THE PHAGOCYTOSIS OF INDUSTRIAL DUSTS IN TISSUE CULTURES

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In the investigation of histological sections of lungs affected by dust it is found that one of the most characteristic appearances is the phagocytosis of the dust particles. This tissue reaction is observed in all cases and is quite independent of the nature of the inhaled material and of the stage of development of the disease. It may occur in the alveoli or in the interstitial tissues.

Regarded generally as a pathological phenomenon it is noted that the dust may derive from living or dead material and, if the fate of the particles after inhalation is studied, then it becomes clear that only those smaller than 10μ are found in the alveoli. On the passage over the terminal reticulum in the alveolar walls, the irritation affects the terminal capillaries with consequent peristatic hyperaemia and slight contraction of smooth muscle. At the same time the phagocytes in the alveolar walls become detached and the dust particles aggregated on the alveolar walls are phagocytosed. In this process polynuclear leucocytes play no part.

In the next stage, as a result of diapedesis, uniformed, and later, formed, elements of the blood enter the alveoli, the sequence being one of albumin, globulin, leucocytes, and erythrocytes. This condition may be described as a catarrhal, desquamative, alveolar pneumonia. Only now do leucocytes with lobulated nuclei assume their role in phagocytosis of the dust particles. A small proportion of the alveolar phagocytes reach the exterior with the exhaled air but the greater part enter the lymphatics of the alveoli and interstitial tissue and are carried away. Concomitantly some free dust particles arrive in the interstitial tissue.

It is not our intention to describe the pathology of pneumoconiosis beyond this stage, for what is described will suffice to show that phagocytosis plays an important part in the defences of the body in this disorder. That this is so is further supported by the fact that phagocytosis in the manner we have described may take place in a few minutes, as has been shown in animal experiments. It therefore appears reasonable, without adopting an argument too narrowly teleological, to regard it as of fundamental importance for the process of regeneration. Every experienced histologist of the pulmonary system, and particularly those who have performed animal experiments, will confirm these observations.

We thought it appropriate to study such a characteristic phenomenon as phagocytosis in isolated tissue cultures with regard to the Leitstaub* problem and with special attention to the ingestion of dust particles. In this work we noted that the cellular activities occurring in the lung may be reproduced to a considerable extent in tissue cultures (Figs. 1, 2, and 3). Also, in tissue cultures a large quantity of the dust is phagocytosed by a certain group of cells. It is noted that the capacity of the macrophages to ingest is directly related to the conditions of growth. If the amount of available dust is excessive and the conditions are favourable the macrophages are filled with particles. Those particles which cannot now be ingested remain extracellular, as our experiments with dust counts have shown. In tissue culture aggregations of dust cells and the formation of spaces quite free from dust may also be observed. The appearance is similar to that seen in lungs in which some areas are affected by dust and others remain free.

The similarity of these reactive mechanisms induced us to go further than mere morphological observations and to study phagocytosis quantitatively with various kinds of dusts and dust mixtures.

* "Leitstaub", literally translated means "guide dust", for instance aluminium, used for the prevention of silicosis.
It is important to discover a test which will show, as early as possible, the silicotic action of any given dust. By means of a considerably improved technique, to which we shall return later, it is hoped to establish the relationship between the degree of activity of a dust in provoking silicotic changes and the degree of phagocytosis.

Up to the present most attempts to determine the silicotic action of dusts have been made in experiments with rabbits extending over several years. In any case the period necessary for investigation exceeded at least a year in order to make certain that silicotic changes would be seen. More recently Rüttner (1950) has performed experiments in which dust was applied to the peritoneum of mice and in this procedure silicotic changes could be seen even after as short a time as one month. The histological sections then showed typical concentric nodules of silicosis. An even more rapid method for estimating the effect of dust is the intratracheal injection of dust in rats as practised by Kettle (1932) and which King (1950) later used successfully in extensive tests. A further method of investigation using rabbits is the intraocular application of dusts which may produce silicotic changes very rapidly.

Fenn (1922) was the first investigator to study
the phagocytosis of the most important dust of all, i.e., free crystalline silicic acid. He used suspensions of leucocytes in his experiments and considered that the degree of phagocytosis occurring with any given dust gave information concerning its power to injure the lungs. He thought that the greater the phagocytosis the less dangerous the dust, presuming the dust at least was removed from the primary focus. His experiments seemed to support this view since coal dust is much more readily phagocytosed than quartz dust. These results were not confirmed by Franks and Watt (1934, 1937) and Kasten (1939) who found phagocytosis of quartz as good as that of coal dust.

Since then the general validity of this thesis is no longer accepted and recent experiments have concentrated more on the morphological changes in the cell which is taking up dust particles. Policard (1941, 1944) was the first to use for this purpose tissue cultures derived from chick embryo lungs. Lauche (1931) used embryonic splenic tissue while Franks and Watt (1937) employed suspensions of leucocytes, and later Kasten (1939) made use of splenic macrophages. Up to the present, the results, with regard to the phagocytosis index and the morphological changes observed in the cells caused by various kinds of dust, have not permitted any general agreement to be reached.

We were encouraged to study the same problem on a large scale because, with the help of the phase-contrast microscope, we could expect a better evaluation of the phenomenon of phagocytosis. The first investigations of this kind were made in our institute by Fischer in 1951. Work concerning the phagocytosis index done up to the present has always dealt with the estimation of the relative proportions of total macrophages to those which had ingested particles (Index I). When we got a better view of the dust with the phase-contrast microscope it was possible to count with great accuracy the individual dust particles in the cells and thus establish an index of the total number of phagocytosed particles per 100 phagocytes (Index II).

Our own experiments, which were carried out in association with Fischer, should not only show the differential phagocytosis of various kinds of dust but we also studied the phagocytosis index of quartz dust when mixed with protective dusts, i.e., dusts of mineral origin which themselves are not dangerous to the lung, and which when mixed with quartz dust are supposed to diminish or even prevent its pathological action (Jötten, 1944; Gärtner, 1950).

**Methods**

The investigations carried out in this work were made with spleen macrophages prepared by Carrel's method as described by Fischer. Explants were obtained from the spleen of 13-day chick embryos. This embryonic tissue is particularly rich in phagocytes (Fig. 4). The culture medium consists of chicken blood plasma and embryonic extract which is obtained by centrifuging a finely divided 9-day chick embryo. To prevent coagulation the chicken blood is drawn into tubes coated with paraaffin wax. The plasma fraction of the medium through coagulation forms a very suitable support for the aberrant cells. The cultures are made on cover-slips. The spleen is divided into fragments each the size of a pin head; using a micropipette 0.015 ml. of plasma is put on a cover-slip and a fragment of spleen is put in this to form a mother tissue. Then 0.015 ml. of embryonic extract is added and the two fluids are well mixed with a preparation needle. The drop is drawn out uniformly to a diameter of 13 to 15 mm. The work must be done quickly for coagulation soon occurs. When it is completed the cover glass is picked up with the hollow ground slide, smeared with paraaffin in such a way that the drop lies exactly in the hollow centre of the slide. It is sealed all round with paraaffin wax and the culture is incubated for 48 hours in the incubator at a temperature of 38–39°C. Then it is transplanted into a fresh medium.

The mother tissue is lifted out of the medium, which

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**FIG. 4.—Spleen culture after second transplant and 48 hours' incubation. Central mother tissue with radiating fibroblast formation and peripheral macrophages. (× 175.)**
is now mostly fluid, washed in Ringer's solution for a few minutes and then re-implanted into fresh medium in the manner already described. Although considerable precautions are taken, it is not always possible to prevent bacterial contamination of the cultures. For this reason we add to the medium penicillin in a strength of 50 I.U. per ml. The addition of penicillin has no visible effect on the growth of the culture or on the behaviour of the cells. Only if the strength is increased to 1,000 I.U. there is a slight retardation of growth, whilst complete inhibition of growth occurs when the medium contains 25,000 I.U. and more (van Marwyck, 1949).

The cultures are most conveniently brought into contact with dust by adding a suspension of the dust material in Ringer's solution (Kasten, 1939). To 0.01 ml. of plasma 0.01 ml. of the dust suspension is added; the spleen culture is placed in the mixture and then 0.01 ml. of embryonic extract added. The distribution of the dust on the surface of the coverslip is not much altered in the process of mixing. The macrophages are seen to grow out mainly in this plane whereas at other levels, where many small round cells are observed, there are few macrophages. The size of the dust particles must not exceed 8 μ as those larger than this are poorly phagocytized or perhaps not at all. In silicotic lungs the quartz particles do not exceed 5 μ (Mavrogordato, Böhme, and Lucanus, 1943-44). As a rule they are about 1 μ in size or even less (0.1-10μ Scheid, 1932).

The time of the first subculturing is chosen for the addition of the dust, for it is found that in subcultures the number of round cells diminishes while the fibroblasts increase; the former eventually disappear. The outward growth of the cells begins in a very short time and in six hours it is very active. At this time hardly any phagocytosis is seen but numerous cells are adjacent to the dust particles. After 12 hours the particles have been ingested and the cells have spread over a larger area. The phagocytes, which have already taken up some particles, also move to the periphery and take up any further dust they may meet. Very soon the immediate neighbourhood of the mother tissue is almost free of dust but beyond this the field is full of phagocytes in a broad ring, many of them packed with dust particles. Beyond this again only dust particles are seen but no cellular elements.

For the first 24 hours the cells do not exhibit much fatty change and the dust particles are therefore easily detectable under the phase-contrast microscope. The quartz particles are seen as brightly illuminated angular structures against the darker body of the cells and these again stand out against the medium which is somewhat brighter. Other dusts such as black aluminium in any case give a good contrast as they are so impervious to light. This also holds good for English red (an iron pigment) which has been chosen for special preliminary study as it stands out so well (Fig. 5). The condition of the cultures after 24 hours' incubation is best for the visualization of the individual dust particles, since, at this time, no fatty change of the cells has begun. After 48 hours the cells may be so fatty that smaller quartz dust particles than those seen in Fig. 6 are barely distinguishable from the fatty droplets in brightness and colour and can only be made out because of their angular shape. For these reasons the preparations were always evaluated between 13 and 44 hours. Counts were made at intervals in the circular zone of macrophages and during the period stated. As this zone slowly moves centrifugally and additional dust particles lying distally are ingested, different counts are to be expected when counted at different times. The differences between the figures were eliminated by readings at fixed intervals in each series of examinations. In each case 100 macrophages were counted and of these the number of phagocytes containing dust particles was recorded (Index I). The particles remaining in the free field were also counted. In this way it was possible to compare similar dust-cell fields. No counts were taken into consideration, where no or great masses of free dust particles were to be seen. Furthermore the
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number of particles contained in every cell of 100 dust cells was counted (Index II).

Counts were made in those parts of the cultures where the optimal conditions were found, namely where growth was vigorous and sufficient dust particles were available. It is evident that the degree of phagocytosis observed must be related to the amount of available dust, for if few particles are available few can be taken up, though the capacity of the cells to do so may still be considerable. The migration of the cells is not specifically directed towards the particles since the medium itself acts as a foreign body irritant; merely what lies in the way is automatically ingested.

We chose among the protective stone dusts those which were already used in animal experiments as described by Jötten (1944). These protective dusts, used in mining, comprised the following mineral substances. (1) Limestone (CaCO₃) has been successfully employed by Emmons in test tubes experiments and by Jötten in animal experiments. (2) Sucal powder, a calcium-sulphur combination somewhat like pure gypsum, has been found useful in practical tests carried out in the mines of the Siegerland and also in animal experiments conducted by Jötten. (3) Bolus alba is a pure hygroscopic aluminium silicate which Jötten has also used in animal experiments. (4) Metallic aluminium, in the form of “black aluminium”, so termed to describe the colour of the aluminium powder, which is determined by the size of the particles: using this kind of aluminium powder Denny, Robson, and Irwin (1937a and b, 1939) performed their successful experiments with animals and human beings. (5) Metallic aluminium (silver-bronze aluminium), is chemically the same material as the former one but it is distinguished from it by a somewhat larger particle size. With this material Jötten and Eickhoff (1942) and van Marwyck and Eickhoff (1950) did their animal experiments and stated the pathogenicity of this aluminium dust.

The particle size of all the materials described lay around 3µ, but aluminium black had a particle size of about 1µ. For the tissue culture tests the most suitable quantity of dust was found to consist of 30 to 40 mg in 15 ml of Ringer’s solution.

Results

The results of the principal experiments on the phagocytosis of dust are illustrated in Figs. 7, 8, and 9. The mean values of all counts are given, which were made between the thirteenth and forty-fourth hour. In the examples given in Figs. 7 and 8 the mean curve for quartz is included. This starts with high readings and then gradually climbs.

Fig. 7.—Graph of phagocytosis of quartz and calcium carbonate separately and mixed. Mean curve from all readings taken. Readings between 13 and 44 hours.

Phagocytosis of pure quartz: for every 100 cells counted, 61 have taken up particles, and 100 cells containing quartz have ingested altogether 312 particles.

Phagocytosis of pure limestone (CaCO₃): for every 100 cells counted, 62 have ingested particles. In 100 cells containing these a total of 334 was ingested.

Phagocytosis of the quartz-limestone dust mixture: for every 100 cells counted, 64 have ingested particles. In 100 cells exhibiting phagocytosis there were altogether 370 particles.
The phagocytosis effect (Index II) which is obtained by admixture of the various protective dusts it is noted that the addition of CaCO₃ makes little difference to the number of particles ingested. When quartz dust is mixed with Bolus alba the effect is also not particularly marked, the increase being about 18%. But this figure may well lie within the limits of error of the experiment. When sucal dust is used the figure rises to 38.8%. This result is particularly striking since it must signify an increase in the number of quartz particles ingested, for, after the dusts had been mixed, the sucal dust had dissolved in the suspension and its particles could no longer be enumerated.

The shape of the quartz phagocytosis curve before and after the addition of metallic aluminium black (Fig. 8) gives a striking illustration of the course of phagocytosis which results. This is strong evidence that the curves shown do, in fact, bring out the true facts of phagocytosis. The curve for the pure substance lies high to begin with and then falls slowly, whereas the curve for the mixture begins at a considerably lower level but then rises to a maximum before falling again to the right, as does the quartz curve. It appears therefore that the number of cells containing only a few quartz particles is small after aluminium black has been added; furthermore the number of cells containing numerous dust particles has greatly increased. In fact the increase after the addition of the aluminium black was 58.9%, a figure which we did not obtain with any other protective dust.

In conclusion we will refer to the experiments with silver-bronze aluminium, a material which was probably heavily coated with hydroxide after moist sterilization. An increase of phagocytosis when this substance was used was no more evident than when CaCO₃ was used.

### Variety of Dust

<table>
<thead>
<tr>
<th>Variety of Dust</th>
<th>Degree of Phagocytosis (Index II)*</th>
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<tbody>
<tr>
<td>Quartz</td>
<td>100%</td>
</tr>
<tr>
<td>Quartz + CaCO₃</td>
<td>100%</td>
</tr>
<tr>
<td>Quartz + metallic alum.</td>
<td>100%</td>
</tr>
<tr>
<td>Quartz + Bolus alba</td>
<td>118%</td>
</tr>
<tr>
<td>Quartz + sucal</td>
<td>138.8%</td>
</tr>
<tr>
<td>Quartz + black metallic aluminium</td>
<td>158.9%</td>
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*Fig. 9.—Graph of augmentation of quartz phagocytosis by guide dusts (Index II).*

The results of these investigations, particularly those with sucal and, notably, the complete absence of any demonstrable effect with silver-bronze aluminium, taken in association with the other experimental work in this institute, have great significance. In animal experiments, the addition of sucal powder prevented the development of silicosis whereas, when silver-bronze aluminium dust was used with the same noxious agent, there was not even a retardation of the silicotic changes.

It was only after the war that it became known to us that the Americans, in their animal experiments, had used aluminium black, and that this particular material very much delayed and even prevented the development of silicotic changes in these animals. These results with aluminium black were recently confirmed by King (1950), who also found marked retardation in the development of silicosis in rats.
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In summarizing our results it appears that the greater the power of a protective dust to stimulate phagocytosis of quartz particles, the greater is its protective value against silicosis. Our current experiments with tissue cultures are designed to bring supporting evidence for this thesis by observations of the specific-morphological cell reactions as seen in tissue cultures.

Summary

By using phase-contrast microscopical methods it was possible to enumerate exactly the dust particles taken up by phagocytes in tissue cultures. Based on this fact we used two methods of observation of the phagocytosis index, which were these: (I) the relative proportions of total macrophages to those which had ingested particles; (II) the number of dust particles taken up by 100 dust cells. It was found that the first index, hitherto in use, gives always the same results. This method is not sensitive enough, but the second index gives constant measurable figures and striking differences for the different dusts.

Based on the evidence of Index II we found that the protective mineral dusts increased the phagocytosis of quartz particles as follows: bolus alba, 18%.; sucal (gypsum), 38%.; metallic aluminium (black), 58%.; limestone (CaCO₃) as well as metallic aluminium (silver-bronze) had no effect whatever.

In relating these results to those of our own animal experiments with protective stone dusts, it appears probable that such protective dusts which promote phagocytosis are capable of reducing the silicotic action of quartz.

REFERENCES

Harrison, —, and Uhlenhuth, —. Cited by Fischer, A. Gewerbzüchtung. Munich. (2nd ed. 1927. 3rd ed. 1930.)