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Rabies Pathogenesis

Brief Review

By

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Introduction

Considerations of the invulnerability of rabies virus to mammalian host defense mechanisms and to the effects of postexposure treatments must be based upon an appreciation of the pathogenetic pattern of the infection. For example, antiviral biologics used in postexposure treatment might be anticipated to work best at a stage where they can intercept the virus before invasion of the privileged environment of the peripheral nervous system. If multiple virus growth cycles occur extraneurally at the bite site and only progeny virus gains access to the nervous system, then treatment regimes may have time and multiple chances for interfering with the infection course. Alternately, treatment regimes aimed at interception of virus late, after neuronal infection and dysfunction have occurred, might be expected to be without benefit or even to be the cause of exaggerated immunopathologic disease.

In this laboratory over several years, studies have been carried out in hamsters on the pathogenetic events at the sites of deep or superficial entry of street, fixed, and rabies-like viruses. The local, ascending, central, and centrifugal aspects of infection have been followed by sequential infectivity titrations as well as light, immunofluorescent, and electron microscopy (50, 52, 53, 55, 70). These studies contribute to a large literature built up over many years (1, 4, 7, 8, 19, 24, 25, 27, 33, 40, 41, 59, 63, 64, 65, 66, 67, 68, 89). In this review, the pathogenetic pattern of rabies infection is described as a basis for considering the likelihood of success of the various means of intervention presently in use or anticipated in the future. Whether the passive and/or active induction of antibody, cell-mediated immune activity, interferon, or even chemotherapeutic agents come to be identified as the most efficacious means of terminating rabies infection, the active principle must be delivered at a time and place where the virus and/or infected cell are vulnerable. Our best weapon must be identified but so must the chinks in the armor of the virus and the infection process.

Viral Invasion, Local Infection, and Transit to the Central Nervous System

Following the infection of newborn hamsters by intramuscular inoculation into hind limbs (a route chosen in our laboratory to mimic exposure by deep bite), we found the first evidence of viral replication in nearby striated muscle cells (52, 53). Frozen section immunofluorescence, in addition to providing histologic localization of viral antigen, proved more sensitive than organ titration in detecting the initial stages of infection. At first, single muscle cells contained viral antigen, but this quickly progressed to the involvement of foci of cells. This pattern of initial infection of striated muscle was found with all rabies viruses studied including 12 street virus isolates. This tropism now needs to be investigated in mature animals involved in the transmission of rabies in nature. By electron microscopy, virus particles were found budding in moderate numbers from the plasma membrane (as well as from sarcoplasmic reticulum membranes) of infected but undamaged muscle cells; therefore, this infection site was considered a significant source of virus capable of finding its way to the nervous system. Alternately, this infection site was considered a likely place for the sequestration of virus in long incubation infection. The nature, if not the site, of rabies virus sequestration remains mysterious and has not been clarified by experimental efforts. We do not know what modulates the infection in muscle.

Deep in muscles, the peripheral nervous system is exposed at neuromuscular spindles. These sensory stretch proprioceptors consist of bundles of modified muscle cells wrapped in unmyelinated nerve endings. In our sequential studies, the neuromuscular spindles were the second site of viral antigen accumulation after muscle infection. Both the muscular and neural elements of spindles became infected, and virus particles were found in the extracellular spaces of the neuromuscular junctions. One explanation for the rapid movement of the rabies genome centripetally across cellular junctions is that membrane fusion occurs allowing a subviral genomic moiety to pass through. Our demonstration of whole virus at junction sites does not deny the possibility of membrane fusion, but if rabies transit depends only upon intact virions crossing cellular junctions, then antiviral agents and host defense mechanisms may have continuing chances to intercept.

Neurotendinal spindles, the sensory stretch receptors deep in tendons, offer the same exposure of nerve endings as do neuromuscular spindles and in our studies were also found to be infected early. In rodent footpads, which have been used as an inoculation site in many rabies studies, both neuromuscular and neurotendinal spindles are the probable primary deep sites of viral entry into the nervous system.

Where early infection involves spindles, the viral genome moves centrally in the same direction as the sensory nerve impulses, but there is no fundamental reason to assume that this is necessary. In fact, the whole question of whether rabies virus affects sensory or motor functions preferentially has been argued for many years; the question has been answered with too much anecdotal evidence and too little experimental evidence. Motor nerve endings, that is motor end plates, are distributed within muscles in precise yet dispersed patterns; in our studies, we never saw an immunofluorescent pattern that matched their distribution. However, by electron microscopy, we repeatedly found evidence of virus

infection in neuronal endings of motor end plates and in nearby extracellular spaces. Infection of muscle cells was much more prominent near motor end plate attachments than elsewhere. This neuromuscular contact is intimate, and the neuronal endings are just as unprotected as they are in spindles.

There are other sensory nerve endings which are involved late in infection and which might also play a role in viral entry from superficial exposure. We found that the olfactory end-organ in the nares became heavily infected; its neuroepithelial cells are exposed directly to the body surface and are considered the site of viral entry in aerosol infections such as those that occur in bat caves (14). Fungiform and circumvallate papillae on the tongue with exposed neuroepithelium of taste buds became heavily infected. Such organs may also contribute to viral shedding and transmission late in infection. Superficial exposure through abraded skin or mucous membranes delivers virus to dermal sites especially rich in sensory nerves and their endings. Amplification of the inoculum in this case may involve virus growth in muscle cells in the dermis and even epithelial cells of the basal layers of the epidermis. Recently, we found widespread infection of the stratum germinativum in the skin of hamsters inoculated with street rabies viruses; from this infection, antigen was carried into the outer, squamous layers, even into the corneum. Unmyelinated nerves in basal layers of the epidermis were found by electron miscroscopy to be infected along with nearby epithelial cells. The nature of this nerve-epidermis interrelationship and its possible role in the course of rabies infection has not been further explored.

The delivery of virus to the extremities of the peripheral nervous system initiates a phase of infection in which strict neuronotropism and passive neuronal transit dominate. There is no solid evidence that hematogenous or other modes of centripetal spread have any significant role in rabies infection (40, 63). In our studies, even though immunofluorescent antigen was not detected in peripheral nerves until a time when spinal ganglia and even the adjacent spinal cord were infected, there eventually was a progression of involvement of more and more nerve fibers and continuing antigen accumulation. Late in infection, this accumulation was massive, even though antigen localization in nerve components was not possible even when nerves were dissected and thinly spread before immunofluorescent staining. Virus was found budding only upon axonal membranes by electron miscroscopy. Intraaxonal accumulation of virus particles and inclusions was concentrated at nodes of Ranvier where there were more organelle membranes for viral budding. JENSON *et al.* described the same distribution (39). Elsewhere along axons, fewer virus particles budded upon plasma membranes and were "trapped" in the space between axons and their myelin sheaths. We never found evidence of involvement of Schwann cells or any other supportive cells, even late in infection; ATANASIU has described Schwann cell infection (67), but has not suggested that such supporting tissues play a role in the centripetal movement of virus. One conclusion from these observations is that very little virus is actually released as an antigenic stimulus at the early stages of street virus infection and little viral surface antigen is exposed to the intercellular compartment where it could potentially become the target for specific host defense mechanisms (this is not the case with some laboratory virus strains in mice and hamsters).

The axoplasm is the only route of viral genome transit consistent with ex-

perimental observations (25, 43). Passive centripetal movement of viral genome in the axoplasmic compartment can account for the speed of transit found when fixed rabies virus strains are inoculated in foot pads of mice and rats. In this transit, delivery of viral genome to the junctions of the peripheral and central nervous systems in the spinal cord is the central event, but deviation of some genomic moieties into progeny particles and inclusion bodies along the course of the nerve is clear evidence of the route followed.

Rabies virus genome in its centripetal transit in sensory nerves makes a diversion into the neuronal cell bodies in dorsal root ganglia. Whether this diversion and viral replication in the perikaryon of ganglionic neurons serves as a necessary amplification is not known, but the continuity of the axoplasmic compartment is not interrupted at the site of junction of the axon and the stalk of the cell body, and it is probable that viral genome can move past this junction directly to the central nervous system synapses in the spinal cord. Even though early ganglion cell body infection might not be necessary and viral genome need not be exposed to the extracellular environment when diverted through this cell body, the infection of ganglionic neurons might contribute at a crucial time to the horizontal spread of infection. Ganglionic neurons are not as isolated from each other as are their myelinated processes, despite their envelopment by satellite cells (62), so productive infection could easily spread infection to adjacent cells. We found progressive involvement of ganglionic neurons in our sequential immunofluorescent studies, and it is also well known that inflammatory infiltration is most intense in ganglia.

Central Nervous System Infection

Following the entry of virus into the central nervous system, usually in the spinal cord, its ascending course to the brain is rapid. This is clear from the ascending paralysis seen in many experimental animals and in some human cases and has been confirmed by sequential immunofluorescence studies (4, 64). In rats and mice used in other studies and in the hamsters used in our studies, transit from the lumbar cord to the brainstem took only a matter of hours (7, 19, 53). As in peripheral nerves, progressive accumulation of viral antigen in the cord was so great that it was anticipated that supportive elements would become infected. Electron microscopic studies, however, showed that only neurons and their processes were infected. Others have found infected astrocytes in the central nervous system of inoculated mice (34, 46), and there is widespread glial infection in human rabies (76, and unpublished observations from this laboratory). These findings do not detract from the fact that rabies virus exhibits a primary neuronotropism both during its ascending and spreading phase in the central nervous system. In our studies, the ascending wave of rabies infection in the brain itself was evident as an immunofluorescent gradient; that is, at earlier stages of infection the concentration of antigen decreased from brainstem to forebrain. This infection gradient, of course, was modified in relation to the route of infection. For example, following intranasal infusion, olfactory bulb infection preceded that in other areas (53). SCHNEIDER demonstrated ascending infection in mice by sequential infectivity titration of parts of the brain as well as by immunofluorescence (64, 65).

Precise and consistent topographic localization of rabies infection in the brain is the most likely explanation for the specific signs and symptoms at various stages of clinical illness in man and animals. JOHNSON has reflected: "The greater localization to limbic system with relative sparing of neocortex provides a fascinating clinicopathologic correlate with the alertness, loss of natural timidity, aberrant sexual behavior and agressiveness that may occur in clinical rabies. No other virus is so diabolically adapted to selective neuronal populations that it can drive the host in fury to transmit the virus to another host animal" (40). Clinical signs of limbic infection and dysfunction in nature may actually only reflect a stage in the precise ascending infection, but in very susceptible laboratory hosts, the rate of spread of infection within the brain is usually so rapid that fury is not seen. Studies of the topographic distribution of rabies infection within the brain have suffered from the methods employed. Viral antigen aggregates (analogous to Negri bodies seen by light microscopy) are often largest in the largest neurons, such as in the pyramidal cells of the hippocampus, the ganglionic neurons of pontine nuclei, and the Purkinje cells of the cerebellum (55). Small inclusions occur in small neurons with least cytoplasmic volume, such as in the olfactory bulb and the granule cell layer of the cerebellum. Antigen aggregate size is also affected by virus strain and host species. Topographic study by immunofluorescence has too casually overemphasized large antigen aggregates so that results tend to match histologic studies based upon Negri body localization. In order to test the question of limbic tropism of infection at the time of fury, sequential topographic studies must be undertaken in species important in the transmission of the virus in nature; such studies must employ frozen-section immunofluorescence (without the bias of antigen aggregate size) and thin-section electron microscopy. Nonetheless, it is clear that terminally, rabies infection in man and animals is usually extremely widespread in the brain. Nearly all neurons in most parts of the brain may be infected, and coincident with antigen accumulation, the brain infectivity titer of fixed virus strains may rise to 10^6 infectious units/g of brain and street viruses to 10^3/g (66). Those exceptional instances of failures of rabies virus to cause progressive neuronal disease should not denigrate the general success this virus enjoys once it has gained access to the mammalian brain.

Several ultrastructural characteristics of infection in the brains of experimentally infected rodents have been used to distinguish the pathogenetic patterns of street and fixed rabies virus strains. The first is the occurrence of cytopathic changes in infected neurons. At death, nearly all neurons in the brain and cord of animals infected with street viruses are intact and have few abnormalities in organelle structure or cell-to-cell relationships (49, 51). Street viruses cause similarly subtle damage in species infected in nature, although neuronal necrosis and neuronophagia are not uncommon in some animals. Fixed viruses in laboratory rodents, on the other hand, cause widespread damage to neurons in the brain and cord (multivesiculation, cytoplasmic condensation, increase in lysosome content, frank necrosis, and intercellular edema). This difference must relate to the mechanism of neuronal dysfunction, the mode of virus spread in the brain, and the nature of the stimulus of inflammatory infiltration.

The second ultrastructural difference between virus strains is the spatial relationship between virion production and nucleocapsid inclusion formation. In

street virus infections, virus particles are associated with cytoplasmic membranes adjacent to nearly all inclusions, but in fixed virus infections many infected cells containing inclusions do not contain virus particles (34, 49, 51). This difference may be related to interference phenomena (especially via defective interfering virus) (13, 31). Interference, in turn, may play a key roll in protection against cell damage and so may explain the meager cytopathology in street rabies infections. In contrast, fixed virus growth, unchecked by interference, may directly cause the rapid neuronal cytopathology (3) and the triggering of inflammatory and immune responsiveness.

A third ultrastructural characteristic that was considered to differ according to virus strain was the propensity for budding upon plasma membranes of infected neurons. In mice it was originally believed that street virus budded primarily upon endoplasmic reticulum membranes of neurons (46), whereas fixed virus also budded upon plasma membranes (51). A similar lack of street virus budding upon plasma membranes was also shown in fox brain (20) and in neuronal organ culture (47), but in salivary glands of foxes (20, 54) and skunks (21), nearly all budding occurred upon plasma membranes.

The difficulty in substantiating street virus dissemination within the unobstructed intercellular spaces of the brain in the absence of plasma membrane budding or cytopathology was dealt with in our studies in hamsters infected with rabies and rabies-like viruses [Mokola and Lagos bat (55)] and in further detail by IWASAKI and his colleagues in mice infected with street and fixed viruses (34, 35, 36). Terminally, most virus was found trapped within neurons after budding upon internal membranes, but smaller numbers of particles were present in extracellular space after budding upon plasma membranes of neurons and their processes. IWASAKI and CLARK (34) showed that earlier in the infection course, a high proportion of virus is formed upon plasma membranes, a situation matching that in infected cell cultures. As virus is delivered into the intercellular spaces of the brain, it can move in two nonexclusive ways. First, spread over relatively long distances through intercellular spaces would be promoted by interstitial fluid movement. As stated by IWASAKI and CLARK, these spaces appear narrow or closed in fixed specimens, but actually in the dynamic living state, fluid channels are large enough to pass virus (12, 17, 34, 55, 62, 66, 82). Second, short distance extracellular passage of virus from an infected cell directly to contiguous nerve cells could also result in the same long distance movement of the infection because of the multiple, long, intertwined neuronal processes. Just as with peripheral nerve axons, neuronal processes in the brain would deliver viral genome to distant synaptic endings. This mode of spread would "avoid the potential risk of inactivation of virus by humoral antibody" (34).

The histopathology of street rabies encephalitis contributes little to our understanding of viral pathogenesis. Typically, there are no gross pathologic changes other than a variable degree of cerebral edema (33, 60). Microscopic changes of perivascular mononuclear inflammatory infiltration, neuronophagia, and neuronal degeneration are sparse in relation to the extent of the terminal infection (80). Inflammatory response is better developed in spinal and cranial nerve ganglia (Gasserian ganglia particularly) and in the brainstem and cord in cases with long clinical courses. Perhaps the inflammatory stimulus in rabies infection is less than

that in many other viral encephalitides, but in any case, histopathologic changes in the central nervous system do not match the drastic dysfunction seen clinically. Moreover, histology has not helped establish whether the timing, the quality or the quantity of the inflammatory response has any predictive value in judging whether host defense, insufficient as it is, derives from humoral, cellular or combined immune systems.

Centrifugal Infection and Virus Transmission

Although the central nervous system is certainly the ultimate target of rabies virus, the pathogenetic description is not complete without description of the infection in organs capable of yielding virus-laden oral secretions for bite transmission (19, 20, 21). The centrifugal movement of rabies virus uses the same axoplasmic route as centripetal passage; it seems most likely that the same passive transit of viral genome allows virus to reach the furthest limits of the sensory, motor, and autonomic nervous systems. Death of the host may interrupt this dissemination at any stage. For example, in our studies, terminal infection of the retina involved only ganglion cells in some animals, whereas rod and cone neuronal layers were also infected in others (52, 55). Similarly, Meissner and Auerbach autonomic plexus infection in the intestine was inconsistent. In contrast, the uniformity of infection in sensory nerve endings in the skin of the head (short centrifugal course from central nervous system) of many species including man, has allowed development of an antemortem diagnostic method based upon frozen section immunofluorescence of skin biopsies (71).

Virus delivered from peripheral nerve endings might, from anatomic considerations, seem able to infect most tissues, but in reality, the ultimate distribution of infection is extremely precise (52, 55). In our studies, we never saw an immunofluorescent cell in the parenchyma of some organs (*e.g.*, liver, spleen, kidney, lung), whereas in others, infections was consistently present (*e.g.*, pancreas, brown fat). Some of these sites of infection, such as the pancreas, must be insignificant from a pathogenetic standpoint, and others, such as myocardium (53), cannot be evaluated from present data. Some infection sites occur where the interrelationship of nerve endings and parenchymal cells is especially intimate (*e.g.*, chromaffin cells of the adrenal gland), but others receive little innervation (*e.g.*, pancreatic exocrine cells). Some infection sites might directly involve a crucial cell population with immediate functional loss (*e.g.*, again, chromaffin cells of adrenal gland) whereas other important cell populations may be affected secondarily via loss of innervation (*e.g.*, striated muscle paralysis). In most cases, the importance of these functional deficits in extraneural organs is moot because of the supervening lethal encephalitis.

Centrifugal spread of virus to sites involved in bite transmission involves target cells directly and indirectly exposed to body surfaces. Salivary gland mucous epithelium is the major source of virus shed into secretions in species which maintain rabies in nature; these include the dog, fox, skunk, and bat (14, 20, 21). Directional virus budding upon plasma membranes of mucous acinar cells facing the salivary space delivers virus into secretions and not toward basal areas where host defenses are concentrated. The sequestration of virus is also favored by normal acinar structure wherein infected epithelial cells are surrounded by a

basement lamina. This anatomic barrier may account for viral shedding after antibody has filled all interstitial spaces of the salivary gland.

Two other commonly infected sites may deliver virus directly into oronasal secretions. In our hamster studies, the olfactory end-organs in the nares were heavily infected; they were also found to be infected in bats (14, 55). Similarly, the exposed neuroepithelium of taste buds in tongue papillae was massively involved in late stages of infection. Infection sites separated from the body surface in the oronasal cavity by only a thin epidermal layer included the many poorly differentiated sensory nerve endings. It is not clear whether virus could be delivered to oronasal secretions from such sites, but they should be investigated further in any species involved in rabies transmission where salivary gland infection is not found.

Viral Replication Strategy in Relation to Genome Transit

The molecular events in the replication cycle of vesicular stomatitis virus (VSV) have been studied in detail and most of the overlapping and interrelated steps leading to the production of progeny virus are understood, at least in an *in vitro* setting. This subject has been reviewed in detail (11, 72, 83). In the case of VSV, there seems little reason to doubt *in vivo* analogies. However, how fair it is to extend these principles to rabies infections remains to be proven since it is not certain that all rhabdoviruses replicate similarly. With this caution in mind, some of the characteristics of VSV infection are transplanted into the setting of rabies virus infection of neurons as a means of exploring the relation of molecular events to pathogenetic events.

Following rabies virus adsorption to the plasma membrane of a neuron at or near its exposed distal ending, it is believed that either the viral envelope fuses with the host cell membrane and the ribonucleocapsid (RNC) is injected into the cytoplasm (83), or that viral entry occurs via endocytosis (*i.e.*, a phagocytic-like process). There seems to be ample proof that the fastest movement of viral genome from the inoculation site centripetally is passive (*i.e.*, by axoplasmic flow and diffusion rather than by multiple replication cycles); this proof comes from amputation and neurectomy experiments in rodents inoculated in footpads (4, 7, 8, 19). With fixed rabies virus strains the inoculated limb has to be amputated within 4 to 10 hours to prevent lethal infection. With street virus, the time during which virus remains at the inoculation site may be extended greatly (6), but this does not detract from the conclusion reached with fixed viruses. Likewise, immunofluorescent observations do not indicate a wave effect with massive antigen accumulation distally and a decreasing amount centrally. Rather, peripheral nerve immunofluorescence, from its earliest detection, appears constant in mass along long nerve fiber profiles. So following the introduction of rabies RNC into the distal end of an axon, what happens? Some central questions must be asked: *What element transits the length of the cell to the spinal cord? Which parental virion constituent is able to undertake the long transit without being diverted into anatomic or physiologic deadends? Are some few replicative cycles immediately necessary to amplify the infection and deliver enough progeny genome moieties to the central end of the nerve so that the infection may continue onward to the brain?* Experiments designed to answer these questions have not been successful, and there are theo-

retical considerations which argue that experiments using electron microscopic autoradiography or immunoelectron microscopy must necessarily be too insensitive to allow use of inocula doses that mimic natural exposure.

Passive genome movement through axoplasm could involve one of the following forms of parental (negative strand) RNA: a) its free form, b) RNC, c) a RNC complex containing transcriptase (10), or d) a complementary (plus strand) RNC present as a minority species in the inoculum (11). If some minimal replicative steps need be initiated immediately upon entry, then transit might involve a) a replicative form, b) a replicative intermediate, or c) another moiety containing complementary (plus strand) RNA (11, 83). One difficulty in extrapolation from the VSV model is that there has been difficulty in identifying the VSV replication apparatus; however, recent experiments indicate that there may be a VSV replicative intermediate containing complementary RNA in a nucleoprotein complex (58, 73) wherein the nucleoprotein protects RNA throughout its replication cycle. Although we have no direct evidence, the most plausible candidate for the rabies transit moiety is one of the RNC complexes with attached transcriptase. This could be a parental or progeny complex. A rabies virion transcriptase enzyme has not been isolated, although its activity has been demonstrated (11). At the central end of the nerve cell, transcription of mRNA (32) would be coordinated with replication (11, 70) to provide balanced accumulation of virion constituents and ultimately RNC assembly and coiling into nascent budding virions (83). This growth cycle would deliver virus to the junctions of the peripheral and central nervous systems and place virus in a position to start causing crucial dysfunction in the very next cells to be infected, spinal cord neurons. Even limited repetitions of this cycle within the spinal cord would deliver virus to the brainstem.

The mechanism of preferential genome transit through an axonal cytoplasmic environment which would seem suitable for diversionary replicative and transcriptive cycles is not known. Likewise, the mechanism involved in sequestration at or near the bite site before genome transit is not known. The propensity for viral growth may vary between cells more than we realize. There may be differences in the microenvironment of extraneural sequester sites and of the distal and central ends of nerve cells which would favor genomic quiescence (or replication at an undetectably low level) at one place, continued movement at another, and multiple growth cycles elsewhere. Host cell factors and synthetic machinery may affect viral replicative events; for example, it has been hypothesized that host cells may contribute a constituent of the viral replicase enzyme, and concentration of this enzyme may vary at different sites (11).

Similarly, host cell ribosome concentration varies greatly in different parts of neurons, being particularly sparse in internodal axoplasm where little viral maturation has been found (34). Host cell modulation of viral replication could, of course, be mediated through several other mechanisms, and viral replication may also be self-modulating (13). An example of the latter involves viral defective interference, a phenomenon now widely thought to have a significant role in the course and outcome of *in vivo* infections (31, 84). In this case, defective viral RNA is amplified and competes successfully for replication of viral RNA while yielding non-infectious virus. Other physiologic factors must also be considered in relation to viral genome transit and growth modulation. Most importantly, retrograde

axoplasmic flow rates must be measured in relation to the speed at which genomic moieties are swept centrally (22, 43, 44, 57, 61). This crucial area of *in vivo* molecular biology remains to be experimentally studied.

Host Response to Rabies Infection

The slow course of rabies infection would seem to provide opportunity for the full spectrum of host defense mechanisms to be initiated, but the single most characteristic attribute of this response is its nearly uniform failure. Can anything be so different between rabies infection and other viral infections of the central nervous system that might explain the exceptional case/fatality ratio? One approach to this yet unanswered question is to consider the interaction between the virus and the host's reticuloendothelial and immune responses. This is best done by experimental "dissection" of the constituents of host response: antibody synthesis, cell-mediated (T cell) immune response, antibody-dependent cellular (K cell) immune response and reticuloendothelial (monocyte-macrophage) activities. Separately, interferon activity must be dissected. Because few studies of rabies infection and immunity have been done in this way, generalizations must be recognized as speculative.

The host reticuloendothelial system may be nonspecifically stimulated by the trauma and injection of foreign materials by the bite of a rabid animal, but little viral antigen may be presented for specific stimulation in the early stages of street virus infection. There is a limited amount of viral budding from plasma membranes of infected muscle or other cells at the bite site. Budded virus is probably removed in the process of infecting further cells. There is no cytopathology which would release larger amounts of viral antigen and also stimulate inflammatory processes. Later, when infection first progresses to the central nervous system, there is still no cytopathology, and the majority of virus formed upon intracytoplasmic membranes of neurons remains *in situ*. Much of the small amount of virus formed upon plasma membranes of neuronal processes is entrapped permanently by surrounding myelin sheaths. Moreover, because the central nervous system does not have a lymph drainage system (62), there can be no flow of viral antigens to reticuloendothelial (and lymphatic) organs as would be the case elsewhere in the body. All in all, the triggering of an immune response via reticuloendothelial action has reason to be delayed and to be of low activity in the early stages of infection. Similarly, the massive amount of antigen produced late in infection and its eventual accessibility to the reticuloendothelial system should result in an exuberant immune response. These "predicted" characteristics are matched in nature. In clinical rabies in man and in domestic animals, neutralizing antibody is often absent at the time of onset of symptoms even though central nervous system infection is widespread by this time. Antibody titers often remain low until the terminal phases of illness and then reach high titers at death. However, this evidence of reticuloendothelial triggering is not matched by an exuberant inflammatory infiltration into target organs. In fact (as stated above) in many species including man, the amount of inflammation seen in the central nervous system at necropsy is remarkably small (60). An explanation of these characteristics based upon any "intrinstic quality" of rabies viral antigens is improbable

since the crude suspensions of infectious material are considered "good antigens" at least when used to elicit antibody by peripheral inoculation (1).

From the time of PASTEUR (59), active (post vaccinal) immunization and, more recently, passive (equine and now homologous globulin) immunization have focused our attention upon neutralizing antibody as the key to postexposure protection of man and prophylactic protection of man and domestic animals (15, 26, 27, 42, 88). Even if long-term prophylactic protection is attributable to active antibody production alone (without further argument), there really is insufficient evidence to conclude that antibody is the only active principle in the early phases of postexposure treatment. The effects of administering hyperimmune globulin are measured in terms of immediate serum antibody levels and homologous or heterologous globulin decay curves. The effects of active immunization are measured in terms of a) time of initial antibody production, b) peak antibody titer, c) duration of antibody titer maintenance, and d) uniformity of antibody response among individual recipients. The protective value of the antibody level achieved has been evaluated by virus challenge in experimental animals and by various indirect assays *in vivo* and *in vitro*. Direct virus challenge tests may seem straightforward, but the methods actually are crude and insensitive. For example, antibody value is measured against large doses of challenge virus chosen to assure uniformity of response in controls (13). Lethality endpoints are used because they are convenient even though they measure only the end event of the interaction between virus and antibody. Advances in this area are rapidly improving this situation, but ultimately, the real value of antibody must be appraised in a time frame and in the sites in which infection can be contained. In this regard, little is known about delivering antibody to the peripheral nervous system, and there is continuing skepticism about the relation between serum and cerebrospinal fluid (or brain interstitium) antibody levels.

BAER and CLEARY (6) showed that passively administered antibody prolongs the survival of mice inoculated with rabies virus, but such treatment does not alter the ultimate mortality rate. Recently, a man died of rabies in the United Kingdom 14 months after his last possible exposure to virus (16); does this case represent a human analogue of the experimental phenomenon? The present practice of passive-active antibody protection is used to provide both immediate [via infiltration of globulin around the bite site (27)] and lasting effects, but if passive antibody only forces a temporary sequestration of virus until globulin decay is advanced, then it is possible that active antibody induction only sequesters virus for a longer time. There is no evidence to support this concern, but in this context what might humoral immunosuppression do at times after which rabies virus is considered to have been eliminated?

Antibody to rabies virus has also been incriminated in immunopathologic phenomena *in vivo* and *in vitro*. When monkeys with low levels of antibody were challenged with street virus during the course of vaccine trials, they died earlier than animals which had no prechallenge antibody. SIKES and his colleagues coined the term "early death" for this phenomenon (69). Similarly, TIGNOR and his colleagues found that monkeys and mice which had low levels of rabies antibody as a result of immunization with rabies-related viruses (Mokola and Lagos bat viruses) died earlier than controls after rabies challenge (77, 78; TIGNOR and SHOPE,

unpublished but cited in 79). TIGNOR and his colleagues continued their studies by giving immunosuppressed mice Lagos bat viral antiserum at 8 days after virus inoculation (79). The homologous antiserum caused paralysis and death within 24 hours; this disease, unlike the unmanipulated infection, was marked by severe inflammatory changes in the central nervous system. In these studies, the phenomenon was not duplicated with rabies virus because of difficulties relating to the choice of viral strains; it appears that a precise balance has to be struck between viral infection in the brain and the dose and timing of antibody in order to reproduce the "early death". When antibody is given early it is protective or can delay disease onset as described above. It is likely that this antibody-mediated disease is an analogue of immune cytolysis produced in rabies infected cell cultures. At the time when virus is being formed upon the plasma membranes of cultured cells, the introduction of viral antibody in the presence of complement causes rapid cytolysis (85). The question has been raised whether adverse reactions of similar nature may be caused by conventional postexposure treatment of man. This question is timely now that higher potency treatments are becoming available, but a moot disclaimer must be added: that is, the viral antigen targets for antibody mediated immunopathologic disease are the same neurons which are in jeopardy from viral replication directly. Whatever the mechanism, destruction of this crucial cell population would have the same lethal consequence and the only significant difference may be the time of cell death. This may be important — the earlier a host cell is killed the shorter the time available for virus release. Both immunopathologic and direct viral damage to neurons must be end events which would be better to avoid than attempt to cure.

Cell mediated (T cell) immune response is the most important aspect of host defense in many viral infections. Experimental or natural modulation of this response can affect susceptibility to infection and the pathologic consequences of infection. An early means of dissecting cellular immune responsiveness involved the use of heterologous antilymphocyte (or antithymocyte) serum (ALS) (29). ALS caused host response to be diminished via T effector lymphocyte death and, secondarily, via diminution of T lymphocyte helper functions in the B lymphocyte-plasma cell response. If ALS exacerbated a viral disease, then it was presumed that cellular immunity is important to the protection of the host, and if disease was ablated by ALS, it was presumed that cell mediated immunopathologic events contribute to the disease processes. When ALS had no effect, it was presumed that cell mediated immunity is either not important or that other arms of the host defense system predominate in the given infection.

When HIRSCH, in this laboratory, gave ALS to mice which had been inoculated intracerebrally with rabies virus, there was no effect upon survival time, the clinical signs, nor the mortality rate (30). DAVID-WEST and OSUNKOYA (18) had the same results with the rabies-related virus, Mokola. However, it has proven difficult to affect morbidity and mortality in many viral systems when intracerebral inoculation has been used, so TURNER (81) carried out similar studies using the intramuscular route of rabies virus inoculation. Despite this variation, death patterns were still the same in animals which received ALS as in control infected animals. In Turner's study, ALS given together with rabies vaccine suppressed antibody production so much that recipients died when challenged with virus.

The *in vivo* activities of ALS have now been shown to be broader than originally thought, so interpretation of experiments has become clouded. Confirmatory evidence of the role of T lymphocytes in the outcome of viral diseases now usually stems from *in vitro* experiments and *in vivo* use of general immunosuppression with selective reconstitution via adoptive transfer of sensitized lymphoid or reticuloendothelial cell subpopulations.

WIKTOR *et al.* (86) sensitized mice with rabies virus (ERA strain) and sequentially collected spleen cells for use in a cytotoxicity assay with ^{51}Cr-labeled target cells. Virus-specific target cell lysis was detected at 4 days after immunization; it peaked at 6 days and was gone by 14 days. A potent cytotoxic response was also evoked with inactivated virus immunization—a most unusual finding. The cytotoxicity was attributed to T lymphocytes since activity was abrogated by antithymus ("anti-θ") antiserum and complement but not by removal of adherant cells (B lymphocytes and reticuloendothelial cells) by passage through nylon wool columns. This activity was prevented by cyclophosphamide or prior administration of antibody to immunized mice. In contrast to findings with several other viruses, this rabies specific cytotoxicity was rather high when T lymphocytes and target cells did not share H-2 histocompatability genes. These *in vitro* experiments clearly indicate that T lymphocyte sensitization can occur in rabies infection or in rabies immunization, but they do not predict whether this response contributes to protection or to immunopathologic disease.

Few *in vivo* experiments have dealt with T lymphocyte mediated immune reaction in rabies. Turner immunized mice with killed rabies vaccine and later transferred spleen cells or serum from these animals into recipients which were then challenged with virus intramuscularly (81). Adoptive immunization with 5×10^7 spleen cells failed to protect recipients, but serum (containing a high antibody titer) protected very well. IWASAKI (37) approached this area differently; he infected athymic ("nude" or nu/nu) mice with a temperate rabies virus strain (a temperature sensitive mutant, *ts*2). The virus caused a lethal paralytic disease in 80 per cent of phenotypically normal littermates but caused a uniformly lethal encephalitic disease in the athymic mice. Severe inflammation of the central nervous system with spongy degeneration and parenchymal focal necrosis occurred in the normal littermates, but the inflammatory reaction was much milder and necrosis was absent in the athymic mice. It was concluded that the neuronal degeneration occurring in both types of mice was probably a direct result of virus replication but that tissue necrosis was mediated by host immune response via thymus dependent inflammatory mechanisms. The outcome of infection was partly regulated by host immune responsiveness, and this responsiveness was beneficial.

For some time, the characteristic paucity of inflammatory mononuclear infiltration into rabies infected central nervous system tissues was used to argue against significant cell mediated immune responsiveness in the unmanipulated disease. Mononuclear infiltration is usually exuberant in most viral encephalitides, whether caused by direct viral cytopathology or by immunopathologic mechanisms. However, this premise has been weakened recently by demonstration in several viral diseases of immunopathologic lesions occurring with only minimal evidences of inflammation. It would be prudent to interpret the implications of histopathologic changes cautiously.

As was stated above in the case of antibody mediated cytolysis of rabies infected cells, any amplification of T lymphocyte mediated reactivity that might be brought about by the use of newer, more potent postexposure treatment regimes might be inconsequential. Does it matter whether T lymphocytes or rabies virus infection per se kills a neuronal cell population which is essential for life processes unless the time of cell killing affects virus yield and the progression of infection?

K cells, the mediators of antibody-dependent cellular cytotoxicity (ADCC), have been studied primarily in *in vitro* systems and only once in relation to rabies infection. WIKTOR *et al.* (86) interacted antibody, sensitized spleen cells, and rabies infected target cells in a cytotoxicity assay but failed to demonstrate any ADCC.

Interferon sensitivity of rabies virus replication and the capacity of rabies virus to induce interferon synthesis are well proven (2, 23, 74, 75). Because interferon suppresses viral replicative events so quickly and does not interfere with active immunization, it has been widely studied for its potential value in the postexposure rabies treatment regimen. In mice, Baer found that exogenous interferon significantly reduced mortality when administered into the same footpad previously injected with virus (5). In further studies, partially purified human leukocyte interferon was given to monkeys 24 or 72 hours after intramuscular challenge with street rabies virus (28). The mortality rate in this group was lower than the rate in a challenge control group, and this was achieved without supplementary active immunization. The use of interferon inducers (poly I·poly C, or poly I·poly C·poly L-lysine) has also been successful in reducing rabies mortality in mice and, when used together with a potent vaccine, protection has been achieved up to 5 days postchallenge (38, 45, 56). JANIS and HARMON (38) found that, in mice, the muscle injected with the interferon inducer contained 4 to 8 times more interferon than the contralateral muscle. At this time, interferon and/or inducers seem to be the most promising additions to postexposure rabies treatment regimens. It will be especially exciting if efficacy at the bite site is confirmed so that local infusion may be used just as it is with immune globulin now. Because some experimental high potency rabies vaccines induce interferon in recipients (9, 87) and some inducers also act as immunologic adjuvants (45), further efforts to amplify and control this potential will be undertaken. MERIGAN (48) has warned that the wide dosage range needed for efficacy in preliminary interferon trials in human viral infections (cytomegalovirus, varicella-zoster, hepatitis B) will require that careful empiric studies be carried out before determining the value of interferon in human rabies infection.

Finally, the interaction between rabies virus and host defenses must be assessed in relation to terminal central nervous system pathophysiology. Whether as a result of direct viral replicative events or immunopathologic damage, neuronal dysfunction is the cause of death, usually by way of respiratory arrest. We know little of the terminal pathway to death at the neuronal cell level or at the organ level (80). In the absence of severe neuronal cytopathology, it has been too easy to dismiss neuronal dysfunction as an "electrical shutdown" or nerve impulse conduction breakdown with no morphologic attribute. This dismissal should be so dissatisfying that it provokes further research on brain physiology during rabies infection. Perhaps this tack will mesh with efforts to understand the failure

of host defenses. In this way, our understanding of rabies pathogenesis may allow us to better predict the outcome of the various means of modifying host responsiveness for prophylactic and therapeutic purposes.

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