INCLUSION BODIES IN TRACHOMA*

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THE epithelial-cell inclusion, which is characteristically found in the early stages of trachoma was first described by Halberstaedter and Prowazek (1907). Since that time, a considerable literature concerning the nature of these bodies has accumulated. It is generally conceded that they consist of virus particles together with material elaborated by the virus and/or produced by the parasitized cell as a result of interference with its metabolism.

The concept of developmental phases in the life-history of the inclusion body was first described by Lindner (1910). At an early stage the inclusion body is composed of larger particles ($0.6-1.6\ \mu$) staining dark blue with the Giemsa mixture—the “initial bodies”—which are associated with a basophilic matrix—the so-called “plastin” material of Halberstaedter (1912). The initial bodies are considered to fragment in some way, thereby giving rise to the smaller particles called “elementary bodies” ($0.25\ \mu$), which stain in a manner similar to that of nuclei with the Giemsa mixture, *i.e.* magenta-red (“Romanowsky effect”). Lindner’s observations have in general been confirmed by those of Thygeson (1934a), and have received indirect support from the parallelism afforded in the maturation stages described for the inclusion body in inclusion conjunctivitis (Thygeson, 1934b), and also by the somewhat similar cyclical changes described for the inclusion bodies in psittacosis (Bedson and Bland, 1932; Bland and Canti, 1935), and lymphogranuloma inguinale (Findlay and others, 1938). Indeed it is largely because of this similarity in the pleomorphism of their inclusion bodies that the large viruses of these four diseases are grouped together taxonomically.

Rice (1936) and later Thygeson (1938) demonstrated the presence of a carbohydrate matrix in which the virus particles lie; using histochemical methods they identified this substance as glycogen.

Grossfeld (1950), using the Feulgen technique, found the elementary bodies to contain desoxyribose nucleic acid (this author considered the initial bodies and elementary bodies to be similar in nature, differing essentially only in size and in their degree of basophilia).

In this present study, histochemical methods were used to demonstrate both types of nucleic acids (desoxyribose nucleic acid (D.N.A.) and ribose nucleic

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acid (R.N.A.) and also carbohydrate in the inclusion bodies. The results of these investigations were obtained alongside the tinctorial properties of the inclusions revealed by using the classical Giemsa method, and the method of Poleff (1951, 1952) which has recently come into general use.

Material

Some 300 cases of early trachoma (Stages I-II according to the MacCallum classification) were studied in schoolchildren in the Marjoyoun district of Southern Lebanon. A flat thin celluloid strip 1cm. wide, with bevelled corners, was drawn across the unanaesthetized palpebral conjunctiva of the everted upper lid. The material so collected was smeared slightly on grease-free slides, which, after air-drying, were further fixed either in absolute methanol or by heat.

Technique

As will be described later, the inclusion bodies exhibit a variety of forms and staining properties; it was therefore necessary to submit a given inclusion body to a number of histochemical staining methods in sequence so that comprehensive information concerning that particular inclusion body could be obtained. The procedures were so arranged that later methods were not invalidated by previous ones; in addition, it was necessary to remove the colour(s) produced by one technique before proceeding to the next. Inclusion bodies were first tentatively identified by staining with Giemsa, and then each inclusion was treated in turn with some or all of the other techniques described below.

Giemsa Stain.—Smears were stained in a 1/40 solution of Giemsa for 1 hour. Subsequent decolourization was achieved by immersion in 70 per cent. alcohol for a few hours.

Poleff's Stain.—This stain, devised to emphasize the contrast between inclusion particles and the epithelial cells, is a citric acid-methylene blue mixture (pH about 2.7) which is applied to heat-fixed smears for 3 minutes. It can be removed from smears by treatment with acid/alcohol.

Nucleic Acids

Ribose nucleic acid (R.N.A.) was identified by Brachet's method. Briefly, a loss of basophilia (e.g. to methylene blue) after incubation in the enzyme is considered to be due to removal of R.N.A.

Desoxyribose nucleic acid (D.N.A.) was identified by the Feulgen method (the "direct Schiff" reaction was used as control). The colour produced by this method was removed by excessive hydrolysis for 5 to 10 min. in 1N.HCl.

Glycogen.—Three histochemical methods supplemented by salivary digestion tests were used for the identification of glycogen:

Iodine Reaction.—Rice's method was adhered to in some instances, but the following modification of Rice's original method was found preferable. Smears were stained with Lugol's solution for 1 minute and then thoroughly air-dried after removal of the excess solution with blotting paper. The smears were cleared in toluene and mounted in "permount" dissolved in toluene. This method has the following advantages:

- (a) facilitating oil-immersion observation and microphotography;
- (b) increasing the contrast between the glycogen and the background;
- (c) being permanent for at least 6 months so that slides can be examined at leisure.

The iodine stain is readily removed from rehydrated smears by washing in water.

Best's Carmine.—The stained smear can be freed of this dye by washing briefly in distilled water.

P.A.S.—This technique was used last in the series of tests because the colour produced cannot be easily removed. Some inclusions were submitted to salivary digestion for 30 min. to 1 hour; a negative P.A.S. reaction following this procedure was attributed to removal of glycogen.

An example of the somewhat complicated technical procedure is partly illustrated (Figs 1a, 1b, 1c). The inclusion illustrated was first identified in a smear stained with Giemsa; it was then ringed and drawn to facilitate subsequent identification. The slide was placed in 70 per cent. ethanol and the dyes dissolved out. The smear was now stained with Lugol's iodine and the result is shown in Fig. 1a. The rehydrated slide was washed in water and then subjected to Feulgen hydrolysis followed by Schiff's aldehyde reagent. Fig. 1b illustrates the result of this test. Further hydrolysis of the smear irreversibly destroys the Feulgen reaction and the slide was now subjected to the P.A.S. routine—Fig. 1c.

Observations

Conjunctival epithelial cells and polymorphs are the chief cells present in smears from trachomatous eyes, although lymphocytes, monocytes, and mast cells may also be seen. The Giemsa mixture stains chromatin a reddish-purple ("Romanowsky effect"), nucleoli deep blue, while the cytoplasm of epithelial cells is stained a paler shade of blue. Azurophil granules, the granules of mast cells and blood basophils are stained red to purple. In addition, macrophages containing basophilic debris (Leber's cells) are present in many of the smears.

The following description of the development of the inclusion body is necessarily presumptive (since division forms were not observed). The smallest—presumably the youngest—inclusions are visible as small aggregations of initial bodies (two to four in number) which are usually associated with an intensely basophilic matrix or ground substance presumably identical with the "plastin" material. This ground substance is homogenous and very sharply defined from the cell cytoplasm. It is upon this substance, which stains deep blue (Giemsa), that the shape of the inclusion at this stage depends; often irregular it may be rounded, crescentic, reniform, or moulded on to one pole of the nucleus like a cap. More than one such inclusion may be seen in the same epithelial cell (Fig. 9; up to four have been noted). The inclusions, while always intracytoplasmic, are frequently closely associated with the nucleus, which at this stage is apparently healthy.

Not infrequently the nuclear membrane of a healthy epithelial cell is ruptured in the smearing process; the intracytoplasmic herniation of chromatin thus caused gives only a superficial resemblance to the smaller inclusion bodies—it has no initial bodies, "plastin" is absent; moreover this phenomenon may be readily observed in smears from healthy eyes.

In the larger inclusions the initial bodies are replaced by the elementary bodies, the "plastin" becomes amorphous and finally so attenuated that only a few fine strands and islands remain. It is, at this later stage, an inconspicuous component of the inclusion body. The elementary bodies stain a reddish-purple tint (Giemsa) and are by now fairly evenly separated from each other. Between them there is glycogen*, which was demonstrated by the three techniques described above. It

*In this connexion it is worth noting that glycogen is not demonstrable by histochemical means in the epithelial cells of the normal palpebral conjunctiva.

FIG. 1(a).

FIG. 1(b).

FIG. 1(c).

FIG. 2(a).

FIG. 2(b).

FIG. 2(c).

FIG. 3.

FIG. 4(a).

FIG. (4b).

FIG. 5.

FIG. 6.

FIG. 7.

FIG. 8.

FIG. 9.

could be completely removed from the inclusions after salivary digestion at 37° C. for 30 min., whilst boiled saliva under these conditions was without such an effect. Glycogen was not detected in the majority of the earliest inclusions.

FIG. 1(a).—Trachoma inclusion stained with Lugol's iodine (by method described in text) showing hollow spheres of glycogen. $\times 950$.

FIG. 1(b).—Same inclusion as in 1(a)—dark central areas surrounded by clear zones. Feulgen reaction. $\times 950$.

FIG. 1(c).—Same inclusion as in 1(a) and 1(b). After excessive hydrolysis the Feulgen reaction has been destroyed and the slide has been subsequently submitted to P.A.S. routine. $\times 950$.

FIG. 2(a).—Rice's iodine technique: showing glycogen component of an inclusion body occupying upper portion of cell. $\times 550$.

FIG. 2(b).—Same inclusion as in 2(a): elementary particles almost completely fill cytoplasm. Giemsa. $\times 950$.

FIG. 2(c).—Same inclusion as in 2(a) and 2(b). Now stained in Best's carmine to show glycogen component of inclusion body. $\times 950$.

FIG. 3.—Rice's iodine method, showing glycogen component of a crescentic inclusion body which had previously been shown to contain initial bodies. $\times 550$.

FIG. 4(a).—Elementary bodies. Giemsa. $\times 950$.

FIG. 4(b).—Same cell as in 4(a): glycogen component of inclusion body. P.A.S. method. $\times 950$.

FIG. 5.—Ovoid inclusion, the particles of which are of the initial body variety, surrounded with glycogen, although this is not demonstrated here. Giemsa. $\times 950$.

FIG. 6.—Two inclusions in one cell. Although elementary bodies predominate, initial bodies are also present. Dark masses in right-hand portion of upper inclusion are residual islands of "plastin". Giemsa. $\times 950$.

FIG. 7.—Initial bodies lying in a bean-shaped plaque of "plastin". Giemsa. $\times 950$.

FIG. 8.—A large macrophage which has apparently phagocytosed a neutrophil and a degenerating epithelial cell, the pyknotic nucleus of which is surrounded by inclusion particles. Giemsa. $\times 950$.

FIG. 9.—An early stage: two inclusions are seen at opposite poles of the nucleus. The initial bodies cannot be seen clearly because of the densely stained "plastin" background. Giemsa. $\times 950$.

Although the above morphological description is true in general, it must be added that small inclusions composed of elementary bodies, large inclusions composed of initial bodies and numerous intermediate types are also seen from time to time.

Although both Thygeson and Rice considered the glycogen in their inclusions to be distributed as a matrix (and this appearance is certainly obtained when smears are "wet-mounted" in Lugol's iodine): the impression gained in this study was more often that of a capsular arrangement—each inclusion-particle being enveloped in a thin film of glycogen. This latter appearance was especially evident when some of the particles were found lying away from the main mass isolated in the cytoplasm or just outside the cell.

Occasionally it appeared as if the elementary bodies had "overflowed" into the general cytoplasm; in such inclusions the underlying dark contour (*e.g.* seen in Figs 1a, 2c, 4a) probably represents more exactly the original line of demarcation of the inclusion; such an appearance is possibly an artefact produced by the smearing process. Small cracks and fissures present in some of the larger inclusions are doubtless artefacts, probably produced by the methods of fixation.

The larger inclusions vary considerably in shape. They may be crescentic,

ovoid, or bonnet-shaped, as can be seen in the illustrations; they are usually sharply demarcated from the surrounding cytoplasm—all of which they finally occupy. In the cells occupied by the larger inclusions the nucleus is usually markedly pyknotic and often distorted in conformity with the shape of the inclusion (Fig. 5). Free initial bodies were not observed in these studies, although the presence of initial bodies in such large inclusions as shown in Fig. 5 suggests that they may be seen from time to time. Free elementary bodies were seen only occasionally and even then were not identified with certainty unless situated near a ruptured inclusion.

Both the initial and elementary bodies gave a strong Feulgen reaction—but the direct Schiff reaction was negative in both cases. It is therefore concluded that they both contain D.N.A.

The “plastin” substance was removable with ribonuclease and is therefore presumably R.N.A.

The surface cells of the normal palpebral conjunctiva possess a very fine carbohydrate-containing cuticle (P.A.S. positive): it is noteworthy that in none of the parasitized cells examined could this be demonstrated.

The inclusion particles noted in these studies were stained only very faintly with Poleff's method and then in the orthochromatic shade of methylene blue. However, this method did reveal cells which contained intense reddish-purple granules (*i.e.* exhibited the alcohol-resistant or “gamma” type of metachromasia). These cells had healthy nuclei; their chromatin pattern as revealed by the Feulgen technique was more coarsely trabeculated with “knots” in contrast to the finer punctate nuclear pattern of epithelial cells which also have one or more true nucleoli. The granules themselves were Feulgen negative and were unstained with the iodine method for glycogen, while Best's carmine stained them only faintly, and the P.A.S. reaction although positive for the granules was unaffected by previous salivary digestion. The granules were, however, well stained with mucicarmine. It was concluded from the above results that these cells are in fact tissue basophils (mast cells). The cytoplasm and granules of such cells are often disposed in a crescentic form around the nucleus leaving one or other pole “naked”—an appearance admittedly not characteristic of mast cells in connective tissue spreads and sections but understandable in smears.

Mast cells were found to be a normal constituent of the lamina propria of the palpebral conjunctiva at all ages often lying close to the basement membrane. Their presence in smears and curettings from the palpebral conjunctiva is therefore to be expected.

Discussion

Some of the difficulties of diagnosing true trachoma inclusions have already been described by Stewart (1939). In evaluating Poleff's method in terms of the histochemical properties claimed for the inclusion bodies, it is shown that this method primarily demonstrates mast cells, while the true inclusion particles are relatively unstained. The microscopic diagnosis of trachoma is best made by using thin smears of the palpebral conjunctival epithelial cells rather than scrapings: a wider area of superficial epithelial cells is thus encompassed and the ratio of epithelial cells to mesodermal elements is thereby increased, so that a greater number of epithelial cell inclusion bodies

can be expected. Rice's iodine method is most useful as a preliminary diagnostic procedure, but if negative it must always be followed by the classical Giemsa stain, because all inclusions do not contain glycogen and some contain so little that they are likely to escape detection by the iodine method.

The great bulk of the mature inclusions is made up of glycogen, which may serve as nutritive material for the virus; it makes thus a considerable contribution to the volume of the inclusion and may therefore also be a factor in the ultimate dehiscence of the mature inclusion body. It would seem that the glycogen is intimately attached to the inclusion particles; this may contribute to their diameter, making it greater than the measurement calculated from Giemsa preparations. It would be of obvious interest to determine the *in vivo* action of malt diastase or ptyalin on these glycogen capsules.

The origin of the glycogen present in these inclusion bodies is not known. If produced by the virus it would imply a high degree of enzymatic organization; if produced as a result of altered cellular metabolism it could be due to inhibition of glycolysis. It appears that the "plastin" ground substance, a conspicuous component of the smaller inclusions containing initial bodies, is as described by Prowazek a product of the cell rather than of the virus, because it disappears completely in more mature inclusions whether the particles ultimately present are of initial or elementary nature.

The results of this histochemical analysis together with the morphological picture obtained are entirely consistent with the idea that the inclusion body is a virus-containing structure, and they thus provide strong evidence against opposing views, such, for example, as that of Grüter (1938), who claimed that the inclusion body in this disease was merely an enlarged and altered Golgi net.

Summary

(1) Observations on the morphology of the Halberstaedter-Prowazek inclusion bodies in trachoma are described.

(2) Using histochemical methods in sequence, it is shown that both initial and elementary bodies contain desoxyribose nucleic acid, while the "plastin" substance of Prowazek is identified as ribose nucleic acid; glycogen is intimately associated with the elementary bodies and may also be associated with initial bodies.

(3) Poleff's method is evaluated in terms of morphological, tinctorial, and histochemical criteria which are established for the true inclusion bodies; on this basis it is found to be quite unsuitable for identifying the Halberstaedter-Prowazek inclusion body.

(4) The need for care in identification of the characteristic inclusion bodies in trachoma is again emphasized; a mixture of chemical and tinctorial methods is profitably used to identify the inclusions, but this is still a somewhat tedious procedure.

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