Antibodies Against Melanin

THE SIGNIFICANCE OF NEGATIVE RESULTS *

H. P. WASSERMANN AND J. J. VAN DER WALT, *Section of Clinical Immunology and Rheumatology, Departments of Internal Medicine and Anatomical Pathology, University of Stellenbosch and MRC Pigment Metabolism Research Unit, Karl Bremer Hospital, Bellville, CP*

SUMMARY

This study reports on unsuccessful attempts to produce antibodies against melanoprotein in rabbits. Available evidence suggests antibodies against melanocytes in the aetiology of vitiligo, but there is no convincing evidence for antibodies against melanin *per se*. It is suggested that the demonstration of antibodies against an immune serum, obtained from rabbits after injection of a melanoma homogenate (which contained cell debris), may be due to antibodies against unmelanized or incompletely melanized melanosomes.

S. Afr. Med. J., **47**, 7 (1973).

Negative results of experiments are usually not reported. They are usually mentioned incidentally in papers the titles of which give little indication that negative results are to be found in the publication. Apart from the difficulty in bibliographic research, contradictory findings may gain prominence without critical assessment of the methodology.

The interest in vitiligo as an auto-immune disorder results from (*a*) the acquired and progressive loss of functionally active melanocytes in the affected areas, and (*b*) the clinical association of the disorder with other auto-immune diseases.[1-3] No direct evidence of an antimelanocyte auto-antibody is, however, available. The demonstration of such an antibody, if found, could be the result of melanocyte destruction rather than the cause of the vitiligo.

The report by Langhof *et al.*[4] of antimelanin antibodies is important because of (*i*) its possible relationship, as often quoted, to an auto-immune aetiology of vitiligo; (*ii*) its significance regarding the current problem of melanin structure, whether it is a homopolymer, formed by the linkage of a single monomer (indole-5, 6-quinone) or a poikilopolymer, formed by the irregular random bonding of several types of monomers.[5,6] Our interest in the problem arose from the possible use of antibodies against melanin for an immunofluorescent technique, to identify extracutaneous melanin. This report concerns our attempt at the production of antibodies against natural melanoprotein, and a consideration of our negative findings.

METHOD I

Fourteen albino rabbits were used in the first experiment. Melanoprotein extracted from human hair[7] was suspended in Freund's adjuvant and injected subcutaneously into the groin of 10 rabbits, the 4 controls receiving Freund's adjuvant only. Cardiac blood for protein electrophoresis was obtained before, and at the conclusion of, the experiment. The initial injection was repeated at weekly intervals for 4 weeks. After a further 3 weeks melanin, dissolved in triethanolamine, was injected intravenously through the marginal vein of the ear. All injections contained about 5 mg melanoprotein.

Results

The rabbits showed no clinical reaction to the melanoprotein injections. The electrophoretic spectra showed no significant change, at the conclusion of the experiment, from the initial values. No antibodies against melanoprotein dissolved in triethanolamine, pyridine or dimethylsulphoxide (DMSO), or suspended in water could be demonstrated by precipitation tests, or on agar plates after 7 and after 14 weeks. Intradermal injection of a melanin suspension at the conclusion of the experiment showed no delayed (cell-mediated) hypersensitive response.

METHOD II

Because the extraction procedure may have changed the melanoprotein molecule, the experiment was repeated with melanin granules obtained from the pigment layer of baboon eyes. (The baboons were used in experimental surgery projects and were those which died because of the surgical procedure.) The melanin was obtained by lysis of the pigment cell layer in distilled water and differential centrifugation.

The only reagent used was distilled water, and the extract was microscopically examined to ensure that it was free from cell debris.

The same protocol as in method I was followed in the 4 rabbits who served as controls in the previous experiment. The results were again negative.

AUTOPSY FINDINGS

At postmortem examination most of the injected melanin, whether administered intravenously or subcutaneously, was found in macrophages in the lung, usually in the form of discrete collections of these cells (Fig. 1).

*Date received: 22 September 1972.

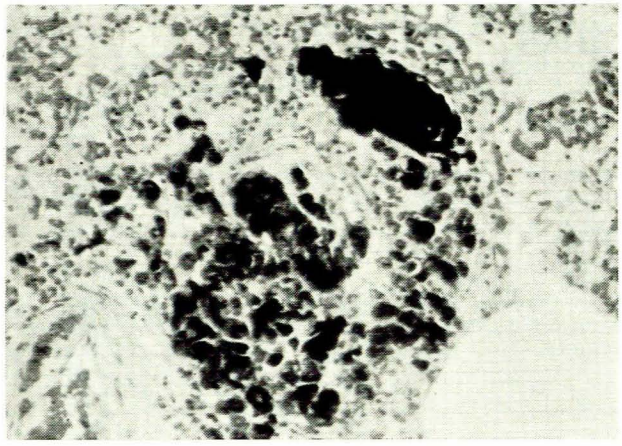

Fig. 1. Injected melanin in macrophages in the rabbit lung. This was seen with intravenously or subcutaneously injected melanin (× 150).

An interesting phenomenon is seen in Fig. 2, which shows injected melanin in the media of a pulmonary arterial branch. At the subcutaneous injection sites there was surprisingly little residual melanin, except in the initial experiments where hair shaft melanin was used. Here clumps of melanin could be found free, surrounded by neutrophils (Fig. 3). This is probably due to the relative crudeness of our initial melanin preparation. With the later more refined preparations using baboon eye melanin, very little residual melanin could be demonstrated subcutaneously. The usual appearance was that seen after injection of Freund's adjuvant, with large spaces, foam cells, occasional giant cells and epithelioid type granulomas (Fig. 4).

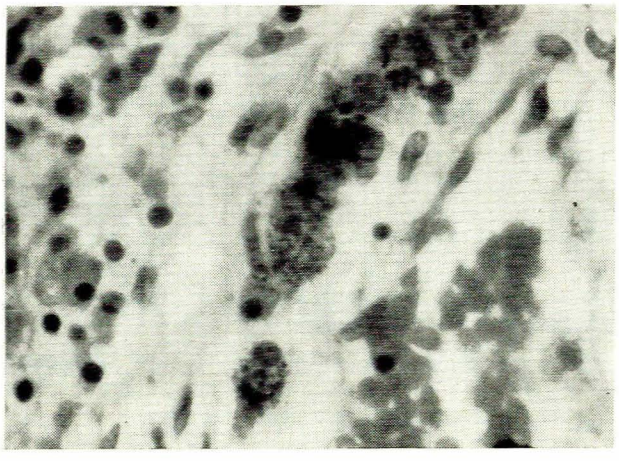

Fig. 2. Injected melanin in the media of a pulmonary arterial branch (× 600).

Regional lymph nodes were difficult to find in the rabbit, but in those located there was little melanin to be seen.

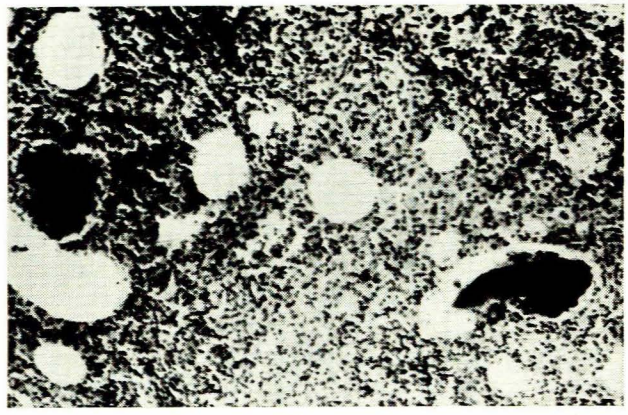

Fig. 3. Subcutaneous injection site after crude hair melanoprotein surrounded by neutrophils (× 150).

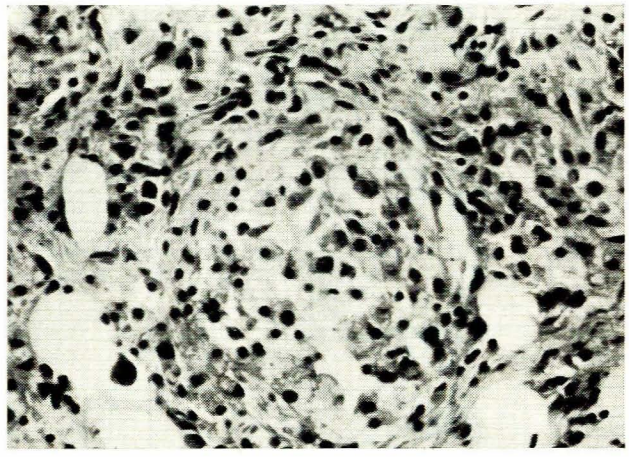

Fig. 4. Subcutaneous injection site after refined baboon eye melanin. Very little residual melanin; note absence of neutrophils (× 600).

Although the bulk of the injected melanin appears to have been filtered out in the lungs, considerable amounts passed through the lungs and could be demonstrated with ease in the reticulo-endothelial system, especially the spleen and bone marrow. This was especially true of those rabbits in which baboon eye melanin was used, as seen in Fig. 5, which shows melanin in the spleen.

Fig. 6 shows the injection site in a rabbit's ear, where melanin dissolved in DMSO had been administered intravenously. Obviously there had been some leakage from the vessel, and melanin is present free in the tissues, as well as in macrophages. One is again impressed by the absence of an effective inflammatory response to the melanin.

The postmortem findings thus indicate that the injected melanin was taken up by phagocytes, processed by macrophages and was widely present in the reticulo-endothelial system. There would seem to be no reason

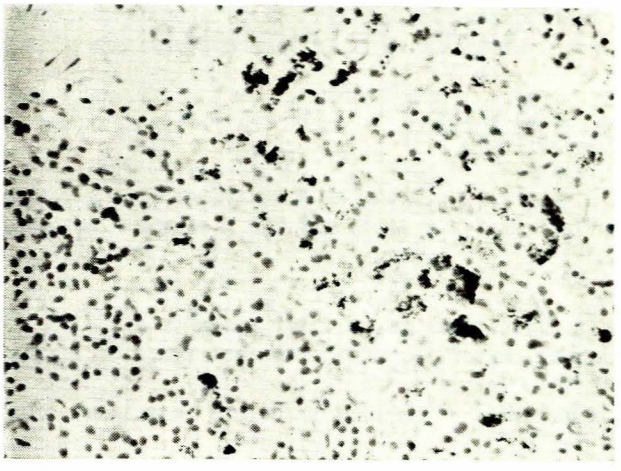

Fig. 5. Injected baboon eye melanin in the rabbit spleen (× 375).

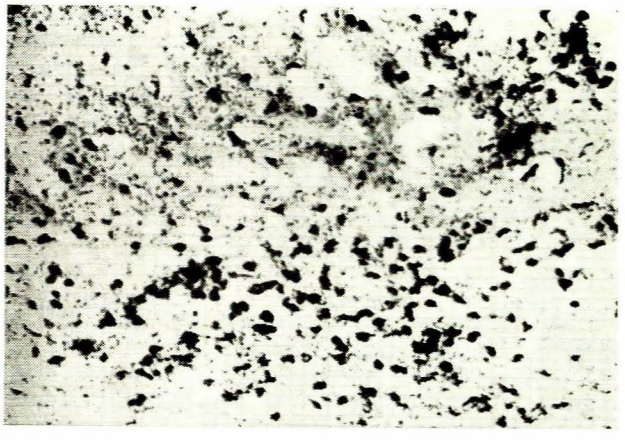

Fig. 6. Intravenous injection site of melanin in DMSO in a rabbit's ear. Melanin occurs free in tissues and in macrophages (× 375).

why demonstrable antibodies should *not* be formed against the melanoprotein. Especially puzzling in this context, was the apparent ability of Langhof *et al.*[4] to demonstrate such antibodies.

POSSIBLE EXPLANATIONS FOR OUR NEGATIVE FINDINGS

Melanin may, in fact, not be antigenic. In the case of synthetic melanin, prepared from DL-dopa with a ^{14}C label in the 2-carbon position, Blois[8] obtained negative results in his attempt to produce antibodies in the rabbit. Antibodies were sought in the sera by agglutination, precipitation, agar gel diffusion and passive transfer into guinea-pig skin. Furthermore, Blois[8] found that the clearance of an intravenous dose of labelled melanin by the experimental animal was no greater than in the control. This study also supports the poikilopolymer theory of melanin structure as pointed out by Blois:[8] due to the random polymerization of several monomers, antibody formed against one molecule of melanin would not be able to 'recognize' a second molecule.

Langhof *et al.*[4] demonstrated antibodies, but not necessarily against melanin. Rahi[9] found antibodies reactive against the patient's own melanoma cells in 7 of 21 cases of uveal melanoma. Of the autochthonous antigens 'melanin is perhaps not one of the antigens because sera from patients with vitiligo containing anti-melanin antibodies do not react with melanoma cells'.[9] Re-examination of Langhof *et al.* shows that the melanin preparation used contained many cellular elements ('. . . des Ausstrichpräparates dieses MP ergab Fehlen cellulärer Elemente.')[4]

Antibodies selectively produced against a melanosomal component. The protein moiety of melanoprotein would be expected to produce antibodies in rabbits. In the synthesis of melanin on melanosomes, they become progressively melanized and subsequently lose their discernible internal structure (stages I - IV).[10] The synthetic melanin is apparently non-antigenic.[8] In dark ethnic groups, especially in hair, melanosomes are nearly exclusively fully melanized melanosomes. It is possible that in their study Langhof *et al.*[4] produced antibodies against the exposed protein moiety of incompletely melanized melanosomes. When fully melanized the antigenic protein moiety and tyrosinase may be occluded by the non-antigenic melanin. We have no further data to support this suggestion, because we did not, at the time, examine the melanin by electron microscopy.

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