Uveitis in Melanomatous Swine: Lack of Evidence for Humoral Immune Melanocyte Destruction

Lynette Feeney-Burns, Margaret Alspaugh,* Robert P. Burns, and Chun-Lan Gao

A strain of miniature Sinclair swine that have cutaneous malignant melanomas destroy their tumors via an immunologic process. Destruction of apparently normal melanocytes of the uveal tract occurs concurrently with tumor cell lysis. The authors investigated possible involvement of humoral antibodies in uveal melanocyte lysis. Uveal antigens solubilized by a variety of reagents were tested by immunodiffusion against autologous and homologous sera; no precipitation lines were found. Western blots of electrophoresed uveal proteins when tested against serum from an animal with a great tumor load showed no antigen–antibody reactions. No specific humoral antibodies were detected. Sera tested for immune complexes also were negative. Invasion of the uveal tract by mononuclear cells was quantified in 1-μm-thick sections of tissue taken at various stages of ocular depigmentation. Disappearance of melanocytes was preceded by an increase in mononuclear cells and followed by an increase in melanin-containing macrophages. The ultrastructural features of the invading cells are presented. The authors conclude that cell-mediated uveal melanocyte destruction is the most likely basis for the uveitis and other damaging sequelae. Invest Ophthalmol Vis Sci 26:551–560, 1985

Cutaneous malignant melanomas of the miniature Sinclair swine are destroyed by an immunologic process[1] that also destroys normal melanocytes of the uveal tract.[2] Disintegration of melanocytes appears to initiate or intensify an inflammation of the uveal tract. This uveitis is followed by band keratopathy, cataract development, and apparently also death of some photoreceptor cells,[3] as the skin and hair of the affected animals changes from black to white[4] and the uveal tract depigments.

Previously we[3] described histologic changes in the uveal tract of a few swine in early and late stages of tumor regression and ocular depigmentation. We noted that during melanocyte destruction the uvea becomes abnormally populated with mononuclear cells that resemble lymphocytes and monocytes. Macrophages containing melanin and other debris increased in number. Melanocytes eventually were entirely lacking, and the uvea showed much fibrosis. Sometimes occlusion of the choriocapillaris was observed. While these findings suggest a cell-mediated mechanism for melanocyte destruction, the possibility of involvement of the humoral arm of the immune system required examination. For example, were the mononuclear cells summoned to the uvea by immunoglobulins or by immune complex deposition?

This article described initial experiments aimed at detecting possible humoral components of the melanocyte cytotoxicity phenomenon as well as further studies of the cellular infiltration of the uveal tract.

Materials and Methods

Sinclair swine were examined clinically every 2–4 weeks for more than a year, as described previously.[3] Serial observations of iris and fundus pigmentation were recorded for pigs having no visible tumors, pigs with flat black spots, and pigs with raised tumors. Linear regression analysis of the data was done by Dr. Ernest Hilderbrand of the Academic Computing Center of the University of Missouri.

Serial blood samples were obtained from tumor-bearing and non-tumor-bearing animals. Serum was separated and stored at −20°C or −70°C and later tested for antibodies, for immune complexes, or other purposes.

In 12 animals, one eye was enucleated early in the study and the second eye at a later time. In most other animals (n = 18), both eyes were enucleated the same day. Eyes were bisected in the superior/temporal to inferior/nasal plane. The superior temporal quadrant of the eyeball was bisected and used for histologic, ultrastructural, and pilot immunofluorescent studies. One half was placed in aldehyde

From the Mason Institute of Ophthalmology and Department of Medicine,* University of Missouri–Columbia School of Medicine, Columbia, Missouri.

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Reprint requests: Lynette Feeney-Burns, Ph.D., Mason Institute of Ophthalmology, University of Missouri–Columbia School of Medicine, 1 Hospital Drive, Columbia, MO 65212.

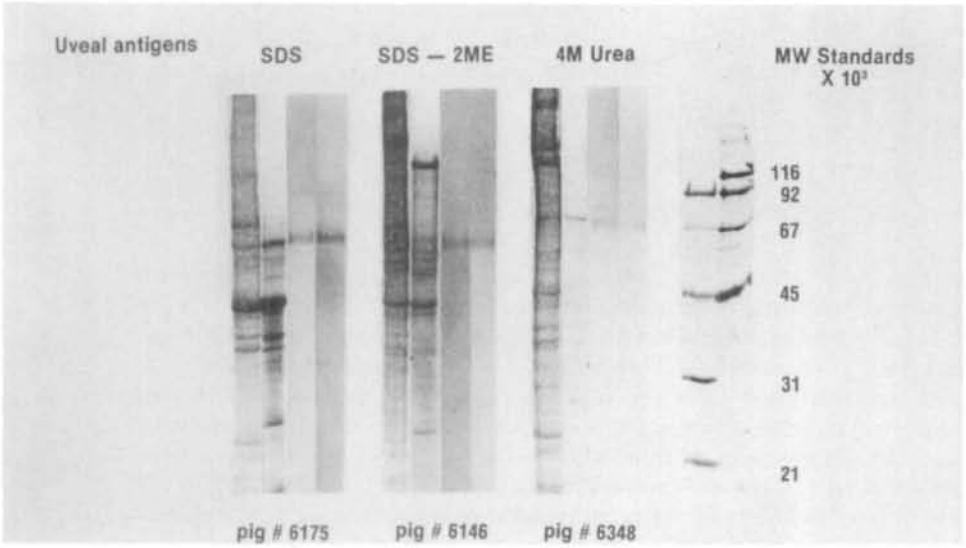

Fig. 1. Immunoblot (Western blot) analysis of uveal extracts of three melanomatous pigs. Pig # 6175, SDS extract. Pig # 6146, SDS plus 2 mercaptoethanol. Pig # 6348 4M urea. Lane 1, PAGE pattern stained with Coomassie blue; lane 2, transferred proteins stained with amido black; lanes 3 and 4, immunoblot reactions. Lane 3 is incubated with serum from pig with heavy tumor burden. Lane 4 is incubated with normal serum; blot stained with diaminobenzidine following incubation of immunoblot with peroxidase-labeled rabbit anti-swine. No differences are seen between normal and "tumor immune" sera staining.

fixative and processed for light and electron microscopy as described previously.[2] The other half rapidly was frozen in isopentane cooled in liquid nitrogen for immunologic studies described below. Normal eyes of six Sinclair Farm pigs of the non-melanomatous lineage also were prepared for histologic and immunofluorescence studies.

Preparation and Testing of Uveal Tract Proteins

The remaining three-fourths of the globe was freed of vitreous and neural retina, the pigment epithelium of the retina and iris were brushed off, and the uveal tract was separated from the sclera along the suprachoroidal plane. This yielded uveal tract contaminated only with pigmented and nonpigmented epithelia of the ciliary body; no further effort was made to remove these. For some experiments, the anterior uvea was kept separate from posterior uveal specimens. The uveal tissue was placed in 2 ml cold phosphate-buffered saline (PBS) and sonicated. The sonicate was centrifuged at 104,000 g for 60 min. The supernatants were concentrated by ultrafiltration. Ouchterlony immunodiffusion[5] tests were performed using 36 different supernatants against autologous and homologous swine sera or normal swine sera to test for possible antibodies to solubilized ocular antigens. Protein concentrations of all the extracts ranged from 4 to 50 mg/ml; antigens were presumed to be within a detectable range because two of the extracts tested at low protein concentrations showed detectable Sm antigen[5] when reacted with anti-Sm serum from a lupus erythematosus patient.

The pellet of the uveal tissue homogenate was subjected to one or more of the following solubilization procedures for proteins: 4 M KCl, 2% sodium dodecyl sulfate (SDS), 2% SDS plus 4.7% mercaptoethanol (ME), 3 M glycine-HCl buffer, pH 3.2, or 4 M urea. The tubes were sonicated again and centrifuged at 104,000 g; the supernatant was saved and concentrated, and the pellet was discarded. These supernatants also were tested in immunodiffusion plates. Twenty-eight sera were tested against 23 extracts. Each serum was tested against at least two extracts, and many were tested against six, including autologous extracts.

Aliquots of the solubilized uveal tract proteins also were electrophoresed in 10% polyacrylamide slab gels to determine the number of proteins present and their approximate molecular weights.

In other studies, uveal tract proteins were electrophoresed and transferred via the electroeluting technique (Western blot) to a sheet of nitrocellulose.[6,7] One set of lanes was stained with amido black stain for visualization of bands, and other sets were used for immunoperoxidase staining as follows: One set of Western blot uveal proteins was incubated with serum from a pig (#6769) with a heavy tumor burden, ie, an animal that would be expected to have the putative antimelanocyte/antimelanoma antibodies in its serum. Another set of uveal proteins was incubated with serum from a healthy normal pig (#6776). Positive controls, consisting of Sm antigen[5] and anti-Sm serum, were included with each slab gel and blot. The blots then were incubated with peroxidase-labeled anti-pig IgG immunoglobulin (Miles Laboratories, Inc., Elk-

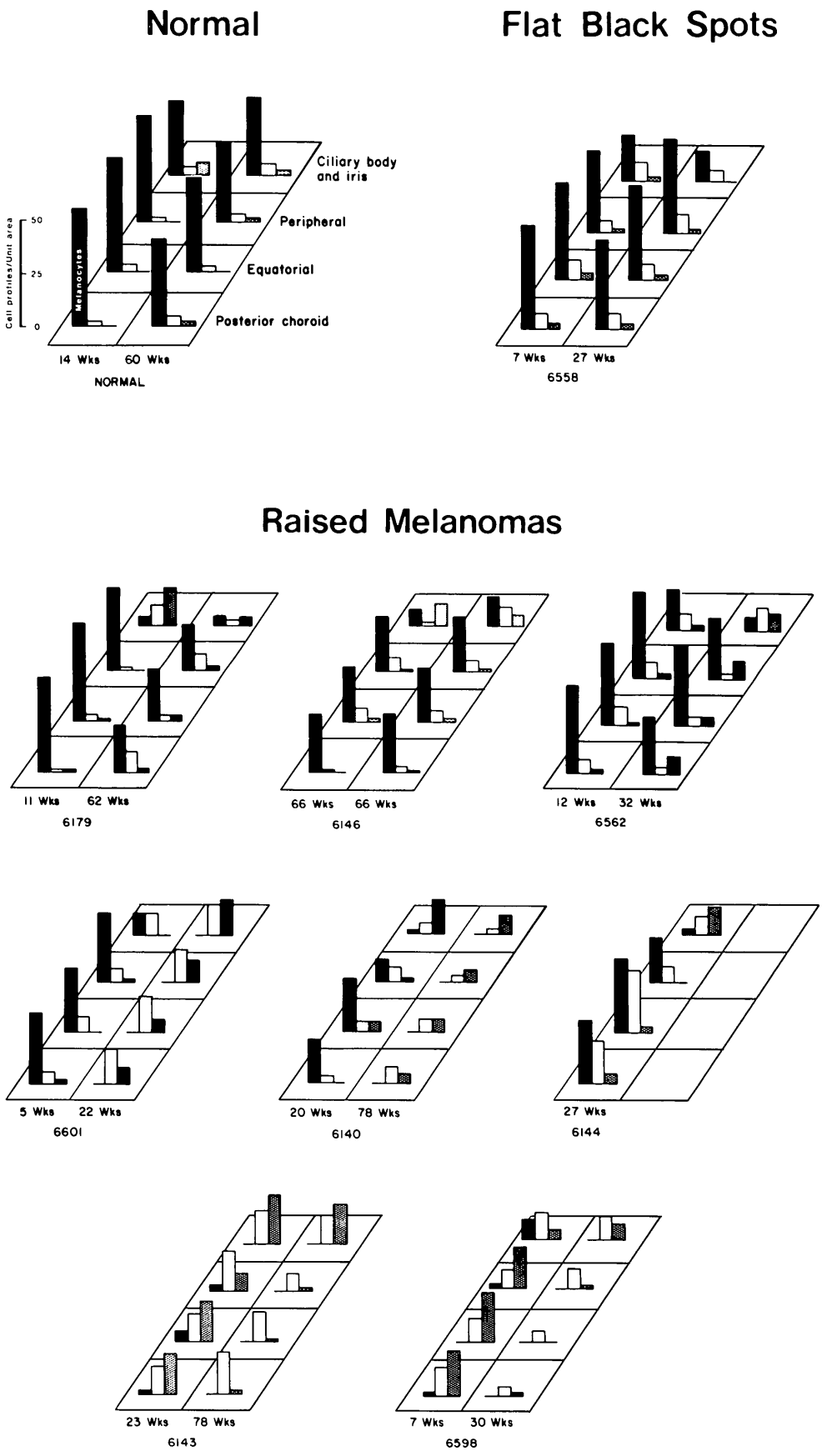

Fig. 2. Histograms depicting the number of melanocytes (solid), mononuclear cells (open), and macrophages (cross-hatch) in sections of posterior, equatorial, and peripheral choroid and ciliary body–iris tissue of two normal and eight of the melanoma-bearing pigs in this study. Age at which eyes were enucleated is given in weeks. A representative normal non-melanoma-bearing animal cell profile also is shown.

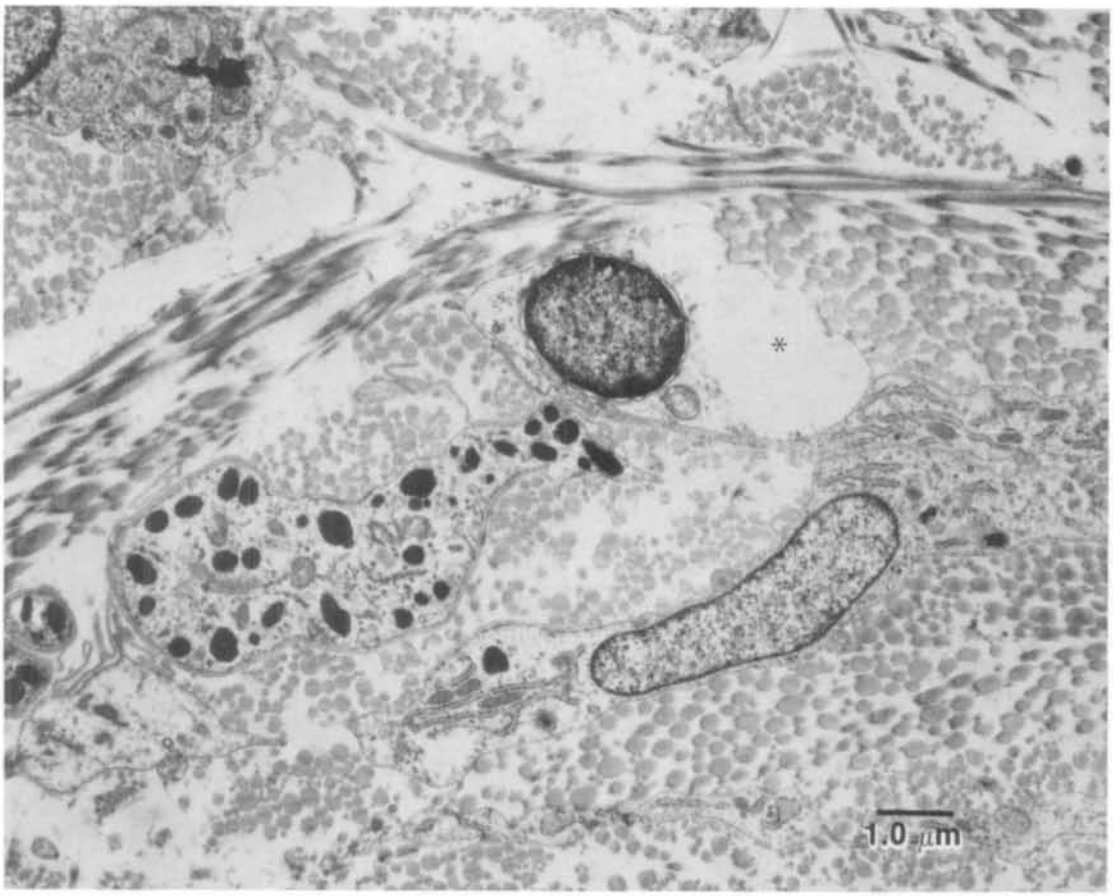

Fig. 3. Early stage of lymphocytic invasion of the uveal tract. Osmotic disturbance (swelling) of the lymphocyte cytoplasm commonly is seen (asterisk). Neither the fibroblasts nor the melanocyte are similarly disturbed (×10,000).

hart, IN). Following an extensive wash in buffer, the peroxidase was visualized using diaminobenzidine (Sigma Chemical Co., St. Louis, MO).

Immune Complex Determinations

The polyethylene glycol precipitation test[8] was performed on the sera of 10 melanomatous and eight normal Sinclair Swine in the laboratory of Dr. Ramesh Gupta, Tulane University.

Invasion Index

The superior/temporal quadrant of each pig eye was subdivided into four blocks, an anterior block containing the iris and ciliary body and three blocks containing the peripheral, equatorial, and posterior choroid. One-micron-thick plastic embedded sections were cut and stained with toluidine blue O. Using a micrometer eyepiece cells within an area of uveal tract measuring 6,750 μm^2 were counted in a Zeiss microscope using a 40× objective. Cells were identified and tabulated as melanocytes, fibroblasts, macrophages, and mononuclear cells (no distinction could be made between lymphocytes and monocytes). Endothelia, smooth muscle, Schwann cells and axons, and cells in vessel lumens were not counted.

Immunofluorescent Studies

A segment of one or both eyes of seven melanomatous pigs and one normal farm pig was used for pilot studies aimed at detecting immunoglobulins in the uveal tract. Eyes that had clinically visible uveitis at the time of sacrifice of melanomatous animals were selected (pigs #6146, 66 weeks; #6147, 62 weeks; #6179, 62 weeks; #6240, 52 weeks; #6334, 39 weeks; #6553, 26 weeks; #6559, 27 weeks). The tissue rapidly was frozen in isopentane cooled in liquid nitrogen. Tissue was stored at −70°C for up to 12 months. Following verification of the presence of mononuclear cell infiltrates in the uveal tract by examination of 1-μm-thick plastic-embedded sections, the frozen segments of these eyes were sectioned at 8 μm. Swine lymph nodes similarly were prepared for a positive control. The effects of a 3-min fixation of the sections in acetone or methanol at 4°C compared with phosphate-buffered saline were evaluated. Also, a range of

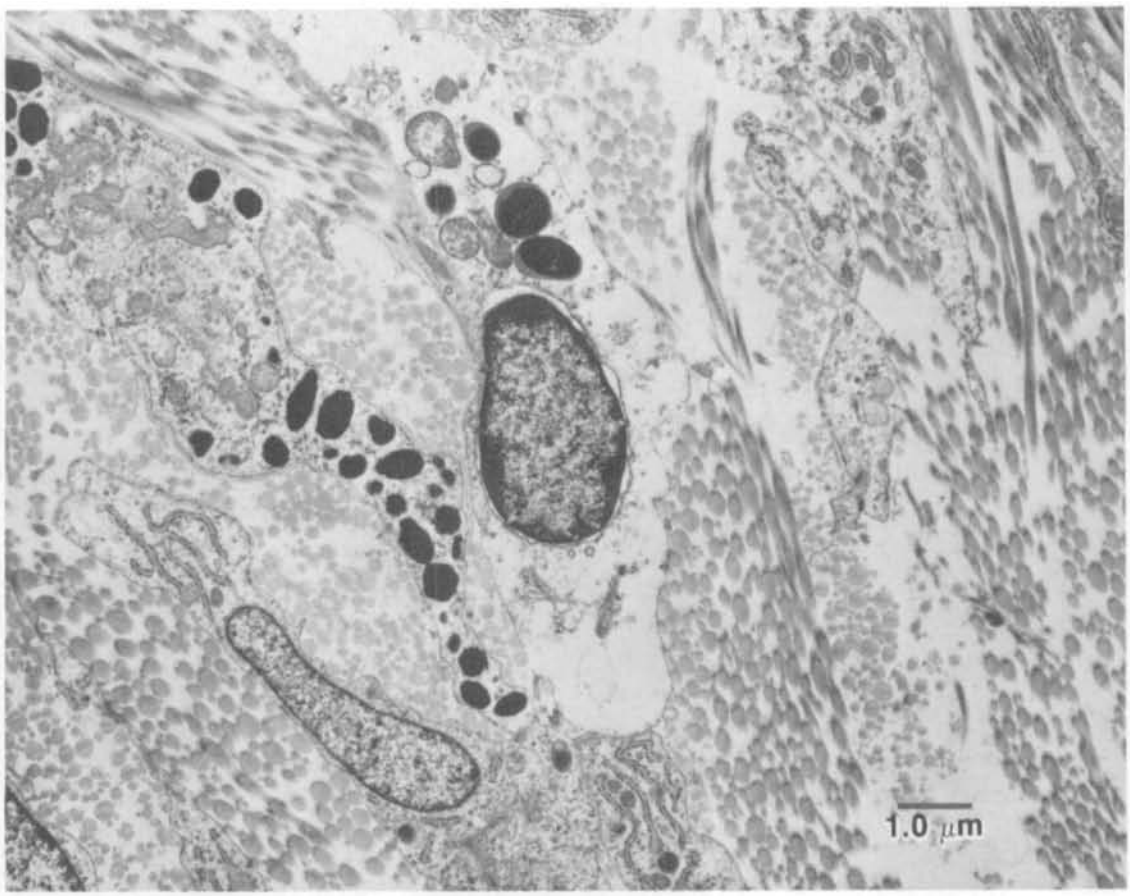

Fig. 4. Early stage of mononuclear cell invasion. This cell has nuclear morphologic features of a lymphocyte, but it has phagocytized melanin, thus it probably is a monocyte–macrophage. Note the osmotic disturbance (swelling) of this cell and the normal appearance of fibroblasts and the macrophage (×10,000).

dilutions for all subsequent reagents was made and tested. Sections were incubated in 1:30 dilution of rabbit anti-swine gamma globulin, rinsed, and then incubated in 1:50 dilution of rabbit FITC-labeled goat anti-rabbit gamma globulin (Antibodies Inc., Davis, CA). Sections incubated in preimmune rabbit serum prior to the FITC-labeling were included in each experiment as a negative control. A positive control for staining was rabbit anti-swine albumin (Cappel Laboratories, Cochranville, PA). Sections were examined in a Zeiss fluorescence microscope using epi-illumination (excitation band pass [BP] filter 450–490 nm, reflector BP 520–560 nm).

The investigations utilizing animals, as described in this manuscript, conform to the ARVO Resolution on the Use of Animals in Research.

Results

The clinical observations regarding pigmentation required statistical analysis because some pigmentary modulations occur with age in normal animals. When the data on the three groups of animals (tumors, flat black spots, no tumors) were analyzed, there was a highly significant ($P = <0.001$) correlation in the rate of depigmentation of the iris and fundus in pigs having cutaneous tumors compared with pigs that had no tumors. Pigs with flat black spots likewise did not depigment their eyes as rapidly as the tumor-bearing animals. Ocular depigmentation was found to precede maximum tumor volume in the few pigs that had cutaneous and metastatic tumor volumes measured at autopsy.[4]

Ouchterlony immunodiffusion tests of PBS extracts of uveal tract all were negative. Immunodiffusion assays of proteins from the other five extraction procedures also produced no precipitation lines.

The polyacrylamide gel electrophoretic profile of uveal proteins differed with each solubilizing reagent (Fig. 1). Urea produced more than 20 bands, whereas glycine–HCl gave about 10 bands (not illustrated). The molecular weight range of these proteins was 10,000 to over 200,000 daltons.

When the proteins in the various extracts were electrophoresed then transferred to nitrocellulose and tested for their immunoreactivity with "tumor im-

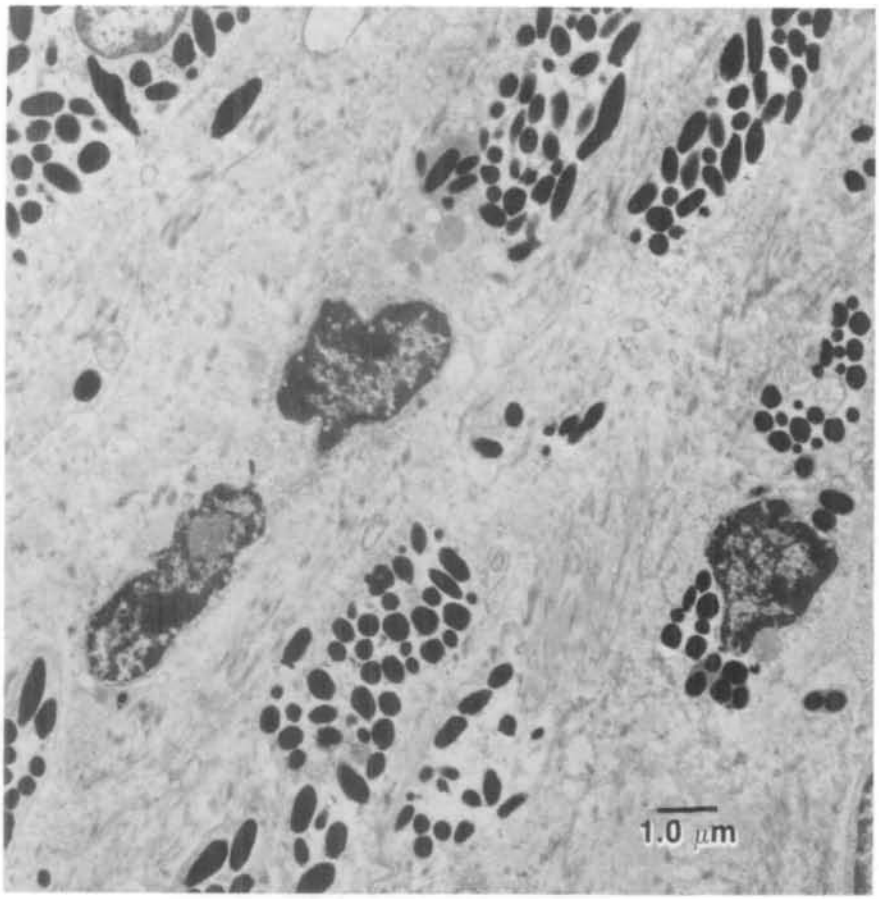

Fig. 5. Mononuclear cells that have invaded the ciliary body prior to melanocyte destruction. Nuclear morphology, larger size, and presence of lysosome-like bodies suggest these may be monocytes prior to full conversion to macrophages (×7,500).

mune" serum, no specific reactions were found with any of the 12 extracts tested. However, as can be seen in Figure 1, there was nonspecific binding by proteins both in normal and "tumor immune" sera to a common uveal protein of approximately 67,000 daltons.

Immune Complex Determination

The polyethylene glycol precipitation technique failed to reveal immune complexes in the sera of 10 melanomatous animals tested.

Invasion Index

Figure 2 shows histograms of the three relevant types of cells (fibroblasts not graphed) of the uveal tracts of a representative normal pig, a pig with flat black spots, and eight of the melanomatous pigs. The number of melanocytes, mononuclear cells, and macrophages are graphed together for the posterior, equatorial, and peripheral choroid plus the ciliary body and iris of each animal. In most animals depigmentation was detectable in the ciliary body prior to its detection in the choroid. A decrease in the number of melanocytes is apparent after an increase has occurred in the number of mononuclear cells. Macrophage numbers increase rapidly after the monocytic cell invasion. There is an inverse relationship between melanocytes on the one hand and macrophages and mononuclear cells on the other among the gray or depigmenting animals. No plasma cells, mast cells, or basophils were seen, and few neutrophils or eosinophils were found in any of the specimens.

Ultrastructure of Invading Mononuclear Cells

The earliest invading cells have the characteristics of lymphocytes and monocytes, ie, at least two subgroups of cells are distinguishable by differences in nuclear morphology. One type has a smaller nucleus, more heterochromatin, and less euchromatin, characteristic features of a lymphocyte (Fig. 3). Both kinds of cells have prominent nuclear envelope cisternae and nuclear pores. The cytoplasm of the smaller lymphocytic cell is scant, has few organelles except for monoribosomes, and a small Golgi complex and few mitochondria. The larger cell with less compact nucleus has inclusions resembling lysosomes and suggests differentiation of a monocytic cell into a

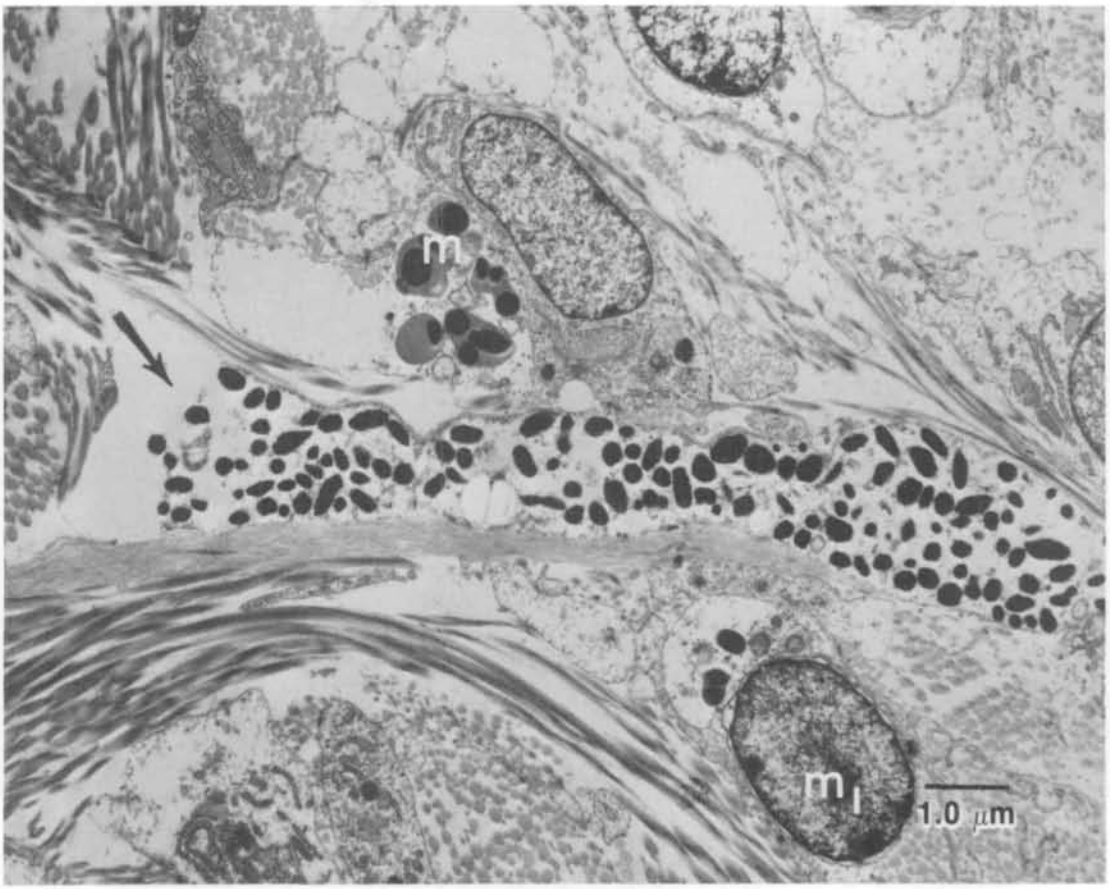

Fig. 6. Early stage of antimelanocyte attack by invading cells. Some melanin has been phagocytized by mononuclear cells (m, m_1). The plasma membrane of the melanocyte is ruptured (arrows) (×10,000).

macrophage (Figs. 4 and 5). These retain many of the nuclear morphologic attributes of the cells of the first type.

Both types of invading monocytic cells consistently show morphologic signs of osmotic stress, ie, they are swollen and appear leaky. This contrasts with neighboring fibroblasts and melanocytes that are well preserved (Fig. 6). Evidence of leaky melanocytes can be found that are assumed to be the early stages of melanocyte cell death (Figs. 6–8).

Immunofluorescence Studies

No specific fluorescence was found in the ocular tissues examined so far using the indirect methods described herein.

Discussion

The aim of these studies was to learn why the apparently normal melanocytes of the eye are destroyed, precipitating a uveitis during the immunologic attack on cutaneous melanoma cells. After examining a large number of pig litters from melanoma-affected sire and dam matings, we found that animals born with tumors begin to depigment their eyes in the first 3 months of life. The ocular phenomena parallel the vigorous systemic cytotoxic activity[2] and give a more accurate assessment of the lymphocytic attack on the tumors than does direct measurement of tumor mass; much of the cutaneous tumor volume is due to invading macrophages rather than proliferation of malignant melanoma cells.[9] We now can anticipate the onset of the cytotoxic events in the eyes and select pigs for immunologic studies in a stage of disease most likely to reveal the initial mechanism of the anti-melanocyte attack.

The hypothesis has been that normal melanocytes have antigens on their surfaces that are the same or similar enough to those on the melanoma cells that both kinds of cells fall victim to the anti-tumor immune response.[10] We attempted to detect possible anti-uveal tract antibodies in sera of individual animals by preparing uveal extracts of various kinds and testing them against their own or homologous sera. The PBS extraction procedure is presumed to remove

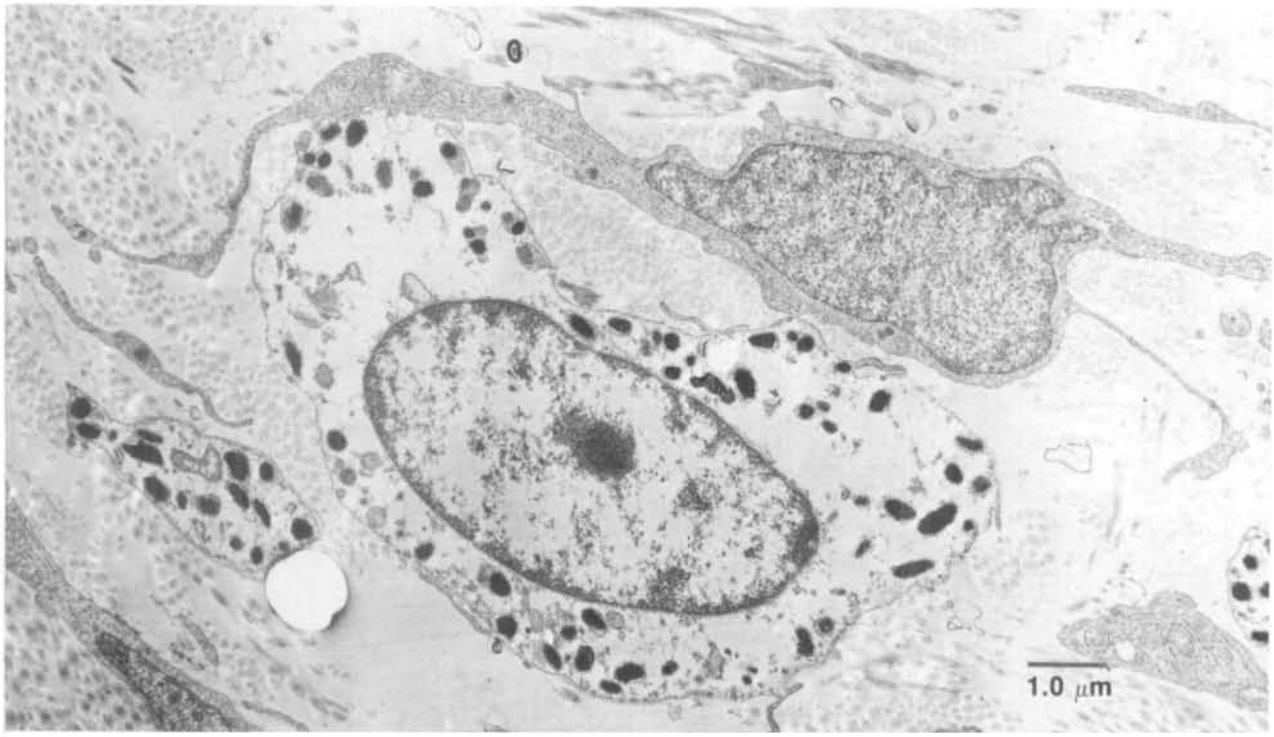

Fig. 7. Ciliary body melanocyte in apparent early stage of disintegration. Note that fibroblasts are normal appearing. Pig #6601, 5 weeks of age (Original magnification, ×11,700).

highly soluble and/or loosely bound proteins and make them available for antigen-antibody reactions. The PBS pellets contained PBS-insoluble proteins (antigens); these were solubilized using five different reagents that unfold polypeptides in different ways. Presumably, the antigens(s) of interest in this study would be in or on the uveal melanocytes, and the solubilization procedures should have made these available for reaction in the ouchterlony assays. No antigen–antibody interactions have been found when extensive testing was performed by a rather insensitive immunodiffusion technique or in a smaller study using the highly sensitive Western blot method. Our initial studies suggest that the humoral arm of the immune system may not be playing a role in cytolysis of the uveal melanocytes, but further studies with the Western blot method at earlier stages of disease are required.

Another immune mechanism known to initiate an inflammatory response and to cause uveitis is immune complex deposition in vascular beds.[11] Such deposition activates the complement cascade and leads to invasion of the affected area of inflammatory cells. Sera from 10 pigs undergoing tumor regression and depigmentation did not show immune complexes in their sera. Moreover, the cells invading the uveal tract were almost exclusively of the lymphocyte or monocyte types rather than of the granulocytic series. These initial data suggest that the cellular invasion may not be elicited by the immune complex/complement mechanism.

Tabulation of cell types in the uveal tracts during the time of depigmentation and tumor regression revealed measurable shifts in cell populations. Individual variation was seen histologically in the rate and amount of uveal melanocyte destruction (Fig. 2). The melanomatous animals whose cell count histograms are in the top row had fewer or late-developing tumors and slower depigmentation than the pigs whose histograms are in the lower two rows. The pigs with the most rapidly depigmenting uveal tracts were those born with many tumors and that showed skin and hair depigmentation in the first months of life. Thus, the uveal melanocyte destruction rate paralleled the rate of depigmentation and regression of the cutaneous melanomas.

Some cells invading the swine uvea are morphologically similar to human T-lymphocyte clones[12]; however, differential studies on the infiltration of B- and/or T-lymphocytes cannot be performed, since reagents that can distinguish B- and T-cells have not been developed for the swine species. Nonetheless, useful data have been obtained for timing the first wave of invading cells. The population of mononu-

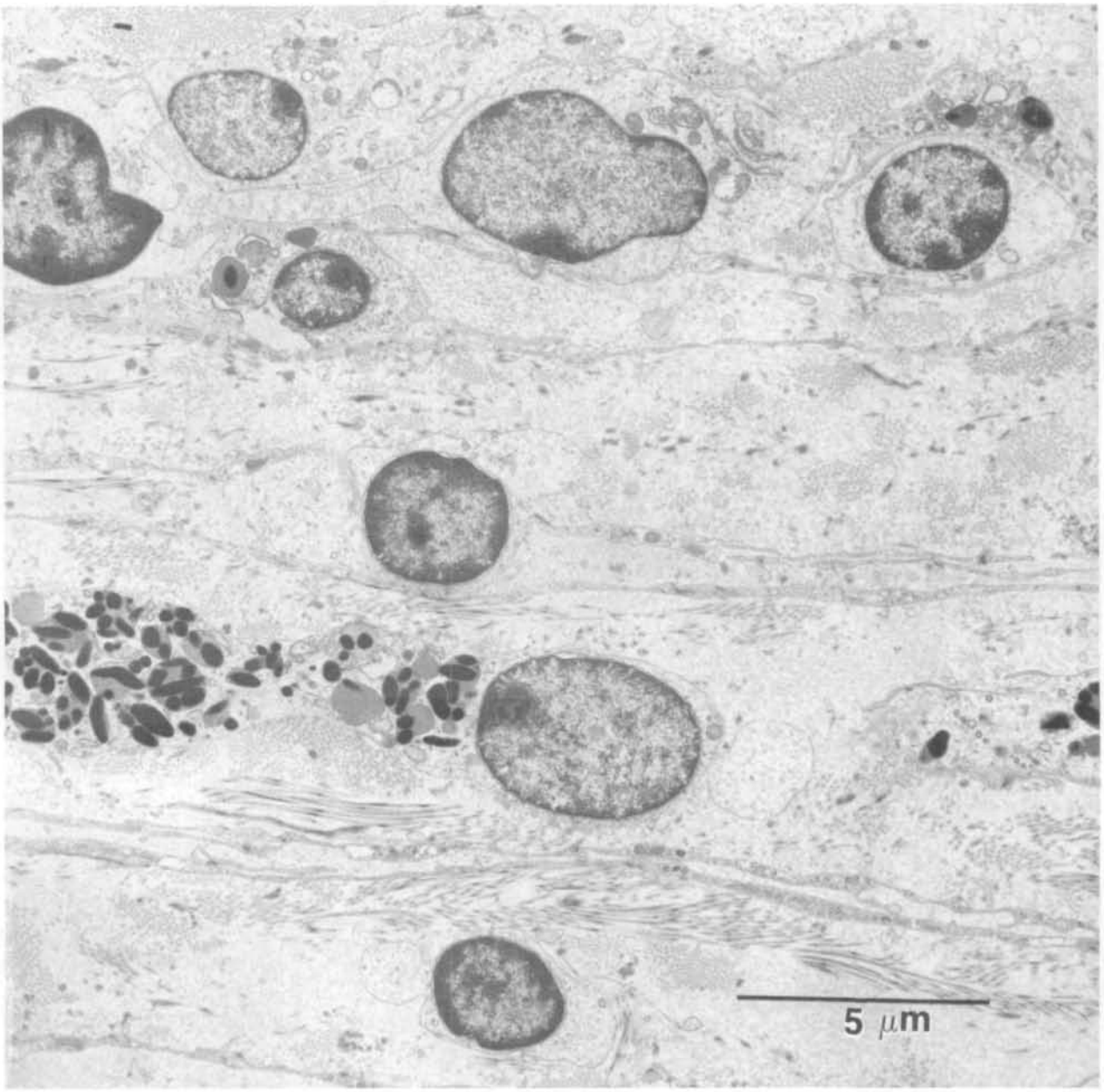

Fig. 8. Choroidal specimen of #6598, 7 weeks of age. Melanocytes are gone. Mononuclear cells and one large macrophage are visible (Original magnification, ×8,200).

clear cells does not increase greatly prior to the fall in melanocyte numbers, possibly because a fraction of the mononuclear cells become phagocytic and thereafter are counted as macrophages. These phagocytic cells enlarge greatly and seem to be unable to exit from the uveal tract. Their perivascular location gives the fundus its tigroid appearance in late stages of ocular depigmentation.

We have been unable to demonstrate immunoglobulins on cells of frozen sections of swine uveal tract using indirect immunofluorescent staining with anti-swine sera. Since the eyes used in this pilot study had full-blown uveitis, these results may reflect only late-stage phenomena, and we should examine earlier stages before ruling out participation of immunoglobulins in this disease.

Key words: cytotoxic lymphocytes, immune complexes, malignant melanoma, uveal antigens, Western blot, Sinclair swine

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