ISOLATION OF A WILD AVIAN POX VIRUS INDUCING BOTH CYTOPLASMIC AND NUCLEAR INCLUSIONS

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Natural infections with viruses of the pox group are characterized in general by hyperplasia of dermal epithelium accompanied by the appearance of intracytoplasmic inclusions. This paper will report the isolation of a wild strain of avian pox virus from a slate-colored junco (*Junco hyemalis*), its cultivation in series on the chorio-allantoic membrane of embryonic chicks, and the occurrence of intranuclear inclusions in addition to the pathognomonic inclusions in cytoplasm. The occurrence of intranuclear inclusions in cells infected with this junco pox virus is of especial interest because within the group of pox diseases, only in human and simian smallpox and in an unidentified pox of monkeys have nuclear inclusions been reported to occur along with those in cytoplasm. Councilman, Magrath and Brinckerhoff,[1] in 1903, described intranuclear as well as cytoplasmic inclusions of smallpox in both the natural disease in man and experimental infection in monkeys. During a recent epidemic of pox disease in a colony of caged monkeys, Sauer, Prier, Buchanan, Creamer and Fegley[2] found both nuclear and cytoplasmic inclusions in monkey skin. The propagation of sheep and swine pox viruses in tissue culture has been described to induce intranuclear changes, but whether these are comparable to well-recognized intranuclear inclusions does not as yet seem clearly established.[3,4]

In the course of trapping and banding wild birds, a wintering slate-colored junco with rough, warty nodules on all the toes of both feet, reminiscent of infection with fowlpox virus (Fig. 1), was trapped in Jackson, Mississippi. Some of these nodules were solitary, some in a ring form around a toe, and the surface of one footpad was involved. The nodules were about 2 mm. thick and 2 of them extended 3 to 4 mm. in length. Nodules for histologic examination and culture were cut from the toes with a sterile scalpel, and 11 days after its capture the junco was banded and released in good condition. When the same bird was retrapped 4 weeks after release, all the nodules had disappeared; its

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feet were entirely healed but were without pigment at the sites of the lesions.

Microscopic examination of the nodular lesions from the junco revealed a densely hyperplastic epithelium with almost every cell at numerous foci containing a large pink-staining cytoplasmic inclusion pathognomonic of infection with avian pox viruses (Fig. 2). Individual cells were swollen and rounded but not detached from each other. Cytoplasmic inclusions were globular, like those of fowlpox, but often seemed less compact.

In addition many infected cells contained well-defined nuclear inclusions which appeared as acidophilic, smooth, compact, usually oval bodies lying in a clear nuclear space (Fig. 10). Nuclear chromatin stained densely against the nuclear wall and nucleoli were demonstrable. This pathologic appearance differed in at least one respect from that of other known avian pox diseases; the following observations on the behavior of the virus isolated from this lesion describe in some measure its pathogenic nature and help to define it as an entity.

Technical Methods

Tissues for histologic examination were fixed in Zenker's fluid plus 5 per cent acetic acid and stained with hematoxylin and eosin. The chorio-allantoic membranes of 9- to 12-day old developing chicks were used for isolation and cultivation of the virus. Inoculations were made through a window cut in the shell following techniques described by Goodpasture and Buddingh.[5] Serial passage was maintained by rubbing small bits of infected membranes over exposed chorion. Suspensions of ground infected tissue in dilutions up to 1:10,000 could be used for passage. A recently isolated field strain of fowlpox was used for comparison and the immunization of chicks.

Observations

Isolation

A small piece of the lesion freshly cut from the junco was moistened with sterile saline, ground to a paste and inoculated onto the plucked scalps of 3 adult house sparrows (*Passer domesticus*) and three 5-day-old chicks, but microscopic evidence of viral infection could not be demonstrated.

Other portions of the original lesions were ground in a mortar, suspended 1:200 in saline containing 500 μgm. of streptomycin and 500 units of penicillin and allowed to stand about 2 hours. Chorio-allantoic membranes of six 9-day chick embryos were inoculated with 0.1 cc. of this suspension. Incubation continued at 37.5° C.

By the sixth day, colorless, translucent foci on the chorio-allantois had developed opaque centers. These areas of apparent infection enlarged during the following 48 hours. On the seventh day one lesion

measured about 2 cm. in diameter; other foci of infection were solitary and thickened. Material from harvested membranes cultured on blood agar plates and Sabouraud's slants was free of contamination. Parts of the membranes were fixed for histologic study and the remainder frozen.

A 1:20 suspension in saline of ground infected membrane was used to establish the second serial passage on the chorio-allantois. Between 72 and 96 hours all membranes showed evidence of infection and several had developed opacity in an interesting honeycomb pattern (Figs. 3, 4 and 6). Subsequently the virus has been easily maintained through 20 passages by serial inoculation of embryonic membranes.

Smears of infected membranes stained by Morosow's[6] technique showed numerous elementary bodies comparable to those of fowlpox virus (Fig. 5). In histologic sections stained for fat by Sudan IV and by osmic acid, the presence of lipoid substance associated with the cytoplasmic inclusions was indicated. This further related the junco virus to fowlpox.[7] A 6-day membranal lesion gave a negative periodic acid-Schiff (PAS) reaction. Lesions at other hour-intervals were not tested for PAS reaction.

Gross Membranal Lesion

Twenty-four to 48 hours after inoculation, embryonic membranes showed vascular congestion and swelling with little clouding. Fairly translucent foci with opaque centers could be seen. At 72 and 96 hours, isolated foci appeared as pocks, or a noticeable honeycomb pattern of opacity developed.

At 96 hours and thereafter, isolated foci appeared circumscribed, hemispheroid and opaque. The honeycomb pattern of the gross membranal lesion became striking. Between the opaque trabeculae of this pattern, relatively thin, hyperemic areas formed a contrasting part of the gross pattern (Fig. 4). The whole network of hyperplastic epithelium could be lifted from the membrane almost intact. Even after fixation *in situ* the network could be shifted from its initial position. Bits of the freed matrix could be used for inoculum. This peculiar pattern appeared to develop independently of a chance distribution of virus in the inoculum. Very dilute suspensions of infected tissue produced only isolated foci of infection, but with low dilutions (1:10, 1:100) or bits of tissue used as inoculum the characteristic "honeycomb" developed.

From the fifth through the seventh day, membranal lesions continued to thicken and become more opaque. Isolated foci might become so globular as to appear like tiny, white beads on the membrane. Sometimes a bleb-like character appeared, but this was not marked. There might be a spread of light cloudiness over the "holes" of the honeycomb, and

often the incorporation of infected epithelium by an overgrowth of mesoderm could be detected grossly.

Microscopic Membranal Lesion

Twenty-four hours after inoculation, epithelial cells had not begun to proliferate markedly. Many ectodermal cells were rounded and entirely free of any attachment to the mesoderm. Small early cytoplasmic and a rare small intranuclear inclusion could be found in these rounded cells. A minimal increase in eosinophilic leukocytes was seen scattered through the mesoderm, but there was never a prominent inflammatory reaction.

In sections cut across a honeycomb pattern at 4 or 5 days, small foci of infected hyperplastic epithelium, mounded to a depth of 8 to 10 cells, usually dropped sharply at the margins (Fig. 8). Other areas of thickened ectoderm extended laterally, forming a strand or a plaque (Fig. 7). Between sharply delimited edges of infected ectoderm the mesoderm was usually denuded of epithelium and appeared to be overgrowing infected foci. Islands of infected ectoderm could be seen within the mesodermal layer (Figs. 8 and 9). There was congestion of capillary circulation to the ectoderm. A general detachment of the ectodermal layer from mesoderm was not to be confused with a process of vesiculation. Thick plaques of epithelium showed necrosis and beginning vesiculation within its own stratum.

This microscopic pattern of the developing lesion suggested that a significant number of initially infected ectodermal cells became rounded, lost mesodermal attachment, and died without proliferation, leaving areas of denuded mesoderm that became the "holes" of the honeycomb. Ectodermal cells in other areas proliferated and piled up in thickened strands elevated above denuded spaces so that the gross lesion presented a peculiar pattern.

Ectodermal cells in infected areas were greatly swollen and a large proportion contained one or more acidophilic inclusions in the cytoplasm that increased in size as the lesion progressed. Large, loosely constructed inclusions often became irregular in shape or had a ground glass appearance. Nuclei of such cells were also swollen; their chromatin was usually condensed and dispersed. Nucleoli lay against the nuclear wall, and large, usually oval, red-staining inclusions in clear nuclear spaces were a conspicuous feature of these older lesions (Figs. 12 and 13). The nuclear inclusions showed a smooth contour and stained more homogeneously than cytoplasmic ones.

Though this whole infectious process was largely ectodermal, mesoderm and endoderm were not inactive. After 48 hours the endoderm

might undergo marked hyperplasia with ruffling of its edge (Figs. 7 and 8). To a limited degree endodermal cells developed inclusions in both cytoplasm and nucleus. Mesodermal cells appeared packed together beneath areas of ectodermal thickening. Beyond 96 hours mesoderm usually showed a marked edema that accounted in part for the astonishing bulk and gelatinous consistency of older lesions.

Junco Pox in Baby Chicks

As stated above, the single attempt to transfer infection directly from junco to baby chicks failed. However, chick scalps inoculated with infective material from the second membranal passage of the virus developed papular nodules that showed both cytoplasmic and nuclear inclusions. Tissue from the third serial passage was ground, moistened with saline to make a paste and used to inoculate plucked scalps of 12 chicks 2 to 4 days old. On the eighth day, scalps were much thickened and weeping. Dry crusts formed and healing followed. On about the tenth day 8 of 9 remaining chicks showed many secondary lesions on tarsi, toes, and footpads; one had lesions on the comb and eyelid. In sharp contrast only 2 papules appeared on the leg in 1 of 4 baby chicks similarly inoculated with fowlpox virus.

Eighteen days after inoculation one of the junco-pox-infected chicks, moribund with extensive nodules about the head and on every toe, was sacrificed. The trachea and crop did not appear to be affected. Of the internal organs only the thymus, which was bilaterally enlarged, pale and yellowish, appeared abnormal. Microscopic examination showed both cytoplasmic and nuclear inclusions in thymic cells, as well as in epithelial cells in lesions on the feet.

In this limited observation of infection in chicks the junco virus appeared to have expressed an especial infectivity for epithelium of the feet. Involvement of the thymus would indicate a hematogenous dispersal of the virus. It may be also noted that involvement of the thymus in chicks was reported in a modified strain of fowlpox virus which Goodpasture described in 1959.[8]

Comparison with Fowlpox

Gross aspects of the natural lesion in wild birds and chickens, and the occurrence of characteristic cytoplasmic inclusions containing Borrel bodies relate the junco virus to that of fowlpox. However, certain differences in membranal reactions to the two viruses have been noted. In our experience fowlpox virus induces a uniform proliferation of chorionic ectoderm. The honeycomb pattern of opacity of junco virus infection has not appeared with fowlpox. Hyperplastic epithelium in the

fowlpox lesion is not movable on the membrane. The surface of the membranal lesion in fowlpox appears moist and at times glistening, as compared with a drier-appearing junco virus lesion.

Microscopically one does not see a separation of hyperplastic ectoderm from underlying mesoderm in membranal lesions of fowlpox, nor does one find areas of mesoderm denuded of ectodermal epithelium. Compared with those of junco virus, cytoplasmic inclusions of fowlpox usually appear more compact and stain in a more hyaline fashion.

Three groups of baby chicks were actively immunized against junco, fowlpox, and vaccinia viruses, respectively. The immunity of each group to each virus was tested. The results of this experiment were not definitive. Suffice it to say that this preliminary test did not indicate an immunologic relation between either junco pox virus and vaccinia virus or between fowlpox and vaccinia virus. Fowlpox virus appeared to immunize chicks against junco virus, but the amount of protection against fowlpox resulting from junco virus infection was not clear.

Pox Virus from Other Wild Birds

During the course of these observations we had opportunity to observe active pox infection in 9 additional species of wild birds: Titmouse (*Parus bicolor*), mockingbird (*Mimus polyglottos*), robin (*Turdus migratorius*), wood thrush (*Hylocichla mustelina*), starling (*Sturnus vulgaris*), cardinal (*Richmondena cardinalis*), towhee (*Pipilo erythrophthalmus*), chipping sparrow (*Spizella passerina*), and field sparrow (*Spizella pusilla*). In each instance histologic sections showed intracytoplasmic inclusions characteristic of avian pox virus. Only in a recently obtained lesion from a wood thrush were nuclear inclusions found. Virus from this lesion was isolated and rapidly passed through 5 passages. Its potential for forming nuclear inclusions seemed stable.

General Discussion

An infection characterized by rough nodular lesions on the feet of wild birds has long been recognized by banders who trap and handle birds in significant numbers. This clinical entity has been nonspecifically referred to as "foot disease" and sometimes "epithelioma contagiosum." Such a disease in birds has been observed to occur in epidemic form among winter flocks of ground-feeding species and though it is usually self-limited, may be deforming or fatal. Its occurrence has been reported in a fairly wide range of species, but little experimental work has been done with its etiologic agent. Musselman[9] demonstrated the infectious nature of the disease by transmitting it from lesions on the toes of chipping sparrows to the feet of uninfected ones. Tyzzer,[9] by his-

tologic examination of lesions from the feet of chipping sparrows, identified the infection as a bird pox and described its viral etiology.

In 1944 Syverton and Cowan[10] transmitted infection from a sooty grouse (*Dendragapus fuliginosus*) to chickens, passing the virus serially in that host. They found chickens immunized against the grouse virus refractory to fowlpox infection. McGaughey and Burnet[11] infected canaries with virus from "wild sparrows" and were able to show that a filtrate from canary lesions was infectious for embryonic membranes. More recently Worth[12] reported transmitting a virus from a junco to chickens, isolating the strain from chickens onto embryonic membranes and re-infecting juncos with membranal virus. Among these investigators, Tyzzer described cytoplasmic inclusions characteristic of pox infection in the tissue of a wild bird, while Syverton, Burnet and Worth each described cytoplasmic inclusions typical of the fowlpox group of viruses in tissues of experimental lesions induced by virus from a natural infection. None of these investigators, however, reported nuclear inclusions in any of the material they examined. It is to the occurrence of intranuclear inclusions along with cytoplasmic ones in cells infected with the currently isolated virus that we have given special consideration.

Cytoplasmic inclusions and elementary bodies in all the pox viruses have fundamental biological and biochemical similarities which establish a unity within this group. The exceptional occurrence of intranuclear inclusions in 3 of these same pox infections calls for interpretation. By the use of ordinary staining methods and light microscopy, vaccinia virus does not appear to induce the formation of nuclear inclusions, nor is intranuclear involvement described in avian pox disease of fowl, turkeys, canaries, or pigeons.

Councilman, Magrath and Brinckerhoff[1] observed nuclear inclusions in addition to Guarnieri bodies in human epithelial cells at the base and edges of vesicular lesions of smallpox. They and Calkins[13] judged that the round, oval or ringlike bodies appeared in the nuclei relatively late in the evolution of the skin lesion and represented a maturing phase in the cycle of the etiologic agent of variola. Even though these authors did not have a clear conception of the biologic nature of the infectious agent in smallpox, they did conceive of an ordered relation between the cytoplasmic and nuclear activity of that agent as it induced disease. Tyzzer[1] was never able to find nuclear inclusions in calves or the cornea of rabbits infected with smallpox virus. The chorio-allantois of chicks is susceptible to infection with smallpox virus, but neither Buddingh[14] nor Downie and Dumbell[15] demonstrated intranuclear inclusions in membranal epithelium. Smadel,[16] in his review of smallpox in Rivers' "Viral and Rickettsial Infections in Man," says of the intranuclear in-

clusions in variola, ". . . their relation to the infective particle of the virus is unkown. Although characteristic for the variolous lesion, they may be independent of the viral agent itself."

Though not always easily demonstrated,[17,18] nuclear inclusions of smallpox appear as well-stained eosinophilic bodies within a clear nuclear space (Fig. 11). Nuclear inclusions of the junco virus under consideration readily stained bright red with hematoxylin and eosin following Zenker-acetic acid fixation. Unlike those in variola, the nuclear inclusions of junco virus usually occurred in cells that contained large cytoplasmic inclusions. Though a few nuclear inclusions could be found in very early membranal lesions, they became larger, more numerous, and definitely more prominent as the infection progressed.

Sauer[2,19] and his associates observed both nuclear and cytoplasmic inclusions in the epidermal cells of monkeys during an epidemic of pox infection in a caged colony. The virus was isolated and propagated in tissue culture where inclusions were found in both cytoplasm and nuclei. The authors noted that intranuclear inclusions occurred but rarely, and then in cells without concurrent cytoplasmic ones. This virus was cultivable on chick membranes and showed an antigenic difference when compared with vaccinia virus. This mammalian virus, like the junco virus, appears to fall definitely within the pox group, but for neither virus has its antigenic relationship within the group been clearly defined.

Although the pathognomonic sign of infection with sheep pox or swine pox virus in their natural hosts does not include intranuclear inclusions, recent cultivation of each of these viruses in tissue culture has elicited demonstrable nuclear alterations. Plowright and Ferris[3] cultivated sheep pox virus in cells from sheep testis and observed the appearance in nuclei of "finely granular or homogenous material, which was frankly eosinophilic." Nuclear chromatin was condensed peripherally, but the authors could not interpret what they found as intranuclear inclusions. Likewise, Kasza, Bohl and Jones[4] questioned whether the intranuclear "vacuoles" which they observed in porcine tissue cultures infected with a freshly isolated swine pox virus could be interpreted as inclusions. On the other hand, Blakemore and Abdussalam[20] interpreted as inclusions the round or ovoid "empty spaces" in the nuclei of swine epithelium infected with swine pox virus. These "inclusions" did not stain and were described by the authors as "chromophobic."

Thus, it appears that few viruses of the pox group induce the formation of clearly and regularly demonstrable inclusions in both the cytoplasm and nucleus of infected cells. The same can be said of viruses in the other groups. The particulate composition of cytoplasmic inclusions in fowlpox and the infectious potential of the particles (Borrel

bodies) was demonstrated by Woodruff and Goodpasture.[7] The isolation of nuclear inclusions and a demonstration of the infectivity of a part or the whole of a single nuclear body is indeed more difficult. The particulate nature of some viruses that form only intranuclear inclusions, as those of herpes simplex and varicella, can be demonstrated, and in such instances the nucleus is recognized as participating in the multiplication of the infectious agent. Investigation as to whether the nuclear entity associated with junco virus is particulate becomes of immediate interest. Such studies are currently in progress in this laboratory by Dr. David Beaver. In light of the fact that other strains of avian pox viruses do not show nuclear inclusions by the usual staining techniques, we cannot disregard the possibility, in this instance, that two distinct viruses may exist. On the other hand, this recently isolated agent of junco pox may lend itself exquisitely to an examination of the relation of an abnormal nuclear function to the infective particles of pox diseases. By the application of the modern techniques of fluorescent and electron microscopy one might well inquire (a) what part, if any, the intranuclear material visualized as a stained inclusion body plays in the normal activity of viral multiplication; (b) whether it can be isolated and defined as an adjunct to or a degradation product of the long-recognized infectious particle of pox diseases; (c) whether it may be identical with that particle or an accidental association of an unrelated agent. These observations also serve to renew a consideration of the relation of cytoplasmic and nuclear inclusions in smallpox.

Summary

A virus that induced both cytoplasmic and nuclear inclusions was isolated from a slate-colored junco. This virus was infectious for chickens and was maintained serially on embryonic chick membranes.

Intranuclear inclusions were recognized in the original lesion from the junco, and this manifestation of the infectious process was consistently present and readily demonstrable throughout the experiments.

The gross lesion of junco pox and the cytologic features of its development on chick chorio-allantois have been described.

A discussion of intranuclear inclusions associated with other pox viruses, with special reference to those of smallpox, emphasizes the fact that these inclusions have not been clearly interpreted. The junco virus may be useful in the study of abnormal reaction in host cells and normal activity in the growth of pox viruses.

A brief review of the establishment of pox disease in wild birds as a viral infection points out that little experimental work has been done with this infectious agent.

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Sections of human smallpox at the Armed Forces Institute of Pathology were available for study, and a photograph of Accession 279939 has been reproduced. An infected mockingbird was submitted for examination by courtesy of Mrs. Amelia R. Laskey.

The constructive criticism of Dr. W. J. Cheatham and Dr. David Beaver during the final preparation of the paper is gratefully acknowledged.

[*Illustrations follow*]

LEGENDS FOR FIGURES

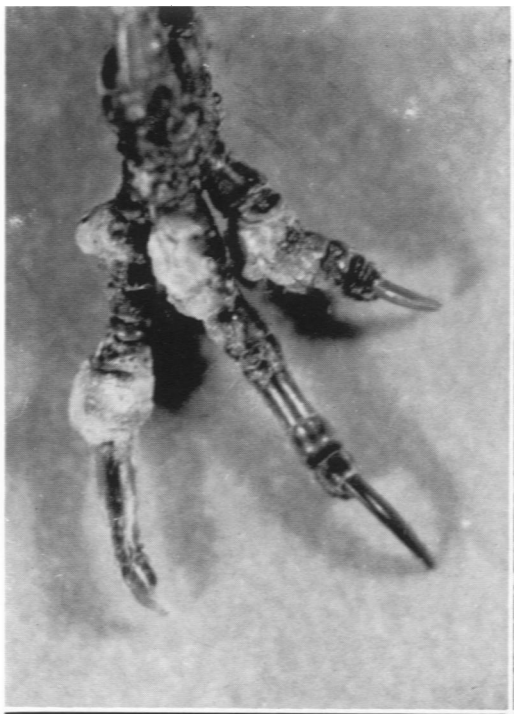
Tissue photomicrographs were prepared from sections stained with hematoxylin and eosin. Tissues were fixed in Zenker's fluid plus 5 per cent acetic acid.

FIG. 1. The foot of a slate-colored junco shows the gross lesions of pox infection on the toes of the wild bird.

FIG. 2. A nodule from the foot of the junco. Most of the cells in the hyperplastic epithelium are swollen and contain cytoplasmic inclusions characteristic of pox disease. × 128.

FIG. 3. A gross lesion of junco virus on the chorio-allantois of the developing chick. A 5-day lesion was photographed *in situ*.

FIG. 4. A higher magnification of a 5-day membranal lesion. × 2.5.



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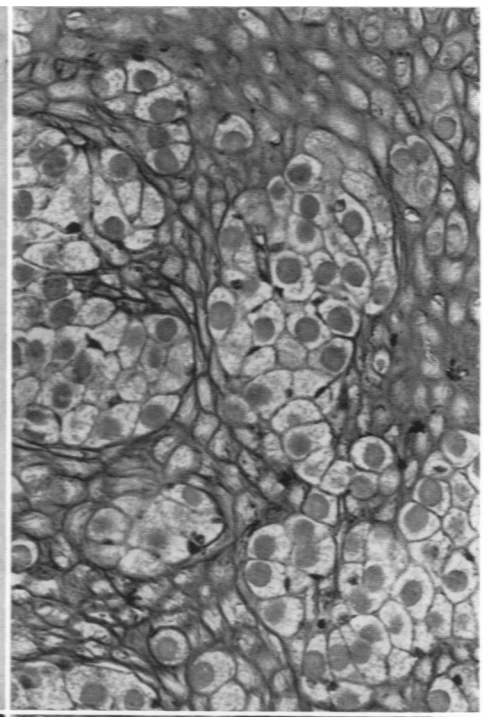

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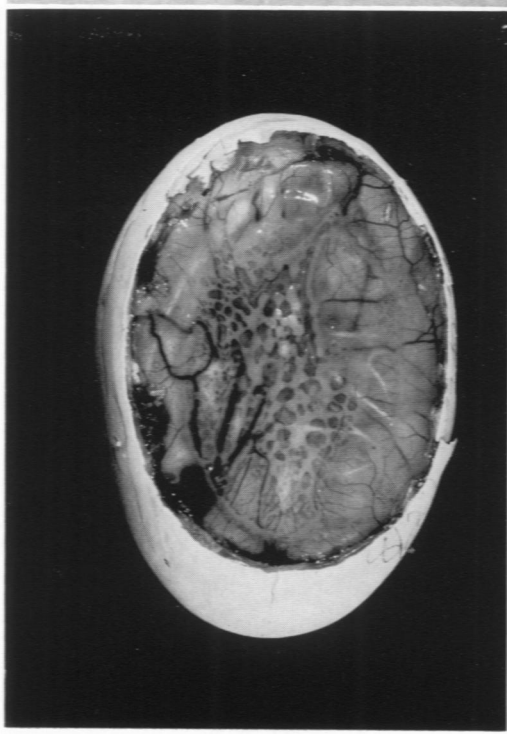

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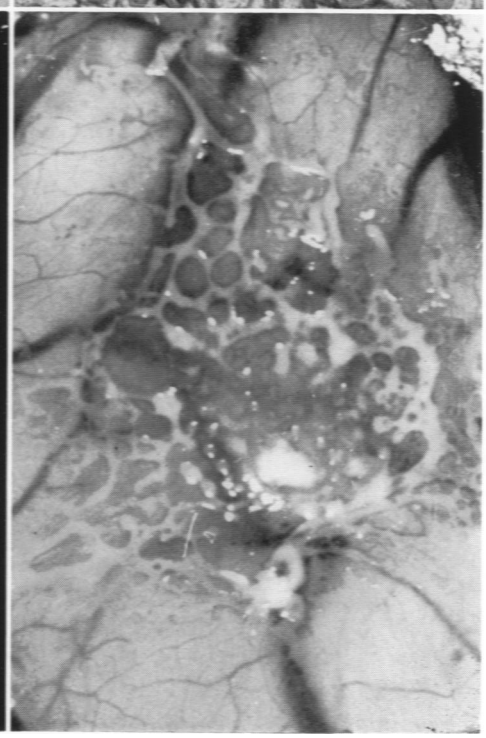

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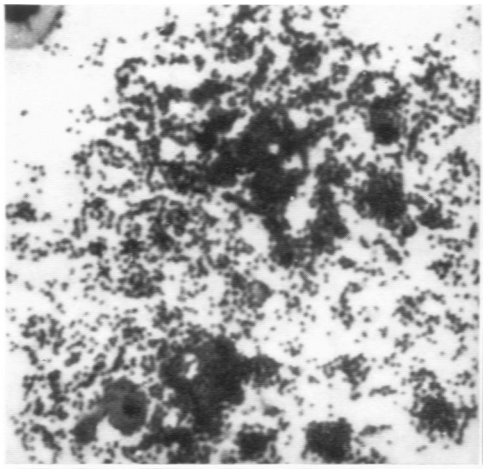
FIG. 5. A smear of a membrane infected with junco pox virus. The smear is stained by Morosow's technique and demonstrates the presence of elementary or Borrel bodies characteristic of pox viruses. × 1,177.

FIG. 6. An infected membrane removed from an embryo shows a 5-day lesion in ninth serial passage. The honeycomb pattern of infection is manifest. (The central opacity represents tissue which was rubbed over the surface in the process of inoculation.) × 2.

FIG. 7. A section across a membrane similar to that seen in Figure 6. Six-day infection in a third serial passage. There is marked hyperplasia of a normally single-celled layer of ectoderm. Dark dots represent cytoplasmic inclusions. Note the tendency of the ectoderm to separate from the underlying mesoderm which is thickened. The endoderm is also hyperplastic and thrown into ruffles or folds. × 20.

FIG. 8. A 6-day infection in the ninth passage of junco virus. The mesoderm of embryonic membrane is denuded between sharply marginated foci of infected hyperplastic ectoderm. An infected nodule at the right may represent either a mounded pock or a narrow strand of ectoderm which, with the denuded surface, forms a honeycomb. Mesoderm has overgrown several foci of infected ectoderm. × 25.6.

FIG. 9. The tendency of denuded mesoderm to push between foci of infected ectoderm and to overgrow is manifest. Individual pocks show central necrosis. There is marked swelling of the mesoderm. × 25.6.



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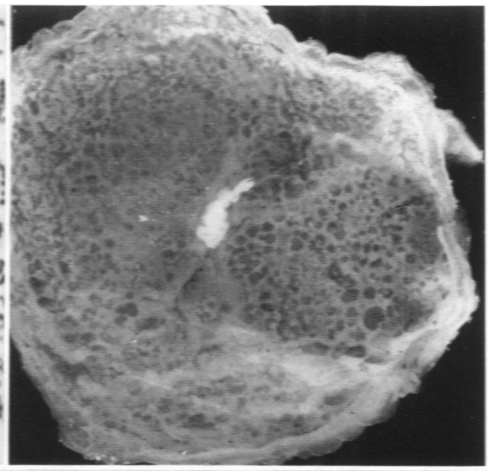

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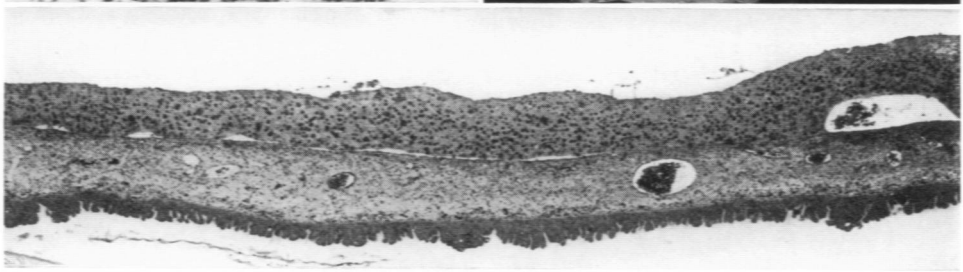

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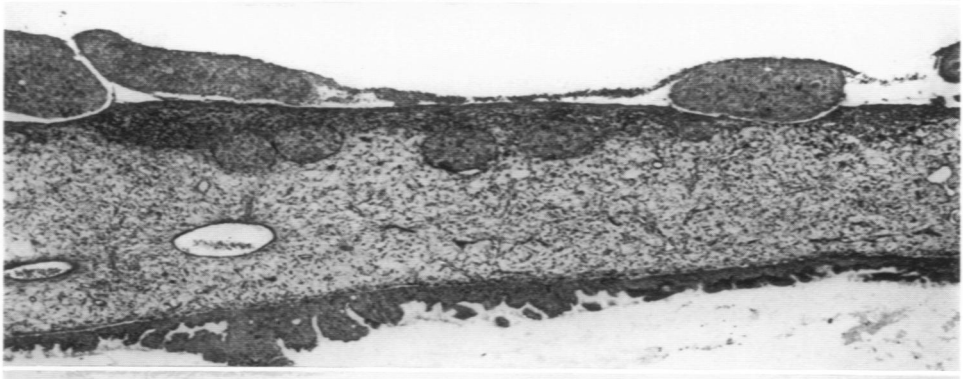

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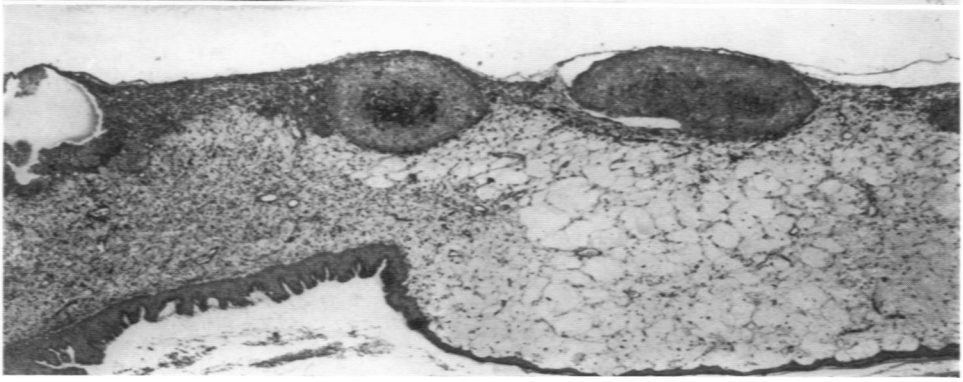

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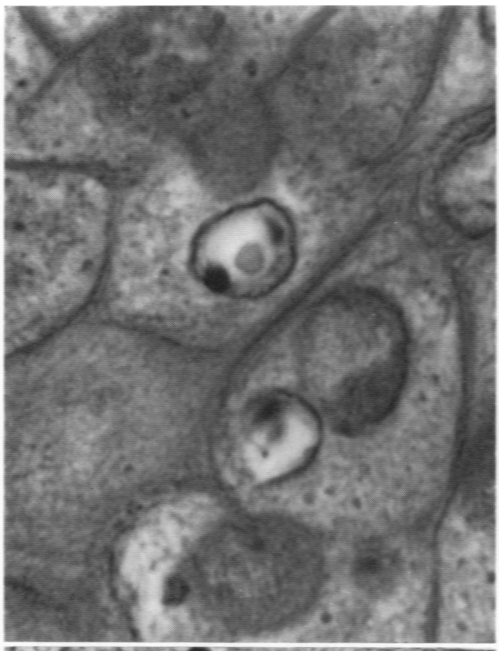
FIG. 10. The ectodermal epithelium from the toe of the junco contains both cytoplasmic and intranuclear inclusions. × 900.

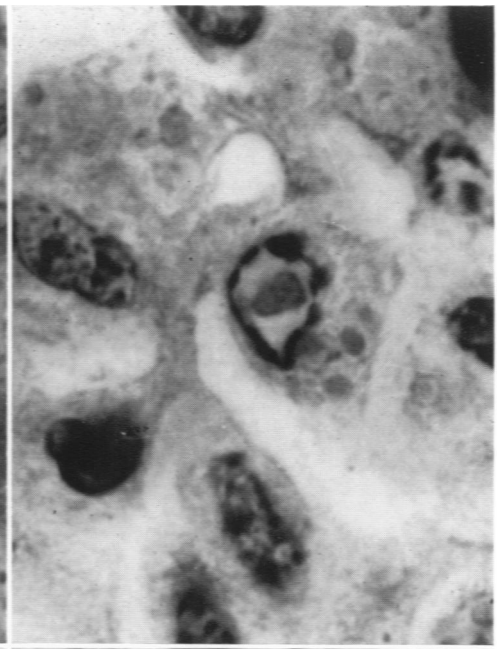
FIG. 11. The epithelium of human skin at the edge of a smallpox vesicle exhibits an intranuclear inclusion of variola virus. Condensation of chromatin at the nuclear wall and the well-defined body within a clear nuclear space are evident. AFIP Accession 279939. × 1,024.

FIG. 12. Intranuclear and intracytoplasmic inclusions of junco pox virus in the chick chorionic ectoderm. Note the irregular, loose construction of the cytoplasmic inclusions, condensed nuclear chromatin, the nucleolus against the nuclear wall, and the well-defined intranuclear body. × 512.

FIG. 13. The chorionic ectoderm exhibits both cytoplasmic and nuclear inclusions of junco pox virus. Condensed nuclear chromatin, resolvable nucleoli and a clear space about an intranuclear body may be noted. Single cells are swollen and contain both types of inclusions. × 800.



10



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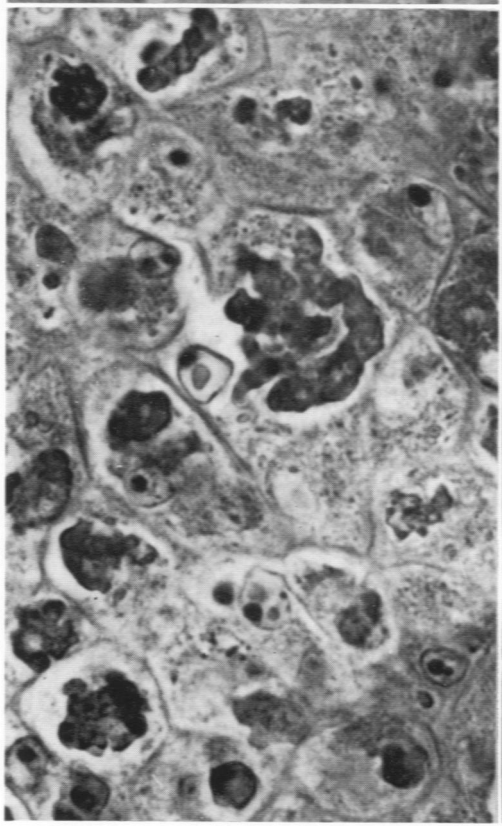
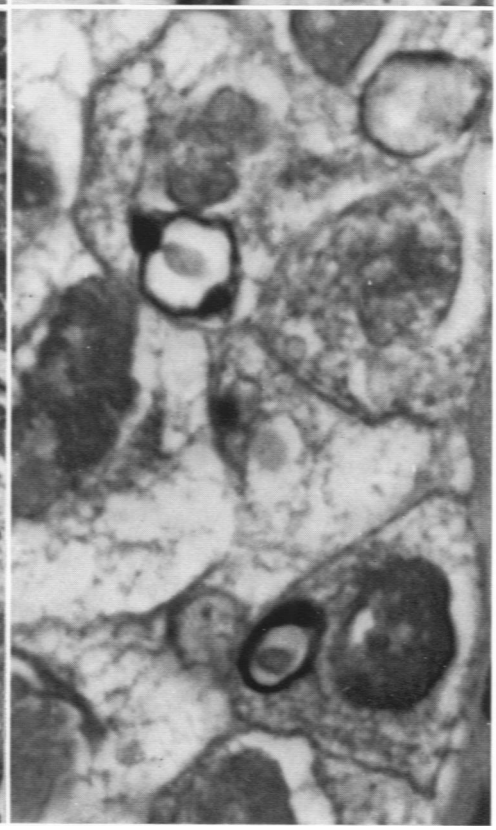


12



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