STUDIES ON MELANIN-LABELLED CELLS IN THE HUMAN SKIN WINDOW

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The physiological functions of leukocytes are performed extravascularly. The physiological events in which leukocytes participate are manifested by the inflammatory response and the healing of wounds. Tissue-culture methods do not reproduce either the physiologic milieu of inflammation or the temporal sequence of cellular migration and thus fail to provide an insight of interrelationship of the various leukocytes participating in the inflammatory reaction.

A method employed by Downey[1] in 1917 for the study of inflammation, was modified by Rebuck[2] for application to man and is now generally known as the 'Human skin-window technique'.[15] The technique allows serial sampling from the inflammatory response with very slight disturbance to the vital process being studied. Several American[3-7] and Continental workers[8-10] have made use of this technique for their studies.

It has been observed that melanin is acquired by the cells participating in the inflammatory response of Bantu and Cape Coloured volunteers.[12] The finding has made possible a study of the melanin-handling by cells in the inflammatory exudate,[11] and it was observed that such melanin-labelled macrophages could fuse to form giant cells.[13] A study on the phagocytic ability of the LE cells in

a Cape Coloured patient, was also performed.[14]

This is a communication on certain differences observed in the ability of various cells to become labelled with melanin and some differences observed in the inflammatory response in White and Bantu subjects. The effect of antigen employed in exciting the reaction, is also re-assessed.

Method

The skin-window technique consists of scraping away a small area of epidermis on the sterilized volar aspect of the fore-arm (or any other convenient site). The correct depth is indicated by fine bleeding points and a sero-sanguinous ooze. *On* this area a drop of antigen is applied to stimulate inflammation. The lesion is covered by a chemically clean, sterile, coverslip surmounted by a cardboard splint and firmly taped to the skin.[15] After approximately 30 minutes neutrophils start to migrate into the lesion and some become attached to the undersurface of the coverslip. The coverslips are removed at hourly intervals and replaced by fresh coverslips. The removed coverslips are quickly air-dried and stained by May-Gruenwald-Giemsa procedure (or after fixation in formalin-vapour histochemical procedures may be applied[15]). These preparations are mounted cell-side down on glass-slides and are permanent preparations which allow study under oil-immersion magnification.

Material

Normal Bantu, Cape Coloured and White subjects were studied as well as several patients without any apparent haematological disorder. The findings to be discussed below are the result of observations from over 100 lesions in more than 60 subjects and comprise more than 2,000 preparations.

OBSERVATIONS

Melanin-labelling

Matsumoto[16] in 1918 was apparently the first to use melanin in a study of phagocytosis. In tissue cultures Smith[17] found melanin to have some definite advantages over other substances then currently in use for the study of phagocytosis.

In the study of phagocytosis by the skin-window technique we found, as did others,[9] that the application of Indian ink to the lesion elicits a new initial granulocyte invasion of the inflammatory field. This constant phenomenon was circumvented in the studies of Riis[9] by applying Indian ink after the 24th hour of inflammation.

In the case of Cape Coloured and Bantu subjects homologous melanin granules are present from the initiation of the lesion, and result in minimal, if any, disturbance of the normal sequence of events.[11, 12] Visible granules were never observed in the cells before the 3rd to 4th hour of inflammation. The different cells label differently with melanin:

The neutrophils: The first cells to appear in the exudate are initially larger than in the blood, with oedematous-looking nuclei (Fig. 1). From the 3rd hour onwards small dark granules appear and at a subsequent stage they condense to form larger granules (Fig. 2). Approximately 20% of cells may show melanin granules.

Basophils and eosinophils. We have never observed ingested melanin granules in basophils and eosinophils though in some instances the response had a strong basophilic or eosinophilic composition.

Monocytes and tissue macrophages. Large clumps of melanin may be observed in these cells giving a striking appearance to the exudate, at an early stage. Monocytes and tissue histiocytes could not be differentiated with confidence. They strengthen the neutrophilic reaction between the 4th and 9th hour (Fig. 3).

Lymphocytes. The acquisition of melanin by these cells has been described in detail elsewhere.[11] Small granules are

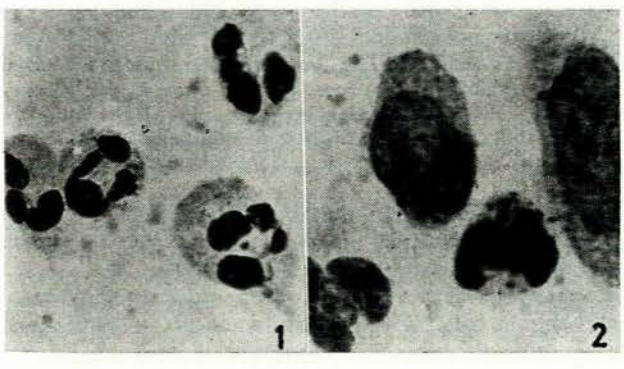
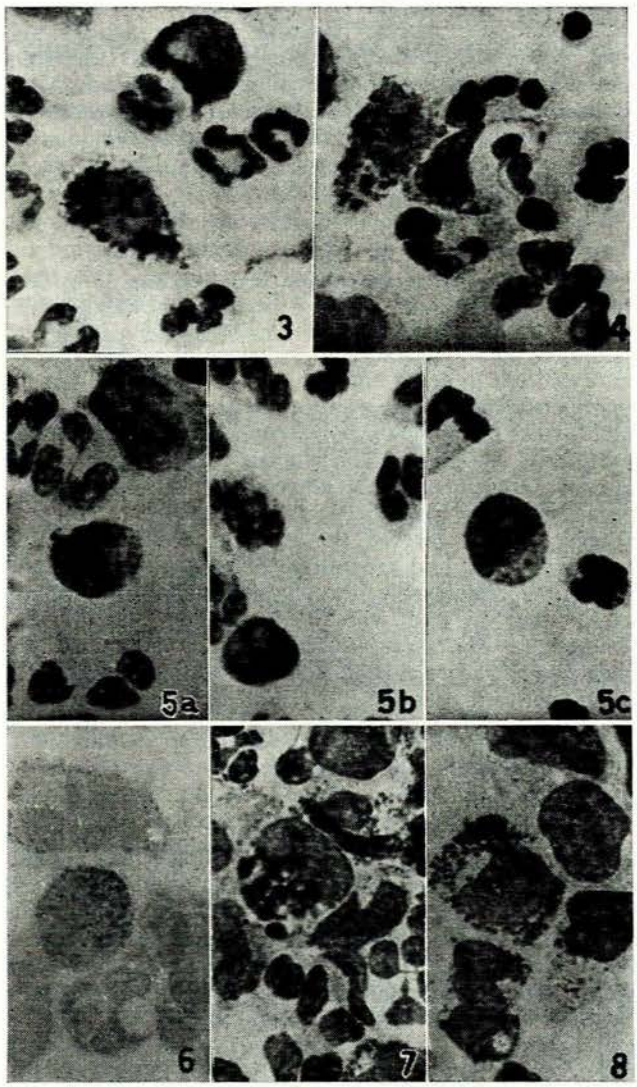


Fig. 1. First-appearing neutrophils, 4th hour of inflammation—Bantu subject. Specific granules sparse, but contain dark green-black melanin particles.
Fig. 2. Shrunken neutrophil (12th hour) showing clumped and larger melanin granules. The two mononuclear cells are hypertrophied (monocytoid) lymphocytes. Note clumped nuclear chromatin.



Lymphocytic and monocytic type of labelling contrasted: *Fig. 3.* Response with antigen (11th hour). Lower left: monohistiocytic cell-type with large clumps of melanin, above: centre, slightly hypertrophied lymphocyte with small discrete melanin granules. *Fig. 4.* Same as Fig. 5. The monocyte with wavy nuclear chromatin contains clumped particles while the lymphocyte in the centre is engaged in phagocytosis of a neutrophil. The thin rim of cytoplasm contains minute, discrete, melanin granules. *Fig. 5 a, b, c.* Typical melanin-labelling in early-appearing lymphocytes, still morphologically similar to the blood lymphocyte, i.e. between 9th and 12th hour (small discrete particles). *Fig. 6.* Monocytic type of melanin-labelling, about *6th hour* of inflammation. (Dopa-stain, nuclei counterstained with haematoxylin.) Large clumps in early-appearing mononuclears. *Fig. 7, 8.* Lymphocytogenous macrophages with melanin granules (18th hour of inflammation).

acquired at a stage where these cells are morphologically similar to lymphocytes in the peripheral blood (Figs. 4, 5 a, b, c). Free melanin granules were not observed in the exudate except in the vicinity of disrupted cells and it is believed that all or most of the melanin granules in lymphocytes are acquired from neutrophils. This does not exclude the possibility that some of these granules were phagocytosed from the exudate. Melanin-labelling furthermore only becomes obvious from the stage where neutrophil-lymphocyte interaction becomes marked.

Hypertrophying lymphocytes. These cells acquire progressively more melanin as they hypertrophy through a monocytoid stage to macrophages, but in each individual studied the number of lymphocytes, monocytoid lymphocytes and macrophages containing melanin remain fairly constant from hour to hour. Monocytoid lymphocytes, in general, label similarly to monocytes (Figs. 6. 7 & 8).

THE INFLAMMATORY REACTION TO VARIOUS ANTIGENS

The normal inflammatory reaction has been adequately documented since the early studies of Metchnikoff[30] as well as the observations in the skin window.[5, 8, 18]

One of the characteristics of the reaction is the regular sequence of morphologic events which occur.[5] During the first few hours neutrophils appear, strengthened at about the 4th to 6th hour by haematogenous monocytes and tissue histiocytes and occasional lymphocytes. This phase suddenly yields to an influx of small to medium-sized haematogenous lymphocytes which hypertrophy to become, eventually, macrophages. This sequence of events will not be considered in detail but it should be mentioned that the hourly transition of the average cell population constitutes the evidence for lymphocyte-macrophage transition and not transitional cell-forms, which occur at all stages of the reaction. Transitional cells intermediate between lymphocytes and macrophages occur at all stages of the reaction. At every hour, however, the average cellular population shows a gradual and progressive differentiation from predominantly lymphocytic, through a predominantly monocytoid stage to one with predominantly macrophages. Changes in the average cell-population constitute the evidence for transformation, and not transitional cell-types, which represent cells lagging behind or being in advance of the average population as regards their stage of development.

Without Antigen

In 24 Cape Coloured and Bantu and in 3 White volunteers a lesion was made and no antigen was applied, the stimulus to inflammation being only the trauma and the irritation of the coverslip. In White subjects the granulocytic phase yields to a mononuclear response which appears to be mainly monocytic and histiocytic in type. The degree of cellularity tends to be poor to average and the lesion heals between 18 and 24 hours.

In the Bantu, a more cellular exudate was obtained with stronger neutrophil participation. More lymphocytes, from the 6th hour, could be observed than in Whites, but the dominant cell-type was a mononuclear one which resembled monocytes rather than lymphocytes, being approximately the size of large lymphocytes in the peripheral blood. These cells cannot be distinguished from tissue histiocytes but this histio-monocytic cell-line is easily distinguishable from the small and medium lymphocytes as observed in the peripheral blood, by a blue-grey rather than sky-blue cytoplasm and a more wavy nuclear chromatin rather than the clumped chromatin pattern of lymphocytes. These cells acquire large clumps of melanin very early in the response and so distinguish them from the lymphocytic type of labelling.

With Non-immune Antigen (egg-white)

In Whites the response follows the same general pattern as that described by several investigators,[3, 5, 8] resulting first of all in a granulocytic exudate, enhanced by monocytes and tissue histiocytes from the fourth hour, but being suddenly replaced by a lymphocytic infiltrate at approximately the 12th hour. These cells resemble the small and medium lymphocyte in the peripheral blood. After phagocytosing small amounts of neutrophilic cytoplasm these lymphocytes undergo hypertrophy to form first monocytoid lymphocytes and later macrophages.

The Bantu reaction differs from the White in a richer initial granulocytic phase and an earlier appearance of lymphocytes (about the 9th hour). The granulocytic response yields to lymphocytic influx in the same way as in the White but not as completely. The granulocytes persist throughout the response and small lymphocytes are found up to the 18th hour, though the average lymphocyte population undergoes the usual hypertrophy.

The melanin-labelling of cells clearly distinguishes this type of reaction from the monocytic-histiocytic type, referred to above.

The granulocyte appears to be a prerequisite for collecting the melanin granules and transferring them to the small lymphocyte, since, with the decline of the neutrophilic phase and the increase in lymphocytes, the number of labelled lymphocytes and hypertrophied forms remains approximately constant.

With Immune Antigen

This was studied in three subjects: A White, (on whom the other two types of reaction were previously studied), was studied after taking his first oral polio-vaccine. This subject did not have previous Salk vaccinations. Sabin live-virus vaccine was applied to the lesion and his peripheral blood showed antibodies to all three types of polio virus (SAIMR). The reaction was basically the same as observed to egg-white, except that the lymphocytic phase occurred earlier (9th hour) than before.

In two Bantu subjects, one having a Mantoux test positive to 1/100 but negative to 1/1,000 tuberculin (J.D.), and the other positive to 1/1,000 (N.F.), were studied using tuberculin as antigen. The same basic response occurred as to egg-white in the case of J.D. except for a very strong lymphocytic participation at the 10th hour of inflammation. In the case of N.F. an extremely cellular preparation was obtained showing the lymphocytic influx at 5¼ hours with hypertrophied (monocytoid) lymphocytes at 8½ hours. From this stage onwards there was a marked eosinophil participation in the response. The effect of antigen on the cytology of these lesions are shown graphically in Fig. 9. These studies revealed that in all circumstances the Bantu show a stronger and longer sustained participation of neutrophils in the reaction, in contrast to the Whites where lymphocytes sharply replace the neutrophilic phase. Furthermore, the lymphocytic phase appears earlier in the average Bantu preparation than in the case of Whites.

DISCUSSION

The finding of melanin granules in lymphocytes, which may hypertrophy to macrophages as observed in this study, establishes the small lymphocyte as a reticulo-endothelial cell with latent potentialities in the blood; these potential abilities becoming manifest on physiological stimulation.

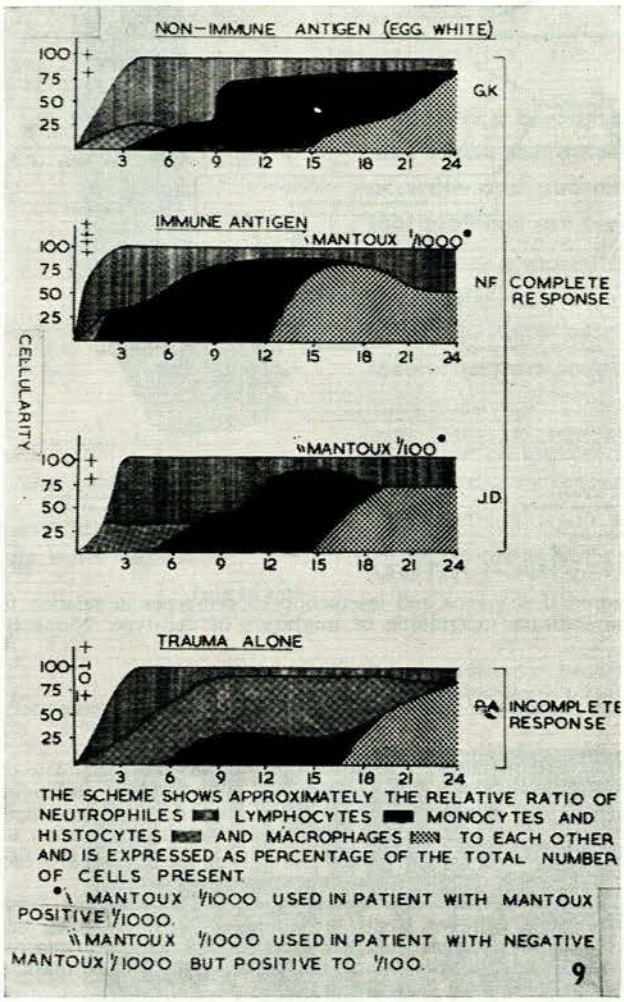


Fig. 9. Schematic representation of the cellular events elicited by different antigens: GK.=Non-immune antigen. N.F.=Immune-antigen (1/1000 tuberculin pos. patient to tuberculin). J.D.=Immune-antigen (1/1000 tuberculin neg., 1/100 tuberculin pos. to tuberculin). P.A.=No antigen. 'Incomplete' response.
Cellularity of response arbitrarily gauged + to ++++. Based on differential counts at hourly intervals. (Reproduced by permission.[12])

The role of neutrophils in activating this hypertrophy (or differentiation) of lymphocytes was first suggested by Rebuck,[18] and was confirmed by Page and Good[5] by the above technique in a patient with cyclic neutropenia, and also in animal studies. Our studies suggest that an equally essential role pertains to the phagocytosis of particulate matter (melanin) whereby neutrophils collect and condense the smaller particles for subsequent transfer to the lymphocyte,[12] which, by virtue of being activated by the neutrophils, hypertrophies to a macrophage.

The delineation of the reticulo-endothelial system (RES) depended on the demonstration of the ability of such cells to collect certain vital dyes. In their studies, culminating in the description of the RES, the lymphocyte was left out by Aschoff and Kiyono because of its failure to take up such vital dyes.[19]

The lymphocyte studied in tissue culture and in the inflammatory reaction does, however, take in the vital dyes as confirmed by Tschachin, Downey, Vierling, Maximov, Seki and Dougherty, and these studies were critically reviewed by Rebuck and co-workers.[19] With a silver-impregnation method, Marshall[20] classified cells as metalophile and non-metalophile, lymphocytes resorting under the latter group while the former belongs to the RES. By the skin-window technique Rebuck *et al.*[19] confirmed the metalophilic properties attained by lymphocytes from the ninth to twelfth hour, (a phase which coincides with the acquisition of melanin granules in our Bantu and Cape Coloured series), as well as its ability to store vital dyes.

The effect of the antigen on the inflammatory reaction had to be assessed. Our studies agree with the findings of Rebuck[18] and associates[21-23] who claim a complete response when antigen is used and an incomplete response without antigen. This is contrary to the findings of Perillie and Finch.[4]

As the latter authors specifically refer to *large* lymphocytes in their study, we are of the opinion that these cells, which in our study label with melanin in a way similar to monocytes and tissue histiocytes, are indeed small monocytes. This difference in melanin-labelling between monocytes and lymphocytes allows an important conclusion to be made regarding the type of mononuclear cells present. One of the main problems, and usual criticisms, of any study on the mononuclear cells in the inflammatory reaction, is whether or not such cells are lymphocytes or small monocytes. The antigen-antibody reaction is one of the strongest challengers to RES function, and our studies clearly show, as do those of Rebuck *et al.*[18, 23] that a challenging antigen markedly influences the nature of the reaction (Fig. 1). Without antigen the stimulus to inflammation appears to be adequately met by neutrophils, monocytes and histiocytes, but antigenic stimulation requires an additional contribution by small lymphocytes.

The influx of small and medium lymphocytes occurring on the antigen-stimulated lesion, not only exhibits morphological characteristics of peripheral blood lymphocytes with similar (measured) nucleo-cytoplasmic ratios, but also labels with fine discrete melanin granules, in contrast to the clumped granules of mono-histiocytic cells of the lesion not stimulated by antigen.

The failure of Perillie and Finch[4] to differentiate the small monocytes (or large lymphocytes) in a lesion without antigen, from the small lymphocytes which dominate the antigen-stimulated lesion, is a pitfall which led to a faulty interpretation in our early studies on Whites. The studies on non-White volunteers caused difficulties in interpretation and led to this more extensive study to confirm or refute the interpretation of Rebuck.

A further aspect of importance is the fact that even without antigen the Bantu exhibit a response which shows a stronger lymphocytic participation than in the studies on Whites. In all experiments the persistence of neutrophils in larger numbers than in White subjects is also very obvious. This finding may be the result of: (1) greater RES activity in the Bantu than in the White, or (2) the presence of a larger amount of melanin which continually requires an active RES component (similar in effect to the application of carbon particles as Indian ink in the skin-window in Whites).

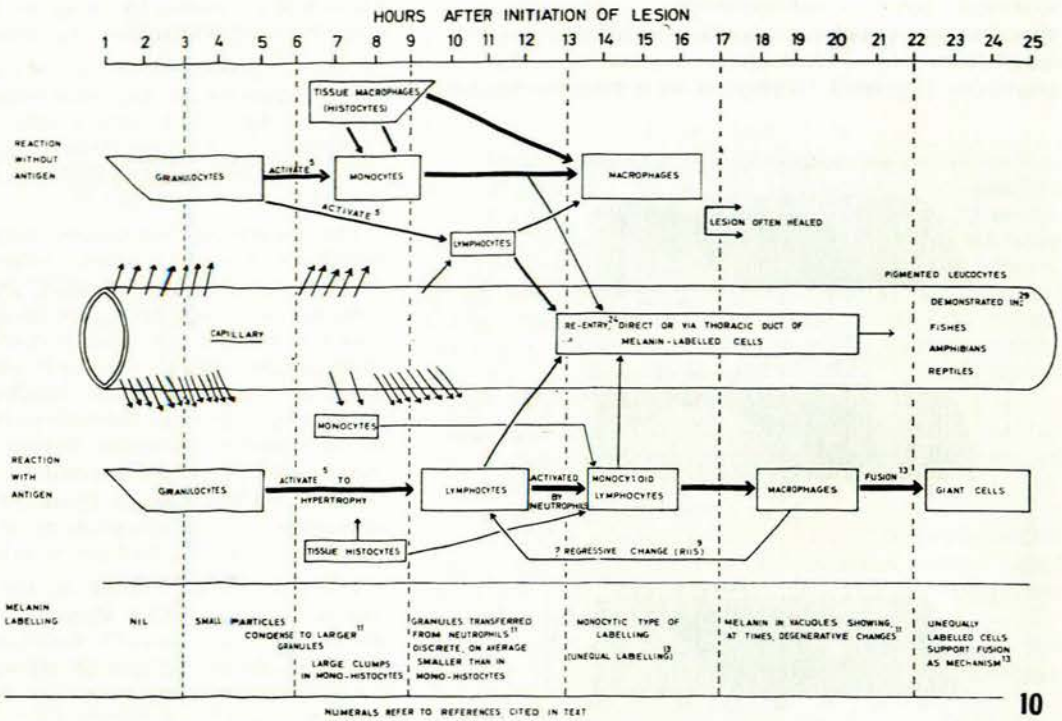


Fig. 10. Diagrammatic representation of temporal sequence and interaction of cell-types in relation to their melanin-labelling. Spacing of arrows indicates magnitude of migration of cell-type. Numerals refer to references cited in text.

Studies currently in progress at this laboratory favour the first explanation, but do not necessarily exclude the latter possibility.

Melanin-labelling of cells in the inflammatory reaction is used as a basis for constructing the diagram of the inflammatory reaction shown in Fig. 10.

In his studies by this technique, Riis[9] presented evidence for a regressive transformation of the inflammatory macrophage into a type of cell which appears to be morphologically identical with the lymphocytes of the peripheral blood. As a recirculation of lymphocytes and monocytes is strongly suggested by studies on the life-history of these cells,[24] melanin-labelled mononuclear cells would be expected to occur in the peripheral blood in normal Bantu and Cape Coloured subjects and would constitute definite proof of such a recirculation. Pigmented monocytes have been reported twice in the past century in the blood of man with melano-sarcoma.[25, 26] In such cases direct invasion of blood vessels by the tumour remains a possibility: if this could be excluded, then the monocytes must re-enter the circulation. It remains to be established whether or not such monocytes represent monocytoid hypertrophied lymphocytes or monocytes leaving and re-entering as such. Macrophages containing melanin have been found in the bone marrow of patients with melanoma[27] and increased melanin-deposition in the placenta was observed more frequently in women with a history of skin-lesions during pregnancy than in those without such a history.[28] A re-entry of mononuclear cells would allow such cells to transport melanin.[11] The demonstration of melanin-containing leukocytes in the blood of man would constitute definite proof of such a re-entry.

SUMMARY

1. Melanin-labelling of cells in the inflammatory exudate of non-White subjects studied by the skin-window technique allows early phagocytic events to be studied in a physiologic milieu and is superior for this purpose to the application of Indian ink which invariably reverts the reaction to a granulocytic phase.

2. The lymphocytes acquire melanin by transfer from neutrophils and are labelled by smaller granules which are subsequently clumped together as they hypertrophy to macrophages, while monocytes and tissue-histiocytes acquire massive clumps of melanin early in the reaction.

3. The skin-window as a 'window' on early reticulo-endothelial activity reveals the important role of the neutrophil, not only for the subsequent differentiation of small lymphocytes to macrophages, but also for the collecting and transfer of melanin to the lymphocytes, thus enhancing also the phagocytic properties of the cells. The effect of antigenic stimulation on the ensuing exudate could be studied, with results confirming the observations of Rebuck *et al.* of an 'incomplete' and 'complete' type of reaction, but does not fully agree with Perillie and Finch's finding of a complete type of reaction without antigen.

4. Cells labelled in the tissues, should be found in the bloodstream, if they re-enter. Evidence is quoted to support such a suggestion.

This study (and others referred to) points to the fact that melanin constitutes an additional pigment handled by the RES in Bantu and Cape Coloured subjects. This may be related to evidence of greater RES activity observed in the non-White reaction but it could not be ascertained whether this is a local phenomenon or holds true for the whole RES of the Bantu.

Some of the material discussed forms part of a thesis submitted for the M.D. degree at the University of Stellenbosch. I wish to thank my promoter, Prof. A. J. Brink, for his valued criticism and continuing interest in these studies.

REFERENCES

1. Downey, H. (1917): Anat. Rec., **12**, 429.
2. Rebuck, J. W. (1940): *Ibid.*, **76**, 46.
3. Rebuck, J. W. and Crowley, J. H. (1955): Ann. N.Y. Acad. Sci., **59**, 757.
4. Perillie, P. E. and Finch, S. C. (1960): J. Clin. Invest., **39**, 1353.
5. Page, A. R. and Good, R. A. (1958): Amer. J. Path., **34**, 645.
6. Boggs, D. R. (1960): Blood, **15**, 466.
7. Hu, F., Fosnaugh, R. P., Bryan, H. G. and Jacks, D. (1961): J. Invest. Derm., **37**, 409.
8. Braunsteiner, H., Paertan, J. and Thumb, N. (1958): Blood, **13**, 417.
9. Riis, P. (1959): *The Cytology of the Inflammatory Exudate*. Copenhagen: Munksgaard.
10. Wulff, H. R. (1962): Acta haemat. (Basel), **28**, 86.
11. Wassermann, H. P. (1963): J. Invest. Derm., **41**, 377.
12. *Idem* (1961): ‚Die effek van 5-hidroksitriptamien op die ontwikkeling van die limfosiet soos waargeneem in die inflammatoriese respons', M.D. thesis, University of Stellenbosch.
13. *Idem* (1963): J. Invest. Derm., **40**, 73.
14. *Idem* (1963): S. Afr. J. Lab. Clin. Med., **9**, 142.
15. Rebuck, J. W. (1958): *Methods in Medical Research*, vol. 7, p. 161. Chicago: Yearbook Medical Publishers.
16. Matsumoto, S. (1918): J. Exp. Zool., **27**, 37.
17. Smith, D. T. (1921): Johns Hopk. Hosp. Bull., **32**, 240.
18. Rebuck, J. W. (1947): 'Cytology of acute inflammation in man as demonstrated by two original technical procedures with especial reference to the role of lymphocytes', Ph.D. thesis, University of Minnesota.
19. Rebuck, J. W., Boyd, C. B. and Riddle, S. M. (1960): Ann. N.Y. Acad. Sci., **88**, 30.
20. Marshall, A. H. E. (1956): *An Outline of the Cytology and Pathology of the Reticular Tissue*. Edinburgh: Oliver & Boyd.
21. Rebuck, J. W., Smith, R. W. and Margulis, R. R. (1951): Gastroenterology, **19**, 644.
22. Rebuck, J. W., Monto, R. W., Monaghan, E. A. and Riddle, J. M. (1958): Ann. N.Y. Acad. Sci., **73**, 8.
23. Rebuck, J. W. and Lo Grippo, G. A. *in* Heller, J. H., ed. (1960): *Reticulo-endothelial Structure and Function*. New York: Ronald Press.
24. Yoffey, J. M. (1960): *Quantitative Cellular Haematology*. Illinois: Charles C. Thomas.
25. Liebman, E. (1929): Schweiz. med. Wschr., **59**, 597.
26. Goodall, P., Spriggs, A. I. and Wells, F. R. (1961): Brit. J. Surg., **48**, 549.
27. Sundberg, R. D. (1956): Anat. Rec., **124**, 368.
28. Ishizaki, Y. and Belter, L. F. (1960): Amer. J. Obstet. Gynec., **79**, 1074.
29. Jordan, H. E. *in* Downey, H., ed. (1938): In *Handbook of Haematology*, vol. 2. New York: Hoeber.
30. Metchnikoff, E. (1883): Biol. Centralbl., **3**, 560.