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Inclusion Bodies

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Inclusion Bodies

How specific are inclusion bodies for the identification of virus diseases?

Alfred M. Lucas, A. B., Ph. D.*

In order that we may both have in mind the same thing during this paper it is probably well to set forth a brief description of inclusion bodies. In a broad biological sense the term applies to any formed mass of material, such as secretion granules and plastids, but it is not in this sense that the pathologist uses the term; for him it has come to mean those masses of material which are associated with virus diseases. Those in the cytosome, when they are small, have practically no unique characteristics which will readily distinguish them from cytoplasmic accumulations of non-virus origin but as they grow larger their size becomes a valuable criterion. Those in the nucleus are more easily identified in that they are usually acidophilic and they usually seek a position in the center of the nucleus. There they lie free from the chromatin which migrates toward the nuclear margin. The existence of an object which appears to be an inclusion body is not proof of the presence of a virus but merely an indication that a virus should be considered if no bacterial agent can be found. If all virus diseases produced a similar type of inclusion body the problem would be simple in that one could seek out that element common to all of them; but some are associated with inclusion bodies in the cytosome, some in the nucleus, some in both nucleus and cytosome and many produce no inclusion bodies at all. Representative examples are given in Table 1.

Most of the virus diseases which produce inclusion bodies in the cytosome belong to the pox group and a steady accumulation of facts for the past 35 years has now culminated in a reasonably satisfactory understanding of the relationship of virus to inclusion body for this group of diseases. The development of this

TABLE I

Viruses Producing Inclusions In:

<table>
<thead>
<tr>
<th>Cytosome Only</th>
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<tbody>
<tr>
<td>Vaccinia</td>
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<tr>
<td>Sheep pox</td>
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<tr>
<td>Fowl pox</td>
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<tr>
<td>Molluscum contagiosum</td>
</tr>
<tr>
<td>Infectious myxomatosis of rabbits</td>
</tr>
<tr>
<td>Trachoma</td>
</tr>
<tr>
<td>Rabies</td>
</tr>
<tr>
<td>Louping ill</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Nucleus Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Varicella (chicken pox)</td>
</tr>
<tr>
<td>Vesicular stomatitis</td>
</tr>
<tr>
<td>Infectious warts of dogs</td>
</tr>
<tr>
<td>Yellow fever</td>
</tr>
<tr>
<td>Rift Valley fever</td>
</tr>
<tr>
<td>Virus III of rabbits</td>
</tr>
<tr>
<td>Disease of parrots and parakeets</td>
</tr>
<tr>
<td>Herpes simplex</td>
</tr>
<tr>
<td>B-virus</td>
</tr>
<tr>
<td>Pseudorabies</td>
</tr>
<tr>
<td>Borna's disease of horses</td>
</tr>
<tr>
<td>Poliomyelitis</td>
</tr>
<tr>
<td>Fox encephalitis</td>
</tr>
<tr>
<td>Jaundice of silkworms</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytosome and Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variola (small pox)</td>
</tr>
<tr>
<td>Alastrim (mild small pox)</td>
</tr>
<tr>
<td>Submaxillary gland disease of guinea pigs</td>
</tr>
<tr>
<td>of ground moles</td>
</tr>
<tr>
<td>Equine encephalomyelitis</td>
</tr>
<tr>
<td>No Cell Inclusions</td>
</tr>
<tr>
<td>St. Louis encephalitis</td>
</tr>
<tr>
<td>Mumps</td>
</tr>
<tr>
<td>Rous sarcoma of fowls</td>
</tr>
<tr>
<td>Sac-brood disease of bees</td>
</tr>
<tr>
<td>Many plant viruses</td>
</tr>
</tbody>
</table>

* Department of Zoology, Iowa State College.
knowledge will be reviewed briefly because it serves as a challenge to what we should be able to do with the intranuclear inclusions. Many years ago the elementary bodies were discovered in smears and it was suggested and later proved that these represented the actual virus agent. Their size was at the limit of microscopic visibility and filtration experiments have since showed that the poxes average about 0.150 micron. When stained they are considerably larger than this. With the ultra-microscope objects as small as 0.1 micron may be distinguished and the new electron microscope will probably make some of the smaller viruses visible. But to identify particles as small as these within a cell and be convincing about it, is rather more difficult. Had the granules remained uniformly dispersed in the cell they probably would not have been recognized. One of the early cytological studies (1) of vaccinia virus came to the conclusion that the basophilic inclusion body was an aggregation of materials produced by the cell but that under the stimulation of the virus abnormal amounts were produced and these clumped together. A contrary opinion (2) to this was rendered for fowl pox when the cytoplasmic inclusion bodies were separated from the cell by digestive enzymes. The inclusion bodies were washed repeatedly and then inoculated into the feather follicles of the fowl. One inclusion body would produce the disease. Efforts to handle the inclusion bodies of molluscum contagiosum (3) in a similar manner were not successful because the bodies fell apart into their separate elementary units. Recently, by the growing of rabbit cornea in tissue culture (4), it has been possible to observe the multiplication of vaccinia elementary bodies and their coalescence into clumps within the cell. Consequently, there no longer is any doubt for this group of viruses, at least, that the inclusion body is an aggregation of virus particles, but this is not the complete story; the cell contributes something also. In fowl pox (5) it was noted about 15 years ago that fats elaborated in association with the Golgi apparatus moved toward the developing inclusion body and gave to it a strong fat reaction. It was this protecting substance which made possible the separation of the inclusion from the cell by digestive enzymes and its absence in molluscum contagiosum (3) explains why the method would not work for this virus. It has already been mentioned that corneal cells add to the developing vaccinia inclusion body a basophilic material (1) so that interestingly enough we are led again to the earliest interpretation of cytoplasmic inclusions expressed by Prowazek when he named them Clamydozoa and explained that it was a cloaked organism—that the cell contributed a mantle over the infective agent. Thus, for the pox group of viruses one can say that finding an inclusion body is comparable in diagnostic importance to finding the organism in anthrax or tuberculosis.

Not all cytoplasmic inclusions have revealed their secrets so well and the opposite extreme is represented by the Negri bodies associated with rabies. The lyssa bodies and Negri bodies are sufficiently distinctive in their structural organization that they have been used satisfactorily for diagnostic purposes but what they are is still completely unknown as evidenced by the wealth of suggestions. Here are some: that the basophilic center is the organism and that the acidophilic mantle is contributed by the cell; that the Nissl substance becomes modified into the Negri bodies; that the neurofibrils are converted into these bodies and finally that they represent extruded nucleoli. Their value for diagnostic purposes would probably be increased if one knew what they are.

Turning to the problem of the intranuclear inclusions we find that progress has been slow because the inclusions stain like oxychromatin which is a normal constituent of the nucleus and because the idea that an organism would elect to reside in the nucleus rather than in the cytosome has relatively little analogous data to support it. We do have the rickettsias, however, as one good example of a group of small organisms which are known to aggregate in the nucleus. Yet, in spite of our limited knowledge on the subject, the idea developed that the presence of
an intranuclear inclusion was a fair indication that a virus was the etiological agent, even though the inclusion body in the nucleus may be merely a cell product. Strong support for this idea came from the fact that none of the bacteria produced cellular reactions of this type and injections of various non-living substances usually failed to produce them. Then followed a period when many intranuclear inclusions were discovered apparently unassociated with any virus disease. The threatened agnosticism was circumvented when Cowdry proposed that intranuclear inclusions be classified into two groups called type A and B (6). Type A included those (a) which were granular and (b) which produced a severe reaction in the cell leading to death. Type B were those (a) which were amorphous irregular masses or hyaline spheres and (b) which produced only mild reactions in the cell. It was unfortunate that two criteria were set up for each of the types because severity of reaction and a peculiar morphological configuration for the inclusion body do not always go hand in hand. It has been suggested, therefore, that it would probably be more helpful to the pathologist if types A and B connote merely their respective morphologies and not indicate any degree of pathological reaction (7). Under this plan types A and B would each include the following:

**Type A**

Herpes simplex  
B-virus  
Yellow fever  
Rift valley fever  
Disease of parrots and parrakeets  
Pseudo-rabies

**Type B**

Borna disease  
Poliomyelitis  
Submaxillary gland disease of guinea pig, ground mole, and man  
Fox encephalitis  
Canine distemper  
Inclusions associated with some chemical agents

This list is not complete for all of the described intranuclear inclusions. Many of the published accounts are too sketchy and very few photographs are sufficiently clear to show such small objects as part of a nucleus for the reader to form an independent opinion. Those papers, in which the author has prepared drawings or even diagrams, are usually satisfactory. Even examination of but one slide may be misleading and it is often necessary to make a fairly thorough study of the inclusion bodies with good optical equipment to determine even the simple question of whether they are granular or amorphous in type.

Of the two types, A has proven to be much more indicative of a virus than B (8) and the question always follows, “Are the intranuclear inclusions aggregations of virus particles as they are in the poxes?” Attempts have been made to handle them as Goodpasture did fowl pox but no success has as yet been reported (9). Therefore, our approach for the present must be indirect and the evidence circumstantial.

The Beams ultracentrifuge has been a useful tool in this work because with it the substances within the nucleus along with the inclusion body may be stratified and thus it can be shown whether the inclusion body has the same specific gravity as some constituent of the normal nucleus or if it forms a distinct stratum of its own. The granular type A inclusion produced by herpes simplex, which has been inoculated into the corneal cells of the rabbit, has been centrifuged and proven to be lighter than any of the parts of a normal nucleus (10). The sequence of layers from the lighter to the heavier pole is, inclusion body, nuclear sap, and mixed oxy- and basichromatin. The tentative conclusion now reached and supported by the work of Nicolau and Kopciowska (11) is, that in the case of herpes the particles represent the virus elementary bodies. Contra-wise centrifugation of several type B inclusions have always stratified them at the same level as the oxychromatin. This material separates out to form a layer between nuclear sap and basichromatin. This is true of fox encephalitis (7), submaxillary gland disease of guinea pigs (12) and inclusions produced by the subcutane-
ous injection of aluminum oxide (13). We have some information on the infective potency of these inclusions in the submaxillary gland disease of guinea pigs. The potency is very low when the monocytes bearing intranuclear inclusions are passed to uninfected animals (14) and becomes very high for the duct cells after the cytoplasmic inclusions make their appearance, but not while they contain only intranuclear ones. Further and still more convincing evidence that at least some of the type B inclusions are not aggregations of virus, that their presence does not automatically indicate the existence of a virus, and finally that they may be merely abnormal accumulations of oxychromatin, is furnished by the experiments of Olitsky and Harford (15,16). They produced characteristic type B inclusions by subcutaneous injections of aluminum oxide and hydroxide, iron hydroxide, carbon and various other substances. They eliminated the possibility of a latent virus. Centrifugation of inclusions produced by aluminum oxide (13) gave the same stratification series as for type B inclusions associated with virus agents.

The production of inclusions by chemical agents is not new. It has been associated with several heavy metals (17,18,19), arsenic, mercury, and lead, and the inclusions produced all belong to type B. In only one case has it been claimed that a type A inclusion was developed by a non-virus agent. Belt (20) found in the liver of persons who died from severe burns that the intranuclear inclusions and Councilman-like bodies in the cytosome were indistinguishable from those produced by yellow fever. He suggested the toxin as a possible cause for the production of the inclusion body but a number of years ago Findlay noted that cells in yellow fever looked as if they had been dehydrated and the work of Underhill and his associates (21) have shown that severe burns may cause the formed elements of the blood to be concentrated as much as 38 percent and become so viscous that the blood fails to flow through the vessels.

Although the information of the true nature of the inclusion bodies progresses slowly yet contributions can be made which will aid the pathologist to analyze better the inclusion-bearing cells which he sees in his routine or research preparations. For example, there are some data on the relative merits of tinctorial reactions. We have no specific stains for intranuclear inclusions. The many techniques in the literature, in most cases, merely make more evident things which can be distinguished by most of the usual staining procedures. For a while detailed studies were made of the comparative tinctorial reactions with a variety of stains but it has been shown in the submaxillary gland disease of guinea pigs and especially well in fox encephalitis that the inclusion bodies may vary from eosinophilic to basophilic, that the inclusion is composed of two parts and that the reaction merely depends upon the proportion of the two staining elements. On the other hand, type A inclusions are more consistently eosinophilic. The analysis of inclusions by microchemical techniques, thymonucleic acid reaction, test for masked iron, Millon test, microincineration and reactions to various chemicals have been reviewed in publications by Cowdry and by Findlay.

More important than staining reactions, per se, is the use of stains to differentiate and to follow the reactions of oxy- and basichromatin within the nucleus. It is this aspect of cytopathology which seems to have been neglected, except in a few cases, and which should in the end be of some practical value to the clinician. The most important point to be emphasized in our thinking about the cytopathology of inclusion body formation is to separate cellular reactions in conjunction with the inclusion body formation from reactions leading to the death of the cell. The formation of an inclusion body does not automatically lead to cell death but the confusion occurs from the fact that the virus agent which may stimulate the development of the inclusion body, often at the same time, produces an area of tissue destruction so that the inclusion-bearing cells may be involved.

Many descriptions of inclusion body formation merely mention the presence of

(Continued on page 45)
Lt. W. S. Monlux, '37, formerly in the Pathology Department at Cornell University, is now stationed at East St. Louis (Collinsville), Ill.

Dr. Robert L. Alkire, '36, formerly with the B. A. I. at Cumberland, Md., is now at Station Hospital, Langley Field, Va.

Capt. F. M. Bolin, '29, is serving with the Vet. Prov. Med. Sq., 2nd Cav. Div., Fort Riley, Kan. Dr. Bolin was formerly with the Veterinary Dept., North Dakota Agricultural College, Fargo, N. D.

Dr. L. E. Stanton, '25, formerly at Jackson, Minn., is now located at the Medical Field Service School, Carlisle, Pa.

The address of Lt. R. W. Rushmore, '39, formerly of Columbus, Iowa, is now 824 Richmond Ave., Silver Springs, Md.

Pvt. Robert E. Storm, '40, is now serving with the Army at Camp Grant, Ill.

Deaths

Dr. Robert Miller, '38, was called to active duty in the army at Ft. Snelling, St. Paul, Minn., in July. Shortly after being called to duty, a son was born to Dr. and Mrs. Miller. Three days after the birth of the son, Mrs. Miller died. Dr. Miller was to have been transferred to Carlisle Barracks, Carlisle, Pa.

Dr. W. Wallace Boggie, '21, died Sept. 22 at Madison, Wis.


Dr. G. F. Starkey, '91, died July 3, 1941.

INCLUSIONS  (Continued from page 14)

an inclusion body surrounded by a halo and the chromatin as margined against the nuclear membrane. True, this is a conspicuous stage and one commonly found but if the cell pathology is to be of further diagnostic value it is desirable to have all of the events from the earliest to the latest, and whenever possible to have them separated from death changes. The following remarks will indicate what is meant. When the inclusion body first appears in the submaxillary gland disease it hardly disturbs the normal organization of the nucleus. Then follows a breakdown of the linin net-work and the pulling away of the chromatin toward the nuclear membrane. After the chromatin reaches the margin, it reverses its movements and crosses the halo to rejoin the inclusion body to which it attaches so firmly that centrifugation will not separate it. It requires ten days to two weeks for the duct cells to attain this condition. They become greatly hypertrophied in the process and it is significant that the cell maintains a normal nucleo-cytoplasmic ratio (if one deducts the volume of the inclusion body from the nucleus). In fox encephalitis a peculiar reaction occurs in the early stages. At the time when the basichromatin is marginating toward the nuclear membrane, the oxychromatin separates from it and moves in the opposite direction, namely, toward the inclusion body which it encompasses. Thus, the inclusion body of fox encephalitis is not quite the same as that of the submaxillary gland disease although they are both of type B. As a third example, the inclusions associated with the subcutaneous injections of aluminum oxide resemble in their early stages a plasmosome nucleolus but later become free of all basichromatin and are

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cytologically similar to type B inclusions of virus origin.

At any time during the course of inclusion formation steps leading to cell death may be superimposed upon inclusion bearing cells. One can summarize the principal steps in autolysis as follows: 1. A change in the chromatin to make more than normal amounts of oxychromatin visible and a tendency for the chromatin granules to show a range in size greater than normal. 2. Frequently a slight liquefaction may occur so that the nuclear sap shows a slight chromatin reaction and sometimes accompanying this there is slight hypertrophy of the nucleus. 3. A coalescence of basophilic granules, destruction of the linin framework except for a few strands and shrinkage of the nuclear membrane. 4. A further shrinkage of the nuclear membrane which draws the chromatin masses together, the nuclear sap disappears and a pycnotic nucleus is the end result. This series of reaction is reversible in its early stages.

Sometimes the inclusion bodies in two closely related diseases are similar but use can be made of the fact that their occurrence is specific for certain tissues as has been done by Green and Evans in the separation of fox excephalitis and canine distemper. Woodruff's (22) differentiated vaccinia virus and fowl pox in the same animal on a cytological basis.

To save-guard against over confidence in the diagnostic value of all inclusion bodies, it is well to keep in mind the work of Wolf and Orton (23) on the inclusion bodies of poliomyelitis in which they found what cytologically appeared to be identical bodies in gliomas and diseases in which the polio virus could play no part. The same attitude is probably correct in the evaluation of inclusions, both nuclear and cytoplasmic, of equine encephalomyelitis.

In conclusion it is apparent that when we have learned the real nature of all the cellular bodies associated with virus diseases, then their use for diagnostic purposes will be greatly enhanced. Until that time comes, however, inclusions should be studied with consciousness on the part of the observer that the inclusions on a fixed and stained slide are merely “stopped action” of a process which progresses from an early to a late stage. In addition, it should be kept in mind that these series of events may be cut short at any time because superimposed upon them there may be other events which lead to death of the cell before the inclusion body series has had time to be completed.

BIBLIOGRAPHY