The pathologic changes produced in chick embryos by yolk sac inoculation of group A Coxsackie virus

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Plates 1 and 2

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Since the discovery of the Coxsackie group of viruses announced by Dall-dorf and Sickles in 1948 (1), various investigators (2–5) have described and illustrated the characteristic acute myositis produced in suckling mice by infection with some of these viruses. In 1950 Huebner, Ransom, and Beeman (6) succeeded in infecting embryonated eggs with one type (Albany-2) of Coxsackie virus. In accomplishing this they used an alternating mouse-to-egg passage technique, but they later found that this mouse-to-egg alternation was not necessary to continue propagation of an egg-adapted strain of virus. Serial passage succeeded readily when the inoculum was injected into the yolk sac, but attempts to propagate the virus by means of inoculation of the chorioallantois were unsuccessful and the virus disappeared on the fourth passage. Although the inoculum was injected into the yolk sac, almost all the demonstrable virus was found localized in the tissues of the embryo (6) and in the amniotic fluid (7). After ten passages, embryo tissue when diluted 10⁻⁷ and injected into suckling mice produced paralysis and death with the characteristic pathologic lesions of acute myositis that differed in no visible particular from those produced by injection of mouse tissue infected with the parent virus.

Since the primary purpose of the above experiment was to establish infection by a strain of Coxsackie virus in the chick embryo and propagate it by serial passage, no special attention was devoted to examining the lesions produced by the infection in the developing embryo. It was noted, however, that of a group of 6 embryos inoculated on the 7th day of incubation and allowed to proceed to the hatching stage, 4 survived for 21 days but failed to hatch and when opened were found to be small and poorly developed. Also a few sections of chick muscle tissue taken at random along the passage series

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for histologic examination showed early necrosis and myositis similar to that seen in mice. Since it thus appeared that the virus not only propagated in the embryonated egg but also produced visible tissue alterations in the embryo, the following experiment was set up to provide a detailed study of the lesions produced in the chick embryo by infection with a Coxsackie type virus of the "A" group—a virus now believed to cause a prevalent summer illness of children known as herpangina (8).

Material and Methods

Stock virus suspension for the experiment was prepared from chick embryos infected with material from the fifteenth egg passage. Heads, feet, and viscera were removed and the infected carcasses were pooled and preserved by freezing until needed. Thawed carcasses were weighed and ground in a Waring blender with sufficient distilled water to make a 20 per cent suspension. After grinding, glass beads were added and the suspension was shaken for 1 hour on a mechanical shaker at 4°C. This more completely homogenized suspension was then spun for 1 hour at 1500 R.P.M. in a refrigerated centrifuge and the supernatant fluid collected and stored at 4°C until used. Before inoculation, penicillin (500 u./ml.) and streptomycin (250 μg./ml.) were added to the fluid and it was allowed to stand at room temperature for 1 hour. Each fertile egg received 0.5 cc. of this fluid injected into the yolk sac. These antibiotics in the dosage used were shown to be entirely innocuous when injected with sterile tissue extract into control embryonated eggs. All inocula were cultured for 7 days at 37°C in fluid thioglycolate media and were found to give no growth of bacteria.

In order to study in detail the pathogenesis of lesions caused by the Coxsackie virus, and the modification of the lesions caused by increasing maturity of the chick embryos at time of inoculation, groups of eggs that had been incubated for 6, 8, 10, 12, 14, 16, and 18 days were inoculated. Sufficient numbers were prepared in each group so that one or more from each could be sacrificed every 12 to 24 hours after inoculation up to the estimated time of hatching at 20 to 21 days. The embryos tolerated the inoculation very well and only approximately 5 per cent were found dead at time of examination. Two eggs inoculated on the 18th day of incubation were allowed to hatch and the chicks died on the 10th and 16th days. A series of uninfected eggs, maintained in the same incubator, were sacrificed at the same intervals to furnish normal control material. All eggs were incubated at a temperature of 35°C.

At the times selected, embryos were removed from their shells, freed of the membranes and yolk sac, their gross appearance noted briefly, and the embryos immersed whole in a suitable quantity of fixative, usually 10 per cent formalin but occasionally Bouin's fluid. A few of the larger specimens were skinned to facilitate penetration of the fixative. After fixation for a week or longer complete transverse slices 4 mm. thick were cut with a razor blade across the greatest diameter of the head, horizontally through the skull base and neck and transversely through the thorax, upper abdomen, lower abdomen, and pelvis. These slices were then hardened for 3 days in 2.5 per cent aqueous potassium bichromate, decalcified for several more days in 5 per cent aqueous formic acid, and embedded in the usual manner in paraffin. Sections were stained as routine with Lillie's azure-eosin (9), and for special purposes with hematoxylin-eosin, phosphotungstic acid-hematoxylin, and Wilder's method for reticulum.

RESULTS

Since the series of eggs inoculated on the 6th day of incubation provided the longest period of observation and the greatest development and variety of lesions, they will be described in detail. The effect of age of the embryo at
time of inoculation on the development of lesions will be noted more briefly in the later series.

Muscle Lesions.—The first clearly recognizable muscle lesions were seen at 2½ days after inoculation, at about the same time that lesions caused by the Coxsackie virus usually become microscopically visible in suckling mice (10). Earlier than this a few single oxyphilic and possibly necrotic muscle fibers were present, but their significance is doubtful as similar altered or necrotic muscle fibers were also seen in the developing muscle of non-infected controls, and have been described as part of the normal process of histogenesis of striated muscle by Maximow (11). Such rare single necrotic fibers were observed in normal controls as late as the 12th day of incubation, and, along with the general difficulty in obtaining perfectly uniform fixation and staining of striated muscle tissue, they emphasize the need for caution in interpreting early and scanty histologic evidence of infection in the chick. Even the earliest lesions evidently develop with great rapidity, as already at their first appearance at 2½ days they were widespread throughout all the larger muscle masses.

Two types of degenerative changes in muscle cells could be recognized which were not consecutive, but roughly parallel. The common and predominant degenerative change, seen in some degree throughout the entire series, was the dense hyaline and oxyphilic necrosis of muscle fibers (Fig. 1) already familiar in suckling mice infected with Coxsackie virus. The other consisted of interstitial edema of affected muscles with acute hydropic distention of the fibers, granular disintegration of the cytoplasm, and rupture of the sarcolemma (Fig. 2). This lesion appeared clearly only in the earliest stages of the youngest series of chicks. Though swift in development and apparently of short duration, this degenerative change may nevertheless have accounted for the very rapid loss of bulk of muscle between the 3rd and 4th day.

The massive destruction of skeletal muscle, though not progressing at a perfectly uniform rate, was nevertheless very rapid and almost complete. As early as the 6th day of infection in some specimens, striated muscle had completely disappeared, leaving behind a loose and edematous mesenchyme containing scattered thin-walled dilated vessels and loose clusters of eosinophilic leucocytes (Fig. 3). After the 7th day of infection only a few scattered strands of surviving muscle were to be found in any specimen. Some of these muscle fibers were of very uneven caliber, suggesting continued development or even hypertrophy of some surviving muscle fibers. The subcutaneous and fascial mesenchyme continued to grow with the embryo, and became quite bulky and edematous with numerous large, thin-walled vascular channels. By the 11th day islands of subcutaneous adipose tissue began to appear and these, as in mice infected with the same strain of virus, showed no lesions. At the same period bundles of smooth arrector muscles attached to the feathers made their appearance and continued to develop without apparent injury from the virus.

Regenerative changes in the damaged muscles were small in amount and made very little apparent progress toward completion. At 5½ days after infection, when muscle destruction was far advanced, occasional swollen fibers appeared with clusters of nuclei along the sides or at their ends (Fig. 4). Often the cytoplasm of such fibers was lumpy and apparently partly necrotic and enclosed masses of mucoid material. Other multinucleated fibers in later specimens appeared more probably viable, with clear basophilic or partly oxyphilic cytoplasm. At no stage, however, did they occur in sufficient numbers to suggest any effectual replacement of the lost muscle tissue. In addition to these regenerative changes in surviving muscle fibers, there appeared irregularly from 6½ days onward, especially in areas of almost complete destruction, clusters of cells resembling primitive myoblasts (Fig. 5). These were round or polygonal cells of medium size with large single vesicular nuclei, prominent nucleoli, and a moderate amount of homogeneous deeply basophilic cytoplasm. Sometimes they were mingled with
moderate numbers of eosinophilic leucocytes. Occasional mitotic figures were seen among them, but they showed no progressive tendency to assume spindle form or develop myofibrils in their cytoplasm, nor did they exhibit any evidence of injury by the virus. Their true identity is accordingly uncertain. The mass loss of muscle was followed by marked stunting and deformity of the skeleton with bending of the carina and overlapping and collapse of the ribs. Ossification of the cartilaginous skeleton was delayed and irregular but not suppressed.

In addition to the characteristic destruction of striated muscle, certain other important lesions were observed which have no counterparts in infected suckling mice. Of these the most striking were alterations in the epidermis and feathers. They were so severe and so regular in occurrence that it seems probable that they too were caused directly by the virus, and represent the development of dermatropism by the virus growing in a new and highly specialized animal host.

Epidermal Lesions.—The earliest lesions, consisting of scattered small areas of coagulation necrosis and shallow ulceration of the epidermis, were seen at 3 days after inoculation when extensive muscle damage was already apparent. The small developing feather buds were still intact. During the subsequent 2 to 3 days the epidermal erosion increased somewhat in extent, and developing feather buds often appeared edematous and abnormally cellular, with coagulation necrosis of a few cells. Feathers continued to develop, but irregularly and in greatly reduced numbers. By the 7th day some of the abnormal feather buds began to coalesce into irregular and partly cystic cellular masses in the subcutis (Fig. 6). Feather shafts often became covered with unevenly thickened, partly necrotic, and desquamating masses of epithelial cells (Fig. 7), and feathers became partly fused into deformed tufts. From the 8th day onward some specimens showed nearly total loss of both epidermis and feathers. In some, remnants of epidermis appeared as small masses of pale edematous squamous cells partly buried in the subcutaneous tissue. In others, the epidermis survived only as thin and partly macerated patches. In the swollen and partly necrotic epithelial cells covering feather shafts there were occasional spherical brightly erythrophilic “inclusions.” In a few later specimens, 12th and 13th day, the coalescing and degenerating feather buds in the subcutis were surrounded by a fairly marked granulomatous monocytic reaction (Fig. 8), but otherwise there was very little inflammatory response to the extensive loss of epidermis. There was also virtually no evidence of epidermal regeneration in the form of mitotic division or epithelial thickening at the edge of the eroded areas. Another noteworthy feature was that the epithelium of the cornea and conjunctiva was invariably intact even though the epidermis covering the head and eyelids was often completely destroyed.

In addition to the two major types of lesions described above, other lesions were noted in random single specimens. These could not be attributed to the direct action of the Coxsackie virus, but are nevertheless deserving of brief mention.

Incidental Lesions.—One specimen obtained 5½ days after inoculation showed a small superficial ulcer in the mucosa of the proventriculus with infiltration and spilling into the lumen of a small amount of monocytic exudate. Another small gastric mucosal erosion was seen at 10 days. A 6½ day chick had a small shallow ulcer of the tongue with its base partly covered by fibrin and leucocytes. Multiple small foci of early coagulation necrosis of the liver appeared in a 9 day specimen, and more extensive liver necrosis in a 10 day specimen.
In a second 10 day specimen there were both focal necrosis and bile stasis, while in a 13 day specimen focal liver necrosis was in some areas surrounded by a marked granulomatous reaction. In a single 10 day specimen scattered tubules in the metanephros were distended by masses of faintly fibrillary or crystalline material not stainable by bacterial stains. The adjacent tubular epithelium was partly necrotic, with moderate monocytic infiltration. It may be noted that all the above incidental lesions were found in living embryos. The two of the series which were dead when harvested showed no visceral lesions to account for their demise.

The complete absence of visible damage in a number of important organ systems was striking. Outstandingly, as is the case with suckling mice infected with group A Coxsackie virus, no lesions were seen in the brain, spinal cord, or dorsal root ganglia. Even peripheral nerves escaped damage. In some late specimens apparently unaltered sciatic nerves traversed the thighs from which all muscle had disappeared (Fig. 3). Presumably the motor end-plates, and possibly the terminal branches had degenerated, but special stains to determine this point were not undertaken in the present study. No lesions were seen in the heart, lungs, spleen, pancreas, and bowel. Though occasional necrotic muscle fibers have been observed in the esophagus of infected suckling mice, none was seen in the chick, nor were there any lesions in the massive and somewhat specialized muscle of the gizzard.

The embryonic membranes were not systematically studied. Those of the later specimens in the gross appeared to be somewhat tough and less translucent than normal. Microscopically they showed on the ectodermal surface scattered areas of ulceration and some heaped-up foci of epithelial proliferation. It was not believed that these lesions were due to Coxsackie virus as they are similar to those observed following a variety of inoculations, or even after simple opening and mechanical disturbance of the developing egg.

**Gross Changes.**—The severe histopathologic lesions were accompanied by equally striking gross changes in the embryos (Figs. 9 and 10). Already by the 4th day of infection and 10th day of incubation the infected chicks were noted to be slightly translucent and lagging behind in size when compared with the corresponding normal controls of 10 days' incubation. From this point on the infected embryos were obviously increasingly stunted, and at 20 days they weighed on the average only about one-third as much as the corresponding normal controls. Feathers were few in number and poorly formed, so that even the most mature embryos appeared nearly naked. The most characteristic change, however, resulted from the massive loss of striated muscle. After the 8th day the body wall and limbs of the embryo became almost transparent and the viscera and stunted skeleton were clearly visible through the edematous and jelly-like subcutaneous tissue.

In spite of these extremely severe lesions, the virus was not directly lethal, and only two of the 6 day series of embryos, one each on the 4th and 15th day, were found dead when harvested.
The general effect of increasing age at time of inoculation was greatly to reduce the extent and severity of the muscle lesions and to delay somewhat their initial appearance; but within the limits of the present experiment, susceptibility to infection apparently did not entirely disappear.

Effect of Maturation of the Embryo.—The first sharp reduction in severity of the lesions became manifest in the series of embryos inoculated on the 12th day of incubation. In this group the initial lesions, consisting of scattered necrotic fibers, chiefly in the thighs, with loose focal leucocytic infiltration, appeared as usual on the 3rd day of infection. These lesions, however, showed comparatively little tendency to progress, and in the 7 succeeding days there appeared only a slightly increased number of necrotic muscle fibers, singly and in small groups, and scattered small leucocytic foci from which necrotic fibers had presumably disappeared. In the final two specimens of the 12th day series, whose infection had lasted 9 and 10 days respectively, there were single small focal losses of muscle in the neck and at the knee with moderate proliferation of fibroblasts enclosing a few slender surviving fibers. In embryos inoculated on the 14th and 16th day of incubation a few scattered necroses of muscle fibers with scanty leucocytic reaction appeared first on the 4th or 5th day of infection, and then increased only slightly in number in the remaining 2 days of incubation. This minimal reaction represents the opposite extreme to the explosive appearance of widespread muscle lesions in the 6 day chick after 2½ days’ infection, and their swift progress to almost complete destruction of skeletal muscle by 5½ days.

The two final embryos of the series were inoculated on the 18th day of incubation, and hatched spontaneously on the 24th day. One was found dead 4 days later or 10 days after inoculation. The immediate cause of death was not apparent, but the muscle fibers were of somewhat irregular caliber and density, and in the deep muscles of the neck, a site of predilection noted in earlier specimens, there were a few small foci of leucocytes presumambly the mark of a mild and self-limited infection. The other chick was found dead on the 11th day after hatching or 16 days after inoculation. Again no immediate cause of death was found, and the striated muscle showed only some irregularity of fiber size and density of doubtful significance.

The necrosis and degeneration of feathers and epidermis, tentatively attributed to the direct action of the virus, also diminished abruptly with the increasing maturity of the embryos at the time of inoculation, and approximately simultaneously with the diminution of muscle lesions. Epidermal erosion and superficial necrosis of feathers with subsequent disappearance and the formation of a few small subcutaneous granulomas containing pigment were still quite prominent features in the embryos inoculated on the 10th day of incubation. In the 12th day series, however, there was only superficial necrosis of scattered single feathers in the middle three daily specimens, while the last four showed no definite lesions. In the 14 day embryos only a single necrotic feather was seen in one specimen after 5 days of infection. No abnormalities of the feathers were observed in the embryos inoculated on the 16th and 18th day of incubation.

A few tests for the presence of antigen and virus in the amniotic and allantoic fluids were made on the series of embryos inoculated on the 6th day of incubation. Complement-fixing antigen was demonstrable in the amniotic fluid on the 5th, 6th, and 7th day after inoculation, but thereafter disappeared. None was detected in the allantoic fluid. Virus, determined by the production of paralysis in 50 per cent of inoculated suckling mice, was present in dilution of $10^{-4}$ in the amniotic fluid from the 5th to the 9th day, and in lesser amounts
thereafter. In the allantoic fluid a smaller but fairly constant amount of virus was present throughout the series.

DISCUSSION

The most interesting result of this experiment is the proof that not only can a strain of group A Coxsackie virus be propagated in the embryonated egg, but that in a host widely differing from the suckling mouse it produces the identical basic type of lesion,—widespread destruction of striated muscle. As with suckling mice, the first visible muscle necrosis appeared between 48 and 72 hours after inoculation. However, in the more immature chick embryos it appeared to progress more rapidly, possibly because of the relatively undifferentiated and fluid character of the embryonic muscle. In addition to the usual oxyphil coagulation of muscle there appeared in the younger series a number of fibers seeming to undergo an acute hydropic swelling and degeneration. Muscle destruction in the early series of chicks was much more complete than is ever observed in the mouse, probably because the embryo within the intact egg makes no use of striated muscle for either respiration or nutrition and hence survives for a prolonged period. Leucocytic reaction, however, was generally scanty, especially in the earlier specimens, while in the more mature embryo it appeared quite sharply focal and abundant relative to the very small number of necrotic fibers present. Regenerative phenomena in the form of interstitial proliferation of cells presumed to be myoblasts were relatively scanty when compared to the mouse. Restoration of damaged fibers by amitotic nuclear division was also scanty, and neither type of regeneration made much progress toward completion. There seemed to be a tendency for less severe and generalized lesions to show some selective localization in the deep flexor muscles of the neck and the distal ends of the thighs. The selective sparing of the intrinsic muscles of the tongue, so noticeable in the suckling mouse (10), was not demonstrable because of the rudimentary development of the tongue in these very immature birds.

The necrotic lesions of the epidermis and feathers, so severe and extensive in the most immature series of embryos inoculated, constitute the one striking deviation from the standard pattern of lesions associated with infection with group A Coxsackie virus in suckling mice. Such epidermal lesions may be the result of one of the following mechanisms. Either they may be caused by the direct action of the virus on the epidermis and adnexa, or may be consequent on soaking and maceration in an amniotic fluid presumably containing toxic breakdown products of muscle necrosis excreted through the kidneys. The available evidence is not conclusive, but generally favors a direct virus causation for the skin lesions. The necrosis of epidermis and feathers is first noted about 72 hours after inoculation, simultaneously with or only slightly after the first appearance of definite muscle necrosis, and roughly parallels the muscle
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lesions in rate of development and severity. Like the muscle lesions, the extent of necrosis of epidermis and feathers is markedly less in embryos inoculated at the 12th day of incubation; but, in contrast to lesions in the muscle, visible damage to the epidermis and feathers is entirely lacking in embryos inoculated on the 14th, 16th, and 18th day of incubation. This parallelism between muscle and epidermal lesions favors a direct virus effect, because if the epidermal lesions were secondary to injurious breakdown products excreted into the amniotic fluid, a delay might be expected in the appearance of epidermal necrosis until a damaging quantity of these products had accumulated in the fluid surrounding the embryo. Also favoring a direct virus causation is the persistence of degenerative changes and inflammatory reaction in the buried roots of the feather follicles, apparently not in direct contact with the amniotic fluid. A further observation also tends to support direct virus causation of the epidermal lesions. This is that the epithelium of the conjunctiva and cornea is invariably intact in spite of most severe damage to the epidermis and feathers. As the eyes open early and the cornea is directly bathed in the amniotic fluid it is difficult to see why the corneal epithelium should not show the effect of an injurious substance contained in the fluid. The actual findings seem analogous to those in smallpox, in which even the most severe skin lesions are only rarely accompanied by damage to the exposed surface of the eyes.

The other visceral lesions, the most frequent of which was acute coagulation necrosis of areas of the liver, are for the present considered to be unrelated to the action of the Coxsackie virus, because they were few in number and of quite random distribution, without relation either to the duration of infection or the age of the embryo. It is worthy of note that, as already observed in suckling mice infected with group A Coxsackie virus, not a single embryo showed any lesions in the central nervous system, sensory and sympathetic ganglia, or peripheral nerves. Also no lesions were observed in the heart muscle.

SUMMARY

A series of embryonated eggs incubated from 6 to 18 days have been successfully infected by yolk sac inoculation with a strain of group A Coxsackie virus. The resulting pathologic changes are here described. The principal lesion consisted of widespread acute necrosis of striated muscle, apparently essentially identical in type and development with that characteristically produced by the virus in suckling mice. The infection was not itself lethal; some embryos with virtually complete destruction of skeletal muscle were found alive. The older the embryo at the time of inoculation, the less the severity and extent of the lesions; but susceptibility was sometimes not entirely lost in embryos 18 days old. Two embryos inoculated at this time hatched and lived for 4 and 11 days respectively. One showed minimal specific muscle
lesions, and the other only some irregularity in muscle fiber size. The lessening of severity of the lesions was especially marked and abrupt in embryos inoculated on the 10th and the 12th day of incubation.

In addition to the muscle lesions characteristic of infection with group A Coxsackie virus, there were regularly present in the more immature embryos extensive necrosis of epidermis and feathers and degeneration of feather follicles, believed to be probably a direct dermotropic effect of the virus infection. Other visceral lesions, chiefly liver necrosis, were occasionally observed but seemed not to have been due to direct virus action.

As in suckling mice infected with group A Coxsackie virus, no lesions were observed in the central or peripheral nervous system nor in the heart muscle. No lesions deemed specific were seen in the few specimens of chorioallantoic membranes examined.

BIBLIOGRAPHY

7. Ransom, S. E., and Huebner, R. J., unpublished data.
EXPLANATION OF PLATES

PLATE 1

Fig. 1. Oxyphilic coagulation necrosis of muscle. Chick inoculated on 10th day of incubation and killed 4 days later. Azure-eosin. × 400.

Fig. 2. Acute hydropic degeneration and edema of muscle. Chick inoculated on 6th day of incubation and killed 3 days later. Hematoxylin-eosin. × 400.

Fig. 3. Lower thigh showing complete loss of muscle, leaving loose mesenchyme and apparently intact sciatic nerve. Three stunted feather follicles are present along the upper edge. Chick inoculated on 6th day of incubation and killed 5½ days later. Azure-eosin. × 61.

Fig. 4. Muscle regeneration in form of multinucleated strap cells. Across the center are two sarcolemmic sheaths containing pyknotic nuclei and mucoid fluid. Chick inoculated on 10th day of incubation and killed 6 days later. Hematoxylin-eosin. × 400.

Fig. 5. Muscle regeneration in form of clusters of cells resembling myoblasts. One in mitosis. Chick inoculated on 6th day of incubation and killed 9 days later. Hematoxylin-eosin. × 895.
(Peers et al. Yolk sac inoculation of group A Coxsackie virus)
PLATE 2

Fig. 6. Coalescence of degenerated feather follicles and superficial necrosis of epithelium. Chick inoculated on 6th day of incubation and killed 9 days later. Hematoxylin-eosin. $\times$ 60.

Fig. 7. Necrosis of feather epithelium and bending and collapse of shaft. Chick inoculated on 10th day of incubation and killed 7 days later. Hematoxylin-eosin. $\times$ 75.

Fig. 8. Granulomatous reaction about degenerated feather follicle in the subcutaneous tissue. Chick inoculated on 6th day of incubation and killed 11 days later. Hematoxylin-eosin. $\times$ 90.

Fig. 9. Uninfected control chick, 18 days incubation. Natural size.

Fig. 10. Infected 18 day chick inoculated on 6th day of incubation. Almost complete loss of muscle and feathers. Stunted skeleton visible through transparent body wall. Chick alive when removed from shell. Natural size.
(Peers et al. Yolk sac inoculation of group A Coxsackie virus)