CELL-INCLUSIONS IN THE BLOOD OF A CASE OF BLACKWATER FEVER.

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Our knowledge of the etiology of blackwater fever is still far from clear, in spite of the numerous laborious and careful researches which have been carried out in connexion with cases occurring in all parts of the Tropics; it appears therefore worth while to put on record the following observation. There is but slight ground for assuming that the bodies described below have any causative connexion with the disease, but others, more favourably situated as regards clinical material, may possibly be led to look for them and to follow up a line of investigation which might, conceivably, throw light on the dark places in our knowledge of this disease.

The material on which this note is founded is admittedly of the scantiest nature, as it consisted only of three unstained blood-films from a case of blackwater fever which occurred in Uganda, and which were sent to me by the Principal Medical Officer, Dr. A. D. P. Hodges, to whom I would here express my warm thanks. No clinical details were furnished, but such have been asked for and, if they should throw any useful light on the matter, will form the subject of a further note.

On first glancing at the stained films I was struck with an abnormal feature in the shape of the presence, in large numbers, of cells of an unusual type. These cells displayed considerable variations in shape and were of exceptional size, the average diameter being about 25 microns. They were of mononuclear type but differed
from the usual hyaline leucocytes, not only in their greater size but in the character and position of the nucleus and in the staining of the protoplasm. As regards the first of these points, the appearance of the nucleus, this but rarely showed any tendency to lateral indentation or to the horse-shoe appearance common in hyaline leucocytes, and, more often than not, it was situated eccentrically and even pressed up against the side, while occasionally it extended completely across the cell. To save a more lengthy description a glance at the plate will show some of the types encountered and figs. 2, 4, 5, 6, 7, 9, 10, 14 and 15 demonstrate this particular feature. It is also noteworthy that the nuclear contours were always quite sharp and well defined, and that there was no evidence of any degenerative change of the nature of karyolysis or karyorrhexis; the nuclei stained well, though in different degrees of depth according to the looseness or otherwise of the nuclear network.

The protoplasm of these cells stained a pale blue but did not exhibit the transparent appearance so distinctive of the hyaline leucocytes; it also frequently showed a reticular appearance more suggestive of the protoplasm of tissue-cells than that of leucocytes.

In the majority of instances the protoplasm was devoid of granules, but minute granules were found in a few, in very varying numbers and most unevenly distributed throughout the cell. As far as could be judged from the stain employed, that of the writer, these resembled the neutrophile granules of the ordinary polymuclear leucocyte or the neutrophile myelocyte, except that they seemed smaller and, in some cells, were of a pinker hue than the latter. The possibility of their being of another nature will be dealt with later.

I cannot speak with certainty as to the true nature and origin of these cells, but for various reasons I am inclined to regard them as endothelial cells which have been disrupted from the walls of the blood-vessels or lymphatics, and have been washed into the circulating blood. They appeared to resemble very closely the endothelial cells which are found free in the spleen, and in the capillaries of the liver in cases of kala-azar and which, in that disease, are more or less heavily infected with the characteristic parasites. I have not myself encountered them before in films of blood from blackwater cases, but similar cells have been noted and carefully described by Christophers and Bentley in the blood of the cases which they

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To illustrate "Cell-Inclusions in the Blood of a Case of Blackwater Fever."

By Lieut.-Col. Sir William B. Leishman, F.R.S., R.A.M.C.
investigated in India, and they, too, regard them as endothelial in origin.

No information is available as to the actual number of leucocytes present in the blood, but, to judge from the films, a moderate degree of leucocytosis must have been present.

The relative proportions of the various cells were as follows:—

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polynuclears</td>
<td>41.5%</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>6.0%</td>
</tr>
<tr>
<td>Hyalines</td>
<td>4.5%</td>
</tr>
<tr>
<td>Eosinophiles</td>
<td>0.5%</td>
</tr>
<tr>
<td>Mast cells</td>
<td>0.5%</td>
</tr>
<tr>
<td>Transitional</td>
<td>8.5%</td>
</tr>
<tr>
<td>Neutrophile myelocytes</td>
<td>2.5%</td>
</tr>
<tr>
<td>Turk's cells</td>
<td>2.5%</td>
</tr>
<tr>
<td>Endothelial (?) cells</td>
<td>33.5%</td>
</tr>
</tbody>
</table>

A few normoblasts were found and, in one of the three films, an undoubted megaloblast. No striking changes were manifest in the red cells, which stained evenly and well and exhibited no evident deficiency of haemoglobin; basophilia was not seen and there were no poikilocytes or cells of exceptionally large or small size.

Careful search was made for malarial parasites, always a point of interest in view of the widely held views as to the connexion of malaria and blackwater, but none were found. At the same time, three or four pigmented leucocytes were found containing clumps of what appeared to be melanin, so it seems probable that parasites were either present or that the patient had very recently suffered from an attack of malaria.

Turning now to the cell-inclusions which are the chief feature of interest of this blood, these were altogether confined to the large endothelial cells. They were far from numerous, only about one cell out of twenty containing them, but they could hardly have been missed as they formed very clear and arresting features. It is not possible to say whether they would have been made manifest by the customary five minutes’ application of the writer’s stain, since each film was stained deeply by half an hour’s contact with the mixed stain and water. By employing the stain in this fashion and subsequently washing the film with 60 per cent. alcohol, every trace of deposit left by the stain is dissolved off; this is essential for bringing out the details of fine structure and has the further advantage that we can be certain that we are not dealing with artefacts due to the deposition of stain.

In all cases the inclusions were seen to be contained in the
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cell protoplasm, none were seen on or in the nuclei and only rarely were they seen in close contact with the nuclear membrane.

A lengthy description of the bodies may be dispensed with by referring to the coloured plate. The sixteen cells there depicted include more than half of the total number of cells in the three slides which were found to harbour inclusions. It will be seen that the inclusions range in size from a diameter of 1 to 5 microns, that they show a definite tendency to the circular in contour and that, while the majority present themselves under the aspect of ring forms, with deep-staining periphery and fainter hued centre, the smaller forms are homogeneous in their colouring. In all cases the inclusions took on a more or less pronounced pink or red colour, while many, as will be seen, showed the deep red reaction usually attributed to chromatin.

The two large inclusions seen in fig. 13 were remarkable in appearance and showed a dark-staining centre and an irregular but very deeply stained membrane or capsule; one other inclusion of the same type was found in another cell which is not figured as it was accidentally destroyed by a scratch before it could be sketched.

In several of the cells containing the inclusions well defined vacuoles of varying size were seen in the protoplasm, and since some of the smaller inclusions were found to be lying in similar clear areas, as for instance in figs. 9 and 13, it is not improbable that the empty vacuoles originally held similar bodies and that such vacuoles were not signs of degeneration of which indeed there was no other evidence in the cells in question.

The remaining feature of the sketches to which attention may be directed is the occurrence of fine granules in some of the cells; these have already been alluded to, and examples are afforded by figs. 4, 6, 11, 12, 15, and 16. It will be seen that they are extremely minute, but naturally it was not possible to depict them with any very precise regard to their real dimensions; they approached in many cases the limit of visibility, which is considered to approximate 0.1 micron.

The Possible Nature of the Cell-Inclusions.

It will be evident that these must fall under one of the following categories: (1) Artefacts; (2) products derived from changes in the nuclei or cytoplasm of the cells; (3) material which has been phagocyted by the cells; (4) micro-organisms. Each of these will be considered in turn.
(1) Artefacts.—The method of staining employed has already been mentioned, and it may safely be asserted that the inclusions were not derived from deposit or other change induced by the staining fluid. The writer’s experience in connexion with this method enables him to speak with confidence on this point. Neither does it seem possible that the inclusions are artefacts due to fragments of foreign matter which had settled on or under the cells; the films were well made and free from dirt, and careful focusing showed that the bodies were actually inside the protoplasm of the cells, and were not lying either on or below them.

(2) Products of Nuclear or Protoplasmic Change.—It has been mentioned earlier that the nuclei of the cells appeared perfectly normal, and showed no signs of any degenerative change, such as karyolysis or karyorrhexis; their contours were always perfectly sharp. It seems, then, improbable they should have been extruded from the nuclei. At the same time it is possible that they may have had such an origin, in spite of the nuclei showing no direct evidence of this at the time the samples were taken. The appearance and staining reactions of the inclusions did not recall to me any products of nuclear change such as I have frequently encountered in other conditions. The possibility that they were products of changes, metabolic or other, in the cell cytoplasm also appeared unlikely, although this, too, cannot be absolutely excluded.

(3) Phagocyted Material.—The most likely objects to be taken into cells which are endowed with the property of phagocytosis are foreign particles of any sort, whether of organic or inorganic nature, and other cells. As regards the former the commonest material which is phagocyted in cases of chronic malarial infection, such as form the large majority of cases of blackwater fever, would be malarial pigment; as already mentioned, such pigment was found in leucocytes in this blood, but none in any other type of cell. Other extraneous matter was not in evidence outside the cells, and the inclusions noted in the endothelial cells appeared too regular in shape and structure to be regarded as of this nature. A more obvious explanation in this disease would be that the inclusions were only altered red cells, since phagocytosis of red cells is well known to be a striking feature in blackwater, although by no means limited to this disease. The most careful study of this feature of the disease, as far as I am aware, is that of Christophers and Bentley, already alluded to. These investigators record the
frequency with which the various cells of the blood and spleen exhibit the phenomenon and describe the various appearances which the ingested cells may assume. Among others, they describe the phagocytosis of red cells by the endothelial cells which are in question in this instance. Such cells they have found frequently in the blood during the acute stage of the disease, although the cells disappear rapidly when the haemoglobinuria is passing off, and in many of them they observed evidences of phagocytosis of red corpuscles. Unfortunately their description was unaccompanied by plates or sketches, so it is not easy to contrast what they found with the cell-inclusions in this case, and I can only express my opinion that the inclusions are not to be accounted for in this manner. I am quite familiar with the appearance and staining reactions of phagocyted red cells in various stages of intra-cellular disintegration, but I have never observed them to take such an appearance as is to be seen in the accompanying plate.

A further observation is recorded by Christophers and Bentley, in which they noted in the blood of one of their cases on the fourth day of the disease a number of small mononuclear cells which had a deeply staining mass of nuclear-like substance lying within the protoplasm near the periphery of the cell; these bodies were small, averaging 1 to 2 microns in diameter, and they were inclined to regard them as nuclear extrusions. They also mention, in the case of large macrophages found in the spleen, that, among other inclusions such as red cells and pigment, some showed particles of a substance staining like chromatin, and somewhat resembling blood-plates. They had, however, observed these particles in other diseases than blackwater and were not inclined to attribute any special importance to them. Again, one may regret the absence of sketches, since it appears from the description that these latter bodies might perhaps have been similar to those found in the present instance. However that may be, the writer at all events has not observed them in specimens of splenic blood from any other disease.

(4) Micro-organisms.—Of these, bacteria may, I think, be safely excluded, at all events the inclusions do not bear the slightest resemblance to any known organisms of this nature. Blastomyces might appear a more probable explanation, especially in connexion with the large forms shown in fig. 13, but the smaller bodies do not resemble any stage of the growth or multiplication of this class of micro-organisms.

As regards protozoa, at first sight these would appear to be even
more definitely excluded than bacteria were it not for the recent information which has come to light as to the probable life-history of the class of organisms to which Prowazek has given the name Chlamydozoa, and which he considers to be referable to the protozoa. The possible bearing of this information on the inclusions will be considered below.

The above short analysis of the various possibilities will at least show how numerous are the difficulties of ascertaining the true nature of the inclusions and how unjustifiable it would be to make any dogmatic assertions as to their origin. It appears to me, however, as at least possible that the inclusions in question may be due to the presence within these endothelial cells of Chlamydozoa. It need hardly be added that, even if this should prove correct, it would be a very long step between that knowledge and the proof that there was any causal relationship between these organisms and blackwater fever. Be that as it may, it would at least be an observation of considerable interest that organisms of this class should be present in this mysterious disease.

Since knowledge relating to the Chlamydozoa is not yet widely diffused—indeed the name itself is probably unknown to many—it may not be inappropriate to include in this note a brief outline of the present state of the subject in order that the reasons for my suggestion may be more readily followed. Apart from this, much of the work in question suggests developments of the greatest importance in the near future in connexion with the causation of some of the most dangerous diseases of man, and has thus an interest of its own.

The organisms to which this name was applied by Prowazek, in 1907, have in most instances two features in common: they are capable of passing through the usual bacterial filter candles, at all events such as are fine enough in their grain to keep back the smallest known bacteria, and, second, in the diseases in which they occur "cell-inclusions" have been noted as a constant feature. Prowazek's original list included the following diseases: small-pox and vaccinia, rabies, trachoma, molluscum contagiosum, contagious epithelioma of birds, foot-and-mouth disease, and certain diseases of fish, dogs and silkworms. Since then Prowazek has modified this list and, according to Hartmann, who gives a good summary of the subject,\(^1\) would only definitely include variola and vaccinia, trachoma, molluscum contagiosum and bird epithelioma. Evidence

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about the remaining ones he considers too incomplete to permit of their being retained in the same class at the present moment. In addition, however, to the above list it appears far from improbable that such diseases as scarlet fever, measles, chicken-pox, rabies and perhaps yellow fever may eventually be shown to be due to viruses of a similar class.

The longest known of the cell-inclusions are those found in molluscum contagiosum, but those associated with variola and rabies are more widely known and are, respectively, named after their discoverers, "Guarnieri's bodies" and "Negri bodies." When first found and described they were taken to be the actual parasites of the respective diseases in which they occurred, and various generic and specific names were attached to them, which it is unnecessary to recapitulate. Although demanding in most cases special staining methods for their demonstration, these inclusions were not difficult to find and recognize and, in spite of some reports to the contrary, it was soon recognized that they were specific to the diseases in which they were found. At the same time, there was much that was puzzling in their morphology and in their distribution in the tissue-cells; for instance, Negri bodies were chiefly found in the grey matter of the cornu Ammonis and the cerebrum and in Purkinje's cells of the cerebellum, while they were found to be scanty or absent in other parts of the nervous system which experiment had shown to contain the virus of rabies in concentrated form. Again, Negri bodies are constantly found in rabid animals, in what is known as the "virus des rues," while they are as constantly absent in the nervous system of animals infected with "virus fixe." Another fact which seemed inexplicable in view of the large size of the inclusions was that, in practically all the diseases enumerated above, the virus has been shown to be capable of passing through very fine filter candles, the filtrate, cell and germ free, proving as virulent for animals as the unfiltered tissue products.

The non-parasitic nature of the inclusions appeared to be finally established when it was shown that Guarnieri's bodies of variola were soluble in strong saline solutions, and were broken up by both peptic and tryptic digestion. The inclusions then came to be looked upon as mere products of cellular reaction in response to the influence of the still unseen and unknown virus. They were, however, still looked upon as specific in the sense that they were only to be found in the particular disease, and might therefore have diagnostic value. As a matter of fact, in some Pasteur Institutes, it is now the custom to search the tissues
of a suspected rabid animal for Negri bodies, and to give a positive diagnosis if these are found; animal inoculation being only resorted to in the event of this examination proving negative.

A fresh impetus, however, has been given to their study by the discovery of very minute granules in some of the diseases in question, more particularly in trachoma, variola, and molluscum contagiosum, in association with the cell inclusions. More recently a similar association has been found in contagious epithelioma of birds, in rabies, in varicella and in both human and experimental scarlatina. The granules in question are extraordinarily minute, and many approach the limit of visibility, which, as has been said, postulates an object of 0.1 micron in diameter. Modern methods of staining, improved lenses and new methods of illumination have permitted the recognition of these minute particles either in the fresh state, where they are best observed by dark-ground illumination, and are seen to have very active oscillatory movements, or in dried films or sections where they are brought out by special methods of staining, of which some variety of Romanowsky is most frequently employed. Much of the work which has led to these results was carried out by inoculating the virus into the cornea of rabbits, where the subsequent appearance of both inclusions and granules has been observed and studied.

The most weighty evidence, however, as to the nature of the granules comes from the investigations of Prowazek and Aragão during a small-pox epidemic at Rio de Janeiro. They found the granules were capable of passing through a Berkefeld filter, and that the sterile filtrate was still virulent, but on filtering the same material through a special filter coated with agar, an "ultra-filter" as they term it, the granules were retained and the filtrate found to be no longer virulent. On examining the surface of the ultra-filter great numbers of the granules were found, while the filtrate contained none, a marked contrast to what had occurred in the case of filtration through the Berkefeld candle, where the granules were as abundant in the filtrate as in the diluted lymph before filtration.

These and other observations which are constantly being reported appear to lead more and more to the conclusion that these minute microscopic granules are the veritable causes of the diseases in question. Owing to their minute size it is impossible

to ascertain accurately their life-history, or to be certain whether they should be included among the bacteria or, as Prowazek suggests, among the protozoa, but what appears to occur is something of the following nature. A small granule gains entrance to a cell—for example, a conjunctival cell in trachoma, a nerve cell in rabies, or an epidermal cell in small-pox—and causes a reaction of the cell which is expressed by the throwing out of some reaction product in the shape of a capsule or mantle of secretion which surrounds the invading particle. (Hence the name Chlamydozoa, which is framed on the word, χλαμύς, a cloak or mantle.) In some instances this covering mantle attains a very considerable thickness, and the body is conspicuous as the cell-inclusion known as a Guarnieri's body, a Negri body, a molluscum body, and so forth. The original granule, which may or may not be visible within the enveloping mantle, has been called the "initial body." The initial body then proceeds to divide, and from it are formed great numbers of the extraordinarily minute little particles which may eventually escape from the inclusion and fill the cytoplasm of the cell; these derivatives of the initial body are known as "elementary bodies," and it is held that it is in this form that the virus extends to other cells or to fresh hosts and that, by reason of their minute size, they are able to pass through filters, as has been described.

It will be seen, therefore, that in this view the recently discredited cell-inclusion is to be regarded as an evidence of the reaction of the cell to the true virus, the chlamydozoon granule, and that it acts as an enveloping cover or capsule to the latter which multiplies within it, forming the elementary bodies which are capable of transmitting the disease further afield.

Turning once more to the cell inclusions found in this case of blackwater fever, it is with the utmost reserve that I suggest that they, too, may bear the same relationship to a minute parasite of the nature of a Chlamydozoon and that there is no insuperable objection to the theory that such a parasite may prove to be the cause of blackwater fever. The total number of inclusions found in these three films is far too small to permit of any definite views being advanced as to a cyclical development, such as has been described above, but it is possible that the minute granules depicted in some of the cells may bear a relation to the inclusions similar to that which appears to obtain in the case of small-pox, rabies and molluscum contagiosum.

Assuming that those Chlamydozoa which have already been
described are the actual causes of the diseases with which they are associated, it will be noticed at once that from the clinical point of view there is little in common between these diseases, while, if we include among them the other diseases which may be suspected of having a similar kind of virus, such as scarlet fever, foot-and-mouth disease, measles and perhaps yellow fever, the clinical dissimilarity appears even more striking. Objections therefore founded on such dissimilarity between the above diseases and blackwater fever would not have much theoretical weight. Certain facts, however, which appear to be well established from the accumulated experience of blackwater fever, may be briefly considered in the light of the tentative suggestion which I have put forward. The majority of the diseases attributed to the Chlamydozoa are known to be infectious, and they frequently spread in epidemic form; epidemicity is certainly rare in the case of blackwater fever, if it occurs at all; at the same time, instances of the apparent epidemicity of the disease have been recorded, although the evidence in connexion with these has not been very complete. The question of infectivity would, on closer inspection, also appear not to be an insuperable difficulty when it is realized that in the large preponderance of the diseases mentioned the virus is obviously localized to a large extent in the skin lesions or in the secretion of mucous membranes; in blackwater, on the other hand, no superficial lesions are in evidence, and the virus, if a specific one exists, is probably situated more deeply in the organs or tissues. The mass of evidence which goes to show that blackwater fever is only contracted by those who are the subject of frequently repeated malarial infection and is practically confined to those districts which are known to be intensely malarious might, with as great propriety, be urged as a reason for suspecting that a specific virus may be transmitted by the bites of mosquitoes, or other insects, as in support of the view that the disease is largely attributable to chronic malarial infection. In this connexion the parallel of yellow fever might be adduced, as it is apparently the case here that a mosquito is the transmitter of a filter-passer, while a similar example might be quoted in the case of the still undiscovered filter-passing virus of pappataci or three-day fever.

Such speculations, however attractive, are of little value in comparison with positive evidence, and I will only put forward one more, which appears to me to count against the theory of a Chlamydozoan being responsible for blackwater fever. In the
majority of the diseases mentioned as being apparently due to these parasites an attack if recovered from confers a considerable immunity against subsequent infection; in blackwater, on the other hand, this is known not to be the case, as second and third attacks are not infrequent and may prove fatal.

Where all is indefinite it would appear out of place to end this note with the customary "conclusions"—these are still to seek. The main object has been to call attention to these bodies in the hope that further search may demonstrate whether there is any foundation for my suspicion—it is little more—that they represent an invasion of the endothelial cells of the visceral blood or lymph vessels by parasites of the nature of Chlamydozoa.