Studies on the Non-specific Resistance Against Rabies Virus

BY

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This is to certify that the entire research work presented in this thesis entitled "Studies on the non-specific resistance against Rabies Virus" has been authentically carried out by Shri Sasanka Kumar Haldar himself under my supervision and guidance.

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C O N T E N T S

INTRODUCTION

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Since the very inception, human beings in their day to day existence are facing various challenges of nature, out of which the irony is probably the greatest threatening coming from the community of microbes. But surprisingly enough negligible members are falling helpless prey to the hands of this natural calamity.

This partiality of nature, why some and not one and all are becoming the victims, stimulated eternal inquisitiveness of human mind. Even in early days people could observe the ability of refractoriness of an individual to a particular infection, once experienced. Later, Jenner's profound achievement, the famous variolation, laid down the foundation of immunity. Ever since, Pasteur, Von Behring, Kitasato etc. have illuminated specific immunisation and is being practised in prophylactic measures to combat various diseases till to-day.

But specific immunisation has not spoken always a successful answer in prophylaxis. Immunologists have put their heads together to think of solid immunity against virulent pathogens possessing poor antigenicity e.g. rabies virus, salmonella. Failure has been experienced in eliciting satisfactory immune response with viruses having

multiplicity in antigenic character like foot-and-mouth disease and influenza viruses. Convincing explanation is still wanted how agammaglobulinaemic patients develop resistance against certain agents, e.g. measles, pox, mumps. And, indeed, the role of antibody in latent virus infection is perplexing.

These problems, where specific immunogen is laging behind, compelled immunologists to search for an alternative and the idea of non-specific resistance took birth following the historic observation "phagocytosis" by Elie Metchnikoff (1905). Since then, substances unrelated to specific immunogen attracted the attention of workers for the use of provoking non-specific resistance. Many a substance besides mycobacterial preparation have been earmarked as non-specific resistance inducer. Among mycobacterial preparation, Mycobacterium phlei (M. phlei) was claimed to be highly effective in modifying host resistance to various infections (Biozzi et al., 1960; Fox et al., 1966; Pilet and Goret, 1966).

Since very early days, rabies virus, being poorly immunogenic poses a tough problem. And, though eminent scientists had left no stone unturned to develop a satisfactory therapeutic or prophylactic measure, but so far unfortunately hardly any significant achievement could be

noticed after the epoch making work of Pasteur in this discipline. A look in the Annual Report of the Pasteur Institute of Southern India, Coonoor (1969) compiled from 1907 to 1969 revealed death of 1454 persons out of 4,78,105 fully treated cases apart from encephalitic reaction in many others. Similarly 919 died out of 1,00,031 antirabies treated animals during the period from 1923 to 31st March, 1970.

Keeping in view the above anomalies, Pandey and Mallick (1970) initiated the work on non-specific resistance using M. phlei as a tool and noted encouraging results against rabies virus. Stimulated with the above finding Srinivasan and Mallick (1971) elaborated the work further.

In continuation of the above finding an attempt was made in this presentation to study the resistance induced by M. phlei in different species namely mouse, rat, guinea-pig and rabbit. Intramuscular route, the portal for natural infection of rabies, was selected as the route of challenge in this work unlike previous workers.

So, the humble objective of this study is to pave the way for the possibility of evolving a satisfactory immunising procedure against rabies, poor viral immunogen with the production of non-specific resistance.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The role of non-specific resistance against disease is a well established fact. No doubt, an extensive work has been undertaken in this area of immunology, but for the sake of brevity, only pertinent work are being reviewed here, related to the subject of study under the following major headings:

I. A peek on non-specific resistance.

II. Non-specific resistance against viruses.

III. Mechanism of non-specific resistance against viruses.

IV. Gel-diffusion test.

I. A Peek On Non-specific Resistance

It is needless to define "non-specific resistance" as the same has in many ways been ornamented by various authors. But inclusion of a brief sketch may not be impertinent here.

An individual is resistant to infection non-specifically due to various factors, in addition to specific antibodies. But specific antibodies which is conventionally active against a particular infection, appear generally

later in comparison to the non-specific resistance (MacLeod, 1960).

By and large, non-specific resistance is of two types:

1. Innate or natural resistance.
2. Artificially induced resistance.

1. Innate or natural resistance

(i) Genetic or inherent factors

The role of genetic or inherent resistance is well appreciated by the fact that a particular species is refractory to certain infections e.g. horse against foot-and-mouth disease, cattle against swine-fever, man against canine distemper.

(ii) Non-specific factors

One and every individual is in the possession of various non-specific factors which have got a definite important role in defending the individual against infection (Carpenter, 1965).

(a) Mechanical barrier- The fact that skin, mucous membrane, various juices of gastro-intestinal tract (Lysov and Trautner, 1967) act as barrier against microbial invaders is well known.

(b) Serum and other body fluids- The presence of antimicrobial substances in the serum and other body fluids is a fact beyond doubt and some of the important factors playing the key role in this phenomena are:

Complement

It is a factor present in normal serum empowered with destructive influence on micro-organisms (Nuttal, 1888; Buchner, 1889; Bordet and Gengu, 1901, etc.).

Properdin

The antimicrobial property of properdin, a high molecular protein, was first observed by Pillemer et al. (1954) and since then many workers have been emphasising its non-specific defensive role (Ross et al., 1955; Landy and Pillemer, 1956; Nelson, 1958, etc.).

Beta-lysin

Von Behring (1892) observed a multiple enzyme system, beta-lysin in coagulated whole blood having a non-specific defensive role.

Lysozyme

The detrimental property to micro-organisms of lysozyme, a mucolytic enzyme, found in different body fluids except cerebro-spinal fluids, aqueous humor, sweat and urine, was first reported by Fleming (1922).

Tissue metabolites

The harmful activity against micro-organisms of a number of substances has been reported e.g. basic polypeptides (Bloom et al., 1947), short and long chain fatty acids (Vally, 1928; Dubos, 1953), spermine and spermidine (Hirsch and Dubos, 1952), protamine and histone (Tomcsik and Guesholzer, 1954), leukin, phagocitin (Metchnikoff, 1905), lymphokines (Dumonde et al., 1969), hematine and mesohematine (Skarnes and Watson, 1957).

Hormones

The non-specific defensive role of some hormones was illuminated by Aycock (1936), Sprunt and Medearman (1940), Weiss (1952) and Cass (1960).

Phagocytes

Since Metchnikoff (loc.cit.), phagocytes are recognised as a great tool in non-specific defence mechanism and later many workers have substantiated its importance (Wright, 1927; Gregg and Robertson, 1953; Rogers, 1960,etc.).

Besides these factors, body temperature (Rich and McKee, 1936; Enders and Shaffer, 1936; Linnell and Norden, 1954; Bennett and Nicastri, 1960) and nutritional factors (Dubos and Schaedler, 1958; Schaedler and Dubos, 1959; Schneider, 1960) have also been found to act in defence mechanism.

2. Artificially induced resistance

The possibilities of inducing non-specific resistance artificially have been explored in the past and have created profound interest to many present-day workers with the help of various microbial and non-microbial substances.

For the sake of convenience, only suitable literature has been picked up for this review and will be dealt under the following headings:-

(i) Saprophytic bacteria other than mycobacteria.

(ii) Endotoxin and other bacterial components.

(iii) Mycobacterial preparation.

(A) Mycobacterium tuberculosis

(B) Saprophytic mycobacteria

(i) Saprophytic bacteria other than mycobacteria

The reports on bacterial non-specific resistance are few and far between.

Kepinov (1924) observed non-specific resistance by inoculating the cultures of Vibrio cholerae, Bacillus prodiogiosus and Bacillus proteus against cholera.

In French West Africa, Chassigneux (1945) experienced failure in fixing the rabies virus of Senegal. He also noted the loss in virulence of the fixed virus (Paris strain) at the fourth passage level in rabbit. As the

material was found to be contaminated with Bacillus subtilis he postulated that this contaminating organism prevented the development of expected result. Later it was supported by experimental evidence. Afterward, Lanfranchi (1942) observed increase in resistance to Pasteurella septica and Salmonella typhimurium on inoculation of B. subtilis polysaccharides with glycolipids of these organisms in mice.

Gryaznivaet al. (1967) noted augmented resistance against Escherichia coli strain 94 and Staphylococcus aureus Gouren strain, with lipopolysaccharides of actinomycetes.

Armani (1969) observed resistance in guinea-pig against diphtheria toxin when Corynebacterium avidum was inoculated with Freund's incomplete adjuvant.

(11) Endotoxin and other bacterial components

Since Boivin (1933), the use of endotoxin as antibody inducer is in vogue but later besides these, the ability of endotoxin in inducing non-specific resistance has been explored.

(a) Resistance against bacteria

Greenberg and Fleming (1947, 1948) marked four-fold enhancement of antitoxin production against diphtheria

toxoid when administered subcutaneously, along with Bordetella pertusis or typhoid-paratyphoid vaccine.

According to Rowley (1956), the lipopolysaccharide fractions of the cell-wall of Gram-negative bacilli was responsible for the increased resistance. Subsequently, various workers have further elaborated the role of lipopolysaccharides in non-specific protection to many Gram-negative, Gram-positive and viral infections (Landy, 1956; Shilo, 1959; Munoz, 1964; Frigerio et al., 1966; Gryazniva et al. loc.cit.).

Berger et al. (1968) used a non-toxic proteinaceous substance, protodyne, generally found in the protoplasm of E. coli which is quite distinct from lipopolysaccharide, as non-specific resistance inducer in mice against Salmonella typhimurium, Sal. typhosa, Pseudomonas aeruginosa, Klebsiella pneumoniae and Streptococcus mastidis.

(b) Resistance against viruses

Increase in resistance to viral infections e.g. Columbia-SK encephalomyelitis and ectromalia was reported by Meier and Kradolfer (1956) and Kradolfer et al., (1957) using lipopolysaccharides.

Kabatova and Popisil (1967) noted a reduction in the influenza virus titre by 3 logs by the administration of a polysaccharide fraction isolated from Candida albicans in 10-day old chick embryo.

(c) Mode of action

As evidenced by published work, the endotoxin exerts its non-specific effect in many ways (Munoz, loc.cit.). Biozzi et al., (1955) and Thomas (1959) were of the opinion that the increased resistance is due to the effect of endotoxin on the reticulo-endothelial system. But Eckman et al. (1958) and Munoz (1961) thought that the increased effect was due to marked changes in the permiability, whereas Egdahl (1959) and Nadel et al., (1961) claimed that this property of the endotoxin was due to the effect on the adrenal gland. Rowley (loc.cit.) observed the augmentation of non-specific resistance in laboratory animals against E. coli infection is due to the increase in properdin level.

(iii) Mycobacterial preparation

(A). Mycobacterium tuberculosis. The observation that tuberculous animals generally become resistant to other microbial infections led a group of workers to think the utility of mycobacterium as a non-specific resistance inducer.

(a) Resistance against bacteria

As early as 1924, Lewis and Loomis noted that living virulent tubercle bacilli could augment the antibody formation against various organisms.

Boquet et al. (1934) observed destruction of anthrax bacillus when inoculated at the site of tuberculin reaction.

Weisfieler (1935) came across non-specific allergic reaction in tuberculous animals against staphylococci whereas against enterobacteria was observed by Bordet (1936).

Pullinger (1936) had proved experimentally that brucell infection was resisted in tuberculous animals.

Freund and Bonanto (1944) claimed that the antigenicity of typhoid bacilli could be accentuated with the incorporation of lanolin and killed tubercle bacilli.

Williams and Dubos (1959) reckoned non-specific resistance in a group of albino mice inoculated with different methanol extracted fractions of tubercle bacilli against staphylococci.

(b) Resistance against viruses

The use of Myco. tuberculosis as adjuvant has also created interest to virologists. It has been observed that virus incorporated with mycobacteria have a marked stimulatory effect on antibody response (Weigle et al., 1960; Shelpel and Klugerman, 1963).

In 1967, Gizatullin et al., considered an increased non-specific immunogenic activity on administration of Freund's adjuvant prior to or together with foot-and-mouth disease vaccine.

Gorhe (1967) noted that multiplication of foot-and-mouth virus was inhibited by Wax D_{15} fraction of Myco. tuberculosis.

(c) Mode of action

Various workers have tried to work out the mechanism of action of Myco. tuberculosis in non-specific resistance but still not yet completely explored.

White et al., (1958) were of the opinion that the peptidoglucolipids is the key fraction for widespread stimulation of immature and mature plasma cells.

Lachmann in the year 1968 considered that Freund's adjuvant not only increased the circulating antibody but also had a marked effect in the manifestation of delayed hypersensitivity.

White et al. (1964) tried to correlate the adjuvant effect with the chemical structure of Wax D of mycobacterium and subsequently in the year 1969 Shinozaki et al. supported it.

(B) Saprophytic mycobacteria

Quite a few workers have explored the probability of non-specific resistance using saprophytic mycobacteria as inducer.

Freund and Walter (1944) claimed M. phlei as effective as killed tubercle bacilli in the field of non-specific resistance.

In a comparative trial of several strains of Mycobacteria, Biozzi et al., (1960) noted a particular strain of M. phlei to be most effective.

(a) Resistance against bacteria

Fox et al., (1966) observed non-specific resistance for prolonged period after administration orally or parenterally a cell-wall fraction of M. phlei in mice and guinea-pigs to experimental infection of Sal. enteritidis and in mice to Staph. aureus.

Pilet and Goret (1966) reported that a state of resistance could be achieved with live or trypsin treated Myco. cheilonensis and M. phlei by inoculating into different routes and in different doses against experimental brucella infection in rats and mice.

Mishra and Mallick (1970) and Jana and Mallick (1971) in a comparative experiment between specific antigen and

M. phlei against Sal. enteritidis noted a resistance of considerable degree and claimed M. phlei to be a better inducer.

(b) Resistance against viruses

In the field of virology, Pandey and Mallick (1970) studied the use of M. phlei as non-specific resistance inducer against rabies infection in comparison to specific immunogen. They conducted experiments in mice and came across a high degree of resistance at single inoculation level against virulent fixed rabies virus. They also found in sera of mice inoculated with trypsinised M. phlei preparation, an enhanced virus neutralizing activity.

Subsequently, in the following year Srinivasan and Mallick (1971) conducted experiment in a similar pattern and confirmed the observation of previous workers. In addition, they observed better resistance at multiple inoculation level in mouse and sheep.

(c) Mode of action

As reviewed earlier, the peptidoglycolipid fraction of Wax D of Mycobacterium sp. is responsible for inducing resistance. According to White et al., (1964) this particular fraction is also present in an appreciable quantity next in order to the human strain of Myco. tuberculosis.

In a histopathological study Fox et al., (1966) noted hyperplasia of Kuffer and lymphoid cells following the administration of cell-wall preparation of M. phlei.

II. Non-specific Resistance Against Viruses

An outline of the basic concept of non-specific resistance against infections has been covered already. Now specific work related to virus will be dealt with.

Webster (1933, 1937) established one strain of mouse resistant to mouse encephalomyelitis virus and correlated it with genetic or inherent immunity.

Casals (1936) reported that 7-9 days old mice are more susceptible than older mice to infection of fixed or street virus by any route.

The role of humoral factors in non-specific resistance have been studied as early as 1936 by Aycock. He observed increased susceptibility to poliomyelitis virus of ovariectomised monkey and administration of oestrogen in those animals augmented the resistance.

Existence of physiological factors e.g. diet, heridity in resistance was illuminated by Church (1939).

In an experiment with vaccinia virus, Sprunt and McDearman (1940) worked out the role of oestrogen in augmentation of resistance.

In the same year, Hyffengger observed slow development of vaccinia in cattle experienced with foot-and-mouth disease and also noted that pox vaccinated cattle, showed some resistance to foot-and-mouth disease.

Some heat labile substances of sera having the power of neutralizing the vaccinia, variola, Rouse sarcoma, Western equine encephalomyelitis, mumps, influenza A and B, Newcastle disease, herpes simplex, dengue and measles, were observed by several investigators (Mueller, 1931; Morgan, 1945; Sabin, 1950; Broidy et al., 1951; Chu, 1951; McCarty and Germer, 1952).

Koprowski (1946) reported inactivation of yellow fever, Japanese B encephalitis, St. Louis and West Nile encephalitis viruses by a substance found in rodents and marsupials.

Casals and Olitsky (1947) found a lipid factor in sera of mice, hamsters, rabbits, and horses which can inactivate neurotropic viruses namely Russian Far East, St. Louis and Japanese B. encephalitis virus.

Again in 1948 Casals and Olitsky extracted a lipid factor from mouse brain and recorded its inactivating effect against Russian Far East virus.

Utz (1948, 1949) found in serum a lacithin like heat stable substance having an inhibitory role on Newcastle disease and influenza viruses.

The importance of age and hormone in resistance was stressed by several workers (Kilbourne and Horsfall, 1951; Sigel, 1952).

The cell-walls of mouse stomach and small intestine were found to contain a mucopolysaccharide fraction which has got an inhibitory action on theilers encephalomyelitis virus GD VII strain (Mandel and Backer 1953a, b).

Wedgewood et al. (1956) elaborated the ability of properdin to inactivate viruses. He reported that Newcastle disease virus could be neutralized. Later Finkelstein et al. (1958) found inactivation of influenza A and B and herpes simplex virus with properdin. Afterwards more information was added about inactivation of viruses e.g. bacteriophage, mumps, vaccinia, variola, dengue and measles with this substance (Cowan, 1958; Mueller Loc.cit., Morgan, loc.cit., Briody et al., loc.cit., and McCarty and Germer, loc.cit).

Holland and McLaren (1959) found a substance, lipoprotein in nature, in primate cells grown in vitro having inhibitory effect on the infectivity of poliomyelitis virus. They also noted that the same substance could not be extracted from non-primate culture cells.

Since Magrassi (1935), enormous work on interference has been undertaken by various workers from different parts of the world (Hyffengger, loc.cit., Peacock, 1966; Beard, 1967; Kazar, 1969).

In 1957, Isaacs and Lindenman came out with their remarkable finding "interferon". Since then much elaborate and extensive work has been conducted on interferon (Burkie and Isaacs, 1958; Sutton and Tyrrel, 1961; Isaacs, 1962; Baron and Buekler, 1963; Ho, 1964; Stewart and Sulkin, 1966; Merigan, 1967; Lamnicsi, 1968; Fayaz et al., 1970).

III. Mechanism of Non-specific Resistance against viruses

The mechanism of non-specific resistance against viral diseases is yet to be known clearly. There are many hypotheses put forward by various workers.

The fact that attachment of virion is the start of infection, is beyond any confusion. From the work of Hirst (1942) it was later known that the mechanism of attachment particularly of influenza virus was due to the presence of a receptor, mucopolysarrharide in nature, on the surface of the ciliated epithelium of the respiratory tract. It was further observed that the attached virus get eluted after sometimes due to the presence of the receptor destroying enzyme, neuraminidase. Thus, the host cells being receptorless become resistant to further infections (Humphery and White, 1970).

Francis et al. (1938) and Straub (1940) observed in an experiment in ferrets and mice, desquamation of the superficial epithelium, following intranasal instillation of influenza virus and before regeneration of the ciliated epithelium a low transitional type of cells, resistant to virus, appeared.

The role of phagocyte in viral defence mechanism still requires elucidation. Smorodinsev (1964) reported that rapid inactivation of influenza virus into the cytoplasm of leucocytes in the peritoneal exudate of white mice. Gallily et al. (1967) remarked that the genetic resistance of mice to the mouse hepatitis virus lies in the macrophages.

The possible mechanism of interference has been studied in details in viral infections. By generally three ways the first virus interferes with the propagation of the second virus (i) destroying receptors on the host cell surface, (ii) producing changes in the metabolic activities and (iii) stimulating the production of interferon (Fenner, 1968; Dianzani et al., loc. cit.).

IV. Gel-diffusion Test

Gel-diffusion test has attained a place of pride in the array of diagnostic field since Oudin (1946) and Ouchterlony (1948). A good number of papers have been

published by various workers on the use of this test for identification of rabies virus or rabies immune serum (Lapine, 1966).

Villemot and Provost (1958a) employed this test with known hyperimmune rabies serum against unknown antigen and obtained two precipitation lines within 24 to 72 hours. On further trial, they observed that rabies hyperimmune serum is necessary for conducting this test and also found no cross-reaction between rabies virus and canine distemper virus (Villemot and Provost, 1958b).

In 1960, Theiry confirmed the specificity of this test. He used 2 ml of agar gel in microscopic slide and noted precipitation band within 8-12 hours.

Grasset and Atanasiu (1961) emphasized the use of concentrated antigen and throughout their experiments mostly 100-fold concentrated tissue culture rabies virus antigen was employed.

Atanasiu (1963) later reported the appearance of two precipitation lines with $BHK_{21}C_{13}$ cells infected with either street virus or fixed rabies Pasteur strain in the presence of hyperimmune specific serum.

As M. phlei has been used as non-specific immunogen in this study related work on this test using mycobacterial preparation as antigen is being reviewed briefly.

The application of gel-diffusion technique in Myco. tuberculosis has been carried out by various workers (Boyden and Sorkin, 1955; Seibert and Soto-Fginerea,1957; Lind, 1960).

In 1962, Gimple and Weissfeiler did not obtain any serological difference between virulent and attenuated human and bovine mycobacterial strains but noted significant difference between this strain and saprophytes.

But no published report is available on cross-reaction between non-specific immunogen (M. phlei) and any specific immunogen treated serum.

MATERIAL AND METHODS

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MATERIAL

1. Virus strain used for the experimental work

A. The Paris strain of fixed rabies virus (RFV, Paris)

The Paris strain of Rabies Fixed Virus was obtained from the Rabies Laboratory, I.V.R.I., Mukteswar. The virus strain was procured by the Laboratory from W.H.O. on 21st October, 1957. Since then regular monthly passage in rabbits was being done to maintain the virus. The infected brain was preserved in 50% glycerinated buffer saline and kept at 4°C in the refrigerator. This was used as a seed virus for the preparation of immunising antigen as well as precipitating antigen.

B. The challenge virus strain (CVS) of fixed rabies virus for serum neutralization test

The challenge virus strain of rabies virus for the serum neutralization test was obtained from the same laboratory. This strain was maintained by serial passage intracerebrally in Swiss albino mice. A 20% suspension in 50% glycerine-buffered saline of infected mouse brain was filled in ampoules, 1 ml each and stored at -5°C.

C. The challenge virus strain of fixed rabies virus for intramuscular challenge

The CVS for intramuscular challenge in different species of animals viz. mouse, rat, guinea-pig and rabbit were prepared separately by giving four serial intramuscular passages in respective species except in rabbit where rat intramuscular CVS was used. Here also 20% suspension in 50% glycerine-buffered saline of respective infected brain was filled in 1 ml or 5 ml ampoules and stored at -20°C. The mouse CVS was used as seed virus in case of mouse intramuscular CVS whereas for other two RFV Paris was used.

2. Carbolised antirabic vaccine (5%)

This was obtained from the Biological Products Division, I.V.R.I Izatnagar (Brew No. 7/71/72).

3. Normal horse serum

This was obtained from the Rabies Laboratory at Mukteswar for the preparation of 2% serum saline solution for challenge virus dilution in serum neutralization test. The normal horse serum was inactivated at 56°C for half an hour.

4. Strain of Mycobacterium phlei for the preparation of non-specific immunogen

A chromogenic strain of Mycobacterium phlei was obtained from Bacteriologist (Chronic Diseases) at Mukteswar.

This strain was maintained in glycerine egg-yolk slants in the Laboratory of Professor of Bacteriology. This was cultivated in two lots, incubator dried and stored at 4°C until preparation of non-specific immunogen.

5. Arcton 113 (I.C.I.)

The fluorocarbon was supplied as a gift sample by M/s Imperial Chemical Industries, London. This was used for purifying the rabies virus from the infected rabbit brain material.

6. Agar Gel

One per cent Ion-agar No.2 (Oxoid) incorporated with methylorange and merthiolate was employed.

7. Laboratory animals used for experiment

(1) Mice

Healthy Swiss albino mice of either sex were used for production of challenge virus strain of fixed rabies virus both for serum neutralization as well as intra-muscular challenge tests. For experiments also same mice were used. Mice of 3-8 weeks of age were used and these were obtained from the Veterinary Section of the Institute.

(ii) Rats

Male healthy albino rats were obtained from the Veterinary Sectionof the Institute and used for the experiment as well as for the production of intramuscular challenge virus strain for rat and rabbit. Rats of 2-2½ months of age were used.

(iii) Guinea-pigs

Healthy male guinea-pigs weighing 300-600 gms. obtained from the Veterinary Section of the Institute were used for the experiments as well as for the preparation of intramuscular challenge virus strain of the rabies fixed virus for guinea-pig.

(iv) Rabbits

Healthy indigenous rabbits of either sex weighing over 600 gms. obtained from the small animal house of the Institute were used for the experiments and for the preparation of immunising and precipitating antigens.

METHODS

1. Preparation of immunising antigen

A. Preparation of heat inactivated 20% suspension of rabies fixed virus (RFV, Paris) infected rabbit brain

(i) Preparation of virus inoculum

A portion of the infected rabbit brain preserved in 50% glycerine-buffered saline was weighed and washed in

saline. It was then emulsified with 9 parts of 2% normal horse serum saline and centrifuged at 1000 r.p.m. for 5 minutes. The supernatant containing 10^{-1} dilution of virus was further diluted to 10^{-2} and used as inoculum.

(ii) Inoculation of rabbit

Healthy rabbits weighing over 700 gms. were taken. Twenty such rabbits were inoculated intracerebrally with 0.25 ml of the inoculum each. The inoculation was made with a tuberculin syringe and 24 gauge 3/8 inch needle after a puncture was made on the skull.

(iii) Development of rabies in rabbits

Inoculated rabbits dying within 5 days were discarded considering these to be non-specific. All the rabbits started showing paralytic symptom within 6 to 7 days and completely paralised by 8th day.

(iv) Harvest of brain

The completely paralysed animals were sacrificed by introducing air through the marginal ear vein. Then the brains were harvested with all sterile precautions after removing the skull and immediately kept at -20°C.

(v) Emulsification of brain

A total of 130 gms. of the above brain material was homogenised in a sterilised waring blender with 520 ml of physiological saline for 5 minutes to make 20% infected

brain emulsion. Streptomycin sulphate (2.0 mgms./ml.) and 200 international units (I.U.) of penicillin G-sodium per ml were added to this emulsion.

(vi) Virus titration

A portion of the brain suspension was taken and centrifuged at 1000 r.p.m. for 5 minutes. Ten-fold dilutions of the supernatant were made serially up to 10^{-8} with 2% serum saline. Five mice weighing between 12-15 gm. were inoculated 0.03 ml intracerebrally from each dilution of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} respectively. The mice were then observed for 14 days for the development of symptoms like rough coat, tremor, flacid or complete paralysis and finally death. The mice which died within 5 days after inoculation were not taken into account. The 50% lethal dose (LD_{50}) was calculated with the help of Karber formual (Roydhowdhuri and Thomas, 1967). The LD_{50} titre was found to be $10^{-6.5}$.

(vii) Inactivation of the brain emulsion

The 20% brain emulsion was then subjected to light centrifugation (1000 r.p.m. for 5 minutes) and the supernatant was inactivated at 56°C for half an hour in a water-bath. The whole immunising antigen was then transferred in amber coloured bottles and kept in deep freeze (-2°C) until use.

Sterility and safety tests were performed and the material was found to be sterile and safe.

This 20% immunising agent was reconstituted with equal volume of physiological saline before use to make it 10% brain tissue suspension. During single inoculation experiments 0.5 ml in mouse, 1.0 ml in rat, 1.5 ml in guinea-pig and 2 ml in rabbit were used, while 1 ml daily in rabbit was used during multiple inoculation experiment.

2. Preparation of challenge rabies virus (CVS) strain

(i) Challenge virus strain for serum neutralization test

The CVS was obtained regularly from the Rabies Laboratory of Mukteswar in sealed 1 ml ampoules. The titre of the CVS was $10^{-7.17}$.

(ii) Intramuscular challenge virus strain (CVS) for mouse for challenge test

From Rabies Laboratory, Mukteswar, one ampule of 20% mouse CVS was taken and was reconstituted with 1 ml of 2% horse serum saline to give the dilution of the virus 10^{-1}. This was then subjected to light centrifugation (1000 r.p.m. for 5 minutes). Then the supernatant was diluted to 1 in 20 virus suspension.

Ten Swiss albino mice weighing about 12-14 gms. were inoculated intramuscularly each with 0.06 ml of the 1 in 20

virus dilution. These were then kept under observation. Those showing symptoms of rabies 5th day after inoculation were separated, their brains were harvested aseptically 24 hours after the onset of the symptoms and preserved in 20% glycerine buffered saline at 5°C.

From this infected brain material, 1 in 20 dilution of the virus was prepared after the addition of antibiotics @ 2.0 gm. of streptomycin sulphate and 200 I.U. of penicillin G-sodium per ml. This was then inoculated to another ten mice as above. Total four such passages were given when a regular mortality pattern through intramuscular route was noted.

Finally, 60 mice were inoculated with the 4th passage level virus intramuscularly and their brains were harvested 24 hours after the onset of symptoms as mentioned already. Immediately after collection, the brains were transferred at -20°C.

All the harvested brains were washed in chilled physiological saline. Total weight of the brain material was 12.5 gms. A 20% emulsion of the brain material was then made in 50% glycerine buffered-saline in a waring blendor for about 5 minutes. After the addition of antibiotic in the proportion mentioned earlier, the brain emulsions were ampouled in 1 ml quantity and properly labelled. It was

kept at -20°C. Two ampoules were taken and after mixing titrated for determining mouse LD_{50} as before. The LD_{50} per 0.03 ml in mice was $10^{-7.3}$.

This CVS was used for intramuscular challenge test in mice throughout the work.

(ii) Intramuscular CVS for rat challenge test

For preparing this CVS also, 4 intramuscular passages in rat were given when a regular mortality pattern was noted. The dose was 0.2 ml of 1 in 20 virus dilution.

Finally, with this 4th passage level virus, 30 rats were inoculated intramuscularly and 20% infected brain emulsion was prepared in the same way as mouse CVS and ampuled in 2 ml and 5 ml quantities and kept at -20°C. This CVS was titrated in mice as before. The LD_{50} per 0.03 ml in mice was found to be $10^{6.7}$. This CVS was used for intramuscular challenge test in rat and rabbits and also in treatment trial in rabbit throughout the work.

(iii) Intramuscular CVS for guinea-pig challenge test

The guinea-pig CVS was also prepared in the same way as mice and rat CVS. The mice CVS was given four intramuscular passages in guinea-pigs to get regular mortality through this route. The dose was 0.25 ml of 1 in 20 dilution.

Ten guinea-pigs were inoculated with 4th passage level virus and their brains were harvested in time. Total weight of the brain was 16.3 gm. From this brain material, 20% guinea-pig CVS was prepared and ampouled in 2 and 5 ml quantities. This was titrated in mice and the LD_{50} per 0.03 ml was found to be $10^{-7.3}$.

This CVS was used for intramuscular challenge test in guinea-pigs.

3. Preparation of precipitating antigen

Twenty-two grammes of infected rabbit brain (RFV,Paris) was homogenised in McIlvaine's buffer with pH 7.4 @ 10 ml per 2 gms. of brain material in a waring blendor. It was then centrifuged (1000 r.p.m. for 5 minutes) and the supernatant was inactivated at 56°C for half an hour. To this whole material, 55 ml of Arcton 113 (ICI) was added and again homogenised for 10 minutes at 10000 r.p.m. This Arcton treated material was subjected to centrifugation at 2000 r.p.m. for 10 minutes when three separate layers having the uppermost very thin insoluble lipid, the middle aqueous layer containing virus and the bottom jelly-like layer containing non-viral proteins and soluble lipids. The middle aqueous layer was pipetted out and stored at -20°C till use. The method described by Srinivasan and Mallick (1971) was followed with slight modification.

This precipitating antigen was used throughout the work.

4. Preparation of bacterial adjuvant

The method described by Mishra and Mallick (1970) was followed with slight modification.

A chromogenic strain of Mycobacterium phlei maintained in the Professor of Bacteriology Laboratory, at Mukteswar was used as seed material. Twenty Roux flaks containing 120 ml of 5% glycerine (Analar) broth (pH 7.2) each were inoculated with the seed and incubated for 15 days at 37°C. The pigmented and luxuriant surface growth was harvested by titration through muslin under sterile precautions and dried in bacteriological incubator, for consecutive 10 days. This dried material was then powdered in sterile pestle and mortar and kept in the refrigerator till trypsinisation.

Procedure for Trypsinisation

Powdered M. phlei was added in the proportion of 30 mgms. per ml with 0.15 per cent trypsin (Difco 1:250) solution prepared in veronal buffer with pH 7.2 (Meyer et al., 1948). The material was then subjected to continuous stirring for 48 hours on a magnetic stirrer at 37°C. It was then treated with moist heat (100°C) for half an hour for avoiding

the effect of trypsin and kept at -20°C after distributing in aliquots in McCartney bottles. Before use, it was diluted with the veronal buffer solution to get the desired amount of M. phlei powder per ml of the preparation required for the particular experiment.

5. Preparation of Gel

The method adopted by Lepine (loc. cit.) was followed with partial modifications:

Ion-agar No.2 (Oxoid)	=	10 gms.
Methyl orange	=	0.03 gm.
Merthiolate	=	0.2 gm.
Distilled water	=	1000 ml.

All the above ingredients were dissolved in running steam. Then 6 ml and 2 ml of the molten agar were poured on petri dishes (5 cm.) and microscopic slides respectively and kept in the refrigerator (5°C).

A. Experimental Procedure

In all six series of experiments were conducted as described below:

Ist Series

Three hundred mice of 6-8 weeks of age were used and divided into 3 groups of 100 mice each. Each group was inoculated as follows:-

Group I. Each mouse was inoculated with 0.5 ml of heat inactivated 10% rabbit brain tissue infected with RFV, Paris. The inoculation was given intra-peritoneally.

Group II. In this group each mouse received 0.5 ml of trypsinised M. phlei suspension (3 mgms. of dried M. phlei powder), intraperitoneally.

Group III. Each mouse of this control group received 0.5 ml of veronal buffer intraperitoneally.

IInd Series

Sixty rats of 2-2½ months of age were taken and divided into 3 groups of 20 each. These were inoculated as follows:

Group I. Each rat of this group was inoculated with 1 ml of heat inactivated 10% rabbit brain tissue infected with RFV, Paris. The inoculation was made intraperitoneally.

Group II. In this group each rat was given 1 ml of trypsinised M. phlei suspension containing 15 mgms. of dried powder intraperitoneally.

Group III. This group served as control. Each rat was given 1 ml of veronal buffer intraperitoneally.

IIIrd Series

In this experimental series 21 guinea-pigs were taken and divided into three groups. Each group consisted of 7 guinea-pigs.

Group I. Each guinea-pig of this group was given 1.5 ml of heat inactivated 10% rabbit brain tissue infected with RFV, Paris, intraperitoneally.

Group II. In this group each guinea-pig was inoculated with 1 ml of trypsinised M. phlei preparation containing 30 mgms. of dried M. phlei powder. The material was injected intraperitoneally.

Group III. Each guineapig of this control group was inoculated intraperitoneally with 1.5 ml of veronal buffer.

IV th Series

In this series 21 rabbits weighing between 70 to 850 gms. were taken and divided into 3 groups of 7 each. Each group was inoculated as follows:-

Group I. Each rabbit of this group was inoculated with 2 ml of heat inactivated 10% rabbit brain tissue infected with RFV Paris. The route of inoculation was intraperitoneal.

Group II. In this group each rabbit was given 2 ml of trypsinised preparation of M. phlei containing 60 mgms. of dried M. phlei powder. The material was injected intraperitoneally.

Group III. Each rabbit was inoculated with 2 ml of veronal buffer to serve as control.

Vth Series

In this experimental series 21 rabbits weighing between 750 to 850 gms. were taken and divided into 3 groups of 7 each. Here multiple inoculation of the immunising agents were given as follows:-

Group I. Each rabbit of this group was inoculated with 1 ml of 10% heat inactivated rabbit brain infected with RFV, Paris, daily for 7 days. The immunogen was injected intraperitoneally.

Group II. In this group each rabbit received 1 ml of trypsinised M. phlei containing 30 mgms. of dried M. phlei powder daily for 7 days. The route of inoculation was intraperitoneal.

Group III. In this control group each rabbit received intraperitoneally 1 ml of veronal buffer.

VIth Series

In this series 20 rabbits weighing between 750 to 850 gms. were taken and divided into 3 groups. All the rabbits were inoculated intramuscularly with 41,70,000 LD_{50} of rat CVS in the morning. Treatment started from the same evening with specific and non-specific immunogen as follows:-

Group I. Each rabbit was treated with 1 ml of 5% carbolised antirabic vaccine daily for 7 days. The route of treatment was intraperitoneal.

Group II. In this group each rabbit was treated with 1 ml of trypsinised M. phlei containing 30 mgms. of dried M. phlei daily for 7 days. The injections were given intraperitoneally.

Group III. In this control group, the rabbits were inoculated daily for 7 days with 1 ml of veronal buffer. The route of injection was intraperitoneal.

B. Bleeding of experimental animals and collection of sera samples for serology

(i) Pre-inoculation bleeding

Before starting any series of experiment, preinoculation bleeding was done.

(ii) Post-inoculation bleeding

Ist Series

Here bleedings were conducted on 10th, 20th and 30th post-inoculation days.

Mouse bleeding was done by puncturing the retro-orbital venous plexus with pasteur pipette. Blood from 15 mice was pooled for collecting sera samples. The sera collected in all the experiments were inactivated at 56°C for half an hour and kept at -20°C.

IInd Series

Post-inoculation bleedings were done on 10th, 20th and 30th post-inoculation days.

Rat bleeding was done by puncturing the retro-orbital venous plexus with thick pasteur pipette. Blood from 8 rats was pooled for collecting sera samples. The sera samples were inactivated and kept as above.

IIIrd Series

Bleedings were done on 10th, 20th and 30th post-inoculation days.

Pooled samples of blood from 2 guinea-pigs were used for collecting the sera. Bleeding was done directly from the heart. The sera samples were inactivated and kept as usual.

IVth Series

Here also, bleedings were conducted on 10th, 20th and 30th post-inoculation days.

Intra-cardiac bleeding was made in case of rabbits. Blood from two rabbits was pooled for collecting sera samples. The sera thus collected was inactivated and preserved in the same way as described above.

Vth Series

In this series of multiple inoculation bleedings were done on 10th, 20th and 30th post-inoculation days after 7th inoculation.

Sera were separated, inactivated and preserved as already described.

C. Single dilution Serum Neutralization Test

The method followed here was the same as that described by Raichowdhuri and Thomas (loc.cit.) with slight modification.

Serum virus mixture

The preserved CVS was diluted in 2% inactivated horse serum saline to get 25 LD_{50} in 0.03 ml.

From two CVS ampoules each containing 0.8 ml of infected brain suspension having LD_{50} titre $10^{-7.17}$, 1 ml was taken and mixed with 1 ml of diluent. This gave 10^{-1} dilution. To get 25 LD_{50} per 0.03 ml further dilutions were made as follows:

Details	Tube Numbers				
	1	2	3	4	5
Diluent (ml)	4.5	4.5	4.5	4.5	4.9 ml
Virus (CVS) 10^{-1} dilution	0.5 →	0.5 →	0.5 →	0.5 →	1.0 ml
Final dilution of the virus	10^{-2}	10^{-3}	10^{-4}	10^{-5}	25 LD_{50}

Separate pipettes were used while transferring virus suspension from one tube to the other during dilution.

The sera samples from all experiments were mixed with equal amount of 25 LD_{50} CVS. The serum-virus mixture will contain 12.5 LD_{50} per 0.03 ml. These samples were then kept in a water-bath at 37°C for 90 minutes. At the end of this period, the serum-virus mixture were kept on ice-bath till its inoculation into mice.

Now, for each serum sample a group of 8 mice of 3-5 weeks old (12-15 gms.) were inoculated intracerebrally with 0.03 ml of serum virus mixture. For each sample separate sterilized tuberculin syringe and 26 gauge needle were used.

Each group of inoculated mice were kept in separate galvanised iron cages labelled with the details of the experiment and observed for 14 days. Death within 5 days after inoculation was considered to be non-specific and was not incorporated in the result. But death of mice after 5 days showing symptoms like rough coat, tremor, complete or flacid paralysis were taken as due to rabies and recorded.

D. Virulent challenge

The route of virulent challenge for all series of experiments was intramuscular in the hind calf muscle. Pre-inoculation challenges were also done before the inoculation schedule in case of all the experiments.

In the Ist series of experiment a group of 8 mice was challenged on 10th and 20th post-inoculation days and 10 mice were inoculated on 30th post-inoculation day with mouse intramuscular CVS. The dose of challenge was 0.06 ml containing 6,61,000 mouse LD_{50}. The results were recorded in the same way as described earlier but here the period of observation was 21 days.

In the 2nd Series of experiment a group of 8 rats was challenged on 20th and 30th post-inoculation days with 0.25 ml of rat intramuscular CVS containing 6,92,000 mouse LD_{50}. The rats were observed for symptoms for 21 days and the results were recorded as usual.

In the 3rd series of experiments 7 guinea-pigs in case of groups I and II and 5 guinea-pigs in case of group III were challenged on 30th post-inoculation day with 0.25 ml of guinea-pig intramuscular CVS containing 20,90,000 mouse LD_{50}. The results were recorded as usual.

In the IVth and Vth series of experiments 7 rabbits of each of the three groups were challenged on 30th day after 1st inoculation and 7th inoculation respectively with 1.5 ml of rat intramuscular CVS containing 41,70,000 mouse LD_{50}. The results were recorded as above.

In VIth series of experiments all the rabbits were inoculated with 1.5 ml of rat intramuscular CVS containing

41,70,000 mouse LD_{50} and then treated with commercial antirabic Semple vaccine as well as with non-specific immunogen (M. phlei preparation) separately for 7 consecutive days. These rabbits were observed for 21 days and results were recorded as above.

E. Gel-diffusion Test

On the prepared petri dishes and slides containing gel, mentioned earlier, wells were made as per pattern drawn on paper with a cork borer. The central well was surrounded by 6 and 4 wells in petri dish and slide respectively having 4 mm diameter. The distance between the central and the peripheral wells was kept constant as 4 mm.

After proper sealing, the central well was charged with the antigen while with sera, collected at different definite intervals, in case of peripheral wells. The wells were recharged with respective material at twelve hours intervals for 7 days. All the charged plates and slides were incubated at 37°C for the first 8 hours and then at room temperature for 15 days. These were observed daily throughout the period for precipitation band.

RESULTS

RESULTS

For the assessment of the non-specific resistance, six series of experiments were conducted. The following *in vitro* and *in vivo* immunological reactions were observed in the 1st to 5th series of experiments, while the 6th series deals with treatment trial.

1. Serum neutralization
2. Refractoriness to virulent challenge
3. Gel-diffusion

1. Serum neutralization Test

(i) First Series

The effect of specific and non-specific immunogens at single inoculation level in mouse was measured here.

The specific immunising antigen induced a high increase of neutralizing antibody till 20th day and then a slow fall up to 30th day was noted (Table 1, graph I).

The non-specific immunogen shows parallel result inducing an increase in neutralizing substance on 20th day followed by slow fall on 30th day (Table 1, graph I).

In control group, cent per cent mortality was noted on 0-day and 20th day while 14.3% and 12.5% survivals were noted on 10th and 30th day (Table 1, graph I).

TABLE 1

EFFECT OF SINGLE INOCULATION IN MOUSE

Result of Serum Neutralization Test

Experiment	No. of animals used	Pre-inoculation			10th day			20th day			30th day		
		No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival
Group I													
Specific Immunogen	100	7	0	0	8	2	25	8	6	75	7	4	57.2
Group II													
Non-specific Immunogen (*M. phlei*)	100				8	2	25	8	6	75	8	4	50
Control	100				7	1	14.3	8	0	0	8	1	12.5

(ii) Second Series

The effect of single inoculation in rat was determined in this experiment.

In the specific immunogen group a steady rise of neutralizing antibody till 20th day and then a slow fall on 30th day was observed (Table 2, graph II). In the non-specific immunogen group though the percentage of survival was less on 10th day but more on 20th day and still more on 30th day than specific immunogen (Table 2, graph II).

In the control group, cent per cent mortality was noted on 0-day, while 12.5% survival on 10th and 20th day and 14.3% survival on 30th day was observed (Table 2, graph II).

(iii) Third Series

This part contained single inoculation experiment in guinea-pigs.

As depicted in table 3 and graph III, the survival percentage in both cases of both specific and non-specific immunogen was exactly identical. A sharp rise in titre was noted on 10th day itself which was maintained till 20th day followed by a gradual increase up to 30th day.

The control group showed cent per cent mortality on 0-day and 20th day whereas 12.5% survival on the 10th and 30th days (Table 3, graph III).

TABLE 2

EFFECT OF SINGLE INOCULATION IN RAT

Result of Serum Neutralization Test

Experiment	No. of animals used	Pre-inoculation			10th day			20th day			30th day		
		No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival
Group I													
Specific Immunogen	20				8	3	37.5	7	4	57.2	8	4	50
Group II													
Non-specific Immunogen (*M. phlei*)	20	8	0	0	8	2	25	8	5	62.5	8	6	75
Control	20				8	1	12.5	8	1	12.5	7	1	14.3

TABLE 3

EFFECT OF SINGLE INOCULATION IN GUINEA PIG

Result of Serum Neutralization Test

Experiment	No. of animals used	Pre-inoculation			10th day			20th day			30th day		
		No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival
Group I													
Specific Immunogen	7				8	4	50	8	4	50	8	5	62.5
Group II													
Non-specific Immunogen (M. phlei)	7	8	0	0	8	4	50	8	4	50	8	5	62.5
Control	7				8	1	12.5	8	8	0	8	1	12.5

(iv) Fourth Series

The data presented in Table 4, graph IV relates to single inoculation experiment in rabbit.

After a sharp rise of antibody titre in specific immunogen on 10th day, the maximum was noted on 20th day which was maintained up to 30th day.

In the non-specific group a significant augmentation of neutralising substance was observed on 10th day followed by a slow fall and then a gradual increase on 20th and 30th days respectively.

Cent per cent mortality was found on 0-day while 14.3% survival on 10th day and 12.5% on the last two days of experiments in the control group.

(v) Fifth Series

The effect of multiple inoculation in rabbit was assessed in this experiment.

It is evident from table 5 and graph V that with specific immunogen there was 50% survival on 10th day which led steadily to 100% on 30th day.

But in case of non-specific immunogen the maximum of survival percentage noted was 75 on 20th day starting from 62.5 on 10th day and came down to 50% on 30th day.

In the control group cent per cent mortality was noted on 0-day and 20th day whereas 14.3% and 12.5% mortality was noted on 10th day and 30th day respectively.

TABLE 4

EFFECT OF SINGLE INOCULATION IN RABBIT

Result of Serum Neutralization Test

Experiment	No. of animals used	Pre-inoculation			10th day			20th day			30th day		
		No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival
Group I													
Specific Immunogen	7				8	4	50	8	5	62.5	8	5	62.5
Group II													
Non-specific Immunogen (M. phlei)	7	8	0	0	7	5	71.5	8	4	50	8	6	75
Control	7				7	1	14.3	8	1	12.5	8	1	12.5

TABLE 5

EFFECT OF MULTIPLE INOCULATION IN RABBIT

Result of Serum Neutralization Test

Experiment	No. of animals used	Pre-inoculation			10th day			20th day			30th day		
		No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival
Group I													
Specific Immunogen	7				8	4	50	8	6	75	7	7	100
Group II													
Non-specific Immunogen (*M. phlei*)	7	8	0	0	8	5	62.5	8	6	75	8	4	50
Control	7				7	1	14.3	6	0	0	8	1	12.5

2. Refractoriness to Virulent Challenge

In order to see the efficacy in protection level produced by specific and non-specific immunogens, this challenge test was conducted.

(i) First Series

The results of single inoculation in mouse are tabulated in table 6 and graph VI. All the mice were challenged intramuscularly with 6,61,000 mouse LD_{50} of mouse CVS.

The survival percentage with specific immunogen was noted as 12.5, 25, 50 and in case of non-specific immunogen it was 25, 37.5 and 70 on 10th, 20th and 30th days respectively. Survival percentage in control group was nil on 0-day, 10th and 20th days while it was 20 on 30th day.

(ii) Second Series

The results of single inoculation in rat challenged intramuscularly with 6,92,000 mouse LD_{50} of rat CVS are shown in table 7, graph VII.

In specific immunogen group the survival percentage remained same (50%) on 20th and 30th day while in non-specific group 50 and 37.5% was noted on 20th and 30th days respectively. The control group showed 12.5% and 25% survival on 20th and 30th days respectively.

TABLE 6

EFFECT OF SINGLE INOCULATION IN MICE

Result of Virulent Challenge (intramuscular) with mouse CVS*

Experiment	No. of animals used	Pre-inoculation			10th day			20th day			30th day		
		No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival	No. inoculated	No. survived	% survival
Group I													
Specific immunogen	100	5	0	0	8	1	12.5	8	2	25	10	5	50
Group II													
Non-specific Immunogen (*M. phlei*)	100				8	2	25	8	3	37.5	10	7	70
Control	100				8	0	0	8	0	0	10	2	20

* Dose 6,61,000 mouse LD_{50}

TABLE 7

EFFECT OF SINGLE INOCULATION IN RAT

Result of Virulent Challenge (intramuscular) with rat CVS*

Experiment	No. of animals used	Pre-inoculation			20th day			30th day		
		No. challenged	No. survived	% survival	No. challenged	No. survived	% survival	No. challenged	No. survived	% survival
Group I										
Specific Immunogen	20	3	0	0	8	4	50	8	4	50
Group II										
Non-specific Immunogen	20				8	4	50	8	3	37.5
Control	20				8	1	12.5	8	2	25

*Dose 6,92,000 mouse LD_{50}.

(iii) Third Series

Table 8 and graph VIII reveal the effect of single inoculation in guinea-pigs as a result of intramuscular challenge with 20,90,000 mouse LD_{50} of guinea-pig CVS.

On 30th day the survival percentage noticed was 14.3 and 56.8 with specific and non-specific immunogens respectively.

No survival was observed in control group.

(iv) Fourth Series

The effect of single inoculation in rabbit is given in table 9 and graph IX. The animals were challenged with 41,70,000 mouse LD_{50} of rat CVS intramuscularly.

With specific immunogen the survival percentage was 42.9 and 57.2 with non-specific immunogen on 30th day. There There was 14.3% survival among the control group.

(v) Fifth Series

The effect of multiple inoculation in rabbit is given here. The animals were challenged with the same dose of CVS as in 4th series of experiment.

On 30th day the survival percentage was 71.5 and 85.8 with specific and non-specific immunogens respectively.

Among the control animals 28.6% survival was recorded

The results of this series are presented in table 10.

TABLE 8

EFFECT OF SINGLE INOCULATION IN GUINEA PIG

Result of Virulent Challenge (intramuscular) with guinea-pig CVS*

Experiment	No. of animals used	Pre-inoculation			30th day		
		No. challenged	No. survived	% survival	No. challenged	No. survived	% survival
Group I							
Specific Immunogen	7				7	1	14.3
Group II							
Non-specific Immunogen	7	3	0	0	7	4	56.8
Control	7				5	5	0

* Dose 20,90,000 mouse LD_{50}

TABLE 9

EFFECT OF SINGLE INOCULATION IN RABBIT

Result of Virulent Challenge (intramuscular) with rat CVS *

Experiment	No. of animals used	Pre-inoculation			30th day		
		No. challenged	No. survived	% survival	No. challenged	No. survived	% survival
Group I							
Specific Immunogen	7				7	3	42.9
Group II							
Non-specific Immunogen	7	2	0	0	7	4	57.2
Control	7				7	1	14.3

* Dose $41,70,000$ mouse LD_{50}

TABLE 10

EFFECT OF MULTIPLE INOCULATION IN RABBIT

Result of Virulent Challenge (intramuscular) with rat CVS *

Experiment	No. of animals used	Pre-inoculation			30th day		
		No. challenged	No. survived	% survival	No. challenged	No. survived	% survival
Group I							
Specific Immunogen	7				7	5	71.5
Group II							
Non-specific Immunogen	7	2	0	0	7	6	85.8
Control	7				7	2	28.6

*Dose 41,70,000 mouse LD_{50}

3. Gel-diffusion Test

An attempt was made to know whether there is any cross precipitating antigenic relationship between the rabies virus and M. phlei. Four sets of experiments were conducted as follows:-

(i) First set

In this test specific precipitation reaction between Arcton purified rabies antigen and heat inactivated RFV,Paris infected brain suspension treated sera from all the first five series of experiments were studied (Table 12).

The antigen, Arcton purified infected brain emulsion, was tested with known hyperimmune serum where two precipitation lines were noted (Fig. 1 and Plate I).

No precipitation line was noted with single inoculation experimental sera whereas one line was observed in multiple inoculation experiment on 20th and 30th day (Fig. 2).

(ii) Second set

Specific precipitation reactions between trypsinised M. phlei preparation as antigen and this non-specific immunogen treated sera from all the series of experiments were studied (Table 13).

Two precipitation lines with mouse and guinea-pig sera and 4 lines with rat and rabbit sera were found (Fig. 3, 4, 5 and 6) in single inoculation experiment.

Serum from multiple inoculation experiment in rabbit also revealed 4 lines (Plate II).

TABLE 12

GEL DIFFUSION TEST

Specific Precipitation Reaction with Rabies System

Days of collecting sera	Single inoculation experiment				Multiple inoculation experiment
	Mouse	Rat	Guinea-pig	Rabbit	Rabbit
	(Number of bands)				
10th	-	-	-	-	-
20th	-	-	-	-	1
30th	-	-	-	-	1

TABLE 13

GEL DIFFUSION TEST

Specific precipitation with M. phlei system

Days of collecting sera	Single inoculation experiment				Multiple inoculation experiment
	Mouse	Rat	Guinea-pig	Rabbit	Rabbit
	(Number of bands)				
10th	2	4	2	4	4
20th	2	4	2	4	4
30th	2	4	2	4	4

(iii) Third set

Here cross precipitation reaction between rabies antigen and M. phlei treated sera from all series of experiments was done. No line was observed in this test (Table 14).

TABLE 14

GEL-DIFFUSION TEST

Cross precipitation reaction between rabies antigen and M. phlei treated sera

Days of collecting sera	Single inoculation experiment				Multiple inoculation experiment
	Mouse	Rat	Guinea-pig	Rabbit	Rabbit
10th	-	-	-	-	-
20th	-	-	-	-	-
30th	-	-	-	-	-

(iv) Fourth set

Study of cross precipitation reaction with trypsinised M. phlei as antigen and the heat inactivated RFV, Paris, infected brain emulsion treated sera was also negative (Table 15).

TABLE 15

GEL-DIFFUSION TEST

Cross precipitation reaction between M.phlei antigen and heat-inactivated rabies infected brain emulsion treated sera

Days of collecting sera	Single inoculation experiment				Multiple inoculation experiment
	Mouse	Rat	Guinea-pig	Rabbit	Rabbit
10th	-	-	-	-	-
20th	-	-	-	-	-
30th	-	-	-	-	-

Fig. 1 Gel-diffusion test with rabies hyperimmune serum and Arcton purified virus.

1. Hyperimmune serum.
2 & 3. Arcton purified virus.
4 & 5. 20% rabbit infected brain.
6. Normal rabbit brain.

Fig. 2 Rabies system with rabbit sera from multiple inoculation experiment.

1. Arcton purified virus.
2. 20th day serum.
3. 30th day serum.
4. 20th day control serum.
5. 30th day control serum.

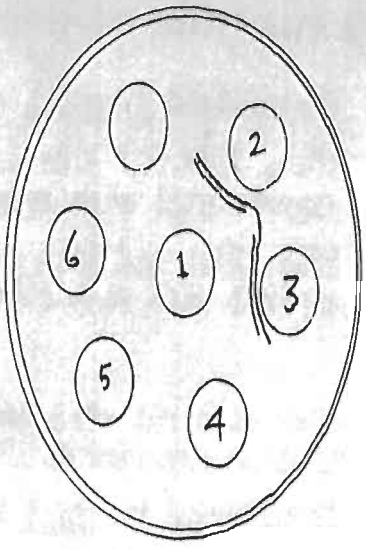


FIG - I

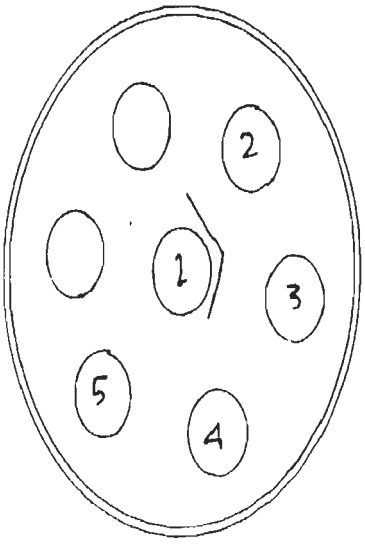


FIG - II

Fig. 3 *M. phlei* system with mouse sera from single inoculation experiment.

1. Trypsinized *M. phlei* antigen.
2, 3 & 4. 10th, 20th and 30th day experimental sera respectively.
5, 6 & 7. 10th, 20th and 30th day control sera respectively.

Fig. 4 *M. phlei* system with rat sera from single inoculation experiment.

1. Trypsinized *M. phlei* antigen.
2, 3 & 4. 10th, 20th and 30th day experimental sera respectively.
5, 6 & 7. 10th, 20th and 30th day control sera respectively.

Fig. 5 *M. phlei* system with guinea-pig sera from single inoculation experiment.

1. Trypsinized *M. phlei* antigen.
2, 3 & 4. 10th, 20th and 30th day experimental sera respectively.
5, 6 & 7. 10th, 20th and 30th day control sera respectively.

Fig. 6 *M. phlei* system with rabbit sera from single inoculation experiment.

1. Trypsinized *M. phlei* antigen.
2, 3 & 4. 10th, 20th and 30th day experimental sera respectively.
5, 6 & 7. 10th, 20th and 30th day control sera respectively.

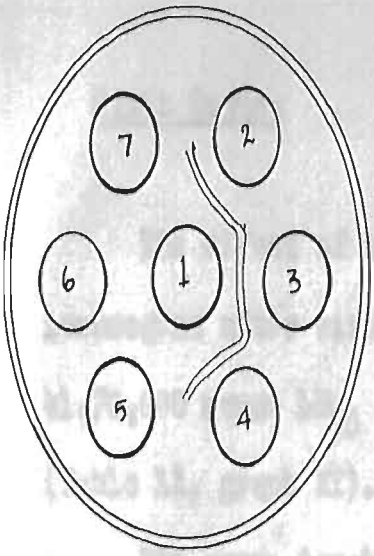


FIG - 3

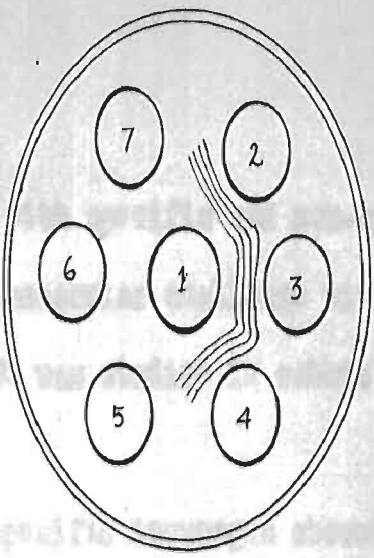


FIG - 4

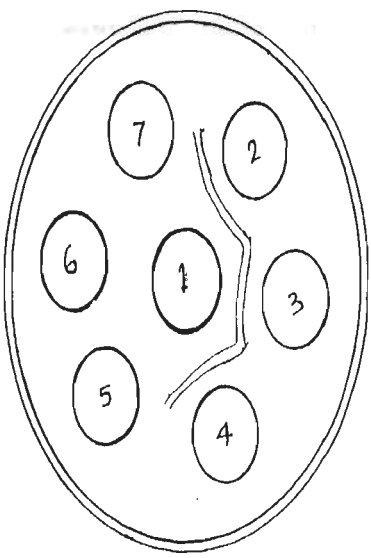


FIG - 5

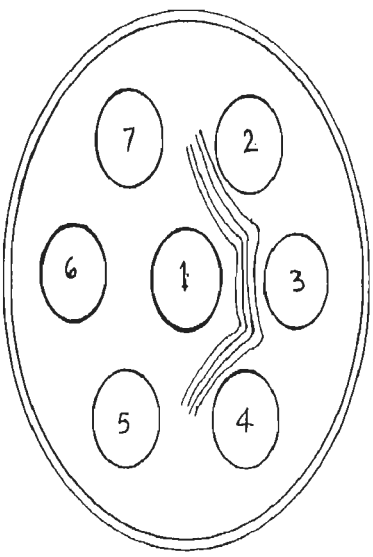


FIG - 6

Sixth Series

The effect of treatment with specific and non-specific immunogens after virulent intramuscular challenge with 41,70,000 mouse LD_{50} of rat CVS was studied in rabbit (Table 11, graph XI).

The group treated with specific immunogen showed 72.5% survival while in non-specific group 57.2% survival was noticed. Survival in the control group was 28.6 per cent.

TABLE 11

RESULT OF TREATMENT IN RABBIT(WITH Vaccine and M.phlei) AFTER CHALLENGE WITH RAT CVS *

Experiment	No. of animals treated	No. survived	% survival
Group I			
Specific Immunogen	7	5	72.5
Group II			
Non-specific Immunogen	7	4	57.2
Control	7	2	28.6

* Dose 41,70,000 mouse LD_{50}

DISCUSSION

DISCUSSION

The ability of the substances of microbial origin in modifying host resistance against heterologous infection is an established fact. Endeavours are being made by various workers to explore this phenomenon in the field where successful prophylaxis is lagging behind. And, the disease, rabies is the one where prophylaxis or therapy is still as cubersome as was in Pasteur's days. The explanation lies in the fact that the rabies virus is a very poor immunogen.

As a sequelae to the encouraging results obtained by Pandey and Mallick (loc. cit.) and Srinivasan and Mallick (loc. cit.) in the use of M. phlei in provoking non-specific resistance against rabies virus, further work has been pursued. They studied the effect of single and multiple inoculations of specific and non-specific immunogens in mice and sheep and assessed their protective efficacy by serum neutralization test, complement fixation test and virulent intracerebral challenge. In the present study an attempt has been made to determine non-specific resistance in four species of animals e.g. mouse, rat, guinea-pig and rabbit using intraperitoneal route for immunization and intramuscular for virulent challenge.

A trypsinised preparation of M. phlei has been used as non-specific immunogen. The importance of this substance in stimulating resistance has already been reviewed. Trypsinised preparation was of choice because on a comparative trial it has been found to be better than live or sonicated preparation (Pillet and Goret, 1966).

To study the effect of the non-specific immunogen, four species of laboratory animals were selected. These small animals were chosen in these preliminary studies because of certain advantages such as less expensive, easy to handle etc. over large animals. Many workers (Fox et al., 1966; Pandey and Mallick, loc.cit., Srinivasan and Mallick, loc.cit.) employed generally mouse and guinea-pig but here rat and rabbit have also been included. Moreover, it has been stated that generally rabbits produce maximum antibody much more quickly than guinea-pig (Raffel, 1961).

The intraperitoneal route was used for immunisation to evaluate over other routes e.g. subcutaneous, intramuscular which have been tried by Pandey and Mallick (loc. cit.), Mishra and Mallick (loc. cit.), Srinivasan and Mallick (loc. cit.) and Jana and Mallick (loc. cit.). Furthermore, Fox et al., (loc.cit.) also used intraperitoneal route for non-specific immunisation. Jana and Mallick (loc. cit.) also found intraperitoneal route to be the best for inducing

non-specific resistance among all the conventional routes.

It may also be pointed out referring to "instructional" hypothesis that the intraperitoneal route would be better for non-specific immunogen (Jawetz et al., 1968).

The experimental animals were challenged by intramuscular route, being the natural route of infection in case of rabies. So the CVS was standardised by passaging intramuscularly in different species as described in detail in "Material and Methods".

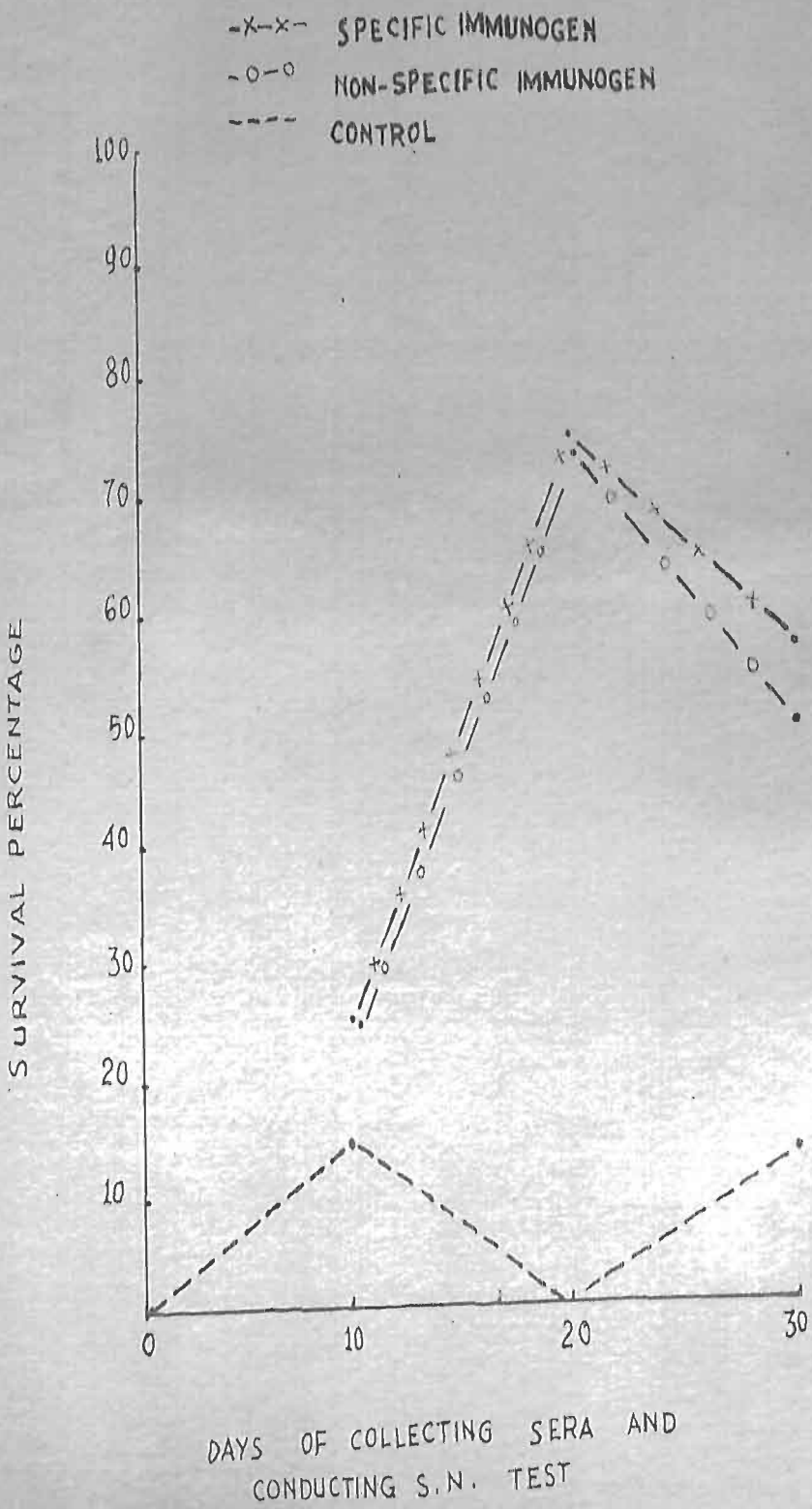
The neutralizing antibody/substance induced by both specific and non-specific immunogens in mouse was almost parallel. The maximum titre 75% attained at a later period, 20th day, followed by slow fall, 50% in case of non-specific and 57.2% in case of specific on 30th day (Graph I). The result differs to some extent from the findings of the previous workers (Pandey and Mallick, loc. cit.) where they observed early appearance and better resistance with non-specific immunogen.

The resistance status induced by single inoculation in mouse was judged by challenging with 6,61,000 LD_{50} of intramuscular mouse CVS. The percentage of survival with non-specific immunogen was found to be more than specific and the difference gradually widens till 30th day (Graph VI). However, level of protection at different stages with specific

GRAPH - 1

SERUM NEUTRALIZATION TEST

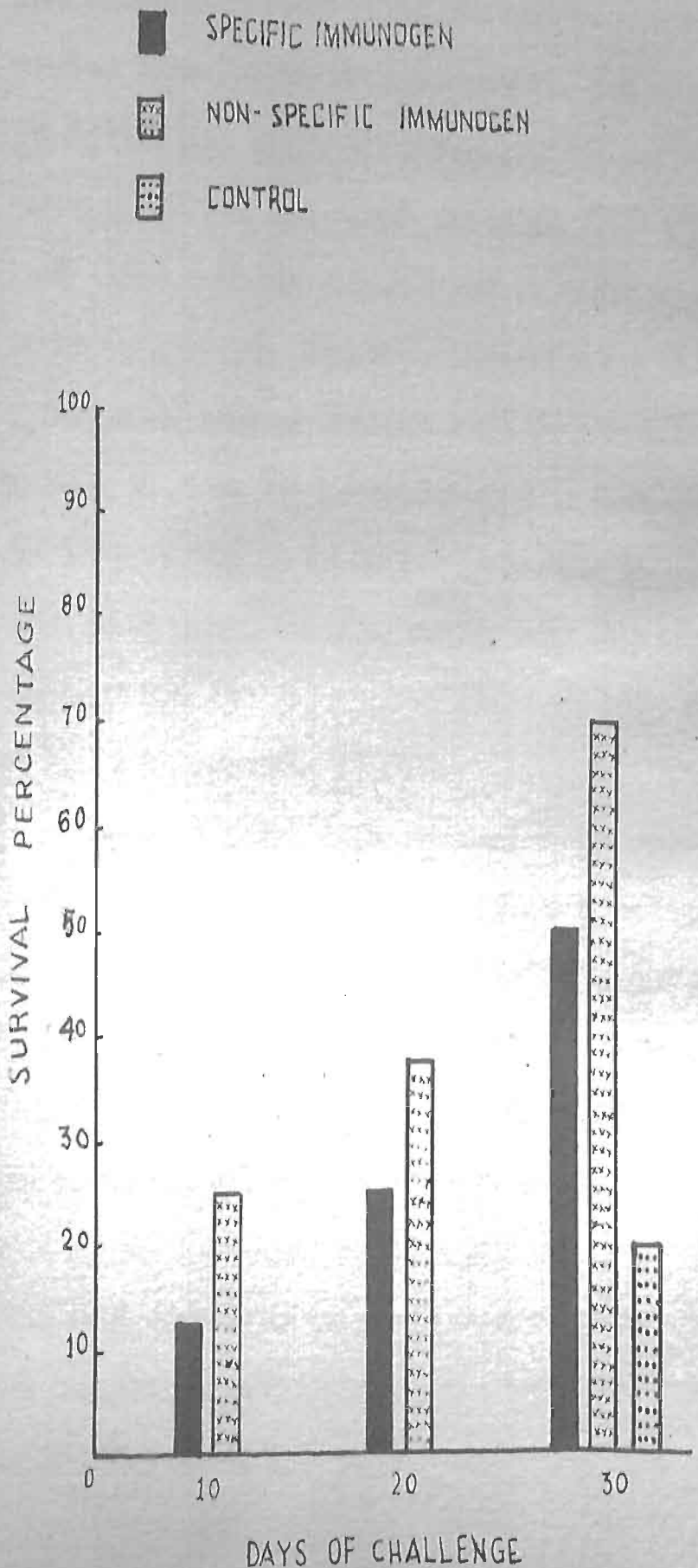
EFFECT OF SINGLE INOCULATION IN MOUSE



GRAPH - VI

CHALLENGE TEST

EFFECT OF SINGLE INOCULATION IN MOUSE



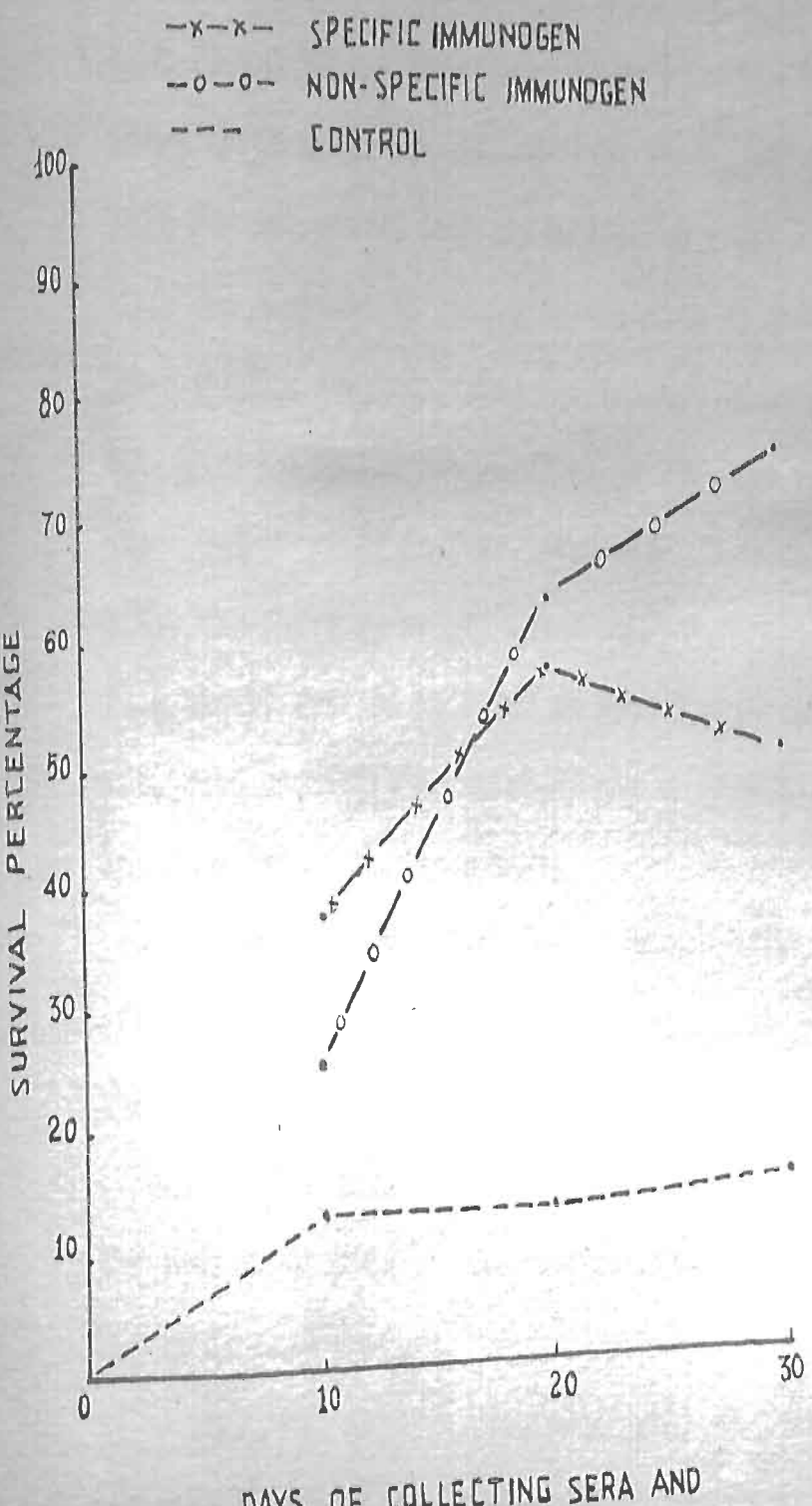
immunogen was in accord with the findings of the above workers, but although a better protection was noted with non-specific immunogen in the present study. The maximum protection was found to be on 30th day which confirms the work of Srinivasan and Mallick (loc. cit.), but differs from the observations of Pandey and Mallick (loc. cit.), Fox et al. (loc. cit.) and Landy (1956) where the increased protection was demonstrated in the early stages. This better resistance may be due to the intramuscular route of challenge where the possibility of the pathogen in facing the induced non-specific factors is more. The 20% protection in the control group found on 30th day seems to be unusual but other workers using this route of challenge have similar experience (Galloway, 1934; Gribencha et al., 1969; Schindler, 1961).

In rat, received single inoculation, the neutralizing antibody with specific immunogen was found to be highest on 20th day which came down very slowly to 50% on 30th day. While the neutralizing substance evoked by non-specific immunogen behaved ina different way. On 10th day the titre was comparatively lower which attained steadily to 62.5% on 20th day and then slowly to 75% on 30th day (Graph II). This is in contrast to the result obtained in mouse where the neutralizing antibody/substance attained its peak (75%) on 20th day.

GRAPH - II

SERUM NEUTRALIZATION TEST

EFFECT OF SINGLE INOCULATION IN RAT



It was noticed on challenge test (6,92,000 LD_{50} of rat CVS) that the percentage of survival was exactly the same (50%) with both on 20th day but on 30th day the protection with non-specific immunogen lowered down to 37.5% whereas the percentage with specific immunogen remained constant (Graph VII). This seems to be peculiar that though the titre in SN test with non-specific immunogen was maximum on 30th day but showed less protection on challenge test.

In response to single inoculation in guinea-pig, the neutralizing antibody/substance induced by both specific and non-specific agents ran parallel in the same manner as in mouse but differed in some respects. Here on 10th day, the titre was found to be 50% which maintained till 20th day and then slowly reached to 62.5% on 30th day (Graph III). Unlike rat, both neutralizing antibody and substance showed gradual increase from 20th day onward.

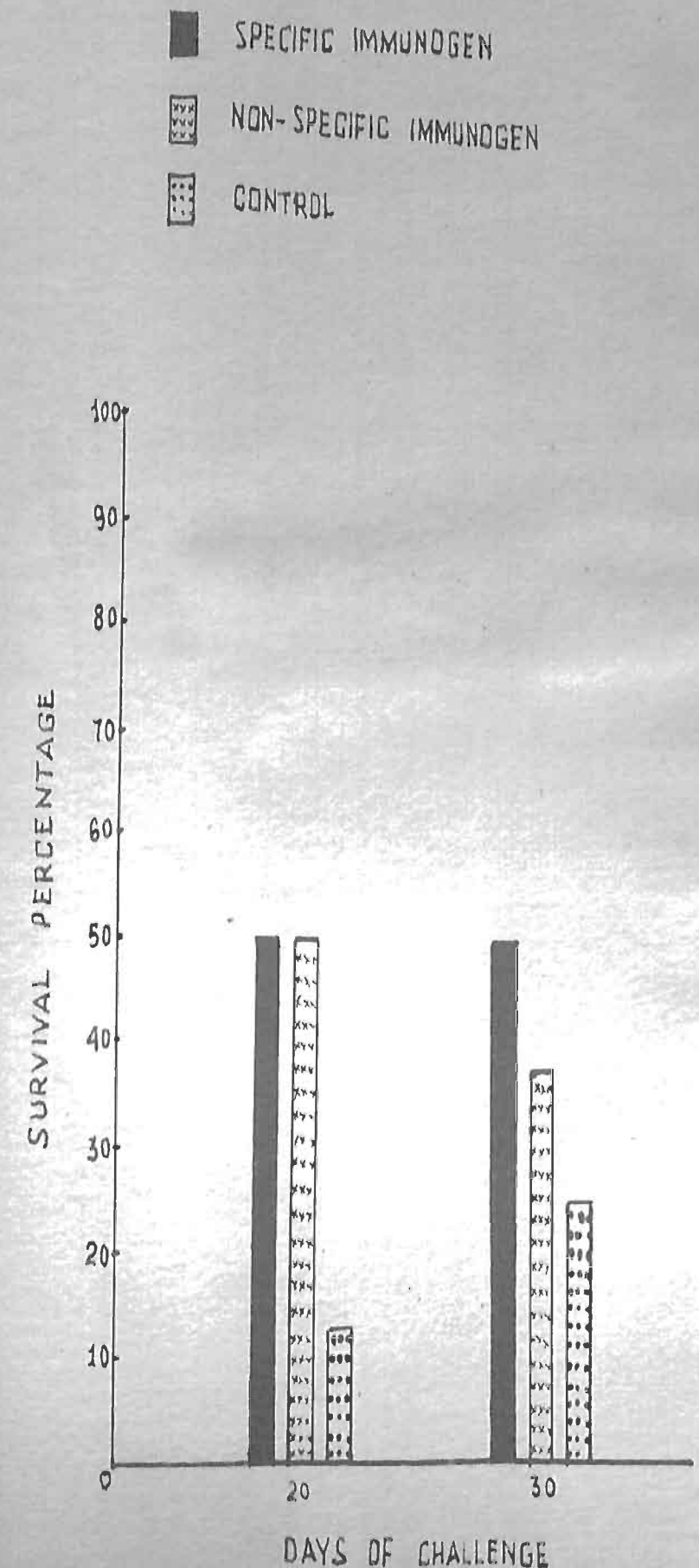
Guinea-pig inoculated with non-specific immunogen on challenge (20,90,000 LD_{50} of guinea-pig CVS) on 30th day showed considerably higher protection than the specific one (Graph VIII). This simulates the result found in mouse but the resistance given by non-specific immunogen was considerably higher in this species.

Challenge test was performed only on 30th day due to the non-availability of the required number of guinea-pigs and rabbits.

GRAPH - VII

CHALLENGE TEST

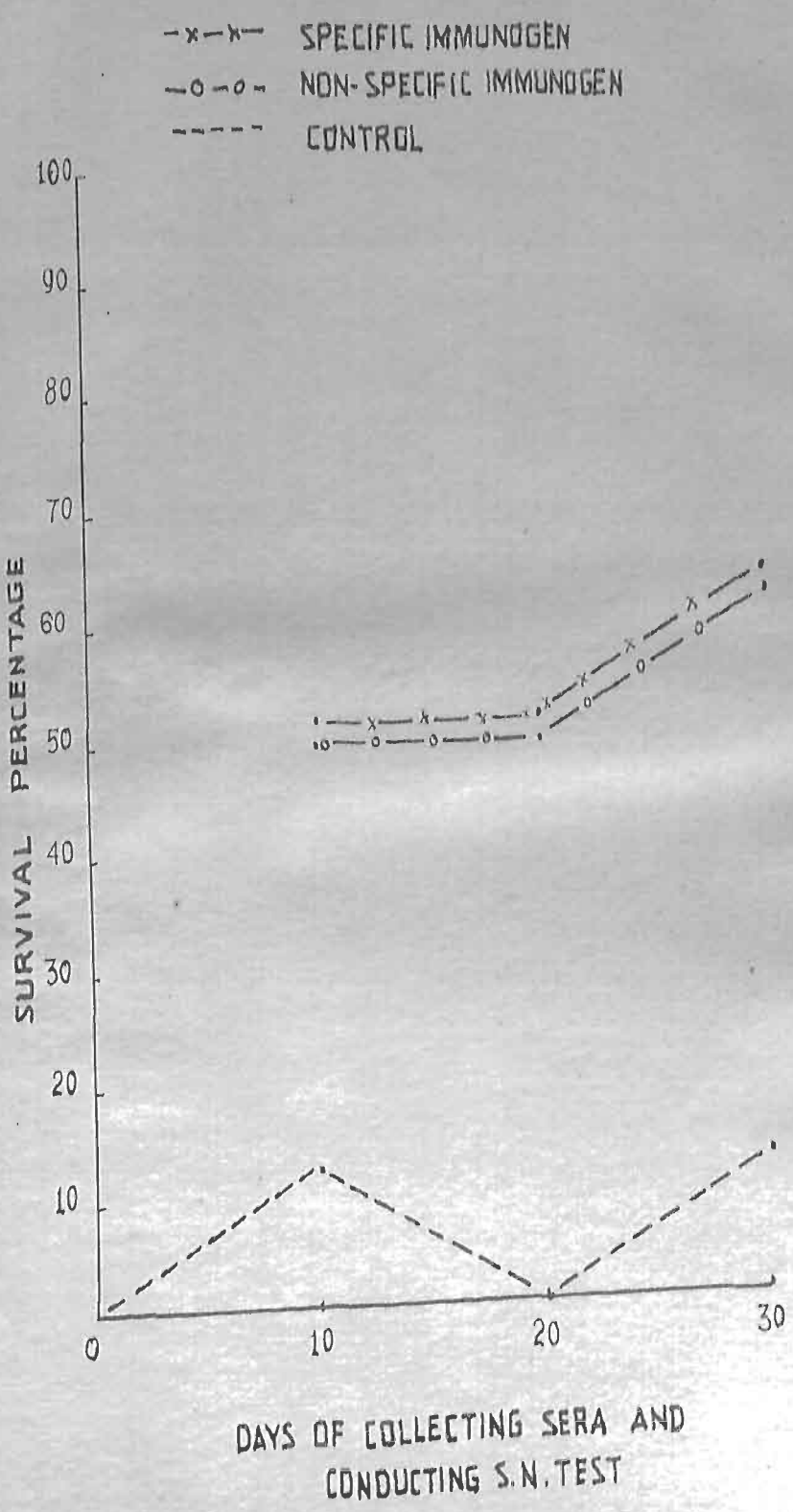
EFFECT OF SINGLE INOCULATION IN RAT



GRAPH - III

SERUM NEUTRALIZATION TEST

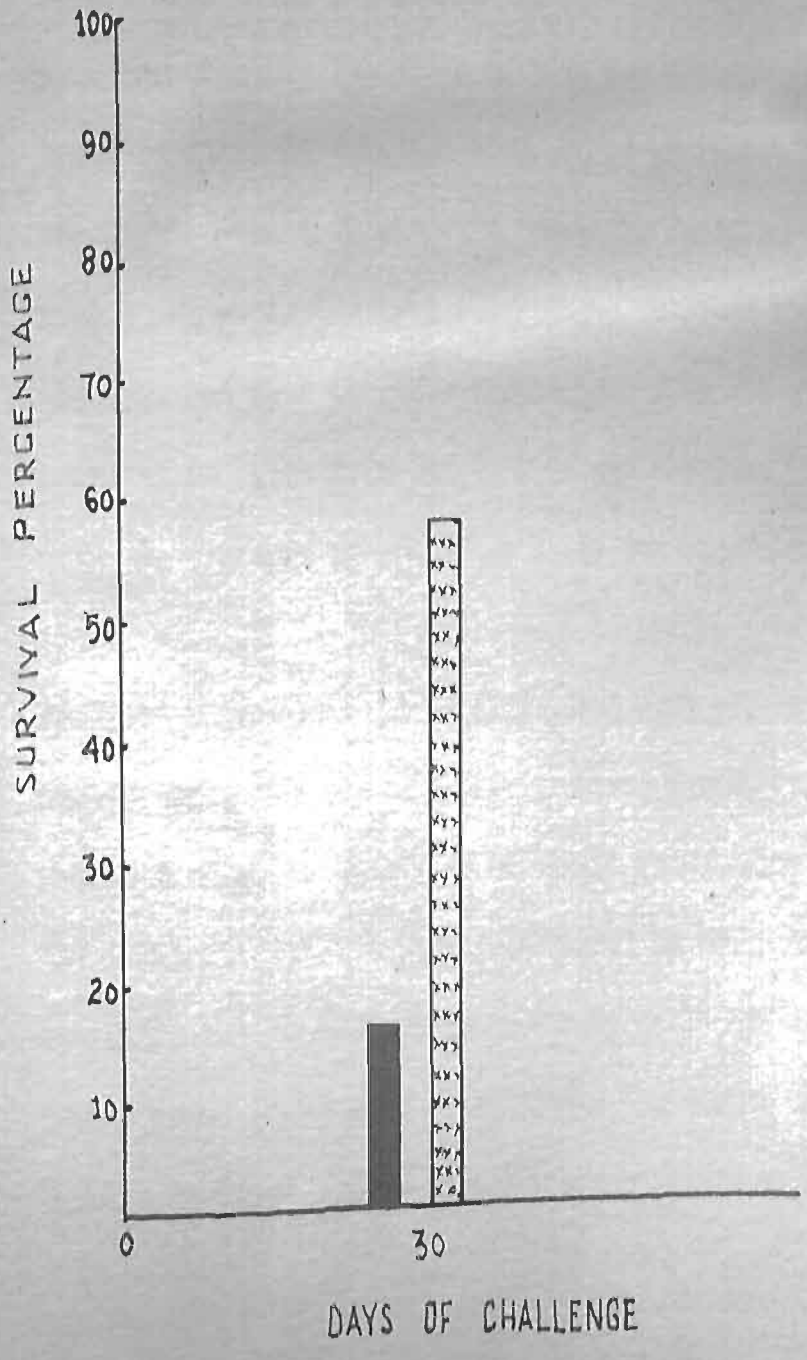
EFFECT OF SINGLE INOCULATION IN GUINEAPIG



GRAPH - VIII

CHALLENGE TEST

EFFECT OF SINGLE INOCULATION IN GUINEAPIG



Rabbit on single inoculation experiment in SN test with non-specific agent showed significant titre, 71.5% which after a fall (50%) on 20th day went up to 75% on 30th day. With specific, the titre started with 50% on 10th day, attained its maximum 62.5% on 20th and remained stationary till 30th day (Graph IV). Among all the species, maximum immunological response was noted in rabbit.

On virulent challenge (41,70,000 LD_{50} of rat CVS), rabbit like mouse and guinea-pig revealed better protection with non-specific immunogen on 30th day (Graph IX).

Multiple inoculation of M. phlei in rabbit yielded comparable result as achieved in single inoculation experiment in SN test. In both these cases, maximum titre recorded was 75%. But with specific immunogen a slow and steady increase in titre from 50% to 100% was observed (Graph V).

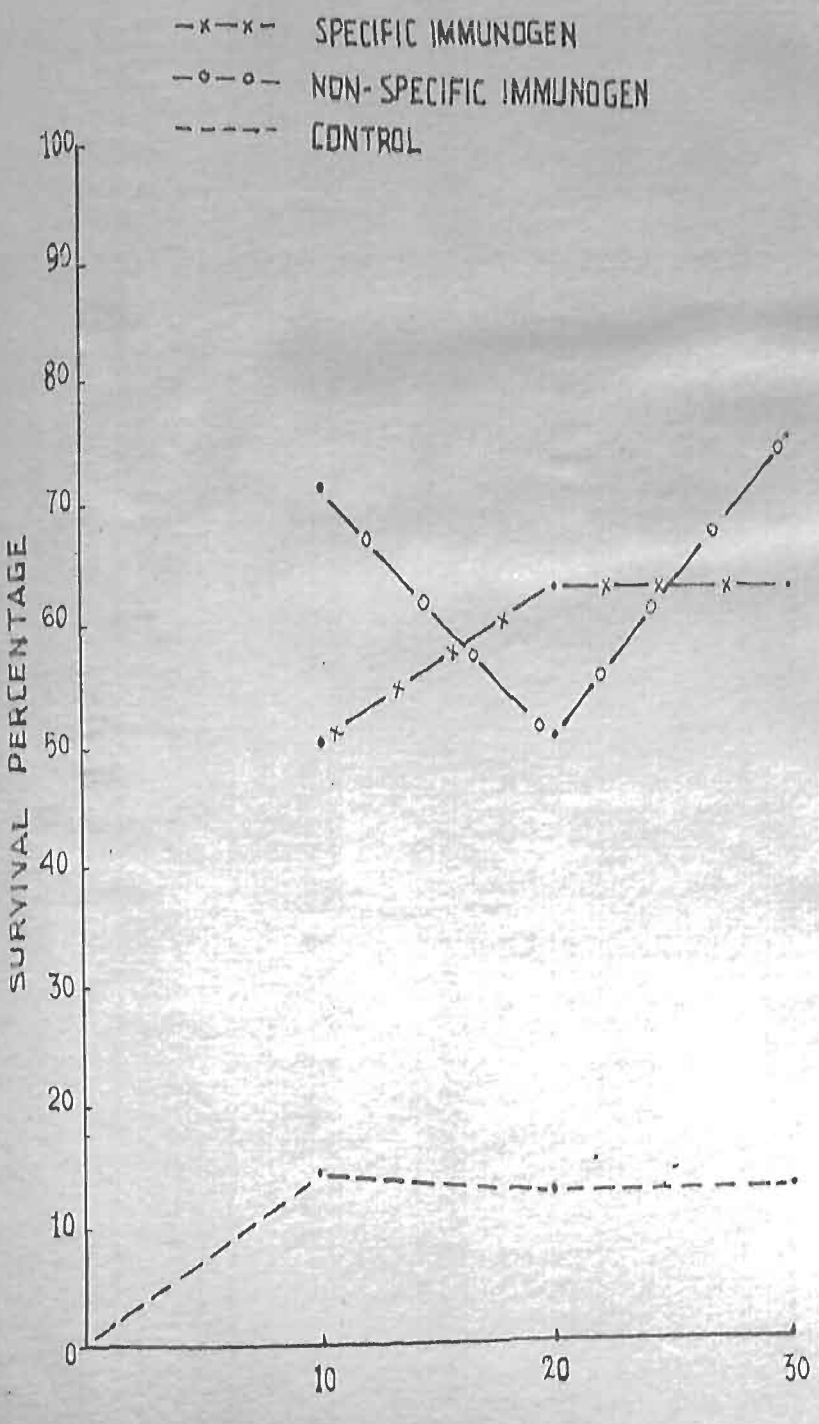
As assessed by challenge test, the non-specific immunogen on multiple inoculation showed better resistance than specific immunogen. It is also noted that both specific and non-specific immunogens elicited better response at 7th inoculation level than that of single inoculation (Graph X).

Gel-diffusion test was initiated in order to study any antigenic relationship between the specific and non-specific immunogens. To be sure of antigenic potency of antigen (Arcton purified rabies virus) tests were set up with

GRAPH. IV

SERUM NEUTRALIZATION TEST

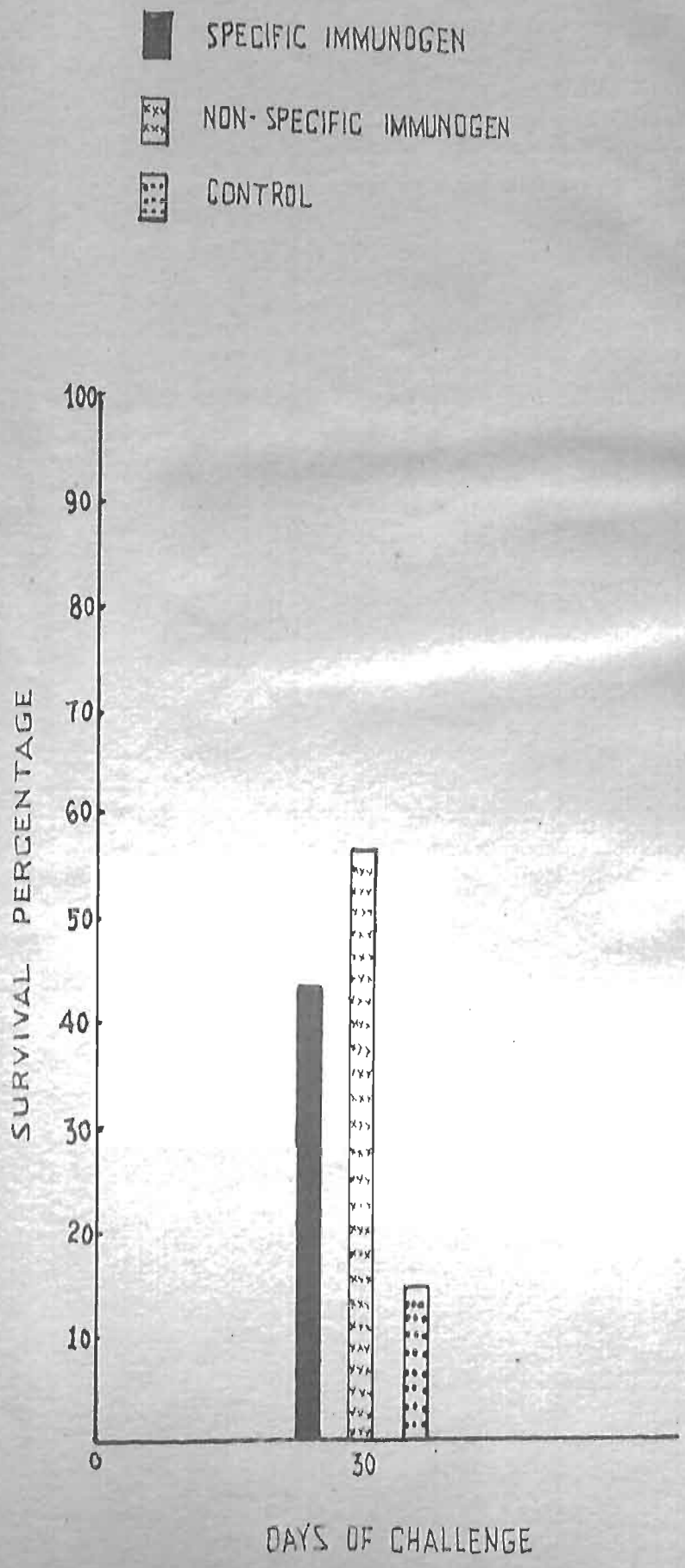
EFFECT OF SINGLE INOCULATION IN RABBIT



GRAPH-IX

CHALLENGE TEST

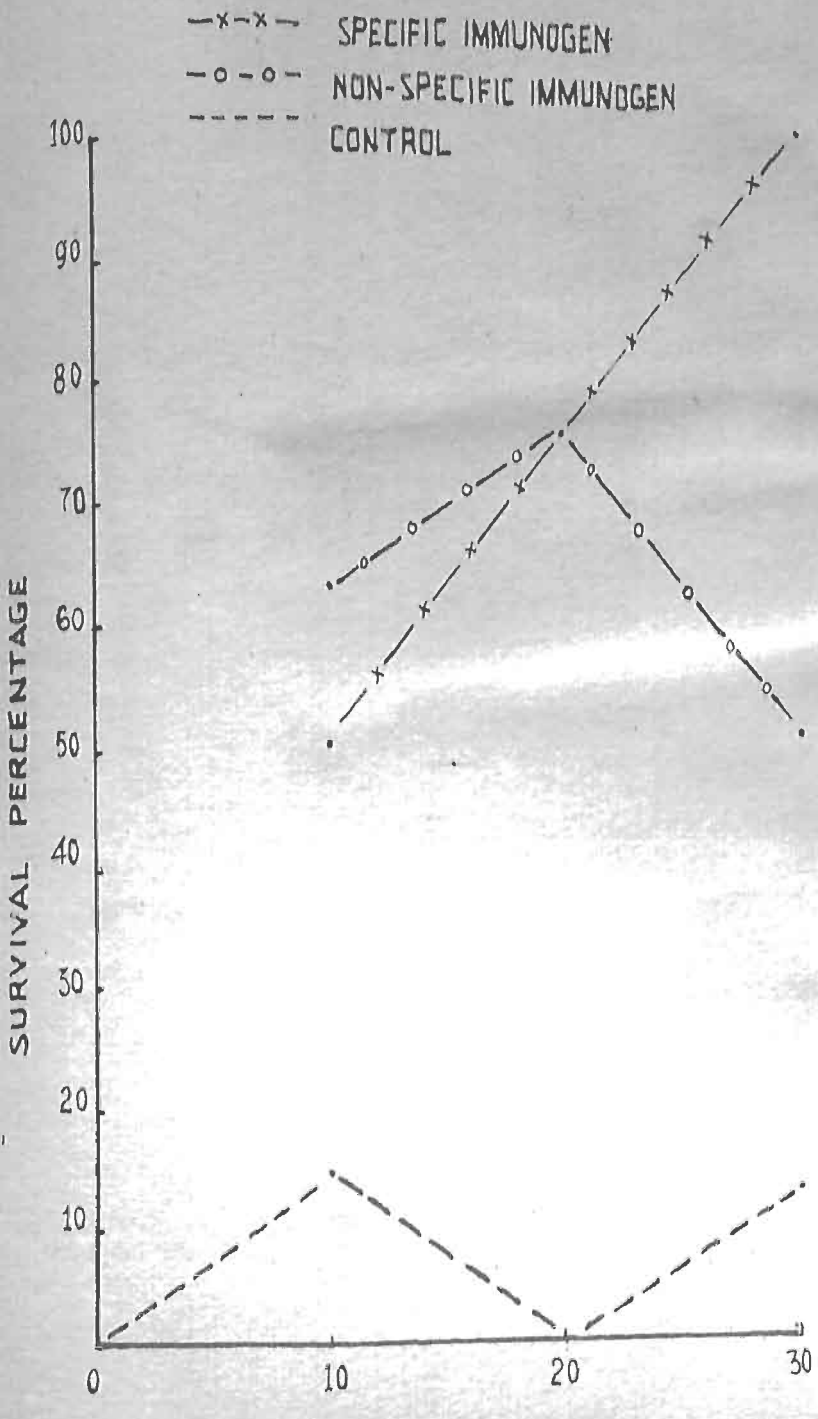
EFFECT OF SINGLE INOCULATION IN RABBIT



GRAPH. V

SERUM NEUTRALIZATION TEST

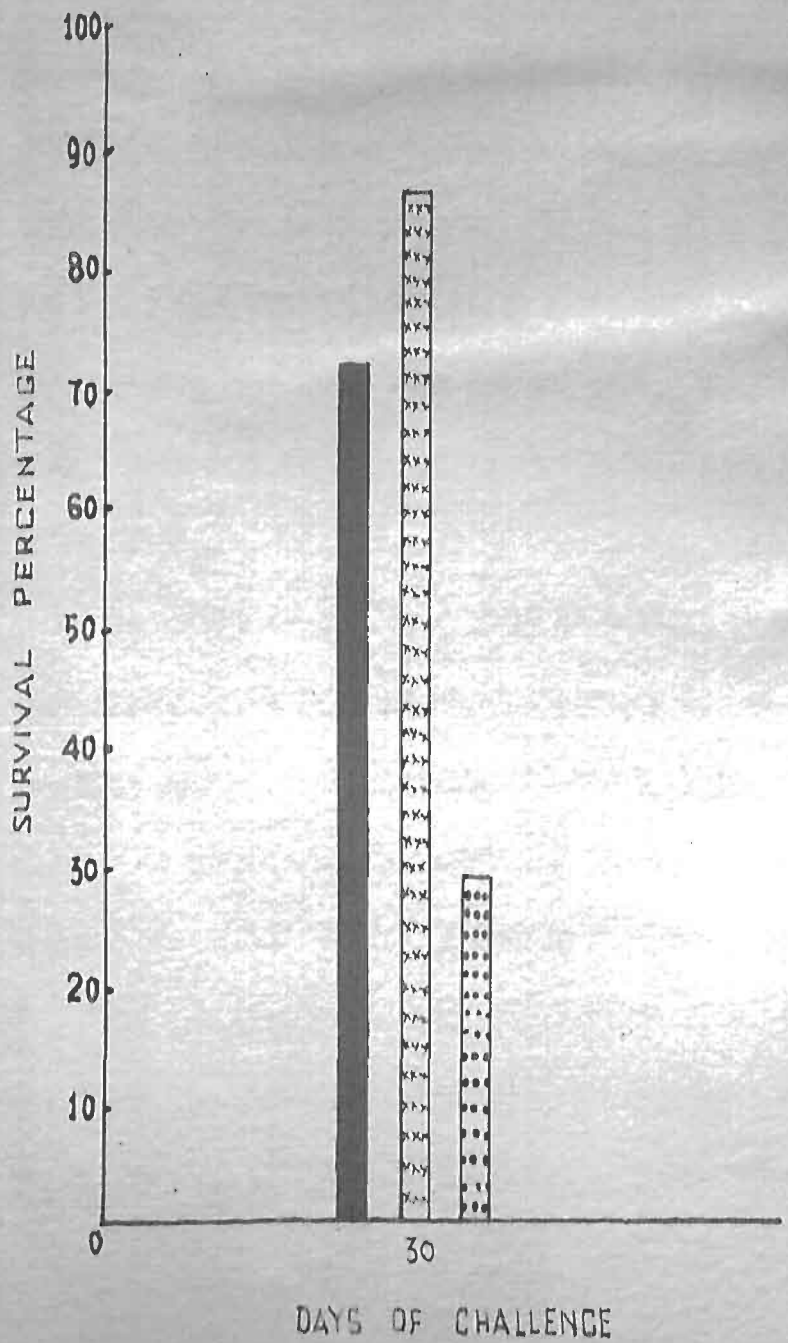
EFFECT OF MULTIPLE INOCULATION IN RABBIT



GRAPH-X

CHALLENGE TEST

EFFECT OF MULTIPLE INOCULATION IN RABBIT



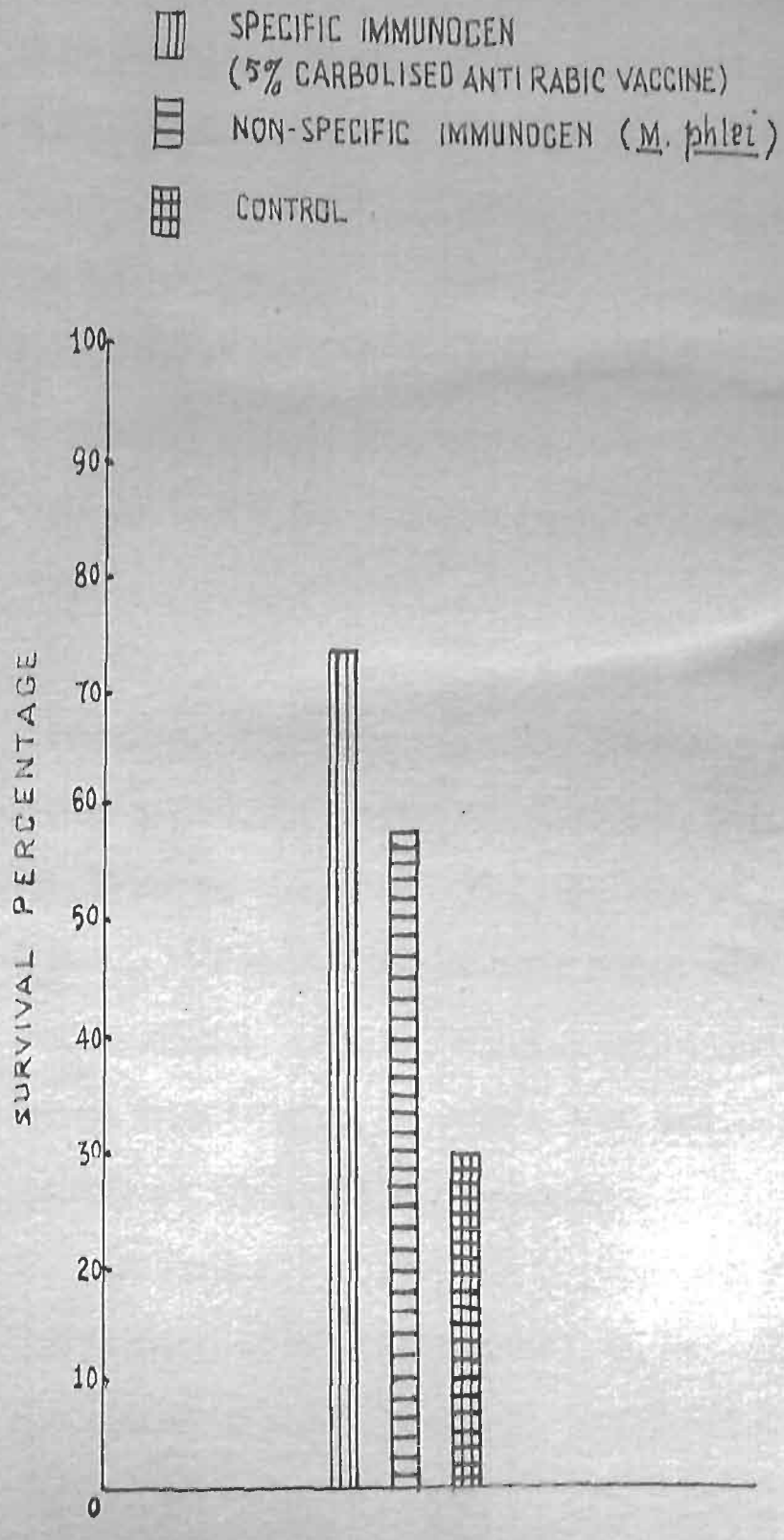
homologous hyperimmune sera and two precipitating bands appeared. This goes with the finding of Villemot and Provost (1958a). They used crude brain preparation as antigen. This standardised antigen was used throughout against homologous and heterologous experimental sera collected at different intervals. Only one precipitating band was demonstrable with multiple inoculated homologous sera of 20th and 30th day. But no line developed with single inoculated sera. Gresset (1966) suggested that precipitating lines with rabies system are not always demonstrable either because they are absent in certain conditions or they are present in too small quantities to be detected. In case of M. phlei system, two bands were noticed with mouse and guinea-pig experimental sera whereas four bands with rat and rabbit single inoculated sera. Sera from multiple inoculated rabbit gave four precipitating lines.

No precipitating lines could be noticed in the heterologous system which indicates that there may not be any precipitating antigenic relationship between M. phlei and rabies virus. So apparently, it seems that there is no correlation between protection and precipitating antibody/substance.

The possibility of employing non-specific immunogen in treatment was attempted. For this, rabbits were exposed with 41,70,000 LD_{50} of rat CVS intramuscularly and subsequently

GRAPH-XI

EFFECT OF TREATMENT AFTER CHALLENGE IN RABBIT



were considerably high on 30th day unlike previous workers (Pandey and Mallick, loc. cit., Srinivasan and Mallick, loc. cit

In this study efforts were made to understand the mechanism of resistance in rabies induced by M. phlei and heat inactivated rabies virus with the help of single inoculation serum neutralization, gel-diffusion and virulent challenge tests. Intramuscular route being the natural route of infection was taken for challenge unlike previous workers (Pandey and Mallick, loc.cit., Srinivasan and Mallick, loc. cit.). Exact assay of the serum neutralizing antibody/substance by conventional multiple serum dilution neutralization test could not be done due to obvious reasons explained in the text.

The mechanism of resistance to rabies infection awaits elucidation. Though Koprowski (1967) has claimed correlation between neutralizing antibody and protection, it is known that this is not the only factor. It is also observed by Lubke (1966) in mice against foot-and-mouth disease virus infection that non-specific substance induced by heterologous spleen extract inoculation and low specific serum neutralizing antibody act synergistically in protection.

The exact role of interferon in the process of protection in rabies is not assessed clearly but judging on the structure of the virus, Turner (loc. cit.) firmly believed

that interferon or interference might have some role. Fayaz et al. (1970) explained the protection of rabbits against rabies as a result of interference produced by pre-inoculation of parainfluenza 3 virus, mediated probably by interferon. Gorhe et al. (1968) suggested stimulation of interferon when an appropriate adjuvant Myco. tuberculosis, was inoculated in an experiment with foot-and-mouth disease virus in mice. So the possible role of interferon in protection induced by non-specific immunogen (M. phlei) in the present study cannot be simply ruled out. But a systematic study in this direction could not be taken up due to limited facilities.

Tompkins et al. (1970) indicated that both lymphocyte and macrophages participate in cellular immunity to viruses. It was reported that non-specific immunogen elicited lymphoid hyperplasia of spleen and Kuffer cells proliferation in liver (Fox et al. loc. cit.) and an increase in polymorpho-nuclear leucocytes in blood picture (Bandyopadhyay and Mallick, 1970). Mallick et al. (1970) found increase in betaglobulin (transferin) in mice which according to them might have some role in non-specific resistance. Detailed studies on these aspects could throw some light in the process of non-specific resistance

It may be emphasised from the present preliminary investigation that the non-specific immunogen afforded better resistance against rabies in majority of the species under

study in contrast to that of specific immunogen.

Hence, further studies on the following aspects may be extended:

1. Exact assay of neutralizing antibody/substance by multiple serum dilution test.
2. To pin-point the fraction of non-specific immunogen responsible for the resistance.
3. Characterisation of the non-specific substance(s).
4. The role of interferon in the resistance, if any.
5. Correlation between cellular changes and protection.

SUMMARY

SUMMARY

The present study has been conducted to find out the efficacy of a non-specific immunogen against rabies virus in mouse, rat, guinea-pig and rabbit with the help of serum neutralization, virulent challenge and gel-diffusion tests.

The non-specific immunogen used in this study was trypsinized M. phlei preparation.

The Paris strain of rabies fixed virus was employed in this investigation, as specific immunogen. For serum neutralization test, mouse CVS was used. But for intramuscular challenge test, CVS was standardised in different species of animals by giving four serial passages in mouse, rat and guinea-pig. In rabbit, rat intramuscular CVS was used.

Throughout the work intraperitoneal route was used for immunization while intramuscular for challenge.

In serum neutralization test, both specific and non-specific immunogens induced considerable antibody/substance in all the species. But non-specific immunogen afforded better resistance in rat and rabbit whereas the effect is parallel and little less in guinea-pig and mouse respectively than specific immunogen.

In challenge test better protection was observed with non-specific immunogen in mouse, guinea-pig and rabbit while rat exhibited almost parallel protection in comparison to specific immunogen.

Though serum neutralization titre with specific immunogen was slightly higher in some cases, it was interesting to note that almost always a better resistance was recorded with non-specific one in intramuscular challenge test.

With multiple inoculation experiment in rabbit, non-specific immunogen yielded comparable neutralizing substance as achieved in single inoculation experiment in serum neutralization test. But specific immunogen showed higher level of neutralizing antibody than that of single inoculation test.

Challenge test in multiple inoculation experiment in rabbit also revealed better protection with non-specific immunogen as observed in single inoculation test.

In gel-diffusion test with homologous rabies system, one precipitating line was noted in multiple inoculation experiment in rabbit. While with M. phlei system two bands in mouse and guinea-pig and four bands in rat and rabbit were observed in single inoculation experiment. Appearance of four bands was also noted in multiple inoculation in rabbit. No precipitating antigenic relationship could be detected between specific and non-specific immunogens.

In a comparative treatment trial in rabbit better protection was elicited with antirabic vaccine (5% commercial Semple vaccine). However, non-specific immunogen also showed encouraging results.

Thus, it appears, that non-specific immunogen evoked considerable resistance in all the species of animals included in this study.

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Plate I Gel-diffusion test with rabies hyperimmune serum and Arcton purified virus.

1. Hyperimmune serum.

2 & 3. Arcton purified virus.

4 & 5. 20% rabbit infected brain.

6. Normal rabbit brain.

Plate II M. phlei system with rabbit sera from multiple inoculation experiment.

1. Trypsinized M. phlei antigen.

2. 10th day serum.

3. 20th day serum.

4. 30th day serum.

5. Control serum.

2
6
1
3
5
4

2
3
4
5

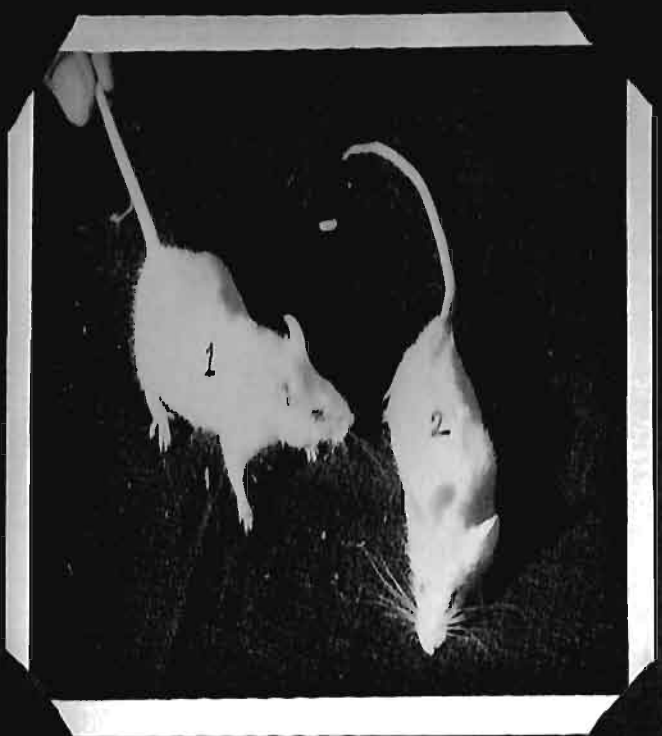
Plate III Mouse No.1, showing no symptoms. It was inoculated with 3 mgms. of M. phlei and subsequently resisted the intramuscular challenge with 6,61,000 LD_{50} of mouse CVS.

Mouse No.2 of control group showing paralytic symptoms on challenge with 6,61,000 LD_{50} of mouse CVS.

Plate IV Rat No.1 showing no symptoms. It was inoculated with 1½ mgms. of M. phlei and subsequently resisted the intramuscular challenge with 6,92,000 LD_{50} of rat CVS.

Rat No.2 of control group showing paralytic symptom on challenge with 6,92,000 LD_{50} of rat CVS.


1
2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2

Plate V. Guinea-pig No.1 showing no symptoms. It was inoculated with 30 mgms. of M. phlei and subsequently resisted the intramuscular challenge with 20,90,000 LD_{50} of guinea-pig CVS.

Guinea-pig No.2 of control group showing paralytic symptoms on challenge with 20,90,000 LD_{50} of guinea-pig CVS.

Plate VI Rabbit No.1 showing no symptoms. It was inoculated with 60 mgms. of M. phlei and subsequently resisted the intramuscular challenge with 41,70,000 LD_{50} of rat CVS.

Rabbit No.2 of control group showing paralytic symptoms on challenge with 41,70,000 LD_{50} of rat CVS.


1
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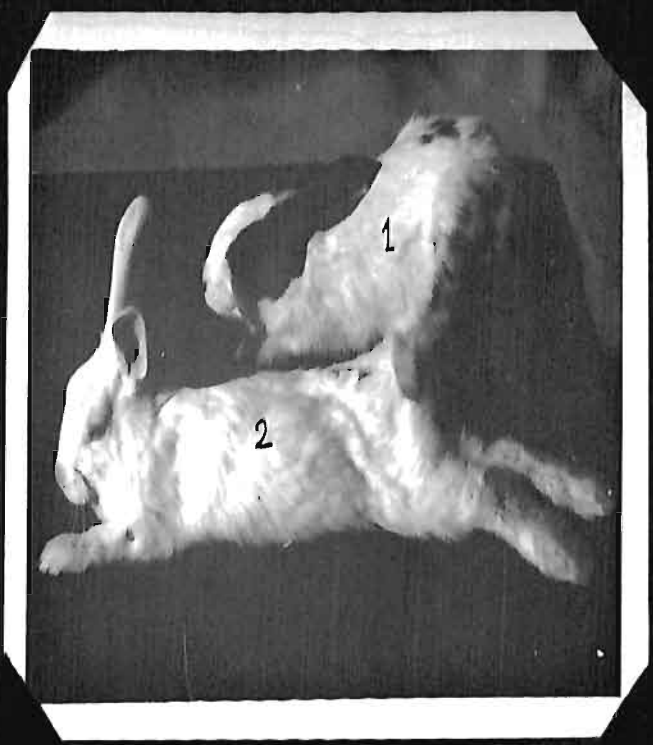
1
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