VACCINAL INFECTION OF THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK EMBRYO *

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Notwithstanding a certain amount of confusion which has at times existed concerning the possibility of an identity of fowl-pox and variola-vaccinia, there are now several studies on record which show that although chickens are susceptible to infection with vaccine, fowl-pox is a distinct disease both immunologically and in the cytology of its lesions (Levaditi and Nicolau, Ledingham, Lowenthal *et als.*, Andervont, Woodruff). Andervont [1] was able to demonstrate Guarnieri bodies in vaccinal lesions of chickens, and this was confirmed by Woodruff,[2] who pointed out essential distinctions between these inclusions and the Bollinger bodies of fowl-pox.

In a recent communication we reported the successful infection of the chorio-allantoic membrane of chick embryos with vaccinia virus, following the method of inoculation described by Woodruff and Goodpasture [3] in their study of fowl-pox of this membrane.[4] In the present report we wish to describe in greater detail vaccinal infection of the membranes of the chick embryo, with especial reference to the cytology of the lesion and the seeming relationship between the Guarnieri bodies and the Paschen corpuscles which constitute the specific elements.

While the Guarnieri bodies are by every investigator of the subject regarded as specific for variola-vaccinia, there is perhaps still skepticism as to the specificity of the Paschen granules and no one as yet has been able to demonstrate a relationship between these two important structures, although many agree with the view of Paschen that the elementary corpuscles represent the actual virus of the disease.[5]

Vaccinal lesions in the chorio-allantoic membrane of embryo chicks afford an unusual opportunity to study both of these morphological elements and, we believe, furnish good evidence that the

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Guarnieri bodies represent, in part at least, colonies or masses of Paschen corpuscles.

Technique

Bacteria-free virus in fresh infected rabbit's testis (Levaditi neurovaccine) was used to initiate the infection. While we have been able to infect the chick membrane with glycerinated virus, the result is more uncertain, and we recommend the use of fresh infected tissue from the rabbit's testis as a constant source of original virus.

Since the infection "takes" rapidly and the lesion evolves readily, requiring only two or three days for its development, we have found it most satisfactory to use chick embryos of 12 days' incubation. At this stage the membrane is well formed and easily accessible. By candling the egg the air-cell and the membrane can be outlined with a wax pencil. A thin coat of melted paraffine is laid over the shell where the window is to be cut. The egg is then placed in a bowl of warm water (40° C) and rested upon a mass of plasticene which has been properly indented to receive it. This holds the egg in the suitable position. The water should come almost up to but not over the paraffined surface. With a hard steel trocar, ground with a triangular end to a sharp point, a window 1 or 1½ cm. square is cut in the paraffined surface. If the sides have been well cut the overlying shell can be easily removed by lifting it at one corner with fine pointed forceps. The exposed shell membrane may be unruptured, though frequently it is more or less injured. A thin coat of melted paraffine (about 45° C) is laid over the cut edges of the shell and the exposed shell membrane with a cotton swab. With the point of a pair of fine curved forceps the shell membrane may be torn from beneath outward on three sides, folded outward and the fourth side cut with small scissors. These procedures tend to prevent infection from broken bits of shell. The instruments used are kept sterile by passing them through the flame of a Bunsen burner.

When the membrane is exposed a bit of infected tissue (rabbit testis or membrane) about the size of a pin-head is placed upon it. A mixture of sterile vaseline and paraffine in a 10 cc. record syringe is used to lay a ring about the opening and upon this a sterile cover glass is placed and pushed down to seal it completely. The egg is then returned to the incubator with the window up. It may be ex-

amined on succeeding days by placing the window under a dissecting microscope.

When it is desired to open the egg to examine and remove the infected area, the cover slip is pulled off and frequently the vaseline comes off with it. If not, it can easily be scraped off with a sterile scalpel. The window is enlarged to the desired size by breaking off the edges with sterile forceps. The infected membrane may then be cut out with small scissors and placed in a petri dish containing sterile isotonic fluid.

The Vaccinal Lesions of the Membrane

After 24 hours the membrane appears somewhat thicker, grayer and more opaque about the bit of inoculum, and after 48 hours there is a zone about 1 cm. wide, thickened, gray, opaque and usually flecked with small hemorrhages. Sometimes almost the entire area is red and hemorrhagic. At this stage there is a gray advancing margin which is best for histological study. In the center there may be a brownish area of necrosis, variable in size. After the lesion has been extirpated a bit of tissue from the thickened and hemorrhagic area is removed with scissors and smears from this are stained by Morosow's method [6] to determine the presence of Paschen bodies. These bodies appear in enormous numbers in the infected membranes at the 48 hour period and after. Their presence is diagnostic of vaccinal infection. Other smears are stained with Loeffler's methylene blue to determine the presence of bacteria. If Paschen corpuscles are abundant and no bacteria are demonstrable, a piece of infected membrane about 0.5 mm. in diameter is inoculated upon the membrane of each of five or six embryos for continuing the passage. Pieces of the remaining infected membrane are then inoculated into culture media and others are kept in the icebox or in glycerol (50 per cent in 0.9 per cent saline). Tissue for histological study is taken from the advancing margin and fixed immediately.

The preparations used in the present histological study were fixed in Zenker's solution (10 per cent glacial acetic acid), and stained in a 2 per cent aqueous solution of acid fuchsin for 10 to 30 minutes, washed and counterstained about 30 seconds with Loeffler's methylene blue, differentiated in absolute alcohol, cleared in xylol and mounted in cedar oil.

Infected chick embryos usually die on the fourth day after inoculation, and we have found the 48 hour period best for transplantation and general study, though 72 hours is often a satisfactory interval.

Serial Transfers

In one series of experiments the virus was carried through eight generations in embryos, in another series it reached the fourteenth generation. For some reason, probably technical, the fifteenth generation failed to "take." In these passages there did not appear to be any diminution in virulence, so far as the appearances of the lesions were concerned.

The virus in the eighth generation was inoculated upon the skin of baby chicks after plucking the down. Macroscopic nodules, about 1 mm. in diameter, corresponding to down follicles, appeared at the site of inoculation within three days. Remaining apparently stationary for two or three days they rapidly receded. Two weeks after their recovery from vaccinia these chicks were inoculated with fowl-pox virus, and infection ensued which ran a typical course. This experiment confirms the result of other observers, that vaccinia in the chick confers no immunity to fowl-pox.

Histology

The infected membrane may be considerably thickened, though not so much so as in fowl-pox. The swelling is due to a variety of causes, least of all to cellular hyperplasia, which is the chief response to fowl-pox.

In the vaccinal infection inflammatory changes and hemorrhage are mainly responsible for the increase in thickness, and this is especially marked in the older areas of involvement. At the extreme advancing edge one finds the latest effects which are characterized by moderate edema, perhaps some capillary hemorrhage and slight hyperplasia, both of ectodermal epithelium and endothelial cells. The entodermal epithelium is slightly, if at all, affected. As the earlier infected areas are approached, ectodermal epithelium is found to be necrotic and its capillaries filled with cellular débris. Inflammatory exudate in the mesodermal layer is increased in abundance and consists largely, in addition to red blood cells, of polymorphonuclear leucocytes. There are also admixed with

these, large rounded mononuclear cells which may be either disoriented endothelium or fibroblasts. Occasionally mitotic figures are found in the large cells. The entodermal epithelium in these areas may show considerable hyperplasia, but usually no necrosis. In the earliest areas of infection these changes are accentuated, and there may be inflammatory cells in the entodermal layer and necrosis. At the advancing margin, capillary endothelium sometimes undergoes active focal hyperplasia, indicated by small isolated groups and whorls of these cells in the form of tiny nodules.

From a histological standpoint the virus seems to affect ectodermal epithelium first and most profoundly, mesodermal cells less markedly and entodermal epithelium least of all. No vesicles are formed. The infection seems to spread diffusely and centrifugally, affecting the entire membrane as it goes. There is no evidence thus far that lesions occur in the embryonic tissues other than at and about the site of inoculation in the chorio-allantoic membrane. We have not yet made a study of the dissemination of the virus in the embryo.

Guarnieri Bodies

In sections stained to demonstrate Guarnieri bodies, very marked changes are to be found in practically all types of cells of the fixed tissue. In order to study these changes to the best advantage one must examine the latest stages in the advancing edge of the infection in the membrane. Injury is profound and the cells rapidly undergo disintegration. It seems probable that there is only a short interval in which the cellular inclusions may be seen to best advantage. The Guarnieri bodies develop in abundance in the cells before there is any evidence of inflammatory cellular exudate.

Guarnieri bodies are best observed in the ectodermal cells. Practically all of these cells in the recently infected areas, when intact, show the "included" material in their cytoplasm. Here the bodies occur in irregular clumps and masses, perinuclear or paranuclear in arrangement. The cytoplasmic masses are granular and rather amorphous. The material stains for the most part with fuchsin in our preparations, though sometimes it is flecked with bluish granules. The granular material is fairly dense when properly differentiated and lies in a clear area. The inclusions, when single, are often triangular in shape with the base next to the nucleus. The Guarnieri

material is very abundant and sometimes almost completely replaces the cellular cytoplasm. In other cells it is more dispersed and is scattered through the cytoplasm in fine and coarse granules. Similar intracytoplasmic masses, often reaching a relatively large size, are found abundantly in endothelial cells and fibroblasts of the mesodermal layer. These masses have the morphology and the staining characteristics of Guarnieri bodies. They are especially well marked in the cells composing the endothelial nodules. Isolated fibroblasts show them quite distinctly. They are to be found typically also in endothelial cells lining veins and capillaries. Adventitial cells, as well as endothelial cells, composing the walls of larger blood vessels, often show these characteristic Guarnieri bodies, and one sometimes gets the impression that they are present in smooth muscle cells as well, but this is still doubtful.

The entodermal cells lining the infected area frequently contain numerous Guarnieri bodies, and here they have more the typical structure of these inclusions as they are usually to be seen in the corneal epithelium of the infected rabbit's eye. That is to say, they are apt to be small, single, discrete, more compact and more densely stained. They lie in a clear space next to the nucleus. In areas of hyperplasia of entodermal cells, however, they may become larger and more granular, simulating those of the ectodermal and mesodermal cells.

One gets the impression that the entodermal cells offer considerably more resistance to the infection than the cells of the other two germinal layers, and this may account for the variation in the morphology of the Guarnieri bodies.

It is to be emphasized that the chief, if not all "included," material within any of these cells is that which composes the Guarnieri bodies. This is of great importance in view of the appearance found in smears from the membranes stained by Morosow's method to demonstrate Paschen corpuscles.

Paschen Corpuscles

If fragments of fresh, infected membrane be placed in distilled water and examined under the oil immersion lens, one frequently sees round or oval masses, apparently within the cytoplasm of cells, which are composed almost entirely of minute, uniform granules oscillating rapidly in Brownian motion. These granules have the

size, uniformity of structure and numbers which correspond to the Paschen bodies so abundantly demonstrable in smear preparations. The masses are not so numerous, however, as Guarnieri bodies are known to be in the same tissue, and we have not been able positively to correlate the two structures in fresh preparations.

Smears made directly from a piece of fresh, untreated membrane and stained by the Morosow method show enormous numbers of Paschen corpuscles diffusely scattered, and also in distinct masses.[6] It appears from these smears that the Paschen bodies occur originally in rather sticky groups which, in the process of making the smear, adhere to each other, forming relatively large clumps of material resembling closely similar masses occurring in smears from fowl-pox. About the edges of these clumps are the discrete Paschen bodies which give the clue to the composition of the whole.

In the smears made directly in this way there are a very few intact cells, so that it is difficult usually to detect any relationship between the Paschen corpuscles and cells. However, if a piece of membrane is placed in 1 per cent trypsin solution for 30 minutes, it becomes softened and smears made with it show many intact cells that evidently have become dislodged from the loosened stroma. Many of these cells show in their cytoplasm one or more masses corresponding in size to the Guarnieri bodies, and in those cells which are spread out or partially ruptured by smearing, it can be seen readily that the intracellular structures are composed of compact agglomerations of uniform, round, minute corpuscles, measuring approximately 0.25 microns in diameter, although enlarged by the staining method. These are the Paschen corpuscles, and from a morphological standpoint we have no doubt that the intracellular masses of them are identical with the Guarnieri bodies found so abundantly and so large in the stained sections. No other observed intracellular structures could account for them.

Another experiment indicates that all the dispersed Paschen corpuscles originate from ruptured cells. If one places a small piece of membrane in 1 per cent acetic acid for 5 minutes, then washes it and makes a smear, there are few if any dispersed Paschen corpuscles, but hyaline masses, for the most part compact, are present within and about the cells; and some of these are to be found smeared out thin enough to see that they are composed of the minute Paschen bodies. The acetic acid evidently fixes the cells, at least partially,

so that in smearing they do not rupture to spread their content of corpuscles. The action of trypsin has the opposite effect, in that it tends to disassociate cells and to rupture them; consequently dispersed Paschen corpuscles are extremely abundant in the smears from tissue treated with it.

Discussion

It has recently been shown that the specific cellular inclusion (Bollinger body) of fowl-pox is composed in large part of uniform granules which correspond in their morphology, numbers and staining reaction with the Borrell bodies previously demonstrated in smear preparations. Furthermore, these specific inclusions have been isolated, washed and inoculated, both whole and in fractions, into chickens, with resulting successful infections.[7] Thus it has been shown that the cellular inclusion of fowl-pox carries the infectious agent; and the evidence is very strong that this agent is morphologically represented by the Borrell granules.

In molluscum contagiosum the specific inclusions have also been shown to be composed of minute granules (Lipschütz granules) which correspond in size, numbers and staining reaction with the Borrell granules of fowl-pox. Owing to the sticky consistency of these intracellular masses it has not been possible to isolate them and prove their infectiveness, although in all other respects, including resistance to tryptic digestion, the Lipschütz granules resemble the Borrell corpuscles.[8]

In these two viral infections, characterized clinically by a pox, it has been determined that the specific cellular inclusions are composed in large part of uniform corpuscles which are small enough to be filterable and numerous enough to account for the infectiousness of great dilutions of original material.

Variola-vaccinia is also characterized by a pox, and there are specific cellular inclusions (Guarnieri bodies) in the lesions. Furthermore, Paschen and others have demonstrated the great constancy of minute corpuscles (Paschen corpuscles) in smears from early lesions. These granules have the same size and staining qualities as those of Borrell and Lipschütz, and they have been shown to be agglutinable by vaccinal immune serum (Paschen, Ledingham[9]). Up to the present time, however, it has not been possible to determine any relation between the Guarnieri bodies and the Paschen

corpuscles, although Ewing [10] was able to demonstrate in Klatsch preparations a granular composition of some of these structures in cells from the rabbit's cornea. By analogy with fowl-pox and molluscum contagiosum one would suspect that the former might be in part composed of the latter. Our experiments seem to show that this is the case.

Observations which support this conclusion depend upon the fact that vaccinal infection of the chorio-allantoic membrane of chick embryos results in the appearance of unusually abundant and large Guarnieri bodies, and in extremely numerous Paschen bodies. In smear preparations from these lesions it has been possible to demonstrate structures which are interpreted to be Guarnieri bodies partially disintegrated within the cells, so that their component granules can be seen readily.

Although Paschen has frequently observed the corpuscles of vaccinia inside cells in smear preparations, he has not been able to relate them definitely to the Guarnieri body. He interprets them, however, to be the infectious agent and judges that they multiply in part, at least, inside the cells.

There is thus strong evidence, not only that the viruses of vaccinia, fowl-pox and molluscum contagiosum are cytotropic in the usual sense of having an especial affinity for cells, but that they are *cytotrophic*, if we may use this term to mean that they require under natural conditions an intracellular environment for their growth.

There is an interesting variation in the cellular affinity of the three viruses under consideration, brought out in the case of two of them by infection in the chorio-allantoic membrane of the chick embryo. Molluscum contagiosum has not yet been successfully engrafted upon a host other than man, but so far as one knows, it affects only and specifically ectodermal epithelial cells in this natural host.

Fowl-pox, readily inoculable upon the chick membrane, affects both ectodermal and entodermal epithelium, although the latter is changed relatively slightly. Vaccinia, on the other hand, affects cells of the three germinal layers, ectoderm, mesoderm and entoderm. In our studies it has been shown for the first time that Guarnieri bodies may occur not only in ectodermal and entodermal epithelium,[11] but also in endothelial cells and fibroblasts. There is thus an increasing latitude of cellular affinity as we pass from mol-

luscum contagiosum, restricted in its growth to ectodermal epithelium, through fowl-pox, which will grow apparently only in ectodermal and entodermal epithelium, to vaccinia, which finds its growth requirements satisfied in cells of all three germinal layers.

This fact no doubt has a bearing upon the cultivability of these viruses in tissue culture. Molluscum contagiosum would probably require a culture of ectodermal epithelium to initiate its growth. We have not succeeded in cultivating fowl-pox in cultures of mesodermal cells of the chick, and the cytology of its lesions suggests that it needs either a culture of ectodermal or of entodermal epithelium. Many investigators, however, have been able to cultivate vaccinia virus through several generations in a medium containing mesodermal cells of the chick, and our investigations confirm, from a cytological standpoint, the possibility of a generation of vaccine in either endothelium or fibroblasts.[12]

The method of infecting the chorio-allantoic membrane of chick embryos with vaccine offers an opportunity for further studies upon the Paschen corpuscles which appear in the lesions in great numbers, and it also provides a means of generating large quantities of sterile vaccinal virus.

Summary

1. Vaccinal lesions of the chorio-allantoic membrane of chick embryos are described.

2. Guarnieri bodies have been demonstrated for the first time in mesodermal cells (endothelium and fibroblasts).

3. Evidence has been presented that the Guarnieri bodies are composed in part of Paschen corpuscles.

4. Similarities between Borrell corpuscles (fowl-pox), Lipschütz corpuscles (molluscum contagiosum) and Paschen corpuscles (vaccinia) are pointed out.

5. It is suggested, on the basis of the cytology of the lesions, that the virus of molluscum contagiosum would require a culture of ectodermal epithelium to initiate its growth outside the body; fowl-pox would require either ectodermal or entodermal epithelium; while vaccinia would multiply in a culture of cells from any of the three germinal layers.

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DESCRIPTION OF PLATES

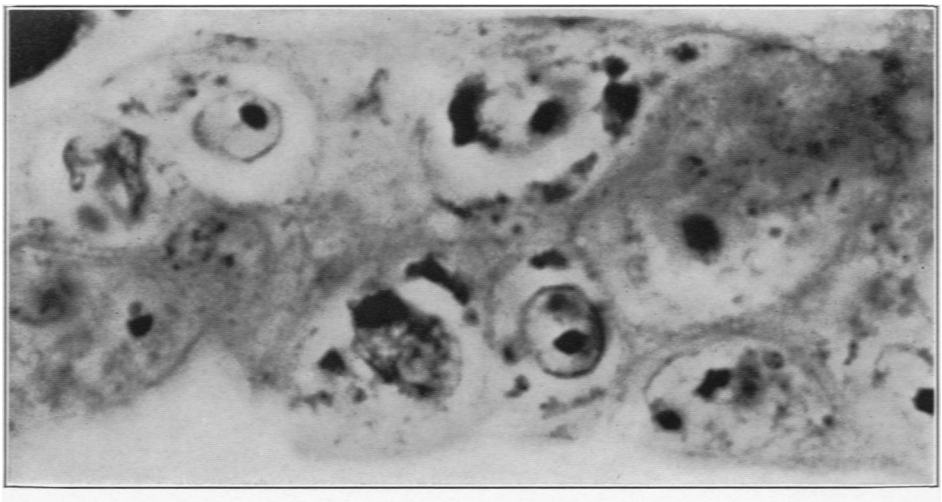
All photomicrographs taken at a magnification of 1800 diameters.

PLATE 42

FIG. 1. Epithelial cells of ectoderm showing large irregular Guarnieri bodies.

FIG. 2. Epithelial cells of entoderm showing more typical Guarnieri bodies.

FIG. 3. Epithelial cells of ectoderm showing irregular Guarnieri bodies diffusely distributed through the cytoplasm.



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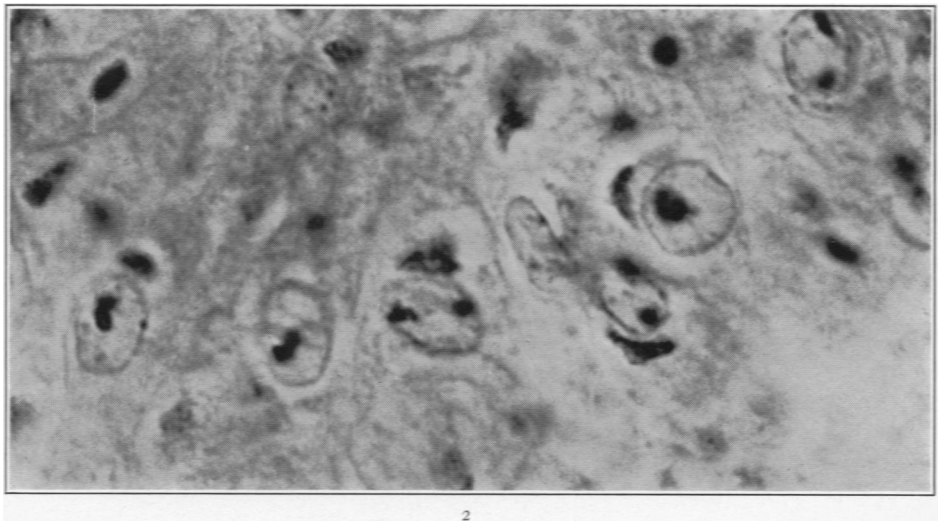

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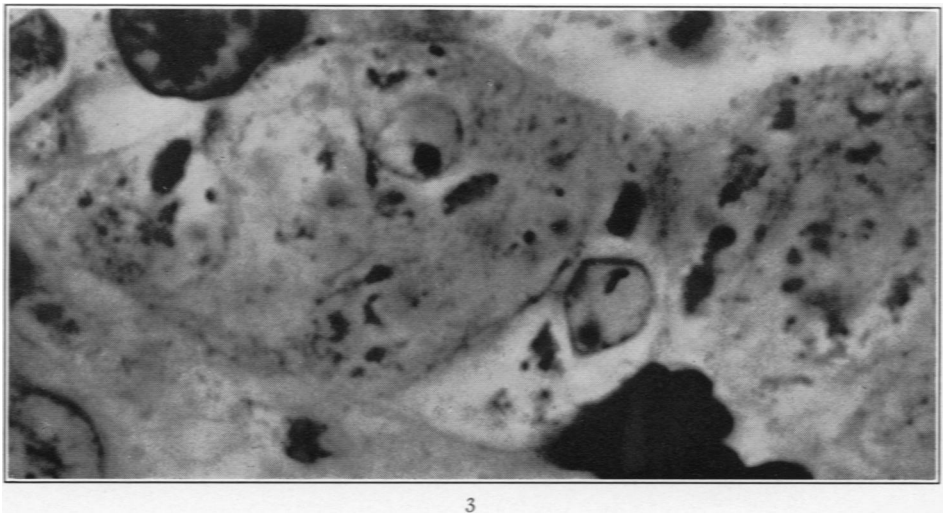

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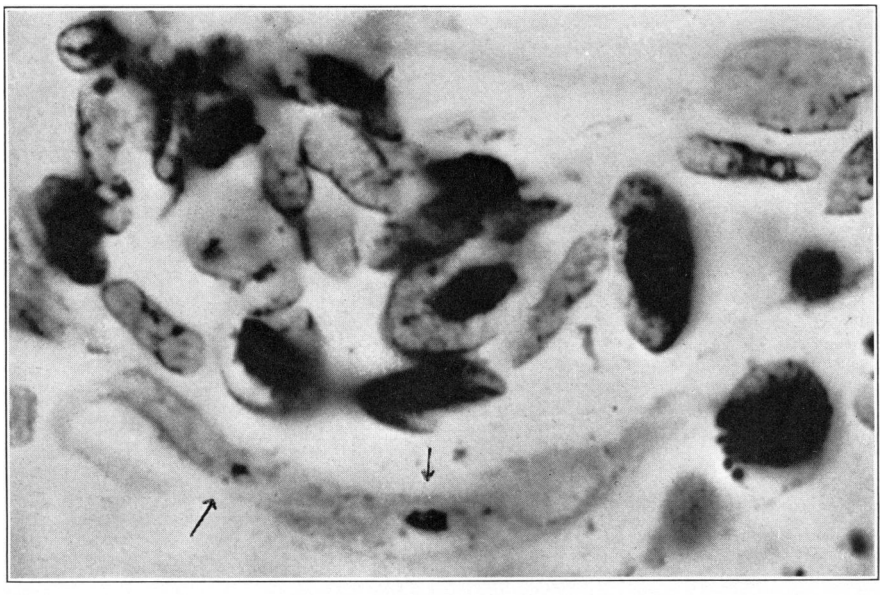
PLATE 43

FIG. 4. Vein showing two Guarnieri bodies within lining endothelial cells.

FIGS. 5 and 6. Guarnieri bodies in capillary endothelium.

FIGS. 7 and 8. Guarnieri bodies in fibroblasts.

FIG. 9. Endothelial "nodule" showing Guarnieri bodies.



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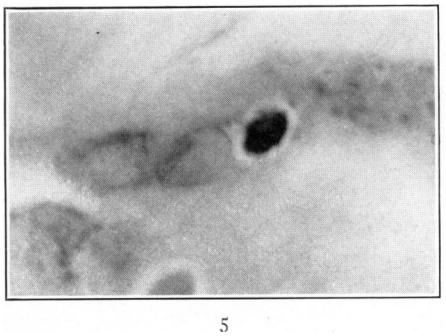

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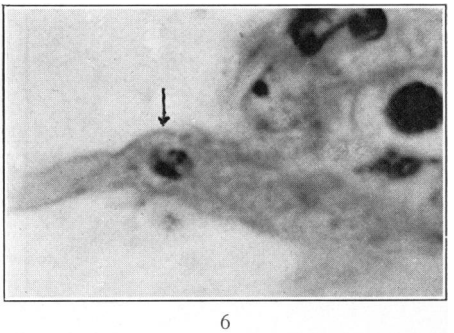

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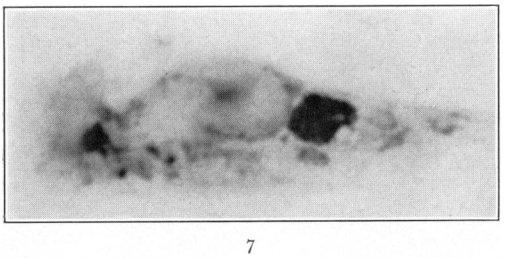

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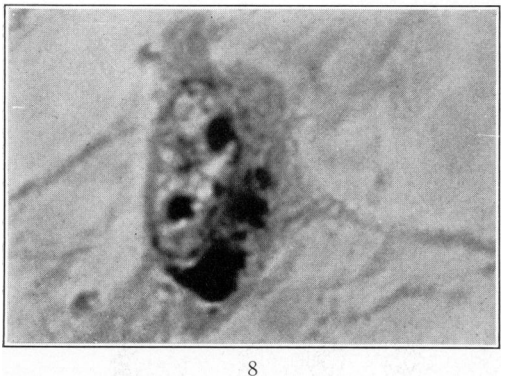

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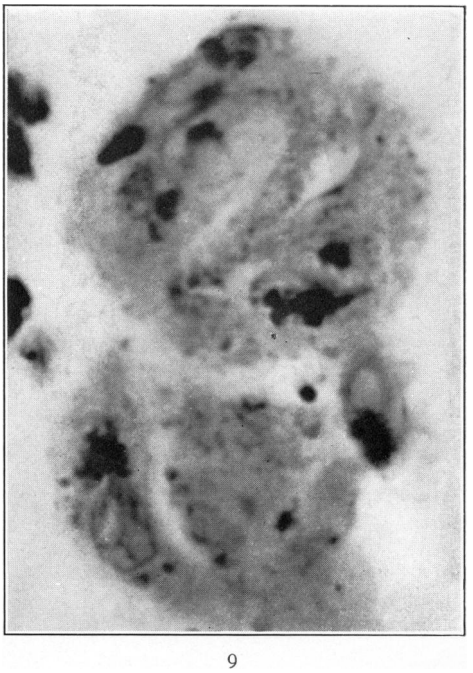

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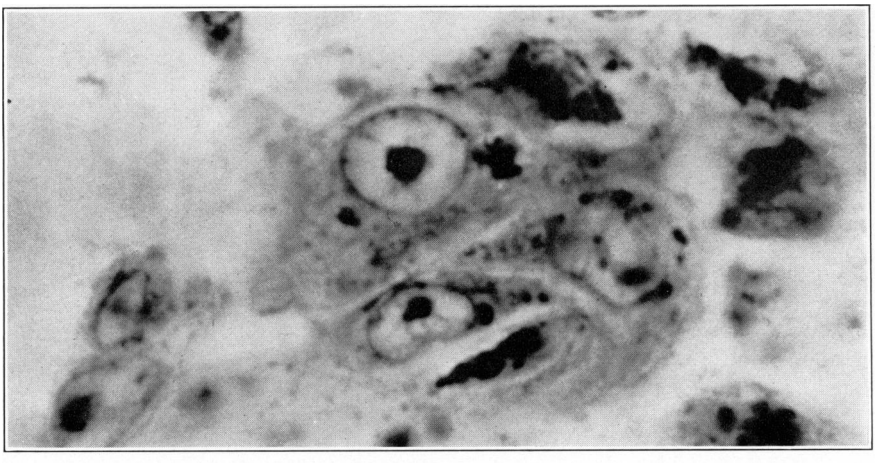
PLATE 44

FIG. 10. Endothelial "nodule" showing Guarnieri bodies.

FIGS. 11 and 12. Cells in smear preparations showing unresolved intracytoplasmic masses, which in thinner portions are seen to be composed of Paschen corpuscles.



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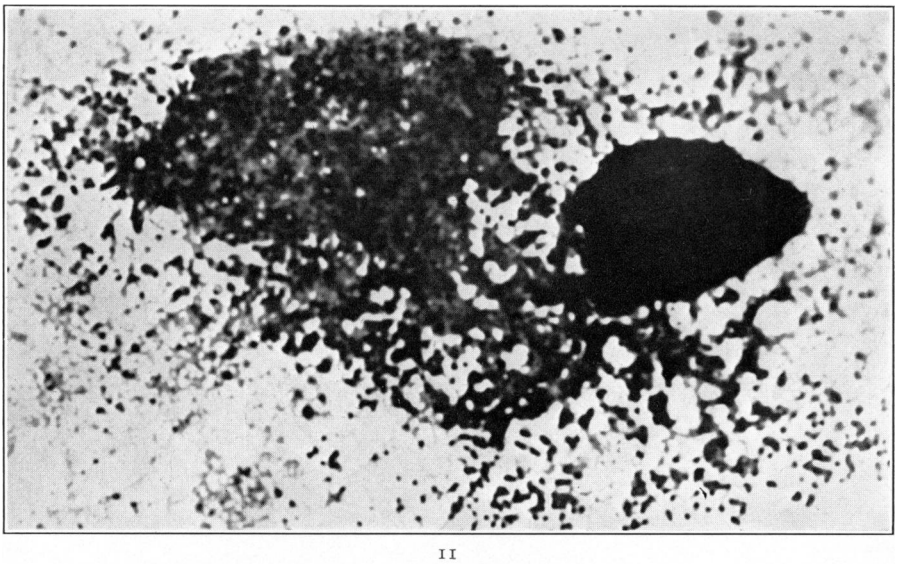

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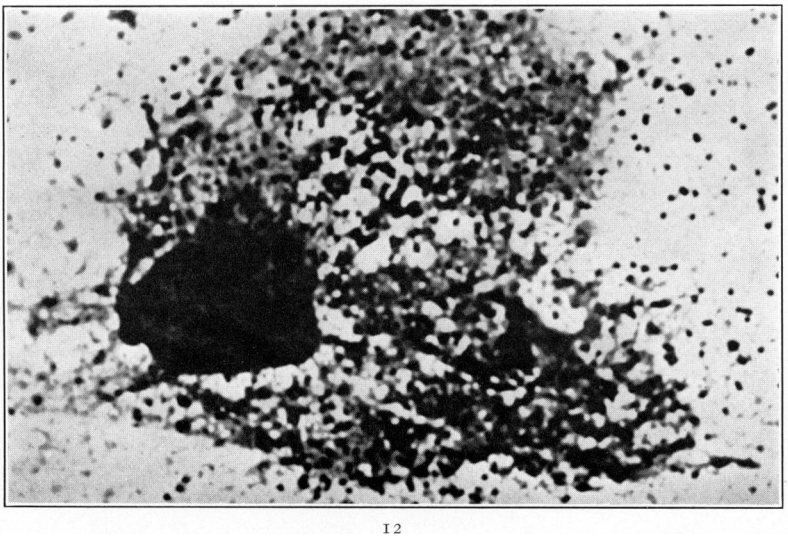

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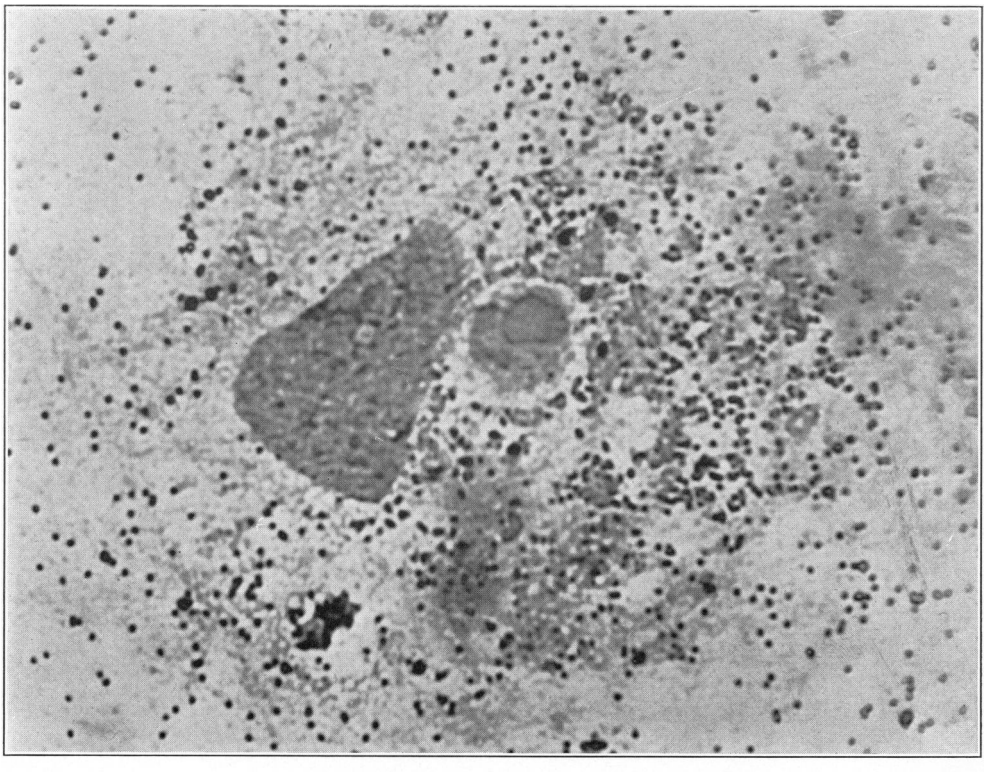
Plate 45

Figs. 13 and 14. Cells from a smear preparation showing unresolved and resolved intracellular groups and masses of Paschen corpuscles. K_2 filter.



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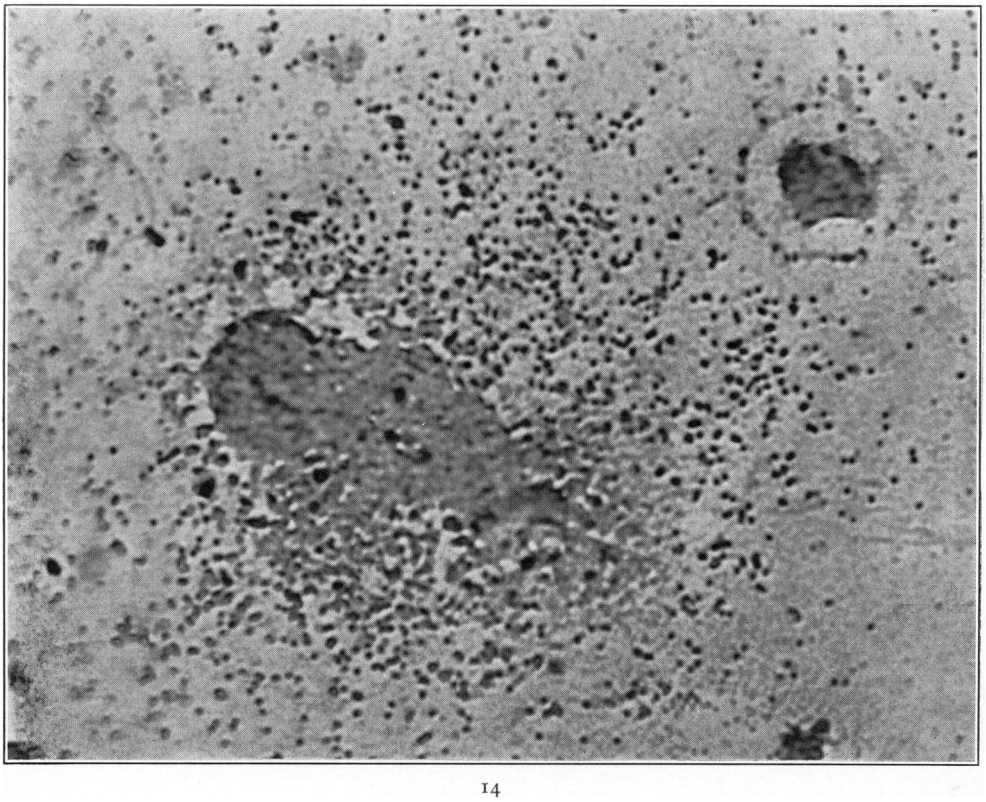

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