PATHOGENESIS OF HERPES SIMPLEX VIRUS INFECTION IN CHICK EMBRYOS *

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The present observations on the pathogenesis of herpes virus infection are presented because they are based on new methods of approach and because they throw additional light both on the potentialities of the virus itself and on the mechanisms of virus infection in general. Previous observations on the behavior of herpes simplex virus under experimental conditions have been of importance in the understanding of the pathogenesis of herpetic and certain other virus infections of the nervous system. For example, evidence of an axonal transmission of herpes virus from a peripheral focus to the brain in rabbits has given a reasonable basis for explaining the phenomena of herpetic encephalomyelitis and constitutes a foundation for present views of the pathogenesis of poliomyelitis, rabies and several other neurotropic virus diseases.[1] The idea of axonal transmission is based on the hypothesis of cytotropism of viruses, that is, the requirement of the internal environment of living susceptible cells for virus multiplication, which is well substantiated.

Experiments with herpes virus heretofore have been concerned for the most part primarily with neuronal transmission and little or no evidence exists for a generalization of the infection by way of the blood stream or by other means. Dawson,[2] Saddington,[3] and others [4, 5] have reported the susceptibility of the chorioallantois of chick embryos to herpetic infection. Dawson maintained the virus on membranes in series and described the gross and microscopic membranal lesions. Burnet, Lush and Jackson [4] reported an increase in the virulence of this virus for embryos, a slight diminution of virulence for mice, but no change in virulence of the HF strain for rabbits during membranal transfers. Burnet and Lush [6] and more recently Schaffer and Enders [5] used embryonic membranes of chicks to titrate antiherpetic serums.

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In all of these studies observations were limited to the membranal lesions and no effort was made to determine infection of the tissues and organs of the embryo proper. My report is concerned especially with an analysis of the mechanisms by which herpes simplex virus induces infection in the bodies of developing chick embryos following various methods of inoculation. In other words it has to do with pathogenesis, or the way in which the infectious agent goes about establishing disease in a susceptible host; and these experiments define various patterns of pathogenesis for herpes virus which follow different methods of inoculation into chick embryos. Special consideration is given to the involvement of endothelium and the dissemination of infection by way of the blood stream. In addition an increase of virulence of the HF strain for chick embryos will be briefly described, as well as a modification of the pathogenicity of the HF strain of virus for rabbits which occurred during serial membranal transmission.

Experimental Procedures

The strain of herpes virus used for these experiments was the HF virus (Rockefeller Institute, New York) whose encephalitogenic property for rabbits is well established. The technic used for exposing embryonic membranes has been described by Goodpasture and Buddingh.[7] A membranal strain of HF virus was easily established by inoculating the chorioallantois of 12 or 13-day old embryos with macerated infected rabbit's brain. Transfers were made by rubbing bits of infected membrane over the exposed surface of other membranes. Twelve and 13-day old embryos were used almost exclusively. A strain of virus thus established has been maintained for 2 years by means of 125 membranal passages uninterrupted except by irregular intervals of storage at 0°–4° C. Of chief importance in these experiments has been the microscopic examination of pathological lesions occurring in 256 autopsied embryos. Gross lesions were routinely noted. It is worthy of emphasis that except in extremely rare instances only live embryos were fixed for histological study. Whole embryos with split skulls and abdomens were fixed in Zenker's fluid (plus 5 per cent acetic acid), and sections for embedding were cut through the embryo perpendicular to the long axis of the body. The hematoxylin-eosin stain has proved en-

tirely satisfactory for the demonstration of herpetic inclusions. The presence of intranuclear inclusions characteristic of herpetic virus has been the rigid criterion for the recognition of infection in fixed tissue.

Infection of the Chorioallantois Following Membranal Inoculation

Although the lesions of the first membranal generations were complicated by the presence of macerated rabbit's brain, they were essentially characteristic. Small opaque foci of infection were recognizable 48–72 hours after inoculation. Of primary importance in microscopic sections is the presence of intranuclear inclusions in ectodermal and mesodermal cells of the membrane. Secondary to the presence of these inclusions is proliferation of ectoderm and mesoderm accompanied by a mild inflammatory reaction. As the lesions progress ectodermal cells tend to become necrotic and to desquamate. Depending on the severity of the infection healing may or may not follow. As serial passages multiplied, the severity of the membranal lesions increased but not in exact direct proportion. For example, the 2nd and 3rd generation lesions were confluent but relatively superficial and proliferative. After the 25th generation the membranal infection caused more destruction than proliferation, and since the 50th generation there has been little change in the character of the lesion. At about the 30th passage the ectoderm over the infected area was being entirely destroyed at 72 hours; there was deep involvement of the mesoderm, and it was grossly and microscopically evident that the circulation was severely interrupted. Petechial hemorrhages and thrombosis of small vessels were present.

Membranes of 9 through 19-day old embryos were found to be susceptible to infection with herpetic virus but the membranal response to infection is different at various stages of development. That the gross membranal lesions at a given hour interval vary with the age of the embryo has been observed by Schaffer and Enders,[5] but they seem doubtful about the characteristic nature of the gross and microscopic membranal reaction to herpes virus. In my experience membranal reactions to the virus of herpes, vaccinia, smallpox, fowlpox or laryngotracheitis are decidedly different when examined either grossly or microscopically at equal

intervals of time after inoculation of comparably aged embryos, but these differences cannot replace the specific cellular inclusion as criteria for the diagnosis of the virus concerned.

Membranes inoculated on the 17th day of incubation and younger develop grossly recognizable herpetic lesions. Gross lesions did not develop after the inoculation of 18 and 19-day membranes. Microscopically herpetic inclusions in ectoderm and mesoderm were regularly demonstrable in significant numbers for 4 or 5 days following inoculation of all membranes from 9 through 16 days incubation. Inclusions developed abundantly in the ectoderm of 17-day membranes but the reaction to infection was purely proliferative without necrosis or spread of the virus to the underlying mesoderm. This would indicate a developmental resistance of membranal ectoderm to infection. Membranes inoculated on the 18th or 19th day of incubation occasionally showed inclusions in ectodermal cells. Two factors must be considered in an analysis of the failure of 18 and 19-day membranes to develop appreciable lesions: one is that the blood at this stage is receding from active circulation through the membrane and the chorioallantois is becoming keratinized and dry in preparation for hatching; the other is that with maturation of the embryo factors of natural immunity which make adult fowls resistant to herpetic infection may be developing.

Infection of the Embryo Following Membranal Inoculation

Brain: From the 1st passage, embryos have been examined for metastatic lesions. Of 28 embryos inoculated on the chorioallantois with infected rabbit's brain, *i.e.* the first membranal generation, only 5 have shown metastatic lesions. Examined microscopically 2 embryos of this series showed an herpetic encephalitis 7 days after inoculation. In 1 embryo the encephalitis was extremely extensive and destructive. A large portion of the brain was necrotic. Most of the remaining neurons, glial cells and ependymal cells contained large intranuclear inclusions characteristic of herpes. There was no evidence of meningitis or myelitis, nor was there any appreciable inflammatory response. The other embryo showed neither hemorrhage nor widespread necrosis. Neurons and glial cells in a localized area contained typical inclusions. It may be of some significance that metastatic en-

cephalitis has been observed only in 1 embryo (3rd passage) beyond the 1st generation.

Heart: Two other embryos inoculated with rabbit's brain showed an herpetic myocarditis. One was fixed 6 and the other 7 days after membranal inoculation. The infected hearts were greatly enlarged and in microscopic sections the myocardial cells in the infected foci are pale staining and are swollen and without evident striations. The nuclei of the cells are enlarged, chromatin is condensed at the nuclear wall, and as in cells of any tissue the inclusions may vary from small condensed acidophilic bodies surrounded by a clear zone to large granular basophilic masses which fill the whole nucleus. The degree of inflammatory reaction appears to vary with the amount of necrosis produced by the infection. Only the lesions in the myocardium appeared repeatedly during the early serial passages and then only after 6 or 7-day membranal infection. After the 10th passage in one series gross cardiac lesions were consistently observed.

Liver: The other 1st passage metastasis was to the liver of a single embryo inoculated on the 11th day of incubation. Only three or four cells in one section were observed to contain inclusions. By the 15th passage the liver was regularly enlarged and showed multiple gross lesions at 5 and 6 days. In another series of passages the virus was not observed to metastasize either to the heart or liver until after the 20th generation. In the latter series the heart was not involved until after the liver began to show gross lesions regularly 5 days after inoculation. Between the 30th and 40th passages of the virus in both series all embryos autopsied 96 hours after inoculation showed enlarged livers dotted with multiple foci of necrosis. The hearts were edematous, pale and flabby with large yellowish plaques in the myocardium. The pericardial sacs and peritoneums were usually filled with a cloudy yellowish fluid.

Microscopically the hepatic lesions vary with the age of infection. At 48–72 hours parenchymal and stromal cells in multiple foci contain inclusions. The infection spreads until sometimes the lesions become confluent and practically every cell in the liver is involved. Necrosis begins at the center of a focus. Actual vesiculation occurs within the liver foci but there is very little cellular response.

Spleen, Kidney, Lung and Other Organs: The spleen has regularly shown gross as well as microscopic lesions but with reference to serial passage they have appeared later than the hepatic and myocardial lesions. The stromal cells in the spleen are the first to develop inclusions. They are small and brightly eosinophilic. The infection spreads and necrosis and vesiculation follow, as in the liver.

Microscopic lesions in the lung, kidney, smooth and striated muscle, subserosa of the stomach and intestines, periosteum, and infection of osteoblasts and chondroblasts were observed repeatedly in microscopic sections. In the lung and subserosa, as in the spleen, connective tissue cells appear to be the first involved and lesions in these organs remain localized. In the kidney the chief lesions are in the glomerular tufts. The infection is bilateral. Both epithelial and endothelial cells of infected tufts are enlarged and contain typical inclusions; necrosis and a mild inflammatory reaction develop secondarily. Well localized interstitial lesions were occasionally observed and in a few instances cells of Bowman's capsule and collecting tubules contained inclusions. From the distribution, frequency, and general character of lesions in the glomeruli, the glomerulonephritis seemed to develop from an initial infection of glomerular endothelium.

Although the mesonephros and the metanephros were both susceptible to infection the mesonephric glomeruli were by far more frequently and extensively involved. It is possible that this is in some way related to the fact that at this stage in development the mesonephros is the more active kidney and is carrying the burden of embryonic excretion.

Endothelium: Of particular interest has been the observation of typical intranuclear inclusions in the endothelium of blood vessels. In an embryo inoculated with virus of the 38th generation and sacrificed after 96 hours the endothelium of the aorta and of a large mesenteric artery was found to contain inclusions. Infected cells were round and slightly enlarged, and many appeared desquamated. The smooth muscle of the media about the intima of the mesenteric vessel was extensively involved. In other embryos the endothelium of the capillaries in the heart, liver and muscle was found to contain inclusions, as were the endothelial cells of the glomerular tufts of the kidney.

Pathogenesis of Embryonic Infection Following Membranal Inoculation

Up to this point all evidence pointed toward the spread of infection from the chorioallantois to various organs of the embryo by way of the blood stream. To test this hypothesis 2 experiments were carried out. Membranes of 12 12-day embryos were inoculated with the 61st generation of chick virus and 12 other embryos received macerated rabbit's brain infected with the original HF virus. At 24-hour intervals 2 embryos from each lot were bled aseptically from the allantoic artery where it leaves the umbilical stalk. Blood from each embryo was immediately inoculated in 0.1 cc. quantities onto other 12-day membranes. After incubation for 72 hours these membranes were fixed. Herpetic lesions were grossly demonstrable in most cases. Microscopically it was determined that the 61st generation virus had been consistently present in the blood stream at 24, 48, 72, 96 and 120 hours after inoculation. The original HF virus was recovered from the circulating blood in 1 instance at 48 hours and regularly at later intervals.

Adult chickens were hyperimmunized, by repeated intravenous injections, with the passaged strain of virus. Serum was collected and injected intravenously in 0.15 cc. amounts into 12 and 13-day old embryos. Within 2 hours after the injection of antiserum membranes of 10 test embryos and 12 control embryos were inoculated with the 107th generation of virus. Deaths of 4 test embryos within 24 hours were attributed to shock or hemorrhage. Each control membrane developed a typically spreading destructive lesion. All embryos of this group except 1 died on the 4th day. Membranes of the test embryos developed well circumscribed, thick purulent lesions with isolated pox at the periphery. All 6 were alive and vigorous at 96 hours. Autopsied embryos of the control group showed marked enlargement and extensive necrosis of the heart and liver with an abundance of cloudy peritoneal and pericardial fluids. Of the test group 1 showed no macroscopic lesion at all in the heart or liver; 4 showed only three or four foci of necrosis in the liver with no enlargement of the liver or heart; and the other showed multiple foci of necrosis in the liver without involvement of the heart or an accumulation of fluid. Microscopically the herpetic lesions of these embryos are small,

definitely circumscribed, and only a very few inclusions can be found. Thus by passive immunization embryos were protected against a lethal herpetic infection; the lesion at the site of inoculation was markedly modified; and metastatic spread of infection was largely prevented. One explanation might be that virus which gained entrance to the blood stream was there neutralized by circulating antibodies passively introduced. To say the least, the modification of the spread of infection by passive immunization adds evidence to the hypothesis of a hematogenous spread of infection.

In an effort to reconstruct the mechanism for the development of a systemic herpetic infection in chick embryos, one recognizes the local multiplication of virus in cells at the site of inoculation on the chorioallantois. The susceptibility of endothelium to infection makes the vascular channels vulnerable to invasion by the virus. The virus can be recovered from the circulating blood or can be neutralized within it by passive immunization. Again the susceptibility of embryonic endothelium to herpetic infection affords a means of establishing a metastatic lesion in endothelium and subsequent spread from endothelium to contiguous susceptible tissues. The demonstration of herpetic inclusions in endothelium makes such an explanation tenable.

One is confronted then with a difficulty in explaining not only quantitative but qualitative differences in the spread of infection during very early and later membranal passages. During serial passage the pathogenicity of the virus becomes enhanced for mesodermal tissue which is inclusive of endothelium. This may account for the greater number of visceral lesions of later generation, but one must look elsewhere for an explanation of the failure of the virus to localize in the brain after its pathogenicity for endothelium has become enhanced.

Infection Following Subamniotic Inoculation

Polk, Buddingh and Goodpasture [8] and Buddingh and Polk [9] described the practicability of infecting chick embryos by making injections of infectious agents into the amniotic sac. In the following experiments 13-day old embryos were inoculated into the amniotic cavity with 0.1 cc. of a 1:10 dilution of ground membranes infected with the 10th generation of chick virus. (In this

series the virus did not metastasize to the liver and heart until after the 10th generation.) Embryos were fixed at 24-hour intervals through 96 hours. At 72 hours the amnion was thickened and the skin of the embryo appeared more opaque and less smooth than normal. Microscopically the ectoderm, mesoderm and muscle cells of the amnion contained herpetic inclusions at 48 hours, as did the epithelium of the skin. Proliferation of cutaneous epithelium at a focus of infection was followed by vesiculation at 72 hours and ulceration at 96 hours. Similar lesions developed at multiple foci in the epithelium of the pharynx. Presumably by direct extension of infection an herpetic peritonitis developed regularly. In a few cases infection extended from visceral peritoneum to liver cells and to the cortex of adrenals.

Attempts to repeat this experiment with virus past the 50th generation have been rather unsatisfactory. Infection of the amnion led to a rapid dissemination of virus by the blood stream and early death of the embryos. Lesions in the pharynx were not observed and the skin of only 1 embryo was infected.

Infection Following Scarification and Inoculation of Skin

A series of 13-day embryos was inoculated on scarified skin of the thigh with 1:10 dilution of ground 10th generation membranes. Working with small forceps through a slit in the chorioallantois the thigh was gently manipulated and steadied so that a needle inserted beneath the amnion could be used to scarify and inoculate the skin with one procedure. Embryos were fixed at 24-hour intervals through 72 hours. Areas of necrosis at the site of inoculation appeared grossly. Microscopically there was extensive necrosis of epithelium and subcutaneous tissue. Intact epithelium and underlying corium were infected. Epithelial hyperplasia resulted in the formation of discrete papules in which all cells contained inclusions. By direct extension the virus invaded striated muscle beneath the superficial lesion. The nucleus of every muscle cell in a limited area contained an inclusion. An herpetic peritonitis developed in these embryos.

Infection Following Subcutaneous Inoculation

Without manipulation 13-day old embryos were inoculated subcutaneously with 0.05 cc. of 1:10 suspension of 10th genera-

tion membranal material. Embryos were fixed at 24-hour intervals through 96 hours. Gross lesions developed at the site of injection. Microscopically these lesions were comparable to those which followed scarification: epithelium, underlying connective tissue and muscle cells contained inclusions. There was an herpetic peritonitis. Remarkable in 1 embryo fixed at 96 hours was the occurrence of a destructive ascending myelitis following subcutaneous inoculation lateral to the dorsal midline at the lower sacral level. In this case there was a specific lesion at the site of injection and a mild peritonitis. The spinal cord at the level of inoculation showed a lesion which was definitely more severe on one side than on the other. Both supporting cells and neurons contained well defined inclusions. At the lumbar level the myelitis was uniformly bilateral and extremely destructive. Practically every cell of the cord in cross section was either necrotic or infected. As the infection ascended it remained bilateral but lessened in intensity and progressed more rapidly in the ventral than in the dorsal columns. At this 96-hour interval the infection had not reached the brain.

Pathogenesis of Embryonic Infection Following Subamniotic and Subcutaneous Inoculations and Inoculation After Scarification of the Skin

The mechanism of the infectious process following these three methods of inoculation is essentially the same. In each case virus is introduced into amniotic fluid and is in direct contact with amnion and cutaneous epithelium. Amniotic fluid is aspirated to bring virus in contact with pharyngeal epithelium. Each epithelial surface involved is susceptible to virus brought in contact with it. Infection is initiated by direct contact and spreads from cell to cell along the epithelial surface and into adjacent tissues of a different type. The herpetic peritonitis likely results from direct extension of infection from the amnion, although the possibility of a primary peritoneal infection following incidental introduction of virus into the communicating extra-embryonic coelom must be considered. Infection of nerves at a local focus will be considered in another connection. The fact that epithelial cells are not readily attacked by virus of the later passages suggests the possibility of a loss of some degree of ectodermotropism.

Infection Following Intracerebral Inoculation

With careful candling eggs were opened over the heads of a series of 13-day embryos and inoculations were made directly into brains without manipulation of the embryos. Five hundredths of a cc. of 1:10 suspension of 10th generation membranal material was injected. Embryos were fixed at 24-hour intervals through 72 hours. At 24 hours there was a localized meningitis at the site of injection and a local infection of the brain. After 72 hours an extensive encephalomyelitis had developed. There were large areas of hemorrhage and necrosis in the cerebrum. Neurons, glial and ependymal cells contained inclusions. All parts of the brain appeared invaded. The infection was generally destructive as it descended the cord but diminished in intensity at the lower levels. As in the ascending myelitis the progress was more rapid in the ventral columns. In the lumbar region a section containing kidney, intestine and gizzard showed that the infection had progressed peripherally by way of the ventral horn cells through the ventral roots to the spinal nerves. Serial sections were studied and by the occurrence of intranuclear inclusions in cells of the perineurium the peripheral spread of infection was followed. Peripheral extension was bilateral through the spinal roots. Neurons of the dorsal ganglia and the dorsal tracts of the spinal nerves did not appear infected. Spread along the somatic branches of the spinal nerve was followed to small radicals of nerves in the striated muscle of the body wall. Infection of these muscles was not observed. Extension of infection along the splanchnic branch to the sympathetic system could likewise be followed. Most of the cells of the sympathetic ganglia at the surface of the gizzard contained inclusions. Perineural cells of smaller radicals and small groups of neurons deep within the gizzard were infected. Here the smooth muscle cells themselves contained inclusions. Ganglion cells about the intestines were also involved in the peripheral spread of infection. In this case there were no lesions in the liver, heart, lungs or kidneys.

Efforts to repeat this experiment by inoculating the 68th generation of the virus directly into the brain were complicated by the fact that virus from incidental membranal lesions rapidly invaded the liver, heart and lungs by the blood stream. The lesions in the

brain appeared less severe. In 1 embryo there was a severe meningitis with a relatively mild lesion in the brain. No destructive myelitis was induced by the 68th generation virus.

Pathogenesis of Infection Within the Nervous System

Transmission of herpes virus from cell to cell along peripheral nerves and within the central nervous system of chick embryos is recognized. Once cells of a nerve or of the cord and brain become infected extension of infection appears to be directed along cells of the nervous system without involvement of adjacent tissues, but these experiments present no evidence of a restrictive spread among particular cells within the nervous system. Cells of the perineurium, glial cells and ependymal cells, as well as neurons, were involved in the spread of infection. A more sensitive susceptibility of neurons is suggested by a more rapid spread along motor neurons within the cord. In another instance the neurons in sympathetic ganglia appeared to be predominantly involved in a centrifugal spread of infection from the cord.

There is no indication that endothelium becomes infected within the central nervous system or that a hematogenous metastasis has resulted from a primary infection of the brain. Virus of later passages, which is judged to possess an enhanced pathogenicity for endothelium, has not localized in the brain from the circulating blood. On the other hand, virus not infrequently localized in the brain from the blood stream during very early passages. It is questionable whether one can adequately explain these two divergent behaviors of virus within the brain on the basis of a quantitative reduction of neurotropism.

Modification of the Virus During Serial Passage

Certain modifications in the behavior of the virus have occurred during membranal passage. An increase in virulence of the passaged strain for embryos is indicated in the foregoing experiments. As the passaged virus became more virulent for chick embryos gross lesions appeared at earlier intervals, and more extensive metastasis and more rapid spread occurred. Twelve or 13-day old embryos inoculated with original HF virus would usually survive 7 days. Embryos inoculated with the 125th generation virus of Series I died regularly at 96 hours. Infection with the

50th generation virus of Series II killed all embryos during the 3rd day. In terms of tissue affinities the virus may be said to have become increasingly mesodermotropic. This tropism is expressed by a more facile infection of endothelium, smooth muscle and connective tissue. Endoderm as represented by liver cells is more readily infected after serial passage of the virus on membranes. Although the present experiments are not conclusive, the possibility of a reduction in virulence for cutaneous ectoderm and pharyngeal epithelium is suggested; also a possible reduction in neurotropism is evidenced by failure of the virus to localize in the brain during late passages and a less rapid spread within the central nervous system after direct inoculation.

As previously reported, the pathogenicity of the HF virus for rabbits became in Series I suddenly modified during membranal passages.[10] After the 20th membranal generation of the 1st series of passages the chick strain has not induced an encephalitis in rabbits following scarification and inoculation of the cornea. It was not possible to recover the virus from the gasserian ganglia or from the brain at the entrance of the fifth nerve 4 and 7 days after inoculation of the cornea. When inoculated intracerebrally into rabbits the original HF virus was infective in higher dilutions than the chick strain. Further consideration of this striking modification is outside the scope of this report. Attempts to induce a similar modification by serial passage of the same (HF) strain have not yet succeeded.

Studies of the modification of the pathogenicity of the passaged virus for hatched chicks are fragmentary. Suffice it to say that in 2 experiments chicks 2 or 3 weeks old developed focal areas of necrosis in the liver following intraperitoneal inoculation with the passaged virus. These focal lesions appeared much like the focal lesions in embryonic livers but inclusions could not be demonstrated. At the present time the strain's pathogenicity is not judged to be so modified as to induce evident infection in hatched chicks.

Discussion

The experiments described above present three mechanisms by which herpes virus has induced extension of infection in chick embryos.

(1) Virus in contact with intact uninjured embryonic epithelium invaded epithelial cells and apparently multiplied within them. Intranuclear inclusions developed along with other cellular changes. There was a stimulus to epithelial hyperplasia. By some means as yet obscure the virus spread laterally to other epithelial cells and vertically to contiguous cells of different tissues. Proliferation, mild inflammation, necrosis, vesiculation and desquamation are parts of the pathological process. This mode of pathogenesis operates to establish infection of the membrane following inoculation of the chorioallantois. It is the essential mechanism of cutaneous and pharyngeal as well as amniotic and peritoneal infections following inoculation into amniotic fluid, because virus in amniotic fluid is in contact with the skin of the embryo, is aspirated into the pharynx, and spreads by extension of infection to peritoneal mesothelium.

(2) The virus may invade the blood stream at the site of a local lesion and be disseminated to establish a generalized herpetic infection in the embryonic host. Invasion of the blood stream was established by recovery of the virus from the circulation. The ability of circulating virus to initiate metastatic lesions was evidenced by the demonstration of intranuclear inclusions in endothelium of both large blood vessels and capillaries. It seems that by successive membranal passage the virus becomes more virulent for endothelium and other mesodermal elements, such as cardiac muscle, as well as for cells of the liver. Further evidence of the infectivity of blood-borne virus is that metastatic lesions may be prevented partially or completely in embryos receiving an intravenous injection of herpetic antiserum. Since the virus is able to enter and escape from the circulating blood the occurrence of metastatic lesions in other organs may be governed to some extent by the susceptibility of the cells of those organs. From these experiments it appears that the parenchyma of the liver, heart muscle and connective tissue cells is most easily invaded. Infections in the heart and liver spread so rapidly one cannot judge whether parenchyma or stroma is first involved. Blood from the chorioallantois of 12-day embryos passes first through the sinuses of the liver and then to the heart before systemic distribution is effected. Thus if the primary lesion is in the chorioallantois the highest concentrations of circulating virus should enter the

liver and the heart. This may explain the early and extensive involvement of these organs rather than a greater susceptibility of these types of cells. The cells of the interstitial tissue of the lungs, the stroma of the spleen and subserosa of the gastro-intestinal tract are the first to develop inclusions in these organs. A single stromal cell was often observed to contain a brightly acidophilic inclusion before spread, proliferation or an inflammatory reaction had occurred. The herpetic glomerulonephritis appears to represent hematogenous metastasis to the glomerular tufts. Whether the lesions in the tubules are of hematogenous or of urinary origin has not been determined histologically.

A consideration of the localization of virus in the brain from the blood stream presents several problems not fully answered by these experiments. The fact that virus localized in the brain in 2 of 7 embryos allowed to go 7 days following membranal inoculation with infected rabbit's brain may be significant. In only 1 other embryo (3rd passage, 7-day infection) was such localization observed. It may be more significant that the virus has not localized in the brain as serial passages have increased. It must be kept in mind that in later generations embryos do not live 7 days after being inoculated and time may be an important factor in the development of the brain lesion. These experiments suggest that the original HF virus and virus of very early membranal generations possess a neurotropism which diminishes for the chick embryo during repeated membranal passage, but no adequate explanation seems to be at hand to explain the mode of infection of the brain in 1st and early passages. On the other hand, during prolonged cultivation of the same strain of virus on the membrane in the absence of any neural elements it is apparent that it has become increasingly mesodermotropic for embryonic tissues and it is not incompatible with the evidence of these experiments to conceive of a concurrent loss of a high degree of affinity for nervous tissue.

(3) The third mode of pathogenesis presented by these experiments is a neural transmission of virus in chick embryos. Both centripetal and centrifugal progress of herpes virus within the central nervous system are illustrated; the one by an ascending myelitis from the sacral level of the cord, the other by a descending encephalomyelitis following an intracerebral inoculation. Proof of axonal transmission along the peripheral nerves to and from

the central nervous system is complicated by the fact that the perineural sheaths of spinal nerves and their radicals are susceptible to herpetic infection. Since perineural cells arise from the same germinal layer as the neurons the same tissue affinities may govern passage along these sheaths as from axon to axon. However, spread within the central nervous system is probably by way of axons. In the cases cited the other two modes of pathogenesis, namely by surface contact and by way of blood transport, can be reasonably ruled out. In neither case is there any metastasis to the liver or heart which would indicate either total absence of virus from the blood stream or its presence in subinfective quantities. In the embryo inoculated subcutaneously there was a mild local lesion at the site of injection with no evidence that the virus had reached the spinal cord by indiscriminate spread from cell to cell. Yet in the cord at the sacral level there was infection which is judged to have resulted from centripetal spread of the virus along the nerve and its sheath from the local lesion. In the other instance centrifugal spread from brain to cord following intracerebral inoculation is obvious and the infection extended bilaterally from the cord by the ventral roots to the spinal nerves. By infection of consecutive sheath cells extension to radicals in striated muscle of the body wall could be followed. In like manner spread by the sympathetic nerves to the smooth muscle in the gizzard was established. The fact that neurons in the gizzard contained inclusions points to spread along the axons themselves.

In considering these different modes of pathogenesis of herpetic infection in chick embryos one becomes aware both of similarities and of dissimilarities to herpetic infection in other animals. For example the vesicular lesions in the skin and pharynx of embryos are comparable to the skin lesions of herpes febrilis and herpetic stomatitis in human beings. Actual vesiculation of lesions in the liver and spleen emphasizes the ability of the virus to stimulate consistently similar reactions. Neural transmission of the virus follows a principle well established in rabbits. On the other hand the presence of herpetic inclusions in vascular endothelium has not to my knowledge been previously described, nor has blood-borne metastasis been definitely established. Herpes virus is rarely demonstrable in the circulating blood of man with natural infec-

tions nor is it usually recoverable from the blood of experimental animals with induced infections. In the experience of Goodpasture and Teague [11] the injection of large amounts of virus intravenously into rabbits was necessary to induce an occasional localization of virus in the brain; and cells of the liver, adrenal and testes in rabbits developed inclusions only if they were injured and locally inoculated. Although this hematogenous potentiality of herpes virus is not usually expressed by herpetic infection in other animals, it is comparable to the behavior of certain other neurotropic viruses. For example, yellow fever virus is known to circulate in the blood stream and to localize in the liver; and the virus of canine distemper manifests an affinity for vascular endothelium and will localize in the liver as well as in the brain of dogs and ferrets.[12] Sabin describes metastases of B virus from the testes to the liver, spleen and adrenals of rabbits [13]; and Burnet, Lush and Jackson observed lesions in the livers of chick embryos following membranal infection with B virus.[6] However, there is no evidence that herpes virus modified by embryonic passage has acquired the property of inducing blood-borne infections in rabbits analogous to virus B.

Certain modifications in the behavior of herpes virus have been observed during serial membranal transfers. The increase in virulence for embryos, as evidenced by a wider dissemination and a more rapid spread of infection, as well as by an enhanced lethal effect, is in keeping with the behavior of many other infectious agents during repeated passage through a single host specie. However, the fact that during serial passage in chick embryos this highly encephalitogenic strain of herpes has lost its ability to induce an encephalitis in rabbits following inoculation of the cornea is of considerable interest. During propagation on an avian host a modification in pathogenicity for a mammalian host has occurred. The nature, stability and immunological aspects of this change will be considered in a subsequent report. Suffice it to point out that modification of an avid neurotropism has occurred. Such modifications of neurotropic viruses might be of importance from the standpoint of practical application to vaccination. However, investigators must be continually aware that undesirable modifications might occur under the same set of circumstances and this emphasizes the importance of adequate histological and

biological controls on the behavior of viruses maintained in an experimental environment.

Summary

1. Three modes of pathogenesis of herpes simplex virus in chick embryos have been described: (1) invasion of epithelium by direct contact and spread by continuity of infection; (2) hematogenous transmission of the virus to establish a generalized herpetic infection; and (3) both centripetal and centrifugal neural transmission of infection.

2. Endothelial cells of large blood vessels and of capillaries of chick embryos were found to contain herpetic inclusions indicating a mode of localization and of metastases through established endothelial foci.

3. Embryonic chick cells of skin, pharynx, peritoneum, amnion, liver, heart, smooth and striated muscle, lung, spleen, kidney, adrenal, subserosa of gastro-intestinal tract, and the central and peripheral nervous system, as well as osteoblasts and chondroblasts, were found to be susceptible to herpetic infection.

4. An increase in the virulence of the virus for chick embryos is expressed by an enhancement of its ability to infect mesodermal and certain endodermal tissues.

5. Evidences of a diminution in ectodermal and neuronal tropisms are presented.

6. During serial membranal transfer the HF strain of herpes virus has lost suddenly on one occasion its ability to induce an encephalitis in rabbits following corneal inoculation. The conditions of this change are not known, and thus far are not repeatable at will.

References

1. Goodpasture, Ernest W. Herpetic infection, with especial reference to involvement of the nervous system. *Medicine*, 1929, **8**, 223–243.

2. Dawson, James R., Jr. Herpetic infection of the chorio-allantoic membrane of the chick embryo. *Am. J. Path.*, 1933, **9**, 1–5.

3. Saddington, R. S. Cultivation of herpes virus, and use of the mouse in its titration. *Proc. Soc. Exper. Biol. & Med.*, 1932, **29**, 1012–1014.

4. Burnet, F. M., Lush, Dora, and Jackson, A. V. The propagation of herpes, B and pseudorabies viruses on the chorioallantois. *Australian J. Exper. Biol. & M. Sc.*, 1939, **17**, 35–40.

5. Schaffer, M. F., and Enders, J. F. Quantitative studies on the infectivity of the virus of herpes simplex for the chorio-allantoic membrane of the chick embryo, together with observations on the inactivation of the virus by its specific antiserum. *J. Immunol.*, 1939, **37**, 383–411.

6. Burnet, F. M., and Lush, Dora. The inactivation of herpes virus by immune sera: experiments using the chorio-allantoic membrane technique. *J. Path. & Bact.*, 1939, **48**, 275–286.

7. Goodpasture, Ernest W., and Buddingh, G. John. The preparation of antismallpox vaccine by culture of the virus in the chorio-allantoic membrane of chick embryos, and its use in human immunization. *Am. J. Hyg.*, 1935, **21**, 319–360.

8. Polk, Alice, Buddingh, G. J., and Goodpasture, E. W. An experimental study of complement and hemolytic amboceptor introduced into chick embryos. *Am. J. Path.*, 1938, **14**, 71–86.

9. Buddingh, G. John, and Polk, Alice D. Experimental meningococcus infection of the chick embryo. *J. Exper. Med.*, 1939, **70**, 485–498.

10. Anderson, Katherine. The encephalitogenic property of herpes virus. *Science*, 1939, **90**, 497.

11. Goodpasture, Ernest W., and Teague, Oscar. Transmission of the virus of herpes febrilis along nerves in experimentally infected rabbits. *J. M. Research*, 1923, **44**, 139–184.

12. DeMonbreun, W. A. The histopathology of natural and experimental canine distemper. *Am. J. Path.*, 1937, **13**, 187–212.

13. Sabin, Albert B. Studies on the B virus. II. Properties of the virus and pathogenesis of the experimental disease in rabbits. *Brit. J. Exper. Path.*, 1934, **15**, 268–279.

DESCRIPTION OF PLATES

PLATE 41

FIG. 1. Gross appearance of infected embryonic chick organs. "A" shows enlargement, multiple foci of necrosis in the liver and spleen, and plaques of necrosis in the heart, as compared with normal organs "B." 38th passage, membranal inoculation, 6-day infection.

FIG. 2. Lobe of an embryonic chick liver showing the extent and distribution of focal metastatic infection with herpes virus. 75th passage, 96-hour infection. × 14.

FIG. 3. Single focus of infection in the liver spreading from a blood vessel. Central necrosis, only slight leukocytic infiltration, and abundance of inclusions in the liver cells at the periphery of the lesion. 81st passage, 96-hour infection. × 120.

FIG. 4. Vesiculation within a single hepatic lesion. Fibrin and leukocytes are present in the area of liquefaction. Narrow zone of necrosis. The inclusions are present in a peripheral zone. 75th passage, 96-hour infection. × 325.

FIG. 5. Two intranuclear inclusions of herpes in parenchymal cells of the liver. One shows an acidophilic inclusion surrounded by a clear zone, with condensation of chromatin at the nuclear wall; the other shows a slightly basophilic granular mass filling the whole nuclear space. 81st passage, 96-hour infection. × 1100.

FIG. 6. Seven intranuclear inclusions of herpes in parenchymal cells of the liver. 81st passage, 96-hour infection. × 1100.

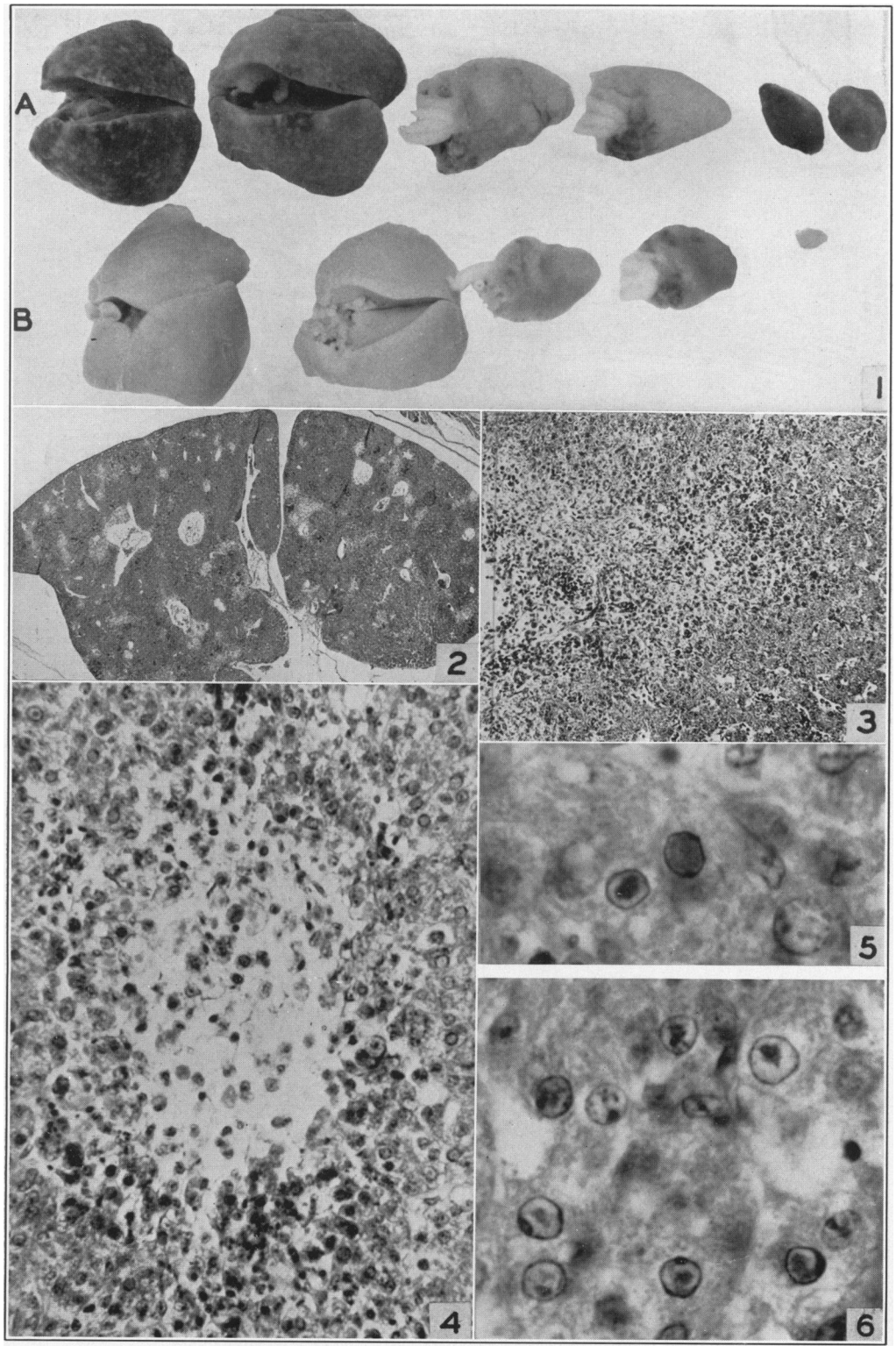
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PLATE 42

FIG. 7. Cross section of an embryonic chick heart showing the focal nature and distribution of herpetic metastases to the myocardium. 23rd passage, 6-day infection. × 18.

FIG. 8. Higher power of the central focus of infection shown in Figure 1. Note the central zone of necrosis with but slight infiltration of leukocytes, and the pale peripheral zone of swollen muscle cells where herpetic inclusion are most numerous. 23rd passage, 6-day infection. × 80.

FIG. 9. High magnification showing specific intranuclear inclusions of herpes virus in three smooth muscle cells of the myocardium. Acidophilic inclusions surrounded by a clear zone with condensed chromatin at the nuclear wall are seen. 23rd passage, 6-day infection. × 1200.

FIG. 10. Four smooth muscle cells of the myocardium containing herpetic inclusions. 23rd passage, 6-day infection. × 1200.

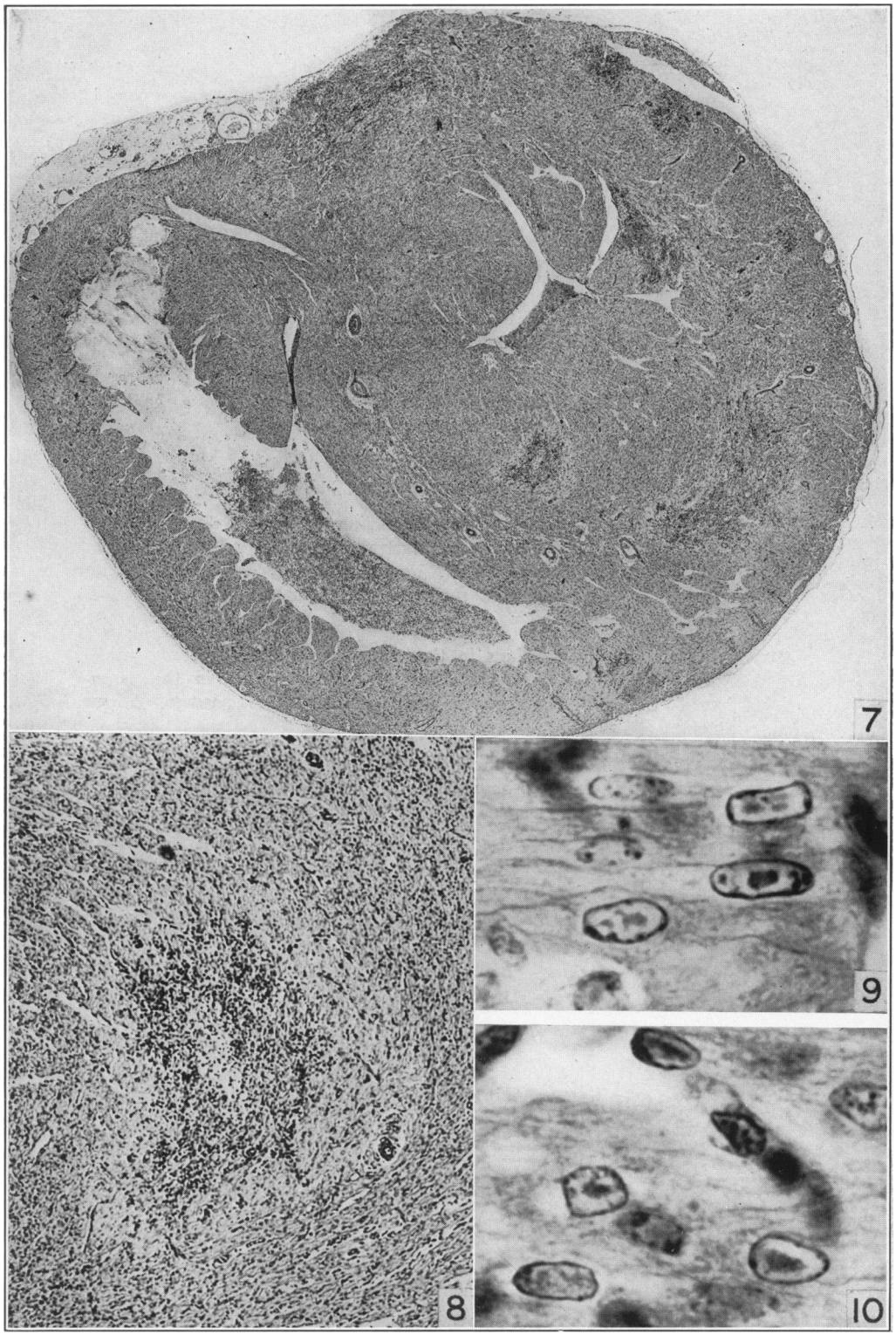
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PLATE 43

FIG. 11. Mesenteric artery of a chick embryo which showed herpetic inclusions in the endothelium and in the *tunica media*. 38th passage, 96-hour infection. × 120.

FIG. 12. Intranuclear inclusions in three endothelial cells from the blood vessel illustrated in Figure 11. 38th passage, 96-hour infection. × 1200.

FIG. 13. Five inclusions in the *tunica media* of the blood vessel illustrated in Figure 11. 38th passage, 96-hour infection. × 1200.

FIG. 14. Herpetic inclusions in the endothelium and five infected smooth muscle cells from the blood vessel illustrated in Figure 11. Note that the endothelium has ulcerated and thrombocytes (black dots) are being deposited at the endothelial surface. 38th passage, 96-hour infection. × 1200.

FIG. 15. Intranuclear inclusions in three capillary endothelial cells in the liver (indicated by arrows). Note also the inclusions in the parenchymal cells both above and below the capillary. 81st passage, 96-hour infection. × 1100.

FIG. 16. Section showing the focal distribution of metastatic lesions in the spleen. 38th passage, 96-hour infection. × 38.

FIG. 17. Two metastatic foci of infection in the lung. 56th passage, 72-hour infection. × 38.

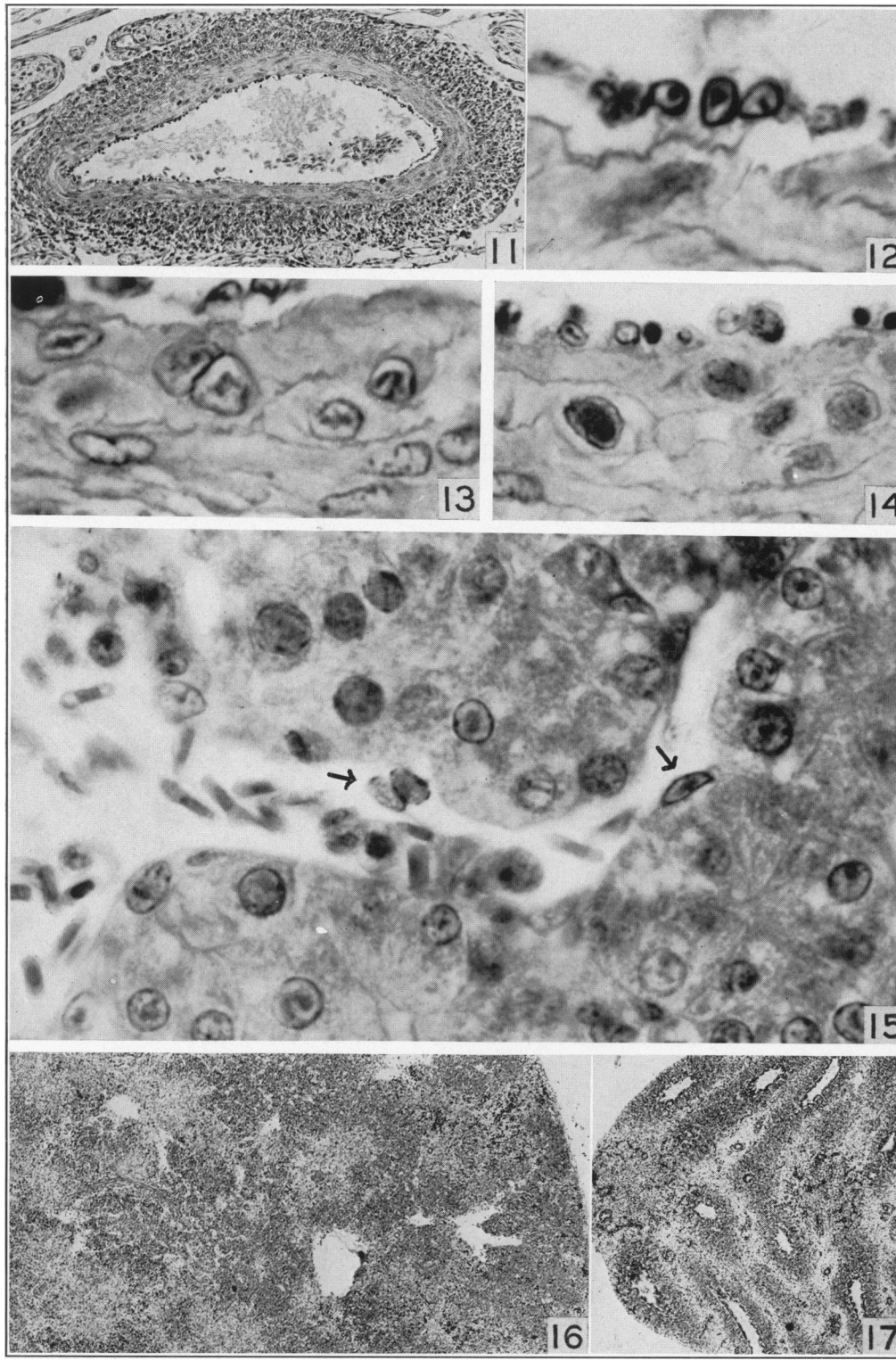
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PLATE 44

FIG. 18. Herpetic infection of a glomerulus. Note the central area of necrosis without appreciable infiltration of leukocytes. Inclusions are also visible within the glomerular tuft. 38th passage, 96-hour infection. × 525.

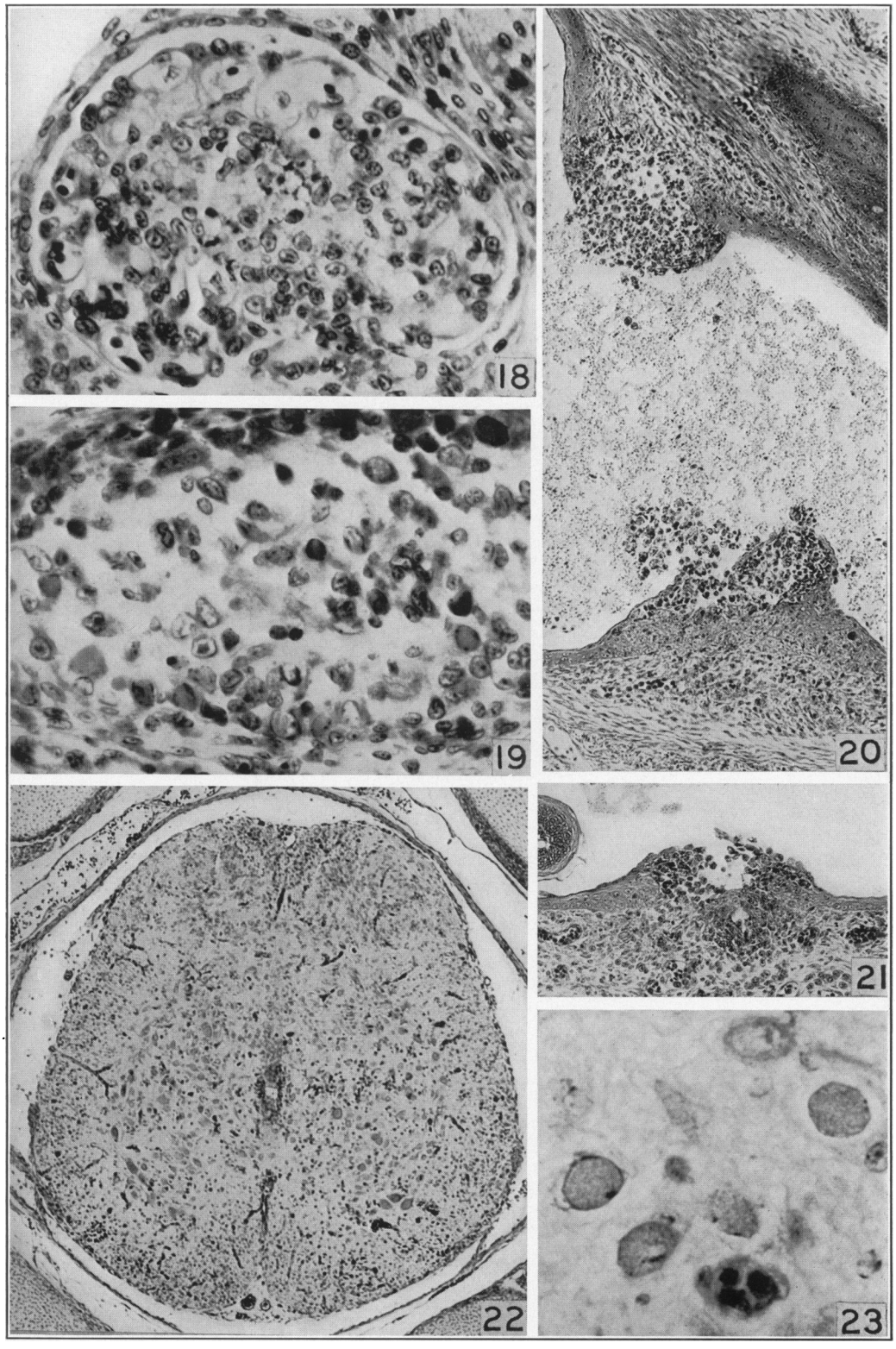
FIG. 19. Herpetic inclusions in the neurons, glial and sheath cells of a sympathetic ganglion at the surface of the gizzard. 38th passage, 96-hour infection. × 525.

FIG. 20. Herpetic lesions in the pharyngeal epithelium. Hyperplasia, vesiculation, ulceration and spread to underlying corium (upper lesion) are seen. 10th passage, subamniotic inoculation, 96-hour infection. × 120.

FIG. 21. Herpetic lesion in the cutaneous epithelium. Hyperplasia and vesiculation are seen. 10th passage, subamniotic inoculation, 96-hour infection. × 120.

FIG. 22. Cross section of the cord showing herpetic myelitis. Every motor neuron, many glial and ependymal cells are infected. A slight amount of hemorrhage and minimal inflammatory reaction are seen. 10th passage, subcutaneous inoculation, 96-hour infection. × 65.

FIG. 23. Herpetic inclusions in four neurons in the brain following intracerebral inoculation. × 750.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