Chapter 2

Review of literatures

Rabies virus

1. Morphology

Rabies virus is a rhabdovirus of the Lyssavirus group. The virus has a bullet - shaped structure, round on one end and flat or concave on the other(12). The average external dimension is 180 nm long and 75 nm wide. The virus core is helical structure of ribonucleoprotein, comprising of single - stranded RNA with a molecular weight of 3.5 to 4.6 10^6 daltons, surrounded by a closely associated layer of protein. The core in turn is enveloped by a membrane. The surface envelope is covered by knob-like spike, 6-8 nm long each, except at the flat end of the particle where the membrane is frequently depressed(13).

2. Biochemical constituents

Purified rabies virions contain 2 to 3 percent of ribonucleic acid (RNA), approximately 67 percent of protein, 26 percent of lipid and 3 percent of carbohydrate (14). Lipid is found only in the outer membrane, which is different in composition from the plasma membrane of the host cell (13). Electrophoretic pattern shows five proteins,i.e., polymerase, glycoprotein, ribonucleocapsid nonstructural and membrane protein, designed as L, G, N, NS and M, respectively. Studies by selective disruption and subsequent isolation of the

fragments have made it possible to attribute function to the individual structures.

2-1. Glycoprotein (G- protein)

Glycoprotein is the largest constitute with molecular weight of 78,000 - 80,000 daltons. It comprises the basic units of the surface projection of the virus (15) and is responsible for conferring immunity against a lethal challenge in animal(16). It is associated with the attachment of the virus to cell surface and also is responsible for the virulence of rabies virus (17), as previously demonstrated in apathogenic mutants selected by the use of monoclonal antibodies against G protein (18,19). Amino acid analysis of this mutant showed a single point mutation at position 333 in glycoprotein sequence (20). The alteration in glycoprotein structure as mentioned above also appears to be critical in controlling viral transport in CNS. The non - pathogenic variant has been shown to differ from its pathogenic parental strain in its ability to infect neurons in vivo and in vitro. Rate of cell to cell spread, number of infected neurons and the degree of cellular necrosis were much lower in the case of non - pathogenic virus (20,21,22).

2-2. Ribonucleocapsid protein (N - protein)

N-protein is linked to the viral RNA to form the helical ribonucleoprotein (RNP). It has a molecular weight of 58,000 - 62,000 daltons representing 31 - 34 percent of the virus protein. Antibodies raised against pure RNP may have no virus neutralizing antibody but can be used in FAT in rabies diagnosis.

However, FAT by using RNP antibody can not differentiate between rabies and other related virus in Lyssa virus gropup (23). The role of nucleocapsid in host defence has to be further investigated. Antibodies to nucleocapsid are detected in the serum of vaccinees who recieved inactivated vaccine (24). Rabies specific T cell lines and clones directed against a preparation of nucleocapsid have been isolated from the inactivated rabies vaccine recipient (25). Antibodies against antigenic domains of the N - protein have been shown to destroy rabies infected cell(26)

2-3. Membrane protein (M - protein)

M and M are the membrane proteins with molecular weight of 35,000 - 40,000 and 22,000 - 25,000 daltons respectively. The M_1 protein has been found in associated with the inner leaflet of the lipid bilayer of the viral envelope, and the smaller M_2 protein is considered to connect the helical nucleocapsid to the viral envelope(27). The viral envelope with or without glycoprotein appears to be associated with hemagglutinating properties. However, its precise nature is still unclear.

2-4. L - protein and NS - protein

L protein is a largest protein with a molecular weight of 190,000 daltons. NS - protein of 47,000 - 50,000 daltons is located with the nucleocapsid protein. These two protein are associated with the enzyme transcriptase activity which is necessary for the replication of rabies virus.

Virus replication

Rabies virus replication is similar to that of other negative stranded RNA virus. Virus particle enters cells either by engulfment or by fusion of the viral envelope and cell membrane. The replication occurs in the cytoplasm of infected cell which usually shows little or no cytological damage. Viral synthesis is accompanied by the accumulation of masses of ribonucleoprotein, producing a granular or linear matrix, known as Negri bodies (28, 29,30), the intracellular presence of which has long been regarded as diagnostic of rabies. When adapted by passage in laboratory animal, the virus becomes fixed in its incubation period. This fixed virus has a shortened incubation time and usually buds from the plasma membrane, while budding of wild type (street) virus usually occurs in the cytoplasmic membrane. Budding has been observed at the surface of cells in muscle, nerve, salivary glands, which contributes to either internal spread or external transmission of rabies virus. Under some circumstances in vitro, there are accumulations of truncated (T)(2) particles. They do not replicate in the absence of infectious virus and they interfere specifically with virus production in cell culture. Their role in the natural disease is unknown.

The virus is quite fragile in the environment and does not survive in dried saliva. However, virus in salivary gland and nervous tissue is very resistant to autolysis and may remain viable in autolyzed brain tissue for 7 to 10 days (2).

The infectivity of rabies virus is destroyed by most organic solvents, oxidizing agents and by surface - active agents.

Infectity is lost when the virus is treated with proteolytic enzyme, ultraviolet and X - irradiation. It is relatively stable between pH 5 and 10. The virus will survive indefinitely when freeze dried or kept at -70 C and remains stable for several days at 0 - 4 C. In saliva it will survive for about 24 hours in temperate climate. It has a half life of approximately 4 hours at 40 C and 30 seconds at 60 C (2).

Rabies virus was until recently believed to be of a single antigenic type. However, several rhabdoviruses in Africa which bear different antigenic variation to each other and to rabies virus have been isolated. These rabies - related viruses include Lagos bat virus, Nigerian horse virus, Mokola virus, Duvenhage virus and the two arthropod - borne, Obodhiang and Kotonkan virus. Classical serological test suggested that these strains shared nucleocapsid antigens with rabies virus which makes them difficult to distinguish in FAT (23). Monoclonal antibody technique could clearly distinguish these viruses from rabies. It is also been demonstrated that there are antigenic variation among rabies virus isolated from different species and from different geographical areas by monoclonal antibody against N and G protein.

Transmission

Rabies virus persists in nature by passage from animal to animal. The animal most often involved in the natural cycle of the disease are dogs, cats, foxes, skunks, wolves, mangooses, vampire bats, and to a lesser extent insectivorous bats, meerkats, raccoons and badgers (31). The altered behaviors of rabid animal are in favor of transmission. The animals will become aggressive,

and tend to attack without reasons. Rabies rarely affects human being, however, this fact is dependent upon a variety of factors, including the low incidence of rabies in domestic pets, the frequent close contact with the wild animal reservoirs of the disease, and the relatively low susceptibility of man to the rabies virus. Transmission usually occurs as a result of the bite of rabid animal. Rabies virus in the saliva is introduced into the bite wounds, into existing cuts or wounds on the skin, or through intact or abraded mucous membrane (31). Transmission can also occur via other nonbite route. Oral infection has been shown experimentally in foxes and skunks following ingestion of mouse carcasses infected with rabies virus (32). Airborne rabies infection of man has occured in laboratories in the two instances (33,34) and was probably the route of transmission in two cases of rabies required in a bat-infested cave in Taxas (35). In several species of mammals, including cattle, bats, and laboratory rodents, rabies has been transmitted across the placenta from mother to fetus. However, this does not seem to happen during human pregnancy. In human every exposure to rabies does not necessarily result in infection, even when such an exposure is from a rabid animal bite.Rabies mortality varied from 35 to 57 percent in unvaccinated bite victims (36,37). There are some biological determinants of transmission. For instance, the saliva must contain an adequate titer of virus. In this connection, infected skunks have been found to develop highest titers in saliva. The bitten animal must be of a susceptible species. In the laboratory, the route of inoculation is also important, with intracerebral being among the most sensitive routes whereas intraperitoneal injection is less

likely to transmit infection. In human, the site of inoculation is clearly important, bites about the head and neck are likely to result in disease earlier than bites at areas more peripheral to the CNS. Rabies virus is present in a variety of human fluids and tissues during the first five weeks of illness (38), but there are no well documented instances of human to human transmission of rabies other than the special circumstances of corneal transplant. Although there is no empiric evidence for human to human transmission by other routes, it remains a theoretical possibility. Study on virus titer in the salivary glands of humans and animals suggests that human presents with a much lower risk, though titers may vary depending on the dose, strain and host animal involved. Salivary gland of experimentally infected dogs and cats with detectable virus have had geometric mean titers of 3,400(39), 9,400 (39) ,24,800 (40),386,000 (41) mouse LD 50/g. Leach and Johnson(42) could not detect rabies virus in the salivary gland of two patients who died on day 2 and 9 of illness, but they did find virus (1,600 mouse LD 50/g) in a third patient, who died on day 4 of illness. Sulkin and Harford (43) found rabies virus (500 mouse LD 50/g) in the salivary gland of a patient who died on day 2 of illness. Therefore, the virus titers found in human salivary gland may vary

Pathogenesis

The knowledge of rabies pathogenesis owes much to the work of Pasteur who demonstrated the CNS as the key organ in the disease. Rabies virus enters a new host most commonly following the bite of rabid animal harboring the virus in saliva. The virus may

either persist extraneurally at local wound sites (44,45) for hours to weeks as an eclipse phase, or follow a relatively rapid centripetal course to the CNS (46). Before entering the nerve rabies virus multiplies locally in muscle cells and is shed into extracellular space (47,48). Rabies virus utilizes acetylcholine receptor as a rabies-binding site (49). This has been confirmed by successful competitive inhibition experiment with alpha-bungarotoxin and d-tubocurarine, both of these bind to acetylcholine receptor and inhibit binding of acetylcholine (50). Monoclonal antibodies to alpha subunit of the acetylcholine receptor also specifically block viral binding in vitro (51). Release of virus from the infected muscle cells and its advance to peripheral and CNS have already been studied in mice. Immunosuppression increased mortality and shortened the time at which virus stays at the inoculation site (52). Thus, it is possible that some immunologic factors play role in the control and release of virus at the inoculation site, and thus influence the duration of this eclipse period. The movement of virus in the nerve was demonstrated by amputation and nerve-section experiments in animal. It was shown that early excision of the inoculated area reduced the mortality rate (53). Rabies virus reach the CNS by passive transport through retrograde axoplasmic flow (54,55). Morphogenic form of the virus whether as an intact virion or as an active ribonucleoprotein complex remains unclear (56,57). Virus may disseminate thoughout the CNS by budding from the plasma membrane into extracellular spaces with potentially extensive distribution via cerebrospinal fluid, or by transsynaptic propagation (56,58,59,60). Viral can only

replicate at nodes of Ranvier , dorsal root ganglia and neurons which contain the necessary apparatus for synthesis of RNA and protein. Replication occurs most commonly within the perikaryon of neural elements. Production occurs to a much lesser degree in glial cells and astrocytes (61). Finally, virus spreads centrifugally from the CNS by axoplasmic transport to a wide variety of highly inervated extraneural sites. Extremely high viral titers are reached by way of efferent secretory nerves to peripheral organs such as the acinar cells of the salivary glands (62) at which time the development of host aberrate behaviors thus enhancing rabies shedding potential and its natural perpetuation. The clinical manifestations of rabies have been considered to be due to direct viral invasion on the nervous system. The relative scarcity of inflammation in the brain tissue with absence of cell destruction point to derangement in function as the main cause of lethality. There is selective vulnerability of neuronal cells to rabies infection in the limbic system with relative sparing of neocortex in the early stage of invasion. These findings readily explain behavioral abnormality, loss of natural timidity, abnormal sexual behavior, and aggressiveness in clinical rabies (63,64). The pathological appearance of the brain in rabies does not by itself, indicates the degree of involvement or the severity of the symptoms. The presence of virus alone may not be the only factor in determining the clinical symptoms and signs (65). Once virus is present in the brain and spinal cord with a high titer, rapid development of symptoms unnecessarily ensues.Numerous evidences indicated that infection by rabies virus may lead to various outcomes such as,

inapparent or abortive infection (66), survival with or without residual signs (67,68), and death. The mechanisms underlying this wide range of clinical outcomes are certainly complex and probably mediated by the combination of factors involving virologic, immunologic and genetic aspects. Study in mice of various strains shows different susceptibility to street rabies virus infections, and suggests that immune reponse under genetic control when develops early is protective or able to abort disease (69). The effect of immune system on rabies is not completely clear. Rabies infected cell in culture can be lysed by antibody in the presence of complement or by T cell. Antibody - dependent cellular cytotoxicity also play some role. Study in T cell deficient nude mice infected with rabies virus suggest that persistence could be established in these animals. Neutralizing antibody alone is insufficient to eliminate viral persistence (70). The effect of immune response is paradoxical. Immunedeficient animals are more susceptible to infection,but have a prolonged illness with different signs from the rampant encephalitis associated with an active immune response (71,72). Passive transfer to rabies immune serum or sensitized cells to infected immune suppressed animals accelerates the appearance of paralysis and death. Like many other viruses, rabies virus also induces interferon production. In experimentally infected animal, interferon is produced most abundantly in brain tissue just before death. Although many experimental evidences showed that rabies virus itself is susceptible to interferon, lethality despite the presence of interferon may indicate that production of interferon in the course of disease is too late to have beneficial effect (73).

The prompt institution of interferon or its inducer at the time of exposure have protected a wide variety of experimental aniamls (73). Defective interfering (T) particles play an important role in rabies pathogenesis, since they can interfere with infection by complete virus and thus may partly explain the long incubation period sometimes seen in rabies. It has been postulated that if an rabies infected animal is inoculated with a high percentage of interfering virus particles, there will be a long incubation period allowing more time for various virologic and immunologic interventions to be effective. Study by Warrell et al did not support this hypothesis.

Incubation period

This is the time elapsing between the moment of contamination and that of the appearance of first clinical signs of rabies. It is highly variable ranging from three weeks to three months in dog (7), and from 1 day to 5 years in human (36) The shortest incubation is observed with bites at the head or hands, which frequently occurs in children.Incubation period also depends on the multiplicity of the wounds and hence on the points of inoculation of the virus, but also on the animal species involved. Long incubation period may be due to the persistence of virus at the inoculation site without further spreading to the nervous system (74,75). This may be a part of the immunologic defense that keeps check on the virus at the bite site.

prevention and treatment

Rabies remains an almost invariably fatal diaease in man

once disease signs develop. Fortunately, with the advent ofeffective prophylactic treatment, a mortality of 35 to 57 percent in unvaccinated person can almost always be prevented. The rationale of treatment is to intercept the virus before it enters peripheral nerve. Because of the long incubation period, active immunization usually produces an adequate antibody level before the virus has entered the peripheral nerve. Postexposure prophylaxis will be required only after careful consideration of all the variable epidemiologic factors involved, such as the presence of rabies in the area, what species are involved, whether the animals are vaccinated, whether the animals bahave normally, and whether the animals excrete virus in the saliva.Since rabid animals can excrete virus in the saliva not only after they become sick but also before that, persons who have been bitten require immediate attention. The most important prevention is washing of the wound. Soap and water, or detergent and water are effective in preventing rabies infection (76). In the case of severe multiple bites or deep wounds a virucidal substance such as rabies antiserum should be inflitrated around the wound. Suturing is not recommended. The patient should concurrently be started on the recommended course of rabies vaccine.

Diagnosis

The history of exposure and typical clinical features are the major criteria for diagnosis of rabies. However,it is sometimes difficult espectially for physicians who are not used to the clinical signs of the disease. Antemortem laboratory diagnosis includes the detection of viral antigen in corneal impression

smear, nuchal skin biopsy using FAT (77).and isolation of virus from saliva and tracheal aspirates. Other methods are demonstrated of a significant rise in titer of antibodies to rabies virus in serum and CSF (78,79,80) in the absence of passive or active immunization. Antemortem diagnosis of rabies is often difficult, no single diagnostic test has been shown to be sensitive, but a positive result on any of the currently used tests is an indication of rabies infection. Antibody response to rabies infection is usually found on day 8 to 10 of the clinical illness and on many occasions may be absent (81). Virus isolation or detection of antigen from various sources may be negative due to in appropriate sampling time (rabies virus may not yet spread to the peripheral organs) or insensitivity of the assay method used (82). The presence of antigen in nuchal biopsy and corneal impression may be extremely irregular. Thus, if rabies is suspected, as much samples as possible should be collected for testing by all currently available diagnostic procedures and repeated samplings may be necessary because the absence of rabies antigen or antibody in early samples does not rule out infection. In some instances, false - positive diagnoses have been obtained by FAT of corneal epitherium (83).

The diagnosis of rabies in biting animal is used to evaluate human exposures for the possibility of having rabies infection.The ideal approach is to determine whether the animal is rabid and excrete the virus in saliva or not.The clinical behavior of an animal is not necessarily a reliable indicator of rabies. These rabid animals do not only excrete virus in the saliva after signs of rabies develop but before as well. Early studies indicated

that this "lag period" varied from 3 to 6 days (7,84).A domestic or pet animal which has bitten a human or another animal but otherwise has no signs of illness , nor had contact with rabid animals, should be confined and observed for 14 days. Both wild and stray animals which have bitten a human or domestic animal should always be considered as rabid and must be killed and sent to the laboratory for examination (85). Each specimen must be accompanied by a detailed history of the animal and nature of human exposures and the name and address of the contact and owners. Also included should be species or breed of the animal,whether the animal died spontaneously or was killed, and its vaccination status.

The laboratory diagnosis is made by killing the animal and examining the brain.When the brain is positive for virus , postexposure vaccination has to be initiated promptly without delay. For routine postmortem diagnosis of the brain of suspected animals, three methods are used.

1. Detection of Negri bodies (by Sellers'stain)(86)

Negri bodies were first described by Adelchi Negri in 1903 ,although at first mistakenly identified as protozoa and as the etiological agent of rabies.The demonstration of Negri bodies in smear or sections of nervous tissue is taken to be pathognomonic for rabies (87,88,89). Negri bodies are considered to form late in the clinical course of rabies. Therefore, they may be absent,few, small, or atypical in animals which are killed or died prior to the development of fully formed inclusion bodies. Negri bodies are not consistently present in animals which died after a characteristic clinical disease (87,90). 58 to 99 percent of dogs

subsequently proven rabid by FAT, were positive for Negri bodies (87). By this method, fresh brain smear is immersed in 1 % solution of basic fuchsin and methylene blue in absolute methanol for 1 - 5 seconds. When examined under high magnification, these stained tissues appear reddish violet to purplish blue. The Negri bodies take up the fuchsin and stained magenta or cherry red and appear as a sharply defined, spherical, oval or elongated body from 0.25 to 27 um in diameter. For the inclusion to be confirmed as rabies specific, basophilic dark-blue staining granules (0.2 to 0.5 um in diameter) must be present on the inclusion bodies.The methods for detection of Negri bodies are simple , fast and economical but the low sensitivity is the major disadvantage.In addition,it may also produce false positive reaction.

2. The Fluorescent Antibody Test (FAT) (92).

The procedure consists of labelling rabies antibody with a fluorochrome (fluorescein isothiocyanate),allowing the labelled antibody to react with specific antigen and observing the reaction with the fluorescence microscope. Fluorescent material may vary from dustlike particles of < 1 um to large masses or threads of 2 to 10 um in diameter. The FA test for rabies is widely used in central or large diagnostic laboratories where facilities and trained personnels are available.The test is specific and rapid. FAT enables detection of rabies antigen in the brain before Negri bodies have developed and in other tissues of infected animal where Negri bodies never form. Experienced personnel can complete the preparation and examination of the specimens within 6 hours or even less. Fresh chilled specimens are most desirable, but thawed

specimen and specimen in which the virus has been inactivated by time because of tissue decay or the presence of antibody may be fully utilized.Although rabies virus may be detected in all parts of the CNS of infected animal, its distribution is frequently uneven(93). It is essential that specimens from several parts of the brain be examined on such animal. This should include the medulla (brainstem), the cerebellum and the hippocampus. Comparative studies of FAT and virus isolation technique have shown a 97 to 98 % correlation (94). Since more tissues are sampled for virus isolation, that technique may be more sensitive when only a very small amount of antigen is present. Therefore, it should be confirmed once the FAT is negative by the inoculation of brain tissue specimens into susceptible animal,especially when human exposure to the submitted animal has occured.

3. The Mouse Inoculation Test (MIT) (95).

The absence of virus as demonstrated by Negri bodies or FAT should be confirmed by the inoculation of brain suspension into suckling or weanling mice as the standard test. Suckling mice are of greater sensitivity than adult mice, but because of the usually adequate reliability of test in weanling mice,the latter are commonly used. 10 - 20 % brain suspension was inoculated by intracerebral route and mice are observed for 28 days for sign of rabies (trembling , humping , paralysis or prostation). A 7 to 20 day incubation period is expected in mice inoculated with street virus, but incubation period of 30 day or more are sometimes observed. The presence of rabies virus antigen in the brain of dying mice is subsequently confirmed by FAT. The disadvantages of

MIT lie in their time requirement, potential hazards for laboratory transmission, and inapplicability when specimens are decomposed or treated so that they are toxic or no longer infectious to the test animals.

No serological test exists of value in diagnosing rabid dogs. Although,an immune assay for detection of anti-rabies IgG (96) and immunoadherence hemagglutination test(97) have been applied successfully to detect antibody in immunized human, IgG antibody requires time to develop and its absence does not exclude rabies.

Since the decision to initiate rabies postexposure vaccination following animal bite is frequently based upon the result of laboratory diagnosis, the procedures used must be fast, specific and very sensitive. FAT has been proved to be such a procedure when performed by an experienced laboratory with high quality reagents.False negative FAT results are not common but can occur(98). Because of the high medical significance of these examination results, virus isolation is commonly used as a back up procedure to FAT in rabies diagnosis.MIT is commonly employed to confirm a negative FAT,espectially in cases of human exposure. Although the MIT is sensitive and easy to perform,it has one major drawback : street rabies virus has a typical incubation period in mice between 7 and 18 days. When treatment of an exposed individual is contemplated,it is not practical to wait for the result of an MIT. Therefore, a routine back up test which gives a more rapid final result than the MIT would be desirable.

In 1980, Rudd, et al has developed the test RTCIT (The

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Rabies Tissue Culture Infection Test) and showed that it was equal to the MIT in sensitivity in street virus strain tested, but yielded final result in 48 hrs(98). The technique of ELISA was developed for detection of rabies antigens in infected brain specimens by Perrin & Sureau (24). It gave a sensitivity of 100%

In recent years, the technique of dot - immunoblot was described to be rapid ,specific and sensitive for the detection of antibody and the identification of virus antigen (99). In 1987,Heberling has successfully develoed serodiagnostic test of neutralizing-rabies antibody by dot immunobinding assay and shown that the test was correlated well with the rapid fluorescent focus inhibition test (RFFIT)(100). Dot-immunoblot for the detection of rabies antigen in various tissue specimens is,therefore,needed to be developed.