ON THE NATURE OF THE CELLULAR ELEMENTS PRESENT IN MILK.

(FOR THE BRITISH DAIRY FARMERS' ASSOCIATION.)

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That cellular elements are found normally in milk has been known for many years, but it is only comparatively recently that any importance from a public health point of view has been attached to their presence. These cellular elements being usually regarded as leucocytes or pus cells, it is natural that their occurrence in milk should be looked on as indicative of an inflammatory process in the udder of the cow or cows from which the milk was obtained. The first observers to make any attempt to arrive at the numbers of these cells in milk seem to be Stokes and Wegefarth [1] in 1897. Their method, however, was very crude, and is not suited for making a true enumeration. Stewart [2] and Slack [3] followed with a very similar method, except that special tubes were used for obtaining the deposit. This "smeared sediment method" as it is called, is, however, capable of great inexactitude. Later observers have, by the use of ordinary blood counters, made the counting of the cells in milk a scientific measurement of reasonable accuracy. To Doane and Buckley [4] belongs the credit for the first published use of such a method. Savage [5] however has made the process still more

https://doi.org/10.1017/S0022172400016326 Published online by Cambridge University Press

accurate by first diluting the milk before centrifugalisation, and also by counting all over the field of the blood chamber, two innovations which are of very great importance, as has been borne out by our investigations. Error is easily introduced if counting is confined to the ruled divisions of the counter. Recently, Trommsdorff[6] has used a method based on the measurement of the deposit obtained on rotation in a graduated tube, and this method, probably on account of its ease and speed, has found much favour in Germany. It has been severely criticised by several investigators, such as Schuppius[7] and one of us[8]. Rühm[9] and some others believe that it is useful if used as a routine control method. We are of opinion that it might be used in such a way as an "indicator," but for the purposes of public health control it is quite hopeless.

The outcome of the elaboration of methods for estimating the number of cells present in milk has been an attempt to diagnose mastitis amongst the cattle supplying any particular milk. For this reason milk has been examined by many observers for the purpose of ascertaining how many cells are normally present. The discrepancies among the results of the various investigators are to be attributed (1) to the different methods used; (2) to a want of appreciation of the sources of error even in the more refined methods; (3) to the use of isolated milks without reference to circumstances; and (4) to a want of careful enquiry into, and supervision of, the milks employed. The most reliable results are those obtained by Russell and Hoffmann[10] working with cows under strict supervision and over long periods, and these results have been fully confirmed by one of us[8] working under similar conditions. Quite recently Savage[5] has given a large number of results obtained from cows both in health and disease, and though the samples are, in many cases, isolated instances, still the conditions were accurately known. As a result of investigation, the possibility of setting a limit to the number of these cells for the purpose of public health control is seen to be very doubtful. A limit of 500,000 per c.c. seems to be tentatively held by many, though at the same time their own results show that much injustice would be done if such a limit were enforced, both by the condemnation of sound milks and by the passing of diseased milks. It has been part of this investigation to make weekly enumerations of the cells found in the milk of several herds under careful veterinary inspection, and as a result it has been possible to show that such a limit of 500,000 per c.c. would often condemn milks from quite healthy sources.

The results of Russell and Hoffmann are particularly interesting, in that they attempted to trace the influence of such factors as parturition and lactation, of feeding and temperature, etc., on the number of cells appearing in the milk without being able to detect any definite connection, and we also have arrived at similar conclusions.

Bergey and Savage have both made enumerations of the cells occurring at the same time in the milk from separate quarters of the udder, and they both find very great variations in the numbers so obtained. The importance of this fact in its bearing on the question of the nature of the cells found in milk is great, and does not seem to have been properly appreciated. The same importance attaches to the now well-known fact that cows which have had mastitis in one or more quarters show a large count of cells from those quarters long after the disease had subsided, and lactation has been re-established in the affected quarters.

So long, however, as this method of examination is confined to an enumeration of the cells only, no particular or special importance attaches to their precise nature, but quite recently there has been a tendency to diagnose the presence of "pus" in milk by a "qualitative" examination of the cells present. This has been done by assuming that the cells present are true leucocytes, and making a differential count in a manner similar to that employed in the case of blood. As such attempts have not been made merely for scientific interest, but have been used for the hygienic control of existing milk supplies, it becomes of paramount importance to ascertain beyond all doubt whether the nature of these cells justifies their classification as true leucocytes, and consequently their differential enumeration, as in the case of blood. In the course of an investigation into the Bacteriology of Garget, undertaken for the Local Government Board, Savage has made use of a classification into polymorpho-nuclear cells, lymphocytes, large leucocytes, and doubtful cells, but with his usual fairness, on account of discrepancies so found, he clearly states that the presence of any particular form of leucocyte cannot be taken as diagnostic of pus.

Before proceeding, however, to discuss the true nature of the cellular elements which come naturally into milk, it will be necessary to consider briefly some of the views which have been held with regard to the formation of milk in the udder, and the general functional activity of that organ. It is necessary clearly to realise the extraordinary secretive power of the udder, the secretion, both in its quantity and complex composition, being without parallel in any other secreting gland of the body.

Attempts have been made to elucidate this from as far back as 1838, when Donné first described the so-called "Colostrum granules," and from that time onward there has been no lack of investigators in this field. Without the least disparagement of the older workers, it is right that we should place more reliance on modern work, on account of the great improvements which have taken place in the technique both of preparation of tissues and of staining methods. Particularly is this of importance, as the real solution of the problem lies in the correct microscopical interpretation of sectional preparations of the udder during lactation.

Much confusion in such interpretations has undoubtedly arisen from a want of appreciation of the fact that the whole of the gland tissue is not in the same state of activity at the same time, so that in sections we shall expect to find some of the alveoli actively secreting, while others, having discharged their contents, are for the moment in a "resting" stage. The varying dimensions and form of the epithelial cells during extension of the alveolar space, and during contraction after expulsion of the contents, make it extremely difficult to recognise the cellular elements as the same in both cases.

As it is impossible to mention the views of the host of observers who have contributed to the solution of this problem, we shall briefly quote the excellent summary of such views made by Pfaundler. He distinguishes four typical glandular tissue conditions, which, however, are always closely connected, and are to be taken as occurring in succession in the actively secreting udder.

(*a*) The alveoli are open, the epithelial cells are cubical and contain clear round nuclei with one or two nucleoli. Mitosis is constantly present in the epithelium; the protoplasm of the cells shows fine granulation and vacuoles. The cells appear indefinite, their outline and limiting surfaces indistinct. There is a marked infiltration of the alveoli with leucocytes (many eosinophile), which are found in such great numbers in the interstitial tissue and the epithelial layer and lumina of the alveoli, that the remaining structure is only recognised with difficulty.

(*b*) The alveoli have very narrow lumina and the epithelium is cubical, the nuclei of which seem shrunken. Mitosis is constantly present. The protoplasm of the cells contains coarse granules and fat drops, and the cell outline is indistinct. The interstitial tissue is rich in leucocytes, but less so than in (*a*). In the lumen of the alveolus leucocytes, colloidal masses and colostrum bodies are present.

(*c*) The alveoli have very narrow lumina and long cylindrical or

pyramidal epithelial cells, clearly marked off from one another, some resting flat on the alveolar wall and some suspended by a narrow process. Each cell contains one to three nuclei, and at the free margin of the cell fat drops are often seen. Many of the cells which hang by a tongue of protoplasm into the alveolar space appear as if torn.

(*d*) The alveoli are dilated. The epithelial cells are flat, and in profile appear as ring-shaped narrow protoplasmic borders with spindle-shaped nuclei, and contain very few fat globules. Mitosis is rarely seen.

Particularly characteristic of this state are certain curious forms, first described by Nissen (*die Nissenche Körperchen*). These bodies consist of nuclei with a characteristic arrangement of chromatin, partly still in the cells and particularly at their distal extremity, and partly in the lumen of the alveolus. The chromatin lies at the periphery of the nucleus in lenticular segmental masses, which ring round the chromatin-free inner nucleoplasm.

It will be noted in this summary that the infiltration of the interstitial tissue with leucocytes, and their extrusion into the lumen of the alveolus, seems to be generally held, but attention is particularly drawn to the pendulous epithelial cells under (*c*), and to the "Nissenche Körperchen," to which reference will again be made.

It is now necessary to consider somewhat in detail the views of Winkler[11]. As the result of a careful histological investigation of the udders of cows during different periods of lactation and before parturition, and in cases of mastitis, he described the general structure of the alveolus as consisting of:

(*a*) The membrana propria, a structureless envelope and a cuticular formation of the interstitial connective tissue.

(*b*) A muscle fibre layer immediately within this, consisting of smooth muscle cells with very delicate elongated nuclei.

(*c*) The epithelial layer lining the lumen of the alveolus.

In these he is in general agreement with most other observers, but between (*b*) and (*c*) he describes a "germinal cell layer," which seems to have been noticed in 1877 by Kolissnikow, who pronounced it to consist of young epithelial cells, and also by Heidenhain in the submaxillary glands, who also describes it as consisting of small round often multi-nucleated epithelial cells. From this germinal cell layer Winkler shows that the epithelial cells of the gland are constantly renewed, and though normally the cells of the germinal layer lie behind those of the epithelial layer, still in sections (on account, perhaps, of the expansion

of the alveolus at the time of taking the tissue from the animal, and the subsequent contraction and distortion brought about by the fixing agent) the germinal cells and epithelial cells often appear to be in the same plane. In epithelial masses found during the colostral period the gradual transformation of these actively multiplying germinal cells into epithelial cells is easily observed. In some cases it would seem that the young germinal cells found in the germinal cell layer develop completely into epithelial cells, though in certain instances a division into an upper and under cell seems to occur, and the upper cell alone becomes an epithelial cell. In the case of the large multi-nucleated germinal cells, and also in young epithelial cells, there is often seen a growing out of one or more of the nuclei, accompanied with little cytoplasm forming a sort of horn which finally becomes separated from the parent cell, and passing through the epithelial layer is pushed out into the lumen of the alveolus. This peculiar action has given rise to an appearance in sections which has caused many to mistake this budding process for a wandering of leucocytes into the epithelium. In the extraordinarily marked nuclear multiplication in this germinal layer lies (according to Winkler) the solution of the much investigated question, "How do the epithelial cells multiply?" when multi-nucleated cells are seldom found in the epithelium, nor does mitosis often occur during lactation. The nuclei of these multi-nucleated cells are usually small, show no differentiation in their nucleoplasm, and so stain evenly and darkly, but in the developed epithelial cell the nucleoplasm exhibits the chromatin in sectors. The former cells also are more resistant and are not dissolved in the milk secretion, as the epithelial cells themselves often are when detached.

From the time of Rauber onwards the view seems to have been often held that leucocytes and lymphocytes are closely connected with milk formation. Rauber's original view that they are destroyed, and in their destruction are converted into the milk secretion itself, is now discarded, but many hold that during lactation there is a strong infiltration of the interstitial tissue by leucocytes, whence they pass through the epithelial cell-layer and enter the lumen of the alveolus. Winkler, however, maintains that the irregularly situated dark-staining nuclei seen in the epithelium are not those of leucocytes, but are the nuclei of "replacement cells," and this is true also of the early stages of mastitis before the secretion has become watery or bloody. The presence of fat globules in many of the cells found in milk clearly shows that these cells are epithelial, and not leucocytes. In these views he is in accord with Michaelis, who also maintains that leucocytes and lymphocytes play

no part in milk formation, and that if they do appear in large numbers it is a proof that suppuration is taking place or injury to the lymphatic vessels has occurred.

We may sum up the views of Winkler and Michaelis briefly by saying that, according to them, "the cells found in normal milk are chiefly young epithelial cells and cells of the germinal layer which have been detached, or thrust out into the lumen of the alveolus and so appear in the milk stream; that over activity of the germinal layer, and consequent increase in the number of these cells, may be the effect of change of food or some disturbance in milk formation, and is not indicative of disease; and, further, that in the early stages of mastitis it is the epithelial layer that is attacked by streptococci, and the destruction of this layer rouses the germinal layer into great activity, with a consequent increase of cells appearing in the secretion, but that no large number of leucocytes and lymphocytes are likely to be found until the mastitis is so far advanced that it shows itself by macroscopic changes in the milk itself; and finally that the multi-nucleated cells found in milk, and usually mistaken for polymorpho-nuclear leucocytes, are really detached young epithelial cells."

With these views we are in complete accord, and on account of the importance now being attached to the presence of multi-nucleated cells in milk, we have considered it advisable to bring these views forward at once, only appending a general statement of facts now in our possession in support of them, leaving the full statement of these until the work now in hand shall be completed.

This confirmation of Winkler's views has been largely brought about by an improvement in the method of staining these cells, which has enabled us clearly to demonstrate that in general structure, etc., these cells for the most part are clearly not leucocytes. That leucocytes do appear is evident from the fact that by this method of staining the presence of true eosinophile cells is easily demonstrated, but they are very few in number, and the vast majority of the cells tally in every particular with those described by Winkler.

Another important point hitherto apparently quite overlooked is that, in spite of the fact that these cells have often been for a considerable time in a liquid containing many bacteria, and under conditions in which normal leucocytes would retain their activity, phagocytosis is practically never seen, and this is true also when an invasion of the epithelium by streptococci has taken place, and destruction of its cells has followed.

We can briefly sum up those facts which support the view that the cells found in milk are for the most part not leucocytes as follows:

(1) The cells present in milk (the so-called leucocytes) are very diverse in nature, and when critically examined, the majority distinctly differ from leucocytes.

(2) However fresh the milk may be, the vast majority of the cells in it never stain like active leucocytes with ordinary blood stains.

(3) Though many multi-nucleated cells are present, the majority of these are distinctly different from polymorpho-nuclear leucocytes.

(4) The cells present in milk, however fresh, are scarcely ever amœboid.

(5) Ingestion of bacteria by the cells present (phagocytosis) is practically absent.

(6) In milk obtained from perfectly healthy cows these cells may occur in vast numbers, and since the mammary gland in structure resembles other glands, it is against analogy that vast numbers of leucocytes should occur in its secretion.

(7) The cause of the presence of a considerable number of cellular elements at times when there is no obvious reason, such as in quarters of the udder which have a previous history of mastitis, etc., but have recovered, is easily explained if these cells are tissue cells and not leucocytes.

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