CUTANEOUS DELAYED HYPERSENSITIVITY REACTIONS TO SOLUBLE MELANOMA ANTIGEN IN PATIENTS WITH OCULAR MALIGNANT MELANOMA

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Abstract We studied the cutaneous delayed hypersensitivity responses to a soluble melanoma antigen in 32 patients with the initial clinical diagnosis of ocular melanoma and in seven control patients. Eighteen out of 19 patients who had pathologically confirmed ocular melanomas were positive to this antigen, as were eight other patients clinically thought to have choroidal melanomas. All seven controls were negative on skin testing with this antigen, as were five patients who were initially thought to have ocular melanomas but who, on extensive work-up, were considered to have other, nonmelanoma, ocular lesions. Thus, patients with ocular malignant melanomas have cell-mediated immunity against an antigen common to systemic malignant melanoma. This delayed hypersensitivity assay may assist in the diagnosis of ocular melanoma, especially in patients with opaque media or those in whom ocular melanoma must be distinguished from metastatic lesions to the choroid. (N Engl J Med 291:274-277, 1974)

THE detection and clinical differentiation of melanomas in the eye may present diagnostic difficulties. Thus, of 737 eyes submitted during 1949-1972 to the Registry of Ophthalmic Pathology with the clinical diagnosis of melanoma, 20 per cent were found on pathological examination to have lesions other than melanoma.[1,2] The diagnosis is especially difficult when cataracts or other opacities in the media prevent adequate fundus examination.

Immunologic methods of diagnosis may be a means of reducing this frequency of enucleations for simulating lesions. There is preliminary evidence of tumor-associated antigens in ocular malignant melanoma,[3-5] in addition to the substantial evidence for their existence in other human tumors.[6] Since cell-mediated immune response to tumor-associated antigens is thought to have a major role in host resistance to tumors, cellular reactivity has been extensively studied.[7] Tests of cutaneous delayed hypersensitivity reactions to tumor extracts have been reported in a number of human neoplasms, including systemic malignant melanoma, Burkitt's lymphoma, and acute leukemia.[8-12] Using both autologous and allogeneic crude membrane extracts as a source of tumor-associated antigens in acute leukemia, we have previously reported that patients give positive reactions only to tumor-associated antigens derived from the same histologic tumor type, and that their response to that tumor antigen correlates with clinical status and prognosis.[9] In a series of skin tests of patients with malignant melanoma,[8] separated soluble antigens of autologous and allogeneic tumors elicited delayed hypersensitivity reactions. One of these antigens appeared in the second peak of the Sephadex G-200 separation of the membrane sonicate and was identified in region A of polyacrylamide gel electrophoresis separation. This antigen appeared to be a melanoma-associated antigen; positive reactions were seen in 17 of 22 patients with early-stage and in seven of 19 patients with late-stage melanoma. This particular antigen produced no reactions in 21 of 22 tests in patients with cancers other than melanoma. In view of the good specificity seen with this assay, and the possible clinical usefulness in ocular malignant melanoma, we have studied a group of patients suspected of having ocular melanoma, along with control patients with other types of malignant processes, to ascertain if skin-test response to the soluble melanoma antigen would be seen specifically in patients with ocular malignant melanomas.

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Materials and Methods

Subjects

All subjects were evaluated at either the Eye Clinic, National Institutes of Health Clinical Center, the Massachusetts Eye and Ear Infirmary, or the Department of Ophthalmology, University of California, San Francisco. All patients had routine ophthalmologic work-up for ocular melanoma, which included: fundus photographs or drawings (or both); transillumination; visual fields; and fluorescein angiography. Twelve also had ^{32}P scans, and five had ultrasound examinations; nine were tested with chloroquine analogue scans. All skin testing was performed by one of us (D.H.C.) after all subjects were given an oral and written explanation of the antigens used and the skin-testing procedure, and informed consent had been obtained.

Subjects tested were in five general categories: nineteen patients tested either before, or at varying times after operation who had pathologically confirmed choroidal or conjunctival melanomas (with nine patients in this category, the investigator performing the skin tests was not aware of the clinical data); eight patients who after extensive work-up were considered to have ocular melanomas, but who are either being followed, or have had cobalt or photocoagulation therapy, so that pathological material is not available for examination; five patients who were referred with the diagnosis of ocular melanoma to the above ophthalmologic centers but who, on the basis of subsequent extensive work-up, are not now thought to have ocular malignant melanomas (in all cases, this clinical judgment was made independently from the skin-test results); two patients with advanced breast carcinomas who had known metastatic lesions to the posterior pole of the eye, with intact cellular immunity; and five subjects with systemic neoplasia other than melanoma, who had no evidence of ocular disease, and had intact cellular immunity as measured by delayed hypersensitivity responses to standard recall antigens.

At the time of testing, no patient with ocular malignant melanoma had clinically obvious metastatic disease. No patients who had had enucleation of the eye and were found not to have an ocular melanoma were tested.

Skin-Test Materials

The details of the methods used in the preparation of soluble

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tumor antigens have previously been described.[8,13-15] Melanoma tissue from patients with systemic malignant melanoma was sterilely obtained at surgery. It was washed in sterile saline, finely minced and pressed through a 60-mesh stainless-steel sieve. The resultant single cell suspension was washed in saline and viability counts were done with use of trypan blue exclusion. The cells were frozen and thawed and then washed in isotonic saline and then progressively more hypotonic saline.[16] The pooled washes were centrifuged at 100,000 × g for one hour. The resuspended pellet was defined as the crude membrane extract. Previous work using this method of crude membrane extraction has shown that the material is composed mainly of smooth membranes, with little contamination by mitochondria or intact nuclei, and that this fraction contains a high yield of HL-A antigens.[17]

The crude membrane extract was then exposed to four sequential 1½-minute periods of low-frequency sonication, with ultracentrifugation at 100,000 × g for one hour and removal of supernatant interposed between the periods of sonication. The supernatants were pooled and separated by Sephadex G-200 column chromatography. Three protein peaks were eluted off the column. Fraction II was used for making the soluble melanoma antigen tested in this study. The centers of each of the protein peaks seen from different specimens of allogeneic melanoma tumor were similar. With standard methods, the approximate molecular weights of the protein peaks were calculated.[14] Sephadex fraction II had a molecular weight of approximately 38,500. The material obtained from Sephadex fraction II was passed through a Millipore filter in addition to being simultaneously plated on blood agar and in thioglycollate broth to ensure sterility. The material was tested for protein concentration by the Lowry method,[18] and adjusted to a protein concentration of approximately 39 μg per 0.1 ml. It was then drawn up, 0.1 ml in each tuberculin syringe, and stored at -70°C until it was tested in patients.

Standard recall antigens were obtained from commercial sources: IPPD (5TU/0.1 ml) (Connaught Laboratories, Willowdale, Ontario, Canada); SKSD (40 U of streptokinase, 10 U of streptodornase, Lederle Laboratories, Pearl River, New York); and mumps (Eli Lilly and Company, Indianapolis, Indiana).

Skin-Test Procedure

Tests with the standard battery of antigens and the soluble melanoma antigen were performed simultaneously by the intradermal inoculation of 0.1 ml of each material, usually in the interscapular region of the patient's back. All skin tests were read at 48 hours, and the average diameter of induration and erythema was measured with a Vernier caliper. A positive reaction was defined as greater than 6 mm of induration at 48 hours. In approximately 100 previous tests done with tumor-derived antigens and standard recall antigens we have biopsied positive reactions.[9] Histologically, the reactions consisted of perivascular infiltration of mononuclear cells in the upper dermis consistent with delayed hypersensitivity reactions. Positive reactions to tumor-derived and standard recall antigens were indistinguishable on pathological examination.

Results

Thirty-nine patients were skin-tested with standard recall antigens and soluble melanoma antigen. All patients showed positive reactivity to at least one of the standard recall antigens, and there was no difference in the reactivity of patients with ocular melanomas to standard recall antigens as compared with 60 normal subjects.[9]

Fourteen patients with pathologically proved ocular melanomas were tested either before or within six months of operation (Table 1, Group 1a). All 14 had skin-test responses to the soluble melanoma antigen greater than 9 mm of induration at 48 hours. Four out of five patients tested from nine months to 3½ years after surgical removal of their ocular melanomas (Group 1b) had positive skin-test reactions to the soluble melanoma antigen. In contrast, all patients without

Table 1. Skin-Test Response to Soluble Melanoma Antigen.

Patient Category	No. of Positive Tests/Total No. of Patients	Induration*	
		Mean†	Range
A. Total ocular melanoma:	26/27	13.2 (0.6)	4.5-18
1. Pathologically confirmed ocular melanoma			
a. Tested before or <6 mo after operation	14/14	14.1 (0.7)	9-18
b. Tested >6 mo after operation	4/5	10.6 (1.6)	4.5-13.5
2. Ocular melanoma, clinical diagnosis	8/8	13.3 (1.0)	9-17
B. Total patients without melanoma:	0/12	2.5 (0.8)‡	0-6
3. Ocular lesions simulating melanoma	0/5	5.0 (0.4)	5-6
4. Breast carcinoma, metastatic to choroid	0/2	2.5 (2.5)	0-5
5. Systemic tumors without ocular disease	0/5		0

*Mm. †± S.D.
‡Significantly < reactivity than patients with ocular melanoma ($p<0.001$).

ocular melanomas (Groups 3, 4 and 5) had reactions of 6 mm or less. Patients with all five pathological cell types of ocular melanoma had positive skin-test reactions to the melanoma antigen. Two patients in Group 1a were of special interest. One patient had a lesion that appeared on clinical examination to be a ciliary-body melanoma, but was negative to ^{32}P scanning by Hagler's criteria.[19] She was found to have a spindle B melanoma of the ciliary body. In a second patient a bronchogenic carcinoma had been resected approximately two years before the discovery of his large, nonpigmented posterior choroidal mass. Clinically, he was considered to have a metastatic lesion of the choroid. A chloroquine analogue scan done at the National Eye Institute was negative for ocular melanoma. He had a 15-mm skin reaction to the soluble melanoma antigen, and was found on pathological examination to have a spindle B melanoma.

Eight of the subjects seen were thought to have choroidal melanomas on the basis of clinical examination, visual fields, transillumination fluorescein angiography, fundus photography or drawings (or both) and, in most cases ^{32}P scanning. All eight of these patients had positive skin reactions to the soluble melanoma antigen. Two of these patients have been treated with methods other than operation (photocoagulation and cobalt). At present we do not have pathological material from any of these patients.

Five patients who were originally referred with the diagnosis of probable ocular melanoma, and were therefore entered into the study, were thought, after more extensive evaluation, to have simulating lesions. In the intervening four to five months since the skin tests were performed, none have increased in size. Two were thought to have choroidal nevi, two choroidal hemangiomas, and the fifth precancerous melanosis. All had skin-test reactions of 6 mm or less to the melanoma antigen. Although nationally the rate of false-positive diagnosis in choroidal melanomas has been reported to be between 5 and 40 per cent,[20-22] at the three institu-

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tions where these patients were tested, and at a comparable institution,[23] that figure is less than 2 per cent (Dr. Taylor Smith, personal communication).

Two patients with breast carcinoma metastatic to the choroid and five patients with no evidence of ocular disease, but with non-melanomatous neoplasia elsewhere, had skin reactions to the soluble melanoma antigen no greater than 6 mm of induration at 48 hours. None of these patients were anergic, and the two patients with breast carcinoma had positive skin reactions to crude membrane extracts of allogeneic breast carcinoma (data not shown).

Patients with ocular melanomas (Groups 1 and 2) had skin-test reactions to the soluble melanoma antigen greater than 9 mm of induration at 48 hours (mean, 13.2 mm) whereas those who did not have melanomas (Groups 3, 4, and 5) had no reactions greater than 6 mm (mean, 2.5 mm). This difference between groups was statistically significant, with a p value less than 0.001 (Student's t-test).

Discussion

Most patients with ocular melanoma had vigorous delayed skin reactions to a soluble melanoma antigen. In contrast, those with other types of ocular lesions or other types of cancer had less or no reactivity. We arbitrarily defined a positive reaction in this study as greater than 6 mm of induration at 48 hours, since this criterion had previously provided good discrimination between groups. Other studies have defined positive reactions to skin-test antigens as either 5 or 10 mm of induration,[24,25] also on the basis of the desired clinical discrimination. It is quite possible that the reactions of 4 and 5 mm observed here were also due to delayed hypersensitivity. We have previously observed that biopsies of some small, clinically negative (i.e., 2 to 3 mm) reactions to standard recall antigens had mild perivascular mononuclear infiltrates in the upper dermis. All patients in the present study who showed some induration had ocular disease, and they may have had some cellular immunity to normal tissue antigens present in the melanoma extract. Alternatively, the reactions in the control patients may have been nonspecific. We have previously found that crude membrane extracts, when tested at greater than 300 μg of protein, can produce nonspecific skin reactions.[17] Possibly, 39 μg of this more purified melanoma extract was too high and resulted in some induration in controls. Although HL-A antigens are present in membrane extracts of tumors, it is unlikely that they contributed to the reactions seen. In previous studies, allogeneic leukocyte extracts, although rich in HL-A antigens, produced virtually no skin reactivity in normal recipients.[9]

The detection of allogeneic reactivity to the soluble melanoma antigen in this study and similar observations in other assays of cell-mediated immunity[3,6,26-28] indicate the presence of common antigens in melanoma. This sharing of antigens between allogeneic melanoma (and between different types of systemic and ocular melanomas) could be the result of many factors. It is consistant with a viral origin of melanoma, since virus-induced tumors in laboratory animals have common antigens.[29] This common antigenicity may also be due to tissue antigens or derepression of fetal antigens. Tissue antigens are not likely to be the cause of all the allogeneic immune reactivity, since patients with choroidal nevi had only low levels of induration. In addition, tissue antigens did not seem to be responsible for the antibodies detected in the serums of patients with melanoma by immunofluorescence assays.[3] Reactivity was seen against choroidal melanoma cells, but not against normal choroidal melanocytes.

Some issues must be dealt with before this test, or similar ones, can be routinely used in a clinical setting. The first is the safety of the procedure. To date, we have not seen any morbidity in approximately 1000 tests of patients with crude membrane extracts or with soluble antigens derived from tumor cells. The soluble tumor antigens should be safer than the crude membrane extracts since the maximum molecular weight of these materials (80,000) makes it highly unlikely that any intact virus particles or nuclear information is present. However, we have avoided testing any of these materials in normal subjects, and therefore cannot say with certainty that there is no possibility of transmission of low-molecular-weight oncogenic information. Although there is no present indication of horizontal transmission in man with nonviable cell materials from human tumors, we do not consider it justified to use this material in any subjects who are unlikely to have a malignant process.

A second issue has been the low yield of soluble antigen obtained from tumor tissue. The technics involved in antigen preparation are tedious and technically somewhat difficult. This drawback appears to be a major limiting factor in their clinical use at present.

A third issue is the accurate quantitation of induration induced by these tumor antigens. Some of the subjectivity in readings can be avoided if the investigator reads a reaction without knowing the clinical data. In tests done in this laboratory, we have had two investigators read a reaction without knowing the other person's results. Their readings have been within 1 mm of each other.

We have demonstrated by an in vivo technic that patients with ocular melanomas have cell-mediated immunity against allogeneic melanoma antigens and that this test may differentiate well patients with melanoma from those with other diseases.

With the increasing clinical use of ^{32}P scans, ultrasound, and fluorescein angiography the rate of false-positive diagnoses of ocular melanoma should be kept at a minimum.[23] However, in patients with opaque media, or in those in whom metastatic lesions are high on the list of differential diagnoses, these diagnostic technics have been demonstrated to have a number of false-positive and false-negative results.[30-33] Twenty-one per cent of choroidal melanomas occur in eyes with opaque media,[22,34,35] and metastatic lesions to the choroid are one of the most common simulating lesions of

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choroidal melanoma.[2] In these two clinical situations, soluble melanoma antigen skin tests may be clinically useful in the diagnosis of ocular melanoma.

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