PRODUCTION AND CHARACTERIZATION OF XENOGENIC ANTI-IDIOTYPE REAGENTS TO RINDERPEST *VIRUS MONOCLONAL ANTIBODY*

Thesis

SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE
OF

Master of Veterinary Science

IN

VETERINARY VIROLOGY

BY

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TO
DEEMED UNIVERSITY
INDIAN VETERINARY RESEARCH INSTITUTE
IZATNAGAR - 243 122 (U. P.), INDIA

1999

Dedicated to

My Experimental Animals.

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Certified that the research work embodied in this thesis entitled" PRODUCTION AND CHARACTERIZATION OF XENOGENIC ANTI-IDIOTYPE REAGENTS TO RINDERPEST VIRUS MONOCLONAL ANTIBODY", submitted by ***Dr. Chandrakant B. Jagtap, Roll.No. 3721,*** *for the award of* ***M.V.Sc.*** *degree in virology of Indian Veterinary Research Institute (Deemed University), is the original work carried out by the candidate himself under my supervision and guidance.*

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Acknowledgement

I wish to express my deepest sense of gratitude to my supervisor Dr. S.K. Bandyopadhyay, for his meticulous supervision, constant encouragement, motivation & constructive councel of the present work.

I am highly indebeted to Dr. R.P. Singh, Dr. Pranab Dhar and Dr. B.P. Sriniavasa, Scientists, Rinderpest laboratory I.V.R.I. Mukteshwar, for their immense help during this present study. I do not find words to express my feelings about them. To them I owe a great deal, not only for kind encourgement and sometimes deservedly blunt criticism, but also for the insight and perspective that only the real experts can give.

I express my intense gratitude to Dr. R. Venkataramanan, PC-AICRP, FMD, Dr. S.K. Das, I/c, N.B.C, & Dr. A.K. Tiwari, Scientist, N.B.C, members of my advisory committee for their constant encourgement and timely help during the entire course of the study.

I am grateful to the I.C.A.R. and Director, IVRI for providing necessary facilities and financial assistance. I am also indebeted to Dr. P.C. Harbola, JD cum Station Incharge, IVRI campus Mukteswar, for providing necessary facilities.

I am thankful to Dr. Sanyal, Dr. Hemadri, Dr. Tosh, Scientists, FMD laboratory and Dr. Mahesh Chander, Sr. scientist, Extention dept. I.V.R.I. Campus, and Dr. Sanjya Pandey, scientist, Economics Dept. I.V.R.I. campus, Mukteshwar, for their constant encourgement during the entire period of study.

I express my indebetedness to Dr. B. Mondal and Dr. Poonam Malik for their cooperation, useful suggetion and moral support throughout this study.

Valuable suggestions given by Dr. Srikumaran, Scientist, Nebraska university and Dr. En Min Zhou, Resrearch scientist, National Centre for Foreign Animal Disease, Canada are also aknowledged.

I have pleasure to thank my seniors Drs. S. Patil, C. Gurumurthy, Manju George, Neeraj Srivastava and Muttu Chelvan for their help, support and inspiration during my entire period of this work. I also extend my heartly thanks to my juniors Anjali, Prabhakar, Kaustav and Pavan for their cheerful company and help in many ways.

I express my indebtedness to Dr. Samadhan Jadhao, Dr. Suraj Sable, Dr. Jayant Korday, Dr. Nitin Muley and Dr. Nitin Kurade for their timely help and constant support during my stay at IVRI.

The good moments of my stay at I.V.R.I. Izzatnagar were shared by colleagues, Kailash, Laxmanan, Raj Gopal, Kiran, Anamika, Rama, Balamurugan and all Maratha's are aknowledged

On the way to completion the true friends to who have shared the moments of laughter and sorrow can never be forgtotten. I can never thank enough Vikas and Dinesh for their care, support and lively company.

My whole hearted thanks are also due to Shri Nayal, Shri Das, Kamar Ji, Miss Suman and Manoj, staff members of R.P. lab and other laboratories of I.V.R.I. campus, Mukteshwar, for their valueable and unstinted help during the entire course of study.

I am also thankful to Sri H.S. Rawat and Sri Mehta, artists and Sri Mohan Ram for doing excellent job of photography and typing the manuscript.

I also whish to extend my heartly thanks to Naveen Sir, hostel staff and other Hostel inmates for their cheerful company at IVRI Mukteswar. My thanks are also due to the staff of library for their co-operation and support.

There are no words to pay regards to my admired parents Anna and Bai who took pains to bring me up to this stage, without their love, inspiration and blessing this dissertation work would have not reached the light of world.

I express my sincere appreciation to my brother Ravi Dada and Kishore Bhau and Tai and Vahini for their deep understanding, patience and moral support throughout my tenure of study. Fond memories of little Umesh, Guddi and Bunti were always with me.

Last but not least, I must thank all those whom I could not mention but who have helped me directly or indirectly during my study at Izatnagar.

Lastly I never forget to thank God without his blessing nothing is possible in this world.

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CHANDRAKANT B. JAGTAP

ABBREVIATIONS

Ab	Antibody
Ag	Antigen
Ab2α	Anti-Id alpha
Ab2β	Anti-Id beta
Ab2γ	Anti- Id Gamma
AGPT	Agar gel precipitation test
Anti-Id	Anti-Idiotype
BSA	Bovine serum albumin
CDV	Canine distemper virus
CIE	Counter immuno- electrophoresis
CMC	Carboxy methyl cellulose
CPE	Cytopathic effect
DMV	Dolphine morbillivirus
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
EMEM	Eagle's minimum essential medium
'F'	Fusion protein
FCA	Freund's complete adjuvant
FCS	Fetal calf serum
FIA	Freund's incomplete adjuvant
FITC	Fluorescein isothiocynate
g	Gravitational force
hr	hour
'H'	Haemagglutinin protein
HIS	Hyper immune serum
HRPO	Horseradish peroxidase
Id	Idiotype
IFT	Immuno-fluorescence test
I/M	Intra-muscular
I/P	Intra-peritonial
I/V	Intra-venous
'L'	Large protein
LRPV	Lapinised rinderpest virus
KDa	Kilo daltons
Kb	Kilo bases
'M'	Matrix protein
M	Molarity
MAb	Monoclonal antibody
min.	minutes
mm	milimeter

CYTOGENETIC EVALUATION OF MURRAH BUFFALOES

BY

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(An abstract of the thesis submitted to C.C.S. Haryana Agricultural University, Hisar in the partial fulfilment of the degree of M.V.Sc. in Animal Breeding).

Fourty five Murrah buffaloes were screened cytogenetically. In somatic metaphase plates it was seen that the diploid chromosome number of Murrah buffalo was fifty, with ten pairs of metacentric/submetacentric and twenty pairs of acrocentric chromosomes. The X-chromosome was the largest acrocentric chromosome. In females there were two X-chromosome and in males there was one X-chromosome and one Y-chromosome. The fifth and the third pairs of autosomes were nearly metacentric in shape.

Relative lengths calculated revealed the contribution of individual chromosome in total genome ranged from 7.0% to 2.0%. The contribution of X-chromosome was 6.25% in females and 5.50% in males. Y-chromosome represented 2.35% of total genome. Morphometric measurements showed 5th and 3rd pairs to be nearly metacentric and 4th, 2nd and Ist pairs to be submentacentric.

The mitotic index was the highest in younger animals. Some metaphase plates showed structural aberrations (5.8% of the total). Eleven plates with polyploidy were also observed but aneuploidy was totally absent. G-bands were present in all the chromosomes. The centromeric regions showed light G-bands in them.

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C-bands were absent in first five pairs of metacentric/ submetacentric chromosomes. Rest of the autosomes had C-band in centromeric regions. X-chromosome had one large C-band in centromere and a small thin band just below it. The Y-chromosome showed C-banding totally over it.

Ag-NOR banding showed nucleolus organizer regions on the terminal ends of chromosome number 3p, 4p, 8, 21, 23 and 24. Most of the non-metaphase nuclei had six nuceoli.

The sister chromatid exchanges frequency ranged from 6.00 to 18.81 per cell cycle. The average SCEs were 11.99 ± 2.99. The longer chromosomes showed more SCEs.

m.o.i.	Multiplicity of infection
N	Normality
'N'	Nucleocapsid protein
nm	nanometer
NMG	Normal mouse immunoglobulin
OD	Optical density
OPD	3, 3' Ortho phenyl diamine hydrochloride
'P'	Phosphoprotein
PBS	Phosphate buffered saline
PEG	Polyethelyne glycol
PI	Percent inhibition
PPRV	Peste-des-petits ruminants
RBOK	Rinderpest bovine old kabete
RP	Rinderpest
RPV	Rinderpest virus
RP-HIS	Rinderpest hyperimmune serum
rpm	revolutions per minute
S/C	Sub-cutaneuos
$TCID_{50}$	50% tissue culture infective dose
VNT	Virus neutralization test
µ	Micron
µg	Microgram
µl	Micro litre
µm	Micrometer
W/V	Weight by volume
W/W	Weight by weight

CONTENTS

INTRODUCTION

INTRODUCTION

Rinderpest, the major killer of animals since ancient times, is an acute, highly contagious and one of the most devastating diseases of cattle, buffaloes and pigs which also affects the small ruminants and most of the wild animals belonging to the order-Artiodactyle. The disease caused many pandemics, killing animal population all over the world, but mainly remained endemic in Asia and Africa for centuries. The strenuous exercise made during this century through various eradication campaigns resulted in the control and eradication of the disease from many countries and the dream of its global eradication to become the first animal disease to wipe out from the face of the earth is not far away.

Classical Rinderpest (*Rinder*-Cattle, *pest*-plague), a scourge of cattle & buffaloes, has a potential to cause more than 90% mortality in a susceptible population. It is clinically characterized by sudden onset of fever, severe depression, necrotic stomatitis, shooting diarrhoea, dehydration and finally death of the suffering animal (Plowright, 1962). The disease mainly spreads by direct contact and through contaminated drinking water. Aerosol transmission of the virus also occurs and is dependent on the relative humidity. The virus replicates both in lymphocytes and macrophages leading to lymphoid necrosis and in the epithelial cells of the gastro-intestinal, respiratory and urinary tracts, endocrine and exocrine glands but not in brain and spinal cord (Hyslop, 1979).

The disease is caused by rinderpest virus. This is an RNA virus belonging to the Order-*Mononegavirales*, Family-*Paramyxoviridae*, Subfamily- *Paramyxovirinae* and the Genus-*Morbillivirus*. The other members of the genus morbillivirus includes measles virus (MV) in man, canine distemper virus (CDV) in canines, peste des petits ruminants virus (PPRV) in small ruminants, phocine distemper virus (PDV) in seals (Osterhaus *et al.*, 1989) and dolphin and porpoise morbilliviruses (Barrett *et al.*, 1993). A morbillivirus of unknown pathogenicity has been identified in American cattle by sequence analysis

(Baron *et al.*, 1994) and an unusual pulmonary infection in man and horses in Australia has been ascribed to a new equine morbillivirus (Gould, 1996).

All morbilliviruses are antigenically closely related and have similar physico-chemical properties and produces characteristic cytopathic effects (CPE) in cell culture (Underwood and Brown, 1974). Morphologically morbilliviruses are pleomorphic, but usually spherical in shape and are of 150 nm or more in diameter although filamentous forms up to 1 μm long may appear.

Rinderpest, which caused havoc for a long time, can be easily prevented by vaccination and is also unequivocally an eradicable disease by mass vaccination. During the 19th and early years of this century, most of the countries eradicated this disease by mass vaccination and slaughter policy. The disease remained endemic in many parts of Asia and Africa, where because of economic and religious reasons stamping out policy could not gain popularity. After the development of caprinised, lapinised and tissue culture vaccines and with the launching of various control and eradication campaigns, these vaccines played a key role in controlling this scourge from many countries of these regions. The disease, which used to take a heavy toll a few decades ago, now has been brought under control to a great extent.

India made a landmark in the history of rinderpest control, when a comprehensive programme with the European Union support- The National Project On Rinderpest Eradication, wiped out the disease from country with no report of the outbreak since 1996 (NPRE, 1997). Accordingly, on 1st March 1998, the whole Indian Territory has been declared "Provisionally free from the disease" (Sinha, 1998). Eradication of rinderpest from the country is only an initial step and maintaining freedom from the disease until all risk of re- entry of the virus has been eliminated, is a real tough task ahead.

Rinderpest is a disease of awesome potential and has the capacity to re-infect territories from which it has been eliminated. Rinderpest free countries, bordering rinderpest-infected countries are always under a permanent threat of transboundary rinderpest incursions. Recently, Pakistan suffered from a very severe outbreak of

rinderpest- one of the worst attributed to rinderpest virus in many years (Rossiter *et al.*, 1998), which subsequently spread to Afghanistan. Therefore, India must be prepared to detect and respond the flare- ups and to differentiate RP from PPR in small ruminants. After declaration of "provisionally free" status and entry into the extremely vigilant "surveillance phase", the handling of virulent as well as vaccine rinderpest virus should also be limited in the country.

To maximize the chances of success, an eradication strategy needs to identify the residual foci of the disease. This is particularly important with mild strains of rinderpest virus, which exert little or no pathological effect on livestock- certainly nothing akin to cattle plague. The obvious solution for this is to develop a strong sero- surveillance system, capable of investigating all parts of the country. This surveillance should seek evidence of persistent rinderpest in yearling animals unprotected by either vaccine or maternal immunity (Mukhopadhyay *et al*, 1999).

For diagnosis of rinderpest a number of serological and molecular biological tests have been standardized. Molecular biological tests like DNA hybridization and RT- PCR are not easily applicable because of their requirement of high infrastructural facilities. Serological tests are economical and easy to perform and are likely to play a key role in this situation. Currently, monoclonal antibody based competition- ELISA (Libeau, 1995) and immuno- capture ELISA (Libeau *et al.*, 1994) are widely used for surveillance of rinderpest in the country. But these serological tests require virus material (purified or sonicated antigen) as a reagent to detect the anti-virus antibodies (c-ELISA) or as a reference antigen (immuno capture ELISA) to confirm the presence of virus in clinical samples.

Handling or widespread use of virus on the face of success in eradication campaign within the country is not acceptable. Since India is following the 'OIE pathway' for rinderpest eradication, it is likely to be restricted soon. Therefore, it is the need of the hour to replace the virus from diagnostics or biologicals with some non-infectious reagent. This can be achieved either by use of recombinant protein (Ismail *et al.*, 1994) or by production of anti-idiotype (anti- Ids) antibodies that mimics the viral epitope (Zhou *et al.*, 1998). The production of recombinant proteins is expensive. The

loss of original conformation because of change in glycosylation pattern- in case of glycoproteins and variable yield of protein in certain expression systems, are some of the problems associated with this approach. Rinderpest virus, because of its close antigenic similarity to PPRV, is indistinguishable in small ruminants using recombinant protein as a diagnostic reagent. Rinderpest virus N protein expressed in eukaryotic system failed to differentiate between rinderpest and PPR positive sera (Sreevastava, 1998).

The idiotype network theory postulated by Nobel laureate Niels K.Jerne (1974) introduced the concept of internal image and stimulated the idea of manipulating the immune system by anti-idiotype antibodies.

Anti-idiotypes that recognize an idiotype and represent an internal image of the antigen have been generated in many systems. Their antigenic mimicry makes them valuable substitutes for infectious agents as immunogens (Kennedy and Dressman, 1983; Regan *et al.*, 1983; Orten *et al.*, 1988; Srikumaran *et al.*, 1990; Chiang and Nicholas, 1996), cell surface receptor probes (Noseworthy *et al.*1983) and immuno-diagnostic reagents (Tackaberry *et al.* 1992; Zhou, *et al.*1998).

The approach of using anti-Ids in immuno-diagnostics has several advantages over current immunoassays. Since anti-Ids are immunoglobulins and are non-infectious they overcome the inherent problems of working with dangerous animal pathogens, facilitate continuous production of the reagents by hybridoma technology and enhance the reproducibility and standardization of the test.

Keeping these things in view, the present study was carried out with the following objectives:

1. To produce anti-idiotype antibodies to monoclonal antibody directed against rinderpest virus.

2. To characterize and evaluate the raised anti-idiotype antibodies as a potential diagnostic reagent for rinderpest.

REVIEW OF LITERATURE

REVIEW

RINDERPEST- HISTORICAL PERSPECTIVES

Rinderpest is one of the oldest and most devastating contagious diseases of cattle and buffaloes. The earliest recognizable description about disease were written in the fourth century AD (Barton, 1956). The disease is thought to be originated in Asia and then spread to Europe and Africa. In about the 1st Century the disease was introduced into Western Asia from China with invasions of Mangols. The disease was recorded as the inevitable sequel to every major military campaign in Europe. In Western Europe many severe outbreaks were recorded during Thirty-year war (1618-48), Nepolenic wars and the First World War. As a result of serious losses sustained during a series of epizootics in France about the middle of the 20th century, the disease was responsible for the establishment of first Veterinary School at Lyon in 1764.

In India the disease is prevalent since centuries. During 1860's the disease was very wide spread resulting in the death of about one million cattle per year. In view of serious losses, the Government of India in 1869 appointed Cattle plague commission. On the recommendation of the commission, the Imperial Bacteriological Laboratory (now Indian Veterinary Research Institute) was set up in 1894 at Mukteswar- Kumaon.

On African continent until 1884, only Egypt knew the disease. The movement of cattle during Italian/Abyssinian war resulted in the disease spreading rapidly over the whole of central and Southern Africa causing enormous losses in domestic cattle and wild animals.

The concept of control of rinderpest by slaughter policy was given by Lancisi in 1714 (Smithcors, 1956). Although unpalatable at that time, this strategy was later on used for the eradication of disease from all European countries, Southern Africa, Australia and Brazil. After the development of effective vaccines in 20th Century, these played a great role in control and eradication of the disease.

PRESENT WORLDWIDE STATUS OF RINDERPEST

Despite various control and eradication campaigns, the disease is still prevalent in some parts of the world. Ultimate success of eradication is continuously threatened by residual foci of RP and lack of efficient follow up system as seen in West Africa during 1980-82 and in East Africa in which the disease is still endemic even after the start of Pan African Rinderpest Eradication Campaign (PAREC) in 1986.

After the resurgence of RP in West Africa during 1980-82, PAREC was started in 1986 in order to control and eradicate the disease. It reduced the incidence of disease within short time and there is no outbreak in West Africa since 1988. Although PAREC has made significant advances in eradication of rinderpest lineage 1 virus from Ethiopia the disease is still endemic in East Africa, in Southern Sudan, South-Western Ethiopia, Northern Eastern Uganda and North-Western Kenya within which the virus continues to circulate, occasionally extending into surrounding areas (Mukhopadhyay *et al.*, 1999).

On the Asian subcontinent the disease is still endemic in Pakistan and its neighboring areas of Afghanistan. Pakistan suffered from a very severe outbreak of Rinderpest in 1994. (Rossiter *et al.*, 1998). Asian lineage of rinderpest virus, which is different from African lineage's, has been spread to trade partners by the movements of infected animals. Oman and the United Arab Emirates are examples of countries that have suffered brief incursions of this type without rinderpest becoming endemic. In contrast, Turkey and Saudi Arabia now have lengthy histories of contamination with

Asian lineage and could have become infected on a permanent basis as appears to be the case in Yemen (Mukhopadhyay *et al.*, 1999).

In India, National Programme of Rinderpest Eradication started in 1992, with the support of European Union, resulted in control of the disease and there is no outbreak since 1996. Recently, the country has been declared "provisionally free" from the disease (Sinha, 1998), which is really a great achievement on the part of Veterinarians and a landmark in the history of the rinderpest.

RINDERPEST VIRUS

Despite its identity as a major killer of animals, especially cattle and buffaloes, the etiological agent of Rinderpest remained unidentified until 1902 when Nicolle & Bey, discovered it as of viral origin. The virus known as rinderpest virus is an enveloped and contains single stranded RNA genome with negative polarity in helical nucleocapsid. Morphologically, it is pleomorphic and ranges from 90-250nm in diameter. Some filamentous particles measure up to 1μm (Plowright, 1962).

The RNA genome of rinderpest virus encodes eight proteins, of which six are structural and two are non-structural. The genome size of RBOK (Vaccine strain) is 15.582 kb. Among the structural proteins the large polymerase (L), the phosphoprotein (P) and nucleoprotein (N) are located inside the virion. The proteins located in the viral envelope are haemagglutinin (H), fusion (F) and matrix (M) proteins. The non-structural proteins C and V are encoded within P gene sequence and are produced in infected cells.

The fusion (F) protein of all Paramyxoviruses is responsible for fusion of the virus envelope with cell to cell fusion, during later stages of infection with the formation of syncytia. It is reported that even in the presence of anti-H neutralizing antibodies, virus could spread from cell to cell because of fusion protein. The H protein of RPV is responsible for the attachment of virus to the surface receptor and so influences the cell

tropism of the virus. Most neutralizing antibodies are directed against this protein. Nothing is known about the nature of the cell surface receptor for rinderpest virus although the lack of neuraminidase activity suggest that they lack sialic acid residues (Barrett *et al.*, 1991)

CONTROL OF RINDERPEST

At the end of 19th century many attempts were made to actively immunize the animals against rinderpest using bile from sick animals and serum- virus mixture. During early years of this century inactivated vaccines also played great role in eradication of rinderpest from Philippines and Thialand. Development of Caprinised, Lapinised and Tissue culture rinderpest vaccine revolutionized the rinderpest control programme. The Caprinised vaccine was mostly suitable for animals with high innate resistance such as African Zebu, the plain Zebu and buffaloes of India, but not for the pregnant and lactating animals. The adverse effects such as thermal reactions, mouth lesions, diarrhoea, decrease in milk yield and abortion in advanced stages of pregnancy, even deaths up to 21.5% has been reported (Scott, 1964). After development of tissue culture vaccine and then adaptation of RBOK strain to the Vero cell line made vaccine production easy and economic (Shishido *et al.*, 1967). This vaccine is currently in use for vaccination of cattle and buffaloes in countries where the disease is endemic.

The tissue culture rinderpest vaccine is thermolabile, which is the main drawback associated with it. It has a poor shelf life under conditions of average storage. The shelf life of vaccine at 22^{0}C is three months, while at 37^{0}C it is three weeks (Plowright, 1972). In most developing countries, where rinderpest vaccination programme is undertaken, the field conditions are harshest and cold chain facilities are poor. Thus, it indicates that under field conditions the rinderpest vaccine could lose its potency at two levels, firstly during improper storage and transportation and in the next phase during reconstitution. Because of these disadvantages, it generated the need to look for other approaches of vaccine production. Live recombinant vector vaccines have been produced for rinderpest

using vaccinia as live vector (Yilma *et al.*, 1988; Belsham *et al.*, 1989). In its first long-term immunity trial, one recombinant is found effective for one year after a single shot vaccination (Inui *et al.*, 1995).

DIAGNOSIS OF RINDERPEST

Field diagnosis of rinderpest in acutely infected animals exhibiting characteristic symptoms and lesions is not difficult. However this is often confusing in endemic areas where outbreaks of milder form occur or in areas where mass vaccination programme has made progress, wherein affected animals may not show typical symptoms or lesions. In small ruminants the PPR virus shows similar symptoms and therefore diagnosis is difficult clinically, so it is necessary in such cases as well for confirmation of provisional diagnosis of rinderpest to resort to laboratory diagnosis.

Various serological as well as molecular biological techniques have been standardized for the diagnosis of rinderpest. Presently, competition- ELISA (Libeau *et al.*, 1992; Anderson & Mckay, 1994; Singh *et al.*, 1998) and Immunocapture- ELISA (Libeau, *et al.*, 1994) are the two serological tests in vogue for the detection of antibody and antigen, respectively.

In c-ELISA, the approach is to measure the competition between the test antibody and detection antibody for binding to the particular antigen. In most cases, the detection antibody is monoclonal which confers specificity to the tests. All c-ELISA developed for RP and PPR uses purified or sonicated virus-infected cells as the antigen. Since the antibody binding reaction is directed against only one viral protein, it would be useful to produce this antigen in large quantities for the production of large number of test kits Recombinant proteins containing particular epitope can be used. But cross-reactive epitopes present on expressed protein makes them unsuitable for differential diagnosis from closely related viruses. Rinderpest virus N protein expressed in an eukaryotic system reacted to both rinderpest virus and PPR virus specific monoclonal antibodies (Sreevastava, 1998). Alternatively peptides comprising the particular epitope can be used

but these are not suitable for the confirmation dependent epitopes. Use of anti- idiotypic antibodies mimicking the particular viral epitope may prove suitable candidate for this. Continuous and bulk production of these are also possible by hybridoma technology (Zhou, 1998).

MONOCLONAL ANTIBODIES TO RINDERPEST VIRUS GLYCOPROTEINS

Rinderpest virus surface glycoproteins, haemagglutinin (H) and Fusion protein (F), plays important role in virus attachment and fusion of host cells and antibodies to these surface proteins probably play a key role in the development of immunity.

Bhavani *et al.* (1989) characterized three antigenic sites (designated as I-III) on haemagglutinin protein of RPV using monoclonal antibodies. Site I & II were found partially overlapping by c-ELISA. Sugiyama *et al.* (1991) characterized seven distinct antigenic sites on haemagglutinin and three on fusion protein. The MAbs to six sites on H protein were able to neutralize the infectivity of RPV. The addition of anti- mouse immunoglobulin increased the virus neutralizing antibody titre of most of the anti- H MAbs, including those lacking neutralizing activity. One of the antigenic sites on H was conserved among morbillivuruses and the MAbs to this site had haemagglutination inhibition activity against measles virus (MV). The anti- F MAb lacked neutralizing activity, but two of the five MAbs did show activity in the presence of guinea pig complement.

McCullough and cowerkers (1986) produced MAbs to all morbilliviruses. They found epitopes on H proteins to be virus specific category while that of on F protein were mostly group specific.

C-ELISA developed by Libeau *et al.* (1995) uses anti- H monoclonal antibody for specific diagnosis of rinderpest. Singh *et al.*, (1998) also developed monoclonal antibodies to rinderpest virus and MAb based C- ELISA. This MAb is also against H protein of RPV.

IDIOTYPE- ANTI- IDIOTYPE NETWORK REGULATION

The immune system of an individual can make millions of different kinds of antibodies- the complex molecules that recognize and bind to alien substances that invade the body marking them for destruction. Each antibody can in turn be the target of other antibodies that recognize its unique molecular characteristics. By means of such antibody -antibody reactions the immune- system interacts with itself. Network reactions seem to modulate the normal immune response and disorders of the networks may underlie auto-immune diseases that stem from an immunological attack on the body itself (Kennedy *et al.*, 1986).

An idiotype or idiotypic determinant (Id) is the antigenic site associated with the variable (V) region of the antibody molecule. The first description about idiotypes came in 1963 from two different groups of investigators. Henry Kunkel and co-workers described a unique set of antigenic determinants on human antibodies (Kunkel *et al.*, 1963) while Jacques Oudin described a similar finding on rabbit antibodies (Oudin & Michel, 1963). In each instance, these investigators generated anti-antibodies that were idiotypic in nature. The findings of Kunkel and Oudin, that the anti-antibodies recognized very unique antigenic determinants, were initially conceived as rare forms of allotypes or individual antigenic determinants on immunoglobulin. Oudin later termed these unique determinants as idiotypes from the Greek words "idios" meaning unique or individual and "typos" designating form.

Evidence that Id and anti-Id may have some biological role in the induction of the immune response came from experiments published in early 1970s. In these studies, it was demonstrated that anti– Id was potent *in vivo*- suppressing agents of antibodies that expressed a given Id (Cosenza & Kohler., 1977, Hart *et al.*,1972). In addition, prior immunization with anti-Id also could enhance specific immune responses to an antigen

(Eichmann & Rajewsky, 1975). This experimental evidence laid the groundwork for developing the network concepts involved in regulating the immune response.

In 1973, Lindenmann coined the term 'homobody' as an anti-Id that demonstrated the biological functions of the antigen and shared complementary three-dimensional structures. In 1974, Niels Jerne postulated a theory of immune regulations based on interactions among antibodies and lymphocytes (Jerne, 1974). These interactions were represented by a series of networks whereby Id and paratope recognition on antibodies and lymphocytes constituted the components of a given network. In Jerne's network theory, the homobody was conceived as an internal image anti-Id, that displayed functional mimicry of the antigen. In network theory, Jerne proposed that the immune system display an auto-regulating behavior via the interactions of a paratope on an Ab2 with its corresponding Id expressed on an Ab-1. The Ab2 also express an Id that can be recognized and regulated by cells that possess antigen receptors with specificity towards either the Id or paratope of the Ab-2. Jerne assumed that the average concentrations of these regulatory antibodies (Ab-2) were low and that the interaction between these antibodies and cells exhibiting their particular antigen receptor for the Id or paratope of the Ab-2 was at a steady state or in equilibrium. The introduction of an antigen would disturb this steady state, and binding of the Ab-1 to the antigen would allow the Ab-1 to escape from the suppressive effects exerted by the Id of the Ab2.

CLASSIFICATION OF ANTI-ID:

According to Jerne there are two types of Anti-Ids- Ab2 α and Ab2 β (Jerne *et al.*, 1982). The Ab-2α binding to its Ab1 may or may not be inhibited by the antigen inducing the Ab1. An Ab2β or internal image anti-Id that is representative of the homobody, has the capacity to serologically mimic the antigen recognized by the Ab1. The basis of this mimicry is that, in certain instances the paratope and Id on the Ab-1 may represent the same or similar sites and thus, the antigen and anti-Id may share complementary structures based on their ability to bind the Ab-1 at its paratopic and idiotopic sites, respectively. The Ab-2β or internal image anti-Id can substitute for the

antigen and can induce an anti-antigen response (Ab-3) that serologically resembles the Ab-1.

Bona & Kohler proposed an alternative classification of anti-Id that subgroups the Ab-2α proposed by Jerne into Ab-2α and Ab-2γ. The Ab-2α recognizes a idiotope located outside the paratope while the Ab-2γ recognizes the Id located within it (Bona and Kohler, 1984). The Ab2α binding to the Ab1 is not inhibited by the antigen while Ab2γ binding is inhibited after the binding of antigen to the Ab1. Although, Ab2β & Ab2γ recognizes the idiotype present inside the paratope, Ab2 β is only the internal image anti-Id while Ab2γ is non-internal image anti-Id.

Kohler and co-workers (1989) classified anti-Ids on the basis of their biological characteristics instead of inhibition of Ab1-Ab2 interaction. They coined the term "Network antigen" to those anti-Id preparations that produce Ab-3 populations that recognize the original antigen. They used a number of conceptual parameters to explain the induction of antigen specific responses *in- vivo* by the non- internal image subclass of anti-Id (Kohler *et al.*, 1989). Of all, the classification given by Bona & Kohler (1984) is most acceptable.

PRODUCTION OF ANTI-IDIOTYPE ANTIBODIES

Various systems have been used to produce anti-Ids namely autogenic, isogenic, allogenic and xenogenic depending on the animal to be used for the production of anti-Ids i.e. whether in the same animal, within an inbred strain, across strain barrier or across species barrier respectively.

Rodkey (1974) developed autogenic anti-Ids in rabbits by separating Ab1 from serum and then injecting into the same animal after some time interval in FCA. This resulted in autogenic anti-Ids production. The production of anti-idiotype response

spontaneously during a normal immune response to antigen was demonstrated by Kelsoe & Cerny (1979), Geha (1982) and Krah & Choppin (1988).

Potter & Liebermann (1970) and Sakato & Eisen (1975) developed isogenic anti-Ids in mice after injecting antibodies from one mouse to another of same inbred line.

For xenogenic anti-Id production most of the workers favored rabbit (Kennedy & Dressman, 1983; Orten *et al.*, 1988; Tsuda *et al.*, 1992; Xue *et al.*, 1991) while guinea pig (Eichman *et al.*, 1975), calf (Srikumaran *et al.*, 1990) and goat (Dandapat *et al.*, 1994) were also used.

ANTI-ID AS PUTATIVE VIRAL VACCINES

A number of studies have implicated the potential use of anti-Id in the induction of protective immunity against a wide variety of infectious agents. Most of the attention of anti-Id based vaccines has focused on Ab-2β preparations that demonstrate serological mimicry to a variety of antigens associated with a number of pathogens. However, studies have also demonstrated that Ab-2α preparations can induce specific immune response without subsequent antigen exposure (Schick *et al.*, 1987).

The first report of an anti-Id inducing an immune response against an infections agent, that would be conceived as exhibiting vaccine potential, was in the Trypanosoma system, by Sack and co-workers (1982). Subsequent studies indicated that the anti-Id was of the Ab-2α class and recognized a restricted Id. The anti-Id was genetically restricted in its ability to induce anti-trypanosoma responses in mice.

In 1983, Kennedy and co- workers, showed the ability of rabbit polyclonal anti-Id, containing Ab-2β population, to induce the antibody response against Hepatitis B virus in mice. Later on, the same group was able to demonstrate the protective immune response of anti- Ids against HBV in chimpanzee- the most relevant animal model for human HBV (Kennedy *et al.*, 1986). During the same year Reagan & co-workers

succeeded in inducing a neutralizing antibody response against rabies virus using an Ab-2α preparation. Since then a number of reports have suggested the potential use of anti-Id as vaccines against a variety of infections viral agents and virally induced tumors. These include the work on Blue tongue (Grieder and Schultz, 1990), Bovine herpes virus (Srikumaran *et al.*, 1990), Newcastle disease (Tanaka *et al.*, 1986), Cytomegalovirus (Keay *et al.*, 1988), Herpes simplex (Lathey *et al*; 1986), HIV (Zhou *et al.*, 1990), Poliovirus (Uytedehaag & Osterhaus, 1985) and Reovirus (Goulton *et al.*, 1986).

Against tumors induced by viruses, it includes maloney murine leukenia virus (Mernaugh *et al.*, 1992), Mouse mammary tumor virus (Raychaudhuri *et al.*, 1987) and Simian virus 40 (Mernaugh *et al.*, 1992).

The most thoroughly studied and successful viral-related anti-Id system is that of reovirus. Green and colleagues used a murine monoclonal antibody that recognized reovirus hemagglutinin type 3 as the Ab-1 preparation to generate anti-Id reagents. These investigators utilized an internal image anti-Id preparation that mimicked reovirus hemagglutinin to identify the cellular receptor for the virus as the β-adrenergic receptor. In addition, mice immunized with the internal image anti-Id induced a protective immune response involving cell mediated as well humoral response (Gaulton & Green, 1986). A high degree of primary amino acid sequence homology was found between the variable region of the anti-Id and the reovirus type 3 hemagglutinin (Bruck *et al.*, 1986).

In poliovirus system, Ab2 immunization led to the production of virus-neutralizing antibodies but did not confer protective immunity (Uytedehaag & Osterhaus, 1985). In the Sendai virus system, a monoclonal anti- clonotypic antibody recognizing the T cell-receptor of a virus-specific T-helper cell clone was used for the first time. Immunization with this anti-clonotypic antibody induced protective immunity against Sendai virus infection by eliciting T cell & B cell immunity (Ertl & Finberg , 1984).

One subtype of Influenza virus, because of its antigenic variation, does not induce neutralizing antibodies against other subtypes, but anti-Ids to Mem 71H-Bel N

virus (H3 subtype) developed neutralizing antibodies against H1 or H2 subtypes along with H3 subtype- a very interesting finding observed by Ander & co-workers (1989). This group also demonstrated that this immune response was not genetically restricted and produced the immune response in different mice strains.

An Ab-2α monoclonal anti-Id developed for HIV, induced antibodies against HIV in mice- syngenic system, but could not produce any detectable anti-virus antibodies in Rabbits- xenogenic system (Zhou *et al.*, 1990). On the other hand, polyclonal rabbit anti-Id developed for pseudo rabies virus induced anti-virus antibodies in mice- a xenogenic system and protected then from lethal challenge (Tsuda *et al.*, 1992).

Tanaka *et al.*,(1986), who raised polyclonal anti-Ids against Haemagglutinin protein of New Castle Disease, found anti-viral antibody response in a syngenic system. Anti-Id induced anti-virus HI titre was found 1:4 as compared to 1:128 shown by the Newcastle disease virus.

ANTI-ID AS CELL SURFACE RECEPTOR PROBES

Anti-idiotype antibodies have been found to be useful in identification of cell surface receptors for certain ligands and viruses. Various studies have demonstrated the usefulness of these anti-idiotype reagents in detection and modulation of specific membrane receptors of certain cell types. Sege & Peterson (1978) raised anti-idiotypes against the retinol binding protein (RBP) and insulin antibodies. The anti-anti RBP antibodies bound specifically to the rat intestine cells, which have physiological surface receptor for RBP. These anti-idiotype reagents abolished the uptake of retinol by these cells in concentration dependent manner. The same authors also found that anti-anti-insulin antibodies inhibited the binding of ^{125}I labeled insulin to isolated rat epididymal cells.

Anti-Ids have played great role in identifying cellular receptors for some viruses. It includes reovirus (Noseworthy *et al.*, 1983), polyomavirus (Marriott *et al.*, 1987),

Epstein-Barr virus (Mernaugh *et al.*, 1992) and murine leukemogenic retiovirus (Mernaugh *et al.*, 1992).

Krah & Choppin (1988) found anti-idiotype antibodies to cell surface receptor specific to measles virus in mice immunized with measles virus, 30-35 days post-immunization. These sera agglutinated erythrocytes which virus also agglutinate, reacted with Vero cells and inhibited virus attachment to Vero cells. This cell surface receptor binding activity was identified to be in the immunoglobulins and was neutralized by sera against the virus.

Orten *et al.* (1988) found MDBK cell surface receptor binding anti-Ids in polyclonal rabbit anti-Id sera developed against Bovine Herpesvirus-1 monoclonal antibody. This binding was demonstrated by indirect fluorescence technique. Srikumaran *et al.* (1990) also found xenogenic polyclonal anti-Ids binding to the BHV-1 permissive cells, as determined by virus infection inhibition study.

Xue and Minocha (1993) identified a 50 KDa protein as a specific receptor for BVDV gp53, by using anti-idiotype antibodies to BVDV monoclonal antibody.

ANTI-ID AS DIAGNOSTIC REAGENTS

Ab-2β, because of its property of antigen mimicry, has a great potential in diagnostics as an alternative to virus material. As anti-Id is an antibody, there is no harm in its use in diagnostics in place of hazardous virus material. Internal image monoclonal anti-Ids ensure the continuous supply of reagent without any tedious processing (Zhou *et al.*, 1998).

Tackaberry *et al.* (1992) developed anti-Id based ELISA to detect the human cytomegalovirus (HCMV). The inhibition-ELISA detected HCMV in a concentration dependent manner from 20 to 0.6 x 10^3 PFU virus/ml, with 50% inhibition at approx. 3 x 10^3 PFU/ml. As the CMV concentration in urine, in active infection stage, ranges from 10^2 to 10^6 PFU/ml, the technique was found useful to detect virus from urine samples.

Zhou *et al.* (1995) produced polyclonal anti-Ids against shared idiotope (IdX) of anti- bluetongue virus MAbs by sequential immunization. This Anti-Id population

reacted with various homologous and heterologous bluetongue virus positive sera and found useful as a potential diagnostic reagent in detection of anti-bluetongue virus antibodies.

There is no published report of anti-idiotype antibodies for rinderpest virus, although Thangvelu (1988) produced anti-Ids to rinderpest virus using anti rinderpest IgG in an allogenic system. These anti-Ids could not found suitable as a diagnostic reagent.

Rinderpest has been declared provisionally free from our country but the disease surveillance will continue for years. For effective surveillance of the disease, we must have quick, accurate and field applicable diagnostic tools in our hand so that they can be widely used throughout the country. The serological tests presently in vogue use the virus material in them as a diagnostic reagent. For large-scale production of diagnostic reagents, continuous propagation of virus is inevitable. Accidental escape of virus although of vaccine strain, may prove dangerous in the wake of increasing susceptible bovine population in the country, after cessation of vaccination.

Therefore it is very much essential to seek the alternatives for the use of virus in diagnostics and biological. Use of anti-Idiotype antibodies mimicking the viral antigen is one of these approaches to replace the virus, in this case.

After the development of Hybridoma technology, it has become feasible to produce anti-idiotype antibodies for a particular epitope of a protein. By selecting a virus specific monoclonal antibody, it is possible to raise anti-Ids, which can be used for specific diagnosis of virus as well as its differentiation from other closely related viruses. Since anti-Ids are non-infectious they overcome the inherent problem of working with dangerous pathogens and also facilitate continuous production of the reagents by hybridoma technology.

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MATERIALS AND METHODS

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MATERIALS

EXPERIMENTAL ANIMALS

Rabbit - Healthy adult New Zealand White rabbits weighing about 1Kg, obtained from LPR section, IVRI Mukteswar, were used in this study.

Mice - Balb/C mice maintained at IVRI, Mukteswar were used for production of ascitic fluid.

VIRUSES

TCRPV - Tissue culture adapted rinderpest vaccine virus (RBOK) between passage level 100-103 maintained in rinderpest laboratory, IVRI, Mukteswar was used in this study.

Lapinised RPV - Lapinised rinderpest virus (Nakamura -III strain) at passage level 997 was used as a challenge virus in rabbits inoculated with anti-idiotype antibodies.

CELL LINE

Vero - African Green Monkey Kidney cell line originally obtained from A.T.C.C., USA and maintained in the Rinderpest laboratory, IVRI, Mukteswar between passage level 125-160 was used for virus propagation, virus neutralization inhibition and cell surface receptor studies.

HYPERIMMUNE SERUM

Anti-Rinderpest virus hyperimmune serum (HIS) raised in rabbits (RHIS) and Bovines (BHIS) at Rinderpest laboratory, IVRI, Mukteswar was used throughout the study.

CONJUGATES

Following antibody conjugates were used in the study:

i). Anti-Rabbit- HRPO (Dakopatts, Denmark)

ii). Anti-Mouse- HRPO (Dakopatts, Denmark)

iii). Anti- Rabbit-FITC (Genie, India)

iv). Anti-Mouse-FITC (Sigma, USA)

REAGENTS AND SOLUTIONS

The detail regarding composition of reagents and solutions are given in the Appendix or mentioned at appropriate places.

EQUIPMENT

Microscopes

1) Inverted binocular microscope, Olympus, Japan.
2) Leitz Orthoplan Universal Largefield Fluorescence Microscope

Centrifuges

1). Biofuge, Heraeus instruments,

2). Hermle Z 360 K, Berthold Hermle AG, Germany

Ultracentrifuge

1). OTD 65D, Sorvall, R. Dunpot Company, USA

Rotor

1). AH629 swing out rotor, Sorvall, Dunpot company, USA

Other Equipment

1). 96 well plastic cell culture plates, Costar, Cambridge. UK

2). ELISA plates and modules, Nunc, Denmark.

3). Vortex mixer, Cyclomixer, Remi, India

4). Magnetic stirrer, Baird and Tatlok Ltd., England

5). Incubator, scientific Instruments, New Delhi, India

6). CO_2 incubator, Forma scientific Inc., Marietta, Ohio

7). Micro- pipette (0-200µl) - single and multi-channel

8). ELISA Reader, Labsystems Multiscan Plus

MONOCLONAL ANTIBODY

Monoclonal antibody (D7 26b) was selected from a panel of anti- Rinderpest virus mouse monoclonal antibodies developed and available at Rinderpest and Allied Disease Laboratory, IVRI, Mukteswar.

METHODS

REVIVAL OF SELECTED MONOCLONAL ANTIBODY (MAb) CLONE

The monoclonal antibody clone D726b already preserved and fully characterized in Rinderpest Laboratory was revived as follows-

The D726b clone preserved in liquid nitrogen was quickly thawed at 37°C and centrifuged at 1000rpm for five min. Supernatant was decanted and the cells were suspended in 1ml of Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FCS. Cells were then added in a 25cc flask containing the same media. The flask was incubated in 5% CO_2 tension at 37^0C incubator. Cells were observed daily. These were expanded in larger flasks at appropriate cell concentrations.

PRODUCTION OF ASCITES

The procedures for ascites production were followed as described by Brodeur *et al.* (1984).

A) **Pristane/ IFA priming**

- Pristan or IFA was used at the rate of 0.5ml/mice as a pretreatment for ascites production. Three mice were used each for pristan and IFA priming.
- Tuberculine syringe was filled with pristan / IFA.
- Mouse was taken by its tail and its feet were allowed to grasp the top of the cage. By grasping the nape of its neck with thumb and forefinger tail was stretched down against the base of the thumb and hold in place with the third and fourth fingers.
- The animal was lifted and the abdomen was disinfected using spirit. Holding the syringe upward at 45° angle, needle (22 gauge, 11/2 inch) was inserted into the abdomen not more than 1cm and injected 0.5ml of either pristan or IFA I/Ply. Animal was replaced in cage and others were inoculated in the same way.

B) **Inoculation of cells**

- The cells (D726b) grown in 75cc tissue culture flask were harvested into 50ml tube and centrifuged at 1000 rpm for five minutes.
- The supernatant was decanted and the cells washed with serum free protein free media (Sigma).

- After second washing with serum free protein free media the cells were re-suspended in same media for inoculation in mice.
- Cells were counted using hematocytometer. Cell concentration was adjusted to 10^7cells/ml.
- Primed mice were inoculated with $5x10^6$cells/mice I/Ply.

C) **Harvesting the ascites fluid**

- After five days of inoculation of cells the animals were observed daily for ascites or any other changes in abdomen.
- When swelling became apparent the fluid was harvested using 18gauge needle in a tube containing EDTA granules.
- It was centrifuged at 1000rpm for five min., supernatant decanted in a screw-cap vial and stored at -20^0c for further use.

PROTEIN A PURIFICATION OF MONOCLONAL ANTIBODY FROM ASCITES FLUID

MAb from ascites fluid was purified by passing through Protein A sepharose column (Sigma). Purification was carried out as per instructions of the kit. Briefly, the procedure is followed as under,

- 1.0 ml of ascites fluid was mixed with 2.0 ml of binding buffer
- Desalting cartridge was regenerated by passing 10ml of HEPES buffer at the rate of 1.0 ml / minute.
- Similarly Protein A cartridge was regenerated by passing 5.0 ml of regeneration buffer at the rate of 1.0 ml / minute.

- After regeneration of Protein A cartridge it was equilibrated with 4.0 ml binding buffer by passing it at the rate of 1.0ml / minute.
- Sample- Binding buffer mixture was then loaded on the Protein A- sepharose column and passed at the rate of 0.5 ml / minute.
- After loading the sample column was washed with 6.0ml binding buffer at the rate of 1.0 ml /minute.
- Desalting cartridge was then attached to the Protein A cartridge and the MAb attached to the Protein A- sepharose column was eluted with 5ml elution buffer by passing at the rate of 0.5 ml / minute.
- Both cartridges were regenerated as above and stored in PBS-Azide until further use.
- Eluted MAb was further tested in ELISA and the protein content was estimated by Lowry's method.

PREPARATION OF Fab FRAGMENTS

The Fab fragments were produced according to Parham (1986) with certain modifications. Briefly, the procedure is described as under-

1). Purified MAb was dialyzed against 0.1M phosphate buffer saline (pH 7.2) and 4.0ml of it containing 1.6mg / ml antibody was taken for digestion.

2). MAb solution was added to the digestion buffer (pH 5.5) containing 0.1M acetate buffer, 0.001M DL-cysteine and 0.002M EDTA in their final concentrations.

3).Papain solution made in 0.1N acetate buffer @ 1.2mg/ml was added. The weight ratio of IgG: Papain used was 20: 1.

4). The mixture was kept at 37^0C under constant shaking for 2hrs.

5). The reaction was stopped by adding 0.4 ml of iodoacetamide (0.2M) and kept at 37^{0}C for 30 minutes. The mixture was then kept at -40^{0}C until further processing.

PURIFICATION OF Fab FRAGMENTS

Digested MAb solution contains the Fc fragments, undigested IgG and other fragments of smaller molecular weight, in addition to the Fab fragments. From this mixture Fab fragments were purified by passing through Protein A-Sepharose column which specifically binds to Fc portion IgG. As Protein A bind to Fc portion it will absorb Fc fragments as well as remains of undigested IgG while Fab fragments will appear in the flow through. Therefore for separation of Fab portion, procedure similar to purification of MAb from ascites fluid was followed. However in this instance, the Fab portions were collected in flow-through fractions first followed by elution of bound Fc fragments and undigested MAb.

EVALUATION OF REACTIVITY OF Fab FRAGMENTS

Purified Fab fragments were evaluated in ELISA to verify the digestion of MAb by Papain and further tested for their neutralizing ability in VNT.

i) Determination of MAb digestion in a Blocking- ELISA

As most of the dominant epitopes are present in the Fc portion of IgG, the anti- species conjugate contains antibodies mainly against these epitopes. If Fab fragment retains its biological activity to bind the antigen the anti-mouse conjugate used for detection will bind to a lesser extent with Fab, when compared to undigested MAb. This principle was used to demonstrate the digestion of MAb D726b in Blocking- ELISA.

- ELISA micro-titre plates were coated with semi-purified TCRPV antigen diluted 1:150 in PBS for 1hr at 37^{0}C.

- After washing with PBS-T for 3 times, Fab fragments (diluted in blocking buffer) were added to the wells in Log_{10} dilutions, while blocking buffer was added in MAb control wells. Plates were incubated at 37^0C for 1hr.

- Pl[illegible] was added in all wells including that of MAb control. Plates were incubated at 37^0C for 1hr.

- Finally after washing the plates, anti-mouse conjugate (1:1000) diluted in blocking buffer was added and plates were incubated at 37^0C for 1hr.

- Substrate buffer containing H_2O_2 and OPD was added to the wells, after washing with PBS-T. Colour development was measured in an ELISA reader at OD_{492}.

Inhibition of colour reaction, in Fab fragment added wells, was expressed as percent inhibition (PI) of colour, using following formula:

$$\text{Percent Inhibition} = \frac{(OD)_{492} \text{ with MAb control} - (OD)_{492} \text{ with Fab Fraction}}{(OD)_{492} \text{ with MAb control}} \times 100$$

All reagents used in this assay were 50µl/ well and assay was performed in duplicates.

ii) **Neutralization of virus by Fab fragments**

Neutralization assay was performed by using 100 $TCID_{50}$ virus per well with Log_{10} dilutions of ascites, Protein A purified MAb and purified Fab fragments.

- Ascites fluid, affinity purified MAb and purified Fab in Log_{10} dilutions were pre-incubated with 100 $TCID_{50}$ TCRP virus for 1hr.

- The virus-antibody mixture was added to pre formed Vero cell monolayer in 96 well cell culture plate. Five wells were used for each antibody dilution. Plates were incubated at 37^0C under 5% CO_2 tension for 7 days.

- Cells were observed for CPE and end point neutralization titre was determined as the highest dilution of ascites/ purified MAb/ purified Fab that inhibited CPE in 50% of the wells.

IMMUNIZATION OF RABBITS BY Fab FRAGMENTS

Two adult healthy rabbits were inoculated with affinity purified Fab fragments (300µg/rabbit) for anti-idiotype antibody production as per the following schedule:

Injections	Days	Adjuvant	Route of inoculation
1)Priming	0	FCA	S/C
2) First booster	14	FIA	I/M
3) Second booster	28	FIA	I/M

Pre inoculation serum was collected from each rabbit before immunization. During each immunization, adjuvant was used in equal volume to that of Fab solution. Blood was collected on 14, 21, 28 and 35 days post immunization for preparation of serum which was then stored in aliquots at -20^0C for further use.

PRODUCTION OF ANTI-Ids

Selection & **Revival of hybridoma clone (D7 26b)**

↓

Expansion of cells

↓

Counting of cells — **Sensitization of mice (IFA/ Pristan priming)**

↓

Inoculation of cells in mice ($5x\ 10^6$ cells) I/P

↓

Observation of mice

↓

Harvesting ascites fluid

↓

Titration of MAb (Indirect ELISA/ VNT)

↓

Purification of MAb- using Protein A- sepharose column

↓

Testing antibody concentration (Lowry's method)

↓

Papain digestion of MAb

↓

Purification of Fab → Protein A- sepharose column → Collection of Flow through

↓

↓

Concentration by reverse osmosis

Assessment of biological activity of Fab
(VNT and Blocking ELISA)

↓

Immunization of rabbits

↓

Collection of sera for anti-Ids at various stages of immunization

PREPARATION OF CNBr ACTIVATED MAbD7 26b COUPLED SEPHAROSE 4B COLUMN

For purification of anti-Ids from anti-Id serum CNBr activated MAb D726b- Sepharose 4B Column was prepared according to the protocol described by Hudson and Hay (1989) with some modifications:

1). **Antibody preparation (MAb D726b)**:

Cell culture supernatant (40ml) of MAb D726b was purified using Protein A Sepharose column as described earlier. The MAb bound to Protein A was eluted with 5.0 ml of elution buffer.

- Affinity purified MAb was dialyzed against 0.1M $NaHCO_3$/0.5M NaCl (pH 8.3) overnight with 3 changes of buffer. This was carried out to remove small molecules containing free amino or sulphydryl groups.
- Dialyzed MAb was centrifuged at 10,000x g at 4^0C for 1hr to remove the aggregates of MAb if any.
- Antibody concentration was determined by Lowry's method.

2).**CNBr activation of sepharose 4B:**

- 5.0 ml of sepharose 4B (Pharmacia) was first taken into a beaker and 3.0 ml of distilled water was added to it. Further procedure was carried out in fume cupboard.
- A stoppered vial was weighed before as well as after adding CNBr (SRL).
- This CNBr was dissolved in distilled water so as to make the final concentration of 50mg per ml.
- While stirring on magnetic stirrer pH of the sepharose beads was stabilized between 11 to 11.5 using 2M NaOH with intermittent mixing.

- 300mg of CNBr dissolved in distilled water was then added to the sepharose beads (6o mg CNBr/ml sepharose) and the pH was maintained between 11 to 11.5 with 2M NaOH for 5 to 10 minutes until the pH became stable.

- Immediately the gel beads were transferred to a filter paper cone in a glass funnel and washed with distilled water followed by borate saline buffer (0.1M, pH 8.3), 30ml each. Beads were transferred to another beaker.

3). **Coupling of MAb to CNBr activated sepharose 4B:**

- 8.0 mg of MAb (1.6mg per ml) dissolved in 5.0 ml 0.1M $NaHCO_3$/0.5M NaCl buffer was added quickly to CNBr activated sepharose 4B and beads were kept under stirring condition overnight at 4^0C.

- Next day beads were loaded into a 5ml syringe in which a piece of glass wool was applied as a filter. The column was washed with 10ml PBS and washings were collected.

- Beads were suspended in 1M glycine (pH 8) at 4°C for 6hrs to block the remaining active sites.

- Finally the column was washed with PBS thoroughly and stored in PBS- Azide at 4^0C after sealing both the ends with parafilm until further use.

The binding of MAb to sepharose 4B was tested in ELISA by comparing the binding of MAb D726b to TCRPV antigen coated ELISA plates, before and after coupling reaction was performed.

PREPARATION OF CNBr ACTIVATED NORMAL MOUSE IMMUNOGLBULIN (NMG) COUPLED SEPHAROSE 4B COLUMN

For absorption of anti-isotypes and anti-allotypes from the anti-Id serum, CNBr activated NMG coupled sepharose 4B was prepared. The same protocol described by Hudson and Hay (1989) was followed.

Blood was collected from BALB/C mice through the inner eye canthus by a very thin pasture pipette. After separation of serum globulin fraction was precipitated out by ammonium sulphate precipitation and finally dialyzed against 0.1M $NaHCO_3$/0.5M NaCl buffer overnight with three buffer changes.

NMG coupled sepharose 4B column was prepared as described earlier for MAb-Sepharose 4B column. The coupling of NMG to column matrix was tested by its ability to remove anti-isotypes and anti-allotypes from rabbit anti-Id containing serum by indirect ELISA.

DETERMINATION OF ANTI-Id ACTIVITY

Indirect ELISA:

1) The MAb diluted 1: 50,000 (ascites fluid) in carbonate- bicarbonate buffer, pH 9.6 was used to coat the wells of ELISA plates, at 37^0C for 1.0hour.

2) After washing the plates with PBS-T for three times the unbound sites were blocked with blocking buffer containing 5% SMP, 0.5% Healthy Goat serum and 0.05% T-20 in PBS for 1hour at 37^0C.

3) The anti-Id sera from both the rabbits, collected at various dpi, were diluted in blocking buffer added to wells and incubated at 37^0C for 1.0hour. Pre-immunization serum was used as control.

4) The wells were washed with PBS-T for three times. Anti-rabbit HRPO conjugate (1:1000) in blocking buffer was added to the wells and plates were incubated at 37^0C for 1hr.

5) After washing the wells with PBS-T, the substrate solution containing OPD and H_2O_2 in phosphate citrate buffer (pH 4.5) was used to develop the colorimetric reaction. Substrate reaction was stopped with 1M H_2SO_4 and OD_{492} of each well was measured in ELISA reader.

End point titer was taken as the OD of highest dilution of anti-Id sera that yields a differential OD of 3 when compared with OD obtained with the binding of the pre-immunization sera. All reagents used in this assay were 100µl/well and assay was performed in duplicates.

For determining the anti-Id activity in the rabbit sera, a modified indirect ELISA, as described by Marriott *et al.* (1987), was carried out. Same steps as described in indirect ELISA were followed with some modifications.

First paratope (antigen combining site) of the MAb was blocked with 10^3 $TCID_{50}$ virus by incubating MAb with virus at 37^0C. This virus adsorbed MAb was used to coat the ELISA plates. As a control un-adsorbed MAb was used to coat the wells and incubated at 37^0C for 1 hr.

Washing and blocking steps were followed as above and anti-Id sera from both the rabbits collected at different dpi were add in same dilutions to virus adsorbed and un-adsorbed MAb coated wells. Plates were incubated at 37^0C for 1 hr.

Rest of the steps were carried out as described above and the difference in the OD_{492} was observed in both set of reaction as a measure of anti- Id activity.

CHARACTERIZATION OF ANTI-Id

Anti-idiotypes are generally categorized into three types- viz., Anti-Id α, β and γ. To categorize the raised anti-Id into one of these types following assays were performed:

(A) ELISA INHIBITION ASSAY -

1) The wells of ELISA plates were coated with partially purified TCRPV antigen diluted 1:100 in PBS at 4^0c for overnight.

2) After washing with PBS-T for three times, unbound sites were blocked with blocking buffer containing 0.2% healthy goat serum and 0.2% T-20 in PBS, at 37^0c for 1hour.

3) Four fold dilutions of anti-Ids or pre-immunization serum in blocking buffer were incubated with equal volumes of ascites MAb (1:50,000) and allowed to react for 1hr at 37^0c. Control consisted of MAb and PBS-T-HGS pre-incubated with PBS-T-HGS.

4) After washing the wells with PBS-T the above pre incubated mixture was added to the wells of ELISA plates and incubated at 37^0c for 1hour.

5) Bound MAb to the TCRPV antigen was detected with HRPO conjugated goat anti-mouse IgG by incubating it for 1hr at 37^0C and adding substrate as described earlier after washing the plates.

Results were expressed as percentage inhibition (PI) of MAb binding, according to the following formula,

$$PI = \frac{(OD_{492} \text{ of MAb control}) - (OD_{492} \text{ of sample mixture})}{(OD_{492} \text{ of MAb control})} \times 100$$

(B) VIRUS NEUTRALIZATION INHIBITION TEST -

1. Vero cells were grown in 96 well tissue culture plate (Costar) in EMEM containing 5% FCS. After complete monolayer formation following steps were carried out.

2. MAb (1:50,000) was pre-incubated with 35 dpi anti- Id sera at various dilutions, for 1hr at 37^0C. Pre-immunization serum was used in the same dilutions as that of anti- Id sera as a control.

3. These pre-incubated mixtures were added to the 96 well tissue culture containing Vero cell monolayer along with 100 $TCID_{50}$ TCRP virus. Plate was incubated at 37^0C in CO_2 incubator and observed for CPE.

(C) COMPETITION FOR CELLULAR RECEPTOR (Virus Infection Inhibition Assay) -

1. Vero cells were grown in 24 well tissue culture plate (Costar) in EMEM containing 5% FCS.

2. After formation of complete monolayer, media was aspirated out and cells were treated with various dilutions of anti-Id sera (35dpi) for 1.0hr at 4°C. Controls consisted of pre immunization at the same dilutions.

3. Cells were washed with sterile PBS and then treated with TCRP virus at 0.5 m.o.i., at 4°C for 1hr.

4. Again cells were washed with sterile PBS and 1.0 ml of 1.5% carboxy methyl cellulose (CMC) containing 5% FCS was added and incubated in CO_2 incubator at 37°C and observed for plaques.

(D) CELL SURFACE RECEPTOR BINDING STUDY -

Anti-Id was tested for its binding to the cell surface receptor for rinderpest virus, as described by Xue *et al.*, (1991). Vero cells were used as target cells for receptor studies.

1. Vero cells were grown on cover slips in Leighton's tube in EMEM containing 5% FCS.
2. At 70%-80% of cell confluency the media was decanted and cells were washed with sterile PBS. Anti-Id serum or pre-immunization serum diluted as 1:8, 1:16 and 1:32 in PBS-BSA (0.2%) was added to cells and incubated at 4^0 C for 1hr.
3. The cells were again washed with PBS and then fixed with chilled acetone/methanol (1:1) for 10mins at 4^0C.
4. Fixed cover slips were mounted on glass slides with DPX mountant and treated with anti- rabbit FITC conjugate (Genei, India) diluted 1:50 in PBS, for 1hr at 37°C.
5. Cells were thoroughly washed with PBS and dried in air.
6. Cells were observed under fluorescence microscope for presence of any fluorescence.

   Alternatively, cell surface receptor binding was also studied on the paraformaldehyde fixed Vero cells at 37^0C.

AFFINITY PURIFICATION OF ANTI- Ids

A). <u>Purification of MAb specific antibodies</u>

MAb specific antibodies i.e. anti-idiotypes, anti-allotypes and anti-isotypes were purified from rabbit anti-Id serum by passing it through MAb coupled sepharose 4B column.

1. 2.0ml of anti-Id serum (35dpi) was mixed with equal volume of PBS (pH 7.2) and passed over the column. Flowthrough was collected and passed repeatedly for 4 times through the column so that all antibodies against MAb get absorbed.

PURIFICATION OF ANTI-Id ANTIBODIES

Preparation of MAb - sepharose 4B coulmn

↓

Passing Rabbit serum (anti-Id containing) through the column

↑

5 cycles ↓

← ← **Flow- through**

↓

Washing column (10 ml PBS)

↓

Elution of anti-MAb antibodies (anti -Iso, -allo &-idiotypes) **with Glycine-HCl** (pH2.5) **& neutralization by $NaHCO_3$**

↓

Collection of Fractions (1ml each)

↓

Determination of elution profile of antibodies in ELISA

↓

Pooling of important fractions

↓

Passing through CNBr activated NMG-Sepharose 4B column

↑

5 cycles ↓

← ← **Flow through**

↓

Collection of final flow through for anti-Id & its concentration- by reverse osmosis

↓

Testing of purified anti-Id for its applicability as diagnostic reagent & an immunogen

2. The column was washed with PBS thoroughly to remove any unbound proteins.

3. Finally the elution of bound antibodies was carried out with 5.0ml 0.1M glycine- HCl (pH 2.5).

4. Eluted antibodies were collected in fractions of 1ml each. 1M $NaHCO_3$ (pH 8.0) was used to neutralize the acidic pH of eluted antibody.

5. Eluted fractions were tested in ELISA for its binding to MAb and those reacting positive were pooled together.

B). **Separation of Anti-Ids**

Anti-Ids were separated from anti-isotypes and anti-allotypes by passing through NMG coupled sepharose 4B column.

Briefly, MAb specific purified antibodies were repeatedly passed through the column and final flow through was collected as anti- Id fraction while the bound anti-isotypes and the anti-allotypes were eluted with 0.1 M Glycine-HCL as previously described.

IMMUNIZATION OF RABBITS WITH ANTI-Id

Purified Anti- Id was tested for its ability to induce anti-viral immune response in rabbits. Rabbits used for immunization were first tested for presence of anti-rinderpest antibodies by c-ELISA. Those negative for anti-RPV antibodies were selected for immunization.

DAY OF IMMUNIZATION	*DOSE / RABBIT*	*ADJUVANT*	*ROUTE*
0	200µg	FCA	S/C
14	100µg	FIA	I/M
28	100µg	FIA	I/M

Sera were collected seven days after each booster. Two control rabbits were inoculated with normal rabbit IgG (NRG) at same dose rate and days interval. All rabbits were challenged two weeks after last injection by lapinised RPV and observed for temperature reaction and symptoms specific to rinderpest. All rabbits were sacrificed when temperature started falling down and observed for RP specific lesions.

INITIAL TESTING OF ANTI-ID AS A DIAGNOSTIC REAGENT

i) Indirect ELISA and competition- ELISA-

Purified anti- Id was used to coat the ELISA plates, in place of viral antigen in Indirect and competition- ELISA. In c- ELISA competition between MAb D7 26b and different sera samples for the binding to solid phase anti- Id was observed. The test was carried out as described by Singh *et al.*, (1998). In Indirect ELISA test sera samples were added to the anti- Id coated plates and antibody binding to the anti- Id was traced by anti- Bovine conjugate.

ii) Inhibition ELISA-

This assay was carried out as a modification of c-ELISA. In this MAb (fixed dilution) was used to coat the ELISA plates. Purified anti- Id (fixed dilution) was pre-incubated with different bovine sera samples and then added to the MAb coated plates. Binding of anti- Ids to the MAb was traced by anti rabbit conjugate. The OD shown by anti- Id pre- incubated with rinderpest positive was compared with that of negative samples.

****

RESULTS

RESULTS

Characteristics of the selected monoclonal antibody

Monoclonal antibody (D7 26b) was selected from a panel available in the laboratory. Selection was based on its virus neutralization ability and reactivity in ELISA (high titre) with TCRPV antigen.

MAb D7 26b was selected because it has strong virus neutralizing ability and showed a comparable OD in ELISA at higher dilutions when compared to other neutralizing monoclonal antibodies. It is of IgG1 isotype and specific to rinderpest virus. It does not bind to PPRV antigen in ELISA and is unable to neutralize infectivity of PPR virus in cell culture. MAb is directed against H protein of rinderpest virus (Singh *et al.*, 1998).

Production of Ascites

Balb/C mice were used for ascites production. After IFA or pristane priming, $5x10^6$ cells were inoculated by intra peritoneal route per mice. On ninth day after inoculation of cells, mice showed noticeable distention of abdomen. Ascites fluid was collected on alternate days in EDTA as anti-coagulant. Maximum volume of the fluid collected in a mouse was about 3.5 ml.

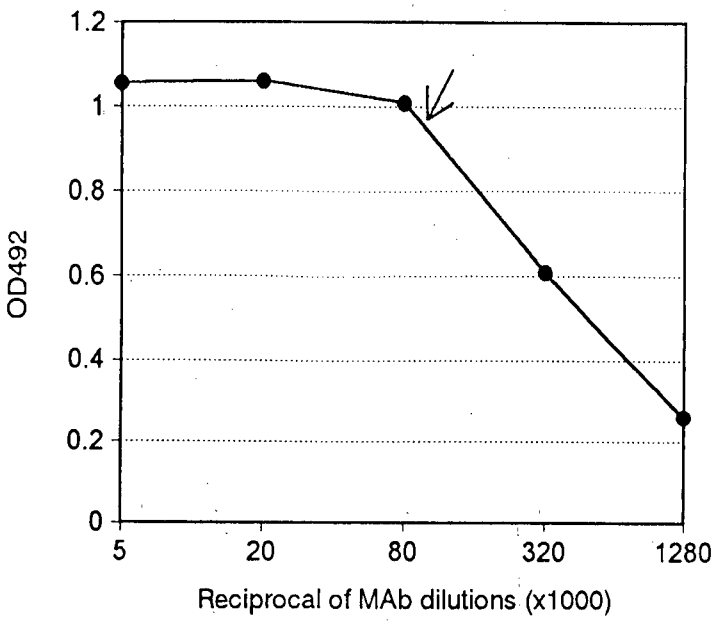
Ascites fluid produced was tested in Indirect ELISA to verify the presence of specific MAb. Antibody titre was taken as the final dilution of MAb on absorbance curve at which OD value started declining steeply. Ascites fluid showed high antibody titre (1:100,000) as compared to the culture supernatant (1:6400) with an OD of approximately 1.0 as shown in Fig.1a and 1b respectively.

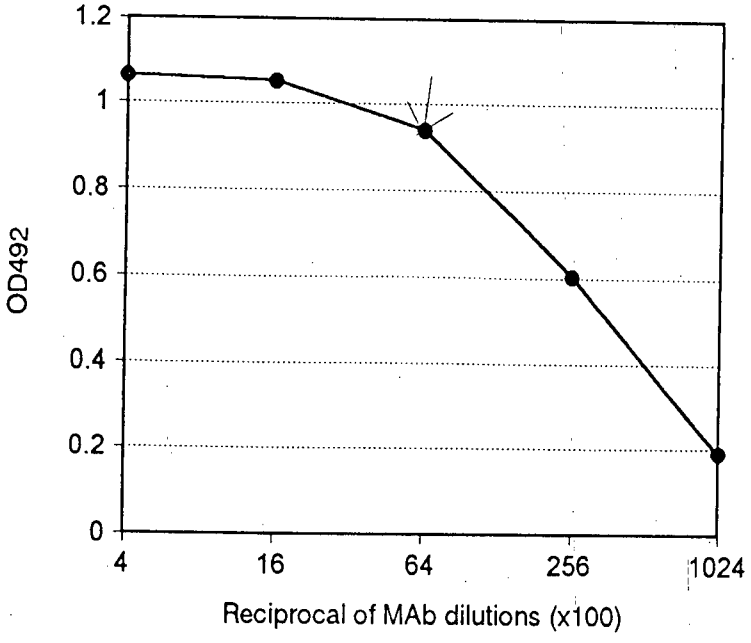
TCRP Virus infected Vero cells (Actone/Methanol fixed)
showing fluorescence after tracing
with MAb D7 26b and then anti mouse FITC conjugate.

Fig.1: Reactivity of MAb D7 26b with TCRPV antigen in Indirect-ELISA

The titre of the MAb was taken as a point (↓) where the OD values started declining steeply.
(a) Reactivity of ascites fluid
(b) Reactivity of culture supernatant

Fig.1a


1.2
1
0.8
0.6
0.4
0.2
0
OD492
5
20
80
320
1280
Reciprocal of MAb dilutions (x1000)


1.2
1
0.8
0.6
0.4
0.2
0
OD492
4
16
64
256
1024
Reciprocal of MAb dilutions (x100)

Virus neutralization titre of ascites fluid was found to be 1:50,000 while that of culture supernatant was 1:3000 using 100-125 $TCID_{50}$ virus per well in micro- SNT.

Purification of MAb from ascites fluid:

One ml of ascites fluid was used for purification of MAb by Protein A sepharose column (Sigma). Purification was carried out as per the manufacturer's instructions. The Protein A- bound MAb was eluted using 5.0 ml of elution buffer. MAb was evaluated in ELISA before and after purification. In the purified fraction, IgG concentration (by Lowry's method) was estimated as 1.5mg/ml i.e. total of 7.5mg in 5 ml-eluted fraction from just one ml of ascites.

Preparation of Fab fragments

Fab fragments prepared by papain digestion were separated from both Fc portion and undigested antibody in a Protein A sepharose column. Flow- through was collected as the Fab fraction (total 12ml). After dialysis against PBS (pH 7.2), it was concentrated by reverse osmosis with the help of PEG 20,000 to 1.5ml. Concentration of Fab in flow-through was 2.0mg/ml, as determined by Lowry's method i.e. total of 3.0mg in 1.5 ml. This shows that, digestion of 6.4 mg of intact MAb yielded 3.0mg of Fab fragments.

Evaluation of Fab fragments

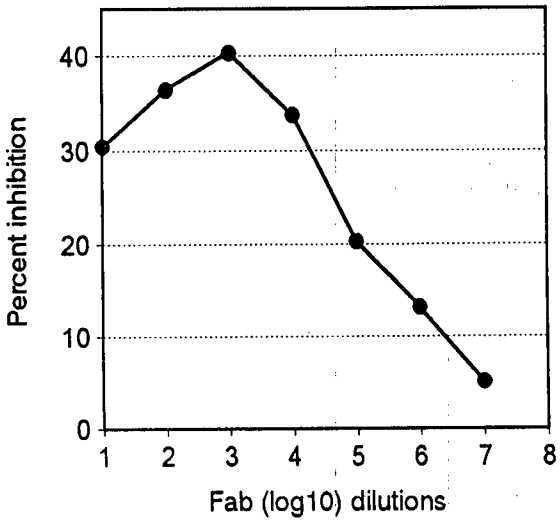
i) Binding of Fab to specific viral epitope by blocking ELISA

Concentrated Fab in log_{10} dilutions was used in a blocking ELISA to block the specific epitope on TCRPV antigen coated on ELISA plates. It showed a concentration dependent inhibition of MAb binding to antigen. About 40% inhibition of colour reaction was seen at higher concentrations of Fab fragment (up to 3 log_{10} dilutions). The binding of MAb increased (less percent inhibition of colour) with the lower concentrations of Fab in a linear fashion (Fig.2).

Fig.2: Percentage Inhibition of colour by Fab fraction in Blocking ELISA

Fab fractions at Log_{10} dilutions were added to the TCRPV antigen coated plates. Unbound sites were blocked with intact MAb (fixed dilution) and then traced with anti- mouse conjugate.

Fig. 2


Percent inhibition
40
30
20
10
0
1
2
3
4
5
6
7
8
Fab (log10) dilutions

ii) Virus neutralization ability

To rule out any structural alteration in antigen binding site of MAb after its digestion by papain, Fab fragments were tested in virus neutralization test. Fab fragments retained the virus neutralizing property of MAb.

Production and characterization of anti-Id in rabbits

I) Determination of immune response and anti-Id activity in rabbit sera:

Anti-idiotype sera raised in rabbits by immunization with Fab fragments were tested in ELISA as described in materials and methods. After first injection, both the rabbits showed antibody response against the original MAb. Antibody response increased strongly within seven days of 2^{nd} and 3^{rd} immunizations. It increased exponentially in rabbit no.3801 with each booster while in the other rabbit there was drop in titre following the 1^{st} booster. In 35 dpi sera inclusive of two boosters, antibody titre reached as high as 1:2560 in rabbit no. 3801(Fig.3).

To determine the anti-Id activity in rabbit sera, a modified Indirect- ELISA was performed in which the MAb (D7 26b) was immuno- adsorbed with TCRP virus prior to addition of anti-Id sera as a tracing antibody. This was compared to that of unabsorbed MAb in the same concentration. Lower OD values were obtained with MAb adsorbed with the virus. This shows that after binding of the virus to the paratope of the monoclonal antibody D7 26 b, the antibodies against the paratope could not bind to it. High OD was obtained in case of virus un-adsorbed MAb as the antibodies directed against the paratopic epitopes (i.e. idiotopes) could bind to MAb in this case. This indicates that antigen and anti-Id can compete for similar binding sites on monoclonal antibody (Fig.4 a & b).

Fig.3: End point titres of anti- Id sera of two rabbits as determined in Indirect-ELISA.

To the MAb coated plates, anti- Id sera were added in different dilutions and binding of the antibodies was traced by anti-rabbit conjugate. End point titre of each serum was taken as the reciprocal of highest dilution of the anti- Id serum, at which it showed three times OD when compared with the pre immunization serum.

Fig.3

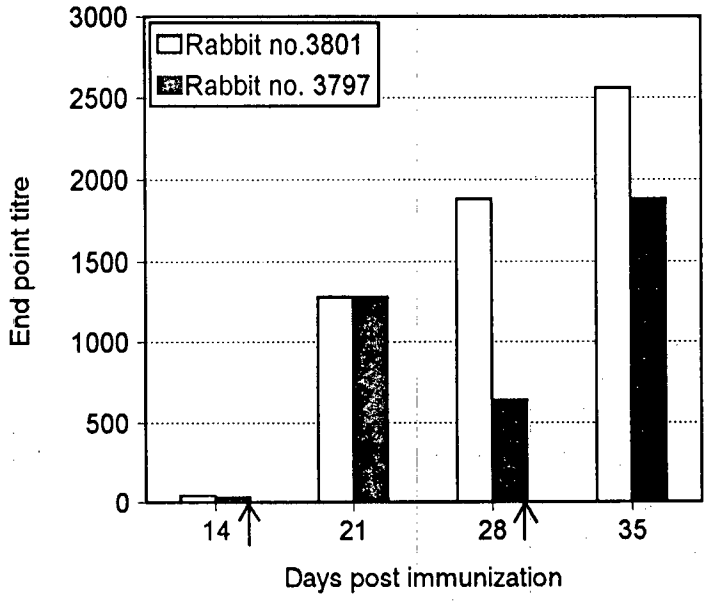

3000
2500
2000
1500
1000
500
0
End point titre
Rabbit no.3801
Rabbit no. 3797
14
21
28
35
Days post immunization

Fig.4: Determination of anti- Id activity in rabbit sera by modified Indirect-ELISA

Anti- Id sera (fixed dilution) were added to the virus-adsorbed and un-adsorbed MAb coated plates. Bound antibodies were traced by anti-rabbit conjugate. Difference in OD shows the presence of anti- Idiotype antibodies in the rabbit sera.

(a) Anti- Id activity in the sera of Rabbit no. 3801
(b) Anti- Id activity in the sera of Rabbit no. 3797

Fig. 4a

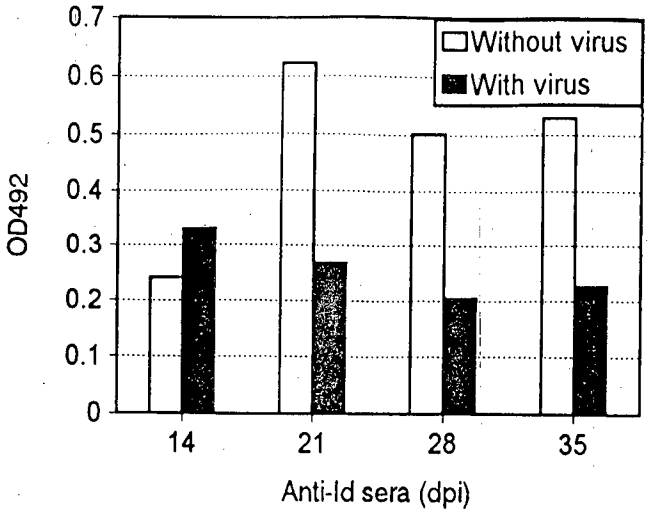
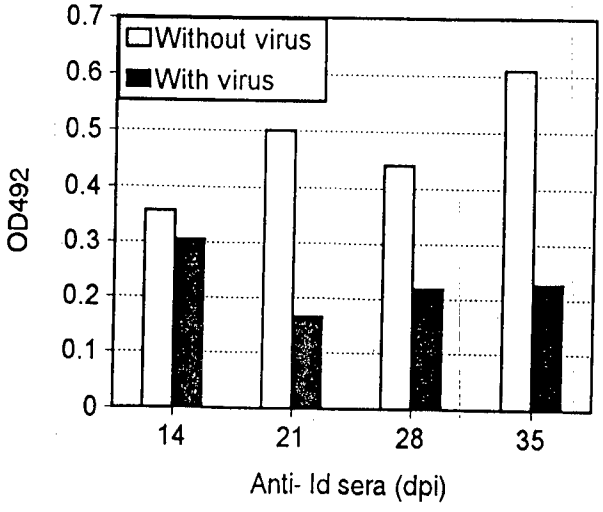

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD492
Without virus
With virus
14
21
28
35
Anti-Id sera (dpi)

Fig. 4b


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD492
Without virus
With virus
14
21
28
35
Anti- Id sera (dpi)

II) Characterization of Anti-Id:

i) ELISA inhibition assay:

This assay was carried out to determine if anti-Ids could inhibit the binding of MAb to the antigen. Anti-Id serum inhibited the binding of MAb to TCRPV antigen in a concentration dependent manner. At higher concentrations of anti- Id, almost total (>95%) inhibition was observed in binding abilities of the MAb to the antigen, which subsequently decreased with the higher dilution of the anti-Id serum.

Percentage inhibition of binding of the MAb to the antigen was directly proportional to the period following immunization. Both the rabbit sera at 14 dpi did not show more than 40% inhibition when diluted 1:8, while longer dpi sera showed >95% inhibition at the same dilution (Fig.5-a & b).

This percent inhibition shown by individual serum was in relation to their respective titres (Fig.3). The 28^{th} dpi sera of rabbit no. 3797 had somewhat less ELISA titre than 21 dpi (Fig.3). The same pattern reflected in the percentage inhibition of MAb binding also.

ii) Virus neutralization inhibition assay:

Virus neutralization inhibition assay was performed by adding, pre-incubated mixtures of MAb (fixed dilution) and anti- Id serum (varying dilutions) to the 96 well tissue culture plate along with 100 $TCID_{50}$ virus. Anti- Id sera inhibited the virus neutralizing activity of the MAb in concentration dependent manner. As shown in Table no.1, inhibition was seen in all the wells up to 1:128 dilution of the anti- Id serum, which decreased with the increase in anti- Id serum dilution.

Fig.5: Inhibition of binding of MAb to the solid phase TCRPV antigen by rabbit anti-Ids, in ELISA Inhibition assay.

Mixtures of MAb (1:50,000) with varying dilutions of anti-Id sera were prepared and pre-incubated for 1hr at 37^{0}C. These mixtures were then added to the wells of ELISA plates coated with TCRPV antigen.

a). Anti- Id sera of Rabbit no. 3801 showing percent inhibition of MAb binding.
b). Anti-- Id sera of Rabbit no. 3797 showing percent inhibition of MAb binding.

Fig.5a

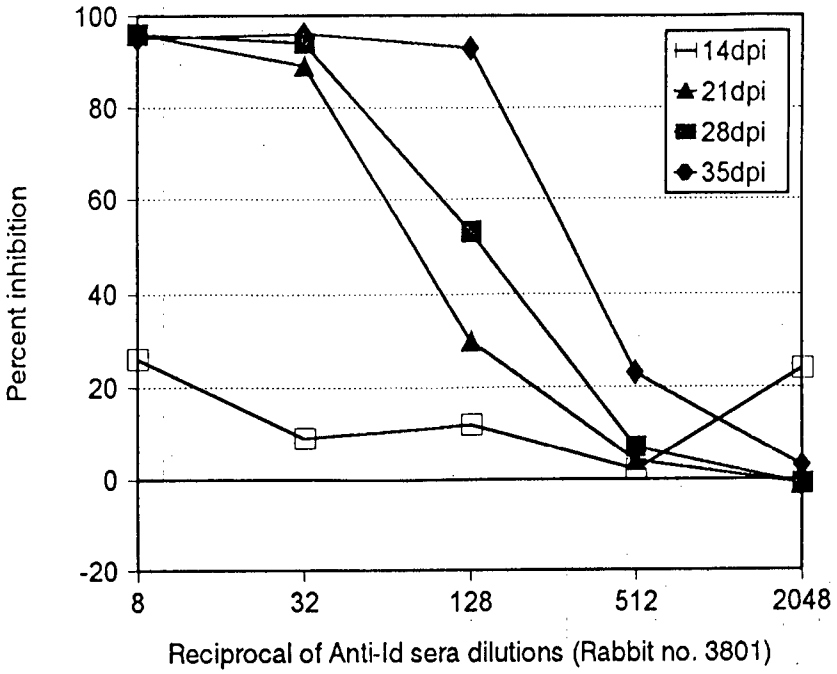

100
80
60
40
20
0
-20
Percent inhibition
14dpi
21dpi
28dpi
35dpi
8
32
128
512
2048
Reciprocal of Anti-Id sera dilutions (Rabbit no. 3801)

Fig.5b

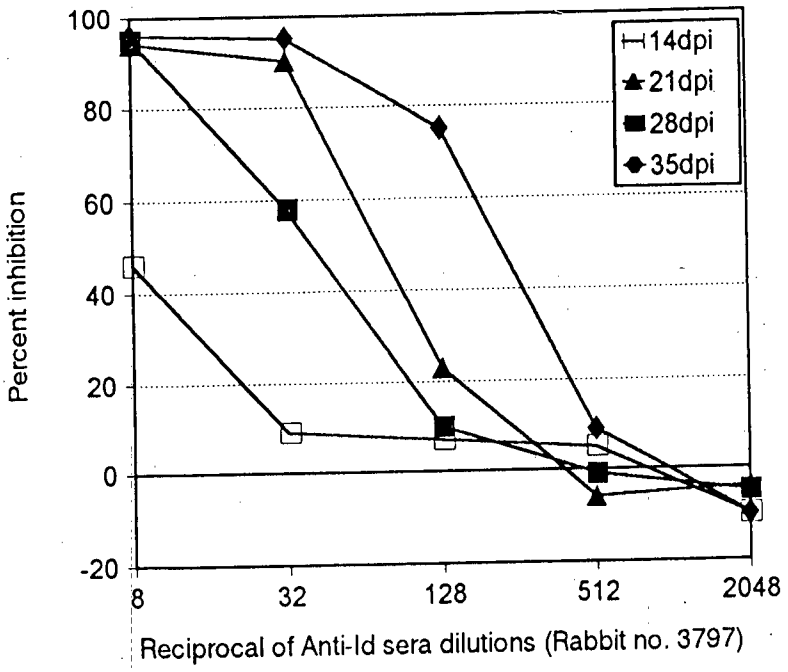

100
80
60
40
20
0
-20
Percent inhibition
14dpi
21dpi
28dpi
35dpi
8
32
128
512
2048
Reciprocal of Anti-Id sera dilutions (Rabbit no. 3797)

Table no. 1

Virus Neutralization Inhibition by anti-Id

Anti- Id dilutions	Pattern of CPE
1:8	CPE in very small plaques
1:16 to 1:128	CPE in all wells
1:256	CPE in more than 50% wells
1:512	CPE in less than 50% wells
1:1024 onwards	No CPE

Note:

- Five wells were used for each dilution of anti-Id.
- Pre-immunization serum could not inhibit virus-neutralizing activity of the MAb.

iii) Cellular receptor binding study:

An attempt was made to find out if the anti- Id can mimic the TCRP virus in binding to specific receptor in Vero cells. For this Vero cells adsorbed with anti- Id sera at 4^0C for 1 hour prior to tracing these with anti-rabbit FITC conjugate following fixation with acetone/methanol. No fluorescence could be seen in anti- Id adsorbed cells. As an alternative method, pre-fixation of cells with paraformaldehyde also did not support binding of anti- Id to Vero cell receptor.

iv) *In- vitro* Virus infection inhibition study:

Virus infection inhibition study was carried out to determine whether anti-Id inhibits infection of Vero cells with TCRP virus. For this Vero cell monolayers in 24-well tissue culture plate were overlaid with appropriate dilution of anti- Id rabbit serum. The plate was incubated at 4^0C for 1hr. Cells were subsequently adsorbed with 0.5 m.o.i. of TCRP virus for 1 hr and after washing with sterile PBS, overlaid with 1ml of 1.5% CMC containing 5% FCS. Cells were observed for plaque formation daily up to eight days. No difference was seen in all, anti-Id as well as pre-.immunization serum treated wells. Anti-Id sera could not inhibit virus infection even at high antibody concentrations. This result is in accordance with the result obtained in cell surface receptor binding study.

Purification of Anti-Id:

Anti-Idiotype antibodies were purified from serum by passing sequentially through MAb coupled sepharose 4B column and then through NMG-coupled sepharose 4B column.

MAb- sepharose 4B column purified the anti-idiotype, anti-isotype and anti-allotype antibodies from rabbit serum, while NMG-sepharose 4B column absorbed anti-isotype and anti-allotype antibodies from above purified fraction letting anti-idiotype antibodies pass in flow through.

In both the cases, sample was loaded in PBS (pH 7.2) while the bound antibody was eluted at acidic pH using glycine- HCl (pH 2.5).

The eluted anti-idiotype, anti-isotype and anti-allotype antibodies bound to MAb -sepharose 4B column were collected in separate tubes as 1.0 ml fractions and these were tested in ELISA. The fractions 3 to 6 showed highest antibody concentration with a peak at fraction 4 (Fig.6). These fractions were pooled for further purification over NMG-sepharose 4B column.

NMG-sepharose 4B column purified anti-Id was then dialyzed against PBS and used either in ELISA–for testing its applicability as a diagnostic reagent or for immunization, after its concentration by PEG 20,000.

From 2.0 ml rabbit serum about 300µg of affinity purified anti-idiotype antibodies were obtained.

Initial testing of anti-Id as a diagnostic reagent and an immunogen:

1) As Diagnostic reagent-

Purified anti- Id was used as an antigen to detect the antibodies to rinderpest in cattle sera in either Indirect or competition-ELISA. But the results were not encouraging in both the assays. An inhibition ELISA was carried out as a modification of c-ELISA. In this fixed amount of purified anti-Id was pre-incubated with various sera constituting rinderpest positive and negative samples. These pre-incubated mixtures were added to the MAb coated plates. Binding of the purified anti- Id to the MAb was traced with anti rabbit conjugate. As shown in Fig.7, anti- Id binding to the solid phase MAb was inhibited by rinderpest positive sera. This inhibition was more with hyperimmune serum as compared to the other rinderpest positive sera.

2) As an Immunogen:

For testing its applicability as an immunogen, purified anti-Id was inoculated into two rabbits. After three biweekly immunizations, it could not induce any detectable humoral immune response. After 15 days of the last inoculation animals were challenged by Lapinised RP virus and observed for thermal reaction with any clinical signs. Control rabbit, which were inoculated, with normal rabbit IgG showed high temperature reaction

Fig.6: Elution profile of anti- MAb antibodies from MAb-coupled sepharose 4B:

Anti- MAb antibodies eluted from the MAb-coupled Spharose 4B column were collected in fractions of 1.0 ml each and tested in ELISA. Eluted fractions (1:40 dilution) were added to MAb-coated plates and traced with anti-rabbit conjugate. Fractions 3-6 show high concentrations of eluted antibodies.

Fig.6

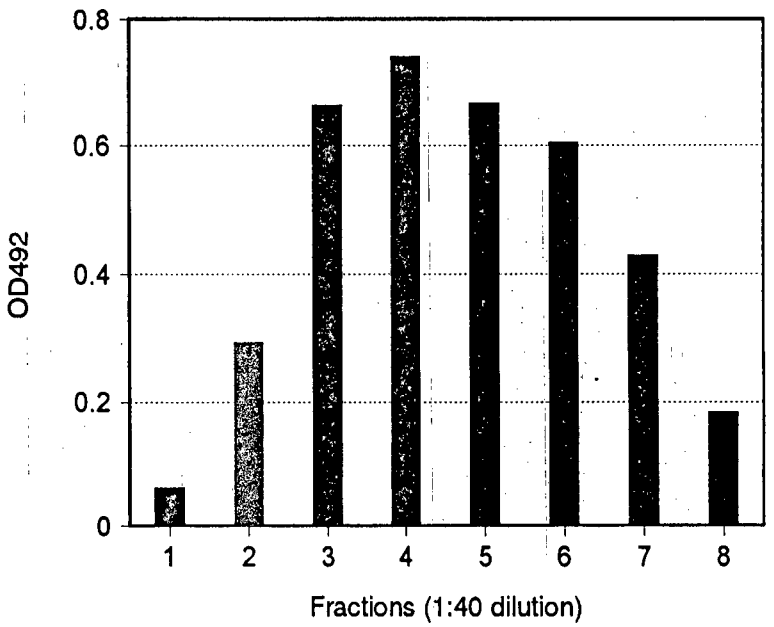


Fig.7: Inhibition of purified anti- Id binding to the solid phase MAb by heterologous (bovine) sera:

To the solid phase MAb, purified anti- Id antibodies pre-incubated with heterologous (bovine) sera was added. Binding of the purified anti- Id to the MAb was traced by anti-rabbit conjugate. Figure shows the inhibition of anti- Id binding to the MAb, when pre-incubated with rinderpest positive sera.

Fig.7

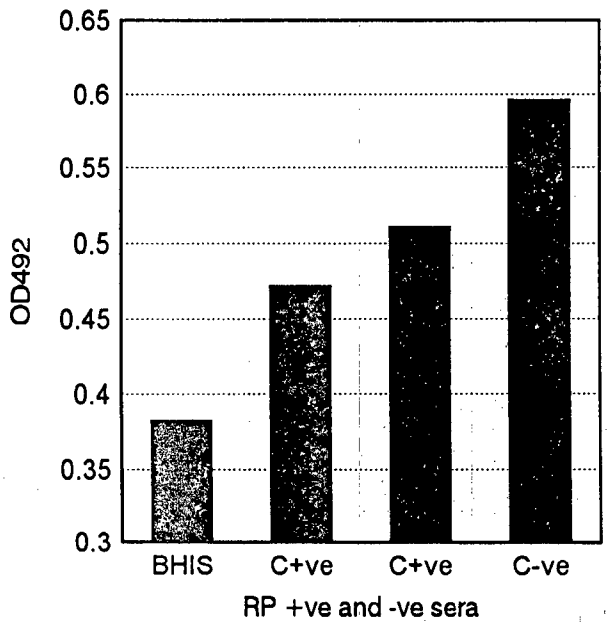
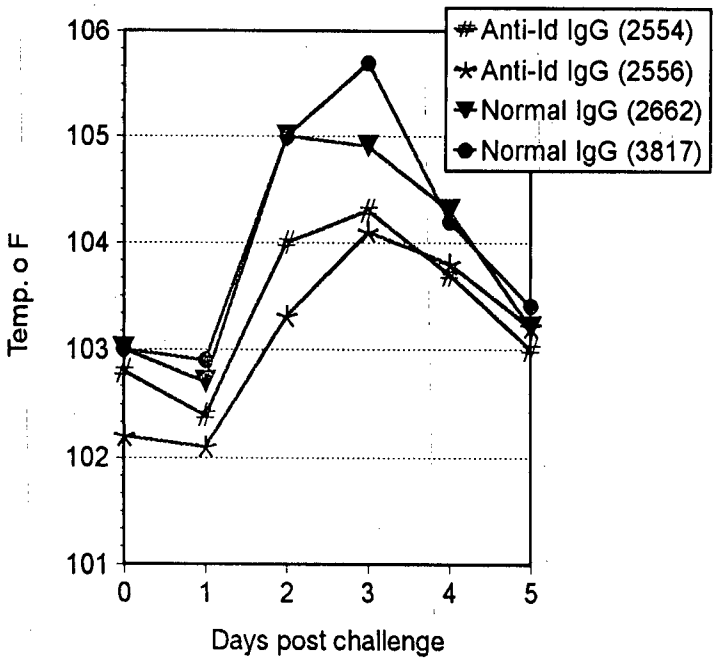

0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
OD492
BHIS
C+ve
C+ve
C-ve
RP +ve and -ve sera

Fig.8: Thermal reaction of four rabbits after the challenge with Lapinised rinderpest virus.

The figure shows that rabbits, immunized with anti- Id antibodies had lower temperature reaction than the controls immunized with normal rabbit serum IgG.

Fig.8


Anti-Id IgG (2554)
Anti-Id IgG (2556)
Normal IgG (2662)
Normal IgG (3817)
106
105
104
103
102
101
Temp. o F
0
1
2
3
4
5
Days post challenge

as compared to anti-Id immunized rabbits (Fig.8). On 5th day after challenge these rabbits were observed for RP specific lesions in saculus rotundus, small and large intestine. Normal rabbit IgG inoculated rabbits showed more severe lesions than anti-Id inoculated rabbits.

DISCUSSION

DISCUSSION

Rinderpest, which plagued the animal population all over the world for centuries is under control as a result of coordinated efforts, made through various eradication campaigns. Now the disease is present only in the form of residual foci in Asia and Africa. Classical rinderpest, one of the oldest and most devastating malady of cattle and buffaloes, bears a potential to cause almost more than 90% mortality in a susceptible population. Although cattle and buffaloes are the most affected species, the disease also occurs in small ruminants, pigs, camel and many wild animals belonging to the Order- Artiodactyla. Although one of the dreaded animal diseases, rinderpest can be easily prevented by the vaccination of susceptible animals and unequivocally is eradicable by mass vaccination. The disease was endemic in India since centuries. The country has recently been declared provisionally free from it (Sinha, 1998). The maintenance of disease free status is more difficult than mere eradication, as it requires a very strong surveillance system to 'chalk- out' any type of incursion of the disease.

Unfortunately, despite of launching various eradication campaigns, the disease is still lurking in some parts of Asia and Africa. These residual foci are a constant threat to the success of eradication programme. One of such foci is our neighboring country- Pakistan, which is of great concern for us in view of transboundary rinderpest incursions.

Emergence of mild strains of rinderpest, because of mass vaccination, has made the diagnosis of disease extremely difficult. The condition has become worse in small ruminants, in which rinderpest and PPR both produce similar clinical symptoms and lesions.

In this critical situation, efficient and widespread surveillance of the disease is of prime importance for the country. Presently, the competition- ELISA (antibody detection) and immuno-capture ELISA (antigen detection) are in vogue for the specific diagnosis of rinderpest and PPRV. These tests use live rinderpest virus, albeit vaccine virus, as one of the reagents in them. Continuous propagation of the virus is obligatory for the large- scale production of these reagents, which involves a considerable risk of escaping the live virus into the environment. With the cessation of vaccination and the resultant increase in the susceptible bovine population, escape of live virus into the environment may sometimes prove dangerous. With the imposition of restrictions on handling of virus, replacement of virus from diagnostics by some non-infectious reagents is the need of the hour.

Keeping these points in view, the present study was undertaken to produce and characterize anti- idiotype antibodies and to explore the possibility of using these as an alternative reagent to viral antigen in diagnostics.

For the production of anti-idiotype antibodies, the idiotype antibody was selected from a panel of anti-RPV monoclonal antibodies available in the Rinderpest Laboratory, IVRI campus, Mukteswar.

The monoclonal antibody was selected on the basis of following criteria-

i) Virus neutralizing ability

ii) OD in ELISA (at various dilutions)

The neutralizing MAb was selected as an idiotype (Id) antibody because of following reasons-

i) In case of rinderpest virus most of the neutralizing MAbs are directed against H protein of the virus. As the epitopes on the H protein of Morbilliviruses are type specific (McCollough, 1986), there are always chances of getting virus specific MAb- if a neutralizing clone is selected. The objective of selecting virus specific MAb (Id) was, to mimic the virus specific epitope with anti- Ids so that such anti-Ids can be employed as diagnostic reagent for specific detection of virus induced antibodies.

ii) Use of neutralizing monoclonal antibody for anti- Idiotype production helps in better characterization of anti- Ids. Anti-idiotype antibodies produced against the neutralizing MAb, if mimic the viral attachment site, can be employed as receptor probes.

iii) Anti-idiotype antibodies produced against the neutralizing MAb, if bear the internal image of antigen, can be employed as an effective immunogen, because of mimicry of the neutralizing epitope.

High affinity MAb shows the high OD in ELISA at higher dilutions and this was taken as the second criteria for selection of MAb.

Monoclonal antibody D7 26b was selected from the panel of anti- rinderpest virus monoclonal antibodies after comparing with other neutralizing MAbs. Culture supernatant of this MAb showed high neutralization titre (1:3000), which was much greater than other neutralizing MAbs and also showed comparable OD in relation to other neutralizing MAbs at high dilutions in Indirect- ELISA. This MAb D7 26b was of IgG1 isotype and against the H protein of RPV (Singh *et al.*, 1998).

After selection of the monoclonal antibody, the clone secreting MAb (D7 26b) was revived and propagated in media with 20% FCS. Balb/C mice were used for ascites production. For the purpose of priming of mice, either FIA or pristane (2,6,10,14 tera-methyl pentadecan) was used. FIA was found comparatively better than pristane for production of ascites as the inoculation of cells could be planned 3-4 days after priming. Pristane requires comparatively longer sensitization period without any obvious advantage.

Ascites is a rich source of monoclonal antibody and the antibody concentration usually ranges from 1.0 -20 mg/ml (Baumgarten & Peters, 1992). In this study also, ascites showed a very high ELISA titre of about 1:1,00,000 and virus neutralization titre of 1:50,000. After purification by Protein A- sepharose column, 7.5mg of MAb were obtained from1.0ml of ascites when tested by Lowry's method (Lowry *et al.*, 1951).

Each antibody contains three types of antigenic determinants on it, viz; isotypes, allotypes and idiotypes. Both isotypes and allotypes are located on the constant region of antibody molecules while idiotypes are located on the variable region. As the isotypes and allotypes are dominant epitopes on antibody molecules, anti-isotype and anti-allotype antibody response dominates over the anti-idiotypes when such antibody is injected into different animal species. Therefore, for anti-idiotype antibody production, if one removes the Fc region of the antibody molecule, most of the anti-isotype and anti-allotype response can be avoided. Orten *et al.* (1988) used $F(ab')_2$ fragments of anti-BHV1 MAb to produce anti-Ids for BHV1 while Marriott *et al.* (1987) used Fab fragments of MAb for raising anti-Ids to polyoma virus.

In the present investigation, Fab fragments were produced by papain digestion of the chosen MAb (D7 26b). Fab fragments were purified by Protein A chromatography. As the Protein A is having high affinity for the Fc region of IgG, it absorbed all undigested MAb and Fc fragments while Fab fragments that appeared in flow through were collected and concentrated by reverse osmosis using polyethylene glycol 20,000. These affinity purified Fab fragments were tested for their reactivity to TCRP virus antigen (in Blocking ELISA) and also for neutralizing activity in virus neutralization test. The Fab fragments retained their binding to native viral epitope and virus neutralizing activity, indicating that there was no structural change in Fab portion.

Blocking ELISA also confirmed the digestion of MAb (D7 26b). In blocking ELISA, Fab fragments at higher concentrations, inhibited MAb binding to viral epitope and showed less OD_{492} (Fig.2) while at increasing dilutions of Fab (less Fab), intact MAb binding increased. Anti species antibody conjugated with enzyme could bind more effectively to Fc portion of MAb, which was lacking in Fab fraction. Therefore, a higher OD value was obtained in the wells with low Fab and the MAb could get the opportunity to bind with viral epitope.

For generation of anti- idiotype antibodies, three biweekly immunizations of Fab fragments were given in two rabbits. The quantity of Fab and time interval between two

immunizations followed in this study was similar to that of used by other workers for production of xenogenic polyclonal anti-Ids in rabbits (Orten *et al.*, 1988; Xue *et al.*, 1991; Dandapat *et al.*, 1994). Increasing titre of anti- MAb antibodies was observed with each booster. This could probably be due to an anamnestic response in the presence of the memory cells.

To detect the presence of anti-idiotype activity in rabbit sera, a modified indirect ELISA was performed as described by Marriott *et al.*, (1987). After blocking the antigen-combining site of MAb with TCRPV, most of the antibodies in the rabbit anti-Id sera could not bind to solid phase MAb. This indicates that the antibodies directed against the idiotypes present inside or near the antigen- combining site could not bind to the MAb after blocking the paratope with homologous virus. This confirmed the presence of anti-Ids in sera. This anti-idiotype activity was very less in 14 dpi sera in case of both the rabbits, which increased with the boosters given afterwards (Fig.4a&b).

Anti-Ids are classified into three types Ab2 α, Ab2β & Ab2γ. Ab2β & Ab2γ are directed against the idiotypes within the paratope while Ab2α is directed against the idiotypes present on the framework regions of MAb (Bona and Kohler, 1984). In order to demonstrate that, the anti-Ids would recognize and bind the antigen-combining site of MAb, an ELISA- inhibition assay was carried out. The MAb binding to antigen is inhibited in the presence of anti- Ids in a concentration dependent manner. This suggests that anti-Ids bind to the paratope of the MAb thereby inhibiting its binding to the antigen. This also confirms the result of the modified indirect ELISA. Similar findings have been reported with anti- Ids for BHV-1 (Srikumaran *et al.*, 1990), BVD (Xue *et al.*, 1991), Cytomegalovirus (Keay *et al.*, 1988) and Herpes simplex (Lathey *et al.*, 1986).

In Virus Neutralization Inhibition Assay, anti-Id sera inhibited the virus neutralizing activity of the MAb. The MAb (fixed dilution), that was pre incubated with the anti- Id sera (at higher concentrations) could not neutralize the virus infection in cell culture. The neutralization inhibition was observed in concentration dependent manner of anti-Ids. Pre immunization serum could not alter the neutralization ability of MAb. The result of

neutralization inhibition test also proves the specificity of anti-Ids against the paratope of MAb.

From the above assays, it became clear that the major population of anti-Id in the sera is of either Ab2β or Ab2γ or both in mixed population. A number of criteria are accepted to differentiate between internal image (Ab2β) and non-internal image (Ab2γ) anti-Ids (Kennedy *et al.*, 1986; Jerne, 1982). These criteria are as follows-

i) Internal image anti-Id antibody can compete with original antigen for binding to the Id (as shown in ELISA inhibition assay or virus neutralization assay).
ii) Internal image anti-Id can compete with antigen in binding to specific antibodies produced in species other than the species from which the anti-Id antibody was derived.
iii) Internal image anti-Id may have identical three-dimensional structure, i.e. amino acid sequence homology, with the original antigen. As shown by Bruck *et al.* (1986) in the reovirus system, the internal image anti-Id MAb directed to a syngenic MAb with specificity for the reovirus hemagglutinin, had an identical tetra-peptide shared with the neutralizing epitope of the hemagglutinin. This anti-Id MAb bound to the cellular receptor site of the reovirus on reovirus susceptible cells and could replace the reovirus antigen.
iv) Internal image anti-Id may mimic the original antigen resulting in the induction of an idiotype immune response.
v) Internal image anti-Id might mimic the physiological properties of the antigen (e.g. receptor binding).

In the present study, although raised anti-Ids fulfilled the first criteria, this does not rule out the possibility of the presence of non-internal image anti-Ids. Therefore for further characterization of anti-Ids, some more assays were performed.

Rinderpest virus H protein is responsible for the attachment of the virus to cell surface receptor (Barrett *et al.*, 1991). The idiotype MAb used in the present study was against the H protein of rinderpest virus. As it is virus-neutralizing MAb, it may be against

the viral ligand that binds to its cell surface receptor. Considering this, the raised anti-Ids were tested in virus infection inhibition study and cell surface receptor binding. In virus infection inhibition assay, anti- Ids were incubated with Vero cells at 4^0C before infecting the cells with TCRPV. The assay revealed no differences in TCRPV infectivity in Vero cells pre-incubated with anti- Id sera or pre-immunization sera.

Grieder & Schultz (1990) also found that monoclonal anti-Id could not inhibit the Blue Tongue Virus infection in MDBK cells pretreated with anti- Id before virus adsorption. The reason for the inability of anti-Id to inhibit the virus infection was considered as the use of multiple receptors by BTV. Xue *et al.* (1990) observed only 25% inhibition of BHV1 infectivity in MDBK cells by polyclonal anti- Ids. This inhibition was not increased in spite of using a high concentration of anti-Id. The possible reason for this was attributed to the involvement of multiple receptors by the virus or low binding affinity of anti-Ids than that of the virus to the cell receptor.

The possibility of utilizing multiple cellular receptors by rinderpest virus can not be ruled out as the measles virus too uses both CD46 and moesin as its cellular receptor (Naniche *et al.*, 1993; Dunster *et al.*, 1994). Also, haemagglutinin protein of RPV may contain other epitopes responsible for attachment of the virus to the cellular receptor.

With the inability of anti- Ids to inhibit virus infection, its binding to the cell surface receptor was studied by indirect immuno-fluorescence test. This was carried out on live and fixed Vero cells at 4^0C and 37^0C, respectively and binding of anti-Id was detected by anti-rabbit FITC conjugate. But in both the cases no specific fluorescence was detected, possibly due to inability of anti- Ids bind cellular receptors. From the results of the virus infection inhibition assay and cell surface receptor binding study, it was evident that there must be a major population Ab2γ in the rabbit sera. There are instances where, it was found that the internal image anti-Id (Ab2β) represents a minor fraction of a polyclonal anti-idiotypic response which was found to be difficult to characterize (Hiernaux, 1988).

The other criteria of internal image anti-Id viz; i) its binding to inter-species cross reactive idiotopes and ii) potential to induce immune response against the original antigen, were also tested in the present investigation.

For this, anti-Id antibodies in serum were purified over MAb coupled sepharose 4B and NMG-coupled sepharose 4B columns. MAb coupled sepharose 4B column purified total anti-MAb antibodies while NMG coupled sepharose 4B absorbed anti isotypes and anti allotypes. The anti-idiotype antibodies were collected in "flow through" and concentrated by reverse osmosis in the presence of PEG-20,000. From 2.0 ml of rabbit serum, about 300µg of anti-Id antibodies were obtained. This yield of anti- Ids was within the range observed by Kennedy & Dressman (1983), who reported ~32-280µg of purified anti-Ids per ml of antiserum. These workers also found acidic elution of antibodies to be more efficient than elution with high pH. They used 1.0-M acetic acid, while in the present study 0.1-M glycine-HCl was used for elution, without any apparent problem.

Purified anti-Ids were used for immunization of rabbits and to test their suitability as a diagnostic reagent in ELISA (ability to bind the inter-species cross-reactive idiotopes).

In indirect- ELISA and c-ELISA, these anti-Ids were used to coat the wells of ELISA plate in place of viral antigen and tested for its ability to detect anti rinderpest virus antibodies from sera-samples of animals with known immune status. In both the assays, purified anti-Ids could not reproduce the results obtained with viral antigen. This shows that, although anti-Ids binds to the antigen combining site of its idiotype MAb and inhibit the antigen binding, it is not the internal image of the antigen. Thus, it was evident that the major population of anti-Ids was of Ab2γ.

We further tested anti-Ids in an inhibition ELISA for its binding to heterologous (bovine) sera. In this experiment instead of coating the anti-Ids to solid phase, these were pre incubated with different bovine sera samples comprising rinderpest positive and negative samples. These pre-incubated mixtures were then added to the MAb coated ELISA plates. Binding of the anti-Ids to the solid phase MAb was traced by anti-rabbit HRPO conjugate.

Anti-Id binding to the solid phase MAb was inhibited when it was pre-incubated with rinderpest positive sera and this inhibition was significant with rinderpest hyperimmune serum (strong positive). When compared to OD obtained with RP negative serum, this inhibition with HIS was about 30%. As observed by Rimmelzwaan *et al.* (1991) in case of Anti-Id γ and Zhou *et al.* (1990) in case of Ab2α, inhibition by heterologous sera was not more than 5%. In the present study this inhibition was about 30%, which shows the presence of small population of either internal image anti-Id or anti-Ids directed against shared epitopes. The inhibition with other rinderpest positive sera (weak positive) was less than the rinderpest hyperimmune serum. The reason for this may be the less concentration of MAb like Ab1 (against the specific viral epitope) in these sera as compared to the hyperimmune serum.

Purified anti-Ids were also used for immunization of rabbits. After three biweekly immunizations, no detectable anti-virus antibody response was observed. These rabbits were challenged with Lapinised rinderpest virus after 15 days of last immunization along with control rabbits, which received normal rabbit IgG instead of anti-Id. Control rabbits showed high temperature reaction when compared to anti-Id immunized rabbits. More severe lesions were observed in control rabbits than in anti-Id inoculated rabbits.

There are several reports of induction of anti-viral antibody response in syngenic system by anti-Ids (Gaulton *et al.*, 1986; Ertt & Finberg, 1984; Zhou *et al.*, 1990; Tanaka *et al.*, 1986). Some of these workers used more than three inoculations of anti-Ids coupled with carrier protein. The dose of anti-Id, adjuvant, route of inoculation and time interval between two immunizations are reported to have great impact on the induction of anti-virus immune response by anti-Ids (Gaulton *et al.*, 1986). The lower temperature reaction and less severe lesions that was observed in anti-Id immunized rabbits following challenge indicate some degree of anti-viral response by anti-Ids, which may be due to induction of cellular immunity as no anti- viral neutralizing antibodies could be detected in post immunization sera.

In conclusion, polyclonal anti- idiotype antibodies were produced in a xenogenic system by inoculating Fab fragments of a murine monoclonal antibody. Anti- Id sera showed high titre of anti- MAb antibodies after two boosters. Anti- idiotype antibodies when pre-incubated with MAb, inhibited MAb binding to the homologous antigen. Anti-Ids also inhibited the virus neutralizing activity of MAb. This shows that anti- idiotype antibodies specifically reacted to the paratopic idiotopes of the monoclonal antibody. These anti- Ids could not inhibit the TCRPV infection in the Vero cells and also did not show any specific fluorescence when observed for cell surface receptor binding. Purified anti- Ids could bind with the anti- rinderpest antibodies from heterologous species, showing the presence of either Ab2β or of anti- Ids against the shared idiotopes. Anti- Id immunized rabbits did not show any detectable anti- virus antibodies but showed low temperature reaction and less severe lesions than control rabbits, after challenge. With these characteristics, it was clear that Ab2γ and Ab2β both constituted the anti –Id sera, but it was the Ab2γ, which dominated as a major anti- Id type.

As polyclonal anti-Ids contain a heterogenous population of antibodies directed against many different idiotopes, its direct application in diagnostics is difficult. Therefore, many workers have used internal image monoclonal anti-Ids as diagnostic reagents in place of anti-Ids. Before embarking on the production of monoclonal anti-Ids, it would be of worthwhile to determine whether the selected idiotype MAb is able to produce internal image anti-Ids. In the present study, MAb D7 26b is found to generate internal image anti-Ids and this can be used for monoclonal anti-Id production. Internal image monoclonal anti-Id, thus generated may prove a strong candidate to replace the virus from diagnostics.

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SUMMARY

SUMMARY

The Idiotype antibody (MAb), for the production of anti-idiotype antibodies, was selected from a panel of anti-rinderpest virus mouse monoclonal antibodies available in the Laboratory. Selection of MAb was done on the basis of virus neutralization ability and OD in ELISA. The selected clone (MAb D72 6b) was revived in cell culture flasks and expanded. The monoclonal antibody (culture supernatant) showed high ELISA titre (1:6400) and neutralization titre (1:3000). The cells were used for the production of ascites in Balb/C mice. IFA or pristane was used for priming of mice. A total number of $5x10^6$ cells per mouse were used for inoculation. Ascites fluid was collected and tested for the presence of MAb. Ascites fluid showed ELISA titre of 1:1,00,000 while neutralization titre of 1:50,000. Monoclonal antibody was purified from ascites fluid using Protgein A- sepharose column. Purified MAb was then digested by papain for the preparation of Fab the fragments. Papain digested MAb was passed through Protein A- sepharose column and the flow- through containing the Fab fragments was collected and concentrated by reverse osmosis with PEG 20,000. Fab fragments were tested for their binding to TCRPV antigen in ELISA and also for neutralizing activity in Virus Neutralization Test. Fab fragments retained their activity to bind the antigen and also could, neutralize the virus infectivity in Vero cells.

Fab fragments were used for immunization of rabbits for the production of anti-idiotype antibodies. Three bi-weekly immunizations were given into two rabbits. Sera were collected at regular interval and assessed for immune response against MAb. High anti-MAb antibody response (>1500 in both the rabbits) was observed after two boosters. These sera were tested for the presence of anti-idiotype activity in a modified Indirect- ELISA. Anti-idiotype activity increased with booster given intermittently.

Anti-idiotype antibodies were characterized by various assays. In ELISA inhibition assay, anti-Id sera pre-incubated with the MAb (fixed dilution), gave inhibition of MAb binding to TCRPV antigen. This inhibition was observed in a concentration dependent manner of anti- Ids. At higher concentrations, anti-Ids inhibited the binding of MAb to the antigen almost totally (> 95%). In Neutralization inhibition assay also, anti-Id sera inhibited the neutralizing activity of MAb. Both these assays clearly indicated that anti-Ids occupies the antigen-combining site of MAb and thereby inhibit their interactions with antigen.

Further, "Virus infection inhibition assay" and "Cell surface receptor binding assay" were carried out to distinguish internal image (Ab2β) and non-internal image (Ab2γ) anti-Ids. In virus infection inhibition assay, pre- treatment of vero cells with anti-Ids at 4^0C did not have any effect on infectivity of Vero cells with TCRP virus. In "Cell surface receptor binding" study also, receptor-binding anti-Ids could not be detected in indirect immunofluorescent tests, carried out on live as well as fixed Vero cells.

Anti-idiotype antibodies were purified from anti-Id serum by passing sequentially through immuno-affinity columns. For this, first anti-Id serum was passed through MAb coupled sepharose 4B column, which purified anti-MAb antibodies from serum. These anti MAb antibodies were eluted from the column at acidic pH using 0.1M glycin- HCl (pH 2.5). Antibodies were collected in 1.0 ml fractions and were tested in ELISA. Important fractions containing high concentration of antibodies were pooled and further passed through NMG-coupled sephorase 4B column. This column absorbed anti-isotype and anti-allotype antibodies while the anti-idiotype antibodies were collected in flow- through. These purified anti-Ids were concentrated by reverse osmosis and used for immunization of rabbits and to test their suitability as a diagnostic reagent in ELISA.

Although, purified anti-Id did not show any significant binding with the anti-rinderpest antibodies in Indirect-ELISA and c-ELISA, the anti- Id binding to the heterologous sera was detected in an Inhibition ELISA, in which the reaction of purified anti-Id and sera sample was carried out in liquid phase separately. The binding of the anti- Ids to

the heterologous sera shows the presence of either internal image anti- Ids or anti- Ids against shared idiotope, in the rabbit sera in small population.

Anti-Id immunized rabbits did not have any detectable anti-virus antibody response even after three biweekly immunizations. After challenge with the Lapinised rinderpest virus, these rabbits showed lower temperature reaction than the control rabbits (inoculated with normal rabbit IgG) and also showed less severe post-mortem lesion in saculus rotundus, small and large intestines.

Since the polyclonal anti- Ids produced against the MAb D7 26b did not work either as a non-infectious diagnostic or as an immunogen, it would be prudent to produce monoclonal anti-idiotypes bearing internal image of the original antigen.

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MINI ABSTRACT

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Rinderpest is a deadly scourge of cattle and buffaloes. The disease has now been controlled and eradicated from many countries. In India, the disease has not been reported since 1996 and thought to be eradicated. To prevent the re-entry of the virus, country must have strong surveillance system. Presently used serological tests require continuous handling of live virus for the production of diagnostic reagents. Therefore, in this critical stage, replacement of live virus by some non- infectious agent is highly desirable. Use of antigen mimicking anti-idiotype antibodies is one of the solutions to this. In the present study, anti-idiotype antibodies were produced, characterized and evaluated as a diagnostic reagent in ELISA.

Ascites was produced in Balb/C mice using the selected monoclonal antibody clone (D7 26b). Monoclonal antibody was purified from ascites. Fab fragments produced after papain digestion were purified from digested MAb. These Fab fragments retained their virus neutralizing activity. Rabbits were immunized with Fab fragments for raising anti-idiotype antibodies. High titre of anti-MAb antibodies was obtained after two boosters. Anti-Id activity in the rabbit sera was determined by modified Indirect ELISA. Anti-idiotype antibodies were characterized by various assays, viz.; ELISA Inhibition Assay, Virus Neutralization Inhibition Assay, Virus Infection Inhibition Assay and Cell surface receptor binding study. The major population of anti-Id was found against the idiotypes present within the paratope. The major anti-Id type was characterized as the Ab2γ. Anti-idiotype antibodies were purified from the sera and evaluated as a diagnostic reagent in ELISA. It reacted with heterologous rinderpest positive sera, indicating the presence of small population of internal image anti-Id (Ab 2β) or anti-Ids against shared idiotope (IdX). When inoculated into rabbits, anti- Ids could not induce detectable anti- virus antibody response, but the rabbits had reduced clinical reaction compared to controls, when challenged with Lapinised rinderpest virus.

लघु सारांश

पशु माता महामारी गाय एवं भैंस का एक विनाशकारी रोग है। यह रोग अब नियंत्रण में है तथा कई देशों से इसका उन्मूलन किया जा चुका है। भारत में इस रोग का सन् 1996 से प्रतिवेदिन नहीं है और यह समझा जाता है कि इसका उन्मूलन हो चुका है। इस विषाणु के पुरःस्थापन के रोक-थाम हेतु एक प्रभावशाली संनिरीक्षण प्रणाली की आवश्यकता है। वर्तमान में प्रयुक्त लसीय परीक्षा हेतु नैदानिक प्रतिकृ के उत्पादन के लिये संतत सजीव विषाणु प्रयोग की आवश्यकता होती है। अतः ऐसे काष्ठा-अवस्था में, सजीव विषाणु का असांक्रामिक कर्ता द्वारा प्रतिस्थापन अतिवांछनीय है। एंटी-इडीयोटाइप प्रतिकाय का प्रतिजन के रूप में प्रयोग इसका एक समाधान है। प्रस्तुत अध्ययन में एंटी-इडीयोटाइप प्रतिकाय का उत्पादन, लक्षण वर्णन एवं मूल्यांकन एलीसा में एक नैदानिक प्रतिकर्तृ के रूप में किया है।

बाल्ब/सी चूहों में चयनित एक कृन्तक प्रतिकाय क्लोन द्वारा जलोदर उत्पन्न किया गया। जलोदर द्रव से कृन्तक प्रतिकाय शोधित किये गये। कृतंक प्रतिकाय के पैपेन पाचन उपरान्त उत्पन्न फॅब अपखण्ड को शोधित किया गया। इन फॅब अपखण्डों में विषाणु निष्फलन क्रियाशीलता प्रतिघृत थी। खरगोशों पर फेब अपखण्डों द्वारा प्रतीकारिजनन कर, एंटी-इडीयोटाइप प्रतिकाय उत्पन्न किये गये। दो बूस्टर के उपरान्त उच्च-इयदील्यांक के एंटी-मॅब प्रतिकाय प्राप्त हुये। खरगोश लसी में विद्यमान एंटी-आईडी की क्रियाशीलता आपरिवर्तित परोक्ष एलीजा द्वारा ज्ञात की गई। एंटी-आईडी प्रतिकाय का लक्षणवर्धन विभिन्न विश्लेषणें नामशः एलीजा निरोधन निश्लेषण, विषाणु निष्फलतन निरोधन परीक्षण, विषाणु संक्रामण निरोधन परीक्षण एवं कोशा तल संग्राहक आबद्ध अध्ययन द्वारा किया गया। एंटी-आईडी बहुसंख्या में पैराटोप के भीतर विद्यमान ईडीओटाइप के विरूद्ध पाये गये। मुख्य-तौर से एंटी-आईडी प्रकार लक्षणवर्णन द्वारा एंटीआईडी-गामा प्रकार के पाये गए। लसी से एंटी-आईडी प्रतिकाय का शोधन कर, एलीजा में नैदानिक प्रतिकर्तृ के रूप में मूल्यांकन किया गया। यह पृथग्धर्मी एंटी-आईडी ने रिन्डरपेस्ट पौजिटिव लैसी के साथ प्रतिक्रिया की, जिससे लघुसंख्या में आंतरिक प्रतिबिम्ब के एंटीआईडी-बीटा या सहभागी ईडीयोटाइप के विरूद्ध एंटी-आईडी विद्यमान होने के संकेत प्राप्त होते हैं। इसका जब खरगोशों में टीका लगाया गया तब एंटी-आईडी उपलम्भन स्तर पर प्रतिविषाणु प्रतिकाय अनुक्रिया प्रेरित करने में असक्षम रहे। लेकिन इन खरगोशों में लाक्षणिक प्रतिक्रिया, नियंत्रण खरगोशों की अपेक्षा कम रही, जब इन्हें लैपिनाइजड रिन्डरपेस्ट वाइरस से चुनौती दी गई।

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APPENDIX

APPENDIX

MEDIA AND REAGENTS FOR CELL CULTURE

Eagle's Modified Minimum Essential Medium(E MEM)

EMEM powder (Sigma)	12.57 gm
Tryptose Phosphate broth (Sigma)	4 gm
L -glutamine	0.29 gm
Penicillin	100 IU/ml
Streptomycin	100mg/ml
Autoclaved Distilled Water	1000 ml

Use sodium bicarbonate to adjust the pH of the medium (7.2-7.4). To prepare growth medium, add 5% fetal calf serum to the above medium.

Trypsin Versene Solution

NaCl	0.8 gm
KCl	0.4 gm
$NaHCO_3$	0.58 gm
Dextrose	1 gm
Trypsin (Sigma)	0.5gm
Ethylene Diamine Tetra acetic acid (EDTA)	0.2 gm
Distilled Water	100 ml

Dissolve by stirring on magnetic stirrer and filter through Millipore filter / Seitz filter

REAGENTS FOR ELISA

Coating buffer (0.05M Carbonate-Bicarbonate buffer, pH 9.6)

Na_2CO_3	0.318 gm
$NaHCO_3$	0.586 gm
Distilled water	200.000 ml

Washing buffer (0.002M PBS, 0.05% Tween-20)

0.1 M PBS	500.00 ml
Distilled water	2000.00 ml
Tween-20	1.25 ml

Blocking buffer

0.1 M PBS	20.000 ml
Tween-20	40µl
Healthy Goat serum	40µl

0.1M Phosphate-Citrate buffer (PCB), pH 5.0

Stock A:	Citric acid	2.10 g
	D. W.	100 ml
Stock B		
	$Na_2HPO_4.2H_2O$	3.56 g
	D. W.	100 ml

H2O2-OPD substrate: (In phosphate citrate buffer)

Ortho phenylene diamine	20.00 mg
Stock A	12.15 ml
Stock B	12.85 ml
D. W.	25.00 ml
H2O2	20 µl

This was prepared just before use.

Stopping Solution (1M Sulfuric acid)

Dilute 55ml of H_2SO_4, 95-97% (18M) in 935ml of DW.

REAGENTS FOR COLUMN PREPARATION

Borate saline buffer (0.1M,pH 8.3)

Boric acid	-	6.18 g
Borax/Na tetraborate	-	9.15 g
NaCl	-	4.38 g
Distilled water	-	1000 ml

Glycine-HCl buffer (0.1 M, pH 2.5):

0.2 glycine (15.01 g/1000 ml)

0.2 M HCl

500 ml of 0.2 M glycine was titrated with 2 N HCl to pH 2.5 then diluted to 1000 ml.

0.01M Phosphate buffered saline (PBS), pH 7.4

NaCl	8.00 gm
KCl	0.20 gm
NaH_2PO_4	1.15 gm
KH_2PO_4	0.20 gm
Distilled water	100.00 ml

REAGENTS FOR MAb DIGESTION:

0.02M DL-Cysteine (20x)

DL-Cysteine	31.4 mg
Acetate buffer (0.1M)	10 ml

0.04 EDTA (20 x)

EDTA	150 mg
Acetate buffer(0.1M)	10 ml

Papain

Papain	1.2 mg
Acetate buffer (0.1M)	10 ml

1M Acetate buffer

A. 0.2 M solution of acetic acid (11.55 ml in 1000 ml)
B. 0.2 M solution of sodium trihydrate (27.29 in 1000 ml)

44 ml of B and 7 ml of A solution were mixed and diluted to 100 ml.

COPPER REAGENT (For protein estimation by Lowry's Method)

2% Sodium carbonate (in 0.1 N NaOH)	:	50 parts
1% Copper sulfate (in DW)	:	1 part
2% Sodium Potassium tartarate (in DW)	:	1 Part

PROTEIN STANDARD (for Lowry)

BSA	1 mg.
0.1 N NaOH	1 ml

1.5% CARBOXY METHYL CELLULOSE (CMC)

CMC	1.50 gm
EMEM medium (autoclavable)	100.00 ml

The solution was dissolved by autoclaving at 15 lbs for 15 minutes and pH was adjusted to 7.2 with sterile bicarbonate solution.

VITA

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