the leading causes of death now-a-days. There are various treatment options for cancer and each has its own side effects because most of the therapeutic agents of cancer are non-selective and may cause destruction of both cancer as well as normal cells. Therefore, search for a highly specific therapeutic molecule is still going on. As it is known that, immune molecules are highly specific in terms of antigen-antibody reactions, so immunotherapy might be a better and more specific choice for cancer therapy compared to conventional molecules in use. Moreover, to handle any emergency cancer cases, there must be instant treatment option through passive immunization. Surface protein antigenic similarity of a 64 kDa *Mycobacterium bovis* BCG surface protein has been reported in the literature. BCG 64 kDa shares a common 64 kDa antigenic determinant with various mouse and guinea pig cancer cells and is cross reactive to one of these cancer cell antigens. The anti-BCG 64 kDa antibody has also been shown to have anti-cancer effects against various solid tumors of experimental animals having cross reactive antigen. However, there is no such report of similar experiment on malignant ascites cell lines. Considering the above facts, this study was undertaken to assess the effects of anti-64kDa antibody on Ehrlich Ascites Carcinoma (EAC) cells. In this study, mouse anti-BCG 64 kDa antibody showed anticancer effect on EAC cell, when compared to control. Animals from experimental group showed increased life span and a plummeted rate of weight gain than negative control. Animals transplanted with EAC cells were treated by two methods. In one method, animals received regular treatment till day 7 and showed 49.2% increase in life span compared to negative control. The other method was cyclic treatment (5 days, treatment, 5 days pause) where life span was increased up to 33.47% compared to negative control. Furthermore, reduced cell growth rate was observed in EAC cell bearing mice treated with anti-BCG 64 kDa containing serum. To confirm the cross reactivity between anti-BCG 64 kDa antibody and EAC cell antigen, whole cell extracts of EAC cells were immunoblotted with BCG 64 kDa immunized mice sera and a 64 kDa band was observed. All these data suggest us that, water soluble BCG 64 kDa surface antigen shares common antigenic determinants with malignant ascites cells and has anti-cancer activity in mice in terms of survival and rate of weight gain. Considering all these results, we conclude that the anti-BCG 64 kDa antibody containing serum could be considered as an effective cancer treatment option.

Contents

List of Figures **V**

List of Tables **VI**

Abbreviations **VII**

Chapter 1: Introduction **1-23**

1.1 *Mycobacterium bovis* BCG 1
- 1.1.1 A brief history of BCG development 1
- 1.1.2 Available BCG strains 5
- 1.1.3 Molecular characterization of BCG 6
- 1.1.4 Physical characterization of BCG 9
- 1.1.5 Biochemical characterization of BCG 11

1.2 EAC cell line 11
- 1.2.1 History of EAC cell line 11
- 1.2.2 Properties of EAC cell line 12

1.3 A brief history of passive immunization 13

1.4 Current scenario of cancer in the world 15

1.5 Available cancer treatments 17

1.6 Use of BCG in cancer immunotherapy 17

1.7 Isolation and characterization of immunotherapeutic subunit from *Mycobacterium bovis* BCG 20

1.8 Objective of the study 23

Chapter 2: Materials and Method **24-40**

2.1 Culturing *Mycobacterium bovis* BCG 25
- 2.1.1 Culture in Ogawa Egg medium 25
- 2.1.2 Culture in 10% Dubos broth 25

2.2 Identification of *Mycobacterium bovis* BCG by Acid Fast staining 26

2.3 Isolation and identification of 64 kDa surface protein from BCG 27
2.3.1 Harvesting BCG cells from Dubos broth 27
2.3.2 Preparation of WEM 27
2.3.3 Estimation of protein content in WEM 28
2.3.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis 29
2.3.4.1 Preparation of gel 29
2.3.4.2 Preparation and loading of sample 30
2.3.4.3 Gel run 30
2.3.4.4 Staining and de-staining of the gel 31
2.3.5 Determining the molecular weight 31
2.3.6 Isolation of 64 kDa protein by blotting 31

2.4 Animal immunization design 32
2.4.1 Immunization of mice and confirmation of antibody production 32
2.4.2 Collection of blood from immunized mice 32
2.4.3 Preparation of serum 33
2.4.4 Confirmation of anti-64 kDa in immunized mice sera by Western blotting 33

2.5 *In vivo* anti-cancer study design 35
2.5.1 Tumor cell transplantation 35
2.5.2 Animal experiment design 37

2.6 Confirmation of cross-reactive property of anti-BCG 64 kDa antibody and EAC cells 39

Chapter 3: Results 41-57

3.1 Culture and identification of *Mycobacterium bovis* BCG 41
3.1.1 Culture of *Mycobacterium bovis* BCG on Ogawa Egg slant media 41
3.1.2 Identification of *Mycobacterium bovis* BCG by Acid Fast staining 41

3.2 Protein concentration estimation in WEM 42

3.3 Determination of desired molecular weight protein 43

3.4 Confirmation of immunization of mice 45

3.5 *In vivo* anti-cancer study with immunized mice sera 46
3.5.1 Group A: After 7 days of continuous treatment, aspiration of intraperitoneal EAC cells for cell count 46
3.5.2 Group B: After 7 days of continuous treatment, observation of mice till death to record survival and rate of weight gain 47
3.5.2.1 Mice survival (%ILS) 47
3.5.2.2 Percentage weight gain of mice 48
3.5.3 Group C: Cyclic treatment and observation of mice till death to record survival and rate of weight gain 51
3.5.3.1 Mice survival (%ILS) 51
3.5.3.2 Percentage weight gain of mice 52
3.5.4 Comparative analysis between Group B and Group C 55
3.5.4.1 Based on survival in days 55
3.5.4.2 Based on percentage weight gain 56

3.6 Confirmation of cross reactivity by Western blot 57

Chapter 4: Discussion **58-63**

Chapter 5: References **64-69**

Appendices **i-vii**

List of Figures

Figure	Title	Page
1.1	Circular representation of *M. bovis* BCG Pasteur chromosome	7
1.2	Mutation in BCG	10
1.3	Scanning electron micrograph of the Line 10 control tumor cells (A) and surface removed cells (B) after water extraction	21
2.1	Methodology of the study	24
2.2	Flowchart showing animal immunization design	34
2.3	Neubauer counting chamber showing the graduation	36
2.4	Flowchart showing animal experiment design	40
3.1	Culture colonies and AFB staining appearances of BCG	41
3.2	Standard curve for protein estimation	42
3.3	Result of SDS-PAGE analysis	43
3.4	Standard curve for determination of molecular weight of desired protein	44
3.5	Result of Western blot analysis	45
3.6	Percentage increase in life span of mice after being treated till 7 days	48
3.7	Percentage weight gain of control and experimental group of mice after being treated till 7 days	50
3.8	Percentage increase in life span of mice after being treated in cyclic manner	52
3.9	Percentage weight gain of control and experimental group of mice after being treated in cyclic manner	54
3.10	Comparison between group B and group C based on survival	55
3.11	Comparison between group B and group C based on percentage weight gain	56
3.12	Result of Western blot analysis to check cross reactivity using whole cell extracts of EAC cells	57

List of Tables

Table	Title	Page
1.1	Available BCG sub-strains	5
1.2	Genomic differences between laboratory and clinical strains of *Mycobacterium bovis* BCG, *Mycobacterium bovis* and *Mycobacterium tuberculosis*	8
3.1	EAC cell count on Day 8 from different mice groups	46
3.2	Results of survival of mice of different groups after 7 days of treatment	47
3.3	Weight of mice in different groups after 7 days of treatment	49
3.4	Results of survival of mice of different groups after cyclic treatment	51
3.5	Weight of mice in different groups after cyclic treatment	53

Abbreviations

The following abbreviations have been used throughout the text.

%	Percentage
et al.	And others
bp	Base pair
BCG	Bacillus Calmette–Guérin
TB	Tuberculosis
rpm	Rotation per minute
nm	Nanometer
WHO	World Health Organization
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy
kDa	Kilodalton
µg	Microgram
ODL	Outer Dense Layer
ETZ	Electron Transparent Zone
EAC	Ehrlich Ascites Carcinoma
TSTA	Tumor Specific Transplantation Antigen
IARC	International Agency for Research on Cancer
UNICEF	United Nations Children's Fund
mA	Mili ampere
Rf	Retention frequency
v/v	Volume in volume
°C	Degree Celsius
FBS	Fetal Bovine Serum
µl	Micro liter
ml	Milliliter
mm	Millimeter
M	Molar
µM	Micro mole
mM	Milli molar
mg	Milligram
gm	Gram
UV	Ultra violet
X	Times
pH	Negative logarithm of hydrogen ion concentration
OD	Optical density
BSA	Bovine Serum Albumin
HCl	Hydrochloric acid
NaCl	Sodium chloride
KCl	Potassium chloride
MST	Mean Survival Time

ILS	Increase in Life Span
Na_2HPO_4	Disodium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
$KHPO_4$	Potassium biphosphate
AFB	Acid Fast Bacilli
WCE	Whole Cell Extract
CAIE	Crossed Affino Immuno Electrophoresis
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
IM	Intramuscular
IP	Intraperitoneal
TAA	Tumor Associated Antigen
WEM	Water Extracted Materials

1. Introduction

Cancer is one of the leading causes of death worldwide now-a-days. The available cancer treatments have their own side effects. Most of the therapeutic agents of cancer are non-selective and causes destruction of normal body cells along with cancer cell. Therefore, search for a highly specific therapeutic molecule is still going on. Immunotherapy of human cancer has drawn the attention because of its high specificity in terms of antigen-antibody reaction. Different types of molecules are currently under investigation, such as, tumor cell lysates, proteins, gangliosides, tumor associated antigens, genes encoding antigenic proteins, etc. (Mondal and Saha, 2011). Proper manipulation of active and passive immunization with immunotherapeutic agents may have prospective role in the treatment of cancer.

1.1 *Mycobacterium bovis* BCG

BCG (Bacillus Calmette Guérin) vaccine is the only available human tuberculosis vaccine that is widely used as a part of the World Health Organization (WHO) Global Expanded Immunization Program., BCG has gained worldwide popularity due to some favorable properties such as widespread safety record in humans, thermal tolerance, low production cost, adjuvant activity (strong inducer of long lasting CD4+ type 1 helper T cell and humoral immunity), ability to provoke mucosal immunity etc. (Zheng et al., 2015).

1.1.1 A brief history of BCG development

It has been more than 100 years since the discovery and development of *Mycobacterium bovis* Bacille Calmette Guérin (BCG) took place (Herr and Morales, 2008; Liu et al., 2009).

It was in 1900 when Albert Calmette, a bacteriologist, and Camille Guérin, a veterinarian, started their research to develop an anti-tuberculosis vaccine at the Pasteur Institute in Lille, France. They started cultivating tubercle bacilli in a medium containing glycerin and potato. Unfortunately, this resulted in non-homogeneous suspension of bacilli. This is due to hydrophobic interactions among cells. And this interaction is mediated by high fatty acid (60%) consistency of the cell wall (Devadoss et. al., 1991). To avoid the tendency of bacterial clumping and adhesion

and optimize animal model experiments, they began to use ox bile, a detergent, to the medium (Liu et al., 2009). Addition of sterile bile allowed the cellular aggregates to turn into state of individual organisms. Surprisingly, they also observed that the virulent property of the organism was lowered. This finding motivated them to commence their long desire of producing vaccine from tubercle bacillus (Luca and Mihaescu, 2013).

Then in 1908, Calmette and Guérin started their long term project with highly virulent bovine strain "lait de Nocard". This *Mycobacterium bovis* strain was supplied by Edmond Nocard, a veterinarian colleague of Guérin in Alfort. He isolated this strain in 1902 from the udder of a cow suffering from tuberculous mastitis (Lugosi, 1992; Yamamoto and Yamamoto, 2007).

Calmette and Guérin cultured the strain in 5% glycerinated potato medium cooked in sterile ox bile and then continued to subculture at roughly three weeks interval (Lugosi, 1992; Michael J Groves, 1992). This eventually led to the discovery of an attenuated vaccine strain named as Bacille de Calmette et Guérin or BCG. First it was named Bacille Bilie Calmette-Guérin upon Guérin's suggestion; later Bilie was omitted (Luca and Mihaescu, 2013).

They observed altered colony morphology along with faster growth rate in the presence of ox bile (Liu et al., 2009). To their surprise, they noticed increased virulence of the organism in biliated medium which was undesired and this was evident by observing the early death of infected guinea pigs. Though it was found that biliated medium induced increased virulence of the organism, continuous subculture on this medium resulted in gradual decrease of virulence. Upon 15 passages on this medium, the organism exhibited strong attenuation in guinea pigs and cattle (Behr and Small, 1999). Even if the organism was cultured on non-biliated potato medium, the virulence was irreversible but colony morphology persisted alterable.

The process of sub-culturing continued. Around after 34th subculture, Calmette and Guérin attained a strain that had desired properties of not triggering tuberculosis in cattle, guinea pig and non-human primates upon high dose administration. This strain was capable of inducing resistance towards virulent tubercle bacilli after 30 days of vaccination.

Later they thought of exploring sub-cellular fractions of the passaged organism for protection against tuberculosis. They prepared acetone and benzene soluble lipid fractions, various tuberculin productions, heat killed bacilli and protoplasts. These subunits were used to immunize eight month old cows. Unfortunately, all these attempts went in vein. Only tuberculin was found to confer little immunity by delaying onset of the disease. This attempt led to the conclusion that presence of live bacilli is directly related to protection against tuberculosis.

The researchers continued passaging at three weeks interval even after obtaining attenuated strain within few months. They maintained this subculture on biliated potato medium for consecutive 13 years (1908-1921). In total, they subcultured the strain for 231 times (Michael J Groves, 1992; Lugosi, 1992; Herr and Morales, 2008; Liu et al., 2009).

The passaging of organism was maintained so strictly that even the First World War did not disturb its continuation. In spite of increased potato price and scarcity of ox bile, the researchers managed to obtain medium components from the veterinary surgeons of the German occupying force (Luca and Mihaescu, 2013).

Finally in 1920, Calmette and Guérin came to a conclusion that the obtained strain was genetically stable and completely avirulent but did not lose its antigenic property, viability and protective action to multiply in the immunized host to confer *premonition* against tuberculosis infections (Lugosi, 1992). They experimented the organism with wide range of animal models which included guinea pigs, rabbits, dogs, cattle, horses, sheep, rats and birds like chickens and non-human primates to establish the safety and efficacy of BCG. They checked its effect in different administration routes like intravenous, intraperitoneal, subcutaneous or oral in diverse doses. All these steps proved the safety and protective ability of the new attenuated strain in all species (Liu et al., 2009).

However, Calmette and Guérin were not sure about testing BCG vaccine in human. A physician from Paris Hospital named Bernard Weill-Halle persuaded them to apply the attenuated strain on a newborn child. The infant was almost certain to develop tuberculosis from his surroundings. So finally on July 18, 1921, the first human trial took place where three 2 mg doses (6 mg total; ~2.4 x 10^8 cfu) were applied by the

oral route on third, fifth and seventh day of life (Liu et al., 2009; Luca and Mihaescu, 2013). The reason of choosing oral route was infection route of tubercle bacillus is gastrointestinal tract, according to Calmette. Later, cutaneous and subcutaneous routes were examined but due to local reactions, oral method was continued. With the success of this incident, BCG was used for treating many more infants, around 664 by the year 1924, without any major side effects (Behr and Small, 1999; Luca and Mihaescu, 2013).

After that, in 1924, the Pasteur Institute started massive production of BCG vaccine and distributed to different laboratories in the world. The vaccine reached to around 34 countries by 1926 and another 26 countries were also enlisted by the end of 1927 (Liu et al., 2009). Then in 1928, the safety of the BCG strain for use in vaccination of animals and human was declared by the League of Nations (Lugosi, 1992).

While distributing vaccine, strong guidelines were recommended on cultivation procedure and usage of the vaccine. Primarily, the vaccine was administered orally but with further recommendation from WHO (World Health Organization), intradermal route was finalized for BCG vaccine as it gives more consistent dosing. Oral route was avoided to conserve the viability of the vaccine from gastric secretion and low pH.

Despite widespread use of BCG vaccine and sharp fall in tuberculosis mortality rate, Calmette and Guérin faced skepticism and questions towards the safety of BCG vaccine. However, Calmette and Guérin were confident enough about the safety of BCG vaccine. Unfortunately, in 1930, a tragic disaster took place in Lubeck. In Lubeck General Hospital, Professor Deycke and Dr. Alstadt were responsible for vaccinating infants. Although the BCG sample was supplied from Pasteur Institute, it was prepared for administration in tuberculosis laboratory of the hospital. The disaster took place after around six weeks. Among 250 vaccinated infants, 73 died of and 135 developed tuberculosis but recovered. This was so tragic and after 20 months of investigation, inattentive handling in Lubeck laboratory was reported which resulted in contamination of the vaccine by virulent tubercle bacilli. This incident spread all over the world and again the questions arose regarding the safety of the vaccine. In Oslo meeting, Calmette defended himself although the popularity of the vaccine had been deemed for a while (Luca and Mihaescu, 2013).

Despite Lubeck incident, BCG vaccination advanced. In 1948, BCG vaccine was declared effective in preventing tuberculosis in First International BCG Congress in Paris. It was estimated that around 10 million vaccinations were done by 1948 (Lugosi, 1992; Yamamoto and Yamamoto, 2007). Even after 2nd World War, to control the severe tuberculosis epidemiology, WHO (World Health Organization) and UNICEF (United Nations Children's Fund) campaigned for BCG in many countries over the world (Lugosi, 1992).

1.1.2 Available BCG strains

Around 80% of world's countries are using BCG vaccination to combat tuberculosis. A single BCG strain was derived from *Mycobacterium bovis* which was then distributed among other countries. Although Calmette and Guérin provided detailed guidelines on the methods of BCG culture maintenance, due to unavailability of lyophilization technique (Zheng et al., 2015) and subsequent passages in modified way, over the years, approximately 21 daughter strains (Table 1.1) have been generated (Moliva et. al., 2015). Different laboratories have maintained culture in modified conditions; resulting in the formation of varied genotypical and phenotypical BCG sub-strains with lower residual virulence level, different viability, immunogenicity and reactogenicity (Lugosi, 1992). To explore the differences among BCG sub-strains, they have been examined in terms of proteomic analyses, biochemical properties, genomics, mycolic acid composition, immunostimulatory effects as Mtb vaccines and as immunotherapy agents for bladder cancer (Zheng et al., 2015).

Origin	BCG strains
Europe	Bulgarian BCG Sophia 222 Czechoslovakian BCG Prague Danish/Denmark Copenhagen strain 1331 French original Bacille Calmette et Guérin strain French Pasteur 1173P2 Polish BCG Poland Romanian BCG Romania 192 Russian BCG Moscow

	Swedish BCG Gothenburg UK Glaxo strain 1077
Asia	Chinese BCG Beijing Chinese BCG Chandan Chinese BCG Lanzhou Chinese BCG Shanghai Japanese BCG Tokyo strain 172
North America	American BCG Birkhaug American BCG Phipps American BCG Tice Canadian BCG Connaught Canadian BCG Frappier Mexican BCG Mexico
South America	Brazilian BCH Moreau

Table 1.1: Available BCG sub-strains (Moliva et. al., 2015)

1.1.3 Molecular characterization of BCG

BCG is a live attenuated bacterium which is derived from *Mycobacterium bovis*. *M. bovis* is a virulent tubercle bacillus, closely linked to *M. tuberculosis*. It is evident that *Mycobacterium bovis* BCG DNA has 99.9% similarity with *Mycobacterium tuberculosis* (Singh et al., 2015).

After 231 subsequent passages, BCG Pasteur strain was developed which never returned to its virulent state. This phenomenon indicates that attenuation of BCG was achieved upon either stable deletions of sequence or genomic regions of duplication or multiple mutations that were not easily revertible (Mahairas et al., 1996; Singh et al., 2015).

After confirmation of BCG safety issues, the strain was disseminated around the world. After that the strain was maintained in different laboratories. Although guidelines were provided for proper handling, due to some changes in handling, various sub-strains were developed. Then in 1960, archival seed lot was introduced and strict restriction was given on number of subculturing from seed lot, that is less or equal to 12 times. Since then, *M. bovis* BCG Pasteur 1173P2 is maintained as

archived seed (1173 refers to passage number). BCG Pasteur strain has circular chromosome of 4,374,522 bp among which 3,954 are protein coding genes (CDS) along with 34 pseudogenes (Figure 1.1). This was revealed using gene prediction and genome comparison approaches. BCG genome is approximately 30 kb larger than its parent *M. bovis* due to presence of two independent tandem duplications, DU1 and DU2. Therefore, BCG Pasteur is considered diploid for 58 CDS and two tRNA genes (Brosch et al., 2007).

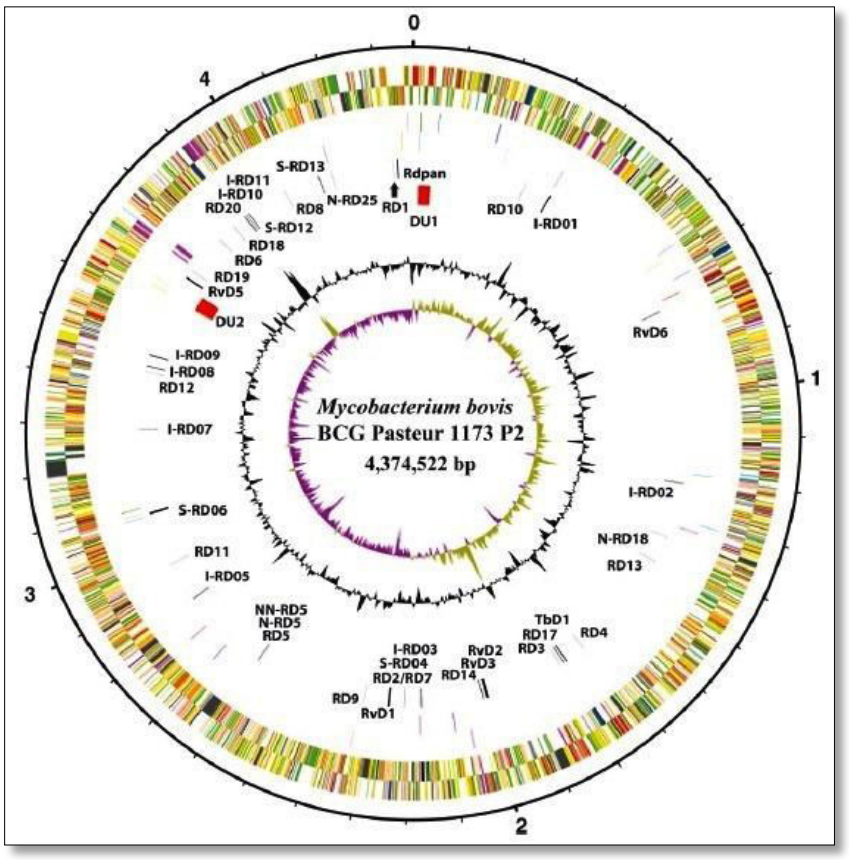

Figure 1.1: Circular representation of *M. bovis* BCG Pasteur chromosome. The outer black circle presents the scale in mega bases. Moving inward, the next two circles show forward and reverse strand CDS respectively, with colors indicating functional classification (Red: replication; Light Blue: regulation; Dark Blue: virulence; Light Green: hypothetical proteins; Dark Green: cell wall and cell processes; Orange: conserved hypothetical proteins; Cyan: IS elements; Yellow: intermediate metabolism; Gray: lipid metabolism; Purple: PE/PEE. The following two circles indicate forward and reverse strand pseudogenes (colors represent the functional classification), the next circle shows RD (black) and DU (red), followed by the GC content (Brosch et al., 2007).

To detect the genetic makeup of avirulent BCG strain, genomic subtraction method was applied. As a result, three genomic regions of difference (RD) were found. Further investigation exposed specific junctions for each of the regions of difference. The comparison was carried out among BCG, *M. bovis* and *M. tuberculosis* (Table 1.2). The identified regions of difference are RD1, RD2 and RD3.

	BCG	*Mycobacterium bovis*		*Mycobacterium tuberculosis*	
		Laboratory	Clinical	Laboratory	Clinical
RD1 (9.5 kb)	Absent	Present	Present	Present	Present
RD2 (10.7 kb)	Present	Present	Present	Present	Present
RD3 (9.3 kb)	Absent	Present	Data not found	Present	Minor case

Table 1.2: Genomic differences between laboratory and clinical strains of *Mycobacterium bovis* BCG, *Mycobacterium bovis* and *Mycobacterium tuberculosis*

It is confirm that RD1 and RD3 are completely absent from all BCG sub-strains whereas RD2 is present in some strains. This occurrence indicates the heterogeneous nature of BCG sub-strains. The primary difference between BCG and *Mycobacterium bovis* and *Mycobacterium tuberculosis* was expression of 10 proteins and some other proteins in higher level even after deletion of genomic RD1 and RD3 sequences. Besides, BCG expresses an acidic protein of around 20 kDa that is not expressed by *Mycobacterium tuberculosis* (Mahairas et al., 1996). Along with that, BCG lacks MPT40 genetic fragment. Moreover, mutations in pncA and oxyR genes also distinguish BCG from *Mycobacterium tuberculosis* (Talbot et al., 1997).

Deletion of RD3 may be of either reason. It is possible that the mother *M. bovis* strain of BCG might lack the RD3 sequence. It is also possible that RD3 was present in *M. bovis* but was deleted during continuous passage (Mahairas et al., 1996).

RD2 deletion was obtained only in sub-strains that are derived from BCG Pasteur strain which pointed to the fact that deletion of RD2 was not a prime feature of original BCG and it was not the result of mutation. RD2 sequence contains a gene named mpt-64 which encodes MPB64 protein. BCG sub-strains those are derived from BCG Pasteur strain before 1925 found to express mpt-64 gene. On the contrary, RD2 is absent in the sub-strains those are originated after 1925 from the same strain,

BCG Pasteur. This led to the conclusion that substantial alterations had been occurred in BCG Pasteur strain after 1925. In southern blot analysis, it was also found that RD2 contains repetitive elements in 10 or more copies along with multiple small repeat sequences at the border of RD2. These sequences are capable of forming hairpin secondary structures and this leads to the root of this deletion (Mahairas et al., 1996).

On the other hand, RD1 was always absent in BCG strain which means that deletion of RD1 is the basis of attenuation (Figure 1.2). To ensure the origin of attenuation, RD1 sequence was restored into BCG. This brought about suppression of at least 10 proteins, down regulation of many cellular proteins and expression of at least three additional proteins (Mahairas et al., 1996; Talbot et al., 1997). The resulted protein profile was much more identical to that of *Mycobacterium bovis* and *Mycobacterium tuberculosis* (Mahairas et al., 1996). Due to deletion of RD1, all BCG strains lack protein secretion system ESX-1. The RD1 locus contains nine genes (*Rv3871-Rv3879c*). Among these nine genes, *Rv3874* and *Rv3875* code for two small, secreted, immune-dominant proteins. One is Culture Filtrate Protein 10 (CFP-10) and another one is Early Secretory Antigenic 6-kDa (ESAT-6) protein. Exportation of these two proteins is mainly facilitated by a secretory apparatus along with one transmembrane protein and two AAA-family ATPases. This secretory apparatus is coded by genes nearby RD1 locus. Several studies deduced that secretion of ESAT-6 and CFP-10 is closely related with RD1 mediated virulence. So the basic idea of attenuation of BCG is primarily deletion of RD1 region (Liu et al., 2009).

1.1.4 Physical characterization of BCG

Mature cell of *M. bovis* BCG apparently looks like cylinder with parallel side and rounded ends. They are usually 2.360 pm and 0.474 pm on average in length and width respectively (Michael J Groves, 1992).

Dried BCG vaccine is a white powder like substance that can be easily dissolved in physiological saline. The pH of this homogeneous suspension is usually within the range of 5.5 to 7.0 (Yamamoto and Yamamoto, 2007).

BCG is primarily present in small micrococcoidal or filterable form which is around 30-50 nm in diameter. Micrococcoidal form is able to survive independently and found in younger growth phase. It is assumed that this form plays a vital role in

maintaining biological activity of the vaccine. This indicates that BCG is dimorphic (bacillary and coccal) in nature and remains in heterogenic condition, depending on culture age (Michael J Groves, 1992).

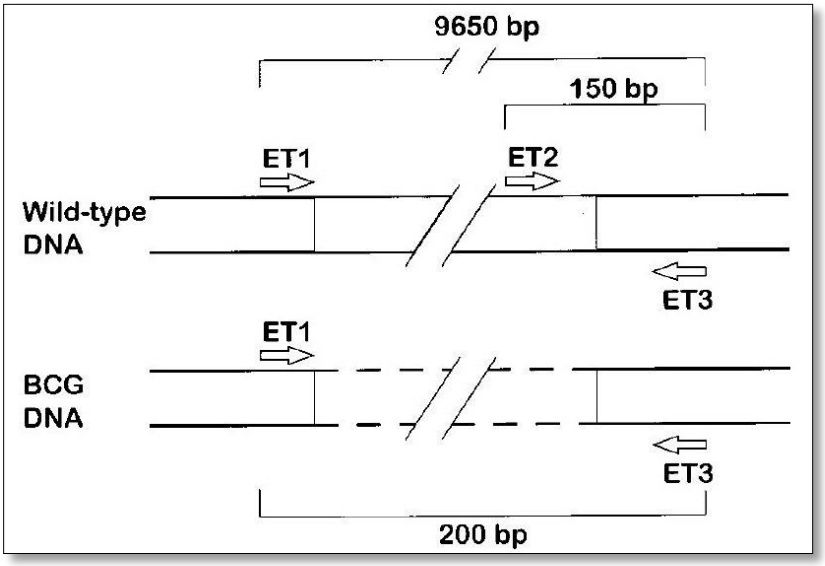

Figure 1.2: Mutation in BCG. The top diagram represented Wild type *M. tuberculosis* DNA. The shaded region indicates RD1 which is deleted in *M. bovis* BCG DNA (represented by dashed lines in lower diagram). The PCR primers ET1, ET2 and ET3 are shown as arrows, oriented in the direction of amplification. Primers ET1 and ET3 are complementary to regions flanking the RD1 sequence. In strains without RD1 (such as *M. bovis* BCG), these primers bind and amplify a 200-bp region. In strains with RD1, these primers bind but the 9,650-bp sequence is too large to efficiently amplify. Primer ET2 is complementary to DNA within the RD1 sequence. Therefore, ET2 and ET3 yield a 150-bp product only in strains with part or all of the RD1 sequence presents (Talbot et al., 1997).

In Scanning Electron Microscopy (SEM), it was found that most BCG cells are aggregated in large clumps. These clumps are covered by thick layer of amorphous material. However, single cells are free from this cover. In this clump, cells are usually aligned parallelly although some cells are oriented in other ways, sometimes perpendicular to majority of bacilli. BCG colonies on agar form a sort of interwoven cords on dorsal surface whereas ventral side comprises of pads or islands. These pads or islands are detached by clefts, going through the colonies (Devadoss et. al., 1991).

Transmission Electron Microscopy (TEM) revealed that BCG cell has trilaminate cell wall structure. This cell wall is made of electron dense cell wall skeleton, cytoplasmic membrane bilayer, a thick electron transparent or diffusely electron dense zone and a

narrow, irregular Outer Dense Layer (ODL). Electron Transparent Zone (ETZ) grows out of the middle wall and pushes the ODL. ETZ from each cell fuses to form a network among them, thus results in amorphous covering material. This covering material consists of acidic groups, carbohydrates and proteins and lacks exposed lipids (Devadoss et. al., 1991).

BCG vaccine is comprised of loosely aggregated cells which have interfacial surface integuments. These integuments are cellulosic in nature. They are mainly released by growing organisms depending on increased oxygen demand in culture medium. Presence of this integument increases the aggregation of cells in culture (Michael J Groves, 1992).

1.1.5 Biochemical characterization of BCG

BCG sub-strains give different results in biochemical tests. Only catalase activity of most of the sub-strains is low. Other biochemical tests, like nitrate reduction, niacin accumulation, urease, Tween 80 hydrolysis, pyrazinamidase, p-amino salicylate degradation and resistance to thiophene 2-carboxylic acid hydrazideetc vary for BCG sub-strain to sub-strain. Even the optimum pH for growth also varies among them (Hayashi et al., 2010).

1.2 EAC cell line

With the aim of better cancer research, animal transplantable tumors have attained a great attention. Among several experimental tumors, Ehrlich Ascites Carcinoma (EAC) cell line from mouse has turned the attention towards it as this cell line is closely similar to human tumors (Ozaslan et al., 2011).

1.2.1 History of EAC cell line

This cell line was first observed in female mouse as breast cancer. Upon this discovery, in 1905, Ehrlich and Apolant started transplanting this tumor tissue subcutaneously from mouse to mouse. Then in 1932, Loewenthal and Jahn entitled this tumor cells as Ehrlich Ascites Carcinoma, based on liquid with cancerous cell, collected from the peritoneum of the transplanted mouse. Later, this cell line was established for qualitative and quantitative cancer studies. Therefore, this cell line was spread promptly all over the world (Ozaslan et al., 2011).

1.2.2 Properties of EAC cell line

EAC cells are undifferentiated and hyperdiploid in nature. They are highly capable of transplantation with rapid growth rate and 100% malignancy although they have shorter life span. These cells do not exhibit any Tumor Specific Transplantation Antigen (TSTA) (Ozaslan et al., 2011). Upon regular microscopy (fixation and staining) or phase microscopy (live cells), EAC cells were characterized. They typically have 8-22 gm diameter with large, eccentric nuclei and large nucleoli. In cell cytoplasm, numbers of mitochondria are present in a bunch on one side (Cassel and McCaskill, 1974).

Ascetic fluid is the discharge in peritoneal cavity which consists of proliferated tumor cells. Upon continuous passage, virulence and proliferation rate of tumor cells increase progressively although differentiation mechanism fades away. This results in uncontrolled growth rate along with ability to heterotransplant, therefore, transformed to ascites form. Ascites liquid is gray or white in color. In some cases, presence of blood makes this fluid light bloody viscous. Usually 10 million tumor cells are present in 0.1cc fluid and cells are homogenously distributed (Ozaslan et al., 2011).

EAC cells can be maintained in the peritoneal cavity of mouse as they are incapable of adhering to synthetic surface *in vitro*. After injecting cells in the peritoneal cavity of mouse, they undergo two phases. First one is proliferating phase where number of cells increases exponentially. Then plateau phase starts followed by resting phase. In resting phase, the number of cells remains consistent. While transiting from proliferating phase to plateau phase, cells undergo some morphological and metabolic changes, like, structural decline, decreased rate of DNA and RNA synthesis due to loss of intracellular purine and pyrimidine nucleotides, nucleosides and bases, lower number of mitochondria, reduction in ATP concentration, low protein synthesis, high rate of thymidine concentration due to lower thymidine kinase activity, low glutathione concentration and high triglycerides, cholesterol esters and free fatty acids (Ozaslan et al., 2011).

During proliferation of EAC cells in peritoneal cavity, cell division takes place along with accumulation of ascites fluid. As a result, high pressure is applied in the peritoneal cavity of the host animal due to increased volume. Moreover, increased number of tumor damages the health of host animal which ultimately results in death

of the host. In a study, conducted by Altun in 1996, it was observed that cell propagation in bone marrow was reserved, depending on the age of tumor in mouse whereas liver regeneration was stimulated by EAC tumor in mouse model (Ozaslan et al., 2011).

1.3 A brief history of passive immunization

Immunity refers to the defense mechanism of the body against infectious diseases which is achieved by an immune response (David Baxter, 2007). Immunity-the state of protection from infectious disease-has both less specific and more specific component. The less specific component provides the first line of defense against infection which is known as innate immunity. Most of these components are present prior to infection and form a set of disease-resistance mechanisms that are non-specific to a particular pathogen. Phagocytic cells, such as macrophages and neutrophils, barriers such as skin, and a variety of antimicrobial compounds synthesized by the host all play important roles in innate immunity. The specific component of immune system does not come into play until there is an antigenic challenge to the organism. This is called adaptive immunity. Adaptive immunity responds to the challenge with a high degree of specificity as well as the remarkable property of "memory". Typically, there is an adaptive immune response against an antigen within five or six days after the initial exposure to that antigen. The major agents of adaptive immunity are lymphocytes and the antibodies and other molecules they produce (Owen et al., 2013).

Adaptive immunity is capable of recognizing and selectively eliminating specific foreign microorganisms and molecules (i.e., foreign antigens). Adaptive immunity can be achieved by passive or active immunization. Short-term passive immunization is induced by transfer of preformed antibodies whereas infection or inoculation achieves long-term active immunization (Owen et al., 2013).

The goal of active immunization is to provoke protective immunity and immunologic memory. After successful active immunization, following exposure to the pathogenic agent elicits amplified immune response that successfully eliminates the pathogen or prevents disease mediated by its products. Active immunization is achieved by inoculation with microbial pathogens that induce immunity but do not cause disease

or with antigenic components from the pathogens. It can be achieved by natural infection with a microorganism, or it can be acquired artificially by administration of a vaccine. In active immunization, the immune system plays an active role by proliferating antigen-reactive T and B cells which ultimately results in the formation of memory cells (Owen et al., 2013).

The ability to transfer immunity from one individual to another by the transfer of antibodies (IgG) is the basis of passive antibody therapy. This indicates development of immunity by receiving preformed antibodies rather than by active production of antibodies after exposure to antigen. In each case, immunity can be acquired either by natural processes (usually by transfer from mother to fetus or by previous infection by the organism) or by artificial means such as injection of antibodies or vaccines. In natural process, preformed maternal antibodies to diphtheria, tetanus, streptococci, rubella, mumps, and poliovirus etc. transfer across the placenta to the developing fetus and provide protection to the developing fetus (Owen et al., 2013). Maternal antibodies present in colostrum and milk also provide passive immunity to the infant. Another type of passive immunity is acquired passive immunity. In acquired immunity, purified, highly concentrated immunoglobulin fractions are provided to protect an immune-susceptible or non-immune individual as medication. These immunoglobulin fractions are produced in human or animals (horse, sheep, and rabbit) by active immunization (David Baxter 2007).

Passive immunization can provide immediate protection to individuals who will soon be exposed to an infectious organism and lack active immunity to it. Passive immunization does not activate the immune system which consequences in no generation of memory response. Thus, the provided protection is transient. Although passive immunization may be an effective treatment, it should be used with caution because certain risks are associated with the injection of preformed antibody. If the antibody was produced in another species, such as a horse, the recipient can mount a strong response to the isotypic determinants of the foreign antibody. This anti-isotype response can cause serious complications (Owen et al., 2013).

The evidence of using immunoglobulin to treat disease was dated back to 19^{th} century. Shibasaburo Kitasato and Emil von Behring used blood products of animals that were recovered from diphtheria infected animal. They heat treated the products and applied

them to immunize guinea pigs. These immunized guinea pigs were then challenged with lethal diphtheria doses and toxins. Soon after this incident, the use of blood products from immunized animals extended to treat diphtheria patients. The trend which was started with diphtheria, with success, the attention drove to cure other contagious diseases like tetanus, small pox, bubonic plague, measles, polio, rabies and infectious hepatitis (David Baxter 2007).

Due to fast emergence of multi-drug resistant organisms, development of non-antibiotic based treatment, especially immunotherapy, is highly recommendable.

In a very recent study, an outer membrane protein, Omp22, of *Acinetobacter baumannii* was targeted as potential antigen to develop efficient vaccine or anti-sera to treat *A. baumannii* infections. The researchers have observed 100% survival of mice and decrease in bacterial load in lung, spleen, liver, kidney tissues and peripheral blood by around 10^5-10^6 folds through passive immunization (Huang et al., 2016).

In a similar study, three proteins (staphylococcal alpha toxin (Hla), staphylococcal enterotoxin B (SEB) and manganese transport protein C (MntC), together termed SAvac-pcAb) from an opportunistic bacterium, *Staphylococcus aureus*, were subjected. These proteins were used for active immunization before. However, due to acute infection caused by *S. aureus*, passive immunization with polyclonal antibody was explored in murine model. After passive immunization of murine sepsis model with rabbit generated polyclonal antibodies, bacterial loads, inflammatory cell infiltration and pathology were decreased at significant rate. Besides, approximately complete protection was provided after infection with lethal dose of *S. aureus* (Zhang et al., 2015).

1.4 Current scenario of cancer in the world

The major impact of cancer on social and personal life is beyond description across the world. Cancer is characterized by the abnormal, uncontrolled growth and spread of body cells which ultimately results in death. Several external (tobacco, infectious organisms, unhealthy diet etc.) and internal factors (inherited genetic mutations, hormones, immune conditions etc.) are responsible for causing cancer and they may work together or sequentially (Cancer Facts and Figures, 2017). Furthermore, some behavioral and dietary habits increase the risk of cancer death, (around 33%) such as,

high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use and alcohol consumption. Also, several infectious agents (especially viruses) are found to be responsible for cancer like, Hepatitis B virus, Hepatitis C virus, Human Immunodeficiency virus (HIV), *Helicobacter pylory* and Human Papilloma virus (HPV). These viruses and bacteria originate cancer causing infection which leads to 25% of cancer cases in low and middle income countries (WHO, 2014). The vital causes of skin cancer (more than 5 million cases annually) are excessive exposure to sunlight and use of indoor tanning devices. Usually it may take ten or more years to cause cancer at detectable level after exposure to external factors (Cancer Facts and Figures, 2017).

Cancer is ranked as second and third leading cause of death in high and middle and low income countries respectively. In 2012, around 14.1 million new cancer cases and 32.6 million people living with cancer (within 5 years of diagnosis) were reported worldwide, according to the International Agency for Research on Cancer (IARC). Among this, 8 million cases belonged to economically developing countries which is around 82% of world population (Global Cancer Facts and Figures, 2015). In 2014, approximately 15,780 children and adolescents (aged 0-19 years) were diagnosed with cancer among which 1,960 died (National Cancer Institute, 2017). Based on regional division, more than 60% new cancer cases take place in Africa, Asia and Central and South America. According to report, the number of total deaths due to cancer was approximately 8.2 million over the world which means about 22,000 deaths per day. Comparatively, cancer death rate is lower in economically developed countries (2.9 million) rather than developing countries (5.3 million) (Global Cancer Facts and Figures, 2015).

In 2016, the most common cancers were breast cancer, lung and bronchus cancer, prostate cancer, colon and rectum cancer, bladder cancer, melanoma of the skin, non-Hodgkin lymphoma, thyroid cancer, kidney and renal pelvis cancer, leukemia, endometrial cancer and pancreatic cancer (National Cancer Institute, 2017). For men, the most common cancers include lung, prostate, colorectal, stomach and liver cancer. In case of women, the list includes breast, colorectal, lung, cervix and stomach cancer (WHO, 2014). In the United States, the annual expenditure for cancer care was nearly $125 billion in 2010 which is supposed to reach up to $156 billion by the end of 2020 (National Cancer Institute, 2017).

It is found in study that cancer mortality rate is comparatively higher in men than women, around 207.9 and 145.4 per 100,000 men and women respectively (National Cancer Institute, 2017). Moreover, cancer mortality rate is dependent on some factors that vary from region to region worldwide. The prime factors are prevalence of major issues, availability and use of medical practices such as cancer screening, availability and quality of treatment and age structure. Based on 2008-2012 deaths, highest cancer mortality rate is seen in African American men (261.5 per 100,000) whereas lowest rate is observed in Asian/Pacific Islander women (91.2 per 100,000) (National Cancer Institute, 2017). In Bangladesh, cancer is considered as the sixth leading cause of death and around 60% patients die within 5 years of diagnosis.

It is assumed that by 2030, the number of new cancer cases will grow to 21.7 million and death number will increase up to 13 million (Global Cancer Facts and Figures, 2015). Therefore, all these statistics indicate cancer as an on-growing global, socio-economic burden.

1.5 Available cancer treatments

Cancer treatment modalities include surgery, radiation, chemotherapy, hormone therapy, immunotherapy and targeted therapy using drugs that interfere with cancer cell growth by targeting specific molecules. Each of these treatments has its own advantages as well as disadvantages. Radiation and chemotherapy are not highly specific, thus result in destruction of normal cells along with cancer cells. Surgery is also not safe due to tumor recurrence at operative site as a result of incomplete removal or spillage of tumor cells (Lamm et al., 1979). A more specific and effective mode to treat cancer is immunotherapy. In this approach, body's natural defense system is boosted to fight against cancer cells. The boosting can be achieved through both active and passive immunization. Usually immune molecules are highly specific regarding binding and this characteristic of immune molecules makes them highly efficient cancer treatment option with less side effects.

1.6 Use of BCG in cancer immunotherapy

BCG has been used as vaccine for tuberculosis for a long time. Besides this, BCG is also used as immunostimulator for the treatment of cancer since 1960 (Lugosi et al., 1992). However, the history of role of BCG in cancer treatment started back to 1929

when Pearl found the link between decreased rates of cancer in tuberculosis patients. Then in 1935, Holmgren used BCG for the treatment of stomach cancer. Subsequently in 1936, stimulatory effect of BCG on reticuloendothelial system was observed by Rosenthal. The most significant study was carried out by Old and Clarke in 1950 where they witnessed that BCG could inhibit cancer progression in animals. Neonates received BCG vaccine had lower risk of leukemia. Moreover, upon intralesional administration of BCG into skin lesion, melanoma could be recessed up to 92% (Gandhi et al., 2013). Several studies have reported the effect of BCG vaccine in leukemia, breast, lungs, intestinal and genitourinary cancers (Lugosi et al., 1992). In prostatic adenocarcinoma, BCG injection creates granulomatous response along with tumor necrosis. However, this practice was suppressed due to risk of fatal septic reactions. In animal model, BCG was found to defeat prostate cancer (Gandhi et al., 2013).

According to Lamm et al. (1979), BCG can also be used to reduce the rate of tumor recurrence at the operative site which may happen due to incomplete removal or discharge of cancer cells during operation. They found decreased rate of tumor recurrence and long term survival in 40% animals, using BCG Tice strain.

Although BCG showed significant success in cancer regression, use of BCG has been faded away with the advance of radiotherapy and chemotherapy (Gandhi et al., 2013). However, in bladder cancer, application of BCG intravesically kept showing promising outcome (Lugosi et al., 1992).

Since last three decades, intravesical instillation of BCG has been considered as the prime treatment of superficial urothelial bladder carcinoma. These tumors remain non-muscle invasive at the primary stage, and can be treated with proper intravesical prophylactic treatment (Begnini et al., 2015). The mechanism of action of BCG vaccine in bladder cancer is not fully discovered yet. However, it can be assumed that a very complex and multifaceted mechanism is involved due to various components of BCG cell (Michael J Groves, 1992). Moreover, being a complex living system, the responses of BCG vaccine towards bladder cancer in human are quite comprehensive and high ranging. An immune competent host, proper BCG dose, close proximity between BCG and tumor cells and low cancer cell number are required for successful immunotherapy using BCG in bladder cancer. The induction routine for successful

BCG therapy includes a 6 week course, usually 2-3 weeks after transurethral resection (TUR). However, for maximum efficiency, the therapy should be continued for 1-3 years. Each dose consists of 2-19×10^8 CFU lyophilized BCG, depending on BCG sub-strains. After making the bacterial suspension in physiological saline, it is instilled into an empty urethral lumen through catheter (Zheng et al., 2015).

For the immunotherapeutic treatment of Non-Muscle Invasive Bladder Cancer (NMIBC), transurethral resection is carried out followed by intravesical instillation of BCG as immunotherapeutic agent. This treatment effectively delays the stage progression of NMIBC to MIBC (Muscle Invasive Bladder Cancer). Both innate and adaptive immune responses are involved in this method. However, the induced response does not evoke development of any memory and that's why therapy is continued for better consequence. After instillation of BCG intravesically, they are internalized into the bladder tumor cells as well as urothelial mucosa cells and adhere to the inner surface of these cells. This binding is mediated by fibronectin and integrin receptors. Due to negative charges on both bacterial cell and body cells, repulsion takes place which indicates the requirement of high doses of BCG. After internalization of BCG, the innate immune response releases several cytokines (TNF-α, IFN-γ, IL-2, IL-6, IL-8, and IL-12) which triggers the release of neutrophils, T lymphocytes, BCG activated killer cells and natural killer cells and thus, results in abolition of BCG containing urothelial cancer cells (Zheng et al., 2015).

Comparisons between BCG and other chemotherapeutic agents (Thiotepa, Doxorubicin, Mitomycin C) revealed that BCG is profoundly better than those agents (Gandhi et al., 2013). Although immunotherapy using BCG has been considered as primary treatment for bladder cancer, this has some major drawbacks. Such as:

1. Presence of installed BCG in urethral lumen for limited time due to frequent bladder discharge
2. Treatment failure due to insufficient or excess BCG instillation (recurrence or progression)
3. Emergence of resistance because of natural resistance associated macrophage protein (NRAMP1) gene polymorphism in patient
4. Presence of unresponsive tumors

5. Moderate to severe local (dysuria, cystitis, and macroscopic hematuria) and systemic (fever, pneumonitis, hepatits, sepsis) side effects due to use of live bacteria
6. Dissemination of infection (BCGosis) to lungs and genitourinary tract in immune-suppressed individual (Begnini et al., 2015; Zheng et al., 2015)

Along with using BCG vaccine for the treatment of bladder cancer, it is also considered for applying against lung cancer and leukemia. *In vitro* and clinical trials are conducted to determine the efficacy of the vaccine against melanoma and colorectal cancer. It was also assumed that BCG has some cytotoxic effects due to its antigens. Among them, mycolic acid is known for cytotoxicity. Studies have been carried out to explore the anti-cancer effect of this substance (Michael J Groves, 1992).

1.7 Isolation and characterization of immunotherapeutic subunit from *Mycobacterium bovis* BCG

Mainly Tumor Associated Antigens (TAA) presented on the membrane of tumor cells has caught the attention in the field of immunotherapy. Since last century, several attempts have been taken to identify, purify and characterize these antigens for using as immunotherapeutic agent to defeat cancer. However, most of the studies have focused on whole cells rather than focusing on surface antigenic proteins. In 1989, Ahsan and Sasaki devised a method, named Water Extraction Method (WEM), to isolate the surface proteins of cells (Ahsan and Sasaki, 1989). This method is the preliminary step of extracting purified tumor associated antigens by isolating specific surface proteins. In a study, Water Extraction Method (WEM) was applied to Line 10 hepatocarcinoma cell and normal liver cell from strain 2 guinea pig (Figure 1.3). A high concentration of TAA containing solution was obtained from Line 10 tumor cell which were not detected in normal liver cells. These TAAs were further studied by different immunological methods to check their composition and characterize their antigenic nature. Most of the TAAs obtained from hepatocarcinoma cells were glycoproteins of different molecular weights (44, 46, 62, 64 and 68 kDa) which were absent in the normal liver cells. Instead, an 82 kDa protein band was obtained in the WEM of normal liver cells (Ahsan and Sasaki, 1989).

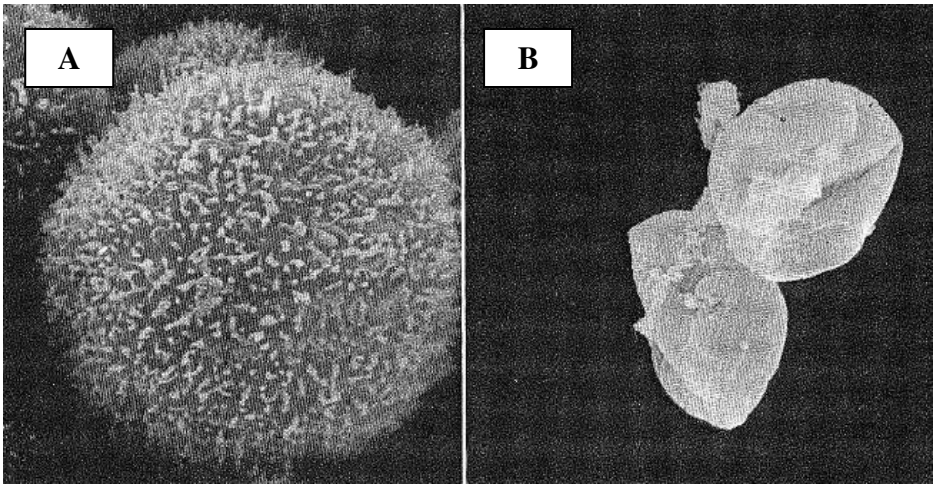

Figure 1.3: Scanning electron micrograph of the Line 10 control tumor cells (A) and surface removed cells (B) after water extraction. The surface structures containing the microvilli projections were prominent in control cells, whereas, after the water extraction, the microvilli projections were completely removed (Ahsan and Sasaki, 1989).

Purification of mycobacterial antigens from antigenic culture mixture has been considered as a difficult procedure due to complex structure of Mycobacterium. This slows down the overall progress of studying immunological properties of mycobacterial antigens (Thole et al., 1987). Several studies have been conducted to purify and characterize species-specific immunologically active mycobacterial antigens. In 1991, Water Extraction Method (WEM) was implied to *Mycobacterium bovis* BCG to isolate surface proteins. The aim was to purify and characterize immunologically active mycobacterial surface antigens that could be used to induce cellular immune response in host. Among various antigens, 64 kDa antigen from *Mycobacterium bovis* BCG has received major response. This antigenic protein is highly soluble and has active immunological properties. Its immunogenic character was shown in animal model by eliciting strong delayed type hypersensitivity reaction and significant increase in IgG antibody level (Ahsan and Sasaki, 1991).

Mycobacterium bovis BCG has been investigated widely for decades to be used as therapeutic agent of cancer in experimental animals. Several attempts have been taken to establish the antigenic relationship between tumor cells and BCG. There is evidence that antigens from human malignant melanoma and acute myeloid leukemia share similar antigens with BCG (Minden et al., 1982). With this aim, four BCG monoclonal antibodies, named 602, 603, 609 and 612 were examined against Meth A,

RL♂1, Colon Tumor 26 of mouse and line 10 guinea pig tumor. Among these monoclonal antibodies, 602 showed broad range of reactivity against all types of tumor cells of sarcoma, colon carcinoma, leukemia, and hepatoma. Other monoclonal antibodies, 603, 609, and 612, reacted more or less with the tumor cells. This study validated the long term assumption of common antigenic determinants between different tumor cells and BCG (Sasaki et al., 1989).

Further analysis was carried out to determine the antigenic relationship between the tumor cells and BCG by SDS-PAGE and Western blotting analysis. This analysis resulted in identification of a band with molecular weight of 64 kDa which was common in both tumor cells (Meth A, RL♂1, Colon Tumor 26, line 10) and BCG. It has been demonstrated that 64 kDa protein from BCG has anti-line 10 activity in immunized experimental animals, guinea pig. This revealed that the BCG 64 kDa and the line 10 tumor cell 64 kDa are identical (Ahsan and Sasaki, 1991). Also in mice immunized with BCG 64 kDa protein, 37% and 50% mice respectively showed complete rejection of further growth of Meth A and CT-26 tumor cell when challenged (Ahsan and Sasaki, 1993). Therefore, all these data together supported the earlier finding and suggested that BCG 64 kDa is probably identical with the tumor specific antigen (Ahsan and Sasaki, 1991).

The evidence supporting immunotherapeutic effect of BCG 64 kDa surface protein warrants further study as a potential therapeutic strategy against cancer. Till date, most of the studies were conducted using tumors such as Meth A, Colon Tumor-26 (CT-26), Line 10 hepatocarcinoma cell and RL♂1 and their experimental method was confined to study anti-cancer activity of BCG and its proteins through active immunization. However, people are rarely aware of developing tumor cells. Therefore, there must be immediate treatment modalities available to treat cancer. Moreover, in immunosuppressed individual, development of active immunity is quite difficult. As a result, they cannot be treated using vaccination strategy. Although use of regular drugs has been advanced a lot, treatment with preformed antibody is still a valid treatment strategy for certain conditions. Hence, there is need of strategy to treat cancer through passive immunization.

1.8 Objective of the study

Till now, there are no reports on *in-vivo* effect of BCG 64 kDa protein or its corresponding antibody on ascetic cells (all the studies were performed on solid tumors). Therefore, our study was conducted with the following objectives:

- Identification and partial purification of 64 kDa water soluble surface protein from *Mycobacterium bovis* BCG.
- Preparation of mouse anti-BCG 64 kDa antibody.
- Performing *in vivo* anti-cancer experiment through passive immunization of EAC cell transplanted mice with mouse anti-BCG 64 kDa antibody.

2. Materials and Method

The methodology of this study can be shortly described in the flowchart below (Figure 2.1):

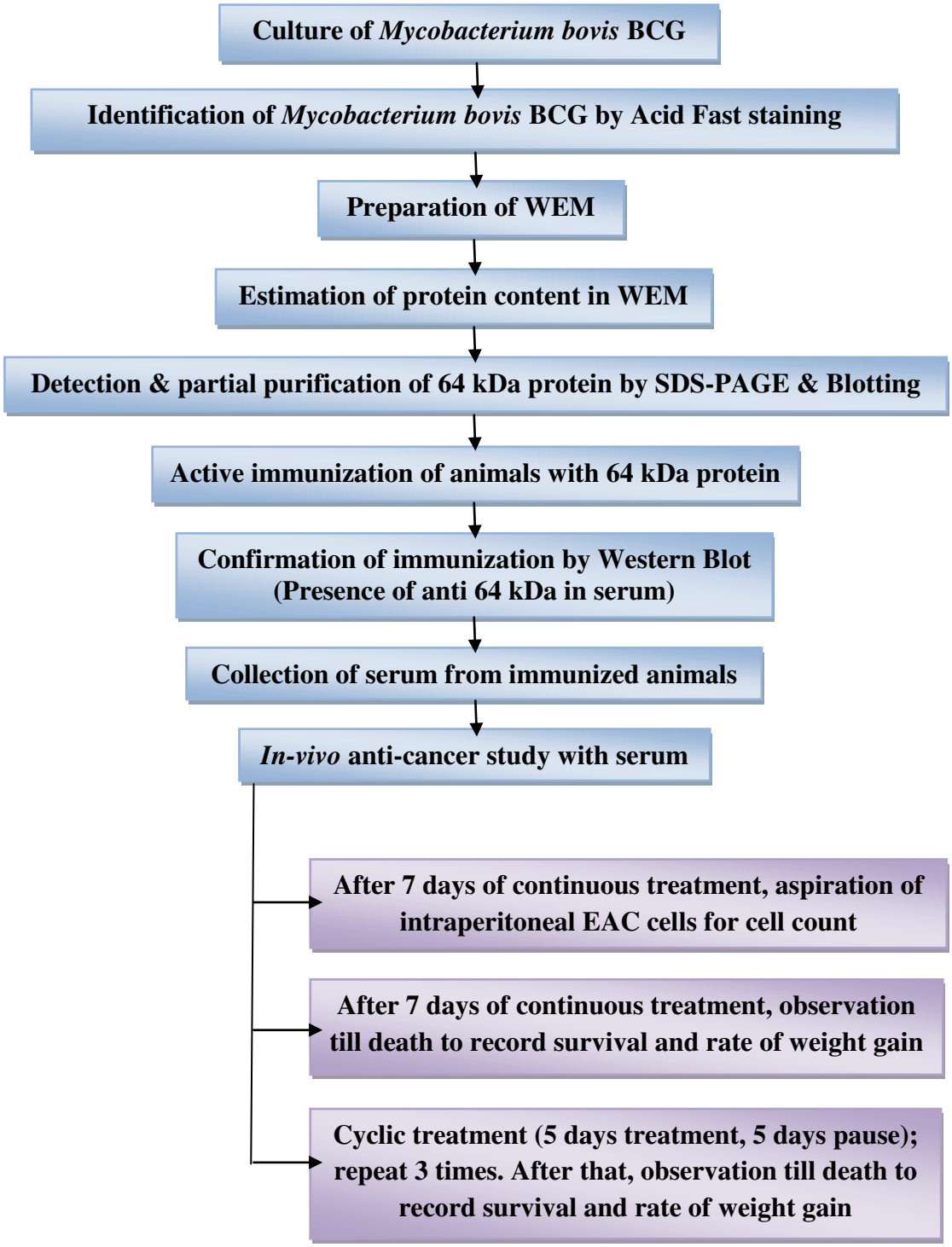

Figure 2.1: Methodology of the study

2.1 Culturing *Mycobacterium bovis* BCG

2.1.1 Culture in Ogawa Egg medium

Lyophilized *M. bovis* BCG (Bulgarian strain SL 222 Sofia) was cultured at first on Ogawa Egg slant medium. This is a selective medium for the culture of *Mycobacteria* as it contains malachite green that inhibits the growth of other bacteria. Presence of sodium pyruvate in the medium (instead of glycerol) induces the quick recovery of *M. bovis* BCG from lyophilized form.

The procedure of inoculation was performed in laminar airflow, maintaining additional aseptic precautions. At first, lyophilized BCG was dissolved in 1 ml sterile physiological saline which is usually provided with the ampoule. The inoculating loop was sterilized by heating red hot, and then cooled to normal temperature. For inoculation of BCG in Ogawa Egg slant medium, sterilized loop was dipped into the suspension of BCG and inoculated by streaking method in zigzag pattern. The cap of the vial was kept loose to permit access of air into the vial as *M. bovis* BCG is an aerobic bacteria. After proper labeling, the vial was incubated at 37ºC for 3-4 weeks and checked every week. After achieving sufficient growth of the bacteria which is indicated by change of color of the medium and appearance of distinct yellowish colonies of BCG, further culture was done in 10% Dubos broth.

2.1.2 Culture in 10% Dubos Broth

After proper cleaning of conical flasks with distilled water, required amount of Dubos broth base (BD Difco^{TM}, USA) powder was measured and dissolved in deionized water by swirling. Next, the homogenous mixture was distributed in equal volumes in conical flasks. This was done in a manner so that the total volume of media in flask occupies 1/5th volume of the total flask volume. The flasks were then sealed using aluminum foil and labeled properly before sending them to be autoclaved. The autoclave was carried out at 121ºC and 15 psi pressure for 15 minutes. Once sterilization was done, the flasks were allowed to cool down to room temperature before adding Fetal Bovine Serum (FBS).

Fetal Bovine Serum (FBS) is usually added at 10% concentration of the medium. FBS was allowed to melt down previously at room temperature. Addition of FBS in

autoclaved medium was carried out in Biosafety Cabinet II. For maintaining aseptic condition, the working area of the cabinet was wiped with 70% ethanol, followed by UV light exposure for 45 minutes. As FBS is an expensive media component, the room UV was also left on for the same duration to avoid additional risk of contamination. After completion of UV sterilization, the lights were switched off and the blower was turned on before lifting the hood. All the required materials (sterile disposable pipette-10 ml, pipette filler, FBS bottle, autoclaved flask containing medium) were then wiped with 70% ethanol and transferred into the cabinet. Under proper aseptic condition, using 10 ml sterile pipette, FBS was added to each flask at 10% (v/v) concentration. Then the flasks were incubated at 37ºC overnight to check contamination.

After overnight incubation, the flasks were checked. Contamination free flasks were taken to laminar airflow chamber. Contaminated flasks were sent to autoclave before discarding the content and then washed. In laminar airflow chamber, under sterile condition, BCG was inoculated in Dubos broth from Ogawa Egg slant medium. Then, the flasks were incubated at 37ºC. After 24 hours, all the incubated flasks were checked for contamination. The generation time for BCG is usually 20-22 hours. Therefore, presence of turbidity in the flask indicates growth of other microorganisms. Contaminated flasks were discarded while rests were incubated for 3-4 weeks.

2.2 Identification of *Mycobacterium bovis* BCG by Acid Fast staining

A clean, grease free slide was taken and labeled. A drop of normal saline was placed on the slide and then a suspension was made by mixing part of bacterial colony in the saline. The suspension was spread widely and heated to prepare a fixed smear. The slide was flooded with heated carbol fuchsin for 5 minutes (primary staining). After removing carbol fuchsin, the smear was covered with 3% (v/v) acid alcohol mixture for 5 minutes (Destaining) or washed alternatively with acid and alcohol till the smear appears colorless or light pink and then washed with deionized water. Counter staining was done by pouring malachite green on the smear and allowed to stand for 2 minutes. The smear was air dried and examined under 100X oil immersion objective.

2.3 Isolation and identification of 64 kDa surface protein from BCG

2.3.1 Harvesting BCG cells from Dubos broth

After 3-4 weeks of inoculation, upon sufficient turbidity of the broth, BCG cells were harvested. For maintaining homogeneity of the suspension, flasks were gently swirled before dispensing. Next, the suspension was transferred to sterile 50 ml centrifuge tubes (40 ml in each tube). The tubes containing medium were balanced before placing them in the centrifuge machine to avoid any damage on the rotor. Equally balanced tubes were then placed opposite to each other in the centrifuge machine, and the centrifugation was conducted at 16,000g for 25 minutes (Ahsan and Sasaki, 1991). Later, the supernatant was discarded and the cell pellet was suspended in sterile physiological saline (0.85% NaCl). This step was done to appropriately wash out medium from the cells. If there were multiple tubes containing cell pellet, all the suspended cells were collected in a single sterile 50 ml centrifuge tube, for convenience as well as to avoid loss of cells. Afterwards, centrifugation at 10,000g for 10 minutes was conducted to discard the saline. This washing step was repeated 2-3 times to completely wash out media components.

2.3.2 Preparation of WEM

After harvesting BCG cells from Dubos broth, the cells were finally suspended in deionized distilled water (usually about 3-5 ml depending on estimated cell volume measured by naked eye). This suspension was transferred to a sterile flat bottomed 100 ml conical flask. The rationale for using a flat bottomed conical flask is to attain the correct speed to hit the flask wall by low volume of suspension during horizontal shaking. Without proper collision force, the surface proteins would not be extracted from the BCG cells. Hence, after placing the flask in horizontal shaker, the speed of shaker was set to 100-120 oscillations per minute while the shaking was continued for 16 hours in room temperature.

The suspension was then collected in a sterile 15 ml centrifuge tube. The tube was centrifuged at 16,000g for 30 minutes; the supernatant was then collected using a sterile 5cc syringe. Due to overnight shaking, the water soluble surface proteins from BCG cells were dissolved in water which is known as WEM. WEM was sterilized through filtration using a 0.2 µm Millipore filter. The filtered material was aliquotted

into a sterile 1.5 ml centrifuge tube and stored at -20ºC. The cell pellet was discarded after autoclave.

2.3.3 Estimation of protein concentration in WEM

Bradford protein assay protocol was used for the estimation of total protein content in WEM. During this process, BioRad protein estimation dye concentrate as indicator dye, and, Bovine Serum Albumin (BSA) as standard protein sample, were used. A 10 mg/ml BSA stock solution was made by dissolving required amount of BSA in deionized distilled water. This stock can be reused within 60 days. After that, it is aliquotted and stored at -20ºC. Usually, while using BSA as standard in this assay, the linear range is 0.2 to 0.9 mg/ml. Therefore, four different dilutions (0.2, 0.4, 0.6, and 0.8 mg/ml) of BSA within the range were prepared.

To make dye reagent in desired concentration, 1 portion of Dye concentrate was mixed with 4 parts of deionized distilled water. After that, to remove particulates, the solution was filtered through the Whatman#1 filter paper. This prepared dye reagent is usable for 2 weeks, if stored at room temperature.

The assay was conducted in triplicate set. After preparing all required reagents (different dilutions of standard solution, dye and sample), 100 µl from each of the diluted standard solutions and sample were pipetted into clean dry test tubes. Next, 5 ml of diluted dye was added to each test tube and properly mixed using vortex mixer. Blank was prepared by mixing dye reagent to deionized distilled water. The solutions were then incubated at room temperature for at least 5 minutes. Incubation longer than an hour was avoided since absorbance of sample increases proportionately with incubation time increase.

Absorbance was measured at 595 nm using a spectrophotometer. After obtaining all the absorbance values of BSA standard solution, they were plotted on graph to produce a standard curve. The absorbance of sample was then plotted on the standard curve to determine the concentration of protein.

2.3.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Preparation of all buffers and solutions required for SDS-PAGE has been mentioned in Appendix II.

2.3.4.1 Preparation of gel

At first, the required materials for a single gel (one short plate, one spacer plate and a comb) were collected. The glass plates were thoroughly cleaned with soap and water and wiped with 70% ethanol. The comb was also cleaned with tap water for removal of any left-over polyacrylamide.

Next, the glass plates were assembled through placing the short plate on top of the spacer plate. This set up was then placed into the holder. Setting it up on melted agarose was carried out to ensure bottom sealing of the glasses. This could be checked by observing the risen agarose gel in between glass plates through capillary action. The seal was also checked for its consistency by pipetting small volumes of water between the glass plates; this water was then removed by filter paper.

The overall setup was conducted in a manner to ensure that the small glass plate was present in front of the investigator. Afterwards, the comb was introduced in the gel chamber to mark the required height of the separating gel. For this purpose, a mark was put approximately 1 cm below the lower end of the comb on the exterior of the small plate.

Following the recipe, a 12.5% separating gel was prepared through pipetting required amount of solutions in a beaker. All the components were pipetted carefully to avoid bubble formation, since presence of bubble inhibits polymerization. Before addition of APS and TEMED, the solution in beaker was swirled gently.

Upon addition of TEMED, polymerization generally initiates. Hence, the gel solution was pipetted to the gel chamber up a bit higher than the mark, so that, when the gel solidifies and retracts, the upper border of the gel reaches down to the marking. After loading the gel solution, butanol saturated water was added at the top until the highest level of the plates, to protect the gel from direct contact with oxygen. Presence of oxygen decays the gel by inhibiting polymerization. Solidification of the gel was

ensured by observing a refraction gradient line at the junction of butanol saturated water and solidified gel.

Next, stacking gel was prepared in the same beaker. Each component was added carefully to avoid bubbling. Soon after addition of TEMED, butanol saturated water was discarded and excess liquid was blotted off using Whatman filter paper without damaging the top layer of the gel. The stacking gel solution was then pipetted on top of the polymerized separating gel, up to the edge of the short plate; the comb was introduced straight on down. While placing the comb in the stacking gel, care was taken to avoid any bubble formation at the bottom of the comb. Solidification of stacking gel was confirmed through observation of gel formation in remaining solution in the beaker.

Once the gel was polymerized, the gel cassette was placed into the electrophoresis tank in a manner that the large glass facing towards the investigator. Running buffer was poured into the both chambers (outer and inner). Then the comb was removed for visualization of the wells. Lastly, trapped bubbles at the bottom of the glass were removed by budging the gel cassette to and fro.

2.3.4.2 Preparation and loading of sample

In order to produce improved protein band pattern, the raw sample was diluted at different concentrations. Afterwards, a 2X sample buffer was added with each sample at a ratio of 1:1. The mixture was then heated in boiling water for 5 minutes. After cooling down, 5-10 µl (depending upon sample mixture volume) tracking dye (Bromophenol Blue) was added to each mixture and mixed properly by vortexing. Once the samples were prepared, 25-30 µl of each sample was loaded into separate wells.

2.3.4.3 Gel run

After completion of sample loading, the lid with proper wire connection was placed at the top of the tank. The other ends of the wires were connected to the power pack. After volt freeing the power pack, the electrophoresis was initiated at 16-18 mA current flow. Formation of bubble at the bottom of the gel cassette indicated the starting of electrophoresis. Downward displacement of sample was observed. When

the dye reached the separating gel, the current flow was increased to 20-22 mA. When the tracking dye reached the bottom of the gel or just flew out of the gel, the power pack was turned off. After detaching all connections, the gel was taken out from the chamber carefully, and the stacking gel was cut down using a gel cutter. The obtained separating gel was then subjected to staining.

2.3.4.4 Staining and de-staining of the gel

The separating gel was placed in a staining solution for observing protein bands. The gel was flooded with the solution and shaken gently for an hour in room temperature. After staining, the solution was restored for future use, and de-staining solution was added. De-staining was conducted overnight. The gel was observed the next morning in naked eye against a bright background.

2.3.5 Determining the molecular weight

The migration distance of each standard band and the tracking dye front was measured using a ruler. This step was repeated for the unknown band in the sample. Then, the Rf value was calculated for each band in the standards, using the following formula:

$$\text{Relative Mobility (Rf)} = \frac{\text{Migration Distance of Protein}}{\text{Migration Distance of the Tracking Dye Front}}$$

After that, a calibration curve was prepared plotting the log (molecular weight) values on Y axis against Rf values on X axis (both values are for standard bands). The equation y = mx + b was generated and used to determine the molecular weight of the unknown protein.

2.3.6 Isolation of 64 kDa protein by blotting

For this purpose, the separating gel was not stained in staining solution after gel electrophoresis. Using Whatman filter paper, gel and nitrocellulose membrane, a sandwich was made where the sequence of these positioned materials were as follows, from cathode (negative: black) to anode (positive: red):

Filter paper>Gel>Nitrocellulose membrane>Filter paper

After placing the materials sequentially, trapped air bubbles within the layers of sandwich were removed by gently pressing with roller. The sandwich was then placed in the blotting chamber and transfer buffer was poured in. The lid was placed and connection wires were connected. The other ends were plugged in power pack which was current freed before running. Transfer was completed overnight at 12-15 volts. The next morning, the power pack was switched off and the membrane was separated from the sandwich very carefully. The membrane was then stained and de-stained with 0.1% ponceau stain and distilled water respectively for visualization of the protein bands to the nitrocellulose membrane.

After calculating Rf value, the desired band was identified and cut. The cut membrane was sonicated to fine particles in physiological saline (0.85% w/v sodium chloride). The sonicated particles need to be small enough to pass through 25G hypodermic needle in order for the suspension to be used for animal immunization.

2.4 Animal immunization design

For the purpose of animal experiment, Swiss Albino mice were used, purchased from ICDDR,B animal facility.

2.4.1 Immunization of mice and confirmation of antibody production

Immunization was done in 2 groups of mice. Each group contained 60 mice, each weighed 15 to 20 gm on average. The groups were designated as Experimental and Control. Immunization was done by 4 intramuscular doses on day 0, day 14, day 28 and day 42 at different sites. The experimental group received sonicated nitrocellulose membrane containing 64 kDa protein whereas the control group received sonicated nitrocellulose membrane only (Figure 2.2).

On day 35, blood was collected from tail veins of mice from experimental group and control group and sera were used to perform Western blot analysis to confirm development of anti-64 kDa antibody.

2.4.2 Collection of blood from immunized mice

On day 49, all the immunized mice from both groups were sacrificed after collection of blood by cardiac puncture.

2.4.3 Preparation of serum

Blood from each mouse was taken into separate, sterile 1.5 ml centrifuge tube; labeling of the respective groups "experimental" and "control" was maintained. The tubes were then kept undisturbed at a slant position at 37ºC in an incubator for an hour. They were next taken out to check blood clotting and transferred to 4ºC and kept in upright position overnight for completion of clotting mechanism. Next morning, the serum was collected with a sterile micropipette and transferred to a properly labeled sterile centrifuge tube. It was then centrifuged at 3,000 rpm for 5 minutes to settle down the remaining erythrocytes. Lastly, sera of 60 mice from each group (experimental and control separately) were pooled in a single 50 ml centrifuge tube and stored at -20ºC for future use.

2.4.4 Confirmation of anti-64 kDa in immunized mice sera by Western blotting

Preparation of all buffers and solutions required for Western blotting has been mentioned in Appendix II.

SDS-PAGE and blotting were conducted by following the above mentioned procedures. The blotted nitrocellulose membrane was then cut into strips. The strips were marked at the bottom of the protein bound surface of the membrane. Following that, the strips were shaken to be blocked in 2% skimmed milk in PBS for an hour. Afterwards, strips were shaken in primary antibody (immunized mouse serum) diluted in different ratios in 2% skimmed milk in PBS for an hour. Later, the strips were washed with 0.1% Tween 20 in PBS for 5 minutes. This step was repeated three times and the final wash was conducted once in PBS for 5 minutes. Next, they were exposed to a secondary antibody (anti-mouse IgG alkaline phosphatase conjugate), diluted at a ratio of 1:10,000 in 2% skimmed milk in PBS for an hour. The strips were washed following the above procedure and in the end, the washed strips were added to a substrate solution in a dark room. Upon mild hand shaking, within 15-20 minutes, the bands were visualized. The strips were rinsed with distilled water twice.

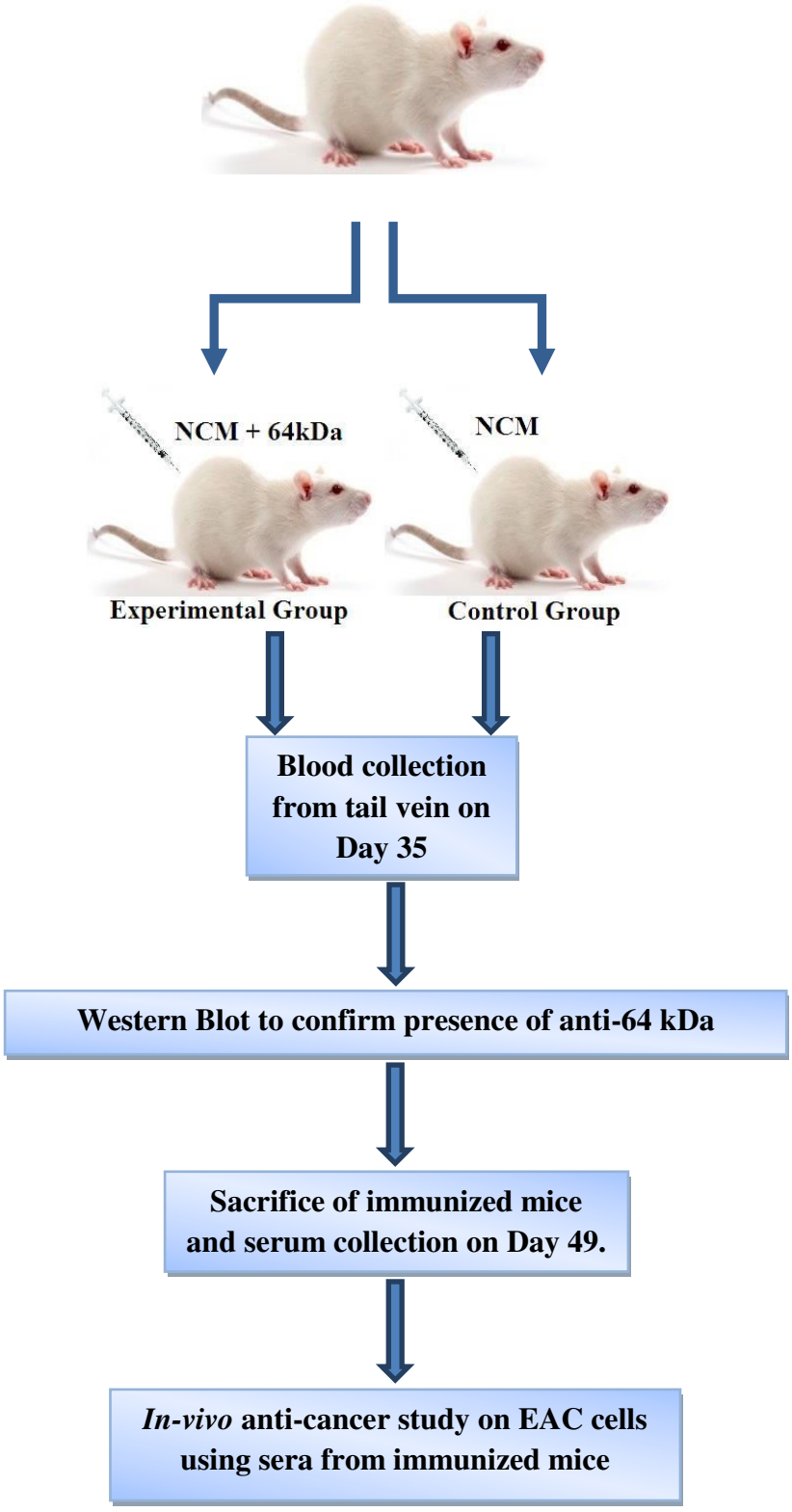

Figure 2.2: Flowchart showing animal immunization design

2.5 *In-vivo* anti-cancer study design

For *in vivo* anti-cancer study, Ehrlich Ascites Carcinoma (EAC) cell line was used.

2.5.1 Tumor cell transplantation

60 mice were taken for *in vivo* anti-cancer study. On day 0, before transplanting tumor cells, body weight of each mouse was recorded.

Each mouse was seeded with 1.3×10^5 cells by intra-peritoneal injection using 100 IU insulin syringe on the same day. Before seeding, number of malignant cells in ascetic fluid was calculated and then diluted in transplantation buffer (appendix II) upon requirement. Cells were counted using Improved Neubauer counting chamber.

The ruled area is divided into 9 large squares. Each of the large squares has a length of 1 mm and width of 1 mm. When a glass cover slip is placed on top of the Neubauer chamber, the ruled area gets a depth of 0.1 mm. Thus the volume of each large square is 0.1 mm^3.

While counting, cells present in 4 large squares were counted. After that, an average cell number was calculated from the total calculated cells in 4 large squares and cellular concentration was expressed as “no. of cells per ml”.

Ascetic fluid was taken from the peritoneal cavity of the mice. After proper dilution of the ascetic fluid, 10 µl of suspension was added to the neubauer chamber after placing a cover slip and the number of cells (marked as A, B, C, D in figure 2.3) were counted.

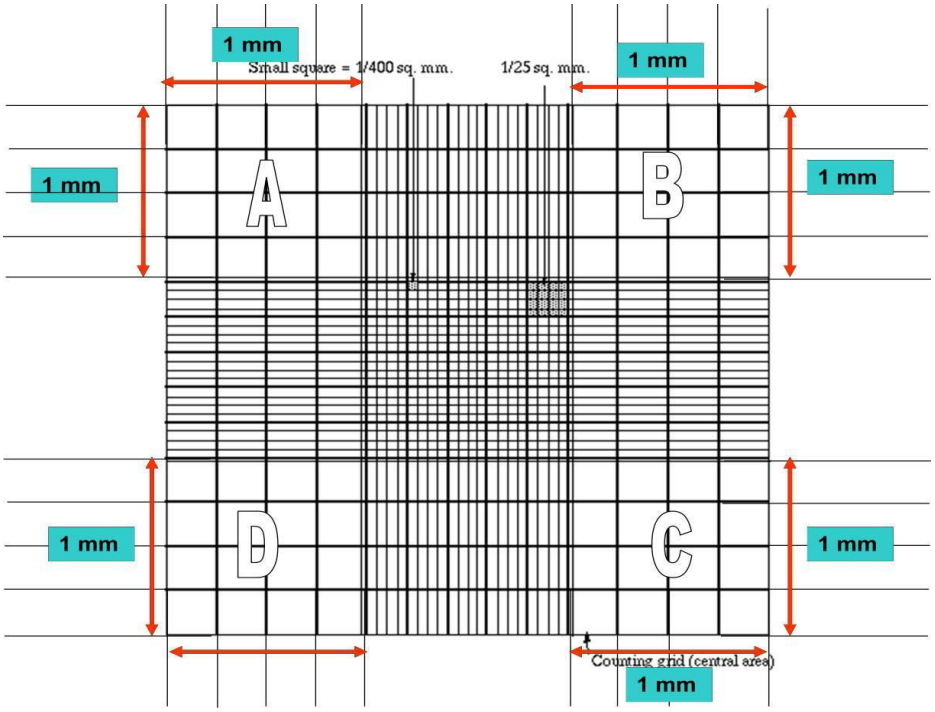

Figure 2.3: **Neubauer counting chamber showing the graduation**. Squares A, B, C, D are designated as large squares and cells were counted in each large square (Having area of 1 mm^2 and volume of 0.1 mm^3) including cells overlying any 2 adjacent margins of the square and excluding those overlying the opposite 2 margins.

Calculation

Following data was obtained from the counting chambers:

A = 134 cells; B = 175 cells; C = 165 cells; D = 200 cells

$$Average = \frac{A + B + C + D}{4}$$

$$= \frac{134 + 175 + 165 + 200}{4}$$

$$= \frac{674}{4}$$

$$= 168.5 \text{ cells} \approx 169 \text{ cells}$$

Therefore, cell concentration = 169 cells per $0.1mm^3$

$= 1690$ cells per mm^3

$= 1690\times10^3$ cells per ml

$= 1.69\times10^6$ cells per ml (Stock suspension)

To seed 1.3×10^5 cells/80 µl/mouse, for 60 mice, 4.8 ml of cell suspension would be needed. Therefore,

$S_1 = 1.69\times10^6$ cells per ml; $S_2 = 1.3\times10^5$ cells per ml

$V_2 = 4.8$ ml; V_1= ?

$V_1S_1 = V_2S_2$

Or, $V_1 \times 1.69\times10^6 = 4.8 \times 1.3\times10^5$

Or, $V_1 = 0.36$ ml

Hence, 0.36 ml suspension was taken from stock suspension and 4.44 ml transplantation buffer was added to make final volume of 4.8 ml.

After preparing the desired cell suspension, 60 mice were injected intraperitoneally in order to seed cell.

2.5.2 Animal experiment design

After 24 hours of seeding Ehrlich Ascites Carcinoma (EAC) cells, anti-cancer study using immunized mice sera was initiated, and the day was marked as Day 1.

Mice were divided into three groups: A, B and C. These groups were further classified into three sub-groups, Experimental, Positive control and Negative control. In the experimental sub-group from group A, B and C, each mouse received 100 µl of serum from the pre-immunized experimental mice group (containing anti 64 kDa) whereas the negative control sub-group received 100 µl of control mice serum. Each mouse in positive control sub-group received Bleomycin at concentration of 0.3 mg/kg body weight/week (Figure 2.4).

In group A, there were 6 mice in each sub-group. Treatment was continued till day 7. On day 8, all mice were sacrificed after collecting EAC cells from the peritoneal cavity. Cells were harvested by aspiration of intraperitoneal material following forceful flush with 3cc ice cold transplantation buffer. Cells in this suspension were counted using Neubauer chamber after proper dilution. Original number of cells in peritoneal cavity was determined by multiplying the cellular concentration with dilution factor. Tumor cells per mouse of the experimental group were compared with those of the negative control group. The cell growth inhibition was calculated by using the following formula (Khanam et al., 2010):

$$\text{Percentage of Cell Growth Inhibition} = (1 - \frac{\text{Tw}}{\text{Cw}}) \times 100$$

Where,

Tw = mean number of tumor cells of the treated group of mice

Cw = mean number of tumor cells of the control group of mice.

In group B, the number of mice in each sub-group was 6. The treatment was continued till day 7. After day 7, the mice were observed till death and their days of survival were recorded. Mean Survival Time (MST) and percentage Increase in Life Span (%ILS) were calculated among sub-groups of mice using the following formulae (Khanam et al., 2010):

$$\text{Mean Survival Time (MST)} = \frac{\sum \text{survival time (days) of each mouse in a group}}{\text{Total number of mice in that group}}$$

$$\text{Percentage Increase in Life Span (ILS)\%} = \left(\frac{\text{MST of treated group}}{\text{MST of control group}} - 1\right) \times 100$$

Another parameter that was studied in this group was rate of weight gain of mice which was implicated after slight modification of a previously described method (Gupta et al., 2004; Islam et al., 2012). Since day 10, weight was recorded at 5 days interval till death of control groups. Percentage increase in weight was calculated using the following formula:

$$\text{Percentage increase in weight} = \left(\frac{\text{Average present weight}}{\text{Average initial weight}} - 1\right) \times 100$$

In group C, each sub-group contained 8 mice. Treatment was carried out in cyclic manner. The cycle was designed to be 5 days continuous treatment followed by 5 days pause, which was repeated 3 times. The treatment started from day 1 and continued till day 30. Later, survival was recorded and data were compared with different sub-groups. Moreover, weight of each mouse in the group was also recorded at 5 days interval starting since day 10. Survival Time (MST), percentage Increase in Life Span (ILS) and percentage increase in weight were calculated following the above mentioned formulae.

2.6 Confirmation of cross-reactive property of anti-BCG 64 kDa antibody and EAC cells

To confirm the cross-reactivity of the anti BCG 64 kDa and EAC cells, whole cell extract of EAC cells was prepared by sonication. This WCE was used as antigen in Western blot. SDS-PAGE and blotting of WCE were carried out in previously mentioned method. Several types of mouse serum were used as primary antibody which included serum from healthy mouse, serum from tumor mouse and serum from immunized mouse. The overall Western blot was conducted following the above mentioned procedure.

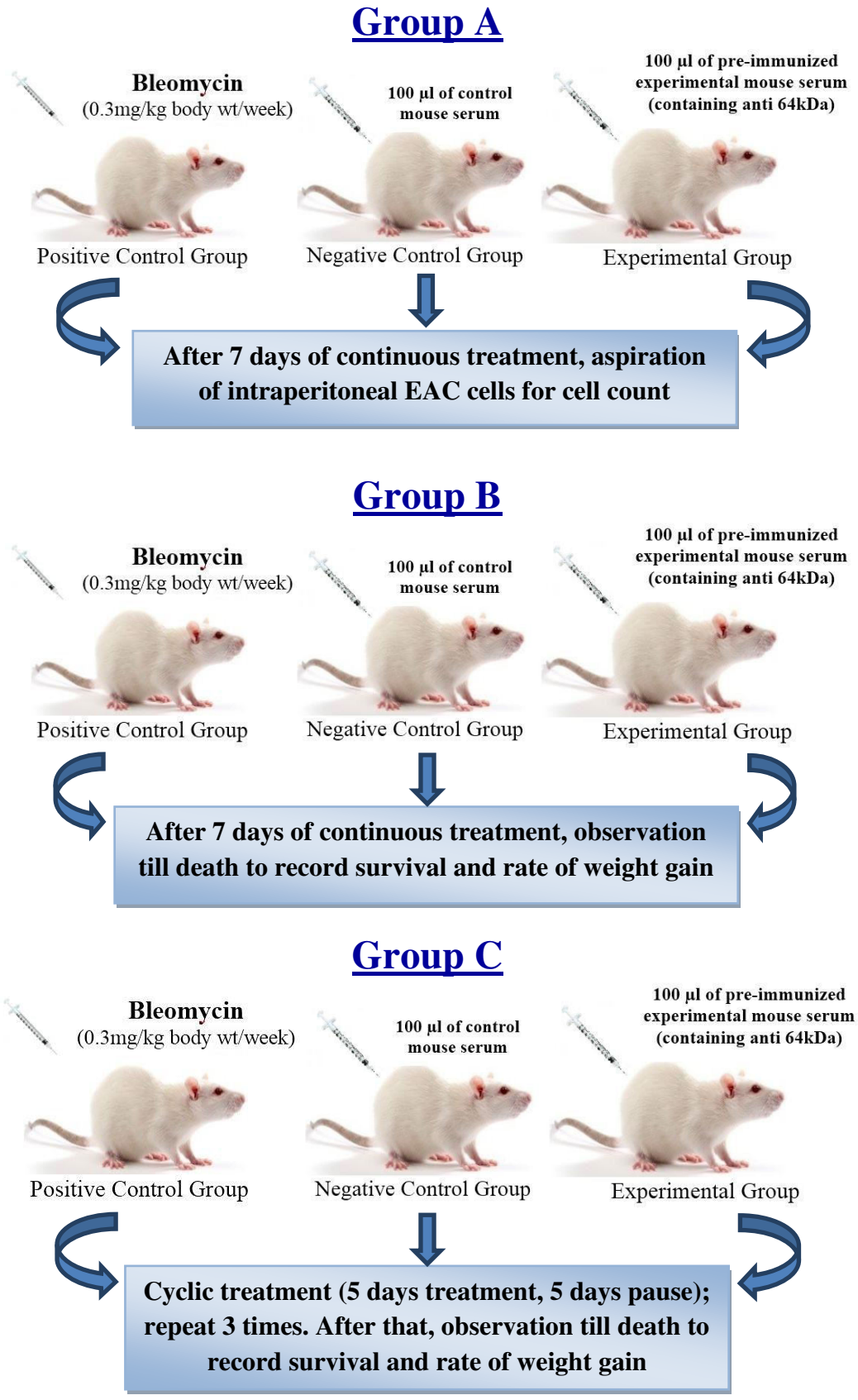

Figure 2.4: Flowchart showing animal experiment design

3. Results

3.1 Culture and identification of *Mycobacterium bovis* BCG

3.1.1 Culture of *Mycobacterium bovis* BCG on Ogawa Egg slant media

Mycobacterium bovis BCG was cultured on Ogawa Egg slant medium and incubated at 37ºC. After 4 weeks of incubation, yellowish white colonies were observed on the slope of the medium. The colonies were distinct and had fragile appearance (Figure 3.1).

3.1.2 Identification of *Mycobacterium bovis* BCG by Acid Fast staining

Grown *Mycobacterium bovis* BCG was stained with Acid Fast Bacilli (AFB) staining and observed under 100X oil immersion objective. Under microscope, red rods (beaded appearance) were observed against whitish background (Figure 3.1).

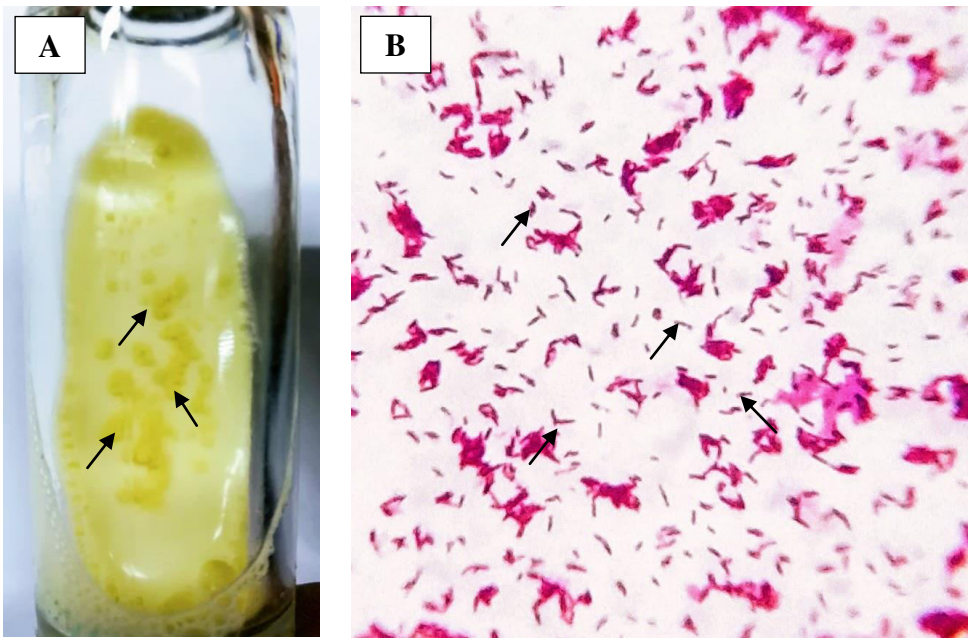

Figure 3.1: Culture colonies and AFB staining appearances of BCG. A. Yellowish culture colonies (marked by arrows) of *Mycobacterium bovis* BCG on Ogawa Egg Slant Medium inside McCartney bottle. B. Reddish rods with beaded appearance (marked by arrows) during microscopy.

3.2 Protein concentration estimation in WEM

The concentration of protein content in WEM was determined by Bradford method. BioRad protein estimation dye concentrate was used as indicator dye. From the standard curve of Bovine Serum Albumin (BSA), the protein concentration of WEM was determined which was found approximately 0.64 mg/ml (Figure 3.2).

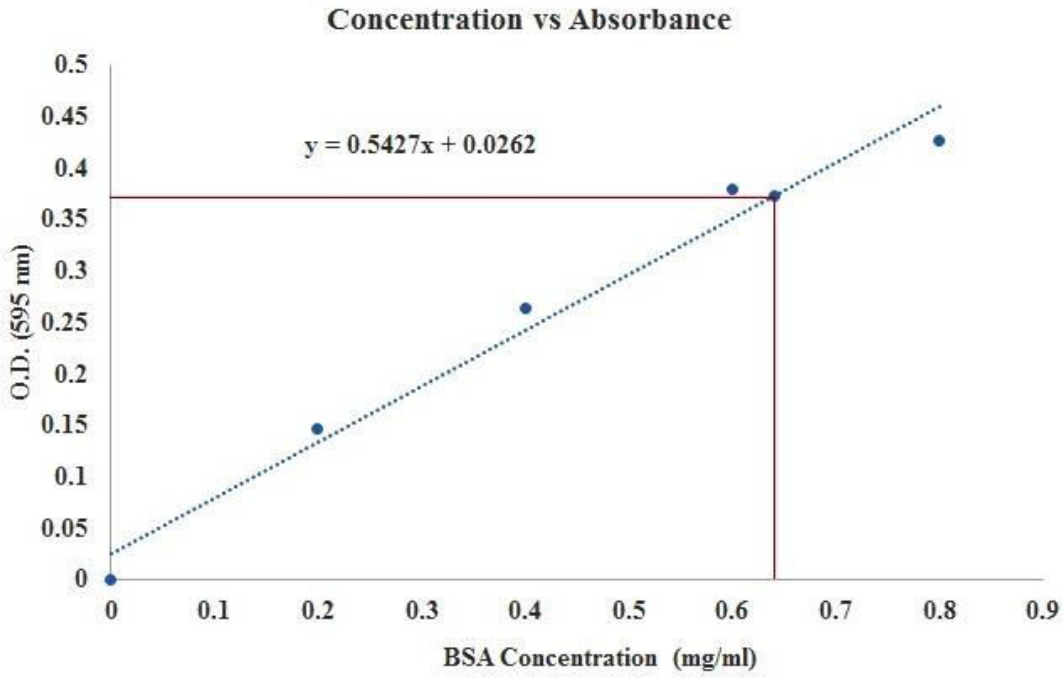

Figure 3.2: Standard curve for protein estimation.

3.3 Determination of desired molecular weight protein

The SDS-PAGE analysis of WEM revealed three distinct protein bands. The molecular weight of the desired protein band was determined by Rf value which was 64 kDa (Figure 3.3).

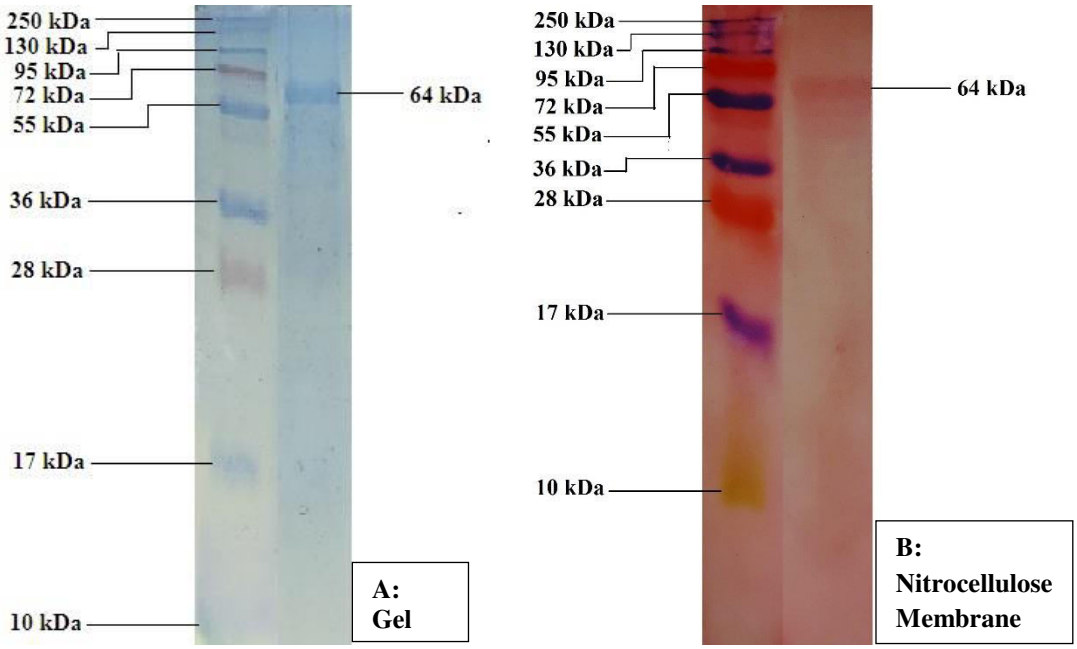

Figure 3.3: Result of SDS-PAGE analysis. Protein markers are the multicolor bands on left side and 1 distinct band on the right side is the protein band in WEM after performing SDS-PAGE using 12.5% separating gel and 5% stacking gel. A. After gel electrophoresis, the gel was stained and destained in staining and destaining solution respectively. B. After gel electrophoresis, the protein was transferred to nitrocellulose membrane followed by staining and destaining with 0.1% ponceau stain and water respectively. The molecular weight of the wide band on the right side was calculated to be 64 kDa by Rf value method. This band along with the nitrocellulose membrane was cut and used for animal immunization.

For the detection of molecular weight, a calibration curve was prepared by plotting Rf values of marker bands on X axis against log molecular weight on Y axis (Figure 3.4).

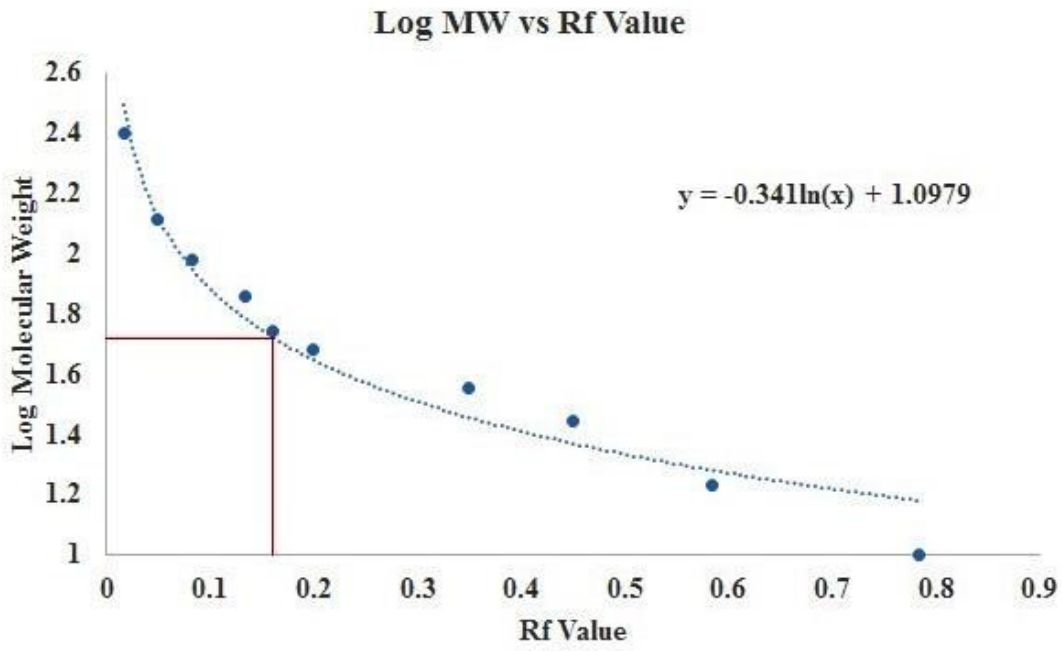

Figure 3.4: Standard curve for determination of molecular weight of desired protein.

After detection of 64 kDa band in WEM, the band was cut along with nitrocellulose membrane, sonicated and used for mice immunization.

3.4 Confirmation of immunization of mice

Successful immunization of mice was confirmed by performing Western blot analysis with WEM as antigen and immunized mice sera as primary antibody. Appearance of distinct 64 kDa band (determined by Rf value) after addition of substrate was the confirmation of development of anti-64 kDa antibody in immunized mice (Figure 3.5).

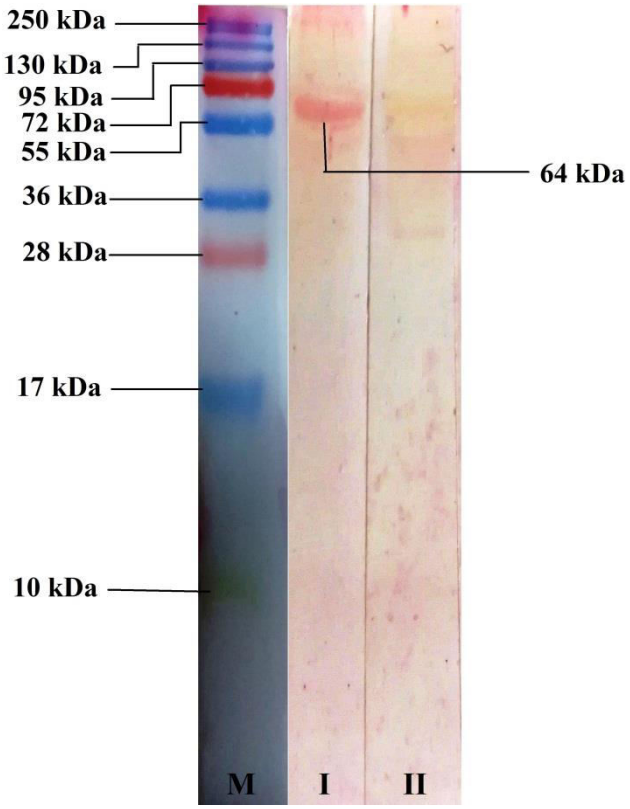

Figure 3.5: Result of western blot analysis. Western blot was performed using WEM as antigen and immunized mice sera as primary antibody. Strip designated as 'M' contains markers of specific molecular weights. The strip 'I' was blotted with serum from mouse immunized with sonicated nitrocellulose membrane, containing BCG 64 kDa protein. The red band on the nitrocellulose membrane strip 'I' has a molecular weight of 64 kDa as determined by Rf value indicating successful immunization. Strip 'II' was blotted with mice sera that were immunized with sonicated nitrocellulose membrane and no band was observed.

3.5 *In vivo* anti-cancer study with immunized mice sera

Anti-cancer effect of mouse sera containing anti-64 kDa was observed on Ehrlich Ascites Carcinoma (EAC) cells. The experiment was conducted in three groups of mice.

3.5.1 Group A: After 7 days of continuous treatment, aspiration of intraperitoneal EAC cells for cell count

Both positive and negative control and experimental mice were transplanted with EAC cells (1.3×10^5 cells/mouse) on day 0 and treated till day 7. All mice were sacrificed on day 8 after aspiration of EAC cells from the peritoneal cavity and then cell number was counted.

Name of groups	Positive Control	Negative Control	Experimental
Cell Count	3×10^6	4×10^8	2.5×10^7
	3.5×10^6	3.5×10^8	2.9×10^7
	6.5×10^6	5.4×10^8	3.5×10^7
	5.8×10^6	5.5×10^8	3.2×10^7
	4.5×10^6	2.2×10^8	4.5×10^7
	7.4×10^6	6×10^8	3×10^7
Average	$5.11\ (\pm1.74)\times10^6$	$4.43\ (\pm1.45)\times10^8$	$3.26\ (\pm0.69)\times10^7$
% of cell growth inhibition	98.85	----------	92.64

SD value within parenthesis

Table 3.1: Table showing EAC cell count on Day 8 from different mice groups

We can see from the table 3.1 that, cell count of the positive control group is 5.11×10^6 cells per ml, negative control group is 4.43×10^8 cells per ml and that of experimental group is 3.26×10^7 cells per ml. So, it can be stated that in experimental group, cell count is $1/10^{th}$ lower than that of negative control group. According to the formula provided in section 2.5.2, percentage of cell growth inhibition was calculated for positive control and experimental group. In experimental group, 92.64% inhibition of cell growth was observed which is quite close to positive control group (98.85%).

3.5.2 Group B: After 7 days of continuous treatment, observation of mice till death to record survival and rate of weight gain

3.5.2.1 Mice survival (%ILS)

Both positive and negative control and experimental mice were observed for survival. Time of survival (in days) of each mouse is given in Table 3.2.

Name of groups	Positive Control	Negative Control	Experimental
Survival of each mouse in days	32	20	30
	35	22	30
	34	22	30
	34	19	31
	33	21	32
	32	20	32
MST	33.33 (±1.21)	20.67 (±1.21)	30.83 (±0.98)

SD value within parenthesis

Table 3.2: Table showing results of survival of mice of different groups in days

We can see from the table that, mean survival time (MST) of the positive control group is 33.33 (±1.21) days, negative control group is 20.67 (±1.21) days and that of experimental group is 30.83 (±0.98) days (According to the formula provided in section 2.5.2). So the percentage increase in life span (%ILS) of experimental group and positive control group compared to that of negative control will be 49.1935% and 61.2903% respectively which is shown in Figure 3.6.

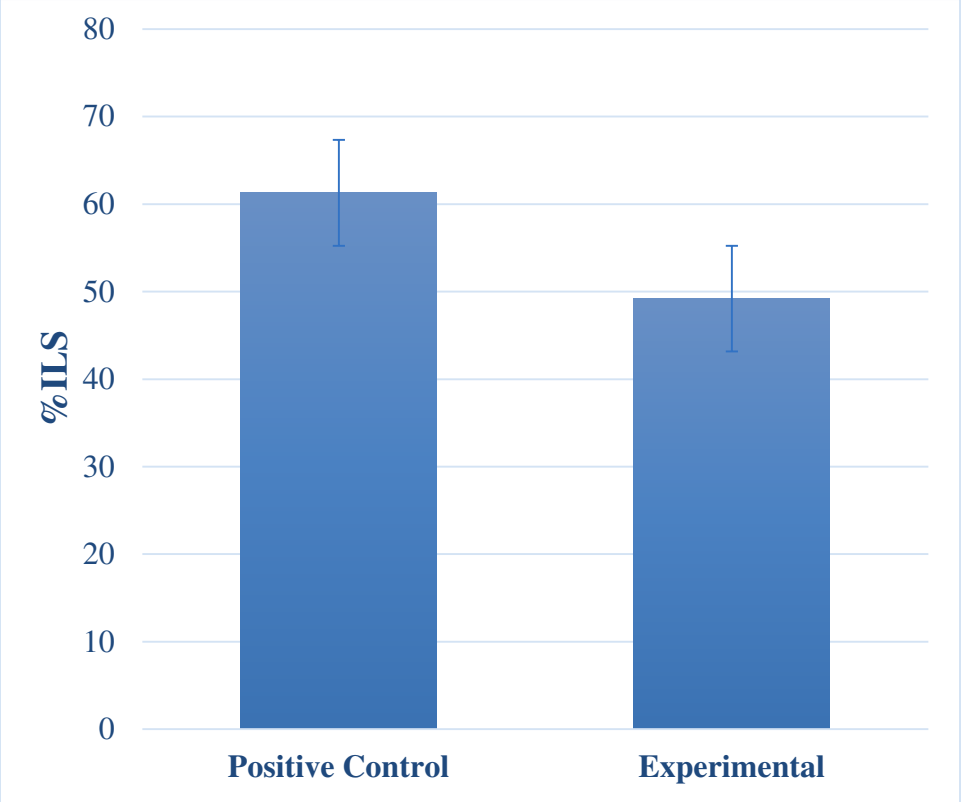

Figure 3.6: Bar diagram showing percentage increased in life span of experimental and positive control group of mice after being treated till 7 days. There was a 49.1935% increase in life span of the experimental group (received sera from BCG 64 kDa immunized mice) compared to negative control group.

3.5.2.2 Percentage weight gain of mice

Weight of individual mouse was recorded on day 0, day 10, day 15, day 20, day 25 and day 30. Table 3.3 shows the weight of individual mouse in a group and the average weight of the group at different time durations of the study. Then calculations were done for percentage weight gain of mice in each group.

Name of groups	Day	Weight of each mouse (gm)						Average weight of mice (gm)
Positive Control	D0	17.04	15.66	18.10	16.80	17.78	16.23	16.94 (±0.92)
	D10	21.1	19.8	19	19.65	20.06	20.58	20.03 (±0.74)
	D15	21.4	20.4	20.1	21.87	20.69	21	20.91 (±0.65)
	D20	26.1	22.94	23.4	24.5	24.78	25.24	24.49 (±1.17)
	D25	35.25	28.9	27.84	31.87	33.5	29.65	31.16 (±2.87)
	D30	40.3	35.47	33.5	38.76	36.6	34.94	36.6 (±2.53)
Negative Control	D0	19.68	15.74	18.86	16.8	17.65	19.06	17.97 (±1.51)
	D10	24.2	19.8	24	22.45	23.68	21.93	22.67 (±1.67)
	D15	27.02	21.3	25.6	24.8	25.03	25.89	24.94 (±1.95)
	D20	34.4	28.3	31.5	Dead	Dead	Dead	31.4 (±3.05)
	D25	Dead	Dead	Dead	Dead	Dead	Dead	---------
	D30	Dead	Dead	Dead	Dead	Dead	Dead	---------
Experimental	D0	17.62	17.41	15.56	16.57	16.29	15.89	16.56 (±0.82)
	D10	21.1	20.89	19.68	21.45	20.78	19.93	20.64 (±0.70)
	D15	22.35	21.15	21.03	23	22.14	21.38	21.84 (±0.78)
	D20	27.73	26.95	25.85	28.03	26.96	26.30	26.97 (±0.83)
	D25	34.04	33.85	32.89	34.76	33.15	32.94	33.61 (±0.74)
	D30	Dead	Dead	37.87	Dead	39.54	38.97	38.79 (±0.85)

SD value within parenthesis

Table 3.3: Weight of mice in different groups at different durations

From above data (Table 3.3), we can predict using the formula provided in section 2.5.2 that, the percentage weight gain in positive control group of mice on day 10, day 15, day 20, day 25 and day 30 were 18.29%, 23.47%, 44.63%, 84.05% and 116.09% respectively. On the other hand, percentage weight gain in experimental group of

mice on day 10, day 15, day 20, day 25 and day 30 were 24.65%, 31.92%, 62.89%, 102.96% and 134.31% respectively. In negative control group, the percentage weight gain of mice on day 10, day 15 and day 20 were 26.23%, 38.83% and 74.78% respectively. From these results, we can clearly predict that, percentage weight gain of experimental mice (Mice receiving anti-64 kDa containing serum) was always slower than that of negative control mice (Mice receiving control serum). A graphical representation of this data has been given in Figure 3.7.

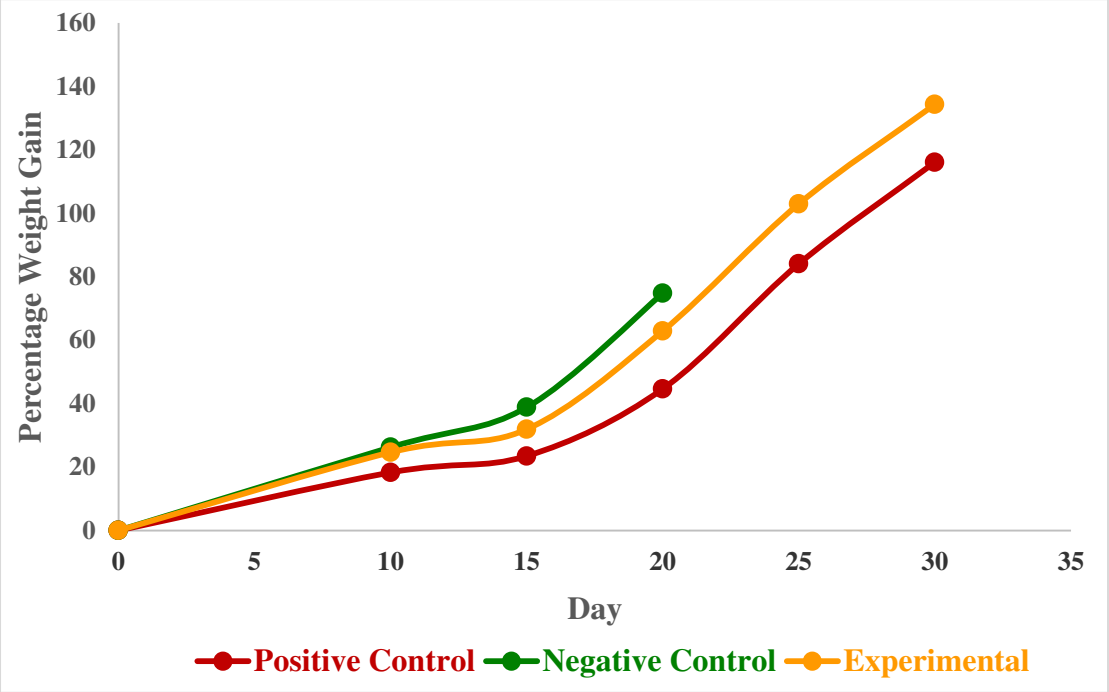

Figure 3.7: Figure showing percentage weight gain of both positive and negative control and experimental group of mice against days. All mice were transplanted with EAC cells (1.3×10^5 cells/mouse) on day 0 and treated till day 7. Weight was recorded from day 10 at a regular interval of 5 days up to day 30. All the weight records were compared with the weights recorded on day 0 and a percentage weight gain was calculated. Throughout the observation period, we can see from the figure that, percentage weight gain of the mice in experimental group was always lower than that of the negative control group.

3.5.3 Group C: Cyclic treatment and observation of mice till death to record survival and rate of weight gain

3.5.3.1 Mice survival (%ILS)

Both control and experimental mice were observed for survival. Time of survival (in days) of each mouse is given in Table 3.4.

Name of groups	Positive Control	Negative Control	Experimental
Survival of each mouse in days	45	30	42
	43	32	42
	43	29	42
	44	31	41
	44	31	40
	45	32	40
	42	30	39
	43	30	41
MST	43.625 (±1.06)	30.625 (±1.06)	40.875 (±1.13)

SD value within parenthesis

Table 3.4: Table showing results of survival of mice of different groups in days

We can see from the table 3.4 that mean survival time (MST) of the positive control group is 43.625 (±1.06) days, negative control group is 30.625 (±1.06) days and that of experimental group is 40.875 (±1.13) days (According to the formula provided in section 2.5.2). So the percent increase in life span (%ILS) of experimental group and positive control group compared to that of negative control will be 33.4694% and 42.449% respectively which has been given in Figure 3.8.

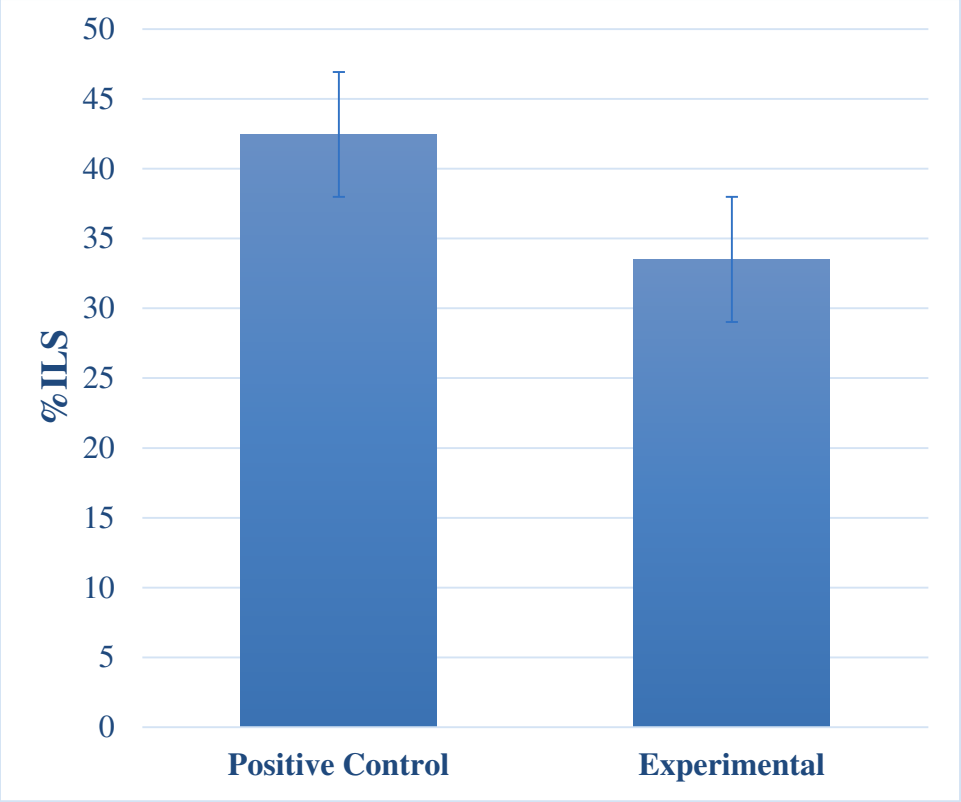

Figure 3.8: Bar diagram showing percentage increased in life span of experimental and positive control group of mice after being treated in cyclic manner. There was a 33.4694% increase in life span of the experimental group (received sera from BCG 64 kDa immunized mice) compared to negative control group.

3.5.3.2 Percentage weight gain of mice

Weight of individual mouse was recorded on day 10, day 15, day 20, day 25, day 30, day 35 and day 40. Table 3.5 shows the weight of individual mouse in a group and the average weight of the group at different time durations of the study. Then calculations were done for percentage weight gain of mice in each group.

Name of groups	Day	Weight of each mouse (gm)								Average weight of mice (gm)
Positive Control	D0	15.82	18.12	16.92	17	17.68	16.35	18.64	17.88	17.30 (±0.95)
	D10	18.1	20.6	17.7	19.4	20.24	18.8	21.04	20.59	19.56 (±1.25)
	D15	19.6	21.74	19.8	21.03	21.58	21.63	22.34	22.05	21.22 (±1.01)
	D20	21.2	23.56	21.05	22.96	23.4	23.87	24.36	24.2	23.075 (±1.28)
	D25	23.06	24.48	22.4	24.02	24.39	24.88	25.6	25.34	24.27 (±1.09)
	D30	24.9	26.95	23.39	27.89	26.76	27	27.69	27.88	26.56 (±1.60)
	D35	26.16	27.04	26.04	30.05	29.89	29.69	30.53	30.75	28.77 (±2.0)
	D40	31.89	32.92	32.54	35.53	33.95	34.17	35.23	35.66	33.986 (±1.43)
Negative Control	D0	15.42	17.7	16.41	15.22	16.78	17.25	16.08	16.56	16.43 (±0.85)
	D10	18.2	20.6	20.3	19.03	20.87	20.88	19.94	20.07	19.986 (±0.94)
	D15	21.17	24.1	23.01	22.89	24.01	24.65	23.42	23.75	23.375 (±1.06)
	D20	25.97	29.21	28.6	27.48	29.23	29.54	28.65	29.37	28.51 (±1.22)
	D25	33.13	35.7	35.12	34.66	34.97	36.02	34.78	35.22	34.95 (±0.86)
	D30	Dead	42.59	40.18	Dead	41.26	Dead	39.97	Dead	41 (±1.20)
	D35	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	--------
	D40	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	--------
Experimental	D0	17.69	17.54	17.9	17.74	17.83	16.93	18.06	18.24	17.74 (±0.39)
	D10	23.6	21.4	21	22.04	21.68	20.87	22.31	23.84	22.09 (±1.12)
	D15	25.4	23.3	24.6	25.67	24.91	23.42	25.88	26.02	24.9 (±1.06)
	D20	29.8	26	29.1	28.36	29.45	26.73	29.38	30.14	28.62 (±1.50)
	D25	34.56	30.19	34.88	32.45	34.26	31.05	35.04	35.48	33.49 (±1.99)
	D30	41.68	37.52	41.63	39.62	41.33	37.69	41.87	42.03	40.42 (±1.89)
	D35	45.03	41.48	45.48	43.7	45.42	42.07	45.93	45.96	44.38 (±1.77)
	D40	50.70	46.93	Dead	Dead	Dead	47.06	49.28	52.18	49.23 (±2.28)

SD value within parenthesis

Table 3.5: Weight of mice in different groups at different durations

From above data (Table 3.5), we can predict using the formula provided in section 2.5.2 that, the percentage weight gain in positive control group of mice on day 10, day 15, day 20, day 25, day 30, day 35 and day 40 were 13.05%, 22.66%, 33.37%, 40.29%, 53.5%, 66.28%, and 96.44% respectively. On the other hand, percentage weight gain in experimental group of mice on day 10, day 15, day 20, day 25, day 30, day 35 and day 40 were 24.53%, 40.35%, 61.32%, 88.76%, 128%, 150% and 177.4% respectively. In negative control group, the percentage weight gain of mice on day 10, day 15 and day 20, day 25 and day 30 were 21.66%, 42.29%, 73.53%, 112.75% and 150.5% respectively. From these results, we can clearly predict that, percentage weight gain of experimental mice (Mice receiving anti-64 kDa containing serum) was always slower than that of negative control mice (Mice receiving control serum). A graphical representation of this data has been given in Figure 3.9.

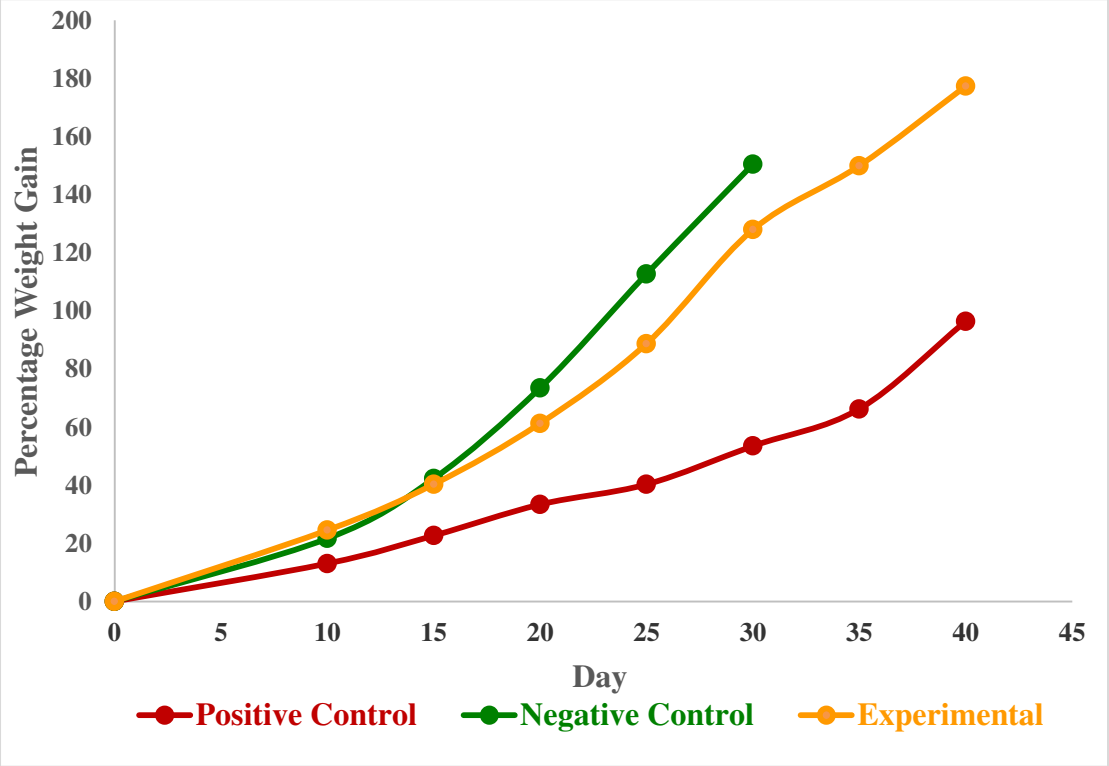

Figure 3.9: Figure showing percentage weight gain of both positive and negative control and experimental group of mice against days. All mice were transplanted with EAC cells (1.3×10^5 cells/mouse) on day 0 and treated in cyclic manner. Weight was recorded from day 10 at a regular interval of 5 days up to day 40. All the weight records were compared with the weights recorded on day 0 and a percentage weight gain was calculated. Throughout the observation period, we can see from the figure that, percentage weight gain of the mice in experimental group was always lower than that of the negative control group.

3.5.4 Comparative analysis between Group B and Group C

To evaluate the effectiveness of the treatment method, survival and weight gain of mice from group B and C were compared.

3.5.4.1 Based on survival in days

To compare effectiveness of regular treatment and cyclic treatment, data from table 3.2 and table 3.4 were combined. Mean survival time of experimental group was 30.83 (±0.98) days in case of regular treatment whereas in cyclic treatment, mean survival time was 40.875 (±1.13) days. So, the percentage increase in life span (%ILS) in cyclic treatment is 32.58% compared to that of regular treatment in experimental group (Figure 3.10).

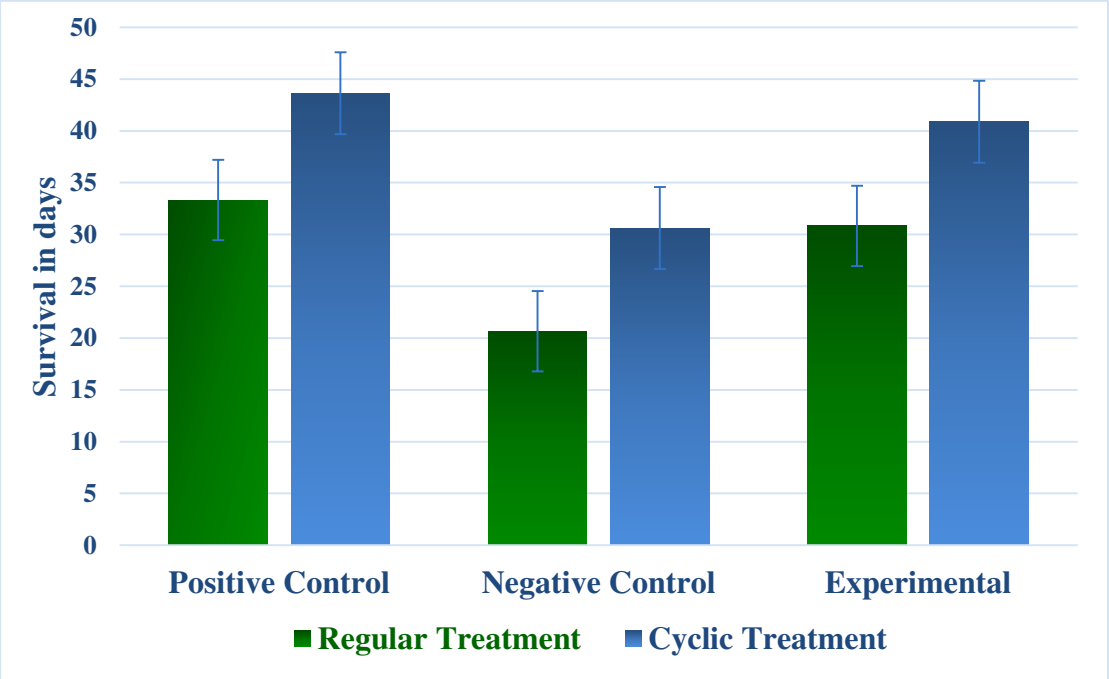

Figure 3.10: Figure showing survival in days of both positive and negative control and experimental group of mice from group B and group C. In each subgroup, outcome of cyclic treatment was better than continuous seven days treatment.

3.5.4.2 Based on percentage weight gain

To compare efficiency of regular treatment and cyclic treatment, data from table 3.3 and table 3.5 were considered. To construct the following bar graph, data were taken up to day 20 as mice from Negative control group of group B did not survive after day 20. Therefore, after day 20, comparison between cyclic and regular treatment could not be possible. On day 20, in regular treatment group, the percentage increase in weight is 62.89% and that of cyclic treatment group is 61.32%. Although the difference is very little, cyclic treatment slowed down the rate of weight gain compared to regular treatment (Figure 3.11).

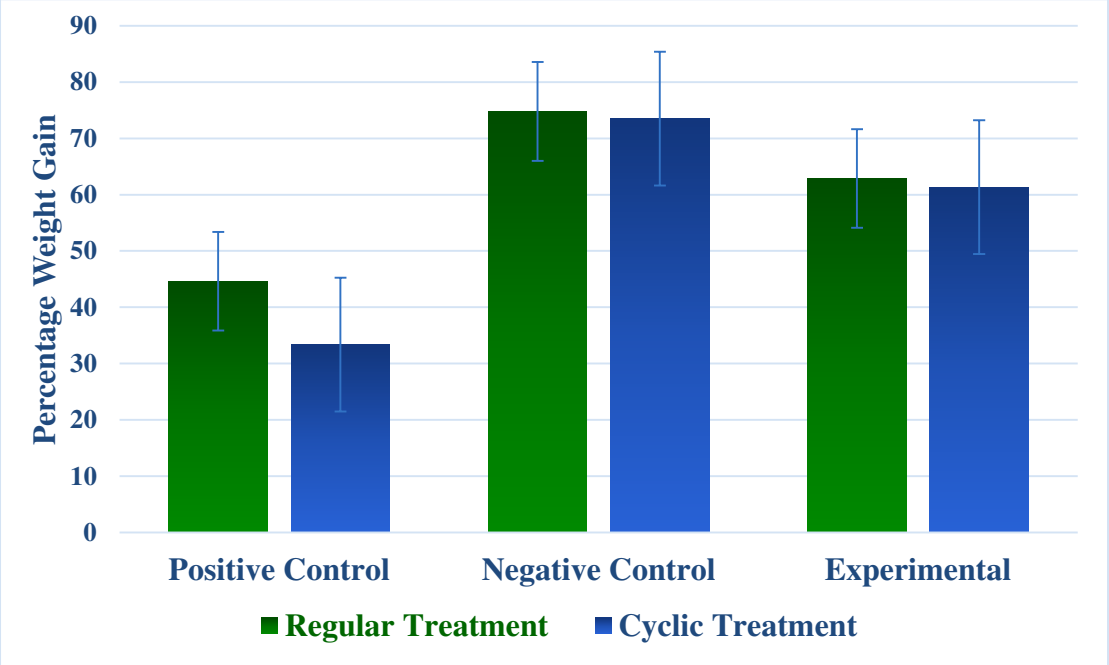

Figure 3.11: Figure showing percentage weight gain of both positive and negative control and experimental group of mice from group B and group C. In each group, affect of cyclic treatment was better as mice from this group showed slower rate of weight gain than continuous seven days treatment group.

3.6 Confirmation of cross reactivity by Western blot

A Western blot analysis was performed using freshly prepared Whole Cell Extract (WCE) of EAC cells as antigen and various mice sera as antibody (serum from healthy mouse, serum from tumor mouse and serum from immunized mouse) (Figure 3.12).

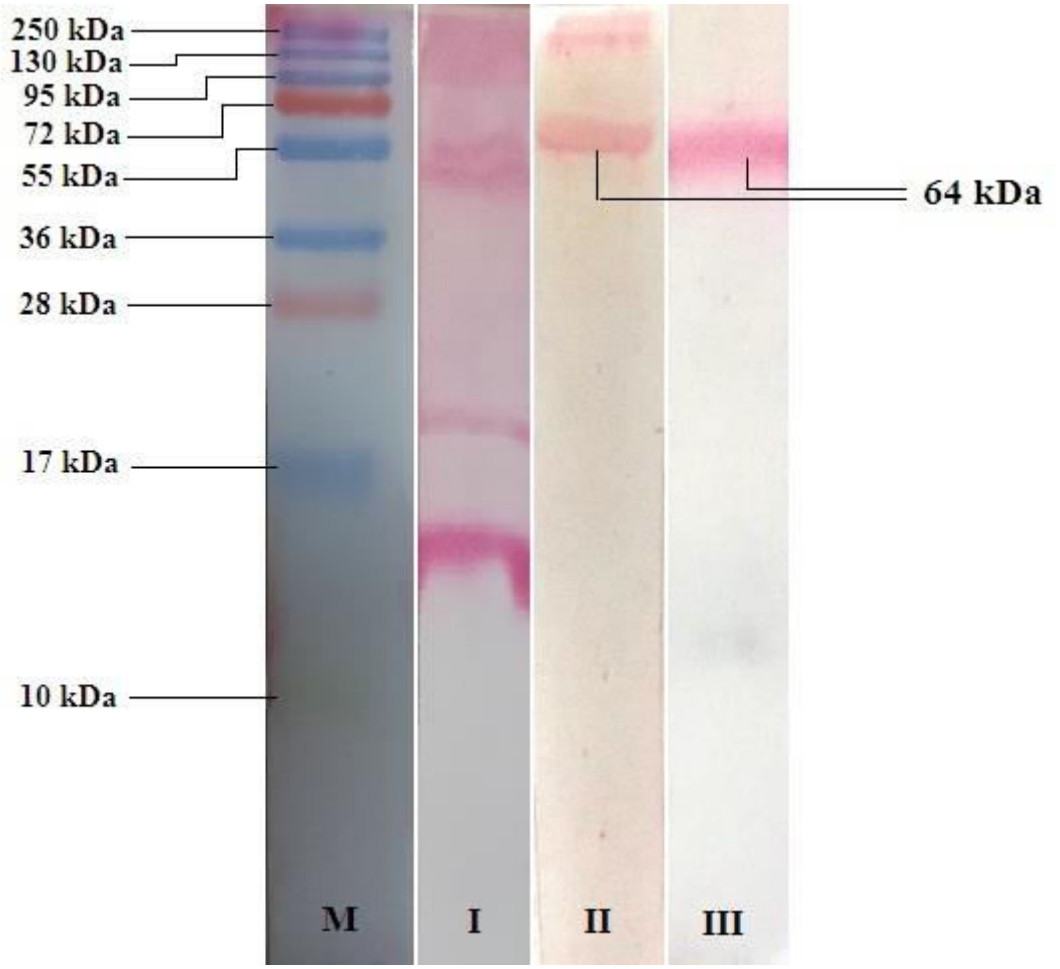

Figure 3.12: Result of western blot analysis. Western blot was performed using WCE of EAC cell as antigen and different types of mice sera as primary antibody. The strip designated as 'M' contains markers of specific molecular weights. Strip labeled as 'I' was blotted with serum from control mouse and shows bands of various molecular weights which are non-specific. Strip 'II' and 'III' were blotted with immunized (BCG 64 kDa) mouse serum and tumor mice serum respectively and show 64 kDa bands.

4. Discussion

Since 1960, whole BCG has been used as therapeutic agent in cancer repression. Along with using whole BCG cell, several studies were conducted with the aim to isolate and purify therapeutic subunit from *Mycobacterium bovis* BCG.

Due to complex nature of mycobacterial antigens in culture filtrate or cell extract, purification of mycobacterial proteins is quite difficult. Thus, to study immunological role of different mycobacterial antigenic proteins, one of the studies attempted to clone and express mycobacterial DNA in a suitable host vector system. They purified BCG proteins from fermentor-grown cloned *E. coli* cells. In order to release proteins from cells, lysozyme treatment was applied followed by sonication to disrupt the cells. This protein mixture was then purified by ammonium sulfate precipitation and anion-exchange chromatography. The major protein content in the purified protein mixture was 64 kDa protein which was called MbaA. In another study, a protein of same molecular weight was determined from culture filtrate of *Mycobacterium bovis* BCG which was designated as P64. To determine antigenic relationship between MbaA and P64, Thole and his co-workers experimented with 10 monoclonal antibodies and both antigens. The result was positive which indicated that MbaA and P64 are immunologically identical (Thole et al., 1987). Moreover, it was found that, monoclonal antibodies to various *Mycobacterium* species reacted with an approximately 64 kDa protein (Kolk et al., 1984).

In the current study, the aim was to identify and characterize this 64 kDa protein of *Mycobacterium bovis* BCG. We have applied Water Extraction Method (WEM) to isolate surface proteins from *Mycobacterium bovis* BCG. This method was established in 1989 in a study conducted by Ahsan and Sasaki (Ahsan and Sasaki, 1989). Their aim was to isolate soluble tumor surface components from the tumor cells without using any chemicals. Before that, various chemical treatments were used for the isolation of cell membrane from cells. Application of 3 M KCl had been used widely for the isolation of tumor-associated antigens present on live tumor cells (Meltze et al., 1971). Although this method was used extensively, it had a major drawback of disruption of cells and nuclei which resulted in contamination of purified antigens (Reisfeld and Kahan, 1971). Commonly used another method of extracting cellular membrane was use of non-ionic detergents in aqueous solutions to facilitate

membrane dissolve (Bjerrum and Lundahl, 1974; Bjerrum and Bog-Hansen, 1976; Gurd et al., 1973; Natori et al., 1978). However, this step was not much significant as no major difference was observed in the number of precipitin line between the detergent-free and detergent containing gels during Crossed Affino Immunoelectrophoresis (CAIE), except an additional line which appeared because of an antigen containing more than one determinant. Therefore, use of distilled water to extract cell surface antigenic protein component appeared as a simple low ionic strength extraction procedure (Ahsan and Sasaki, 1989). Previously, distilled water has been used to release several protein components from bacteria and other sources (Harris, 1971; Oaks et al., 1986). It was found that distilled water easily shed surface structures by repeated pipetting without bursting of cells by osmotic shock (Sasaki et al., 1987). To validate Water Extraction Method (WEM) as promising method, several immunochemical techniques were applied. As WEM does not involve any harsh chemical usage or extreme conditions, the morphological structures of the cells are not altered. Only surface components of the cells are released, thus purification of the surface proteins becomes easier. Using electron microscopy, Ahsan and Sasaki demonstrated that WEM removed only surface materials from the tumor cells as surface-removed cells contained no microvilli structures. FITC-ConA binding test was also done to ensure release of glycoprotein components of tumor cell surface. To prove the immunogenic specificity of the extracted surface proteins, Crossed Affino Immunoelectrophoresis (CAIE) technique was applied. This ensured the native undissociated conformation of the WEM protein. From the WEM of line 10 hepatocarcinoma cell of guinea pig, five major protein bands were prominent having apparent molecular weights of 44, 46, 62, 64, and 68 kDa. On the other hand, in normal liver WEM, these protein bands were absent which indicates that these bands are only specific for the Line 10 tumor cells and probably associated with TAA activity.

Based on this finding, in further studies of Ahsan and Sasaki, WEM was used to isolate surface proteins from *M. bovis* BCG. A major protein band of 64 kDa along with some other low molecular weight protein bands were visualized in SDS-PAGE of water extracted materials. Isolation of soluble BCG 64 kDa by WEM with less or no contamination of other proteins suggested that BCG 64 kDa is a surface protein. Before that, it was demonstrated using BCG-monoclonal antibodies that there are

common antigenic determinants between the surface extracts of Line 10 tumor cells of guinea pig and different mouse tumor cells (including Meth A and CT-26) and BCG (Sasaki et al., 1986; Sasaki et al., 1989). Then it was demonstrated that this 64 kDa surface protein of BCG has antigenic cross reactivity with line 10 hepatocarcinoma cell of guinea pig (Ahsan and Sasaki, 1991). Based on this finding and previous results, it was concluded that the antigenic determinants between the BCG 64 kDa and the 64 kDa major surface antigenic component of Line 10 tumor cells are identical. This finding also led to another milestone that BCG 64 kDa is probably identical with the tumor specific antigen (Ahsan and Sasaki, 1991).

Previous studies demonstrated increased titre of anti-BCG 64 kDa antibody in immunized animal sera which supported the capacity of BCG 64 kDa to induce immune response. Moreover, it was established that BCG 64 kDa has strong antitumor activity. Animals immunized with BCG 64 kDa showed either complete rejection or partial inhibition of the tumor growth when observed after tumor transplantation (Ahsan and Sasaki, 1991; Ahsan and Sasaki, 1993). The immunological explanation behind protective tumor immunity by BCG 64 kDa is activation of lymphocytes or induction of natural killer (NK) cells (Ahsan and Sasaki, 1991).

Inspired from these previous studies, in this study, we aimed to exploit the antigenic cross reactivity of anti-BCG 64 kDa antibody and tumor cells. Antigen- antibody cross reaction may be simply defined as a reaction that takes place between an antibody that was developed in response to a specific antigen and an antigen that is similar in many ways to the antigen in response to which, the antibody was developed. Till date, most of these studies were confined in treatment of cancer through active immunization. In the present study, the main focus was to develop cancer treatment by inducing passive immunity so that immune-competent individuals can be treated. Moreover, this type of treatment modality also helps to combat any situation that may arise without prior notification.

With this aim, we partially purified 64 kDa protein band after performing an SDS-PAGE of the WEM and then staining with Coomassie brilliant blue R250. This 64 kDa protein band was used for animal immunization and after immunization, only a 64 kDa band was visualized on nitrocellulose membrane after Western blot analysis,

indicating isolation of 64 kDa band from WEM without contamination of other proteins. As control group in our study, we have immunized animals with nitrocellulose membrane only. From previous experiments, it was found that nitrocellulose membrane acts as an adjuvant and does not have any immunogenic effect (Ahsan and Sasaki, 1991). Although most of the studies describing passive immunization mentioned about monoclonal antibodies to treat disease, we have implied polyclonal antibodies to check the effectiveness of the treatment first.

As animal model, mouse was used in our experiment. The reasons behind this choice were relatively low cost for purchase and maintenance, ease of handling, better breeding capacity, available knowledge about mouse physiology, anatomy, and its genes, genetic and genomic similarity between human and mouse etc. Furthermore, EAC cell line was used as tumor cell for anti-cancer study which is specific for mouse. Most of the previous studies were confined to solid tumors. Thus, in this experiment, mouse model was used along with mouse specific cancer line.

It is known that when an antigen is presented to body's natural defense system, number of antibody production is comparatively low and the produced antibody is mainly IgM (very low IgG level). When the same antigen is again encountered by the body's immune system, the level of antibody production rises to a high level. This time, main type of antibody is IgG and this antibody level persists for a quite long time. In our study, we have given 4 doses of injection to immunize mouse with BCG 64 kDa protein. Our target was to develop anti BCG 64 kDa antibody in mouse. To ensure high level of anti BCG 64 kDa IgG production, 4 doses of injection were given. As after 2^{nd} exposure to same antigen, IgG production increases and this high level is maintained for longer period, we collected blood from tail vein of mouse after 7 days of 3^{rd} dose to check antibody titre.

For immunization purpose, intramuscular injection was given at right and left thigh muscle of the mouse alternatively. Usually, very low volume of antigen is used for immunization purpose, approximately 50 µg protein per kg body weight (Ahsan and Sasaki, 1991). To administer this type of small volume, we have used 100 IU insulin syringe for injection. As the study was conducted with EAC cells which are present in peritoneal cavity of mouse, intraperitoneal injection was given at the lower left or right quadrant of mouse body during anti-cancer study.

In the current experiment, raw serum from immunized mice was used for anti-cancer study. In normal mouse serum, the level of IgG is 2-5 mg/ml. Along with anti-BCG 64 kDa IgG antibody, there are different types of proteins and antibodies present in raw serum. For the treatment, we applied this raw serum where required antibody is present in a very dilute form. If pure antibody can be used for the treatment, it is assumed that improved result may be obtained compared to current finding. Moreover, the anti-cancer study was conducted only at single dose i.e. 100 µl serum. If we consider the above mentioned IgG concentration, the administered dose was very low. It can be suggested that there might be better outcome if increased concentration of IgG can be used for treatment. For intraperitoneal injection, usually not more than 100 µl volume of liquid is applied. The reason behind this constraint might be implied extensive force due to presence of extra liquid substance. Therefore, we were confined to use 100 µl serum. So, if dose variation needs to be introduced, the prime step would be purification and concentration of IgG. Only after that, different concentration of IgG could be used for treatment purpose within limited liquid volume.

Three groups of mice were taken for anti-cancer study. In one group, all mice were sacrificed on day 8 to compare percentage cell growth inhibition among positive, negative and experimental sub-groups. The experimental group showed lower EAC cell number compared to negative control group which indicates the efficiency of anti-BCG 64 kDa antibody to suppress tumor cell growth.

For another two groups, two different modes of treatment were implied using serum containing anti-64 kDa antibody. One group of animals received continuous seven days treatment and then observed till death and the other group was treated in cyclic manner (5 days treatment; 5 days pause). To compare the outcome of two modes of treatment, we have considered two parameters: percent increase in life span and percent increase in weight. Based on both parameters, cyclic treatment showed better result compared to only 7 days treatment. Tumor cells are known to increase in number exponentially. If the treatment is done in a manner where after certain period, new cells will be destroyed, it is assumed that the treatment will be more effective. So, from this point of view, the better outcome of cyclic treatment can be explained.

To confirm the cross reactive property of the anti BCG 64 kDa and tumor specific antigen, when WCE of EAC cells were immunoblotted with mouse anti-BCG 64 kDa containing serum, a 64 kDa band was visible (Figure 3.12 strip III). This cross reactive property of this protein may have contributed by turns to the *in*-vivo anti-cancer property of the anti-BCG 64 kDa antibody in challenged mice. The most interesting finding of the immunoblot analysis was that, when WCE of EAC cells were blotted with serum from a tumor mouse, a 64 kDa band was visible (Figure 3.12 strip II). This finding suggests formation of an anti-64 kDa antibody in tumor mice which also confirms the cross reactive relationship between anti-BCG 64 kDa and tumor specific antigens. This band was not visible in strip I, in which, WCE of EAC was blotted with serum from control mouse.

From all the findings of our study, we can conclude that, BCG 64 kDa surface protein shares common antigenic determinants with 64 kDa protein from ascetic tumor cell (EAC cells), and as a result, mouse anti-BCG 64 kDa antibody confers protective immunity in EAC cell transplanted mouse.

The current experiment was conducted on small number of animals. To make this treatment available for human, more trials need to be performed on large animal groups. Moreover, effect of anti-BCG 64 kDa antibodies could be checked on different cancer cell lines of other animal species to ensure its effectiveness. As there is always a risk of mounting serum sickness and serious allergic reaction in passive immunization, highly purified immunoglobulin fractions need to be produced for human therapy. Therefore, further research is required to develop anti-BCG 64 kDa monoclonal antibody to increase the effectiveness of the treatment.

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Appendix I

Media Composition

Unless otherwise mentioned, all media were sterilized by autoclaving at 121°C for 15 minutes at 15 lbs pressure. Distilled water was used for preparation of all media. The compositions of media used in this thesis have been given below:

1. Ogawa Egg Slant Medium

Ingredients	Amount
Malachite green	0.4 gm
Sodium Pyruvate	4 gm
Sodium Glutamate	6 gm
Monopotassium dihydrophosphate (KH_2PO_4)	6 gm
Egg suspension	1200 ml
Distilled Water	600 ml

2. Dubos broth (Approximate formula for 900 ml purified water)

Ingredients	Amount (gm/L)
Pancreatic Digest of Casein	0.5 g
L-Asparagine	2.0 g
Monopotassium Phosphate	1.0 g
Disodium Phosphate	2.5 g
Ferric Ammonium Citrate	0.05 g
Magnesium Sulfate	0.01 g
Polysorbate 80	0.2 g
Calcium Chloride	0.5 mg
Zinc Sulfate	0.1 mg
Copper Sulfate	0.1 mg
FBS	100.0 mL

Appendix II

Solutions and Reagents

A. SDS-PAGE Analysis stock solution

i. 30% acrylamide-bis acrylamide solution

Acrylamide	14.5 gm
Bis-acrylamide	0.5 gm
Distilled water	Up to 50 ml

The solution was stored at 4^{o}C. The powder is neurotoxic. Mask was used during handling.

ii. Upper gel buffer (0.5 M Tris HCl, pH 6.8)

Tris-base	6.57 gm [0.5×MW (121.14)/10]
Distilled water	up to 100 ml
pH	adjusted to 6.8 with concentrated HCl

The solution was stored at 4^{o}C.

iii. Lower gel buffer (1.5 M Tris HCl, pH 8.8)

Tris-base	36.34 gm [1.5×MW (121.14)/20]
Distilled water	up to 200 ml
pH	adjusted to 8.8 with concentrated HCl

The solution was stored at 4^{o}C.

iv. 10% SDS

SDS	5.0 gm
Distilled water	Up to 50 ml

The solution was stored at room temperature.

v. 10% ammonium per sulfate (APS)

Ammonium per sulfate (APS)	5.0 gm
Distilled water	Up to 50 ml

The solution was aliquot into 0.5 ml in each micro centrifuge tube and stored at -20^{o}C.

vi. TEMED

Readymade and was stored at room temperature.

vii. Butanol saturated water

Fifty ml of distilled water was taken in a beaker with a magnetic stirrer. Butanol was added to water provided the magnetic stirrer machine was turned on. Addition of butanol was stopped when the solution got saturated. The saturated solution was collected using a dropper and was stored at 4^{o}C.

viii. 0.1% BPB (Bromophenol blue solution) or Tracking dye

At first, 2 ml of 50% glycerol solution was made by mixing 1 ml glycerol with 1 ml dH_2O. Then 2 mg of Bromophemol blue was weighed and 50% glycerol was added up to 2 ml mark. It was stored at 4^{o}C.

ix. 2X Sample loading buffer

0.5 M Tris - Cl (pH 6.8)	0.4 ml
10% SDS	0.4 ml
2-mercaptoethanol	0.04 ml
Glycerol	0.4 ml
Distilled water	0.76 ml

The solution was stored at 4^{o}C.

x. Running/Electrophoresis buffer (pH 8.3)

Tris base	3.0 gm
Glycine	14.4 gm
10% SDS	10 ml
Distilled water	1000 ml

The pH of the solution should be 8.3, which may range from 8.1 to 8.5. The solution was to be stored at 4^{o}C. The bottle was to be marked after every usage. It should not be used more than 8-10 times.

xi. Staining solution

Coommassie brilliant blue R-250	200 mg
Acetic acid	10 ml
Methanol	10 ml
Distilled water	80 ml

The solution was stored at room temperature.

xii. Destaining solution

Acetic acid	10 ml
Methanol	10 ml
Distilled water	80 ml

The solution was stored at room temperature.

xiii. Composition of 12.5% separating gel

Distilled water	1.60 ml
1.5M Tris HCl pH 8.8	1.25 ml
30% acrylamide	2.1 ml
10% SDS	0.10 ml
10% APS	30 µl
TEMED (Added last)	8.5 µl

xiv. Composition of stacking gel

Distilled water	2.137 ml
0.5M Tris HCl pH 6.8	0.937 ml
30% acrylamide	0.625 ml
10% SDS	37 µl
10% APS	22 µl
TEMED (Added last)	8.5 µl

B. Western blot solutions

i. Transfer buffer

Glycine	14.4 gm
Tris base	3.03 gm
Methanol	200 ml
Water	800 ml

ii. Substrate solution (For alkaline phosphatase conjugated secondary antibody)

50mM Tris HCl pH 9.14	10 ml
AS-MX Napthol phosphate	10 mg
Fast Red TR	20 mg

C. Transplantation buffer

To make 0.2 M 400 ml buffer, 150 ml 0.2 M KH_2PO_4 and 350 ml 0.2 M $KHPO_4$ were prepared. At 1(KH_2PO_4):3($KHPO_4$) ratio, KH_2PO_4 and $KHPO_4$ were added. The pH was measured and adjusted to 7.2 by adding either KH_2PO_4 (if pH>7.2) or $KHPO_4$ (if pH<7.2).

KH_2PO_4 (150 ml 0.2 M)	4.083 gm
$KHPO_4$ (350 ml 0.2 M)	12.2 gm

D. Phosphate Buffered Saline (PBS)

PBS was prepared by dissolving 8.0 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na_2HPO_4 and 2.0 gm of KH_2PO_4 in 800 ml distilled water. pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 L by distilled water. The solution was sterilized by autoclaving and was stored at room temperature.

E. Normal saline (0.85% NaCl)

0.85 gm of NaCl, upto 100 ml by distilled water.

Appendix III

Apparatus used

Autoclave	HL-42AE, Hirayama corp, Japan
Balance	Adventurer AR1140, Mettler Toledo EL202
Centrifuge	Eppendorf Centrifuge 5804, Germany
Class II Biosafety Cabinet	Lab Caire, USA
Freezer (-30^{o}C)	Liebherr comfort, Germany, Siemens
Heater/Magnetic Stirrer	Spinot
Incubator	Japan
Inverted Microscope	KYOW Optilab TR-T
Laminar Air Flow	HF – 48 Flow laboratories, Japan
Microcentrifuge	Hettich, Tarsons Spinwin MC-02
Microwave Oven	Butterfly, China
pH Meter	Hanna HI2211
Power Pack	Biometra Standard Power Pack
Refrigerator	Royal Frestech, Vestfrost,
SDS-PAGE Unit	BioRad Mini-Protean II cell
Horizontal shaker	Electro Plus, Schuttelmaschine LS10
Spectrophotometer	Genesys 5
Vortex mixer	IKA MS3 Basic
Water bath	Grant SUB6, England