NUCLEAR INCLUSIONS SUGGESTIVE OF VIRUS ACTION IN
THE SALIVARY GLANDS OF THE MONKEY,
CEBUS FATUELLUS *

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Evidence is fast accumulating that we must recognize a special group of salivary gland viruses. All of them have been discovered by chance. They are so benign that attention was not directed to them by distinctive clinical symptoms. What attracted notice was the extraordinary hypertrophy of certain acinous or duct cells accompanied by the formation in their nuclei of inclusions resembling those caused by viruses.

The first inclusion-laden cells were reported under the heading of "protozoan-like bodies" in the parotids of two infants by Ribbert ¹ and in the submaxillary glands of guinea pigs by Jackson.² Credit is due to Goodpasture and Talbot ³ for recognizing the close resemblance between the bodies in humans and guinea pigs and for pointing out the similarity of both to the intranuclear inclusions described by Tyzzer ⁴ in varicella. Lipschütz ⁵ then rediscovered the intranuclear inclusions in herpes, admirably described and illustrated by Kopytowski,⁶ and emphasized the great importance of these bodies in "inclusion diseases" in general. But it was Kuttner and Cole ⁷ and Kuttner ⁸ who led in the demonstration that the inclusions in guinea pigs are actually caused by a virus. This naturally excited so much interest that investigators, while examining the salivary glands of other animals, have been on the lookout for nuclear inclusions, with the result that at the present time they have been reported in rats,⁹ moles,^{10, 11} mice ¹² and hamsters.¹³ Finally Kuttner and Wang ¹³ have proved that the intranuclear inclusions in hamsters, mice and wild rats are caused by viruses that are very similar to the submaxillary gland virus of guinea pigs. We await with interest proof of virus action in the formation of the inclusions in humans and moles.

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OBSERVATIONS

As in the discovery of the intranuclear inclusions in the other forms mentioned, so also in *Cebus fatuellus* they were encountered almost by chance.¹⁴ A routine examination was being made of all the principal tissues of 2 *Cebus fatuellus* and 18 *Macacus rhesus* monkeys which received repeated doses of irradiated ergosterol. The inclusions were seen in the parotid and submaxillary glands of both of the *Cebus* monkeys but in none of the *Macacus*. Their appearance is illustrated in the plate.

All of the inclusion-laden cells were so much hypertrophied that they could easily be recognized without the use of an oil immersion objective. What usually caught the eye was a large, spherical, eosin-staining mass — the nuclear inclusion — with a clear halo between it and the nuclear membrane. The cytoplasm of the affected cells was distinctly basophilic, owing to the presence of cytoplasmic bodies, and consequently stood out sharply against the neighboring cells which were acidophilic, for the inclusions were limited to the secretory and intercalated ducts. None were found in the secretory acini. The altered cells were spherical in shape and bulged so far into the lumen that in some cases they seemed to occlude it.

Though easily seen, the inclusions were difficult to find by reason of their extreme rarity. A total of 33 was studied; of these 11 were seen in 102 sections of the parotid and 22 in 149 sections of the submaxillary gland. Each section was 7μ thick and about 5 mm. square. The largest number of inclusions ever seen in a single section was 4. After about a month spent in their study they could be found at the rate of 4 or 5 per hour. No inclusions were detected in the sublingual glands.

The size, and indeed all of the structural features, of the inclusions and of the cells containing them were remarkably uniform. The cells, nuclei and inclusions were roughly spherical. The diameters of 30 cells and nuclei and of 29 nuclear inclusions were measured in microns (Table I).

In the case of a binucleated cell the maximum diameter amounted to 25.2μ , but the size of the nuclei and their inclusions remained about the same. Traces of the second nucleus in the binucleated cell illustrated in Figure 6 are seen below and to the left.

All of the nuclear inclusions were strongly acidophilic when viewed

after fixation in formalin-Zenker and coloration with hematoxylin and eosin. They contained no hematoxylin-staining material. When cut through the middle they appeared to be optically homogeneous, but thin slices of their surface showed them to be made up of tiny, acidophilic adherent particles. The outlines of the inclusions were usually quite sharp. Only 1 inclusion was observed per nucleus.

The halo interposed between the inclusion and the nuclear membrane was altogether free of basophilic chromatin near the inclusion, but as one approached the membrane some particles of basophilic

TABLE I
Measurements of Cells, Nuclei and Nuclear Inclusions

Diameters	Cells	Nuclei	Inclusions
Maximum largest diameter.....	19.2	13.2	7.2
Minimum largest diameter.....	12.0	8.4	3.6
Average largest diameter.....	15.9	13.2	7.2

material could be made out — in other words, margination on the nuclear membrane was not usually complete (see Figs. 5 and 6). The width of the halo about the inclusion was approximately the same on all sides.

Nucleoli were identified in all instances where the section included a large amount of nuclear material. Invariably the nucleoli, one or two in each nucleus, left the central nuclear zone and became closely applied to the nuclear membrane. The uniformity in this migration of nucleoli, in their size (approximately 1.5 by 3 μ), degree of flattening and staining reaction was quite striking. Such a marginated nucleolus is illustrated to the left side of the nuclear inclusion in Figure 3 and above it in Figures 4 and 5. The coloration indicated an even mixture of acidophilic and basophilic material, the latter almost masking the former.

Cytoplasmic inclusions were observed in 21 out of 33 nuclear inclusion-laden cells and are represented in Figures 2 to 6. They were distinctly basophilic and most of them were particulate in consistence. The largest exhibited a maximum diameter of 6 μ and a minimum of 3.6 μ ; the smallest was a sphere about 1.5 μ in diameter and the average, likewise spherical, was approximately 2.5 μ in diameter.

These cytoplasmic inclusions occurred in the distal cytoplasm between the nucleus and the lumen of the duct. Most of them were not provided with clear halos. Some, which were of much denser consistence, did have halos.

The epithelial cells next to those in which inclusions had developed showed no particular sign of injury, revealed by the simple technique mentioned, except that they were mechanically pushed aside. It may be that they were very slightly increased in size, as Scott and Pruett¹⁵ found by careful direct measurements to be the case in the submaxillaries of guinea pigs. The intercalated ducts showed no evident alterations, but the secretory ducts, which contained many more cells and in which most of the inclusions were found, did look abnormal. The cells in which nuclear inclusions had not formed did not all present the same appearance. None showed evidence of hydropic degeneration, as illustrated by Pearson¹⁶ in his Figure 12. Some were nevertheless singled out from the rest by the intense acidophilia of their cytoplasm and the pyknosis of their nuclei. Both cytoplasm and nucleus were shrunken. Such cells were observed alone and in groups, but in number they were always less than 50 per cent of the total. The lumen of one secretory duct was crowded with them. If this degeneration and desquamation of duct cells were a normal process, one would expect multiplication of the remaining cells to make good the loss, but no mitoses were seen.

Since the salivary glands of other *Cebus* monkeys, which had not received irradiated ergosterol, were not available for comparison, the best that could be done was to shift to similar tissues of *Macacus rhesus*. All of 18 given the irradiated ergosterol in equal and sometimes greater amounts exhibited the same process of degeneration and desquamation but none of them to the same degree. It was also seen in 4 untreated *Macacus* monkeys. Although these degenerating cells never showed any tendency whatever to inclusion formation, the possibility remains that their presence in such numbers is an accentuation of a normal process of elimination caused by injury to the duct epithelium and related in some way to the formation of nuclear inclusions, despite lack of topographical association between the two. We do not, however, advance this as a suggestion.

Another change in the ducts which should be reported, but not used as evidence, was accumulation of fluid about the bases (proximal poles of the cells) so that they were often quite widely separated

from the usually contiguous acinous tissue. This, also, was less marked in the *Macacus* monkeys. Calcium concretions occurred in both *Cebus* and *Macacus*, but were more numerous and larger in the former. They were probably caused or intensified by the irradiated ergosterol, as will be described in a later paper by Cowdry, Scott and Möller.¹⁷ Proliferation of fibroblasts and slight leukocytic infiltration took place near them, but the nuclear inclusions were not unusually abundant in their vicinity. Lymphocytic infiltration was often seen in the *Cebus* monkeys and less frequently in the *Macacus*. Of the 33 inclusion-containing cells in the *Cebus*, 20 were located in areas of this sort of infiltration. On the other hand, their presence in such areas was the exception rather than the rule because they were so rare and the invading lymphocytes so numerous.

The following tissues, additional to the salivary glands, were examined in each of the 2 *Cebus* monkeys to ascertain how widespread the formation of nuclear inclusions might be in the organism as a whole:

Fundus, ileum, colon, duodenum, pancreas, spleen, liver, adrenal, kidney, urinary bladder, lymph node, skeletal muscle, lung, thymus, thyroid, parathyroid, trachea, esophagus, mucous membrane of cheek, sublingual glands, testis, prostate, jejunum, pituitary, skin of back, heart muscle and bone marrow.

Since no nuclear inclusions at all like those in the submaxillaries and parotids were seen, it is evident that the reaction was restricted to these glands. Rare acidophilic droplets in the nuclei of the acinous cells of the pancreas of 1 monkey and of the pars distalis cells of the pituitary of the other were disregarded. The animals were quite mature, for their long bones had become ossified and their testicles showed active spermatogenesis. They weighed 1510 gm. and 1650 gm., respectively. Each received 6 daily doses of 5 cc. irradiated ergosterol 10,000 x (Mead Johnson and Company). All except the first of these were given with 10 gm. of calcium gluconate in tomato juice. They were killed 3 days after the last dose, at which time the blood calcium and phosphorus of 1 was 9.7 and 9.9, and of the other 9.6 and 5.

Attempts to determine if these salivary gland inclusions are caused by a virus were not undertaken because the inclusions were not discovered until weeks after the animals had been killed. Having in mind the results of transmission experiments with other salivary gland viruses, the chances of success would be good only if the

ground up glands of adults possessing inclusions were injected into animals of the same species so young that they had not acquired a natural infection and were, therefore, susceptible.

The nuclear inclusions in these *Cebus* monkeys did, however, exhibit a close resemblance to those known to be caused by viruses in the salivary glands of the guinea pig, rat, mouse and hamster. Like inclusions in the same location of moles and humans, it can only be said that their presence suggests the possible action of a virus. The nuclear inclusions in moles were more basophilic, but we found that this basophilia was at least partly due to failure of the basophilic chromatin to lose its basophilic properties or to marginate on the nuclear membrane. Instead of doing so, it accumulated in the form of a thin layer about the inclusion. The coating could easily be seen in favorable specimens. No measurements have been made, but the intensity of acidophilia of the core of the nuclear inclusion inside this basophilic layer in moles may closely approach that of the entire inclusion in *Cebus*, to which no basophilic material is attached.

In shape the inclusions are more uniformly spherical than the type inclusions in the guinea pig. The latter are often elongated in cells that are flattened. Thus, we have not found in *Cebus* any departure from the spherical shape of inclusions or cells comparable with that illustrated by Pearson.

The basophilic cytoplasmic inclusions in *Cebus* exhibited a greater range in size than in either the guinea pig or the mole. Consequently they are not so frequently disposed in orderly rows between the nucleus and the lumen. After formalin-Zenker fixation and staining with hematoxylin and eosin, the vast majority of them appeared to be made up of tiny uniform particles which look a little like *Rickettsiae*. These masses of particles were ordinarily not provided with halos. Occasionally, however, an inclusion, much more dense and not visibly particulate, was seen among the others and was limited by a halo. Very rarely all of the cytoplasmic inclusions in a single cell showed this peculiar density plus halos. Clear vacuoles may occur in the inclusions as well as in the cytoplasmic ground substance, but they were rare. When the section was overstained with hematoxylin the cytoplasm was likewise colored blue and the outlines of the inclusions could only be distinguished with difficulty. Structural details were also obscured if the sections were too thick, that is

more than 7μ . The characteristic margination and partial flattening of the nucleolus on the nuclear membrane referred to in the *Cebus* inclusion-laden cells was not encountered so repeatedly in the mole in which the nucleoli often held their spherical outlines and remained in contact with the inclusions.

DISCUSSION

Evidently *Cebus fatuellus* is to be added to the other species mentioned as exhibiting nuclear inclusions suggestive of the action of a salivary gland virus. The salivary gland viruses discovered in

TABLE II
Comparison of Salivary Gland Viruses with Pathogenic Viruses

Salivary gland viruses	Pathogenic viruses
No recognizable symptoms	Severe symptoms
Affect infants	Older individuals, infants often relatively immune
Marked cellular hypertrophy	Little or no hypertrophy
Larger inclusions	Smaller inclusions
Accompanied by basophilic cytoplasmic inclusions	Not so accompanied
Inclusion-laden cells persist for months	Are quickly removed
Active virus remains latent in tissue apparently as long as inclusions persist	Soon disappears or loses potency, for transmissions must be made promptly if they are to be successful
Virus has been transmitted only to same species	In many cases it has been transmitted to a wide range of species
Transmission only to individuals so young (generally under 1 month) that they have not become naturally infected	Adults equally satisfactory for transmission unless previously infected
Incidence in a group high, in some instances 100 per cent	Incidence comparatively rare

guinea pigs, rats, mice and hamsters, and suspected in humans, moles and *Cebus fatuellus*, are probably representatives of quite a large group, for it is likely that many others will be discovered. Even with the evidence now available it is possible to contrast these inapparent salivary gland viruses with pathogenic nuclear inclusion-producing viruses, like those of herpes and yellow fever (Table II).

The association between persistence of inclusions and presence of active virus may be stressed. Our reason for thinking that the inclusion-laden cells persist for months depends upon the age of formation, the age of disappearance and the likelihood of replacement of old inclusions by newly developed ones in the interval.

The age of formation is certainly early. Thus, Löwenstein¹⁸ found inclusions in the parotid of a 2 months old infant; Wagner¹⁹ in the sublingual of a 2 weeks old infant; Cole and Kuttner²⁰ in 3 out of 43 guinea pigs less than 1 month old; and Thompson¹² in 10 out of 70 rats, 2 months old.

Very little attention has been paid to the age of disappearance. In humans Farber and Wolbach²¹ found inclusions in 22 out of 183 infants of 17 months or less. This was a 12 per cent incidence, but 80 per cent were in individuals under 1 year of age. After 1 year they probably disappear quite rapidly, but accurate data on a large series are lacking. In "adult" guinea pigs they have been observed by many workers and in high percentages of 80 or more. It is difficult to estimate the age of adult guinea pigs ordinarily used in the laboratory but it can be taken conservatively as 1 year. Thompson failed to find inclusions in 12 rats 6 months old. Our Cebus monkeys were at least 3 years old but the age of inclusion formation is not known for them or for adult moles. The available information about adult hamsters and mice is not helpful.

With reference to replacement in humans, a statement by Farber and Wolbach is instructive: "One perplexing feature in our study of the inclusion bodies was failure to find small forms which could with confidence be interpreted as stages in formation. If the inclusions were present at all they were strikingly alike and within a narrow range of size and detail." The Rectors¹¹ in their report on moles also remarked upon the uniformity of the inclusions and the absence of young forms. The same feature was exhibited by Cebus.

It is clear from the experiments of Pearson in our laboratory that cells hypertrophied to the maximum degree and charged with nuclear and cytoplasmic inclusions are dead. The nuclear changes alone are so drastic as to appear incompatible with life. All the epithelial cells of the glands possess mitochondria in the usual number except the inclusion-laden ones from which the mitochondria have completely disappeared. Neither do they behave like living cells. When Pearson transplanted the glands of guinea pigs into the

peritoneal cavity, these cells with inclusions differed sharply from all the rest by not exhibiting autolytic changes; we think, because they were already dead.

Dead cells are as a rule promptly disposed of by the organism. It is surprising how these persist, maintaining their distinctive properties without being desquamated, falling a prey to phagocytes or being removed by autolytic enzymes. It is also interesting that we have come to rely upon such dead cells as marking the existence of active virus.

Is the virus bound to the surface of the dead cells or locked away in their cytoplasm or nucleus? If it is simply a case of adsorption on their plasma membranes the association must be a very intimate one amounting almost to chemical combination for, as pointed out by Scott and Pruett, the cells are so placed that their surfaces are being constantly washed by the passage of large volumes of water. In some species they are in acinous cells, in others in duct cells or in both. The duct cells are of the intercalated and secretory variety. It is by transfer through the walls that water is added to the secretory products. No instance is on record of the formation of inclusions by the cells of the collecting ducts, the walls of which are not regularly flushed by fluid tides in this way. Nature has indeed selected one of the worst places in the body for the attachment of virus to cells if this attachment is capable of being loosened by currents of fluid. On the hypothesis that the virus is held in the cytoplasm or in the nucleus, the immunity of the dead cells to the usual forces of disintegration would appear to favor its persistence over long periods.

SUMMARY

Intranuclear inclusions, closely resembling those caused by salivary gland viruses, were found in the parotid and submaxillary glands of 2 *Cebus fatuellus* which had received irradiated ergosterol but which showed no signs of disease. No inclusions were observed in the salivary glands of 18 *Macacus rhesus* similarly treated.

REFERENCES

1. Ribbert, H. Ueber protozoenartige Zellen in der Niere eines syphilitischen Neugeborenen und in der Parotis von Kindern. *Centralbl. f. allg. Pathol. u. path. Anat.*, 1904, **15**, 945-948.
2. Jackson, L. J. An intracellular protozoan parasite of the ducts of the salivary glands of the guinea-pig. *J. Infect. Dis.*, 1920, **26**, 347-350.
3. Goodpasture, E. W., and Talbot, F. B. Concerning the nature of "protozoan-like" cells in certain lesions of infancy. *Am. J. Dis. Child.*, 1921, **21**, 415-425.
4. Tyzzer, E. E. The histology of the skin lesions in varicella. *J. M. Research*, 1905-06, **14**, 361-392.
5. Lipschütz, B. Untersuchungen über die Ätiologie der Krankheiten der Herpesgruppe (Herpes zoster, Herpes genitalis, Herpes febrilis). *Arch. f. Dermat. u. Syph.*, 1921, **136**, 428-482.
6. Kopytowski, W. Zur pathologischen Anatomie des Herpes progenitalis. *Arch. f. Dermat. u. Syph.*, 1903, **68**, 55-80, 387-402.
7. Kuttner, A. G., and Cole, R. Further evidence concerning the significance of nuclear inclusions as indicators of a transmissible agent. *Proc. Soc. Exper. Biol. & Med.*, 1926, **23**, 537-539.
8. Kuttner, A. G. Further studies concerning the filtrable virus present in the submaxillary glands of guinea-pigs. *J. Exper. Med.*, 1927, **46**, 935-956.
9. Thompson, M. J. Intranuclear inclusions in the submaxillary gland of the rat. *J. Infect. Dis.*, 1932, **50**, 162-170.
10. Rector, L. E., and Rector, E. J. Intranuclear inclusions in salivary glands of moles. *Proc. Soc. Exper. Biol. & Med.*, 1933, **31**, 192.
11. Rector, E. J., and Rector, L. E. Intranuclear inclusions in the salivary glands of moles. *Am. J. Path.*, 1934, **10**, 629-636.
12. Thompson, J. Inclusion bodies in the salivary glands and liver of mice and rats. *Am. J. Path.*, 1934, **10**, 676-677.
13. Kuttner, A. G., and Wang, S.-H. The problem of the significance of the inclusion bodies found in the salivary glands of infants, and the occurrence of inclusion bodies in submaxillary glands of hamsters, white mice and wild rats. *J. Exper. Med.*, 1934, **60**, 773-791.
14. Cowdry, E. V., and Scott, Gordon H. Nuclear inclusions suggestive of virus action in the salivary glands of the monkey, *Cebus fatuellus*. *Proc. Soc. Exper. Biol. & Med.*, 1935, **32**, 709-711.
15. Scott, Gordon H., and Pruett, B. S. Studies on the submaxillary virus of guinea pigs. II. The nuclear-cell, nucleocytoplasmic and inclusion-nuclear indices of the affected cells. *Am. J. Path.*, 1930, **6**, 53-69.
16. Pearson, E. F. Cytoplasmic inclusions produced by the submaxillary virus. *Am. J. Path.*, 1930, **6**, 261-274.

17. Cowdry, E. V., Scott, Gordon H., and Möller, Max. Blood and tissue changes in monkeys after treatment with irradiated ergosterol. (In preparation.)
18. Löwenstein, C. Ueber protozoenartige Gebilde in den Organen von Kindern. *Centralbl. f. allg. Pathol. u. path. Anat.*, 1907, 18, 513-518.
19. Wagner, H. Zur Kenntnis der "protozoenartigen Zellen" in den Organen von Kindern. *Beitr. z. path. Anat. u. z. allg. Pathol.*, 1930, 85, 145-164.
20. Cole, R., and Kuttner, A. G. A filterable virus present in the submaxillary glands of guinea pigs. *J. Exper. Med.*, 1926, 44, 855-873.
21. Farber, S., and Wolbach, S. B. Intranuclear and cytoplasmic inclusions ("protozoan-like bodies") in the salivary glands and the organs of infants. *Am. J. Path.*, 1932, 8, 123-135.

DESCRIPTION OF PLATE

PLATE 89

FIGS. 1-6. Photomicrographs taken at a magnification of 1600 diameters of nuclear inclusion-laden cells in the salivary glands of *Cebus fatuellus*. Figs. 1 and 2, and 5 and 6 are of the parotid, and Figs. 3 and 4 of the sub-maxillary gland.