IMMUNOFLUORESCENT, TISSUE CULTURE AND RADIOIMMUNOLOGICAL STUDIES ON THE PATHOGENESIS OF RABIES

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ABSTRACT

Attempts were made to study different methods which might be useful for monitoring the pathogenesis of rabies. Immunofluorescent staining for rabies antigen, determination of blood and cerebrospinal fluid (biochemical) parameters, isolation of street rabies virus from field specimens on tissue culture and radioimmunoassay for quantitation of rabies antibody and antigen were the methods employed.

Immunofluorescent (IF) studies indicated that rabies antigen could easily be identified in the skin of naturally infected animals, particularly skunks and foxes but not in the skin of experimentally infected goats or dogs with short incubation periods. When the IF staining and examination was performed on the same set of skin specimens by 2 observers at different times, the results were comparable ($R = 0.934$). Postexposure immunoprophylaxis did not seem to interfere with the IF results on skin specimens from rabid animals in treated inoculated mice.

IF examination of cranial nerves dissected from goats and dogs experimentally inoculated in the masseter muscle with street rabies

revealed that the negative results obtained were directly related to a paucity of rabies antigen in nerves innervating the skin. The salivary glands from these experimental animals likewise contain little or no rabies antigen despite the presence of large quantities of antigen in the brain.

In another study, brain specimens from rabid and normal animals preserved in 10% buffered formalin was used for direct immunofluorescent staining after treatment with 0.25% trypsin in Hanks balanced salt solution containing additional 0.02% calcium chloride (pH 7.8). The results obtained were comparable to those obtained with direct IF staining of frozen cryostat cut sections or standard smears. Specimens shipped in 10% buffered formalin might minimize risk of exposure to handlers, prevent tissue deterioration and be useful for retrospective studies.

Serum and cerebrospinal fluid samples from goats and dogs experimentally inoculated with street virus were checked for changes in the levels of albumin, globulin, creatine phosphokinase, glutamic oxalacetic transaminase, glucose and chloride. In addition the CSF was checked for total cell counts and total protein. Values obtained for serum chemistry of both the goats and the dogs and the CSF chemistry of the dogs were within the reported normal ranges. The CSF of the rabid goats showed elevation in mean total protein value and albumin, mean CPK values, and mean total cell counts but the absolute values overlapped with the reported normal range of values. The determination of blood and CSF constituents in monitoring the stage of infection with rabies did not produce results which were useful.

Skunk kidney cells, skunk brain cells and raccoon kidney cells were

compared with CER, a baby hamster kidney, a murine neuroblastoma and a dog kidney cell line for isolation and propagation of street rabies virus from naturally and experimentally infected animals. The skunk and raccoon kidney cells did not seem suitable for primary isolation of rabies virus from naturally infected animal brain suspensions but CER, murine neuroblastoma and the skunk brain cells were suitable. Rabies virus could be adapted to grow in all the cell cultures by serial subcultivation of the infected cells.

Solid-phase radioimmunoassay using [^{125}I]-protein A could be used to measure specific binding antibody in the sera of vaccinated rabbits and human beings and in the serum and cerebrospinal fluid samples from experimentally inoculated goats and dogs. Attempts were made to adapt the procedure for quantitation of rabies antigen in the brain suspensions of clinically rabid animals. The results indicated that the method as employed was not suitable for detection of antigen in the brain suspension.

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CHAPTER I

GENERAL INTRODUCTION

After an individual is exposed to rabies, the virus, being neurotropic, is picked up by peripheral nerve endings at the point of exposure. It travels passively in the axoplasm towards the central nervous system (CNS) at a speed of about 0.02 mm per sec (Ercegovac, 1976). The first site to be affected within the CNS depends on the site of exposure. The lumbar spinal cord is first affected following hindleg inoculation, cervico-thoracic cord following foreleg inoculation and the brain stem following masseter muscle inoculation (Baer et al., 1965, 1968). In the CNS, there is virus replication and budding upon the plasma membranes of neurons and their processes. This may be followed by passive movement of the released virus in the interstitial fluid compartment between the cells or by direct transfer of the released virus from cell to cell (Iwasaki and Clark, 1975). Mononuclear cell infiltration, perivascular cuffing, gliosis, satellitosis, neuronophagia and local antibody synthesis follow CNS infection. Then, there is a centrifugal spread of the virus resulting in virus generalization (Schneider, 1975) as the disease progresses. This virus generalization results in a variety of tissues becoming available for the study of virus infection and for diagnosis. While the phenomena of virus spread and mobility are usually viewed from the perspective of the suitability and characteristics of the host, there can be little doubt but that adaptive characteristics of the infecting virus are also intimately involved.

Immunofluorescent staining (IF) and electron microscopy (EM) have contributed significantly to the delineation of the above pattern of

pathogenesis. IF is the most useful diagnostic test currently available (World Health Organization, 1973). For good results, the staining has to be performed on sections cut or smears made from fresh or frozen tissues followed by acetone fixation or on smears of tissues preserved in glycerol without acetone fixation. There is some risk of infection involved in the use of this technique since acetone fixation at -20°C does not completely eliminate infective virus (Fischman and Ward III, 1969); not using acetone fixation poses an even greater risk of laboratory infection.

In many experiments on the pathogenesis of street rabies infection, the quantity of virus present in different tissues might need to be determined. This quantification has to be done in mice since the presently available cell lines do not support efficient growth of virus from field specimens. CER and murine neuroblastoma (NA) cell lines (Smith et al., 1978) are offering new hope; however, there is still need for the development of a cell culture system that could be used for primary isolation of field strains. In pursuit of this goal, it seemed logical to utilize a cell system derived from an in vivo host, known to be a highly efficient reservoir, and to which the field strains of virus are likely to be highly adapted. Such a cell system might be derived from the brain or other tissue of the striped skunk.

Early detection of rabies antigen and antibody is necessary for the understanding of the early events in rabies infection. In the early stages of the infection, only small amounts of antigen are produced. Recently, a Workshop on the Immunopathology of Rabies (Shope et al., 1979) suggested that radioimmunoassay for viral antigen and in situ

hybridization with oligonucleotide probes for viral genomes might be applicable to this problem.

Based on the foregoing information, the main objectives of the work reported in this dissertation were:

1. To determine the reproducibility of results obtained by IF staining for the study of rabies virus antigen in the skin of some infected animals.

2. As a means of explaining the sometimes negative results obtained when examining the skin of infected animals, to study the dissemination of rabies virus into the cranial and other nerves.

3. Because of the ever present difficulties in securing adequate specimens for examination when shipment and preservation is needed, to determine if any modification can make formalin-fixed tissues suitable for examination by IF.

4. To evaluate changes which occur in the cerebro-spinal fluid (CSF) and blood serum early in the course of the infection as an indirect means of antigen detection.

5. Using tissues from a maintaining host species highly favorable for the replication of rabies virus, to develop a new cell culture system which could be used for the primary isolation of rabies virus.

6. To adapt solid-phase radioimmunoassay as a sensitive method for the detection of rabies virus antigen and antibody.

7. To ultimately draw conclusions concerning the pathogenesis of rabies and the sensitivity of detection systems and further the assessment of the adaptive abilities within the host agent relationship.

CHAPTER II

LITERATURE REVIEW

Immunofluorescent Staining

In 1950, Coons and Kaplan reported the application of fluorescein isocyanate and its use to label globulin. This was the birth of immunofluorescent staining. Later, Riggs et al. (1958) reported the synthesis of a more stable fluorescein isothiocyanate (FITC) which could be conjugated to globulin (Marshall et al., 1958). Goldwasser and Kissling (1958) adapted immunofluorescence for identifying rabies antigen in naturally infected animals. This soon became a method of choice for routine diagnosis in many public health laboratories (McQueen, 1959; Etchebarne et al., 1960; Lennette et al., 1965).

Principle of Immunofluorescent Staining

A specific immunoglobulin, purified by precipitation with ammonium sulfate and dialysis against 0.85% saline or by gel filtration with suitable Sephadex, is conjugated with a fluorochrome, a substance which emits light of longer wavelength than that of the excitation absorption (Kawamura, 1977). The emitted wavelength is within the visible spectrum. When the conjugate is applied to tissue sections or impression smears containing specific antigen, there is antigen-antibody complex formation. Excitation with ultraviolet light causes the fluorochrome to emit a visible light. This is the direct immunofluorescent method. In the indirect method, an unlabelled antibody is reacted with a known or unknown antigen. Then the globulin of the resulting antigen-antibody complex is

stained with specific antiglobulin conjugate.

Factors Affecting Immunofluorescent Staining of Rabies Antigen

The quality of the conjugate is very important. This depends on the quality of the antiserum which in turn depends on the quality of the immunizing antigen and the animal immunized. The use of purified antigen, containing a low level of tissue protein, obviates the need for normal mouse brain absorption, allows for the use of a high dilution of the conjugate which reduces the chance of nonspecific staining, and affords a dark background and great contrast (Trimarchi and Debbie, 1974). Nonspecific fluorescence can result from excessively labelled globulin. However, passing conjugated globulins through a DEAE column, collecting various fractions and pooling those with the desired qualities reduces nonspecific fluorescence (Kissling, 1975). Antiserum for conjugate production can be produced in any species of animals. Hamsters (Lennette et al., 1965), equine (Dean and Abelseth, 1973), goat (Forster and Wachendorfer, 1971), rabbit (Trimarchi and Debbie, 1974), bovine antisera and mouse ascitic fluid have been used. FITC has been widely used in rabies immunofluorescence. It has a maximum absorption peak in the blue range at 490 nm and an emission peak of 525 nm which gives a green fluorescence (Nairn, 1969). FITC reduces autofluorescence. Due to negative charges on the conjugate, it is easily adsorbed nonspecifically by tissue sections, therefore the molar ratio of FITC to protein of 1-2 is desirable (Kawamura, 1977).

Good results are obtained in fresh or frozen specimens. Specimens preserved in glycerol give large amounts of autofluorescence

(Goldwasser and Kissling, 1958), do not produce satisfactory impressions unless washed thoroughly in saline (Tierkel, 1973) and are difficult to cut in a cryostat. Elimination of acetone fixation during processing increases the intensity of IF staining of glycerol-saline preserved tissues (Andrulonis and Debbie, 1976).

There is difficulty in attempting to identify rabies virus antigen by IF in partially decomposed specimens. The number of specimens arriving at laboratories in a deteriorated state is small but significant. In the Michigan Department of Health which receives about 2000 specimens yearly, between 40-80 are deteriorated and unfit for examination when received (Lee and Becker, 1972). Deterioration results from the method of killing of the animals (Lee and Becker, 1972), delay before the animals are found, delay during transit to the laboratories or inadequate refrigeration (Lewis and Thacker, 1974). Positive results are reliable but the dependability of negative results is uncertain (Lewis and Thacker, 1974). Tissues which have been used for IF diagnosis of rabies include brain, spinal cord (Lee and Becker, 1972), skin (Smith et al., 1972), cornea (Schneider, 1969b; Larghi et al., 1973) and salivary glands (Goldwasser et al., 1959). The impressions or sections can be stained unfixed, fixed in acetone (Kissling, 1975) or in ethyl alcohol (Fischman and Ward, 1969). Properly fixed smears or sections in acetone fluoresce better than unfixed (Kissling, 1975). Fixation in ethyl alcohol increases nonspecific staining (Kissling, 1975).

Other factors effecting rabies IF are the experience, training and proficiency of the diagnostician (McQueen, 1959; Lennette and Emmons, 1971); use of adequate controls; adequate sampling of the specimen;

proper storage of conjugate; adequate pH control and the use of an adequately equipped fluorescent microscope.

Application of IF in Rabies

As a diagnostic tool IF is sensitive, specific (McQueen et al. 1960), accurate, rapid and inexpensive. It is the most widely used test for rabies (Dean and Abelseth, 1973). The time required for the test can be reduced to 45 minutes by eliminating acetone fixation and incubating the conjugate-impression reaction at room temperature for 10 minutes, without any adverse effect on diagnosis (Larghi and Jimenez CH, 1971). Fluorescing rabies antigen may still be seen in putrid specimens after mouse inoculation and Seller's stain examinations are no longer effective methods of diagnosis (Lewis and Thacker, 1974). IF has contributed significantly to intra-vitam diagnosis of rabies (Schneider, 1969b; Smith et al., 1972; Bryceson et al., 1975) and has been used extensively for the studies of the pathogenesis of the infection (Dean et al., 1963; Murphy et al., 1973; Baer et al., 1965).

The sensitivity of IF has been evaluated by many workers (McQueen et al., 1960; Etchebarne et al., 1960; Lee and Becker, 1973). The review by Jentzsch (1967) updated by Kissling (1975) indicates that 87 (2.9%) of 3,049 cases reported positive by animal inoculation were negative by IF test. This was based on published works from many laboratories and it is conceivable that the workers performing the tests had different degrees of competence or other unknown variables existed. If performed by a competent laboratory worker, IF presumably identifies 98% of rabies infected specimens. Mouse inoculation is slightly more sensitive than

IF but the agreement between the two tests is equally 99% or higher if performed by competent laboratory workers (Kissling, 1975).

Centripetal and Centrifugal Spread

Goodpasture (1925) theorized the neural transport of rabies virus. Dean et al. (1963) first reported the presence of virus in peripheral nerves. Amputation experiments (Dean et al., 1963 and Baer and Cleary, 1972) supported this theory. A sparing effect was obtained when sciatic neurectomy was performed prior to footpad inoculation (Baer, et al., 1965; Dean et al., 1963). After inoculation there is a period of about 3 hours during which the virus cannot be demonstrated (Schneider, 1969a). Hematogenous route of transport of rabies virus is an exception rather than the rule (Dean et al., 1963).

Early Events - Centripetal Spread

After deep intramuscular inoculation with rabies virus, there is a retention and replication of the virus in the muscle cells (Murphy and Bauer, 1974; Murphy et al., 1973; Charlton and Casey, 1979). Following the intramuscular inoculation of striped skunks with a skunk isolate of rabies virus, antigen was detected in the muscle cells at the site of inoculation before it was detected in the central nervous system (CNS). Extrafusal fibers were involved (Charlton and Casey, 1979). Electron microscopic studies have shown virus particles budding from plasma membranes and sarcoplasmic reticulum of muscle cells (Murphy et al., 1973; Charlton and Casey, 1979). Infection of the neuromuscular spindle, neurotendinal spindle and motor end plates have been observed (Murphy et

al., 1973; Harrison and Murphy, 1978). When the virus enters the peripheral nerve, it moves passively, towards the CNS, in the axoplasm at a speed of 0.02 mm/sec (Ercegovac, 1976) or 5 cm per day (Miller and Nathanson, 1977). Viral accumulation at the nodes of Ranvier where a lot of organelles are present (Murphy et al., 1973; Jensen et al., 1969) and virus buildup upon axonal membranes (Murphy et al., 1973) are observed. The virus may travel to neuronal cell bodies in the dorsal root ganglia, and replicate there thus amplifying the infection.

Events in the Central Nervous System

The first portions of the CNS to be affected depend on the site of inoculation. Intramuscular injection in the masseter muscle results in the brain stem being the first area to be affected and intramuscular injection in the limbs results in the initial infection of the spinal cord. The transit from the spinal cord to the brain takes a few hours Dean et al., 1963; (Baer, et al., 1965). In the CNS, infection is mainly in the neurons and their processes (Murphy, 1977) though infected astrocytes have been observed (Iwasaki and Clark, 1975). Glial infection has been observed in human rabies (Sung et al., 1976). Neuronal ribosomes are present mainly in the perikarya and dendrites; thus, the synthesis of viral ribonucleoprotein might take place there. The viral products are then transported from the perikarya to the myelinated axons (Charlton and Casey, 1979; Iwasaki and Clark, 1975). Virions bud from cell surfaces. More budding is seen from dendritic and axonal processes than from the perikarya (Iwasaki and Clark, 1975). Iwasaki and Clark (1975) proposed two potential pathways for the movement of rabies virus in the brain:

1. Transit through intercellular spaces, making it possible for the infection of cells distant from the cells releasing the virus particle.
2. Direct transit of the virus between contiguous cells.

This cell to cell theory is supported by the observation of virus budding on postsynaptic and adjacent plasma membrane with simultaneous viropexis by the presynaptic axon terminal (Charlton and Casey, 1979). The cell to cell transit enables the virus in the CNS to evade the host antibody and phagocytes (Iwasaki and Clark, 1975).

Late Events -- Centrifugal Spread

Baer et al. (1965) injected street virus into a single hind footpad of rats, sacrificed healthy appearing animals 9 to 18 days post-inoculation and observed the virus in both sciatic nerves. During centrifugal spread, the virus also uses peripheral nerves (Schneider, 1975). The movement is passive and also via axoplasmic route (Murphy, 1977). Sensory fibers or specific motor (fusimotor) fibers are conspicuously involved and the release of the virus from these fibers is probably facilitated by axonal fragmentation (Charlton and Casey, 1979). The result of the centrifugal spread is virus generalization (Schneider, 1975) so that at the terminal phases of the disease the virus is present in many tissues in the body. Virus particles have been observed budding upon plasma membranes of mucous acinar cells (Murphy, 1977). Viral antigen is present in the nasal mucosa, neuroepithelial cells of the ureter, urinary bladder and urethra (Debbie and Trimarchi, 1970), corneal cells (Schneider, 1969b) and in the nerve fibers surrounding the hair follicles of the skin (Smith et al., 1972). The main implication of the centrifugal spread is the utilization of the

corneal cells (Schneider, 1969b), the oronasal mucosal scrapings (Schaaf and Schaal, 1971), or frozen skin sections from biopsy (Smith et al., 1972) for in vitam diagnosis of rabies. The presence of rabies virus in the cornea (due to centrifugal spread) was responsible for the 2 recent cases of the human-to-human transmission of rabies by corneal transplant (Houff et al., 1979; Center for Disease Control, 1980).

Tissue Culture Methods in Rabies

Prior to the development of IF for rabies (Kissling, 1958), titration in mice was the main method to study the pathogenesis of rabies. Efforts were made to isolate, propagate and passage rabies virus in vitro. In 1913, Noguchi reported maintenance of rabies in fragments of brain, medulla and spinal ganglia from experimentally infected animals. In the same year, Levaditi maintained the virus in explants of spinal ganglion from infected monkeys. In 1930, Stoel infected embryonic chicken brains and hearts explanted in rabbit plasma clot and maintained rabies virus for five consecutive passages. Rabies had been maintained in rabbit embryo brains in Tyrode solution (Kanazawa, 1936) and in rodent embryo brains (Webster and Clow, 1936; Bernkopf and Kligler, 1937). In 1942, Plotz and Reagan reported in vitro cultivation of street rabies virus. In 1958, Kissling reported the growth of street and fixed virus in hamster kidney cells. This indicated that rabies virus could be propagated in a non-nervous tissue and gave rise to several attempts to propagate rabies in non-nervous tissues. Among the non-nervous tissues that have been used are hamster kidney cell culture (Fenje, 1960; Kaplan et al., 1960), pig kidney cells (Abelseth, 1964), chick embryo cells (Kondo, 1965),

dog salivary gland (Depeux, 1963), bat adipose tissue (Allen et al., 1964), sheep embryo kidney, intestine, rabbit kidney, dog kidney, human embryonic kidney, skin, muzzle and lung (Aksenova et al., 1966), rabbit endothelium cell line (Fernandes, et al., 1964), human diploid cell strain WI-38 (Wiktor, et al., 1964), cell cultures from reptiles and fishes (Clark, 1972), primary dog kidney cells (Hronovsky et al., 1968), pig fallopian tube cell line (Boullaint, et al., 1974), pig kidney cells PK-2A (Strating, et al., 1975), Japanese quail embryo cell culture (Bektemirova et al., 1979), CER and neuroblastoma cells (Smith, et al., 1978). The list is probably longer. In many cases, only fixed virus could efficiently be cultivated.

After the monolayer of cells is inoculated with rabies virus, the virus attaches to the surface, is absorbed by pinocytosis and penetrates into the cytoplasm of the cell. If the multiplicity of infection (MOI) is high the virus could be within the vesicles in the cytoplasm of the cells within 5 minutes (Iwasaki et al., 1973). This phase is affected by the presence of polyions. Addition of diethylaminoethyl (DEAE) dextran (50 mg/ml) and protamine sulfate (1 mg/ml) to the virus inoculum has been shown to enhance absorption and penetration and hence the infectivity (Kaplan et al., 1967). Progeny virus is produced in the cytoplasm of the cell and buds either on the surface membrane or on the plasma membrane (Iwasaki and Clark, 1977). The nuclei are required for the formation of infectious particles since the infection of enucleated TC-7 monkey cells results in synthesis of virus-directed RNA and the production of rabies antigens but not of infection virus (Wiktor and Koprowski, 1974). The formation of virus particles by budding could be seen 72 hours after

infection of baby hamster kidney cells with fixed virus strains (Hummeler, et al., 1967).

First foci (matrices) of synthesis become apparent in the cytoplasm containing virus nucleoproteins, and virus membranes are formed in these foci or at the edges of these foci or matrices. There is organization of ribosomes into polyribosomal aggregates indicating accelerated nascent protein synthesis in the cells. Finally, there is budding on the surface. Later, in the infection, long filaments could be observed (Hummeler, et al., 1967). Infection of cells and the passage of the virus in the cells result in change in the cell and the virus. However, in many cases the morphology of the cells remain undisturbed when seen under the light microscope. Cytopathic effect (CPE) had been observed on pig fallopian tube cell line infected with ERA strain of rabies virus (Bouillant, et al., 1974), and rapidly on Iota cells fused with inactivated Sendai virus and infected with fixed virus (Diaz, et al., 1973). In some cases CPE is observed after prolonged passage (Hronovsky, et al., 1968). Inclusions could be demonstrated in chronically infected cells by staining with May-Grundwald-Giemsa stain after fixation in Bouin's solution (Wiktor, 1973). Cytochemical studies reveal that these inclusions appear to contain viral antigenic protein and small amounts of ribonucleic acid (Love et al., 1964). Plaque formation may be observed after infection of cell monolayers with fixed virus strains. Plaque formation in BHK 21-S13 cells have been utilized for virus titration (Sedwick and Wiktor, 1967) and for antibody assay by plaque reduction (Wiktor and Clark, 1973). Plaquing due to rabies infection has been observed on chick embryo cell culture (Kondo, 1965; Sekine and Yoshino, 1976), porcine kidney cell (PK-2A) cell line (Strating, et al., 1975), and vero cells (Buckley and Tignor, 1975).

Propagation of rabies virus in tissue culture may result in the production of defective interfering (DI) particles (Crick and Brown, 1974; Wiktor, et al., 1977) and the production of interferon (Wiktor and Clark, 1972). Interferon is probably responsible for the cyclical pattern of viral infection seen in tissue cultures (Wiktor and Clark, 1972). Other results of rabies virus propagation in tissue culture are a) carrier type infection (Fernandes, et al., 1963; Wiktor, et al., 1964) in which no cytopathic effect is observed and the virus can be maintained by cell transfer for unlimited number of passages, and b) endosymbiotic relationship between the cell and the virus in which the infected cells have only small intracytoplasmic inclusions, suffer no interference with mechanisms of cellular multiplication and therefore can be propagated indefinitely (Fernandes et al., 1964; Wiktor, 1966). Enhancement of virulence of several strains of attenuated rabies virus after one to five serial passages in murine and human neuroblastoma cells has been reported (Clark, 1978).

Propagation of rabies in tissue culture is not without problems. The major problem is contamination of the tissue culture with other viruses, especially those that do not produce cytopathic effects, or with mycoplasma (Wiktor, 1966).

Solid Phase Radioimmunoassay Using ^{125}I-labeled Protein A

Radioimmunoassay (RIA) is a highly sensitive and widely used technique for the detection of specific antigen or antibody molecules (Premkumar-Reddy, et al., 1977). Double antibody radioimmunoassay has

been used for this purpose. This depends on the finding that the antigenic sites of an antibody are separate from its antibody sites (Hunter, 1973). An antibody molecule may therefore form a complex with its antigen and then itself be complexed to a second antibody. Based on this, a limited amount of the antiserum or its dilution (antibody 1) is reacted with the appropriate radiolabeled antigen, and the resulting antigen-antibody complex is precipitated with a second antibody (antibody 11) directed against antibody 1 (Scolnick, et al., 1973; Stephenson et al., 1973). The resulting precipitates are sedimented by centrifugation, and the amount of labeled antigen in the antigen-antibody complex precipitate, or the bound radioactivity in the supernatant are measured. This method has been used to detect antibodies to rabies virus (Wiktor, et al., 1972).

^{125}I-labeled purified rabies virus was reacted with rabbit anti-rabies antibodies and the resulting virus antibody complexes were precipitated with 1:2 dilution of anti-rabbit IgG. The virus binding capacity of the antirabies rabbit serum was shown to be similar to or higher than the neutralization titer based on plaque reduction test. This technique was more rapid and more sensitive than plaque reduction test. However, simultaneous labeling of both glycoprotein and nucleocapsid might be expected to result in a decrease in the specificity and sensitivity of radioimmunoassay because of instability of the virus (Bruns et al., 1977). This instability has been observed for other labeled viruses and is due to oxidation and radiation damage (Gerloff et al., 1962). Bruns and colleagues (1977) used ^{125}I-labeled highly purified ribonucleoprotein for radioimmunoassay for quantitation of rabies antibodies and antigens and for the determination of serological relationships among the lyssaviruses. Another application of RIA to rabies serology has been in the detection of mono-

clonal antibodies against rabies virus produced by hybridomas between mouse myeloma cells and spleen cells derived from BALB/c mice immunized with inactivated rabies vaccine (Wiktor and Koprowski, 1978). In this case, BHK-21 cells infected with the ERA strain of rabies virus and fixed in 10% formaldehyde solution were used as an immunizing antigen and the antibodies detected using ^{125}I-labeled rabbit antimouse $(Fab')_2$ antibodies. In general, not much work has been done on the detection of rabies antigen and antirabies antibody in infected tissues by RIA. There is a great need for RIA for detection of rabies antigen (Shope et al., 1979).

Solid-phase RIA is widely used for measurement of antibody to a variety of antigens (Marrier et al., 1979) and for quantitation of antigens. The solid-phase may be tubes or plates (polystyrene and polyvinyl chloride plates or tubes) or medium such as cellulose, sepharose or Sephadex (Hunter, 1973). The attachment of the antigen or antiserum to the solid-phase may be by adsorption or by covalent conjugation (Hunter, 1973). In a solid-phase double antibody system for detection of antibody, species specific ^{125}I radiolabeled anti-IgG is used to measure the amount of specific antibody bound to immobilized antigen (Marrier et al., 1979).

Protein A is covalently linked to the peptidoglycan part of the cell wall of Staphylococcus aureus (Sjöquist et al., 1972). It was first described in 1940 by Verwey; Grov and his colleagues (1964) named it *Protein A. It is a single polypeptide chain with a molecular weight of* 42,000 (Sjöquist et al., 1972). The molecule has 4 tyrosine residues and no tryptophan (Sjöquist et al., 1972), making it easy to iodinate. It has 3 FC binding regions, each having 50 amino acid residues (Sjödahl,

1976). Binding is via a FC piece of the immunoglobulin (Kronvall and Frommel, 1970). The intact molecule has at least two sites accessible for binding of IgG (Sjöquist et al., 1972). Staphylococcus aureus Cowan 1 strain is particularly rich in Protein A. It can be isolated from lysostaphin digests of the Staphylococcus by affinity chromatography on IgG-Sepharose (Hjelm et al., 1972).

Human IgG1, IgG2, IgG4 bind protein A strongly (Hjelm, 1975). IgA2 and some IgM can also bind (Saltvedt and Harboe, 1976). Rabbit IgG and some IgM (Goding, 1976), dog IgG and up to 90% of IgM (Warr and Hart, 1979) and antibodies (mainly IgG) from other species of mammals bind to Protein A in varying degrees. Using the inhibition of binding of [^{125}I] protein A to immobilized rabbit IgG by IgG from different species as a criterion, Langone (1978) compiled the order of binding to Protein A by IgG from different species. Rabbit, human and guinea pig IgG were found to bind equally. These are followed by pig, dog, cow, mouse, horse, sheep and goat in that order. IgG from chicken and rats bind Protein A only very slightly.

Protein A has been used in a variety of forms for the study of cell surface antigens and bound or fluid-phase antibodies. It has been used conjugated with FITC (Biberfeld et al., 1975; Ghetie et al., 1974), bound to erythrocytes (Ghetie et al., 1974), labeled with horseradish peroxidase (Atanasiu and Perrin, 1979), and radiolabeled with tritium (Wilder et al., 1979), and iodine (Biberfeld et al., 1975). The most widely used isotope for labeling of Protein A is iodine 125 [^{125}I]. This is because its half life of up to 60 days is relatively long (Hunter, 1973). ^{125}I-Protein A has been used extensively to replace the second

antibody in radioimmunoassays. The Protein A methods give more rapid binding of IgG and immune complexes, lower nonspecific adsorption and higher recovery of antigen after washing than radioimmunoprecipitation (Kessler, 1975).

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CHAPTER III

IMMUNOFLUORESCENT STAINING FOR RABIES ANTIGEN IN THE SKIN OF INFECTED ANIMALS

SUMMARY

Immunofluorescent staining (IF) was evaluated for the identification of rabies virus antigen in the nerves of skin. The results of this examination were obtained by two persons performing the tests independently on the same set of specimens. Skin specimens from 71 skunks, 26 dogs, 16 cats, 1 pig, 103 foxes, 7 cattle, 57 goats and 31 mice were checked for the presence of rabies virus antigen by direct immunofluorescent staining. This method was suitable for the detection of rabies antigen in the skin of naturally infected animals, particularly skunks and foxes, and poor for antigen detection in the skin of experimentally inoculated animals, especially goats. There was high degree of correlation ($r = 0.934$) between the results obtained between the diagnosticians. Postexposure treatment does not seem to interfere with antigen detection in the skin of clinically ill animals.

INTRODUCTION

Clinical rabies has historically been regarded as being uniformly fatal because the diagnosis could only be confirmed postmorten when brain tissue was available for examination. However, abortive and recovered rabies is now a well documented phenomenon (Bell, 1975). Bell listed the criteria for proving that an apparently healthy animal has had rabies. These include a) high titer of neutralizing antibody in the cerebrospinal fluid, b) a positive corneal test in an animal that does not succumb, and c) isolation

of virus from saliva of an animal that does not succumb (Bell, 1975). A positive result obtained from immunofluorescent staining of cryostat cut frozen skin sections should be added to this list. A combination of these techniques will aid in the understanding of the pathogenesis of rabies especially that of abortive or recovered rabies not fitting the classical criteria for diagnosis. Detection of rabies antigen prior to death might be important in decision-making as regards management of a human patient.

Immunofluorescence has been used to detect rabies antigen in the skin of mice inoculated with rabies virus strains of bovine, feline or canine origin up to 4 days before the onset of clinical signs (Smith et al., 1972). It has been used for confirming cases of human rabies prior to death (Bryceson et al., 1975) and for diagnosis in naturally occurring terminal rabies in dogs, cattle, cats, mongooses, human beings and in experimentally inoculated rhesus monkeys (Blenden, 1974, 1978). Howard (1976), examining different tissues in naturally infected skunks, by direct immunofluorescent staining, observed that examination of the skin is as sensitive and specific as examination of the brain for rabies antigen in this species.

The objectives of this study are 1) to evaluate the use of immunofluorescent (IF) staining of skin biopsies in the detection of rabies antigen and to compare the results obtained with the results obtained by examination of brain specimens postmortem, 2) to compare the results of IF staining of skin biopsies between two individuals performing the test independently on the same set of samples, 3) to evaluate the effect

of postexposure immunoprophylaxis on antigen detection in the skin of clinically ill experimentally inoculated mice.

MATERIALS AND METHODS

A. The Basic Techniques

1. Sources of Specimens

Heads from striped skunks (Mephitis mephitis) were obtained from the laboratories of the Division of Health of Missouri, Jefferson City, Missouri. These striped skunks were suspected of being rabid either because of their behavioral changes or because there was a human exposure. Other striped skunks were also trapped in the skunk rabies endemic area of the State of Montana. These consisted mostly of clinically normal and a few sick animals. Their heads were shipped to our laboratory frozen in dry ice.

Skin biopsies were taken from the muzzle or the lateral sensory papillae of the German red foxes (Vulpes vulpes) and shipped frozen to our laboratory from Professor Wachendörfer, Federal Republic of Germany. These specimens were from foxes captured, shot or gassed during the fox depopulation campaign in West Germany.

Dog and cat specimens were from animals suspected of rabies submitted to the Laboratories of the Division of Health of Missouri, from animals suspected of being rabid in the University of Missouri Veterinary Clinic and from experimentally inoculated dogs.

Specimens from pigs and cattle suspected of being rabid were obtained from the Laboratories of the Division of Health.

Mice and goat specimens were from experimentally inoculated animals.

2. *Test reagents*

Normal mouse brain (NMB) - Weanling (3 weeks of age) mice were killed by carbon dioxide anesthesia and their brains were harvested. A 20% suspension was prepared with 0.75% bovine serum albumin (BSA) Fraction V in buffered water (pH 7.6). This suspension was centrifuged at 100 x g for 10 minutes and the supernatant was aliquoted and stored at -70°C.

Infected mouse brain (IMB) - A suspension of brain tissue containing challenge virus standard (CVS), originally obtained from the Center for Disease Control, Lawrenceville, Georgia and maintained in mice in our laboratory, was diluted 1:1000 and injected intracerebrally into weanling mice. The brains were harvested when the mice were moribund approximately 6 days post-inoculation. A 20% suspension of this brain tissue composite was prepared with 0.75% BSA in buffered water, centrifuged at 1000xg for 10 minutes, aliquoted and stored at -70°C.

Antirabies globulin conjugate - Fluorescein isothiocyanate labelled anti-rabies globulin of either bovine or equine origin was obtained commercially (BBL, Cockeysville, MD 21030), the conjugate was routinely titered and used at a dilution of 1:150.

3. Biopsy Technique

Skin biopsies were obtained whenever tactile hairs were present. The favorable sites were muzzle for all species, lateral sensory papillae on the cheek of dogs and sometimes the skin around the eye in cattle and

goats. A stainless steel Keyes dermal punch was used in obtaining the biopsy samples. For mice a scalpel or scissors was used.

4. The Procedure

The biopsies were mounted with the dermis upwards on brass cryostat buttons using a commercial mounting medium (O.C.T. compound, Tissue Tek II, LabTek Products, Naperville, Illinois). The buttons were placed in a cryostat chamber at -20°C for the mounting medium to solidify. Oblique sections 10 μ thick were cut in the cryostat chamber (I.E.C., CTF Microtome-Cryostat, International Equipment Co.). Five slides were prepared from each tissue block and at least 2 consecutive sections were placed on each slide. The sections were cut from different levels of the block to include the hair follicle at different levels. After fixing in acetone at -20°C for 30 minutes, the slides were rinsed in buffered water pH 7.6 for 1 minute to wash off the mounting medium. The slides were then air-dried and each section was encircled using a wax pen (Mark-Tex, Tech-Pen, Mark-Tex Corp., Englewood, New Jersey). Some fluorescein isothiocyanate labelled antirabies globulin was diluted 1:150 in infected mouse brain suspension (IMB) and some to the same ratio in normal mouse brain suspension (NMB). The diluted conjugate was incubated at 37°C for 30 minutes after which 2 drops of 0.1% aqueous solution of Evans Blue were added to each milliliter of the diluted conjugate to serve as a counterstain. One drop of the IMB-diluted conjugate was put on the section near the frosted end of the slide to serve as an inhibition control; one drop of the NMB-diluted conjugate was put on the section(s) further away from the frosted end for examination for specific uninhibited fluorescence. The slides were incubated at 37°C for

30 minutes in a moist chamber to prevent drying. After incubation, the slides were washed twice in buffered water or phosphate buffered saline (PBS) pH 7.6 for 5 minutes each and then rinsed in tap water for 1 minute. The slides were then air dried and examined by microscopy using ultraviolet illumination. The microscope used was a Leitz Ortholux microscope with HBO 100 W mercury lamp and epi-illumination. The filtering system consisted of BG 23 heat, 2XKP490 (=KP500) excitation and K 495 suppression filters. The sections were examined with 10X ocular and 10X objective lenses to locate the hair follicles followed by the use of 10X ocular and 95X oil immersion objective lenses for detailed examination for specific fluorescence. The presence of clear cut rounded dots or strings of apple green fluorescence in the nerve plexus surrounding the hair follicle or in clusters or strings in other regions of the test section and the absence of such fluorescence on the inhibition slide indicated the presence of rabies antigen. A 5-point scoring system was used depending on the quantity and the intensity of fluorescence in the sections examined.

± indicates few fluorescing dots in only one hair follicle in only 1 section out of 5 examined.

\+ indicates few fluorescing dots in 1-2 hair follicles in 2 sections out of 5 sections examined.

++ indicates a moderate amount of fluorescence in 2-4 hair follicles in 3 sections out of 5 examined.

+++ indicates moderate amount of fluorescence in most large hair follicles in all sections examined.

++++ indicates large quantitites of fluorescence in most hair follicles (both large and small) in all the sections examined.

Photographs were taken using a fully automatic microscope camera-orthomat (Ernst Leitz GMBHD 6330, Wetzlar, Germany) and a high speed film 27 Din 400 ASA (Eastman Kodak Company, Rochester, New York 14650).

B. The Effect of Postexposure Treatment on Detection of Rabies Antigen in the Skin of Mice Experimentally Inoculated with Street and Fixed Strains of Rabies Virus

1. Mice

White Swiss mice, aged 4 weeks and weighing 20-25 g, were obtained from the colony at the Rabies Research Laboratory, University of Missouri - Columbia. Only mice of the same sex were put together in a cage. They were housed in groups of 5 per cage and provided with standard diet and water ad libidum.

2. Virus

The Street virus used was obtained from a rabid dog. A 20% suspension was prepared from the brain stem at the region of the corpus trapezoideum in 0.75% BSA in buffered water pH 7.6. This was stored at -70°C until use. It had a titer of $10^{4.03}$ mice LD_{50}. The fixed virus strain used was CVS-27 originally obtained from Center for Disease Control, Lawrencille, Georgia and maintained in mice in our laboratory. A 20% suspension was prepared from whole mouse brains in 0.75% BSA in buffered water and stored at -70°C. It had a titer of $10^{6.6}$ LD_{50}/0.03 ml in weanling mice.

3. Vaccine:

ERA(R), modified live virus, porcine tissue culture origin, high cell passage, street Alabama dufferin strain, was a gift of Connaught Laboratories, Ontario, Canada. Lot number 431 which had been preserved at -70°C was used. Reconstitution was as recommended by the manufacturer and with the diluent provided.

4. Antiserum

Goat antiserum against rabies virus was prepared in a goat that had recovered from clinical rabies during a postexposure treatment experiment. The recovered goat was given 3 boosters of 2 doses of ERA^{R} vaccine one week apart. Blood was obtained by jugular venipuncture on the 8th day after the last booster. The antiserum had a titer greater than 1:15625 by rapid fluorescent focus inhibition test (Smith et al., 1973).

5. Experimental Designs

a. Experiment 1: The mice were divided into 4 groups (A, B, C and D) of 20 each and 3 groups (E, F and G) of 15 each. All the mice in groups A, B, C and D were inoculated intramuscularly in the thigh with 0.15 ml of the 20% rabid brain suspension (LD_{50} in mice = $10^{4.68}$) using 0.25 ml glass syringe and 25 gauge, 3/8 inch needle. Those in groups E, F and G were not inoculated with the virus. The mice in group A were left untreated. Those in groups B and E were treated 48 hours after inoculation with 0.2 ml of ERA vaccine given intraperitoneally (ip); those in groups C and F had 0.2 ml of ERA vaccine ip and 0.1 ml of goat antirabies serum intramuscularly (im) in the same thigh into which the virus was inoculated; and those in groups D and G were treated im with 0.1 ml of goat antirabies serum only.

b. Experiment II: The mice were divided into groups as in experiment 1 except that group A had 25 mice. Groups A, B, C and D were inoculated with 0.1 ml of 10^{-2} dilution of CVS i.m. in the thigh. The treatment schedule was as in experiment 1 except that 0.2 ml of ERA vaccine and 0.2 ml of goat antirabies serum were administered to the corresponding groups 18 hours after virus inoculation.

6. Observation

The mice were observed twice daily for sign of sickness and paralysis. Dead mice were collected individually in small plastic bags, coded and stored at -20°C. The surviving mice were euthanatized with carbon dioxide 30 days after virus inoculation, coded and stored with the rest. Direct immunofluorescent staining was done on the skin samples from the mice without any regard to the grouping.

C. Statistical Analysis

To compare the result obtained in this study with the results previously obtained with the same specimens or with the results obtained with the brain samples, correlation coefficient r was calculated from a 2x2 table using the formula:

$$r = ad - bc / \sqrt{(a+c)(b+d)(a+b)(c+d)} \quad .$$

The prediction power of skin IF as an indicator of brain infection was calculated by Goodman and Kruskal's "Lambda" λ (Hadani, 1978).

$$\lambda = \max(b,d) + \max(a,c) + \max(a,b) + \max(c,d) - \{\max(a+b, c+d) + \max(a+c, b+d)\}/2K - \{\max(a+b, c+d) + \max(a+c, c+d)\}.$$

RESULTS

The results obtained by immunofluorescent staining of the skin of the skunks, foxes, dogs, cats, pigs, cattle, mice and goats are shown in Table 3.1. In the positive cases antigen could be seen in the parafollicular nerve plexuses (Figure 3.1, 3.2, and 3.3). Such fluorescence was not seen on the inhibition side of the slide. Rabies positive foxes and skunks had more fluorescence both in intensity and quantity than other species (Table 3.2).

Data on IF staining for rabies antigen in the skin of the skunks (66), foxes (103), dogs (26), pig (1), cattle (6) and goats (32) were obtained from previous records in our laboratory and compared with the result of the present study. The current result correlated well with the records ($r = 0.934$). Data on brain immunofluorescent staining of some of the samples were also retrieved for comparison and for measuring how skin immunofluorescence predicts clinical rabies (Table 3.3). The correlation coefficient (r) of 0.98 and the predictive power (λ) of 0.98 were obtained when the results of the skin samples were compared with the results of the brain samples of the foxes. A high degree of correlation ($r = 0.93$) and a high predictive power of skin for brain result ($\lambda = 0.90$) was obtained for skunks. The lowest correlation ($r = 0.13$) was obtained with the results of the experimentally inoculated goats.

Experiments I and II show that postexposure treatment did not influence the detection of rabies antigen in skin of mice that had clinical rabies (Tables 3.4 and 3.5).

Table 3.1

Immunofluorescent Staining of the Skin of Animals for Rabies Antigen

Species	Number Tested	Number Positive/ Number Negative	% Positive
Skunk	71	18/53	25.4
Fox	103	53/50	51.5
Dog	26	5/21	19.2
Cat	16	12/4	75
Pig	1	1/0	100
Cattle	7	7/0	100
Mice	31	31/0	100
Goat	57	10/47	17.5

Table 3.2

Distribution of Intensity and Quantity of Fluorescence in Positive Skin Samples by Species

Species	No. Positive	±	+	++	+++	++++
Skunk	18	-	2(11)*	1(5.5)	4(22)	11(61)
Fox	53	-	4(75)	8(15.1)	14(28.3)	27(50.9)
Dog	5	-	3(60)	2(40)	-	-
Cat	12	-	7(58.3)	4(33.3)	1(8.3)	-
Pig	1	-	-	1	-	-
Cattle	7	-	7	-	-	-
Goat	10	4(40)	6(60)	-	-	-

*The number in parentheses indicates the percentage for each group.

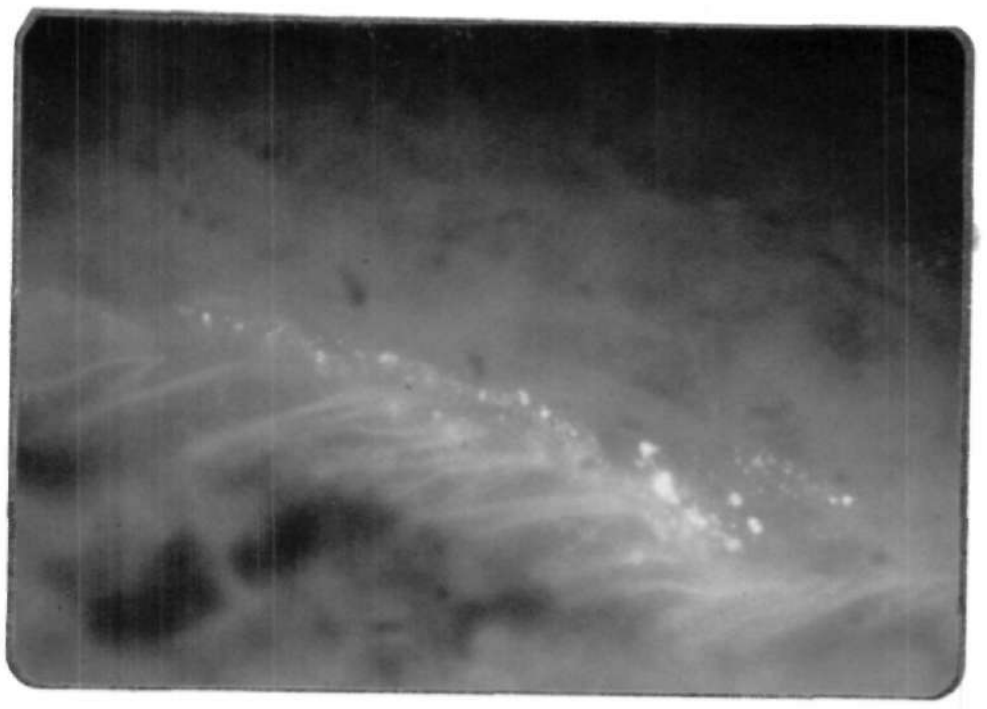


Fig. 3.2. Immunofluorescent staining for rabies antigen in the skin of a naturally infected German fox. The antigen was located in parafollicular nerve plexus of tactile hairs. X950.

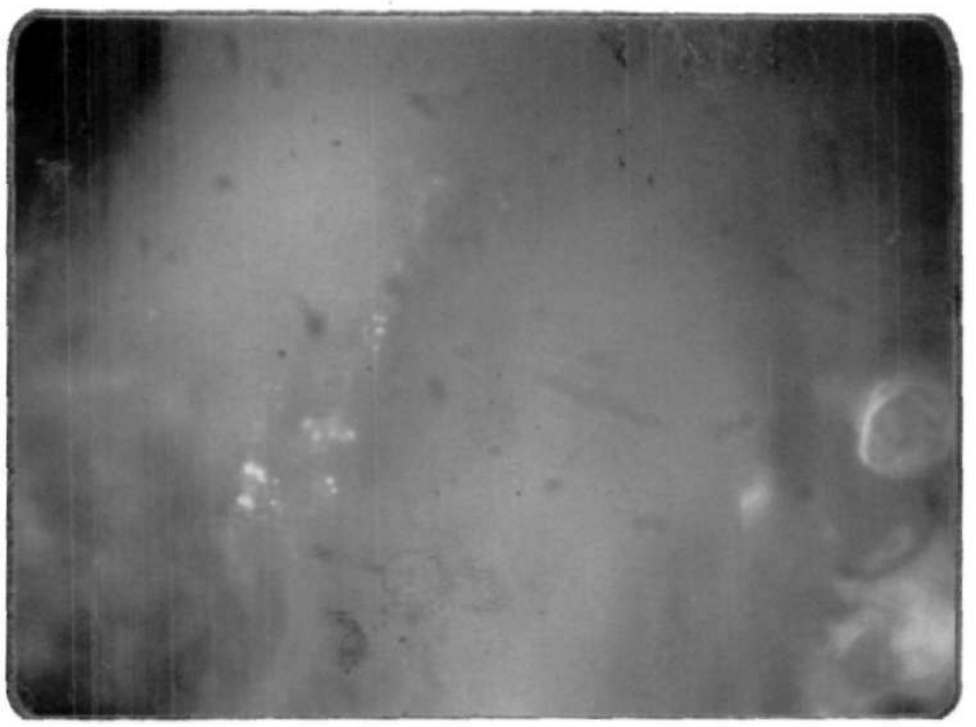


Fig. 3.3. Immunofluorescent staining for rabies antigen in the parafollicular nerve fibers of the skin of the upper lip of a rabid goat. The goat was inoculated in the masseter muscle with street rabies virus. X950.

Table 3.3

Comparison of the Results from IF Staining of the Skin With IF Staining of the Brain for Rabies Antigen

Species	Total	Skin IF Positive	Brain IF Positive	r**	λ***
Skunk	71	18	20	0.93	0.90
Fox	103	53	52	0.98	0.98
Dog	26	5	17	0.36	0.
Cat	16	12	12	NC*	NC*
Pig	1	1	1	NC	NC
Cattle	6	6	6	NC	NC
Goat	57	10	53	0.13	0.

*NC = Not calculated
r** = correlation of the skin with brain result
λ*** = predictive power of skin for brain result.

Table 3.4

Design and Results of Experiment 1 Showing the Effect of Postexposure Treatment on Detection of Street Virus Antigen in the Skin of Experimentally Inoculated Mice

Group	Number	Treatment			Result			
		Challenge Virus	Vaccine	Serum	Mortality	% Mortal.	No. IF Pos.	Median Inc. Period
A	20	+	-	-	17	85	17	15
B	20	+	+	-	11	55	11	13
C	20	+	+	+	2	10	2	11
D	20	+	-	+	6	30	6	10.5
E	15	-	+	-	0	0	0	-
F	15	-	+	+	0	0	0	-
G	15	-	-	+	0	0	0	-

Table 3.5

Design and Results of Experiment 11 Showing the Effect of Postexposure Treatment on the Detection of Fixed Rabies Virus in the Skin of Experimentally Inoculated Mice

Group	Number	Treatment			Result			
		Challenge Virus	Vaccine	Serum	Mortality	% Mortal.	Number IF Pos. Skin	Median Incub. Period
A	25	+	-	-	22	88	22	6
B	20	+	+	-	14	70	14	8
C	20	+	+	+	16	80	16	7.5
D	20	+	-	+	19	95	19	6
E	15	-	+	-	0	0	0	0
F	15	-	+	+	0	0	0	0
G	15	-	-	+	0	0	0	0

DISCUSSION

Inhibition of fluorescence on the sections stained with IMB-diluted conjugate confirms that the fluorescence in the test sections is specific. The specific fluorescence could consistently be observed in the nerves surrounding the hair follicles as observed by Smith et al., 1972. However, in cases as in mice experimentally inoculated with street virus and treated with rabies antiserum, the fluorescence was more widespread and in some cases the cells of the stratum germinativum showed specific fluorescence. Animals naturally infected with street virus showed more fluorescence than experimentally infected animals probably because of short incubation period in the experimentally inoculated animals. Negative IF results are sometimes seen in skin of clinically ill experimentally inoculated dogs (Blenden, 1979, personal communication). It has been observed that in animal experiments, the incubation period usually ranged from 2 to 3 weeks with almost none over a month (Ahuja et al., 1951; Tenbroeck, 1950; Baer et al., 1968) unless there is therapeutic intervention. Thus, there is not sufficient time for the virus to accumulate in the nerves of the skin in easily detectable amounts. Natural infection follows a slower course with longer incubation periods. This probably contributes to the quantity of antigen in the skin since there is more time for virus generalization. In the two natural reservoirs of rabies (skunks and foxes) included in this study more than 75% of the animals that showed IF positive skins had a lot of antigen in the nerves surrounding both large and small hair follicles and in other locations. This could be explained by the fact that these animals are highly

susceptible to infection with rabies virus (Constantine and Woodall, 1966) and therefore allow for rapid virus dissemination in their nerves. In addition there is a possibility of adaptation of the virus to the species. The virus that infects a particular reservoir animal (skunk or fox) is likely to have undergone many intraspecies transfers and have become adapted to that species. This has also been known to occur after several transfers of virus in chick embryo (Koprowski, 1954) in tissue culture (Wiktor et al., 1964). This modification in the reservoir host probably allows for more rapid dissemination and the accumulation of viruses in tissue without a significant effect on the animal for an indetermined period of time. This could be the reason some apparently healthy animals trapped during a surveillance program were IF positive for rabies antigen.

There were good correlations between the results obtained from skunk skin and the brain and between the fox skin and brain. Howard (1976) has reported 100% agreement between the results IF of other tissues including the skin and the brain in naturally infected skunks. The implication is that if the fox or skunk population is to be conserved and if rabies surveillance as an ecological tool is to continue on the conserved population, it would be possible to trap, biopsy, and confine awaiting the laboratory test. The animals can be released if found to be negative for rabies by skin test. This would not be satisfactory with skunks or foxes which have bitten a human being.

In naturally infected domestic animals, antigens were detected during the terminal phases though the quantity of fluorescence was lower than in the natural hosts of rabies since the type of adaptation possible in the wildlife species is not possible in domestic animals in the United States.

The high correlation between the results of this study and the results from previous records on the same specimens indicates that this method gives reproducible results. Thus when the test is performed by experienced diagnosticians, the results are comparable.

Hair follicles are richly innervated. Details of the innervation of the skin of men has been described (Montagna and Parakkal, 1974). Basically every hair follicle is surrounded by nerve fibers from the base of the bulb to its junction with the epidermis. Some nerves lie parallel to the hair follicle and others are in a sacklike net around it. Electron microscopic studies have shown a perifollicular network around the hair follicles at the level of sebaceous glands (Montagna and Parakkal, 1974). Each nerve is arranged in palisade around the follicle close to the outer root sheath. There might be slight differences in this basic arrangement between species. The nerves around the hair follicles in the head are branches of cranial nerves. After exposure to rabies virus, there is a centripetal movement to the brain, replication in the brain and a centrifugal spread from the CNS (Dean et al., 1963, Baer et al., 1965). The virus in the skin is the result of this centrifugal spread. Smith et al. (1972) was able to isolate virus from the skin of experimentally inoculated mice. Electron microscopic studies (Figure 3.4) show that whole virus particles are involved (Umoh and Blenden, 1979, unpublished data). Since the distribution of nerves in the hair follicle is not uniform, it was necessary to section at different levels to account for the variability.

In the experiments to assess the effect of postexposure treatment on antigen detection in the skin, it was necessary to use large amounts

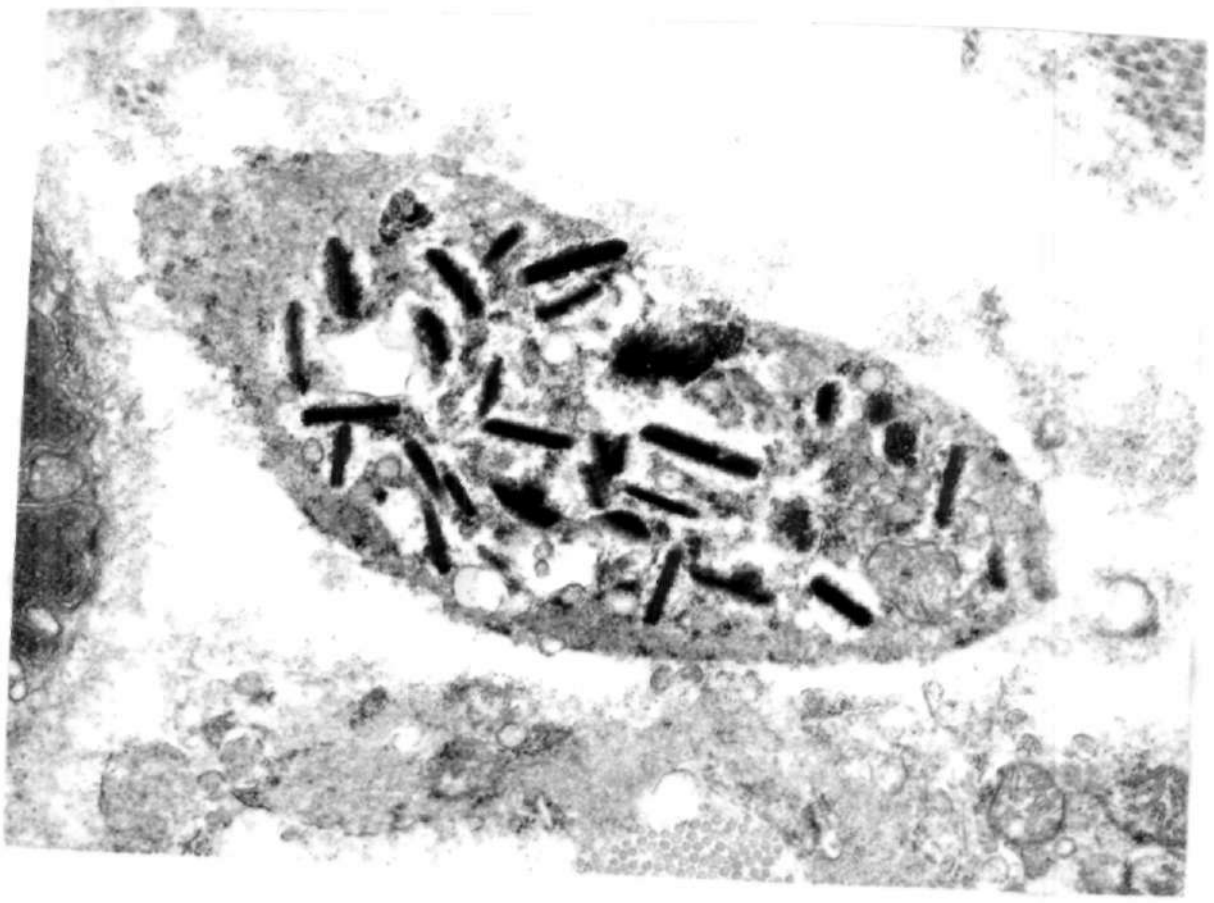


Fig. 3.4. Nerve fiber probably infraorbital branch of the maxillary branch of trigeminal nerve in the muzzle skin of a rabid mouse. Whole rabies particles were observed. X 20,450.

of the challenge virus because no predictable pattern was obtained with lower doses. Treatment was given within a shorter time when CVS was used than when street virus was used because CVS, being adapted to mice, disseminates more rapidly in this species than the unadapted street virus. Intraperitoneal vaccination route was used because trial experiments showed that ERA vaccine was nonpathogenic by this route with the dosage used.

There are many reports of the failure of postexposure treatment to protect against rabies (Habel, 1954; Ahuja et al., 1951; Tenbroeck, 1950; Fenje, 1974). These experiments indicate that skin biopsies examined by IF from clinically ill, postexposure treated patients could be used for differentiating clinical rabies from another disease entity.

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CHAPTER IV

THE DISSEMINATION OF RABIES VIRUS INTO CRANIAL NERVES AND OTHER TISSUES OF EXPERIMENTALLY INFECTED GOATS AND DOGS AND NATURALLY INFECTED SKUNKS

SUMMARY

Direct immunofluorescent staining was performed on cranial nerves and other tissues excised from experimentally inoculated rabid animals to find out why most rabid experimentally inoculated goats and some rabid experimentally inoculated dogs show negative results with immunofluorescent staining of the frozen skin specimens for rabies antigen. It was observed that cranial nerves in these animals contain only slight to moderate amounts of rabies antigen while naturally infected skunks had a lot of antigen both in the nerves and in the skin. Thus the negative results of skin biopsies seen with goats and some dogs experimentally inoculated with rabies virus are related to a paucity of rabies antigen in the nerves to the skin. This in turn is related to the susceptibility of the host, the route of infection, the adaptation of the virus strain to that host species and the length of incubation period. All the cranial nerves checked in the experimentally inoculated animals had virus antigen at points close to the brain and the amount of antigen seemed to depend on the size of the nerve trunks.

INTRODUCTION

Dissemination of rabies virus via peripheral nerves is well documented (Dean et al., 1963; Murphy et al., 1973; Baer et al., 1965; Schneider, 1975a, 1975b). In fact, hematogenous transit is an exception rather than the rule (Dean et al., 1963). In experimentally

inoculated mice, rabies antigen can easily be demonstrated in the skin by immunofluorescence (Smith et al., 1972, Chapter 3). However, in experimentally inoculated goats and certain dogs, negative results are commonly obtained by immunofluorescent staining of the skin (Umoh and Blenden, 1979, unpublished data) despite the presence of a large quantity of virus in the central nervous system. In order to explain these negative results, this study was set up to check the dissemination of rabies antigen in the cranial nerves and other tissues and to elucidate the relationship between the virus titer in the brain and the presence of antigen in the skin. The main hypotheses were that: (1) the ultimate distribution of virus in peripheral nerves is associated with the distribution of the virus in the nerve trunks; (2) the invasion of the cranial nerves by rabies virus after intramuscular inoculation is uniform.

MATERIALS AND METHODS

Animals

Twelve goats, 9 males and 3 females, aged between 4 and 6 months, were purchased through Animal Resources, University of Missouri, Columbia. They were maintained at the Veterinary Research Farm, University of Missouri, Columbia for a week before inoculation. They were fed hay throughout the experiment.

Seven dogs, 6 months old, from the same litter were obtained from Animal Resources, University of Missouri, Columbia. The dogs were born and bred in confinement in the Veterinary Research Farm. The dam had not been vaccinated against rabies. The dogs had not been vaccinated against any disease and had no contact with other dogs.

Virus

A 20% suspension was prepared from the brainstem of a rabid dog in 0.75% buffered bovine serum albumin. This had a titer $MICLD_{50}$ $10^{4.03}$/ 0.03 ml in weanling mice. The dosage for use in the animals was based on the results of previous inoculation of these species with the same virus isolate (Umoh, 1977).

Inoculation

The goats were ear-tagged and 10 of them were inoculated intramuscularly in the right masseter muscle. Five had a dose 5.54 $\log_{10}$ $MICLD_{50}$ in 1.0 ml, 4 had 5.85 $\log_{10}$ $MICLD_{50}$ in 2.0 ml and 1 had 5.94 $\log_{10}$ $MICLD_{50}$ in 2.5 ml, the dosage selected by the size of the goat. Two goats were not inoculated. The dogs were anesthetized by intravenous injection of 4.0% Bio-tal^{R} (Sodium thiamylal, 5.0 ml, Bio-Ceutic Laboratories Inc., St. Joseph, Missouri). Blood was obtained by cephalic or lateral saphenous venipuncture and cerebrospinal fluid by aseptic suboccipital puncture. All the dogs were inoculated with a uniform dose of 5.54 $\log_{10}$ $MICLD_{50}$ in 1.0 ml in the left masseter muscle. In a subsequent experiment the two remaining goats were inoculated with 5.54 $\log_{10}$ $MICLD_{50}$ of the virus via the masseter muscle.

Observation of the Animals

The animals were observed once daily for signs of central nervous system (CNS) disorder. When signs such as excessive muscular tremors, salivation, or paralysis were observed, the goats were anesthetized with Rompun^{R} (Xylazine, 15 mg, BayVet Division, Cutter Laboratories Inc.,

Shawnee, Kansas) and blood was obtained by jugular venipuncture and CSF by suboccipital puncture. The dogs were anesthetized and blood and CSF obtained as described. Prostrate and paralyzed animals were euthanatized by intravenous injection with 5.0 - 7.0 ml (1.3 - 1.8 g) of T-61 euthanasia solution (Taylor Pharmacal Co., Decatur, Illinois 62525). Seven weeks postinoculation the surviving dogs were euthanatized. Heads were removed from the carcasses and stored at -70°C until they were dissected. The sciatic nerves were sampled before the disposal of the carcasses.

Dissection and Sampling

The heads were split sagitally and thawed for easy dissection. A 20% suspension was prepared in 0.75% buffered bovine serum albumin (BSA) from a section taken from the brain stem in the area of corpus trapezoideum of the brain for each animal. This was stored at -70°C for later use in virus titration. A section from the brain stem was mounted on a cryostat button using a commercial mounting medium (O.C.T. Tissue Tek II, LabTek Products, Naperville, Illinois). The brain was removed carefully to expose the cranial nerves. These nerves were identified and pieces cut off as they emerge from or enter the ventral portion of the brain. A piece was cut off the rostral end of the olfactory bulb close to the cribriform plate. The optic chiasma and the cervical spinal cord were sampled. The eyeball was removed to expose the optic nerve, the occulomotor and the trochlear nerve. Pieces were cut off from these nerves. Corneal impressions were made on slides. The cornea and the back of the eye (retina) were excised from the eyeball and mounted. The maxillary branch of the trigeminal nerve was excised after the removal of the nasal concha. In some cases the trigeminal ganglia were sampled. The skin on the face was

removed. The dorsal buccal nerve on the lateral surface of the external masseter and the ventral buccal nerve on the ventral margin of the masseter muscle, both being branches of the facial nerve, were sampled. The levator labii maxillaris muscle was transected and deflected to expose the branches of the infraorbital nerves. These were excised as they leave the infraorbital foramen and further down in the upper lip. The lingual nerve was sampled on the lateral side of the mandible and the mental nerves as they leave the mental foramina. Other structures and nerves sampled were salivary glands, skin of the upper lip, tactile hair follicles in the supraorbital skin, beards where available, hypoglossal nerve and sciatic and/or saphenous nerves.

Heads from naturally infected skunks were obtained from the Division of Health of Missouri, Jefferson City, MO. They were dissected and some cranial nerves and other structures were sampled. All the samples were mounted on cryostat buttons and cut in a cryostat chamber (IEC Model Series CTF AC 3975, International Equipment Co.) at -20°C. The nerves were cut longitudinally since this was shown to be better than tranverse section in revealing the virus antigen by pretest trials.

Immunofluorescent Staining

The processing and examination of the specimen was carried out as described in Chapter 3. The fluorescence was scored as: (-) when no specific fluorescence could be seen, (+) when few specific fluorescing dots are seen after much searching, (++) when few specific fluorescing dots are found in every other field, (+++) when moderate amount of fluorescence could be seen in every field, (++++) when a lot of fluorescence could be seen in every field.

Titration of Virus on CER Cells

Eight-chambered LabTek slides (LabTek Products, Miles Laboratories, Naperville, Illinois) were seeded with 2.5 x 10^5 CER cells in 200 µl of growth medium (Glasgow Minimum Essential Medium G-MEM supplemented with 10% heat inactivated fetal bovine serum (Fbs) and 70 µg/ml gentamicin) and held at 37°C for 18-24 hours. The medium was removed and 100 µl of $\log_{10}$ dilutions in growth medium of the 20% brain suspension in 0.75% buffered bovine serum albumin was inoculated into each chamber. After adsorption at 35°C for 90 minutes, the monolayer was washed with Hanks balanced salt solution at pH 7.4 and overlayed with 400 µl of maintenance medium (G-MEM, 2% Fbs) buffered with TES (55 mM) and sodium hydroxide (56 mM). The chambers were incubated in a humidified incubator at 35°C for 3 days. The cells were washed with Dulbecco phosphate buffered saline (0.1 M) pH 7.5, fixed in cold acetone at -20°C for 10 minutes and stained with fluorescein isothiocyanate labeled antirabies globulin (BBL, Cockeysville, MD 21030) prepared for immunofluorescence microscopy. Twenty fields were examined for each chamber and the number of fields with 5 or more fluorescing cells were recorded. The titer was calculated by the method of Reed and Muench (1938) and expressed as tissue culture infective dose 50 ($TICD_{50}$).

RESULTS

Strong positive results were obtained by immunofluorescent staining of brain stem, cerebellum and the cervical spinal cord from the rabid goats (Table 4.1). Positive IF results were obtained in 4 (33%) of the skin samples from the upper lip, 6 (50%) cornea impression smear samples from

Table 4.1

The Dissemination of Rabies Virus in Some Tissues of the Goat Following Experimental Inoculation

	Goat Identification Number											
	110	111	112	113[R]	114	115	116	117	118	119	120	121
*Virus inoculum	5.54	5.54	5.54	5.54	5.54	5.95	5.85	5.85	5.85	5.85	5.54	5.54
Incubation period	14	17	22	24	21	15	14	14	14	17	19	22
Duration of illness	4	3	3	49	3	2	1	3	5	2	3	4
**Virus titer in brain stem/g	4.28	3.7	3.93	<0.7	4.03	4.30	3.7	3.85	3.90	4.01	4.07	4.31
IF Staining: Brain stem	4+	4+	4+	-	4+	4+	4+	4+	4+	4+	4+	4+
Cerebellum	4+	4+	4+	-	4+	4+	4+	4+	4+	4+	4+	4+
Cervical spinal cord	4+	4+	4+	-	4+	4+	3+	4+	4+	4+	4+	4+
Optic chiasma	4+	3+	3+	-	3+	3+	2+	3+	3+	3+	4+	4+
Olfactory lobe	4+	4+	4+	-	2+	4+	4+	4+	4+	3+	4+	4+
Supraorbital skin	+	-	-	-	-	-	-	-	-	-	-	+
Skin - upper lip	2+	-	-	-	-	+	-	+	-	-	-	+
Skin - beards	-	NA	NA	NA	NA	+	NA	NA	-	NA	NA	NA
Cornea section & smear	2+	-	-	-	-	+	-	-	+	+	+	+
Retina	+	+	ND	-	ND	+	-	ND	ND	ND	ND	ND
Submax. sal. gland	+	-	-	-	-	+	-	-	-	-	-	-
Submax. sal. gland	+	-	-	-	-	+	-	-	-	-	-	-
Parotid sal. gland	+	-	-	-	+	+	-	-	-	-	-	-

* expressed as $\log_{10}$ of $MICLD_{50}$ in weanling mice
**-$\log_{10}$ $TICD_{50}$/g
NA = not applicable
R = euthanatized 7 weeks after onset of illness after partial recovery.
ND = not done.

cornea, and 2 (16.5%) salivary gland samples from rabid goats (Table 4.1). All the cranial nerves contained rabies antigen in close proximity to the brain. In 2 cases the distal branches of the facial nerve (dorsal buccal and ventral buccal) had no demonstrable antigen even though the facial trunks had antigen. In 1 case (116) no antigen could be demonstrated in the maxillary and infraorbital branches of the trigeminal nerve even though the rest of the trigeminal contained antigen. The sciatic nerve was negative for rabies antigen in all the rabid goats (Table 4.1). The goats (110, 115, 117 and 121) that had rabies antigen in their parafollicular nerve plexuses had above average rabies titers in their brainstems (Table 4.1). In general the titers obtained by titration of 20% brain suspensions from the goats were not significantly different from each other at $\alpha = 0.05$ using Wilcoxon signed ranks test (Conover, 1971). Where skin immunofluorescent staining for rabies antigen of skin was positive, the quantity of antigen was low as shown by the paucity of the fluorescence. Positive results were only obtained in 2 goats by IF staining of corneal impression smears while 6 positive cases (50%) were obtained by staining cryostat-cut sections of the cornea.

Of the seven dogs inoculated, only three developed clinical rabies and were euthanatized on the day of onset. Immunofluorescent staining on nerves and other tissues revealed that the quantity of virus antigen present in their brains and spinal cords was low to moderate. A low amount of antigen was detected in the maxillary nerves of all 3, the infraorbital nerve of 1, the facial nerves at the roots of all 3 and the optic nerves of all 3 (Table 4.3). All other nerves, the salivary glands and the skin biopsies were negative for rabies virus antigen. On titration of the 20% brain suspension on CER cells, dog 4-79 had a $TCID_{50}$ of less than $10^{-2.4}$/g,

Table 4.2

The Dissemination of Rabies Antigen in the Peripheral Nerves of Goats by Immunofluorescent Staining

Peripheral Nerves	Goat Identification Number											
	110	111	112	113*	114	115	116	117	118	119	120	121
Optic	2+	+	+	-	2+	+	+	+	2+	2+	2+	2+
Root of trigeminal	2+	2+	2+	-	2+	2+	+	+	2+	2+	2+	2+
Root of facial	2+	+	+	-	2+	2+	+	+	+	+	2+	2+
Hypoglossal	+	+	+	-	+	+	+	+	+	+	+	+
Root of vagus	+	+	+	-	+	+	+	+	+	+	+	+
Spinal accessory	+	+	+	-	+	+	ND	+	+	+	+	+
Occulomotor on eyeball	+	+	+	-	+	+	ND	+	+	+	+	+
Trochlear	ND	+	+	-	ND	+	ND	ND	ND	ND	+	+
Maxillary	2+	+	+	-	2+	+	-	+	+	+	+	+
Infraorbital	2+	+	+	-	2+	+	-	+	+	+	+	+
Lingual	+	+	-	-	-	+	-	+	+	+	+	+
Mental	+	+	-	-	-	+	-	+	+	+	+	+
Dorsal buccal	+	+	-	-	+	2+	-	+	+	+	2+	+
Ventral buccal	+	+	-	-	+	+	-	+	+	+	+	+
Sciatic	-	-	-	-	-	-	-	-	-	-	-	-

* The specimens were taken from the recovered goat (113) 7 weeks after onset of clinical signs.

dog 2-79 had $10^{-2.9}$/g and dog 7-79 had $10^{-3.1}$/g (Table 4.3). The incubation periods for the three dogs were 12, 13 and 13 days for dogs 7-79, 2-79 and 4-79 respectively. In both the goats (Fig. 4.1) and the dogs, the antigen was present as linear rows of tiny dots ("string of pearls") in the nerve fibers. The parenchyma of the salivary glands demonstrated no virus antigen in the goats or dogs. Where antigen was detected in the salivary glands, it was present in the nerves in the glands.

Immunofluorescent staining of tissues and nerves from naturally infected skunks revealed large linear rows of antigen in the maxillary, infraorbital (Fig. 4.2), facial nerves (Figure 4.3), salivary glands, and the skin (Figure 4.4) and corneal impression smears. The virus titers $TCID_{50}$/g in the brain stems of the skunks were $10^{-3.89}$, $10^{-3.91}$, $10^{-4.1}$ and $10^{-2.9}$. These values are not significantly different from the values obtained for the experimentally infected goats.

DISCUSSION

During the terminal phases of rabies infection, the virus undergoes centrifugal spread from the central nervous system resulting in virus generalization (Schneider, 1975b). Using the hamster model and immunofluorescent staining, Murphy et al. (1973) studied this centrifugal spread after the intramuscular injection of rabies virus. They observed that the virus spread to many organs during the terminal phases. Fischman and Schaeffer (1971) also observed extensive centrifugal spread after oral and intranasal administration of rabies virus. They also observed that the cheek mucosa, hair follicles and stomach or intestine from moribund animals inoculated intracerebrally or intramuscularly with rabies virus

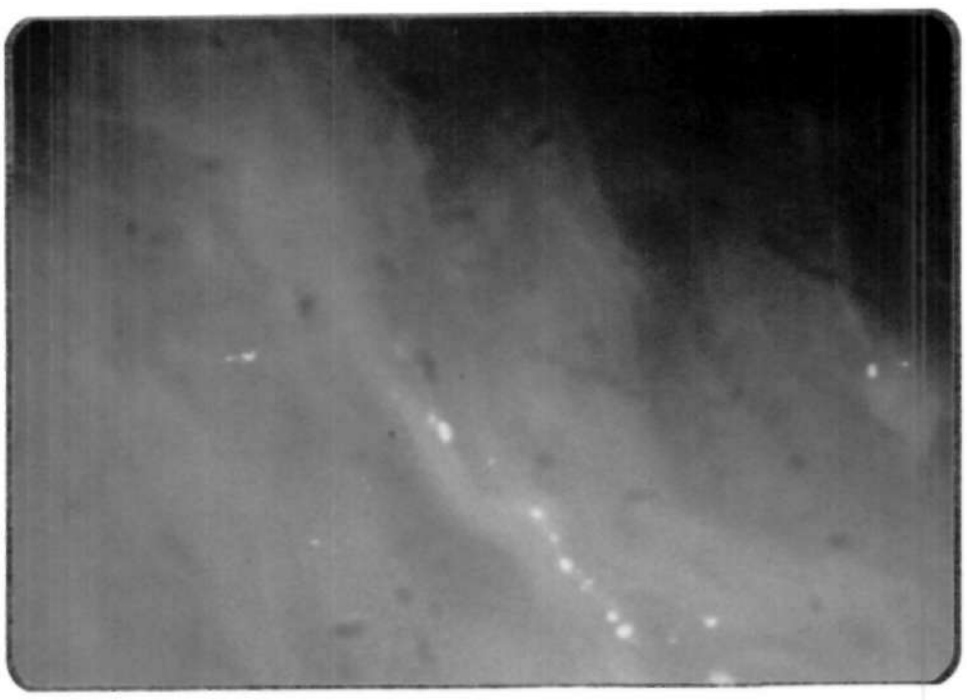


Fig. 4.1. Immunofluorescent staining for rabies antigen in the maxillary nerve (a branch of the trigeminal nerve) of a rabid goat. The goat was experimentally infected with street rabies virus. The amount of antigen present in the nerve was small. X950.

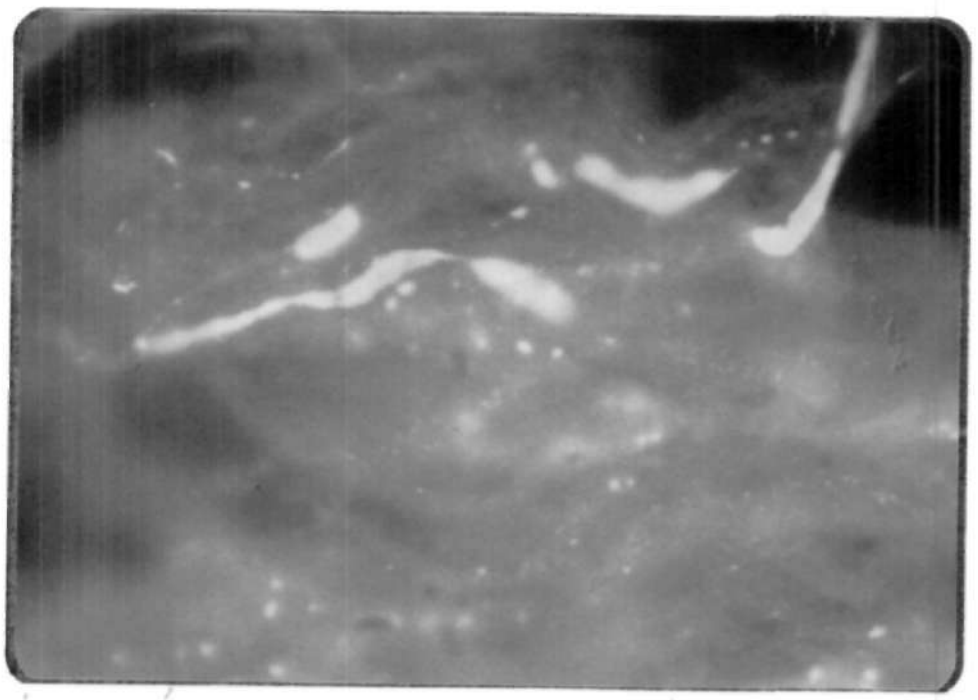


Fig. 4.2. Immunofluorescent staining of rabies antigen in the infraorbital nerve of a naturally infected skunk. X950.

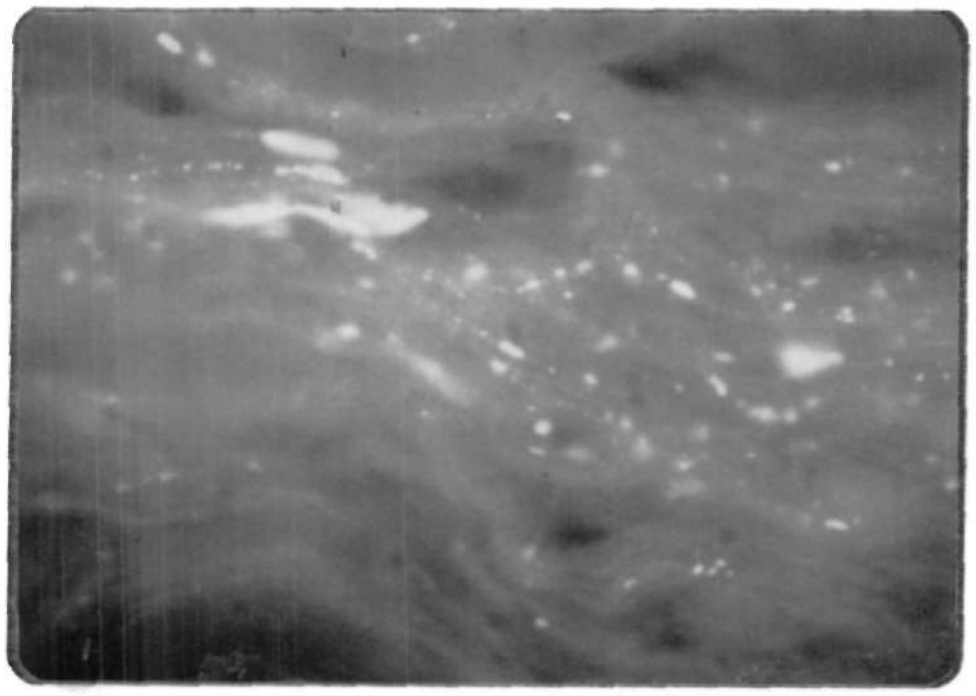


Fig. 4.3. Immunofluorescent staining of rabies antigen in the facial nerve of a naturally infected skunk. The nerve was cut longitudinally. X950.

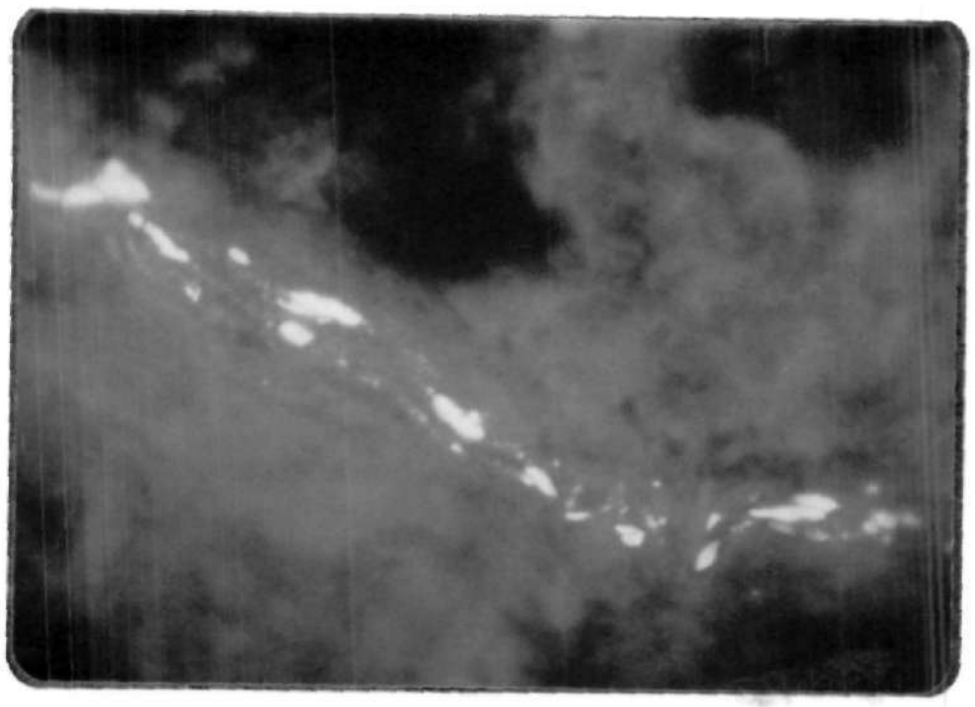


Fig. 4.4. Immunofluorescent staining for rabies antigen in a nerve fiber (probably infraorbital branch of maxillary branch of trigeminal nerve) in the skin of a rabid skunk. The nerve was not associated with hair follicle. X950.

were devoid of fluorescing cells. This observation and the results of this study seem to indicate that intramuscular inoculation does not predictably result in extensive centrifugal spread as evidenced by fluorescence in the major nerve trunks of cranial origin and the cells of the tissue they innervate.

Large amounts of rabies antigen were seen in the nerves of naturally infected skunks in this study and in the study by Howard (1976). This could be explained by the fact that the skunk is highly susceptible to rabies (Constantine and Woodall, 1966), that the virus strain which is spread between skunks is presumably adapted to and highly infective to that species and that the main portal of infection is aerosol (partially intranasal). These factors probably allow for extensive spread of the virus in skunks especially during the terminal stages of the disease.

There is lack of information on the susceptibility of goats to rabies but they are probably less susceptible than skunks. In this study, the goats were infected by intramuscular route with a strain of virus not adapted to the caprine species. This probably results in minimal spread of the virus in the infected animals. Thus little amounts were seen in the nerves.

After inoculation of the Pasteur strain of fixed rabies virus into the masseter muscle of 13 rabbits followed by euthanasia at the onset of clinical signs, only little pathological changes were encountered in the lumbar ganglia except in one case (Field, 1951). In this study, using the same route but with a street virus strain, no antigen was detected in the sciatic and saphenous nerves of the rabid goats and dogs.

Probably the terminal portions of the spinal cord (cauda equina), where the sciatic nerve originates, were not invaded by the virus although this was not evaluated.

Dean and his colleagues (1963) mentioned the difficulty of producing infective salivary glands in dogs after peripheral inoculation so they had to inoculate dogs intracerebrally to obtain infective salivary glands. However, they could easily obtain infective salivary glands with intramuscular injection of virus into the masseter muscle of foxes. In this study the virus was injected intramuscularly in host species less susceptible to rabies than foxes and the salivary glands were negative for rabies antigen except in 2 cases where the antigen was seen in the nerves in the glands. Thus both the route of inoculation and the susceptibility of the host to rabies might be among the factors which determine the presence of antigen in the salivary glands. The absence of antigen in the skin seems to be a reasonable expectation judging from the paucity of antigen in the nerves supplying the lips (the main sites for the specimens), viz., the infraorbital and mental nerves.

The distribution of antigen in the cranial nerves did not seem to be uniform. The optic nerves, which are short and thick, routinely showed large quantities of dustlike fluorescence over the entire surface while others had only scattered areas with linear rows of antigen.

The incubation periods were short in this study (14-24 days for the goats, 12-13 days for the dogs). These short incubation periods probably do not allow time for much centrifugal spread of the virus to have occurred. Hence the quantity of antigen in the cranial nerves and tissues is low despite the presence of appreciable amount of virus

antigen in the brain. The lack of antigen in the parafollicular plexuses is attributed to low content of antigen in the cranial nerves innervating the skin.

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APPENDIX

CALCULATION OF THE VIRUS TITER AFTER TITRATION OF VIRUS SUSPENSION IN CER CELLS

Virus dilution	No. of Non-Fluorescing Fields	No. of Fluorescing Fields	Cum. No. Non-Infected ↓ Flds.	Cum. No. Infected ↑ Flds.	% Infec. Flds.
$10^{-1.7}$	0	20	0	49	100
$10^{-2.7}$	0	20	0	29	100
$10^{-3.7}$	12	8	12	9	43
$10^{-4.7}$	19	1	31	1	3.1

$$\text{difference of logarithms} = \frac{50\% - (\%\text{ of infected fields next below } 50\%)}{\%\text{ infected fields next above } 50\% - \%\text{ infected cells next below } 50\%}$$

X logarithm of dilution factor.

$$= \frac{50-43}{100-43} \times 1 = 0.1228.$$

$\log^{\text{reciprocal}}$ 50% end point dilution = 3.7 - 0.1228

= 3.5772

log 50% end point dilution = -3.5772

$TICD_{50}$ titer = $10^{-3.58}$

$TICD_{50}$/gram = $10^{-3.58} + 10^{-0.6990} = 10^{-4.28}$

CHAPTER V

IMMUNOFLUORESCENT STAINING OF RABIES ANTIGEN IN FORMALIN FIXED INFECTED BRAIN TISSUE AFTER TRYPSIN TREATMENT

SUMMARY

Central nervous system specimens were secured from clinically rabid animals preserved in 10% buffered formalin and then treated with 0.25% trypsin in Hanks balanced salt solution supplemented with 0.02% calcium chloride. The trypsin solution was applied to cryostat cut sections or tiny pieces of the specimen for smears. Direct immunofluorescent (IF) was performed. The results were comparable to those obtained with direct IF staining of fresh frozen cryostat cut sections or smears. Blind coded experiments using brain samples from negative, weakly positive and strongly positive for rabies antigen, indicate a percentage sensitivity of 100 when sections were used and 88 when smears were used. There was no marked difference in intensity of fluorescence when trypsin treatment was carried out at room temperature or at 37°C for periods ranging from 30 minutes to 210 minutes, overnight at +4°C or when the concentration of trypsin was varied between 0.0625% and 0.25% for 1 hour at room temperature. Specificity of the fluorescence was ascertained by the inhibition of the fluorescence following treatment of the trypsin-digested sections or smears with antirabies conjugate diluted in a 20% suspension of challenge virus standard strain of rabies virus infected mouse brain. It is proposed that specimens for IF staining of rabies antigen might be shipped in 10% formalin to minimize the risk of exposure to the handlers and prevent tissue deterioration.

INTRODUCTION

Immunofluorescent staining is the most accurate microscopic test at present available for the diagnosis of rabies (Dean and Abelseth, 1973). For good results, the suspect tissues might be preserved by chilling or freezing or in 50% glycerol-saline solution prior to testing (Andrulonis and Debbie, 1976). Smears or sections from glycerol-saline preserved tissues are not satisfactory for fluorescent antibody examination after fixation in acetone (McQueen et al., 1960) and elimination of acetone fixation has been shown to improve fluorescence (Andrulonis and Debbie, 1976). Delays in the transportation of chilled or frozen specimens often result in the specimens arriving in the laboratory partially or totally decomposed. False negative results are likely to be obtained from deteriorated brain (Lewis and Thacker, 1974) and skin (Blenden, 1978) specimens. Yet the laboratory results may be an important factor in the decision to administer postexposure treatment to an exposed individual.

The routine fluorescent antibody test for detection of rabies is not a safe procedure since smears from infected brains fixed in acetone at -60°C for 4 hours could still be infective (Fischman and Ward, III, 1969). The use of 95% ethyl alcohol to eliminate the infectivity (Fischman and Ward, III, 1969) results in excessive nonspecific staining and poor results (Kissling, 1975). The use of unfixed smears poses even greater exposure risks. Fixation of specimens in 10% buffered formalin renders the specimen safe and prevents deterioration during transportation. Enhancement of immunofluorescent staining of measles and herpes simplex viruses (Swoveland and Johnson, 1979) and a variety of other antigens (Huang et al., 1976) in formalin-fixed paraffin embedded tissues after

trypsin treatment has been reported. In this study, trypsin digestion was used to detect rabies virus antigen in smears and cryostat cut-sections, prepared from tissues which had been preserved in 10% buffered formalin (pH 7.4) for as long as 7 weeks.

MATERIALS AND METHODS

Tissues

Brain and spinal cord samples from goats and dogs were obtained from animals used in previous experiments. These were both rabies positive and rabies negative specimens. Initially 34 specimens were examined as sections and 14 as smears.

Normal Mouse Brain (NMB) Suspension

Weanling mice were killed by carbon dioxide euthanasia and their brains were harvested. A 20% suspension was prepared with 0.75% bovine serum albumin (BSA) in buffered water pH 7.6 and centrifuged at 1000 x g for 10 minutes at -10°C. The supernatant was aliquoted and stored at -70°C.

Challenge Virus Standard Mouse Brain (IMB) Suspension

The challenge virus standard (CVS), originally obtained from Center for Disease Control, Lawrenceville, Georgia and maintained in mice in our laboratory, was diluted 1:100 and injected intracerebrally (0.03 ml) into weanling mice. The brains were harvested when the mice were moribund. A 20% brain suspension was prepared with 0.75% BSA in buffered

water, centrifuged at 1000 x g for 10 minutes at -10°C, aliquoted and stored at -70°C.

Antirabies Globulin Conjugate

Fluorescein isothiocyanate labeled anti-rabies globulin of bovine or equine origin was obtained commercially (BBL, Cockeysville, MD 21030). The conjugate was titrated and a dilution of 1:150 was suitable for use in all the tests.

Rabies Antiserum

A goat which had recovered from an experimentally induced rabies was given 3 boosters of 2 doses each of ERA vaccine one week apart. Blood was drawn on the 10th day after the last injection. Serum was extracted and stored at -20°C.

Preparation of the Tissues

Cut sections from Fresh Frozen Specimens - Fresh frozen brain or spinal cord samples were mounted on brass cryostat buttons using a commercial mounting medium (O.C.T. Compound, Tissue Tek 11, Lab-Tek Products, Naperville, Illinois). Sections 10μ thick were cut in a cryostat chamber (I.E.C. CTF Microtome-Cryostat, International Equipment Co.) at -20°C and 2 sections were mounted on each precleaned glass slide with a frosted end. The slides were fixed in acetone at -20°C for 30 minutes. After acetone fixation, the slides were dipped in buffered water pH 7.6 for 1 minute to dissolve the mounting medium and then air-dried. In some cases smears were made on the slides, fixed in acetone and dried for immunofluorescent staining.

Cut Sections from Formalin-Fixed Tissues - Brain and spinal cord tissues which had been preserved in 10% buffered formalin (pH 7.4) from 3 days to 7 weeks were used. The specimens were removed from the formalin and washed in phosphate buffered saline (PBS, 0.1M, pH 7.5). The specimens were mounted and cut in a cryostat and 2 sections were placed on the slides. The slides were fixed in acetone at -20°C for 30 minutes and then washed with buffered water pH 7.6 for 1 minute. The slides were placed in petri dishes or in pans and covered with 0.25% trypsin (Difco 1:250, DIFCO Labs., Detroit, Michigan) in 1 X Hanks balanced salt solution (H-BSS, GIBCO) containing 0.02% calcium chloride and in which the pH was adjusted to 7.8 with drops of 7.5% sodium bicarbonate solution. Trypsin treatment was for 1 hour at room temperature or overnight at +4°C. After trypsin treatment, the slides were dipped in PBS pH 7.5, refixed in acetone at -20°C for 10 minutes and air-dried for immunofluorescent staining.

Standard Smears from Formalin-Fixed Tissues - After removing the specimens from the formalin, they were washed in PBS pH 7.5 or buffered water pH 7.6, crushed or cut into tiny pieces, placed in test tubes and treated with 0.25% trypsin in H-BSS containing 0.02% calcium chloride (pH 7.8) for 1 hour at room temperature or overnight at +4°C. After the digestion, the tissues were washed twice with PBS before smears were made on slides. The smears were fixed in acetone at -20°C for 30 minutes, air-dried for immunofluorescent staining.

Immunofluorescent Staining

Each section or smear on the slide was encircled with a wax pen (Mark-Tex, Tech-pen, Mark-Tex Corp., Englewood, N.J.). Fluorescein

isothiocyanate (FITC) labeled antirabies globulin was diluted in 1:150 in infected mouse brain suspension (IMB) and some was diluted to the same ratio in normal mouse brain suspension (NMB). The diluted conjugate was incubated at 37°C for 30 minutes. After incubation, 2 drops of 0.1% aqueous solution of Evans blue dye per 1.0 ml of diluted conjugate were added. One drop of the conjugate diluted with IMB suspension was put on the section or smear near the frosted end of the slide for inhibition control and one drop of the conjugate diluted in NMB suspension was placed on the section or smear further away from the frosted end for the test side. The slides were incubated at 37°C for 30 minutes in a moist chamber. After incubation, the slides were washed twice in buffered water pH 7.6 for 5 minutes each and then rinsed in tap water for 1 minute. The slides were air-dried and examined using a Leitz Ortholux Fluorescence Microscope, with HBO 100 W lamp and epi-illumination. The filtering system consisted of BG23 heat, 2 KP 490 (=KP 500) excitation and K 495 suppression filters. The slides were examined with a 10X ocular and 95X objective oil immersion lens. Judgement was based on the presence of apple green fluorescence in strings or clusters in the test side and the absence of such fluorescence on the inhibition control side. A 5-point scoring system was used to record the amount of fluorescence present in a particular slide.

- = no fluorescence seen in all fields examined.
+ = small amount of fluorescence seen in 1 to 4 fields.
2+ = fluorescence present in 25-50% of the fields examined.
3+ = fluorescence present in 50-75% of the fields examined.
4+ = fluorescence present in every field.

Blind Coded Samples

The samples were from the brains of goats and dogs that were experimentally infected with rabies and from naturally infected skunks. Samples (48) with grades of fluorescence consisting of +(9), 2+(3), 4+(20) and negative (16) by previous immunofluorescent staining for rabies antigen were put in 10% buffered formalin. After at least 3 days, the person doing the coding was provided with bottles containing 10% buffered formalin and instructions to cut identical portions into the bottles and code them. The samples were kept at room temperature until processed for immunofluorescent staining both as sections and as smears.

Effect of Digestion Time

Brain samples, which had been shown to be positive for rabies antigen by direct immunofluorescent staining, were placed in 10% formalin for at least a week. The samples were removed from formalin, washed with PBS, mounted and cut in a cryostat and fixed in acetone. The samples were washed in buffered water for 1 minute and treated with 0.25% trypsin in Hanks BSS containing 0.02% calcium chloride for varying times of 30, 60, 90, 120, 150, and 180 minutes at 37°C. Then two slides from each brain sample were processed for immunofluorescent microscopy. Some slides were digested at room temperature for 30 and 60 minutes and overnight at 4°C before processing.

Effect of Concentration of Trypsin

Slides prepared from formalin fixed rabid brains were digested at 4°C overnight with 0.25%, 0.125% and 0.0625% trypsin in Hanks BSS with 0.02% $CaCl_2$ before being processed for immunofluorescent staining.

Effect of Trypsin Treatment on Frozen Specimens

Fresh frozen brain, spinal cord and skin samples were mounted, cut in a cryostat and placed on slides. The sections were treated with 0.25% trypsin in H-BSS containing 0.02% calcium chloride for 1 hour at room temperature. The slides were then fixed in acetone at -20°C and stained for immunofluorescent examination.

Statistical Analysis

The percent sensitivity and specificity calculated as described by Mausner and Bahn (1974), were used to compare IF staining of rabies antigen in trypsin treated formalin-fixed tissues with IF staining of fresh or frozen specimens. The formulas for these calculations follow:

Percent Sensitivity = [true positive/(true positive + false negative)] X 100.

Percent Specificity = [true negative/(true negative + false positive)] X 100.

RESULTS

At the initial stage, only specimens taken from the brain stems of goats and Ammon's horn in dogs were used. The initial results showed a 100% sensitivity and 100% specificity between sections from the fresh frozen specimens and formalin preserved specimens from the same animal. Large and medium size antigen aggregates fluoresce at the same intensity in both cases but tiny antigen inclusions tended to be still masked in many cases though not enough to cause any significant effect on the scoring of the section (Figures 5.1, 5.2, 5.3). The initial results from the smears were comparable to those obtained with smears made from fresh frozen specimens.

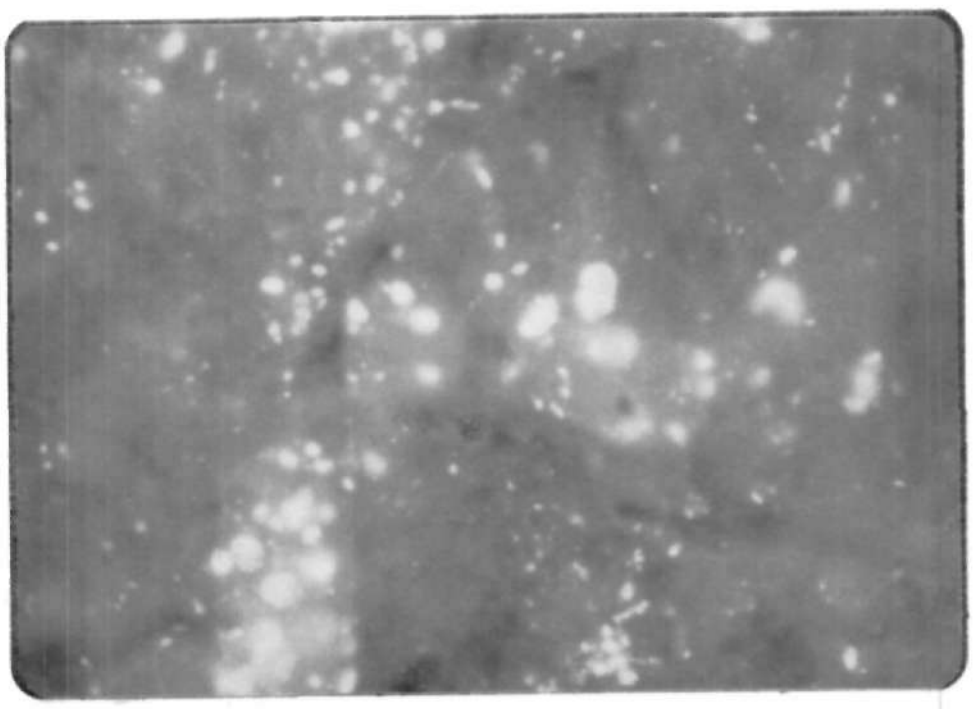


Fig. 5.1. Immunofluorescent staining for rabies antigen in a section of the brain of a goat experimentally infected with a street rabies virus. X950.

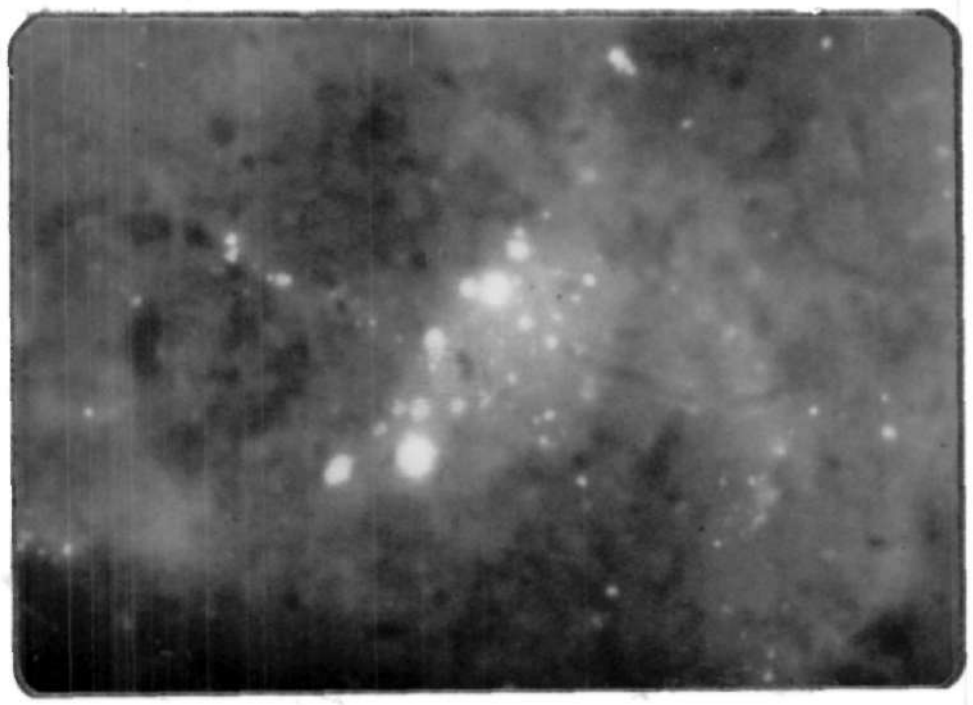


Fig. 5.2. Immunofluorescent staining of rabies antigen in a smear made from infected goat brain tissue which had been preserved in formalin for 10 days at room temperature and then digested with 0.25% trypsin for 1 hour at room temperature. X950.



Fig. 5.3. Immunofluorescent staining of rabies antigen in a smear made from infected goat brain tissue which had been preserved in 10% buffered formalin for 10 days at room temperature. The tissue was digested with 0.25% trypsin overnight at 4°C before the preparation of the smear. X950.

The results of blind coded samples indicated 100% sensitivity when cryostat cut sections of the formalin fixed tissues were examined and 88% sensitivity when smears were examined. The percentage specificity was 100 in both cases.

The results of the effects of the time of trypsin treatment, and concentration of trypsin are shown in Tables 5.1, 5.2 and 5.3.

When fresh frozen tissues were treated with trypsin prior to immunofluorescent staining, the effect of the trypsin treatment was not readily discernible except for slight tissue digestion (Table 5.4). When smears or formalin fixed cryostat cut sections were treated with goat anti-rabies serum after trypsin treatment and before IF staining, no fluorescence was observed in known rabies positive samples.

DISCUSSION

The results of immunofluorescent staining of rabies antigen in formalin fixed brain tissues, taken from the brain stem in goats and Ammon's horn in dogs, after trypsin treatment, are comparable in sensitivity and specificity to those of IF staining of fresh frozen specimens of the same tissues. Thus fixation in 10% buffered formalin of brain specimens for rabies examination might be considered in situations where frozen specimens could not be sent due to distance or other transportation difficulties. The smear method would be useful in laboratories where cryostats are not available, however, care must be taken to prevent the smears from washing off; five or more slides should be prepared and read for each sample.

Prolonged treatment with trypsin at 37°C does not affect the intensity of fluorescence remarkably but could result in tissue disintegration. Satisfactory results were obtained by treating the sections at

Table 5.1

Effect of the Time of Trypsin Treatment at 37°C on IF Staining of Formalin Fixed Brain Tissues for Rabies Antigen

Specimens (Brainstems)	Fresh Tissue	Formalin Fixed Tissue						
		No trypsin treatment	Trypsin treatment time at 37°C					
			30 min	60 min	90 min	120 min	150 min	180 min
Goat 120	4+	-	3+	4+	4+	4+	4+	4+
Goat 118	4+	-	4+	4+	4+	4+	4+	4+
Goat 110	4+	-	2+	4+	4+	4+	4+	4+
Dog 2-79	2+	-	2+	2+	2+	2+	2+	2+
Dog 7-79	3+	-	2+	3+	3+	3+	3+	3+
Goat 113	-	-	-	-	-	-	-	-
Dog 1-79	-	-	-	-	-	-	-	-

Table 5.2

Effect of Time of Trypsin Treatment at Room Temperature on IF Staining of Formalin Fixed Brain Tissue for Rabies Antigen

Specimen	Fresh Tissue	Formalin Fixed Tissue			
		No trypsin Treatment	Trypsin Treatment 30 min	60 min	16 hours
Goat 110 brain stem	4+	-	2+	4+	4+
Goat 117 brain stem	4+	-	3+	4+	4+
Goat 114 brain stem	4+	-	2+	3+	4+
Goat 118 brain stem	4+	-	3+	4+	4+
Goat 120 brain stem	4+	-	4+	4+	4+
Goat 110 Spinal cord	4+	-	2+	4+	4+

Table 5.3

The Effect of the Concentration of Trypsin on IF Staining for Rabies Antigen in Formalin Fixed Brain Tissue Samples After Overnight Trypsin Treatment at +4°C

Specimen (Brainstems)	IF on fresh tissue	Formalin Fixed Tissue Concentration of Trypsin			
		0.0%	0.0625%	0.125%	0.25%
Goat 110	4+	-	4+	4+	4+
Goat 115	4+	-	3+	4+	4+
Goat 118	4+	-	4+	4+	4+
Goat 120	4+	-	3+	4+	4+
Goat 121	4+	-	4+	4+	4+

Table 5.4

Effect of Trypsin Treatment on Immunofluorescent Staining of Rabies Antigen in Cryostat Cut Frozen Specimens (Not Fixed in Formalin)

Specimen	No trypsin Treatment	Trypsin Treatment*
Goat 110 brain stem	4+	4+
Goat 120 brain stem	4+	4+
Goat 117 brain stem	4+	4+
Goat 120 spinal cord	4+	4+
Skunk skin, Mo. Div. Hlth., Jeff. City	3+	3+
Skunk skin, Mo. Div. Hlth., Jeff. City	4+	4+

*0.25% trypsin for 60 minutes at room temperature.

room temperature for 1 hour or overnight at +4°C. Even though lower concentrations of trypsin gave satisfactory results, 0.25% was used routinely because of convenience since the same concentration was being used for the trypsinization of cells. The false negative results observed with smears in the blind coded experiment resulted only from specimens that were known to contain low level of rabies antigen (1+) in the areas examined and was due in part to the initial difficulty in making smears from formalin fixed brain tissues. Later it was observed that grinding the specimens coarsely before trypsin treatment gave very good smears. When these initial problems were overcome, the smear method was fast and gave results comparable to the cryostat cut section of freshly frozen samples. No fluorescence was observed when the conjugate diluted in a 20% CVS infected mouse brain suspension was applied to the trypsin treated formalin-fixed tissue section or smear. Thus the fluorescence on the test side was specific for rabies antigen. Specificity of the test was further ascertained by observation of no fluorescence when the trypsin treated smears or sections were treated with rabies antiserum prior to IF staining.

Formalin fixation probably renders the tissues impermeable to the conjugate. Trypsin presumably acts by causing some disruption of the tissue structure (Curran and Gregory, 1977), and thus increasing the permeability. Trypsin catalyses the hydrolysis of only those peptide bonds in a polypeptide chain whose carbonyl function is donated by either a lysine or an arginine residue (Lehninger, 1975). This probably alters the net charge and eliminates nonspecific binding of the conjugate (Fothergill, 1969).

This technique would be useful in many instances where rabies was mistakenly not considered in diagnosis of an encephalitic condition until after the only available brain tissue had been put in formalin. It might also be useful in developing and underdeveloped tropical countries where poor refrigeration and tropical heat combine to cause rapid deterioration of rabies suspected brain specimens. Fixation in formalin might be a safe way to transport specimens to be used for immunofluorescent staining of rabies antigen or antigens of other dangerous viruses. It might be possible to examine a particular section by Haematoxylin-Eosin staining, then decolorize and process for IF staining.

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CHAPTER VI

CHEMICAL CHANGES IN SERUM AND CEREBROSPINAL FLUID OF GOATS AND DOGS EXPERIMENTALLY INOCULATED WITH STREET RABIES VIRUS

SUMMARY

The use of changes in blood and cerebrospinal fluid (CSF) constituents as an aid in monitoring the presence of rabies virus infection and the progression of clinical rabies was evaluated. Serum and CSF samples from goats and dogs experimentally inoculated with street rabies virus were analyzed for albumin, globulin, creatine phosphokinase (CPK), glutamic oxalacetic transaminase (GOT), glucose and chloride using commercially available kits. CSF cell counts were performed by hemacytometer method and the CSF total protein values were determined by the Coomasie dye binding method.

Values obtained for serum chemistry of both the goats and the dogs were within the reported normal ranges. The CSF of the rabid goats showed elevation in mean total protein value, mean CPK values, and mean total white cell counts. When the absolute values were compared with the reported normal range of values, overlapping results were obtained. The values obtained for the CSF chemistry from the dogs were within reported normal ranges regardless of the inoculation status. The determination of these blood and CSF constituents in monitoring the stage of infection with rabies virus did not produce results which were useful based on change. The fact that values did not change could well be suggestive of rabies when contrasted to other forms of encephalomyelitis.

INTRODUCTION

Examination of the cerebrospinal fluid (CSF) is indicated whenever there is a clinical evidence of central nervous system (CNS) lesions (Cornelius, 1963). Examination of the blood parameters secured simultaneously from the same animal is necessary since the values of certain parameters in the CSF may be a reflection of the values of the same parameters in the blood. CNS lesions may result in pleiocytosis (Cornelius, 1963), elevated protein levels (Schuller, et al., 1979) and elevated enzyme levels in the CSF (Wright, 1980). Total cell counts in the CSF may be normal (Bhatt, et al., 1974) or raised (Hattwick, et al., 1972) in human rabies. In fact, CSF may be normal for 10 days after the onset of rabies in human being (Maton et al., 1976). CSF protein levels may be normal (Ghösh, 1964) or elevated (Porras et al., 1976; Maton et al., 1976) in rabies.

Serum levels of creatine phosphokinase (CPK) are elevated in diseases affecting skeletal and cardiac muscles (Okinaka et al., 1961) cerebral infarction (Acheson et al., 1965; Eisen and Sherwin, 1968) since brain, skeletal and cardiac muscles are rich in this enzyme (Eshchar and Zimmerman, 1967; Dawson, et al., 1967). Various CNS conditions result in a raise in the levels of this enzyme in CSF (Drummond and Belton, 1972; Katz and Leibman, 1970; Wright, 1980). Elevated levels of serum CPK have been reported in human rabies (Hattwick et al., 1972). Elevation of CPK levels in CSF and serum is expected in rabies since the disease affects brain as well as the myocardium (Ross and Armentrout, 1962; Cheetham et al., 1970; Warrell et al., 1976).

The main hypothesis for this study was that there were changes in the total cell counts and total protein levels in the CSF and in the levels of albumin, globulin, glucose, chloride, glutamic oxalacetic

transaminase, and creatine phosphokinase in the CSF and serum samples of goats and dogs that were experimentally inoculated with street rabies virus.

MATERIALS AND METHODS

Animals

Twelve goats, aged between 4 and 6 months, were purchased through Animal Resources, University of Missouri-Columbia. They were maintained in the Veterinary Research Farm, University of Missouri-Columbia for a week before inoculation. Seven dogs, 6 months old, from a single litter, were likewise obtained from Animal Resources, University of Missouri-Columbia. The dogs were born and bred in confinement on the Veterinary Research Farm. None of the goats or dogs had been vaccinated against rabies. The dogs had not been vaccinated against any disease and had no contact with other dogs.

Virus

A 20% suspension in 0.75% buffered bovine serum albumin was prepared from the brainstem of a rabid dog. This suspension had a titer $10^{4.03}$/ 0.03 ml intracerebral LD_{50} in weanling mice. The dosage for use in the animals was based on the results of previous inoculation of these species with the same virus isolate (Umoh, 1977).

Inoculation

The goats were eartagged for identification; 10 of them were inoculated into the right masseter muscle. Five were given a dose of 5.54 $\log_{10}$ $MICLD_{50}$ in 1.0 ml; 4 had 5.85 $\log_{10}$ $MICLD_{50}$ in 2.0 ml; and, 1 had 5.94 $\log_{10}$ $MICLD_{50}$ in 2.5 ml depending on the size of the goat. Two goats were

not inoculated. The dogs were anesthetized by intravenous injection of 4.0% Biotal^{R} (sodium thiamylal, 5.0 ml, Bio-Ceutic Laboratories Inc., St. Joseph, Missouri). All the dogs were inoculated with a uniform dose of 5.54 $\log_{10}$ $MICLD_{50}$ in 1.0 ml into the left masseter muscle.

Observation and Sampling of the Animals

Blood was obtained by cephalic or lateral saphenous venipuncture and the CSF secured from the cisterna magna by an aseptic suboccipital puncture (Cornelius, 1973) just before virus inoculation. The animals were observed daily for signs of central nervous system disorder. As soon as the signs were observed, the goats were anesthetized with Rompun^{R} (Xylazine, 15 mg, Bayvet Division, Cutter Laboratories Inc., Shawnee, Kansas). Blood was obtained by jugular venipuncture and CSF by suboccipital puncture using 20 gauge 4 inch long spinal needle. Lumbar puncture was unsatisfactory for obtaining CSF in goats. The dogs were anesthetized and the blood and CSF obtained as already described. The blood was allowed to clot and serum extracted. Each serum sample was divided into 5 aliquots, some of which were stored frozen and others refrigerated. Analyses were carried out within 5 days of collection.

Examination of Cerebrospinal Fluid

Only clear and colorless specimens of CSF were evaluated. CSF was checked for turbidity, coagulability and cells. A total cell count was performed shortly after collection by hemocytometer method as described (AO Brightline hemacytometer manual, American Optical Corp., Buffalo, N.Y. 14215). Filtered diluting fluid (glacial acetic acid, 10 ml; distilled water, 90 ml; methyl violet, 0.2g) was drawn into a leukocyte-diluting

pipette up to the 1 mark and filled to the 11 mark with CSF. This was mixed thoroughly, a few drops from the lumen of the pipette discarded, and each side of the hemacytometer (Bright-line, American Optical Corp., Buffalo, N.Y. 14215) filled. All the cells within the entire ruled area on both sides of the counting chamber were counted and the result was multiplied by 1.23 (a prescribed conversion factor), the product being the number of cells per cubic milliliter. The CSF was centrifuged at 800xg for 5 minutes. The supernatant was aliquoted and stored both at -20°C and at 4°C for chemical analysis. The sediment was used for a differential cell count. The differential count was performed as described by Choi and Anderson (1979). Smears of the sediment were made on precleaned glass slides and a drop of a filtered staining solution added (toluidine blue 1.0g, 95% ethanol 20 ml, made up to 100 ml with deionized distilled water, and mixed 1:1 with equine serum). The smears were covered with coverslips and were examined immediately using 1000X oil immersion. Five hundred cells were counted. Cells with round nuclei, dense chromatin and agranular cytoplasm were identified as lymphocytes while those with reniform nuclei and few cytoplasmic granules were identified as monocytes.

Chemical Analysis

a) The Total Protein Contained in CSF

The total protein contained in CSF was determined by the Coomasie dye binding method (Bradford, 1976). The working reagent was prepared by dissolving 100 mg of Coomasie brilliant blue G-250 (CBB, Sigma Chemical Co., St. Louis, Missouri) in 50 ml of 95% ethanol. To this was added 100 ml of 85% (v/v) phosphoric acid and the solution was made up to 1000 ml with distilled water. The standard protein was obtained from

Pierce microprotein Rapid^{R} Kit (Pierce, Rockford, Illinois). Protein concentrations ranging from 10 mg to 99 mg in 100 µl were used to prepare standard curves. Five ml of CBB reagent were added to 100 µl of the sample, 100 µl of 0.85% saline solution (blank) and 100 µl of diluted standard protein. These were mixed with a vortex mixer (Vortex Genie, Scientific Industries, Sprinfield, MA). Absorbance of the mixture was measured within 1 hour with a spectrophotometer (Coleman, Junior Spectrophotometer, Coleman Instruments, Maywood, Illinois). Samples with protein concentrations greater than 100 mg were diluted 1:10 in 0.85% saline, the assay was repeated and the resulting concentration was multiplied by 10.

b) Albumin

Albumin was determined colorimetrically at 630 nm based on the affinity of albumin for bromcresol green using a commercially available kit (Sigma Technical Bulletin 630, 1979, Sigma Chemical Co., St. Louis, Missouri 63178).

c) Globulin

Total globulin was determined colorimetrically at 560 nm (Sigma Technical Bulletin No. 560, 1978, Sigma Chemical Co., St. Louis, Missouri). This is based on the reaction between tryptophan and glyoxylic acid in the presence of sulfuric acid producing a violet color which can be measured spectrophometrically.

d) Chloride

Chloride was determined by titrating the sample with a standardized mercuric nitrate solution in the presence of diphenyl-carbazone as an indicator. The quantity of chloride present was expressed as mEq/liter

(Sigma Technical Bulletin No. 830, 1977, Sigma Chemical Co., St. Louis, Missouri).

e) Glucose

A commercially available kit (Sigma Technical Bulletin No. 115, 1978, Sigma Chemical Co., St. Louis, Missouri) was used to determine glucose levels in both CSF and serum. This is based upon the conversion of glucose to glucose-6-phosphate, coupled with the subsequent reduction of nicotinamide adenine dinucleotide phosphate (NADP) to reduced NADP (NADPH) which in the presence of phenazine methosulfate reduces iodonitrotetrazolium violet (INT) to reduced INT. The reduced INT is measured spectrophotometrically at 520 nm.

Creatine Phosphokinase

The quantity of CPK was determined using a commercially available kit (Sigma Technical Bulletin No. 661, Sigma Chemical Co., St. Louis, Missouri). This test is based on the following reaction:

$$\text{ATP} + \text{creatine} \xrightarrow{\text{CPK}} \text{ADP} + \text{phosphocreatine}$$

$$\text{Phosphocreatine} \xrightarrow{\text{hydrolysis}} \text{creatine} + \text{inorganic phosphorus}$$

The inorganic phosphorus was measured colorimetrically at 660 ng (Coleman Junior Spectrophotometer, Coleman Instruments, Maywood, Illinois). The level of CPK was expressed as Sigma units per ml.

Glutamic Oxalacetic Transaminase (GOT)

The level of GOT was determined based on the reaction:

Aspartic acid + α-ketoglutaric acid $\xrightarrow{GOT}$ oxalacetic acid + glutamic acid

The oxalacetic acid was reacted with 2-4 dinitrophenyl-hydrazine as described in the kit used (Sigma Technical Bulletin No. 505, Sigma Chemical Co., St. Louis, Missouri). The absorbance of the mixture was determined spectrophometrically at 505 nm. The level of GOT was expressed as Sigma-Frankel (SF) units per ml.

Statistical Analysis

The results were expressed as arithmetic means and standard deviations. The Wilcoxon signed ranks test (Conover, 1971) was used to compare the values obtained from samples taken prior to virus inoculation with those obtained from the dogs during clinical rabies and 7 weeks post-inoculation. Similar comparisons could not be made for the goat data because samples were not taken before inoculating the goats with rabies virus.

RESULTS

All the cerebrospinal fluid samples obtained from the goats were clear and colorless. CSF samples from 2 rabid and 1 normal goats were viscous but clear. The CSF of the 2 normal goats had total cell counts of 7 and 29 per mm^3 respectively while samples from rabid goats had 15 to 60 cells per mm^3 (mean = 38.7, standard deviation (SD) = 14.8). Differential cell counts performed on CSF samples from the rabid goats showed 80-91% lymphocytes and the rest were monocytes.

Chemical analysis of the CSF of the goats showed elevation in mean total protein, mean CPK level, mean glucose level, and mean chloride

Table 6.1

Chemical Analysis of Cerebrospinal Fluid Samples from Normal and Rabid Goats

Goat No.	Albumin g/100 ml	Globulin g/100 ml	Total Protein mg/100 ml	CPK Sigma Units	Glucose mg/100 ml	Chloride mEq/l	GOT SF Units	Cells per mm^3
120 Normal	0	0	10	15	59.4	119.1	12	29
121 Normal	0	0	6.6	10	53	90.5	24	7
110 Rabid	0.19	0	41.2	15	106.1	130	14	-
111 Rabid	0.46	0	985	-	67.1	110	-	51
112 Rabid	0.05	0	4.4	7	61.1	130	8	15
113 Rabid	0.19	.06	60.2	15	54.9	130	10	-
114 Rabid	0.14	0	40	7	36.8	130	8	40
115 Rabid	1.6	0	-	18	115.9	105	18	-
117 Rabid	0.23	0	30	15	109.8	115	14	42
118 Rabid	0.23	0	6.6	11	60.5	130	10	60
119 Rabid	0.23	0	113.8	89.5	67.1	120	20	35
122 Rabid	.19	.18	60	-	48	122	-	28
123 Rabid	0.45	0.22	106.2	156	61.1	114.6	26	-
Mean±SD (control)		0	8.3±2.4	12.5±3.5	56.2±4.5	104.8±20.2	18.0±8.5	18±15.5
Range (control)		-	6.6-10	10-15	53-59.4	90.5-119.1	12-24	7-29
Mean±SD (test)			144.7±297.5	36.6±51.8	71.7±26.5	121.5±9.3	14.2±6.1	28.7±14.8
Range (test)			4.4-985	7-89.5	36.8-115.9	105-130	8-26	15-60

level above the mean of the values of those parameters for the 2 normal goats (Table 6.1).

The values obtained when the blood serum samples from normal and rabid goats were analyzed for albumin, globulin, CPK, glucose, and GOT are shown in Table 6.2. Except with CPK and GOT, the mean values of each constituent determined for the sera of the rabid goats were not different from the values obtained for the sera from the 2 normal goats.

The CSF samples secured from the dogs were clear and colorless. The total cell counts in the CSF of the dogs taken before virus inoculation ranged from 9 to 14 per mm^3 (mean - 11, SD = 3), and ranged from 13 to 32 per mm^3 (mean 22, SD = 8), in the CSF samples taken at the end of the experiment or during clinical rabies. Differential counts revealed small lymphocytes (76 - 88%), large lymphocytes (10 - 21%) and polymorphonuclear neutrophils (1 - 4%) regardless of the inoculation status.

Chemical analysis of the CSF from the dogs showed only slight increase in the mean total protein and the mean CPK values in CSF taken postinoculation when compared to CSF taken before virus inoculation (Table 6.3).

Chemical analysis did not reveal significant changes in the levels of albumin, globulin, CPK, chloride, or glucose between the sera of dogs taken before inoculation and the sera taken after inoculation or during clinical rabies (Table 6.4). Serum GOT levels were significantly lower ($\alpha = 0.05$) in the postinoculation samples than in the preinoculation samples. Glucose levels in the sera of the two rabid dogs (#s 2-79 and 4-79) were lower than levels in the preinoculation sera from the same dogs.

Table 6.2

Chemical Analysis of the Blood Serum Samples from Normal and Rabid Goats

Goat No.	Albumin g/100 ml	Globulin g/100 ml	CPK Sigma units	Glucose mg/100 ml	Chloride mEq/l	GOT SF units
120 Normal	3.2	2.5	13	106	104.8	30
121 Normal	3.2	2.5	24	78.2	104.8	46.5
110 Rabid	3.2	2.3	71	90.9	110	92
111 Rabid	3.3	1.5	24	37.7	100	96
112 Rabid	2.6	2.6	71	102.7	115	60
113 Rabid	3.1		62			44
114 Rabid	2.7	2.5	24	83	110	92
115 Rabid	3.2	2.3	13	108.5	104.8	72
117 Rabid	2.8	2.3	24	45.7	102.5	72
118 Rabid	3.2	2.0	24	143.3	102.5	60
122 Rabid	3.7	2.7	220		97.6	128
Mean Control	3.2	2.5	18.5±7.8	92.0	104.8	38.5±11.7
Range			13-24	78.2-106		30-46.5
Mean test	3.09±0.34	2.3±0.4	59.2±64.5	87.4±36.6	105.3±5.9	79.6±25.2
Range	2.6-3.7	1.5-2.7	13-220	37.7-143.3	97.6-115	44-128

Table 6.3

Chemical Analysis of Cerebrospinal Fluids From Dogs

Dog No. & Status		Albumin g/100 ml	Globulin g/100 ml	Protein[a]	CPK Sigma units	Chloride[c]	Glucose[a]	GOT[b]
1-79	Preinoculation	0	0	6.6	15	123.8	67	19
	Postinoculation	0	0	7.9	17	123.8	62.5	17
2-79	Preinoculation	0	0	4.4	46	123.8	91	10
	Postinoculation	0	0	13.2	51	119.1	71.3	8
3-79	Preinoculation	0	0	21.9	4	119.0	73	16
	Postinoculation	0	0	ND	4	119.0	107.8	16
4-79	Preinoculation	0	0	10.8	0	119.0	73	22
	Postinoculation*	20.5	0	29.6	7	130.9	68.8	12
5-79	Preinoculation	0	0	10.9	15	123.8	76	24
	Postinoculation	0	7.6	8.2	14	104.8	68.8	25
6-79	Preinoculation	0	0	15.3	7	121.4	83	22
	Postinoculation	0	0	26.3	22	119.1	83.0	19
7-79	Preinoculation	0	0	4.4	16	119.0	76	18
	Postinoculation**	-	-	-	-	-	-	-
Mean	Preinoculation			10.6±6.4	14.7±15.1	121.4±2.4	77±7.8	18.7±4.7
	Postinoculation			17.0±10.3	19.2±16.9	119.5±8.6	77.0±16.5	16.2±5.9
Range	Preinoculation			44-21.9	0-46	119.0-123.8	67-91	10-24
	Postinoculation			7.9-29.6	4-51	104.8-130.9	62.5-107.8	8-25

a = mg/100 ml b = SF units c = mEq/l *Indicates clinically rabid dog

**The animal died before the specimens could be obtained.

Table 6.4

Chemical Analysis of the Serum Samples from Dogs. The Blood was Drawn **Before Inoculation,** During Sickness and 7 Weeks Post-Inoculation

		Albumin g/100 ml	Globulin g/100 ml	CPK Sigma Units	Chloride mEq/l	Glucose mg/100 ml	GOT SF Units
1-79	Preinoculation	2.83	2.13	31	100	78	20
	Postinoculation	2.83	2.3	29	95.2	102	18
2-79	Preinoculation	1.8	1.35	16	90.5	97	16
	Postinoculation*	2.8	3.3	21	104.9	90.6	14.5
3-79	Preinoculation	2.83	2.4	16	109.5	82	18
	Postinoculation	2.85	2.4	16	109.5	134	18
4-79	Preinoculation	2.64	2.32	27	95.2	109	18
	Postinoculation*	3.05	2.88	30	111.9	87.5	12
5-79	Preinoculation	2.35	2.80	61	100	79	44
	Postinoculation	2.4	2.7	56	100	93	14
6-79	Preinoculation	2.17	2.17	5.5	100	84	15
	Postinoculation	2.2	2.2	7	109.5	108.5	12
7-79	Preinoculation	2.81	2.01	31	104.8	94	10
	Postinoculation*	-	-	-	-	-	-
Mean	Preinoculation	2.49±0.4	2.17±0.44	26.8±17.8	100±6.2	89±11.4	20.1±11.0
	Postinoculation	2.69±0.3	2.64±0.42	26.5±16.8	105.2±6.5	102.6±17.2	14.8±2.7
Range	Preinoculation	1.8-2.83	1.35-2.8	5.5-61	90.5-109.5	78-109	10-44
	Postinoculation	2.2-3.05	2.2 - 3.3	7 - 56	95.2-111.9	93-134	12-18

*Indicates clinically rabid dogs.

DISCUSSION

The clear and colorless CSF obtained in most cases indicates no contamination of the CSF with blood which could affect the result of chemical analysis. The coagulated CSF obtained from 3 goats (2 rabid and 1 normal) is difficult to explain since the normal goat showed no signs of illness throughout the period of the experiment. However, there are certain non-inflammatory conditions which can alter capillary permeability and allow fibrinogen and other proteins to enter the CSF resulting in coagulation (Cornelius, 1963). The CSF of the rabid goats showed mild pleiocytosis (normal value = 3 lymphocytes per mm^3, Cornelius, 1963).

There was a slight elevation in the mean total protein levels in the CSF of the rabid goats. This elevation could be due to increased meningeal permeability or to local protein synthesis in the brain since the CSF from the rabid goats were shown to contain antibody against rabies and since the 2 normal goats had only a low level of protein in their CSF. The mean CSF-glucose value and the mean CSF-chloride values for the rabid goats were within reported mean for normal goats (Table 6.5). However, 3 rabid goats (Nos. 110, 115 and 117) had hyperglycorrhacia which could be due to the encephalitis (Cornelius, 1963). The slight elevation of CSF-CPK indicates CNS damage (Drummond and Belton, 1972; Wright, 1980).

Serum albumin, globulin, and chloride for the rabid and normal goats were within the normal ranges (Swenson, 1977; Guss, 1977). Serum glucose values were higher than the reported values of 40-60 mg/100 ml (Guss, 1977) for both rabid and normal goats. Serum GOT was normal (Table 6.5) except for 1 rabid goat which had a value of 128 SF units. Low values were obtained for the 2 normal and for 1 rabid goat (30, 46.5 and 44 SF units,

Table 6.5

Reported Normal Values for Blood and Cerebrospinal Fluid Chemistry of Goats

Constituent	Values	Reference
Serum		
Glucose	40 - 60 mg/100 ml	Guss, 1977
Chloride	100 - 125 mEq/l	Swenson, 1977
GOT	50 - 100 SF units/ml	Guss, 1977
CSF		
Glucose	71 mg/100 ml	Himwich, 1977
Chloride	681 mg/100 ml (116.4 mEq/l)	Himwich, 1977
Total Protein	12 mg/100 ml	Cornelius, 1963 Himwich, 1977

respectively). The mean serum CPK value was higher for the rabid goats than for the control goats. This again indicated CNS damage (Wright, 1980). However, the scarcity of normal CPK values for goats and low number of normal control goats did not permit adequate interpretation of the results for CSF and serum CPK levels.

The total cell counts in the CSF of the dogs taken before inoculation, during the onset of clinical rabies and 7 weeks after inoculation were within the reported normal range of values (Cornelius, 1963; Wright, 1980). However, 1 inoculated but not clinically rabid dog, had mild pleiocytosis (value - 32) 7 weeks postinoculation indicating a mild inflammatory response. The differential cell counts in the CSF of the dogs were within normal range (Cornelius, 1963).

The protein, chloride, glucose and GOT values both in the preinoculation and postinoculation CSF from the dogs were within the acceptable normal range of values (Table 6.6). Even though the mean CSF-CPK values were higher than reported mean values (Table 6.7) regardless of the inoculation status, the absolute values were within the range reported for beagles (Jordan, 1977).

The values for albumin, globulin and GOT obtained from both preinoculated and postinoculated sera of the dogs were within the reported normal values (Table 6.6). Preinoculation serum glucose levels were lower than postinoculation levels in all non-rabid dogs but the 2 rabid dogs had higher values in the preinoculation sera than in postinoculation sera. The rabid animals were probably anorexic and hence had lower serum glucose levels. The serum CPK values obtained for the dogs from both preinoculated and postinoculated blood were higher than the values (0-9 sigma

Table 6.6

Reported Normal Serum Chemistry Values for Dogs

Constituent		mean±SEM*	Minimal-maximal levels	
Glucose	mg/100 ml	108±4.4	90 - 135	Cramer et al., 1969
Chloride	mEq/l	115±1.0	110 - 120	Cramer et al., 1969
Albumin	g/100 ml	3.2±0.06	2.9 - 3.5	Cramer et al., 1969
Globulin	g/100 ml	3.0±0.14	2.2 - 3.4	Cramer et al., 1969
GOT	SF units/ml	25±1.9	18 - 34	Cramer et al., 1969
CPK	Sigma units	2.7±1.1	0 - 9	Cramer et al., 1969

*SEM = standard error of the mean
Only reported values for male beagles were used.

Table 6.7

Reported Normal Cerebrospinal Fluid Chemistry Values for Dogs

Constituent		mean±SEM	Minimal-maximal levels	
Cells/mm^3		14	4 - 24	Cornelius, 1963
Protein	mg/100 ml	27.5	11 - 55	Cornelius, 1963
Glucose		63	56 - 115	Cornelius, 1963
Albumin	mg/100 ml		10 - 25	Benjamin, 1962
Globulin	mg/100 ml	9		
GOT	IU	14.4±10.1	0 - 28	Wright, 1980
CPK	IU	4.4± 4.7		Wright, 1980

units) reported for the species (Cramer, et al., 1969). However, mean value of 37 I.U. (range 8-87 I.U.) has been reported in dogs without neurological symptoms (Wright, 1980), and values ranging from 30-85 I.U. have been reported in normal beagles (Jordan, 1977). Thus the serum CPK values obtained for the dogs could be accepted as normal. Serum chloride levels were slightly below the reported normal values in both preinoculated and postinoculated blood of the dogs (Table 6.6).

Values for CSF albumin and globulin were not reliable because the methods used for their detection were not sensitive enough to quantitate low levels of these chemicals in the CSF.

However, the high levels of CSF-albumin observed for the rabid goats when compared with levels of CSF-albumin for the 2 normal goats could probably be due to increased meningeal permeability. Increase in the CSF albumin levels has been observed during a course of clinical rabies in 3 patients (Schuller et al., 1979). CSF albumin needs a more detailed study since it might be useful in evaluating clinical rabies cases. Quantitation of CSF-albumin might be a useful addition to the usually used CSF protein.

Biochemical values obtained in both the dog and the goat experiments were not distinctly different from normal values especially when the ranges were considered rather than the means. This supports the fact that CSF (and probably sera) may be normal in the early stages of clinical rabies (Maton et al., 1976). Pleiocytosis may be present (Hattwick et al., 1972) or absent (Bhatt et al., 1974) in human rabies. Meningeal lesions generally produce greater changes in the CSF than lesions in the CNS parenchyma (Cornelius, 1963). In rabies, the CNS parenchyma is more

affected than the meninges and hence the lack of remarkable changes in the CSF.

Thus in cases of neurologic diseases in which the clinical pathological parameters in both CSF and blood are normal, rabies should not be ruled out but further analysis of these specimens for antibody to rabies virus should be considered. These biochemical parameters might not be useful in studying the progression of rabies.

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CHAPTER VII

COMPARING PRIMARY SKUNK KIDNEY AND BRAIN CELLS AND RACCOON KIDNEY CELLS WITH ESTABLISHED CELL LINES FOR STREET RABIES VIRUS ISOLATION AND PROPAGATION

SUMMARY

Cell cultures were prepared from skunk kidney, raccoon kidney and whole skunk brains and were grown in Glasgow minimum essential medium in a closed system. These cells were compared with CER, murine neuroblastoma cell line C1300 clone NA, baby hamster kidney cell line (BHK-21 13S) and dog kidney cell line (MDCK) for virus isolation and propagation of street and fixed rabies virus.

The skunk brain cells were suitable for efficient replication of all the virus isolates but the skunk kidney and raccoon kidney cells were not suitable for isolation of field strains of rabies virus without prior adaptation. The skunk brain cells were comparable to the CER and murine neuroblastoma cells for virus isolation and propagation. Any strain of rabies virus could be adapted to grow in any of the cell cultures with serial subcultivation of the infected cells. By the fourth subcultivation of the infected cells, the percentage of fluorescing cells after immuno-fluorescent staining and the virus titers determined on CER cells had increased for all the virus strains and in all the cell systems. Also intracytoplasmic inclusions had started to appear in many cell systems. Any of the cell cultures could be infected in 10 seconds with mouse CVS which contained a large quantity of virus particles. The quantity of virus in the inoculum, the type and the susceptibility of the cells, and previous adaptation of the virus isolate to a host system could contribute to the efficient replication of the virus in any cell culture system. The efficient replication of the virus isolates in the skunk

brain cells (comparable to CER or mouse neuroblastoma cells) and not in the skunk kidney or other cell lines tends to support the idea that the virus of street origin replicates better in a cell line of neural origin, rather than in a cell line from the species to which the virus is felt to be highly adapted.

INTRODUCTION

To confirm the presence of rabies virus in tissues of field cases and for titration of field strains in the studies of pathogenesis of rabies, mouse inoculation as described by Koprowski (1973) is routinely used because street virus does not infect most cell lines efficiently (Smith et al., 1977). However, the CER cell line, developed in Japan, and a murine neuroblastoma NA cell line have been used successfully for the isolation of field strains of rabies virus in 2-4 days (Smith et al., 1978).

Skunks are natural reservoirs of rabies in central United States as are raccoons in the Southeastern part. These animals are very susceptible to rabies. Could a cell culture derived from skunk and raccoon kidneys or the skunk brain be as good as or better than CER and NA cell lines for primary isolation and propagation of street rabies virus? In order to answer this question, attempts were made to grow skunk and raccoon kidney and skunk brain cells and use them for virus isolation and propagation. The susceptibility of these cells to rabies virus was compared with that of CER, neuroblastoma, a baby hamster kidney cell line (BHK21 clone 13S), and a dog kidney cell line (MDCK).

MATERIALS AND METHODS

Cell Lines

A baby hamster kidney cell line (BHK-21 clone 13S) was obtained from Jean Smith, Center for Disease Control, Rabies Laboratory, Lawrenceville, Georgia. It was maintained in Glasgow minimum essential medium (G-MEM) (Grand Island Biological, GIBCO, Grand Island, N.Y.) supplemented with 10% heat inactivated virus and mycoplasma screened fetal bovine serum (fbs) (GIBCO), 10% tryptose phosphate broth and gentamicin (70 µg/ml, Schering Corp., Kenilworth, N.J.). The CER cell line was obtained from Dr. A.L. Smith, Yale University, Department of Epidemiology and Public Health, New Haven, CT). This cell line is antigenically related to BHK-21 cells as determined by immunofluorescence microscopy (Smith et al., 1977). A murine neuroblastoma cell line C1300, clone NA (hypoxanthine-guanine phosphoribosyl transferase deficient) originally prepared by Dr. F.A. McMorris and his colleagues (McMorris et al., 1974) was kindly supplied by Dr. H.F. Clark of Wistar Institute, Philadelphia, PA. Both the CER and murine neuroblastoma cells were maintained on Dulbecco minimum essential medium, D-MEM, (KC Biological, Inc., Lenexa, Kansas) supplemented as for BHK cells above. These cells were grown in the presence of 5% carbon dioxide. The dog kidney cell line (MDCK) was purchased from Flow Laboratories (Rockville, Maryland 20852) and were maintained as for the BHK-21 clone 13S cell line.

Trypsinization of the Cell Lines

BHK-21 clone 13S and CER cell lines were routinely dispersed with 0.25% trypsin (DIFCO 1:250, DIFCO Laboratories, Detroit, Michigan 48232)

in Hanks balanced salt solution (GIBCO) at pH 7.8. The MDCK cell line could only be properly dispersed with 0.25% trypsin-versene. The neuroblastoma cells were dispersed by shaking in the presence of 0.1% trypsin in Hanks BSS pH 7.8.

Preparation of Primary Cell Cultures

The adult skunk used was purchased from a dealer; the baby skunks were obtained through the courtesy of the Missouri Department of Conservation; the adult raccoons used were trapped from the wild. The adult skunk and raccoon were euthanatized by intracardiac injection of T-61 euthanasia solution (3.0 ml, Taylor Pharmacal Co., Decatur, Illinois 62525). The baby skunks were killed by decapitation. The abdomen of each animal was opened aseptically and the kidneys excised and placed in a sterile petri dish. The kidneys were washed 3 times in Hanks BSS pH 7.6 containing gentamicin (70 µg/ml). All subsequent steps were performed under a laminar flow hood (Bellco Model 8000, Bellco Glass, Inc., Vineland, N.J. 08360). The cells were prepared as described by Merchant and colleagues (1964). The capsule of each kidney was removed and the kidney washed again in Hanks BSS. The kidney was then transferred to a sterile petri dish with sterile instruments. The cortex was taken off in strips and transferred to another sterile petri dish containing sufficient Hanks BSS to prevent the fragments from drying. The strips or fragments were further cut into finer pieces, placed in a sterile flask, washed 5 times to remove blood and debris. The washed pieces were transferred to a sterile trypsinization flask containing a teflon bar. Prewarmed (to 37°C) 0.25% trypsin in Hanks BSS (10x volume of tissue fragments) was poured in and the flask was placed on a magnetic stirrer. After 15 minutes, the cell

suspension was poured into a bottle containing 5.0% fbs in Hanks BSS and held at 4°C. A further volume of prewarmed 0.25% trypsin was added and the process repeated every 15 minutes until all of the fragments of tissue were not visible. Each of the resulting cell suspensions was added to the suspension in 5% fbs at 4°C. The cell suspension was filtered through a sterile cotton gauze and then centrifuged at 800g for 10 minutes. The cells were resuspended in G-MEM supplemented with 10% fbs and 70 µg/ml gentamicin, counted and seeded at 5×10^5 cell/ml. The growth medium was replaced 48 hours later and every 3 days thereafter.

The preparation of the skunk brain cells was as described above except that 0.125% trypsin in Hanks BSS pH 7.8 was used instead of 0.25% trypsin. The flasks were seeded with 1×10^6 cells/ml. The cells were cultured in a closed system in G-MEM supplemented with 15% fbs, 70 µg/ml gentamicin and 2.0 mM L-glutamine (Sigma Chemical Co., St. Louis, MO) or in D-MEM supplemented as for G-MEM but grown in 5% carbon dioxide.

Checking for the Presence of Agents in the Primary Cell Cultures

Cytopathologic studies were done by May-Grundwald-Giemsa staining as described by Wiktor (1973). The cells were grown on glass coverslips in a petri dish. The coverslips were harvested after 3 days, and washed 3 times with warm Hanks BSS. The cultures were fixed for 5 minutes in absolute methyl alcohol or in Bouin's solution for 20 minutes and washed in 70% ethanol for 20 minutes. The coverslips were stained in May-Grundwald stain for 15 minutes, then in Giemsa (1:10) for 30 minutes and rinsed with distilled water. They were then treated for 3 minutes each in acetone and acetone-xylol (equal parts v/v) and cleaned in xylol for 5 minutes, mounted on a glass slide with mounting medium (Product No.

T01-0292, Technicon Corp., Tarrytown, N.Y. 10591) and examined using light microscopy for cytopathologic changes.

Hemagglutination studies were done using fluid harvested from the flasks in which the primary cells were grown and checked for the presence of hemagglutinating viruses by testing for hemagglutination of guinea pig erythrocytes.

Electron microscopic studies were conducted using cells in their 3rd to 5th passages which were lysed and examined for the presence of agents by the method of Spradbrow (1968). Briefly, the cells were scraped from the tissue culture flasks using a rubber policeman and centrifuged at 1000g for 10 minutes. The supernatant was decanted and the cells were suspended in about 500 µl of sterile distilled water. A drop of the cell suspension was placed on a carbon coated grid and overlaid with a drop of 2.0% phosphotungstic acid at pH 7.0. Excess fluid was removed with a piece of filter paper and the preparations were examined with an electron microscope.

Plating Efficiency

This was performed for skunk kidney cells in their 3rd subculture. The monolayer was dispersed with trypsin-versene, centrifuged at 1000g for 10 minutes and the cells resuspended in Hanks-BSS. The cells were counted using a hemacytometer method and diluted in Hanks BSS to contain 1000 cells/ml. Five hundred cells in 500 µl were seeded in 8 flasks (25 cm^2). Every two flasks had 5.0 ml of G-MEM, Eagle's MEM, D-MEM or Lactalbumin hydrolysate medium (GIBCO). Each growth medium was supplemented with 10% fetal bovine serum, 2.0 mM L. glutamine and gentamicin (70 µg/ml). All the flasks except those with G-MEM were incubated in 5% carbon dioxide for

15 days. At the end of the incubation period, the clones of cells were counted and the percentage plating efficiency was calculated with the original number of cells seeded (500) as the denominator.

Growth Kinetics of the Skunk Kidney Cells

Kinetic study was carried out on the 3rd passage. The monolayer was trypsinized, counted and resuspended in G-MEM supplemented with 10% fbs, gentamicin (70 µg) and L-glutamine (2.0 mM) to contain 1×10^5 cells/ml. Twenty flasks were seeded with 5.0 ml of the cell suspension each and incubated at 37°C. At 24-hour intervals for 10 days, the cells in 2 flasks were trypsinized, resuspended in 5.0 ml of the growth medium and counted. The cells from each flask were counted twice and the average for 4 counts was calculated.

Rabies Virus Isolation Studies

Table 7.1 shows the sources of virus which were used for isolation attempts. All of the virus sources were known to be positive for rabies virus antigen by immunofluorescent (IF) staining except 1 goat (G-51).

Eight-chambered Lab Tek slides (Lab Tek Products, Miles Laboratories, Naperville, Illinois) were seeded with 2×10^5 cells in 200 µl of growth medium (G-MEM supplemented with 10% fbs 70 µg/ml gentamicin and 2.0 mM L-glutamine) and held for 24 hours at 37°C. In some cases the primary cell subcultures were delayed up to 72 hours for a monolayer to be formed. The medium was aspirated and 20% suspensions of the IF positive brain tissue in 0.75% bovine serum albumin or tenfold dilutions of the tissue in growth medium (for titration) were inoculated on to the monolayer. After absorption for 90 minutes, the cell sheet was washed twice with Dulbecco

phosphate buffered saline pH 7.5 (D-PBS) and overlayed with 400 µl of maintenance medium; viz., G-MEM supplemented with 2% fbs, 2.0 mM L glutamine and gentamicin (70 µg/ml) and containing N-tris [Hydroxymethyl]methyl-2-aminoethane sulfonic acid (Tes, 55 mM) (Sigma Chem. Co., St. Louis, MO) and sodium hydroxide (56 mM). The slides were incubated in humidified incubator for 3 days at 35°C. The cells were then washed with PBS, fixed with cold acetone at -20°C and stained with FITC-labeled immunoglobulin for fluorescence microscopy. For titration, a tissue culture infective dose$_{50}$ ($TCID_{50}$) was calculated by the method of Reed and Muench (1938). A 6 point scoring system was applied to the intensity and quantity of fluorescence observed during the isolation attempts.

- = when no fluorescing cells were observed in all the fields examined.

± = when few fluorescing cells were observed in 1 or 2 fields.

+ = when few fluorescing cells were observed in up to 5 fields.

2+ = when fluorescing cells were observed in 6-10 fields.

3+ = when fluorescing cells were observed in 11-15 fields.

4+ = when fluorescing cells were observed in 16-20 fields.

In all cases, 18 to 20 microscopic fields were examined.

Passage of the virus Isolates on Cells

Growth medium was removed from the confluent monolayer of cells in 25 cm^2 flasks. The monolayer was washed twice with PBS pH 7.5 and treated with 50 µg/ml of DEAE-dextran in growth medium at room temperature for 10 minutes. A 20% suspension of IF positive brain in 0.75% buffered bovine serum albumin (500 µl) or ERA vaccine reconstituted with 2.0 ml of growth medium (500 µl) was put on the cell monolayer. After 90 minutes adsorption

Table 7.1

Sources of Virus Isolates and Brain Specimens Used for Isolation Attempts and Propagation

Host of Origin	Tissue	Source	Our designation
Dog	brain	Buenos Aires, Argentina	BA
Dog	brain	Mo. Division of Health - Jeff City	MO
Goat	brain	Experimentally inoculated with a dog isolate	G-44
Goat	brain	Experimentally inoculated as above	G-45
Skunk	brain	Mo. Division of Health - Jeff City	Sk-A
Skunk	brain	Mo. Division of Health - Jeff City	Sk-B
Skunk	brain	Mo. Division of Health - Jeff City	Sk-C
Mice	brain	Center for Disease Control Lawrenceville, Georgia	CVS
Pig kidney cells	tissue culture fluid	ERA Vaccine Connaught Laboratories	ERA
Goat	brain	normal goat brain	G-51

at 37°C, the monolayer was washed twice with PBS and overlaid with 5.0 ml of G-MEM containing 2.0% fbs and 70 µg/ml gentamicin. The flasks were incubated at 35°C for 4 days. The neuroblastoma cells were grown in D-MEM supplemented as with G-MEM and incubated at 37°C in the presence of 5% carbon dioxide. After the 4 day incubation, the medium was removed and stored at -70°C until used for titration. The cells were trypsinized, counted and seeded using 5×10^5 cells per ml. Infected neuroblastoma cells were scraped from the flasks with a rubber policeman, dispersed by vortexing (Vortex Genie, Scientific Industries, Springfield, MA) and seeded using 1×10^6 cells/ml.

Enumeration of Infected Cells

Infected cells were scraped from the monolayer in the flasks with a rubber policeman. The cells were separated by vortexing, washed with PBS and resuspended in a small volume of PBS. Smears were made on glass slides, air-dried, fixed in cold acetone for 30 minutes and stained with FITC-labeled globulin for fluorescence microscopy. At least 500 cells were counted and the percentage of fluorescing cells was calculated.

Presence of Inclusions in Infected Cells

Infected cells grown on coverslips in petri dishes were fixed in Bouin's fixative, stained by the May-Grundwald-Giemsa staining method (Wiktor, 1973) and examined under a light microscope.

Comparing the Rates of Virus Adsorption by the Cells

The cells were grown on coverslips in petri dishes. The growth medium was removed from a confluent monolayer and the monolayer was washed

with PBS. Then, 1.0 ml of challenge virus standard (CVS) strain of rabies virus containinig $10^{8.5}$ MLD_{50} was put on the monolayer. After incubating for 10, 20 and 30 seconds and 2 minutes, the virus containing medium was removed from 2 petri dishes after each time interval and the monolayer washed twice with PBS. Maintenance media (4.0 ml) was put on, and the petri dishes were further incubated at 32°C for 3 days. The coverslips were harvested, washed in PBS, fixed in cold acetone and stained for fluorescence microscopy. The cells were counted and the percentage of fluorescing cells was calculated.

Statistical Analysis

The percentage of cells exhibiting fluorescence and the titers of virus were compared for all the cell lines used by the Kruskal-Wallis test (Conover, 1971). When the results of the test were significant at $\alpha = 0.05$, multiple comparisons were performed as described by Daniel (1978). No adjustments for ties were made in the multiple comparisons since ties have been demonstrated to have negligible effect on the overall results (Daniel, 1978).

RESULTS

Preparation of Primary Cell Cultures

When the skunk or raccoon kidney cells were seeded at 5×10^5 cells per ml, confluent monolayers were observed in many cases on the 6th day of incubation. The skunk (Fig. 7.1) and the raccoon cells were spindle shaped. These cells were subcultured on the 8th day of incubation. The skunk brain cells grew slowly and a monolayer was obtained after 10 days. The cells were subcultured on the 12th day of incubation. The cells were

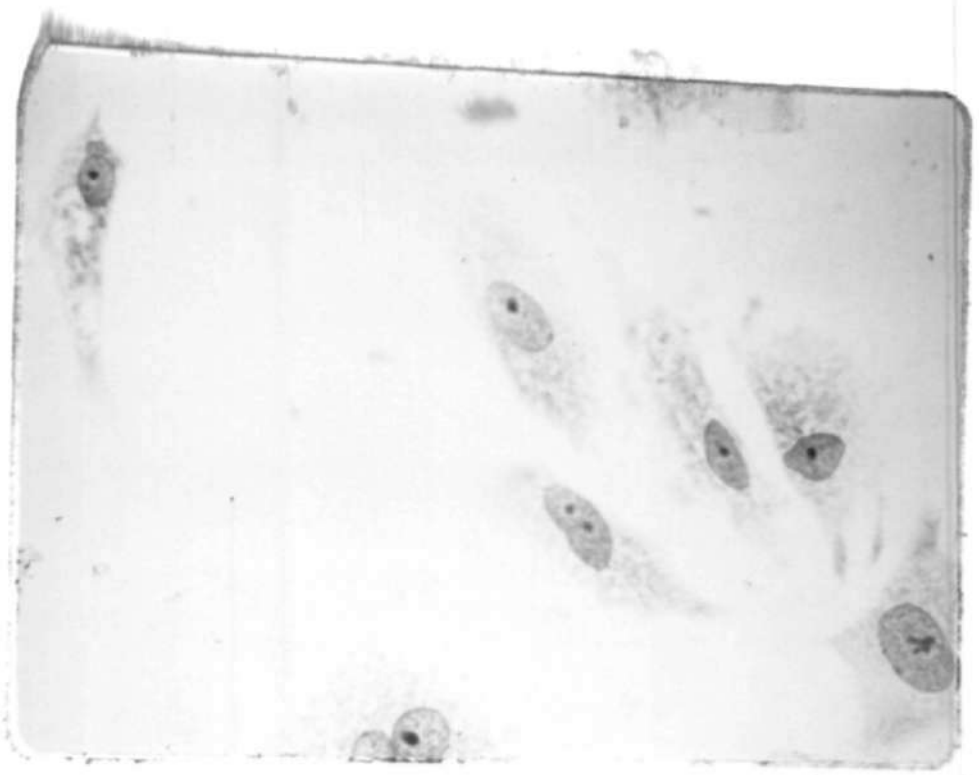


Fig. 7.1. A 48-hour culture of skunk kidney cells at the 2nd passage level. May-Grundwald-Giemsa stain X450.

elongated in many cases and had axons and dendrites (Fig. 7.2). The skunk kidney cells (from baby skunks) were maintained for 12 subcultures, the raccoon kidney cells for 7 subcultures and the skunk brain cells for 6 subcultures after which they started to die in great numbers during subculturing.

Checking for the Presence of Agents in the Primary Cell Cultures

Cytopathological changes were not observed in the cells except in one case where the cells were prepared from an adult skunk (Fig. 7.3A). In this case, some of the cells were large and multinucleated and in some cases the nuclei were arranged peripherally around the cytoplasm (Fig. 7.3B)

Culture fluids from the primary cell cultures and the subcultures did not agglutinate guinea pig red blood cells and indicated the absence of hemagglutinating viruses.

When negative staining was performed on the cell lysates and examined by electron microscopy, no infectious agents were observed except on one occasion where particles similar to rotavirus were observed. These were also seen in the established cell lines. The source of the contamination was not known but the serum used was suspected since this was the only ingredient common to the various media.

Plating Efficiency

It was observed that 83% of the skunk kidney cells seeded grew in G-MEM, 81% in Eagles-MEM, 87% in D-MEM and 76% of the cells in lactalbumin hydrolysate medium. G-MEM was chosen since the cells could be maintained in a closed system in this medium.

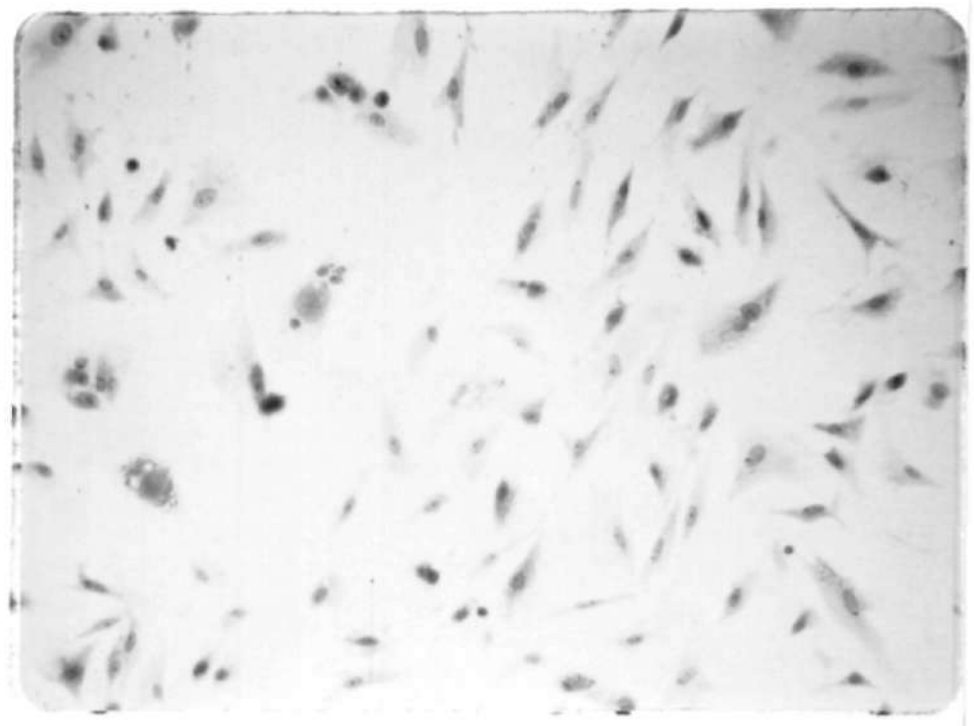


Fig. 7.3a. Cytopathological changes in the cell culture of kidney cells derived from an adult skunk. May-Grundwald-Giemsa stain X100.

Growth Kinetics of the Skunk Kidney Cells

The growth of the cells at the 3rd subculture level was rapid and reached a peak on the 7th day and then started to decline (Fig. 7.4). The number of dead cells as noted by trypan blue exclusion increased steadily after the 7th day of incubation.

Rabies Virus Isolation Studies

All the virus isolates replicated efficiently in CER, NA and SBC. The fixed virus (mouse CVS and ERA vaccine virus) replicated efficiently in all the cell lines (Table 7.2). BSK and RKC did not support efficient growth of field strains of rabies virus on primary isolation. Only 3 out of the 7 field isolates could be identified as having infected BSK or RKC. The results obtained with these cell cultures were similar to those obtained with BHK and MDCK.

Passage of Virus Isolates on Cells and Enumeration of Infected Cells

Each virus isolate had an increase in titer with subcultivation of the infected cell regardless of the cell system used. With many isolates, the titers obtained at the 4th passage with infected NA and SBC were higher than the titers obtained at the same passage level with infected BHK, BSK, MDCK and RKC (Table 7.3). The number of fluorescing cells increased with subcultivation of the infected cells (Tables 7.4 and 7.5), regardless of the virus isolate and the cell system. However, when the percentage of fluorescing cells with a cell system was compared with the percentage of fluorescing cells within other cell systems, significant differences were obtained ($\alpha = 0.05$). By multiple comparisons, it was

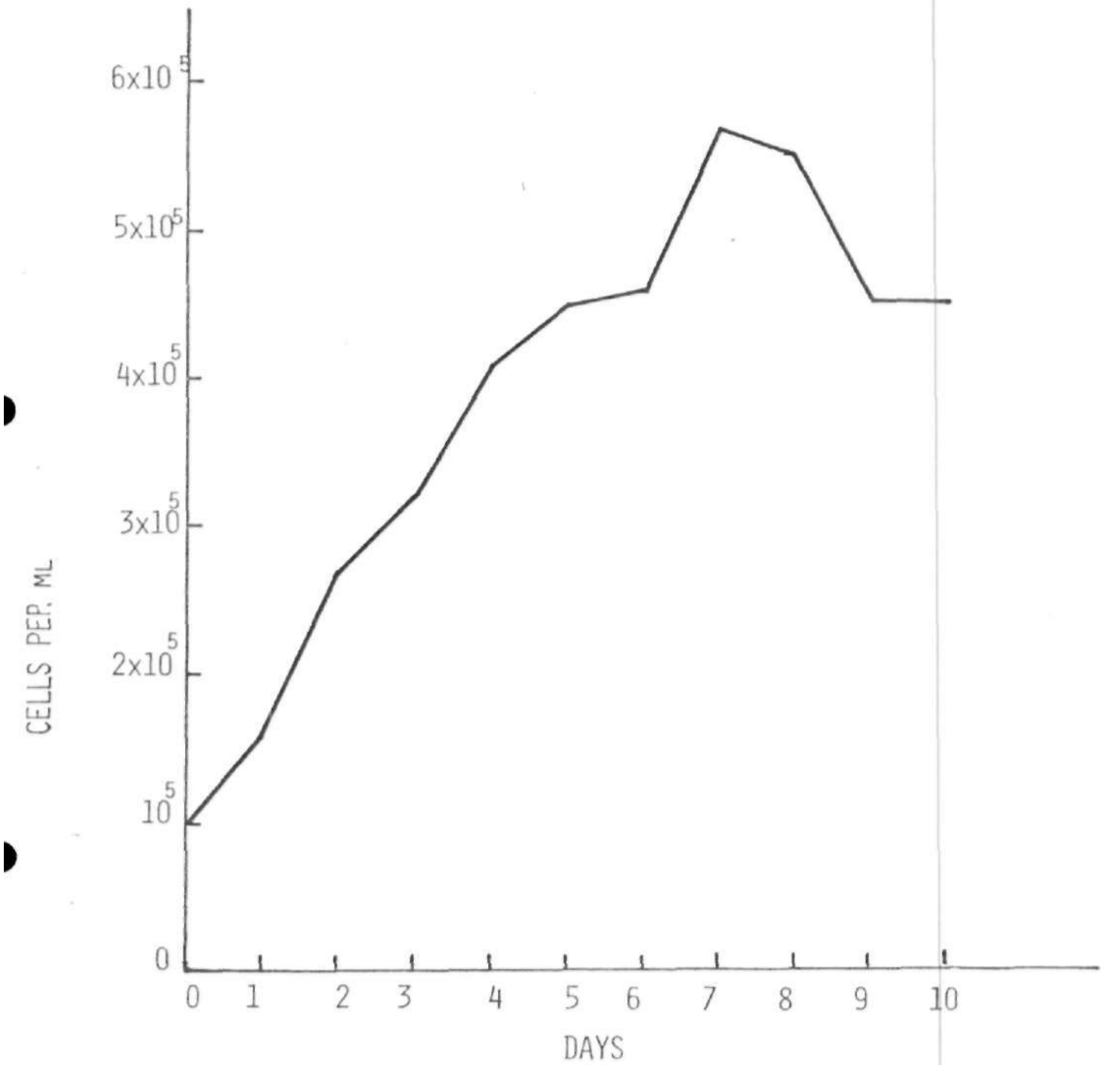

6x10^5
5x10^5
4x10^5
3x10^5
2x10^5
10^5
0
CELLS PER ML
0 1 2 3 4 5 6 7 8 9 10
DAYS

Table 7.2

Results of Virus Isolation Attempts on the Established Cell Lines and the Primary Cell Cultures Determined by IF

Virus Isolates	Cell Cultures						
	CER	NA	BHK	MDCK	BSK*	SBC**	RKC***
BA	+	+	+	+	+	+	+
MO	+	+	-	-	-	+	-
G44	+	+	+	+	+	+	±
G45	+	+	-	-	-	+	-
Sk-A	+	+	-	-	-	+	-
Sk-B	+	+	±	+	±	+	±
Sk-C	+	+	±	±	-	+	-
CVS	4+	4+	2+	2+	3+	4+	2+
ERA	4+	4+	2+	2+	2+	4+	2+
G-51	-	-	-	-	-	+	-

BSK* = baby skunk kidney cells

SBC** = skunk brain cells

RKC*** = raccoon kidney cells

Table 7.3

Results of Titration of Virus on CER Cells

Virus Isolates	Unpassaged and Fourth Passage Level in Various Cells					
	Unpassaged*	NA-IV	BHK-IV	BSK-IV	SBC-IV	RKC-IV
BA	1.7	5.1	4.5	4.5	4.5	3.9
MO	<1.7	4.2	ND	2.7	3.5	ND
G 44	1.7	4.7	5.1	2.7	4.0	3.2
G 45	<1.7	4.5	ND	ND	5.1	1.9
Sk-A	3.2	5.4	4.2	3.6	5.2	2.7
Sk-B	2.2	4.5	3.2	3.2	4.9	3.5
Sk-C	3.2	6.0	4.5	3.8	5.7	3.0
CVS	6.2	7.1	6.5	6.7	7.1	6.5
ERA	4.5	6.7	5.5	5.8	6.5	6.0
G-neg.	<1.7	<1.0	<1.0	<1.0	<1.0	<1.0

The titers are expressed as log TCD_{50}/0.1 ml.
ND= not done
* = primary brain tissue

Table 7.4

Percentage of Fluorescing Cells During Primary Propagation
(The cells were counted 3 days postinfection)

Virus Isolates	Cell Cultures						
	CER	NA	BHK	BSK	MDCK	SBC	RKC
BA	16	14	1	.5	1.3	19	2
MO	6	5	0	0	0	3	0
G 44	9	17	1	2	0.5	12	2.5
G 45	6	13	0	0	0	8	0
Sk-A	6	9	0	0	0	6.5	0
Sk-B	5	11	0.5	0.5	1	4	1
Sk-C	10	23	0.5	0.5	0.5	7	1
CVS	20	42	7	12	6	21	11
ERA	41	81	80	37	28	73	35

Table 7.5

Percentage of Fluorescing Cells at the 4th Passage Level of the Virus Isolates on the Cells

Virus Isolates	Cell Cultures						
	CER	NA	BHK	BSK	MDCK	SBC	RKC
BA	95	100	76	81	79	94	71
MO	92	100	44	35	ND	89	22
G 44	91	100	57	45	65	100	85
G 45	89	97	22	73	59	90	19
Sk-A	79	100	45	49	35	92	31
Sk-B	90	100	55	60	56	91	49
Sk-C	87	95	61	43	ND	63	56
CVS	100	100	100	100	100	100	89
ERA	100	100	100	100	93	100	100

observed that the differences in the percentage of fluorescing cells between CER and BSK, CER and RKC, CER and GHK, NA and BSK, NA and BHK, NA and RKC, SBC and BSK, SBC and BHK, and SBC and RKC were significant. There were no significant differences in the percentage of fluorescing cells between CER and NA, CER and SBC or NA and SBC. Similar results were obtained when the titers of the virus at the 4th passage level in the cells were compared.

Presence of Inclusions in the Infected Cells

May-Grundwald-Giemsa staining did not reveal any cytoplasmic inclusions in the infected cells from the 1st passage of the virus isolates except with NA infected with CVS and ERA vaccine virus. By the 4th passage, all virus isolates passaged on NA, 7 of the isolates passaged on BHK, 5 of the isolates passaged on CER, and 4 of the isolates passaged on MDCK, BSK or RKC produced inclusions (Table 7.6). Because of the difficulty encountered in growing the skunk brain cells on coverslips, the staining was not performed for this cell culture at 4th passage level of the virus. Except for ERA infected cells, the inclusions observed in the cells were small in size. (Fig. 7.5).

Comparing the Rates of Adsorption by the Cells

All the cell cultures could be infected as early as 10 seconds with mouse CVS shown by the percentage of fluorescing cells (Table 7.7). There seemed to be an increase in the number of fluorescing cells with time of exposure. BHK cells exposed to mouse CVS for 30 seconds had 51% fluorescing cells probably due to inadequate washing.

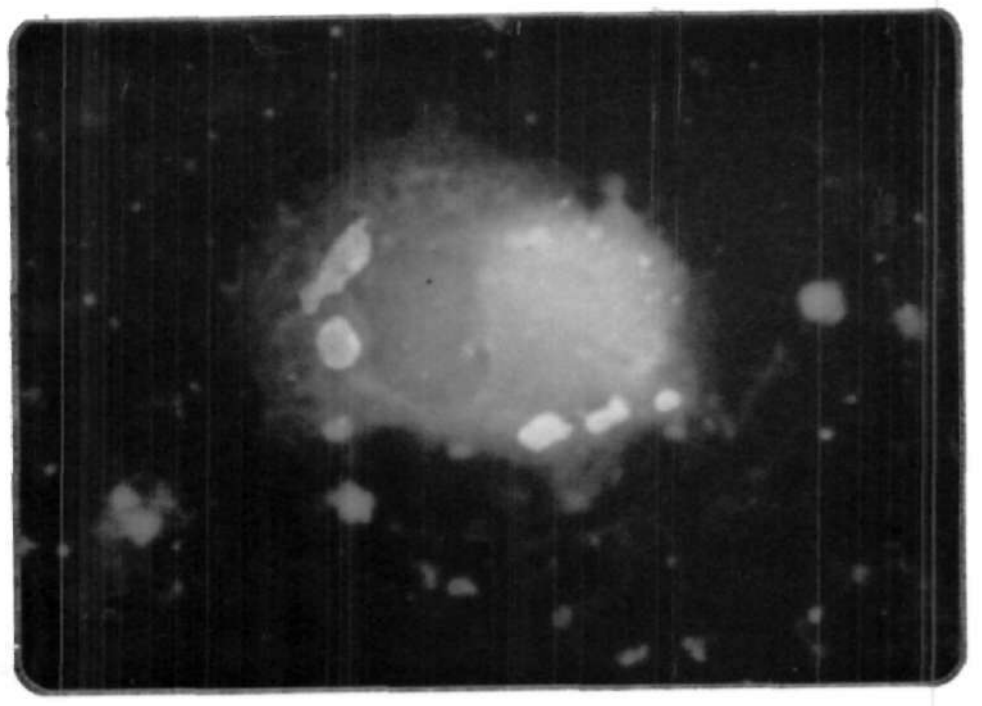


Fig. 7.5. Immunofluorescent staining of rabies antigen in smears made from infected CER cell culture suspension. X950. Large intracytoplasmic inclusions were observed.

Table 7.6

Presence of Inclusions in Infected Cells at the 1st and 4th Passage Levels of the Virus Isolates

Virus Isolate	Cell Cultures											
	CER		NA		BHK		MDCK		BSK		RKC	
	I	IV	I	IV	I	IV	I	IV	I	IV	I	IV
BA	-	+	-	+	-	+	-	-	-	+	-	+
MO	-	+	-	+	-	+	-	+	-	-	-	-
G-44	-	+	-	+	-	+	-	-	-	+	-	+
G-45	-	-	-	+	-	-	-	-	-	-	-	-
Sk-A	-	-	-	+	-	+	-	+	-	-	-	-
Sk-B	-	-	-	+	-	+	-	-	-	-	-	-
Sk-C	-	-	-	+	-	-	-	-	-	-	-	-
CVS	-	+	+	+	-	+	-	+	-	+	-	+
ERA	-	+	+	+	-	+	-	+	-	+	-	+

Table 7.7

Comparing the Rates CVS Virus Strain Absorption by the Different Cell Cultures Using Percentage of Fluorescing Cells

Exposure Time	Cell Cultures					
	CER	NA	BHK	BSK	SBC	RKC
10 sec.	6	12	9	7	6	5
20 sec.	11	15	51	6	21	13
30 sec.	30	40	9	14	17	25
2 min.	27	41	22	25	29	18

DISCUSSION

Skunk kidney, raccoon kidney and skunk brain cells were grown in a closed system in G-MEM. The same medium was routinely used for the growth of BHK-21 and MDCK cells. The primary kidney cells grew rapidly and formed a monolayer within a week especially if the cells were prepared from young animals. The skunk brain cells grew more slowly. The skunk brain cell culture, prepared from whole brains, was a mixed population of cells as evidenced by variable sizes and shapes.

The growth medium used was probably a factor in the degeneration of the skunk kidney cells after 12th passage, raccoon kidney cells after 7th and skunk brain cells after 6th passage. A more detailed study of the nutritional requirements of these cells is required to determine the medium composition for optimum growth if the cells are to be used routinely for virus isolation.

A disadvantage for the use of primary cell culture is the possibility of the presence of infectious agents and pathological changes in the tissues from which the cells are prepared. Pathological changes were seen in one case where cells were prepared from the kidney of an adult skunk but the lysate did not reveal any infectious agents. All the cell cultures used for virus isolation were free of infectious agents.

If G-MEM, Eagle's-MEM, D-MEM or lactalbumin medium is properly fortified it could be used for supporting the growth of a variety of cell cultures. A closed system was used with the hope of reducing the risk of contamination. However, on two occasions all the cell systems were contaminated, first by rotavirus and later by yeast. The contaminated cells were discarded.

The growth curve of the skunk kidney cells in the 3rd passage indicate a rapid multiplication of cells up to the 7th day of incubation. Even though the growth medium was changed once during the experiment (on the 5th day), the growth of the cell declined after the 7th day because contact inhibition prevents rapid multiplication of the cells and because of the depletion of the nutrients which led to the death of cells.

Two field isolates (MO and G-45) of rabies virus did not infect BHK, MDCK, BSK and RKC prior to passage by serial subcultivation of the 'infected' cells because the amount of virus in the unpassaged brain suspension was minimal. Thus the infection of cells with field strains might be related to the quantity of virus present in the inoculum. Evidence of infection could be seen when the cells were infected with field strains which had high titers of virus in unpassaged suspension.

CER, NA and SBC support efficient growth of both field isolates and fixed strains of rabies virus. This result confirms the previous finding that NA and CER could be used for isolation of field strains of rabies virus (Smith et al., 1978) and also indicates the suitability of the use of skunk brain cells for such isolation. The 3 field isolates from the skunks did not grow efficiently in the skunk kidney cells but did so in the skunk brain cells indicating that adaptation to the type of tissue (nervous or non-nervous tissue) was more important than the adaptation to the species as a whole.

If BHK, MDCK, BSK and RKC are to support efficient growth of field isolates of rabies virus, these virus isolates must first be adapted to a cell culture system or passaged in a suitable host to produce high titers prior to infection. Mouse CVS and ERA could be grown in these cells

without prior adaptation because the quantity of virus in the inoculum was high. Serial subcultivation was a suitable method for adapting the virus for efficient replication in the cell systems. This was not carried out beyond the 4th passage and is therefore difficult to say for how many passages the infected cells could have been maintained by this method. Probably autointerference could have prevented continuous rise in the titer in some of the cell systems, especially with the subcultivation of fixed virus infected cells. All the cultures were infected at multiplicity of infection (MOI) of less than 0.1 even with the fixed virus isolates; thus autointerference could not have been a problem. As only cell cultures free of infectious agents were used for virus isolation, the negative results obtained with BHK, MDCK, BSK and RKC with some of the field isolates was not explained as any form of viral interference, but is related to the susceptibility of these cells to rabies virus and the quantity of virus in the inoculum.

By the 4th passage, most of the isolates could be considered adapted to grow in the different systems since more than 50% of the cells had fluorescence at that level. High titers and high percentage of fluorescing cells were obtained when the virus isolates were passaged in NA and SBC indicating the ease of adaptation of the virus to these systems. With a large quantity of virus any of the cell cultures used could be infected within 10 seconds. But with a small quantity of virus, only a few presumably highly susceptible cells could be infected.

NA possess the biochemical and electrophysiologic characteristics of differentiated nerve cells (McMorris et al., 1979) and has been used for the study of rabies virus replication (Iwasaki and Clark, 1977), and isolation of field strains of rabies virus (Smith et al., 1978). The SBC

was prepared from whole brains of baby skunks. A mixed population of cells of the brain was obtained which supported efficient replication of field isolates of rabies virus without prior adaptation. Thus SBC might be a suitable model for the study of rabies infection and the primary isolation of field strains. The quantity of the virus particles in the inoculum, the type and the susceptibility of the cells, previous adaptation to a cell culture or other host system are among the important factors that affect the efficient replication of rabies virus in a cell culture system.

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CHAPTER VIII

RADIOIMMUNOASSAY FOR DETERMINATION OF RABIES VIRUS ANTIBODY IN BLOOD AND CEREBROSPINAL FLUID AND ANTIGEN IN THE BRAIN USING ^{125}I-LABELED PROTEIN A

SUMMARY

A solid-phase radioimmunoassay for rabies antibody and antigen using ^{125}I-labeled protein A is reported. Purified rabies virus, inactivated by β-propiolactone, was adsorbed to polyvinyl chloride microtiter plates. Rabbit, human, dog and goat antibodies were reacted with this antigen, followed by the addition of rabbit anti (goat IgG)IgG for the goat sera and cerebrospinal fluid (CSF). ^{125}I-labeled protein A was then added and allowed to react with the immunoglobulins. With this technique rabies antibodies could be quantitated in both blood serum and cerebrospinal fluid. For the detection of antigen, an appropriate dilution of rabbit anti-rabies serum was reacted with a dilution of normal or infected brain suspension in a tube. The antigen-antibody complex formed was transferred on to the coated plates and the percent inhibition of specific binding by the antiserum was determined. The results indicate that this technique is not yet suitable for the detection of rabies antigen in brain suspensions.

INTRODUCTION

Radioimmunoassay is a sensitive method for detection of antigens and specific antibodies (Skelley et al., 1973; Stephenson et al., 1975). Using purified rabies virus labeled with ^{125}Iodine and reacting it with dilutions of rabies antibody, followed by precipitation with anti-IgG-serum, Wiktor and colleagues (1972) were able to detect rabies binding antibody.

The virus binding ability of anti-rabies serum was shown to be similar to or higher than the values obtained by a neutralization test based on plaque reduction. The disadvantage of this method is that the two major antigens of rabies virus (glycoprotein and nucleocapsid) were labeled simultaneously resulting in a decrease in the specificity and sensitivity of the method (Bruns et al., 1977). Rabies ribonucleoprotein has been labeled with ^{125}Iodine and used for the detection of the ribonucleoprotein specific antibodies (Bruns et al., 1977).

Staphylococcus aureus protein A, a cell wall component of Staphylococcus aureus, demonstrates biological binding properties for certain cellular receptors (Cowan et al., 1979). It reacts to some degree with the IgG of almost all mammals (Kronvall et al., 1970; Lind, et al., 1970). This occurs by binding to the Fc fragments of several subclasses of IgG produced in a variety of species (Kronvall and Williams, 1969; Goding, 1978), especially IgG subclasses 1, 2 and 4 (Forsgren and Sjoquist, 1966). In man, IgM and IgA can also bind to protein A (Harboe and Folling, 1974). In the dog, virtually all normal serum IgG and approximately 90% of normal serum IgM bind to protein A (Warr and Hart, 1979).

^{125}I-labeled protein A has been used for detection of antibodies in fluid-phase (Lagone et al., 1977; Colombatti and Hilgers, 1978; Marrier et al., 1979) and antibodies bound to cell surface antigens (Zeltzer and Seeger, 1977; Langone et al., 1977; Brown et al., 1977; Biberfeld et al., 1975). It has also been used for the detection of herpes simplex virus antigens and antiviral antibodies (Cleveland et al., 1979). Other investigators have used protein A to quantitate rabies antibodies in an enzyme linked immunoassay (Atanasiu and Perrin, 1979).

Early detection of antigen is necessary for the understanding of

the course of rabies infection. However, the amount of antigen in the tissues in the early stages of infection is very limited. Thus there is need for a very sensitive method of detection. A recent workshop on the immunopathology of rabies noted that radioimmunoassay is a possible technique for detection of viral antigen that might be applicable when the quantity of antigen is small (Shope et al., 1979).

This report describes attempts to detect rabies antigen in the brain tissue of rabid animals, rabies specific antibodies in the serum of vaccinated humans and experimental animals, and in the cerebrospinal fluid of experimental animals by a solid-phase radioimmunoassay using ^{125}I-labeled protein A.

This study was based on the hypothesis that solid-phase radioimmunoassay using ^{125}I-labeled protein A is (a) a satisfactory technique for the detection and quantitation of rabies antibody in the blood and cerebrospinal fluid samples from infected animals and the blood of immunized animals and, (b) a satisfactory technique for the detection and quantitation of rabies virus antigen in the tissues of infected animals.

MATERIALS AND METHODS

Virus for Preparation of Antigen

ERA^{R} rabies vaccine, kindly supplied by Connaught Laboratories, Ontario, Canada, was adapted to growth on baby hamster kidney cell line (BHK-21 clone 13S) obtained from Jean Smith, Center for Disease Control, Lawrenceville Facility, Georgia. After 4 passages using a cell mixture technique (i.e., mixing infected cells with noninfected cells) more than 90% fluorescing cells were obtained. The growth medium was decanted and

preserved at -70°C as a source of virus for preparation of virus pool. Challenge virus standard (CVS) strain of rabies obtained from the Center for Disease Control, Lawrenceville, Georgia, and adapted to BHK-21 cells was also used for preparing a virus pool.

Preparation of Virus Pool

BHK-21 clone 13S cells were grown in Glasgow minimum essential medium (GMEM, Grand Island Biological Co., GIBCO, Grand Island, New York) supplemented with 2X L-glutamine, 10% fetal bovine serum (GIBCO), Tryptose phosphate broth (10%), and gentamicin (70 µg/ml). The cells were subcultured twice weekly. After the growth medium was decanted, the 3 day old monolayer was washed twice with Hanks balanced salt solution (HBSS, pH 7.5, GIBCO) and treated with maintenance medium containing DEAE-Dextran (50 µg/ml) for 10 minutes. The maintenance medium was then decanted and the monolayer washed once with HBSS pH 7.5; it was then incubated at 35°C for 60 minutes with 1.5 ml of virus (in 25 cm^2 Falcon plastic flask). Maintenance medium (5.0 ml) containing G-MEM supplemented with 10% tryptose phosphate broth, 0.1% bovine serum albumin (BSA), 3X L-glutamine, N-tris[Hydroxymethyl] methyl-2-aminoethane sulfonic acid (55 mM, Sigma Chemical Co., St. Louis, MO) and sodium hydroxide (56 mM) with a pH of 7.8 was then added to the virus-treated cells. The flasks were incubated for 3 to 4 days at 35°C after which the medium was harvested and stored at -70°C.

Inactivation of the Virus

Inactivation was completed using β-propiolactone as described by Koprowski (1973). Briefly, β-propriolactone (Sigma Chemical Co., St. Louis, MO) was diluted to 1:40 in cold sterile double distilled water and

this was immediately added to refrigerated virus pool to give a final concentration of 1:4000. The mixture was placed at 4°C for 18 hours stirring continuously and then at 37°C for 2 hours for complete hydrolysis of the lactone. Infectivity was checked either on BHK21 clone 13S cells or on CER cells.

Purification of the Virus

This was completed by batch adsorption and subsequent selective elution of the virus on aluminium phosphate gel, followed by ultracentrifugation as described by Schneider (1973). Briefly, the inactivated tissue culture fluid was centrifuged at 1000xg for 15 minutes to remove cell debris. Aluminium phosphate gel was prepared by mixing 50.0 ml of 16% w/v aqueous solution of trisodium phosphate dodecahydrate ($Na_3PO_4.12H_2O$) with 2 litres of sterile distilled water and then 50.0 ml of freshly prepared 10% w/v solution of Aluminium trichloride hexahydrate ($AlCl_3.6H_2O$) was added gradually with gentle stirring during addition. The gel formed was equilibrated with phosphate buffer (0.07M, pH 7.2 $\pm$ 1). The clarified tissue culture fluid was treated with the gel in the ratio of 1:20 (gel: TCF) and held at 4°C for 30 minutes. This was centrifuged at 1800xg for 15 minutes and the sediment washed twice with phosphate buffer (0.07M), using 1/10th the volume of the original tissue culture fluid, followed by centrifugation at 1800xg for 5 minutes. The supernatant was pelleted by centrifugation at 44,000xg for 60 minutes. The sediment was resuspended in 3.0 ml of tris EDTA sodium chloride buffer (TEN, 20 mM tris hydrochloride, 1 mM EDTA, 100 mM Nacl pH 7.6) and centrifuged at 2000xg for 15 minutes. The supernatant then contained the purified inactivated virus.

The presence of virus was verified by placing 2 drops of the purified virus suspension on a slide, airdrying and performing immunofluorescent staining. The protein content was measured by the Coomasie dye binding method (Bradford, 1976).

Radioiodination of Protein A

Protein A (Pharmacia Fine Chemicals, Piscataway, N.J.) was radiolabeled with Bolton-Hunter reagent (Hunter, 1973) as described by Langone et al. (1977). Protein A (250 µg) was dissolved in 100 µl of phosphate buffer (0.1M NaH_2PO_4 - Na_2HPO_4, pH 8.0) and added to a vial in which the benzene in the reagent had been evaporated under a nitrogen stream from 1 millicurie (mci) of Bolton-Hunter reagent (New England Nuclear, North Billerca, MA). The tagging reaction was allowed to proceed for 15 minutes at 25°C followed by the addition of excess glycine to stop the reaction. The ^{125}I-Protein A then was separated from low molecular weight residuals by chromatography on 1.0 x 16.0 cm column of Sephadex G-15 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) equilibrated with phosphate buffered saline (PBS) containing 0.1% gelatin. The effluent was monitored for radioactivity and the first peak was pooled for further use. The stock ^{125}I-protein A solution was diluted with PBS pH 7.5 containing 1% bovine serum albumin (BSA) and 0.02% sodium azide to 20,000 counts per minute/ 100 µl prior to use.

Radioimmunoassay for Rabies Binding Antibody

Sera

Human sera used in this study were obtained from individuals who had

received preexposure immunization against rabies. Specimens of dog serum and cerebrospinal fluid (CSF) were obtained from 2 groups of experimental dogs before they were inoculated with street rabies virus. Sera and CSF were also obtained when the dogs were clinically ill with rabies and just before euthanasia at the end of the experiment. Rabbit sera were obtained by immunization of rabbits with ERA^R rabies vaccine and with a purified tissue culture adapted CVS strain of rabies virus. Normal rabbit serum (control) was kindly supplied by Dr. J. J. Webber, Department of Veterinary Microbiology, University of Missouri, Columbia. Goat sera and CSF were obtained from experimentally inoculated clinically rabid goats on the day of onset and from normal uninoculated controls. Some sera were those collected during a postexposure experiment in goats. Rabbit anti (goat IgG)IgG was obtained commercially (Cappel Laboratories Inc., Cochranville, PA).

Assay Procedure

The previously purified rabies virus (ERA strain) was diluted 1:100 in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (2.8 µg/100 µl) and was used to sensitize polyvinyl chloride microtiter plates (Cooke Laboratory Products, Alexandria, Virginia 22314) by addition of 100 µl per well. This was incubated for 3 hours at 37°C and then overnight at 4°C in a humidified chamber to prevent drying. After incubation, the plates were washed with 200 µl phosphate buffered saline (PBS, 0.1M, pH 7.5) per well and then filled with 200 µl of 1% BSA in PBS per well. The plates were allowed to stand at room temperature (approximately 24°C) for 4 hours, washed 4 times with PBS and stored at 4°C until use. The sera and the cerebrospinal fluid were diluted in 1% BSA in PBS. Respective 100 µl aliquots of the

diluted sera or CSF were put into antigen-coated wells. The plates were incubated for 3 hours at 37°C and then washed 4 times with PBS. Into the wells containing the specimens from human, dogs and rabbits, 100 µl of ^{125}I-protein A, containing approximately 20,000 cpm was added. This was incubated for 60 minutes, washed 4 times with PBS and the washings aspirated. The wells containing goat sera and CSF were treated with 100 µl of 1:500 dilution of rabbit anti (goat IgG) IgG in 1% BSA in PBS. This dilution was predetermined by titration. After 4 hour incubation at 37°C, the plates were washed 4 times with PBS and 100 µl of ^{125}I-protein A was added. The plates were then incubated for 1 hour at 37°C, washed 4 times and the washings aspirated. The plates were dried and the individual wells were cut with a pair of scissors, placed in 12x75 mm polystyrene plastic tubes for counting in a gamma 8000 scintillation counter (Beckman Instruments, Inc., Irvine, CA).

Determination of Neutralizing Antibody

Neutralizing antibodies from the sera and the CSF were determined on BHK 21 clone 13S cells by rapid fluorescent focus inhibition test of Smith et al. (1973).

Radioimmunoassay for the Determination of Rabies Antigen

Test Samples

The street virus isolates used were from the brains of 4 naturally infected skunks, 6 goats and 4 dogs experimentally infected with a dog isolate; and 2 naturally infected dogs. The challenge virus standard (CVS) strain was provided in an infected mouse brain. Suspensions (20%)

were made from the brain samples of the infected animals in 0.75% bovine serum albumin (BSA) in buffered water pH 7.5 and stored at -70°C prior to use. ERA^R vaccine was reconstituted with 1% BSA in PBS pH 7.5 to the 2.0 ml recommended by the manufacturer. Dilutions of the test samples, controls and antiserum were made in 1% BSA in PBS.

Assay Procedure

Equal volumes of the dilutions of the test sample in 1% BSA in PBS and rabbit antirabies serum (reagent antibody) diluted in the same solution were mixed in a 12x75 mm plastic tube. This mixture was incubated at room temperature for 4 hours. After incubation, 100 µl of this solution was added to microtiter wells previously coated with purified rabies antigen. The plates were incubated at 37°C for 1 hour and then washed 4 times with PBS. Finally, 100 µl of ^{125}I-labeled protein A was added to each well and incubated at 37°C for 1 hour. This was followed by 4 washes and the washings aspirated. The wells were dried and cut from the plates with scissors. The bound radioactivity was counted using a gamma 8000 scintillation counter.

Optimization Experiments

The radioimmunoassay was carried out in plates coated with 1:100 dilution of purified inactivated CVS (to be used in subsequent assays) using $\log_{10}$ dilutions of CVS rabies virus in the mouse brain suspension (titer $10^{-6.5}$ $TCID_{50}$/0.1 ml) and twofold dilutions of rabbit rabies antiserum. The conditions for the assay were as already described. Utilizing the results (Table 8.7) of this experiment, a 1:32 dilution of the rabbit anti-rabies serum was used for subsequent assays.

The Use of Protease Inhibitors

When it was determined that the percent inhibition obtained with a normal brain suspension was in many cases almost equivalent to that obtained with a known rabies positive sample, proteolytic activity was suspected. Trasylol (500 units/ml, Sigma Chemical Co., St. Louis, MO), phenylmethylsulphonyl fluoride (250 µg/ml, Sigma Chemical Co., St. Louis, MO), and soybean trypsin inhibitor (250 µg/ml, Sigma Chemical Co., St. Louis, MO) were added to the samples before the assay to inhibit the action of proteases in the brain tissue suspensions. The brain tissue suspensions were treated with trypsin (250 µg/ml) for 40 minutes. The trypsin digestion was stopped with soybean trypsin inhibitor (250 µg/ml) and the suspensions were used for radioimmunoassay.

Acidification

Suspensions of infected mouse brain and normal mouse brain were acidified with 0.04 M citrate buffer pH 2 (ratio 1:1), heated at 96°C for 15 minutes, clarified by centrifugation and then neutralized with 0.35M phosphate buffer. The neutralized infected or normal mouse brain suspension was used for assay for antigen.

Statistical Methods

Antibody levels were expressed as percent binding and net binding. Values were calculated by the following formulae:

$$\text{Percent binding} = \frac{\text{cpm* for the test or control sample}}{\text{cpm in 100 µl } {}^{125}\text{I-PA added}} \times 100.$$

*cpm = counts per minute.

Net percent binding = (% binding for the test sample - % binding for the negative control of the same dilution).

A net percent binding level of 2 or higher was taken to indicate the presence of specific antibody. Another way to express the results of antibody determination was by the calculation of the signal/noise ratio (Goding, 1978) using the following formula:

Signal/noise ratio = cpm for test sample/cpm for the negative control sample.

The presence of specific antibody was determined when the signal/noise ratio was 2 or higher. The Mann-Whitney test (Conover, 1971) was used to compare the radioactive binding (cpm) when the 1:5 dilution of goat test sera or CSF was compared with the same dilution of negative control sera or CSF. The quantity of antigen present in a test sample was expressed as percent inhibition of specific binding of protein A by rabbit antiserum and this was calculated with this formula:

$$\text{Percent inhibition} = \left[\frac{\text{cpm for reagent antibody binding - cpm for test sample}}{\text{cpm for reagent antibody binding}}\right] \times 100.$$

RESULTS

Binding of Human and Rabbit Rabies Antibody

Coated plates were treated with dilutions of antirabies serum produced by immunization of rabbits and the sera of human beings who had received preexposure immunization. The results are shown (Tables 8.1 and 8.2 and Figure 8.1). Based on the signal/noise ratio (cpm of test

Table 8.1

Results of Titration for Antibody of Sera from Rabbits and Human Beings Immunized Against Rabies (Plates were coated with 1:100 dilution of inactivated purified ERA virus; 19,625 cpm in 100 µl protein A was added to each well)

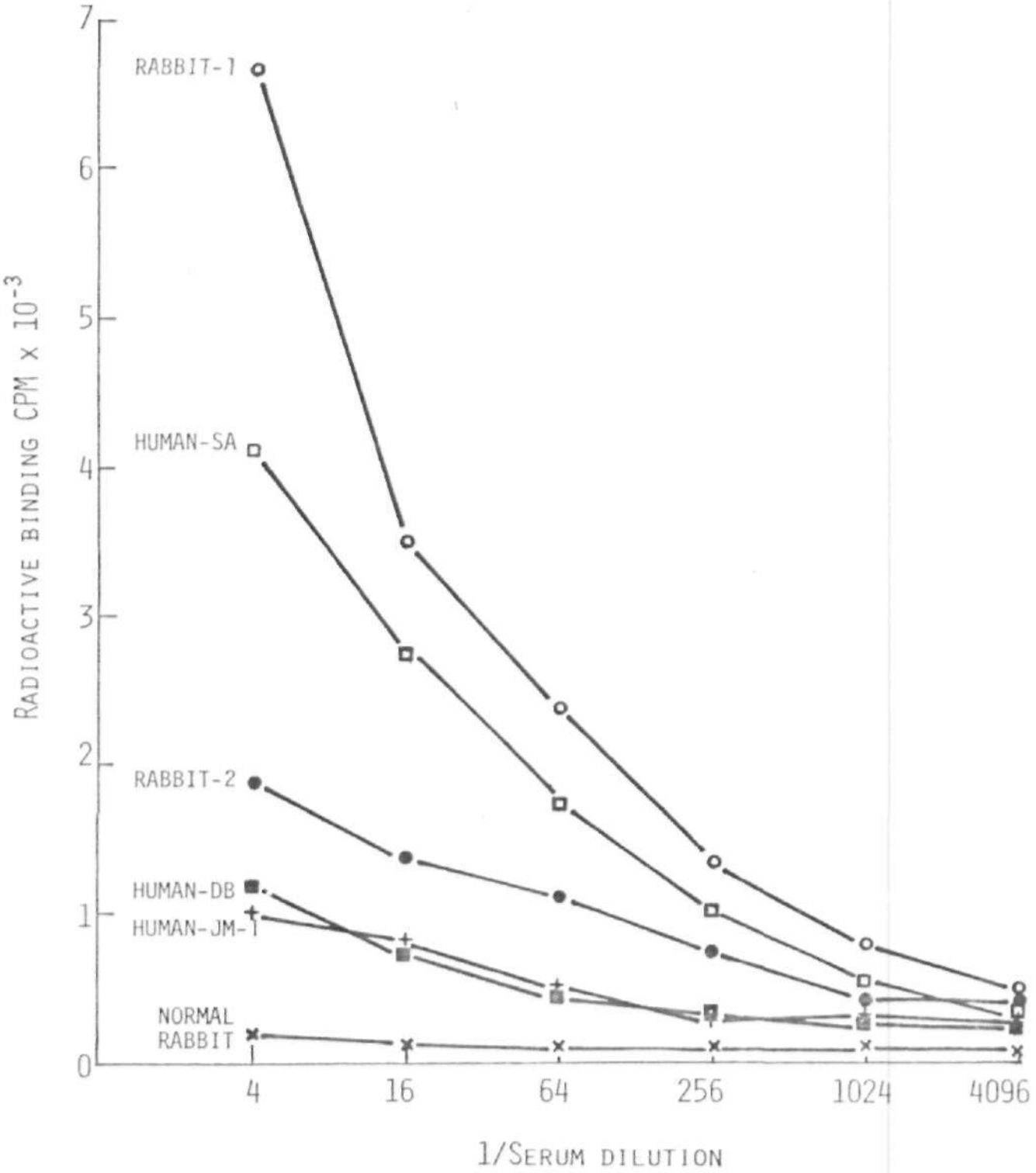
	Neutralization titer by RFFIT	Serum Dilution 1:5	1:25	1:125	1:625	1:3125	1:15625
Normal rabbit <1:5	-	146	106	77	46	50	62
rabbit 1	1:600	4323*(29.6)**	3088(29.1)	1523(19.8)	986(21.4)	494(9.08)	92(1.5)
rabbit 2	1:125	1159 (7.9)	653(6.2)	341(4.43)	156(3.39)	85(1.7)	97(1.50)
human 1	>1:625	2526 (17.3)	1347.5(12.7)	673(8.7)	407(8.85)	139(2.8)	119(1.9)
human 2	1:89	2034 (13.9)	1236(11.7)	605(7.9)	33 0(7.2)	145(2.9)	78(1.3)
human 3	>1:625	2862 (19.6)	1570(14.8)	890(11.6)	460(10)	206(4.1)	113(1.8)
human 4	1:25	928 (6.4)	401(3.8)	122(1.6)	113(2.5)	85(1.7)	63(1.0)
human 5	1:40	1654(11.3)	821(7.7)	303(3.9)	97(2.10)	90(1.8)	64(1.0)
human 6	1:125	1708(11.7)	692(6.5)	283(3.7)	169(3.7)	121(2.4)	78.5(1.3)
human 7	1:13	1219 (8.4)	522(4.9)	262(3.4)	103(2.2)	90(1.8)	88(1.4)
human 8	1:10	1013(6.9)	464(4.4)	168(2.2)	73(1.6)	56(1.0)	73(1.2)

* indicates cpm
**indicates the ratio:cpm test serum/cpm negative serum of equivalent dilution

Table 8.2

Results of Titration for Antibody of Sera from Rabbits and Human Beings Immunized Against Rabies
(Plates were coated with 1:10 dilution of purified CVS. 20,000 cpm/100 µl of ^{125}I-protein A was added to each well)

Specimen	1:4		1:16		1:64		1:256		1:1024		1:4096	
	% Binding	Net-binding (%)	% Binding	Net-binding (%)	% Binding	Net-binding (%)	% Binding	Net-binding (%)	% Binding	Net-binding (%)	% Binding	Net-binding (%)
Normal Rabbit	0.98	-	0.53	-	0.45	-	0.42	-	0.38	-	0.40	-
Rabbit 1	33.3	32.32	17.45	16.92	11.83	11.38	6.61	6.19	3.99	3.61	2.45	2.05
Rabbit 2	9.43	8.45	6.89	6.36	5.55	5.10	3.63	3.21	2.05	1.67	1.91	1.51
Human-JCl	6.44	5.46	4.72	4.19	2.48	2.03	1.59	1.17	1.42	1.04	1.36	0.96
Human-SA	20.39	19.41	13.64	13.11	8.63	8.18	4.99	4.57	2.7	2.32	1.69	1.29
Human-CD	5.74	4.76	3.72	3.19	2.76	2.31	2.12	1.70	2.13	1.75	2.16	1.76
Human-DB	5.95	4.97	3.62	3.09	2.11	1.66	1.64	1.22	1.29	0.91	1.34	0.94
Human-JM-1	5.17	4.19	3.9	3.37	2.27	1.82	1.52	1.10	1.44	1.04	1.42	1.02
Human-JM-2	5.6	4.62	3.71	3.18	2.47	2.02	1.55	1.13	1.44	1.04	1.47	1.07


RADIOACTIVE BINDING CPM x 10^{-3}
7
6
5
4
3
2
1
0
RABBIT-1
HUMAN-SA
RABBIT-2
HUMAN-DB
HUMAN-JM-1
NORMAL RABBIT
4
16
64
256
1024
4096
1/SERUM DILUTION

serum/cpm of negative serum) being equal to or greater than 2 for the result to be positive, the titers obtained were higher than those obtained by RFFIT (Table 8.1). The exact radioimmunoassay titers were not calculated. The binding of the normal rabbit serum used as control was very low (Figure 8.1). The quantity of bound radioactivity decreased with dilution of the antigen used in coating (Figure 8.2) and with the dilution of the test antiserum (Figure 8.3).

Binding of Dog Antirabies Antibody

The results show that some inoculated dogs had antibody response without clinical disease (Tables 8.3, 8.4 and 8.5). Two dogs, WH 78 (4-10-79) and DR 88 (4-10-79), that were inoculated with street rabies virus 4-1/2 months previously had CSF antibody titer greater than 1:3125 by RIA without having clinical rabies (Table 8.3). All the inoculated (Table 8.3) dogs had levels of antibody in their CSF detectable at 1:5 dilution and in their sera detectable at 1:25 dilution (Table 8.4).

In another experiment 2 of 6 dogs (#2-79 and 4-79) which were inoculated with street rabies virus and which had clinical rabies 2 weeks after inoculation, had levels of antibody detectable by RIA in CSF and blood which were taken as soon as the clinical signs of rabies were noticed in the dogs (Table 8.5, Fig. 8.4), CSF and blood taken from the surviving dogs 7 weeks after the inoculation, had cpm values which were higher than the cpm of the normal CSF and blood at a 1:5 dilution.

Binding of Goat Anti-Rabies Antibody

Since the degree of binding of protein A to goat IgG is low (Langone, 1978), rabbit (antigoat IgG) IgG was used on the antigen-antibody

Table 8.3

Results of Titration for Antibody in CSF from Dogs Experimentally Inoculated with Street Rabies Virus

Subjects	Neutralization titer	1:5	1:25	1:125	1:625	1:3125	1:15625
EN 78 11-21-78	<1:2	222	142.5	71	45	40	51
IH 88 4-13-79	1:2	494(2.2)*	157(1.1)	71(1)	54.5	42.5	37
JL 78 4-10-79	1:2	445(2.00)	191.5(1.3)	78.5	60.5	37.5	36.5
WH 78 11-21-78	<1:2	264(1.0)	203	109	73	56	46
WH 78 3-28-79	1:3	593(2.7)	239(1.7)	140	71	52	43
WH 78 4-10-79	1:16	1231(5.6)	778(5.5)	355(5.0)	155(3.4)	88(2.2)	64
HN 88 4-10-79	1:3	689(3.1)	271(1.9)	115	57	48	47
DR 88 4-10-79	1:28	1263(5.7)	1095(7.7)	435(6.1)	201(4.5)	95(2.4)	73
PG 88 2-28-79 +	1:4	728(3.3)	309(2.2)	139(2.0)	83	73	45
ND 88 3-2-79 +	1:25	593(2.7)	381(2.7)	198(2.8)	112(2.49)	61.5(1.5)	63.5
EW 88 2-28-79	1:3	505(2.3)	274(1.9)	139	102	56	47

*signal/noise ratio = cpm test CSF/cpm from known negative dog CSF of equivalent dilution.
+ = clinically ill.
^{125}I-PA added had a radioactivity of 13,499 cpm in 100 µl.
The dogs were inoculated with rabies virus on 11-21-78

Table 8.4

Titration for Antibody of Serum Samples from Dogs Experimentally Inoculated with Street Rabies Virus Expressed as cpm

Date of Serum Collection	Serum Dilution 1:5	1:25	1:125	1:625	1:3125	1:15625
EN 78 11-21-78	282	160	74	51	45	40
JL 78 3-9-79	962(3.4)*	583(3.64)	216(2.92)	106(2.1)	80(1.8)	51
IH 88 2-9-79	987(3.5)	362(2.26)	168(2.27)	58	54	47
HN 88 3-9-79	878.5(3.1)	406.5(2.54)	209.5(2.83)	86(1.69)	68	45
DR 88 3-9-79	1132.5(4.0)	372(2.3)	139.5(1.9)	88	51.5	44.5
PG 88 2-28-79**	972(3.4)	402.5(2.52)	174(2.35)	108(2.1)	58.5	61
EW 78 3-9-79	666(2.36)	309.5(1.934)	144(1.945)	70	54	41
WH 78 3-28-79	659(2.33)	370.5(2.32)	200(2.7)	84	50	36

*signal/noise ratio = cpm of test serum dilution/cpm of known negative serum of equivalent dilution. 13,499 cpm in 100 µl ^{125}I-PA was added.
**The dog was clinically ill; the date of onset of clinical signs was 2-27-79.

Table 8.5

Detection of Levels of Antibody by RIA in the CSF and Sera of Dogs Inoculated with Street Rabies Virus

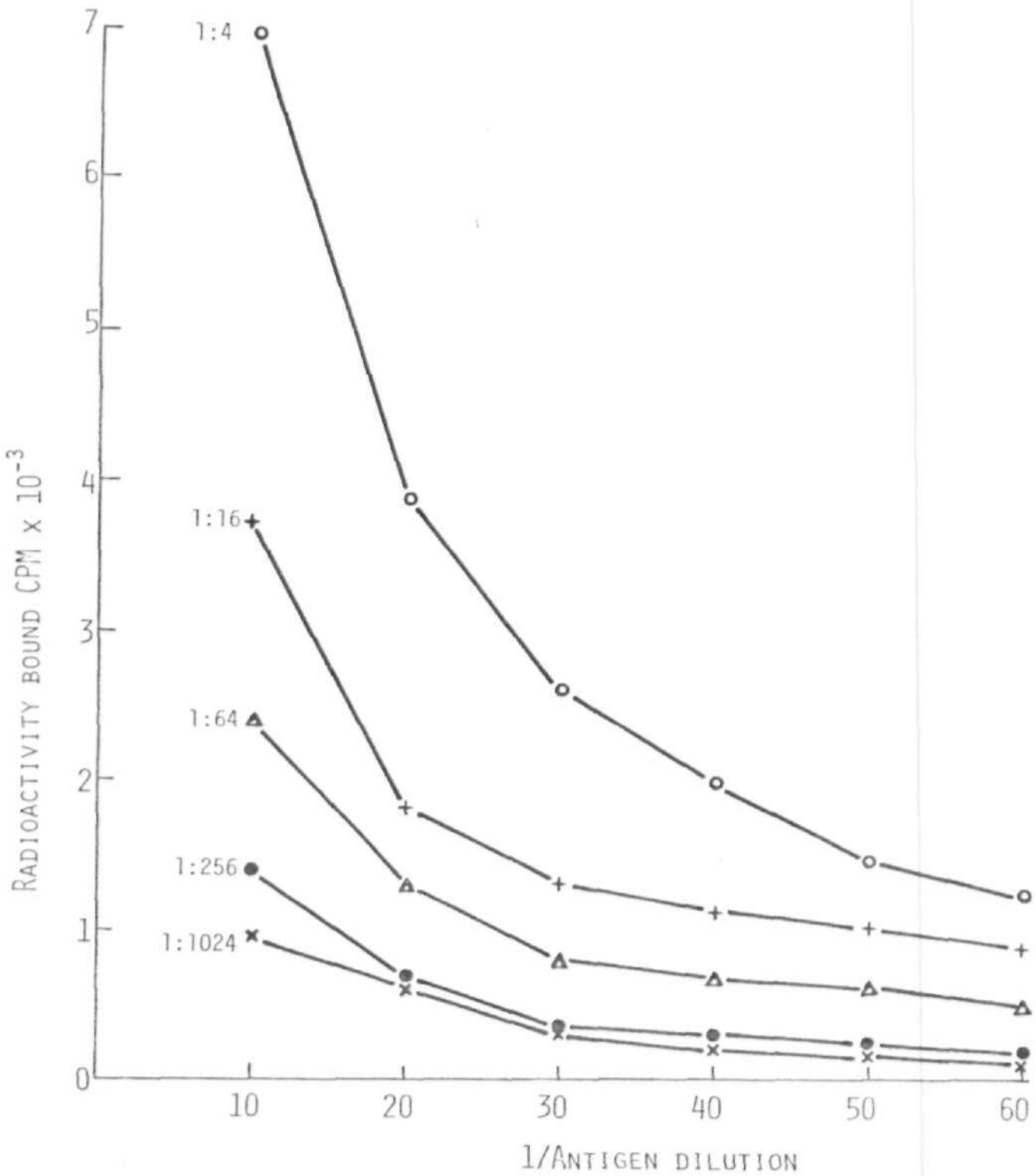
	CSF		Serum	
	mean cpm	signal/noise ratio	mean cpm	signal/noise ratio
Clinical rabies*				
2-79	167	1.8	2996	2.00
4-79	185	2.0	3065	2.05
Subclinical rabies**				
1-79	111	1.2	2004	1.3
3-79	211	2.3	2900	1.94
5-79	160	1.7	2889	1.9
6-79	173	1.9	1813	1.2
Normal	93	-	1494	-

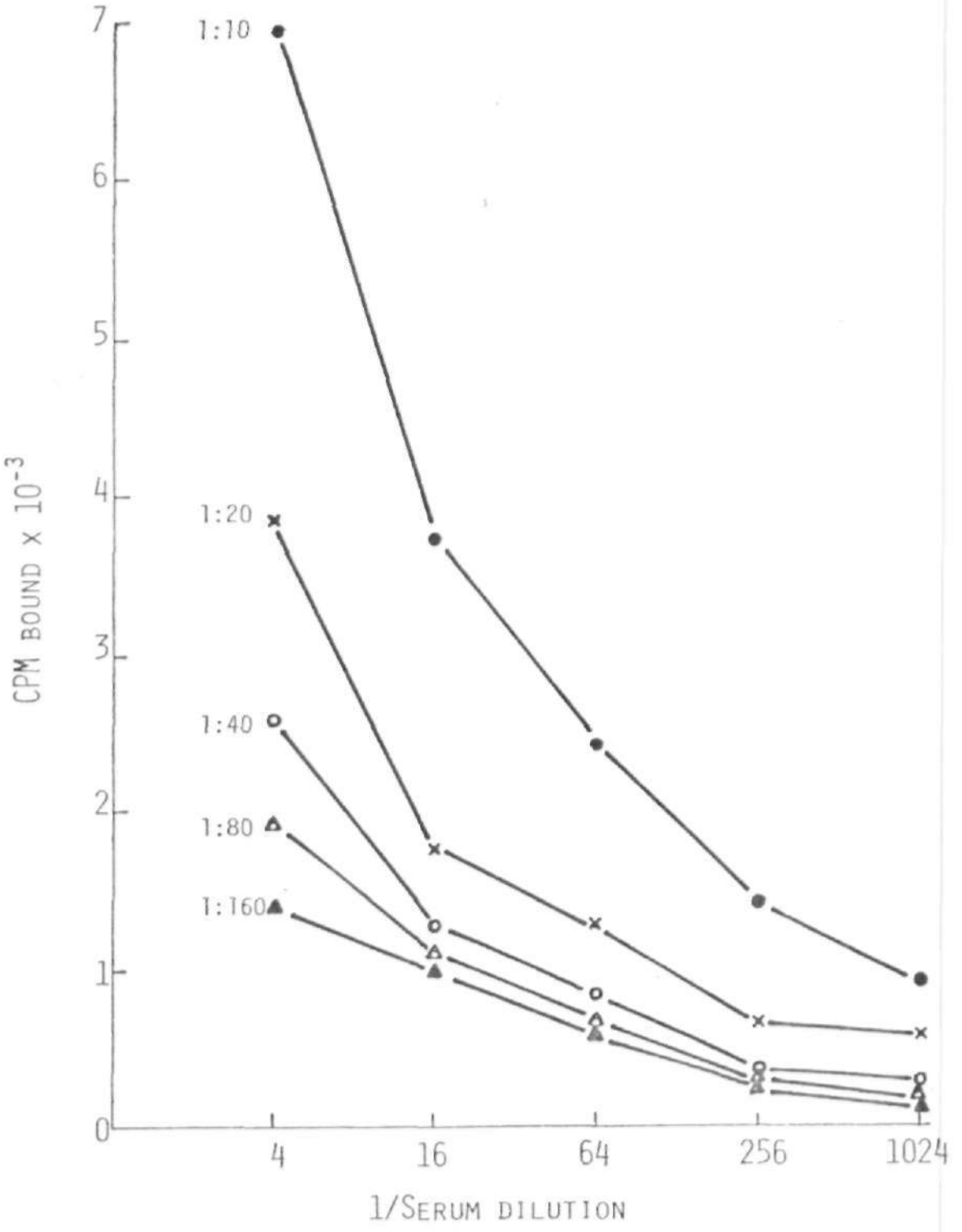
* the specimens were on the day of onset of clinical signs
** the specimens were taken 7 weeks post-inoculation.

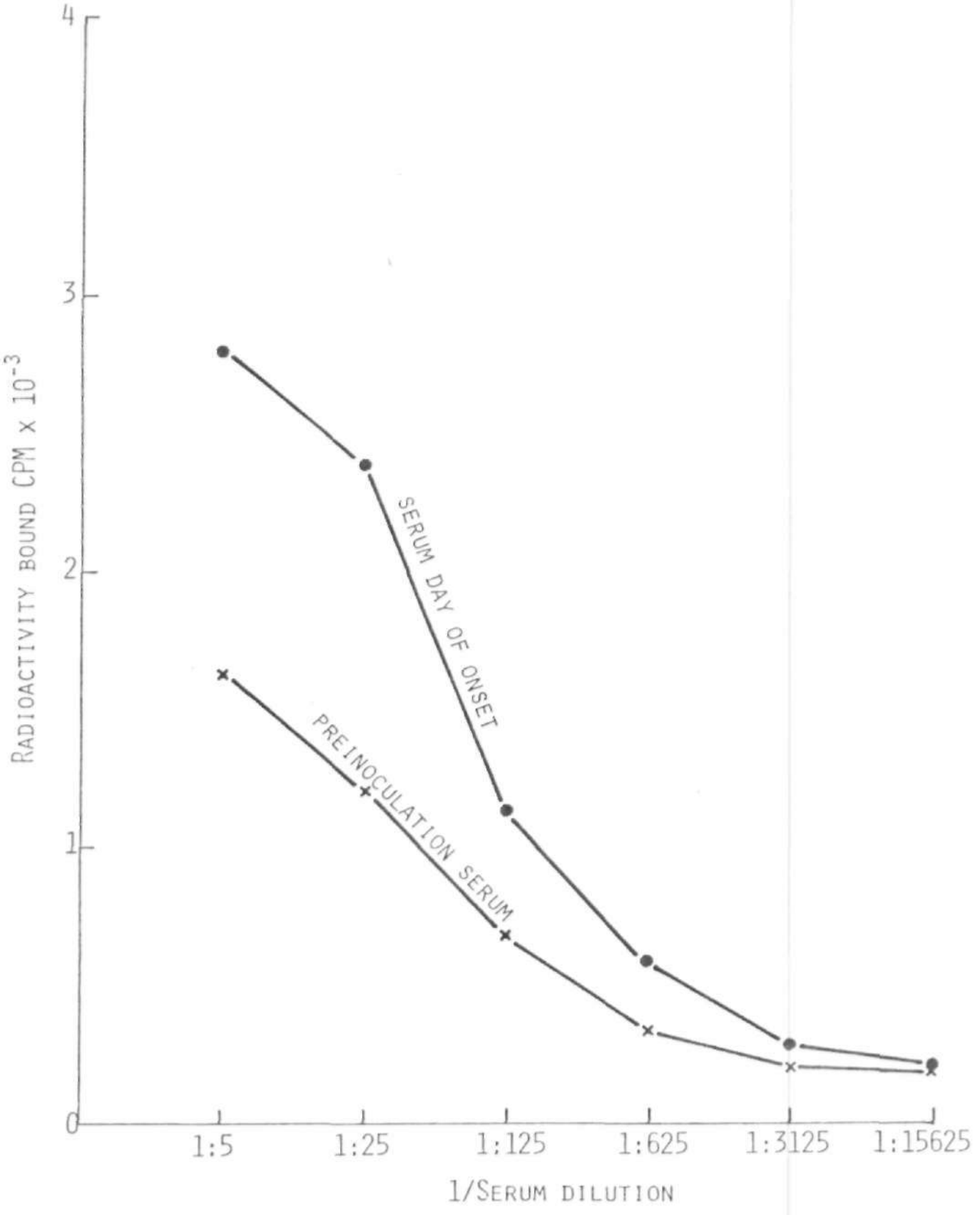
Table 8.6

Results of Titration for Rabies Antibody in the Cerebrospinal Fluid of Goats Drawn on the Day of Onset of Clinical Signs (Plates were coated with 1:100 dilution of purified ERA strain of rabies virus)

	Goat Cerebrospinal Fluid 1:5 Dilution				
Specimen	Mean cpm	cpm test/cpm neg. control	% binding	net binding(%)	Titer by RFFIT
Normal goat	488	-	2.4	-	
115	2069.5	4.24	10.4	7.95	1:36
114	584.5	1.198	2.9	0.5	1:12
117	622	1.27	3.1	0.7	1:4
113	741	1.52	3.7	1.3	1:25
118	532	1.09	2.7	0.3	1:10
119	1091.5	2.23	5.5	3.1	1:28
111	1106	2.26	5.5	3.1	1:12
110	644	1.32	3.2	0.8	1:4


7
6
5
4
3
2
1
0
RADIOACTIVITY BOUND CPM x 10^{-3}
1:4
1:16
1:64
1:256
1:1024
10
20
30
40
50
60
1/ANTIGEN DILUTION


7
6
5
4
3
2
1
0
CPM BOUND X 10⁻³
1:10
1:20
1:40
1:80
1:160
4
16
64
256
1024
1/SERUM DILUTION

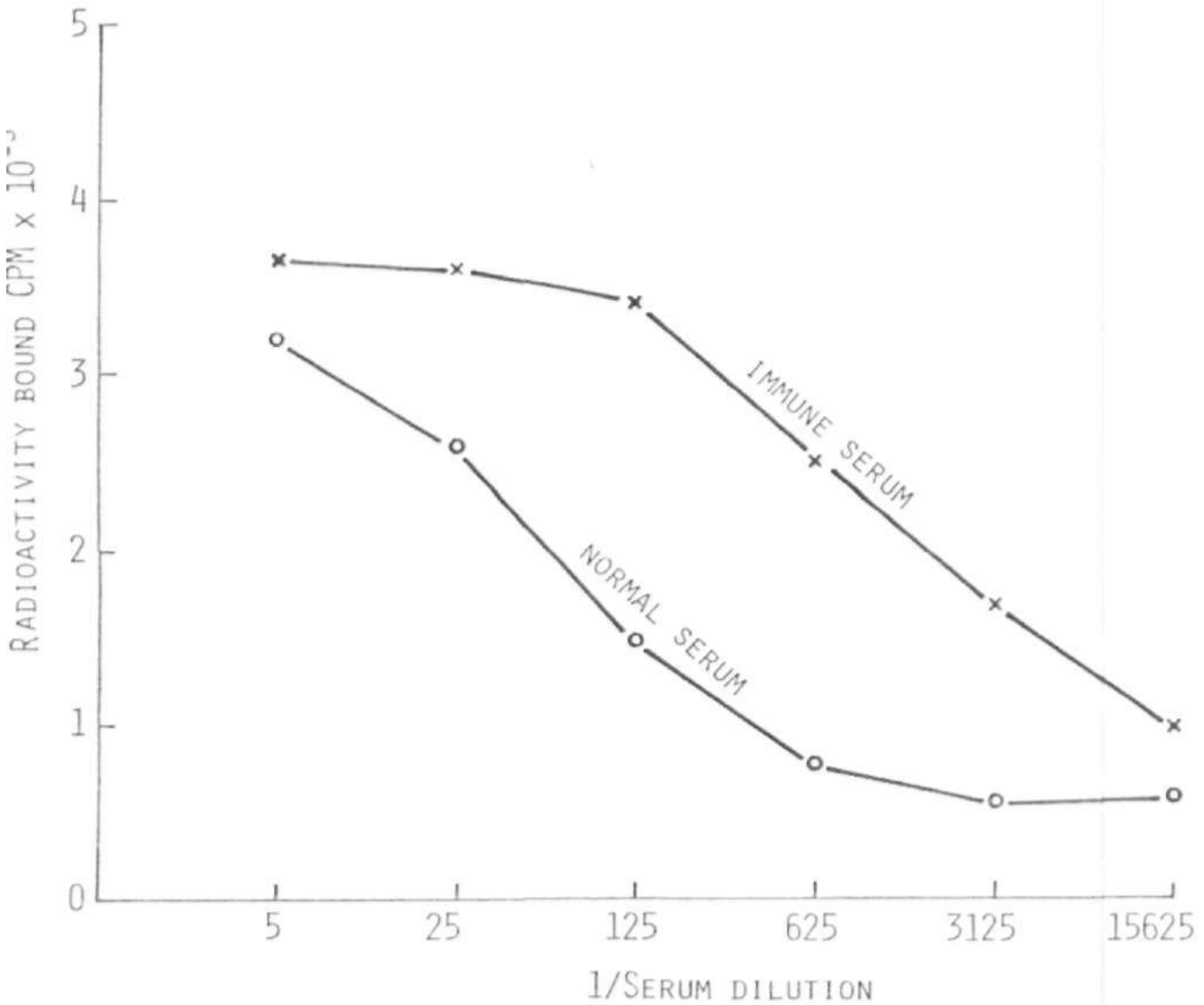

RADIOACTIVITY BOUND CPM x 10^{-3}
4
3
2
1
0
SERUM DAY OF ONSET
PREINOCULATION SERUM
1:5
1:25
1:125
1:625
1:3125
1:15625
1/SERUM DILUTION

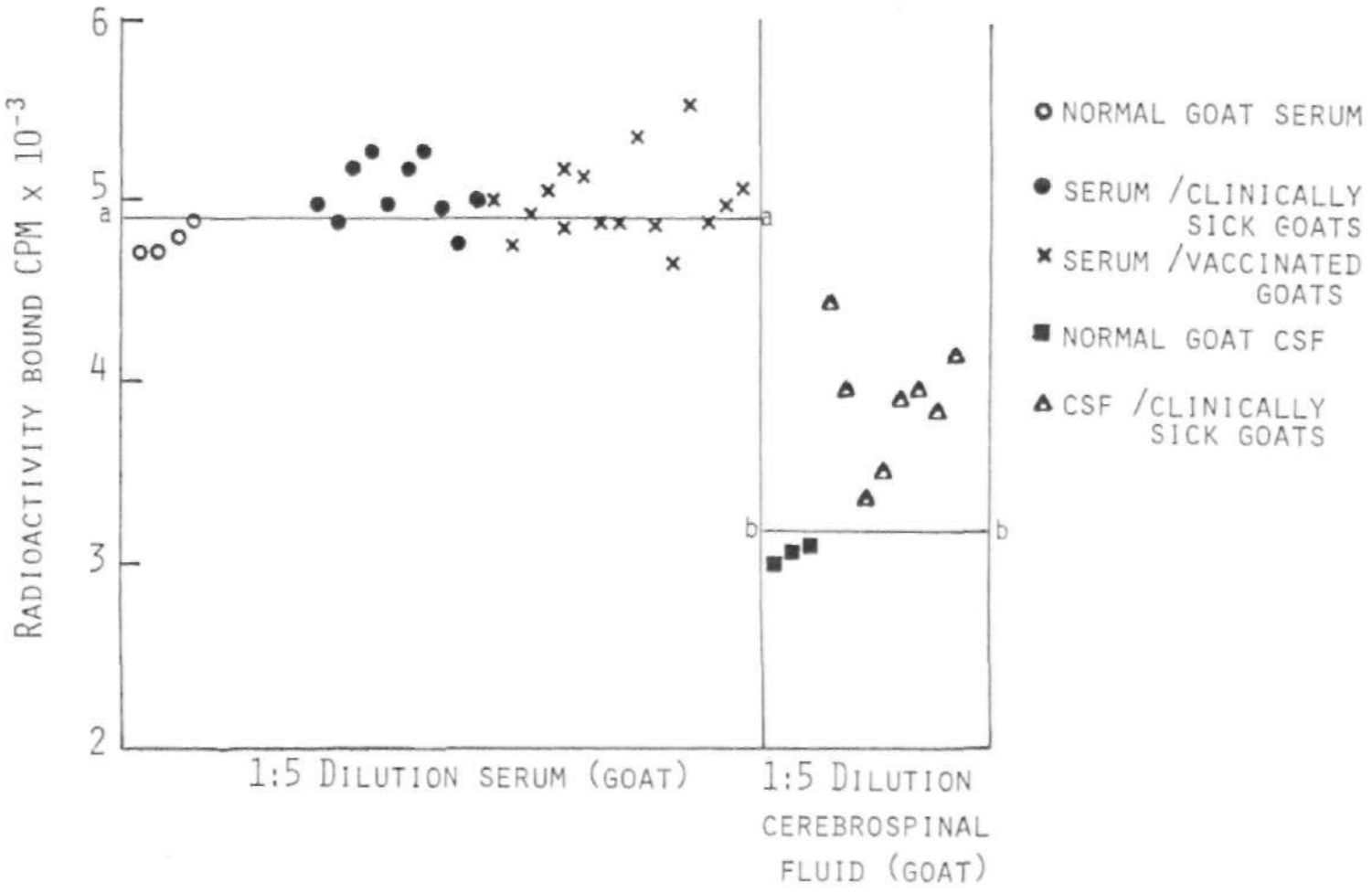
complex before the addition of ^{125}I-protein A. Comparing the results obtained with control sera and CSF, the radioactive binding (cpm) of the test sera and cerebrospinal fluid was significantly different from that of the controls by Mann-Whitney test. At a 1:5 dilution of the goat serum there was a high level of nonspecific binding. Higher dilutions gave better results (Figure 8.5). All the CSF drawn on the day of onset of the clinical signs showed positive net binding (%) (Table 8.6) at 1:5 dilution and 3 CSF samples gave net binding (%) greater than 2. Neutralization tests show that those goats had rabies antibody titers in their CSF ranging from 1:12 to 1:36.

At low dilution (1:5) of the goat serum, the normal control sera gave high radioactive binding and thus low net binding and a low signal/noise ratio. Radioactive binding (cpm) resulting from the assay of the sera and CSF of normal and rabid goats and sera from immunized goats for rabies antibody was plotted (Figure 8.6). Cpm values higher than the highest value for normal serum or CSF were regarded as indicative of the presence of specific antibody.

Detection of Antigen

Initial trials, in which plates coated with ERA vaccine virus and rabbit antiserum (produced by immunization with ERA vaccine) were used to detect rabies antigen in ERA vaccine, indicated that measurement of antigen by ^{125}I-PA radioimmunoassay is feasible (Figures 8.7). In subsequent separate but identical experiments, when brain suspensions from rabid animals were used, the control (NMB) show significant amounts of inhibition (Tables 8.8 and 8.9). In some cases the degree of inhibition


RADIOACTIVITY BOUND CPM x 10⁻³
5
4
3
2
1
0
IMMUNE SERUM
NORMAL SERUM
5
25
125
625
3125
15625
1/SERUM DILUTION


RADIOACTIVITY BOUND CPM X 10^{-3}
6
5
4
3
2
a
a
b
b
1:5 DILUTION SERUM (GOAT)
1:5 DILUTION CEREBROSPINAL FLUID (GOAT)
NORMAL GOAT SERUM
SERUM /CLINICALLY SICK GOATS
SERUM /VACCINATED GOATS
NORMAL GOAT CSF
CSF /CLINICALLY SICK GOATS

was even greater with the normal brain suspension than with the rabid brain suspension. The use of protease inhibitors and acidification with citrate buffer did not improve the result (Table 8.10).

DISCUSSION

This study indicated that ^{125}I-labeled Staphylococcus aureus protein A may be used for the measurement of rabies antibody in a microplate solid-phase radioimmunoassay. Reliable results could be obtained with human, rabbit or dog sera without the use of antiglobulin. This technique does not require live virus since the virus used in coating the plates for the assay was β-propiolactone inactivated. Rabies glycoprotein antigen was not tried but Atanasiu and Perrin (1979) found that good results could be obtained when tubes for enzyme linked immunoassay using horseradish peroxidase labeled protein A was coated with glycoprotein. These preliminary results indicate for the human, rabbit and dog, that the sensitivity of antibody detection by RIA using ^{125}I-labeled protein A was adequate. The RIA antibody titers were higher than the titers obtained by RFFIT. Thus, the conclusion can be drawn that RIA using ^{125}I-PA was a sensitive method for antibody detection. Using ^{125}I-labeled purified rabies virus Wiktor et al. (1972) was able to obtain RIA titers 10-200 times higher than titers seen with a plaque reduction method.

The results indicate that inoculated dogs that were not clinically ill with rabies had antibody in their CSF and serum. Antibody in CSF is indicative of infection (Bell, 1975) and high levels (i.e., > 3125) by RIA as observed in 2 dogs (Table 8.3) are indicative of abortive infection. The assay for rabies antibody in goat sera and CSF required the use of

Table 8.7

Results of Optimization Experiment to Determine the Optimum Antibody Dilution for Subsequent Experiments. (CVS strip of rabies virus in mouse brain with a titer of $10^{-6.5}$ $TCID_{50}$/0.1 ml was used. 1:32 dilution of antiserum was used for subsequent experiments)

Dilution of test antigen:	Dilution of Antiserum 1:16	1:32	1:64	1:128	1:256	1:512
No antigen	356	346	198.5	165	145.5	126
CVS 1:10	192(46.1)*	278.5(19.5)	102.5(48.4)	82(49.1)	67(54.0)	67.5(46.4)
$1:10^2$	246.5(30.8)	187(46.0)	121(39.04)	87.5(47.0)	67(54.0)	79.5(36.9)
$1:10^3$	284(20.2)	195(43.6)	190(4.3)	121.5(26.4)	109.5(24.7)	81(35.7)
$1:10^4$	298(16.3)	224(35.3)	174.5(12.1)	138.5(16.1)	113.5(22.0)	106(15.9)
$1:10^5$	322(9.5)	188(45.7)	179(9.8)	147(10.9)	120(17.5)	111(11.9)
$1:10^6$	417( )	196(43.4)	161.5(18.6)	139(16.0)	125(13.8)	95(24.6)
$1:10^7$	256(28.1)	326.5(5.6)	177(10.8)	144(12.7)	121(17)	108(14.3)

*number in parenthesis = % inhibition

Table 8.8

The Result Obtained in an Experiment to Detect Antigen in Infected Brain Tissue Suspension by RIA Using ^{125}I-PA

Antigen dilution	Percent Inhibition* NMB[a]	CVS[b]	MO[c]	G-44[d]
1:10	15.3	39.6	26.3	21.2
$1:10^2$	2.5	36.2	36.3	22.9
$1:10^3$	8.9	29.9	30.0	28.3
$1:10^4$	14.4	37.9	25.0	20.6
$1:10^5$	28.4	15.3	30.3	21.0
$1:10^6$	28.3	22.0	22.0	15.3

*Percent inhibition = $\left[\frac{\text{cpm for reagent antibody binding-cpm for test sample}}{\text{cpm for reagent antibody binding}}\right]$ X100

a = normal mouse brain
b = challenge virus standard
c = rabid dog brain suspension obtained from the Laboratories of Division of Health of Missouri
d = goat #44 experimentally inoculated with street rabies virus

Table 8.9

The Result of an Experiment to Determine the Quantity of Rabies Antigen in Infected Brain Tissue Suspensions by RIA Using ^{125}I-PA (1:16 dilution of rabbit rabies antiserum was used)

Antigen Dilution	Percent Inhibition* NMB	CVS	MO	BA[e]	G44	SKA[f]	SKB[g]
1:10	40.9	73.8	63.4	74.0	67.0	63.4	61.9
$1:10^2$	48.2	69.5	66.4	73.5	64.9	59.5	63.7
$1:10^3$	61.9	78.6	19.5	76.3	65.8	16.4	58.3
$1:10^4$	64.0	81.3	11.3	18.2	8.6	23.8	1.2
$1:10^5$	35.7	43.2	-	32.7	42.9	38.1	2.97
$1:10^6$	8.9	21.1	-	-	51.8	-	8.3

*Percent inhibition = $\left[\frac{\text{cpm for reagent antibody binding-cpm for test sample}}{\text{cpm for reagent antibody binding}}\right]$ X100

e = rabid dog brain obtained from Argentina
f = rabid skunk brain
g = rabid skunk brain

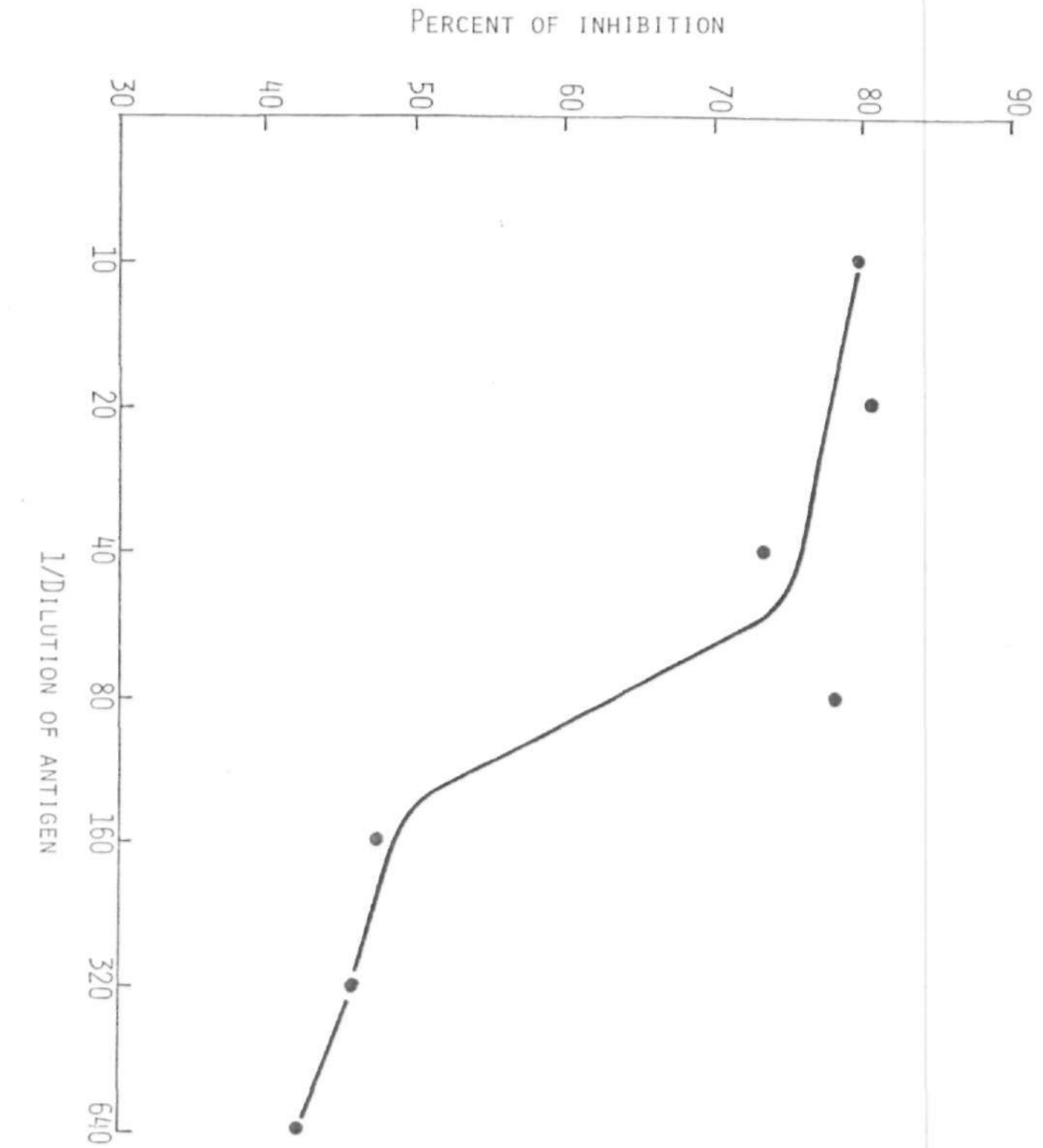
Table 8.10

The Effect of Treatment of the Brain Tissue Suspensions with Citrate Buffer (0.04M, pH 2.0) and Protease Inhibitors Prior to Radioimmunoassay for Rabies Antigen Using ^{125}I-PA

	Percent Inhibition*											
	Untreated		Citrate		Aprotinin		STI*		PSF**		Trypsin	
Antigen dilution	NMB	CVS	NMB	CVS	NMB	CVS	NMB	CVS	NMB	CVS	NMB	CVS
1:10	37.3	53.4	40.3	37.3	42.4	47.1	49.7	65.3	49.6	44.6	29.9	43.6
$1:10^2$	34.2	36.6	39.6	38.7	42.9	41.5	38.7	42.6	33.3	48.2	22.9	32.8
$1:10^3$	32.8	35.0	37.3	35.9	50.1	44.7	40.5	41.9	35.9	42.6	26.8	28.0
$1:10^4$	46.8	38.7	25.4	36.3	40.8	39.4	38.0	45.7	29.3	55.9	37.3	38.7

STI* = soybean trypsin inhibitor
PSF** = phenylmethylsulphonyl fluoride.

*Percent inhibition = $\left[\frac{\text{cpm for reagent antibody binding} - \text{cpm for test sample}}{\text{cpm for reagent antibody binding}}\right]$


PERCENT OF INHIBITION
30
40
50
60
70
80
90
10
20
40
80
160
320
640
1/DILUTION OF ANTIGEN

rabbit (antigoat gammaglobulin) IgG since goat sera binds protein A poorly. At low goat serum dilutions the nonspecific binding was a problem resulting in high radioactive binding (cpm). Therefore, a serum dilution greater than 1:5 would be an adequate dilution threshhold having significance.

The principle of RIA using ^{125}I-PA is that, in the alkaline pH created by the coating buffer, the inactivated rabies virus binds to the polyvinyl chloride plate. The antibody in the test sera reacts with the bound virus through the Fab portion to form antigen-antibody complex bound to the plate. The FC portion of the antibody is available for the binding of the ^{125}I-labeled protein A. The degree of binding depends on the quantity and type of antibody present and on the concentration of the virus used in the coating. Rabbit, human (Langone, 1978) and dog (Warr and Hart, 1979) origin IgG bind protein A well. In dogs over 90% of IgM can bind to protein A (Warr and Hart, 1979). In species where IgM does bind to protein A, it would be possible to assess an early response to rabies vaccination or infection since IgM is the first antibody to be formed following the administration of an antigen (Thomas, 1975). IgM was probably the main antibody measured in the dogs since the specimens were taken on the day of onset.

For the detection of antigen, an antigen(unknown)-antibody complex was formed in a tube and then transferred to standardized antigen coated plates. The antibody having been used up with the unknown antigen is not available for combination with the known coated antigen. The added labeled protein A therefore does not have adequate antibody for combination. This results in decreased retention of radioactivity in the wells. This worked

well when the same strain of rabies virus was used for vaccination of rabbits, for coating the plates and as a test antigen. With brain suspensions, nonspecific inhibition was apparent in using the normal brain tissue making it difficult to differentiate rabies positive brain suspension from infected brain suspension. The presence of proteases, which could result in false positive samples (Osterrieth, et al., 1979) could not explain this since the use of protease inhibitors did not affect the results. Absorption of the reagent antiserum in normal mouse brain likewise did not improve the sensitivity of the assay.

It could be concluded that 1) solid-phase radioimmunoassay using ^{125}I-labeled protein A is a satisfactory technique to detect and quantitate rabies antibody in human beings, rabbits and dogs without the use of anti-globulin and goats with the use of rabbit antigoat IgG,

2) the antibody titers obtained by RIA were higher than the neutralizing antibody titers obtained by RFFIT.

3) the detection of antigen in the brain tissue suspensions while theoretically possible, probably requires a more specific and higher titered antiserum than used in this study, a soluble coating antigen, and identification and elimination of inhibiting substances that might be present in the suspensions.

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VITA

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presented by Jarlath Udo-udo Umoh

a candidate for the degree of Doctor of Philosophy

and hereby certify that in their opinion it is worthy of acceptance.

Leonard C. Bleuden

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Donald A. Schmidt