Studies of Humoral and Cell-Mediated Immunity in Human Melanoma

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INTRODUCTION

The existence of tumor specific antigens in animal systems has now been well established and reviewed (1–4). Although definitive proof of the specificity of such antigens in human tumors has been lacking, there is evidence that tumor associated antigens also exist in humans (5–7); these antigens have now been demonstrated in such human tumors as Burkitt's lymphoma (8, 9), carcinoma of the colon (10), neuroblastoma (11), sarcomas (12–14), and melanoma (15). These studies have also revealed that human subjects do mount an immunologic reaction against their tumors.

Since this paper concerns immunologic studies of human melanoma, it will be appropriate to review several clinical features in the natural history of melanoma, which appear to have clear relevance to the host response to this particular neoplasm: 1. Convincing demonstrations of cases occasionally undergoing spontaneous regression of primary melanoma (16–18), or partial regression of primary cutaneous melanoma (19), or regression of isolated metastatic nodules (20), and the commonly encountered feature characterized by long disease free interval in many patients (21) are all suggestive of some host defense mechanism against their tumors.

2. The auto-destruction of melanotic tumors in the so-called benign "halo nevus" and the occurrence of similar phenomenon noted at times with malignant melanoma also attest to the existence of host immune mechanism against autologous tumor.

3. Further suggestion of host interaction against melanoma may be derived from the frequent histologic evidence of mononuclear cellular infiltrate around tumor nodules (22). In addition, approximately 10% of the patients with metastatic melanoma presents with no identifiable primary site. Some of these patients, however, report spontaneous involution of pre-existing "moles." Biopsy of these presumably cryptic primary sites has revealed mononuclear cellular infiltration but no residual tumor (15).

Besides indirect evidence for tumor immunity in melanoma, more direct evidence for characteristic antigens related to different constituents of melanoma cells, and

circulating antibody directed against such antigens, have been independently reported by several authors over the last 5 yr (23–29). Circulating antibody with cytotoxic property against melanoma cells was demonstrated by Lewis *et al.* (23) and subsequently by Grey *et al.* (24) in a complement-dependent, *in vitro* test system. Using immunofluorescence technique, several authors (26–29) have subsequently confirmed the presence of circulating antibody against cytoplasmic, nuclear, or cell membrane antigens. Though there were differences in the seroepidemiologic data regarding the incidence of antibody in homologous sera, from a consensus of major studies, several general conclusions can be made: 1. characteristic intracellular and membrane bound (cell surface) antigens are detectable in melanoma cells with appropriate experimental technique, 2. autologous sera react with their respective cell constituent antigens more frequently than homologous sera, and 3. incidence and the titre of circulating antibody appear to correlate with extent and stages of the disease (30).

The purpose here is to review some of the major immunologic studies in melanoma and present relevant data from our work in the field of humoral and cell mediated immunity in malignant melanoma.

DETECTION OF TUMOR RELATED ANTIGEN AND CIRCULATING ANTIBODY IN MALIGNANT MELANOMA

A tumor associated soluble antigen capable of stimulating both autochthonous and allogeneic lymphocytes from melanoma patients was detected earlier in this laboratory (25) in the cyst fluid from a necrotic melanoma. This substance was found to be an electrophoretically homogeneous protein with the mobility of a beta globulin. An immunochemically identical substance was isolated from the urine and tumor tissues from three patients. Rabbit antiserum prepared against the lymphocyte stimulating substance reacted with the tumor cell protein and its plasma and urinary counterpart, when tested by Ouchterlony gel diffusion technique.

In a subsequent study (31) we reported that following dimethyl triazeno imidazole carboximide (DTIC) therapy the circulating antigen disappeared from patient's serum when studied on starch block electrophoresis. Posttreatment lymphocytes responded strikingly to pretherapy tumor fluid, however, marked reduction in stimulation was noted when such lymphocytes were incubated with posttherapy tumor fluid. Also, lymphocytes from five other melanoma patients were also stimulated by the pretreatment tumor fluid, while marked reduction in this cross-reactivity was noted with posttherapy fluid. These studies suggested the presence of a cross-reacting tumor associated antigen and inhibition of its synthesis upon treatment with DTIC.

Subsequently, an indirect membrane-immunofluorescence technique has been employed to detect membrane-bound antigen in melanoma cells and circulating humoral immunity against such antigen. Tissue cultures are maintained in monolayer in Ham's F-10 media (Gibco) supplemented by 20% fetal calf serum (Gibco). All cultures are periodically tested for mycoplasma contamination. Trypsinized cells (1×10^5) are incubated in microfuge tubes with test serum (in desired concentration) for $\frac{1}{2}$ hr at 37°C after which the cells are washed in phosphate buffered saline three times, and incubated with a commercially available (Hyland) fluorecein isothiocyanate labeled antihuman globulin (1:20) for another $\frac{1}{2}$ hr at 37°C. The cells are again washed, resuspended in 20% glycerol, mounted on glass slides, and read for specific membrane immunofluorescence. A positive reaction
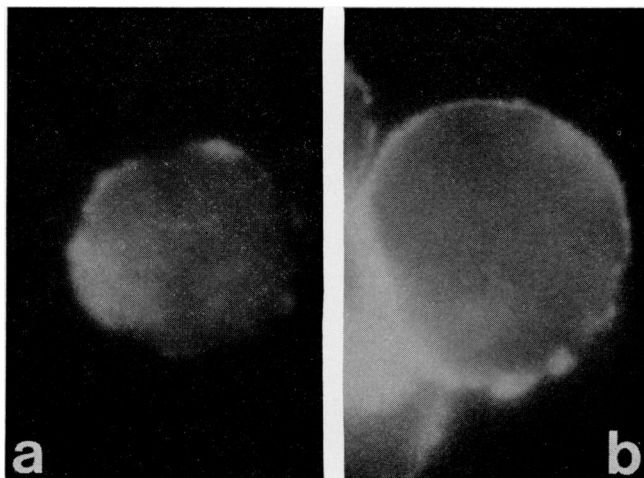
FIG. 1. Membrane immunofluorescent photomicrographs of melanoma cells grown in serial tissue culture (1a), and after heterotransplantation (1b). The cell in serial tissue culture demonstrates discrete areas of fluorescence (1a), whereas a complete ring of fluorescence is observed on the cell derived from transplanted tumor explant (1b).

is characterized by discrete sites of bright apple-green fluorescence or a complete ring of fluorescence (see heterotransplantation section) along the cell membrane (Fig. 1). A diffuse bright green staining is considered indicative of cell death. An immunofluorescence index (I.F. index) is calculated by the following formula: I.F. index = mean proportion of unstained cells with normal control serum minus the proportion of unstained cells with test serum, divided by the former number. An I.F. index of 0.3 or more is considered positive.

Autologous sera react almost always with their tumor cells in culture, whereas positive reaction is noted in 40% of the homologous sera from melanoma patients when tested on one of our reference melanoma cell lines. When a panel of positive sera is tested against several other melanoma cell lines, positive reaction is always noted with some of the reference sera against one or the other line but never consistently with all of them.

Characteristic individual specificity in melanoma antigen. Serologic specificity of the antimelanoma antibody has been previously documented with the usual methods of testing positive reference sera against established culture lines derived from other malignancies (where such tests have been found negative), and also by absorption study where other cell lines, including cells from benign nevi, have been shown to fail to absorb the reactivity from the positive reference sera when cultured melanoma cells successfully did so (27, 28).

Thus, available data and the results of our study suggest that antigens with tumor related specificity are often shared by melanomas from different patients. It is apparent, however, that beside this shared cell surface antigen, individual specificity of cell surface antigen in melanoma also does exist. This is evident from our data and the data of others that while the incidence of circulating antibody against melanoma antigen varies from 40% to 61% in different series, the reactivity (by I.F.) approaches 100% with autochtonous sera. This individual specificity in membrane antigen in melanoma has also been shown by Lewis *et al.* (30) with an *in vitro*, antibody mediated cytotoxicity test. Since antigens bound to cell surface are

capable of eliciting host response that may lead to eventual destruction of tumor cells, these individual specificities of cell membrane antigen are crucial in host defense against melanoma.

Relation between circulating antibody and prognosis in malignant melanoma. It has been suggested that incidence and level of antimelanoma antibody correlate with stage of disease (32). Cytotoxic antibody was detectable by Lewis *et al.* (23, 30) in patients who had only localized disease, while such antibody could not be found in patients with widespread metastatic melanoma. The decline of antimelanoma antibody was found to parallel the progression of disease from localized to generalized form. A reversal of this phenomenon was further demonstrated by the same authors as inoculation of irradiated autogolous tumor cells following extensive removal of disseminated tumor was found to convert seronegative patients to seropositive (by cytotoxicity test). In a subsequent study (33) they demonstrated that sera from seronegative patients (prior to immunization with irradiated autologous cells) contained a "blocking agent," an IgG, which interacted with the antibody (also an IgG) in the postimmunized sera. It should be noted that this phenomenon (where the "blocking agent" was directed against another cytotoxic antibody, or perhaps against an antigen–antibody complex), differed fundamentally from those described in lymphocytotoxicity or colony-inhibition studies (34, 35), where such blocking factor(s) are believed to be directed against the tumor antigen. The phenomenon of loss of humoral immunity is, however, somewhat reminiscent of similar phenomenon of reversible gain or loss of TL antigen (antigenic modulation) in mouse leukemia (36). It is not possible to determine whether the loss of antibody is the cause or the consequence of progression of disease, nor is it known whether induction of antibody by such immunization will have favorable or adverse influence on tumor growth, particularly since the phenomenon of immunologic enhancement is well established in experimental tumor system (37). Further studies are warranted for better understanding of the implication of shared versus individually specific surface antigen, and for identification of the circumstances leading to the formation of the so-called blocking factor(s), both of which will have important bearing on the design of immunotherapy.

CELL MEDIATED IMMUNITY IN MELANOMA

Thus far the discussion has been limited to the humoral immunity in melanoma. Humoral immunity, however, is a relatively ineffective host defense against most tumors and (though not conclusively proven in humans yet) may, in effect, block tumor rejection and cause immunologic enhancement. The evidence thus far strongly indicates that the cell mediated immunity (CMI) is the principle effector mechanism in immune defense against cancer. Thymus derived lymphocytes, believed to be the major participants in CMI, are primarily involved in rejection of both tumor homograft and naturally occurring tumors (8, 38–41). The two rejection processes indeed appear to be mediated through the same principle. The homograft rejection being initiated by tumor specific transplantation antigens (TSTA) and the immune response against spontaneous tumors is also believed to be initiated by tumor specific antigens (both the TSTA in animal tumor and tumor specific antigen in human tumor are, by definition, neoantigens). From their work on cell mediated immunity in various human tumors the Hellstroms (39) have offered an analogy between TSTA and tumor specific antigens in human tumors.

Obviously, transplantation experiments with human tumor is not a realistic possibility, yet autotransplantation and homotransplantation studies (42, 43) have been conducted in humans and it has been shown that "takes" of such transplant is influenced by patient's cell mediated immune response. To obviate the unrealistic approach of transplantation experiments, other *in vitro* experimental techniques have been adopted for the study of cell mediated immunity in human tumors. For our purposes we shall confine our discussion to a brief review of some of these studies in melanoma and describe our own experience. It has been shown that characteristic delayed hypersensitivity reaction can be elicited in guinea pigs, with intradermal injection of soluble antigen extracted from chemically induced sarcoma, when the recipient animal is preimmunized with the same tumor antigen. Furthermore, selective resistance to such tumor transplant has been shown to correlate with positive dermal hypersensitivity reaction (44, 45). These studies have led to the general belief that cell mediated immunity against tumors involve delayed hypersensitivity reaction.

Delayed hypersensitivity type reaction with intradermal injection of cell-free extracts of malignant melanoma has been demonstrated by Fass *et al.* (46). Specificity of this reaction has been established from demonstration of failure to produce such a reaction with extracts from normal tissue or benign pigmented mole. This type of delayed hypersensitivity reaction specific to tumor extract has been shown to correlate with the extent of disease by the authors, who noted positive reaction specific to tumor extract only in patients having localized disease. Anergy to such tumor extract according to the authors was not due to generalized anergy, implying a rather selective defect in response to melanoma antigen.

Besides delayed hypersensitivity against autologous tumor extracts, nonspecific reaction against common bacterial or fungal antigens, or sensitization with chemical hapten such as dinitrochlorobenizene (DNCB) has been shown to serve as a prognostic indicator in melanoma where positive reaction has been equated with favorable prognosis (47), though an earlier study by Ziegler *et al.* (48) failed to make such a correlation, and Pinsky *et al.* (49) subsequently noted that ability to react to DNCB sensitization is often preserved in virtually terminal patients with widespread melanoma.

Since the immune lymphocytes are the mediator cells responsible for delayed hypersensitivity reaction, homograph rejection, and also by implication for tumor immunity, *in vitro* experimental techniques have been employed to study the interaction between immune lymphocytes and tumor cells. Evidence for selective interaction between immune lymphocytes and melanoma target cells has been sought in a bidirectional manner. On one hand, studies are undertaken to document characteristic changes in lymphocytes and on the other hand, attempts have been made to study the consequences of such interaction on the survival of melanoma target cells (cytotoxicity). Either would be indicative of cell mediated immune reaction.

That autologous and sometimes the allogeneic lymphocytes react with melanoma cells or melanoma cell products has been demonstrated by several authors (50, 51). Using a mixed, lymphocyte tumor cell culture (MLTC) technique Nagel *et al.* (51) demonstrated transformation of autologous lymphocytes characterized by increased DNA synthesis when they were allowed to interact with autochthonous melanoma cells. As mentioned earlier, lymphocyte transformation study from our laboratory revealed transformation of both autochthonous and allogeneic lympho-

cytes from melanoma patients with a soluble antigen extract from the cyst fluid of a necrotic melanoma (25), indicating cross-reactivity with a common melanoma antigen. The same antigen was used by Cochran *et al.* (52) in a leukocyte migration inhibition study where the authors demonstrated leukocyte migration inhibition in 10 of 16 disease-free melanoma patients as opposed to 1 of 6 patients with recurrent melanoma and 2 of 16 normal controls. Cochran's study provided further evidence for measurable cell mediated immunity against melanoma.

Besides the demonstration of changes in lymphocytes observed in the interaction of lymphocyte and melanoma cell, cytotoxic property of autologous and allogeneic lymphocytes have been demonstrated by the Hellstroms, using the colony inhibition technique (40, 53), and more recently by several authors with *in vitro* lymphocyte cytotoxicity technique (35, 41, 54, 55). These *in vitro* microcytotoxicity techniques are of considerable interest and have offered a unique opportunity to study the complex interrelationship between humoral and cell mediated immunity in human tumors.

Microcytotoxicity test for cell mediated immunity. Because of the obvious potential of *in vitro* lymphocyte cytotoxicity tests, we have adapted a modification of Hellstrom's microcytotoxicity technique for assay of cellular immunity in malignant melanoma. Details of the technique have been described elsewhere (54). In brief, cultured cells derived from melanoma were used as target cells in 16 mm diam Linbro plates (100/wells). After allowing the cells to attach (over 18–24 hr incubation) test serum in 1:6 dilution is added for 30 min after which the serum was removed. Lymphocytes obtained by Woods' technique (56) were then added to the wells. The plates were incubated on a rocker for an additional 45 min after which the medium was supplemented with 40% fetal calf serum. Then the plates were again incubated on a rocker under CO_2 for 48–50 hr after which they were washed and stained. All incubations were conducted in humid chambers at 37°C. Finally the residual viable cells were counted. Appropriate controls were always included.

Results (Table 1) to date indicate that *in vitro* lymphocyte mediated cytotoxicity may decrease with metastatic spread of melanoma. Levels of cytotoxicity (in excess of 40%) are seen only in patients free of disease after surgery or chemotherapy. It should be noted that our data differs from those of Hellstroms (57), who were only able to show a reduction in the "level" of cell mediated immunity in advanced cases by titrating the ratio of lymphocytes and tumor cells. The Hellstroms

TABLE 1

% Cell-mediated Cytotoxicity (Corrected Values)

	No systemic treatment	BCG or chemotherapy (DTIC)	
Disease-free or regressing	Positive control = 40% SH 36% AF 44% Median = 40%	AP 59% TH 39% Median = 47%	
Progressing	JM 33% D 17/33% Median = 24/33%	ME 23% (NC) KPL 23% LH 32% GR 35% Median = 26% (0's excluded)	BR 20% CY 0*% ET 0*% JB 0*%

TABLE 2

SERUM BLOCKING FACTORS

	Absent or negative	Positive
Disease-free or regressing	5	0
Progressing	2	5

(Patients without significant CMI excluded from table)

(34, 57) have demonstrated that patients with progressive metastatic disease exhibit a circulating factor in their sera which is capable of blocking autologous and allogeneic lymphocytotoxicity *in vitro*. Results of our studies are in agreement with the Hellstroms. Blocking factor was found to be present in sera from five out of seven patients with progressing metastatic melanoma where as none of the five patients clinically free of disease or with regressing tumor exhibited such blocking factor (Table 2). Interestingly, the Hellstroms (58) have recently reported the presence of another serum factor that is capable of canceling the blocking property of sera containing the so called blocking antibody.

It is noteworthy that considerable differences exist in the results of *in vitro* lymphocyte cytotoxicity studies from different laboratories. For example, Currie *et al.* (59) failed to demonstrate the presence of any blocking factor in sera from patients with melanoma and found that autochthonous lymphocytotoxicity in established melanoma was extremely uncommon. Despite such differences, evidence nevertheless is mounting in the literature in favor of a serum blocking factor in bladder carcinoma (60), in neuroblastoma (12), and in human sarcoma (61), where autologous serum is being shown to inhibit lymphocyte stimulation by autochthonous sarcoma cells.

It should be emphasized that while serologic methods for the detection of humoral tumor immunity are relatively well established (62), microcytotoxicity techniques for the detection of cell-mediated immunity are yet to be uniformly standardized. The need for standardization of microtest technique for assay of lymphocyte cytotoxicity has recently been well articulated by the participants of a workshop in human tumor immunology held at Sloan-Kettering Institute for Cancer Research, New York (63). Investigators representing different institutions worked with the same materials (target cells, lymphocytes, sera, reagents, etc.). Significant differences in the results obtained by different investigators were observed. Though malignant melanoma was not studied in this workshop, the methodologic problems observed will nevertheless apply for *in vitro* microtest technique for assay of cell mediated immunity in any human cancer. Several important problems, such as use and screening of serum for plating efficiency; use of target cells with reference to their derivation, need for target cells free of mycoplasma contamination; method of lymphocyte purification; period of incubation, etc., were discussed at great length. Recommendations were made that a cooperative effort be undertaken to standardize some elements in the technique using coded materials supplied through a central laboratory.

Clearly then, before these techniques can be widely applied by different laboratories to obtain reproducible data, these basic problems in the methodology must be resolved. Fortunately, several laboratories are now working in this direction.

HETEROTRANSPLANTATION STUDY
WITH MALIGNANT MELANOMA

No satisfactory heterotransplantation model of human melanoma is currently available. Such a model would offer an experimental system paralleling the *in vivo* condition of human melanoma to a far greater degree than do any *in vitro* systems and could be useful as a model for various immunologic and immunotherapeutic studies. For the purpose of establishing an animal heterotransplantation model, we have attempted to transplant human melanoma in Wistar Furth (WF) rats by inoculating cultured melanoma cells. Antithymocyte serum (ATS)-treated WF neonates have been inoculated with 1×10^7 melanoma cells subcutaneously on their day of birth or on the second postnatal day. The recipient neonates are given further injections of ATS on every other day until tumor nodules become palpable, after which gradual tapering of ATS administration is made. Palpable tumor growth on the site of inoculation is usually appreciable on the tenth day (range 7–21) with continued growth thereafter. Serial passage of such transplants in ATS-treated WF neonates is easily achieved by inoculating finely minced fresh tumor explants. Three cell lines have been transplanted. The transplanted tumor exhibits the same histology as the original biopsy tissue from which the cell line is derived (Fig. 2) and both the transplanted tumor cells and the cultured cells maintain their aneuploid karyotype.

Membrane immunofluorescence studies have revealed a significant augmentation of membrane antigenicity on the animal-passaged cells. This is characterized by a complete, or almost complete, ring of bright fluorescence all around the cell membrane as opposed to isolated discrete sites of immune complexes on the tissue culture cells when tested under identical experimental conditions (Fig. 1). Of further interest, the animal-passaged cells tend to lose this antigenic augmentation as these cells are maintained through continued subcultures. Thus, we believe that the phenomenon of antigenic augmentation by animal passage offers a system for restoring, preserving and augmenting membrane antigen. Besides immunologic studies, this model may also be used for chemotherapeutic, cytokinetic or other relevant studies with melanoma.

HL-A PHENOTYPE WITH MELANOMA PATIENTS

As a part of our study of cell mediated immunity in malignant melanoma, HL-A typing has been undertaken on lymphocytes from patients with malignant melanoma. Initial studies revealed an interesting deficit of the second, sublocus-antigen series characterized by an absence of HL-A5. The study subsequently has been extended and the preliminary result with details of the methodology has been published (64). Thirty-three patients with melanoma thus far tested have failed to reveal the presence of HL-A5, as opposed to 24 out of 29 nonmelanoma cancer patients and 9 out of 62 normal individual controls demonstrating positive HL-A5 (Table 3). Typing data from families of five patients indicate that in one family the parents carried the HL-A5 antigen, suggesting that the deficiency of HL-A5 in the patient is acquired by deletion or by genetic crossing over. The significance of this finding of HL-A5 deficiency in melanoma remains obscure, and further studies are presently underway to examine the relevance of this phenomenon to malignant melanoma regarding its mode of dissemination or host-immune response against such tumors.
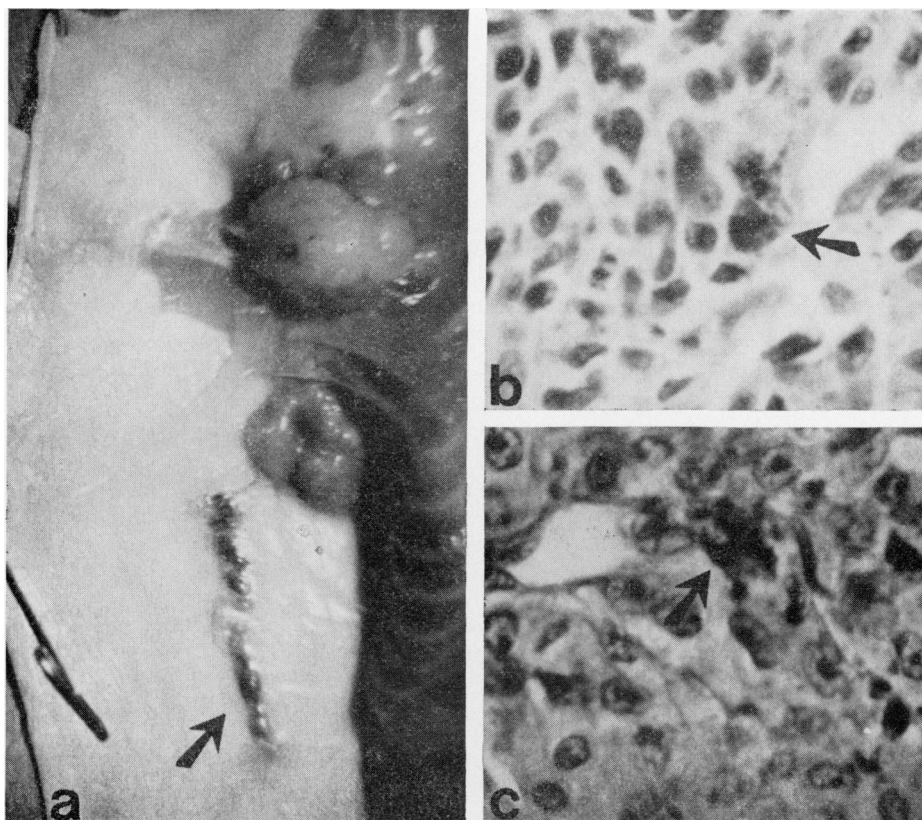
FIG. 2. Photograph of human melanoma tumor transplant (2a) in Wistar Furth rat. The arrow in Fig. 2a represents growth of tumor along the needle track. Figure 2b demonstrates the histology of the biopsy from which the cell line has been derived and Fig. 2c demonstrates the histology of the transplanted tumor explant. The arrows in Fig 2b and 2c indicate pigment production in the tumors.

TABLE 3

PROSPECTIVE STUDY OF HL-A5 DEFICIENCY IN MELANOMA

	No. tested	HL-A5 +	HL-A5 −		
Normal control	62	9	53	$P = 0.480$	
Cancer control	29	5	24		$P^* = 0.017$
Melanoma	33	0	33	$P = 0.018$	

* Fisher's Exact Test.

SUMMARY

The goal of immunologic study of a given neoplasm is twofold. One is to define the antigenic makeup of the malignant cells and the other is to identify host-defense mechanisms involving either humoral or cell mediated immunity. Data from studies reported here has supported the view that tumor-specific or associated antigens are present in human melanoma as identified by a number of assay systems including immunofluorescence, lymphoblastogenesis, leukocyte migration inhibition and lym-

phocytotoxicity. A new system of heterotransplantation which appears to augment tumor cell membrane antigenicity is a new tool for further immunologic study. The observation that the HL-A5 sublocus is deleted in melanoma patients may provide a genetic marker in this tumor system.

ACKNOWLEDGMENTS

REFERENCES

1. Foley, E. J., Antigenic properties of Methylcholanthrene induced tumors in mice of the strain of origin. *Cancer Res.* **13**, 835 (1953).
2. Prehn, R. T., and Main, J. M., Immunity to Methylcholanthrene induced sarcomas. *J. Nat. Cancer Inst.* **18**, 769 (1957).
3. Old, L. J., and Boyse, E. A., Immunology of experimental tumors. *Ann. Rev. Med.* **15**, 167 (1964).
4. Klein, G., Tumor antigens. *Ann. Rev. Microbiol.* **20**, 223 (1966).
5. Southam, C. M., Evidence for cancer specific antigens in man. *In* "Progress in Experimental Tumor Research" (Homburger, F., Ed.) Vol. 9, p. 1. Karger, Basel/New York, 1967.
6. Southam, C. M., Evidence of immunological reactions to autochthonous cancer in man. *Eur. J. Cancer* **1**, 173 (1965).
7. Oettgen, H. F., Old, L. J., and Boyse, E. A., Human tumor immunology. *Med. Clin. N. Amer.* **55**, 761 (1971).
8. Henle, G., and Henle, E., Immunofluorescence in cells derived from Burkitt's lymphoma. *J. Bacteriol.* **91**, 1248 (1965).
9. Klein, G., Clifford, P., Klein, E., and Stjernsward, J., Search for tumor specific immune reactions in Burkitt's lymphoma patients by membrane immunofluorescence reaction. *Proc. Nat. Acad. Sci.* **55**, 1628 (1966).
10. Gold, P., and Freedman, S. O., Demonstration of tumor specific antigens in human colonic carcinoma by immunologic tolerance and absorption technique. *J. Exp. Med.* **121**, 439 (1965).
11. Hellstrom, I., Hellstrom, K. E., Pierce, G. E., and Bill, A. H., Demonstration of cell bound and tumor immunity against neuroblastoma cells. *Proc. Nat. Acad. Sci.* **60**, 1231 (1968).
12. Morton, D., and Malmgren, R. A., Human osteosarcomas: Immunologic evidence suggesting an associated infectious agent. *Science* 162, 1279 (1968).
13. Giraldo, G., Beth, E., Hirshaut, Y., Aoki, T., Old, L. J., Boyse, E. A., and Chopra, H. C., Human sarcomas in culture. Foci of altered cells and a common antigen; induction of foci and antigen in human sarcomas in cultures by filtrate. *J. Exp. Med.* **121**, 439 (1965).
14. Mukherji, B., and Hirshaut, Y., Antigen (S₂) in human sarcomas and fetal tissues. *Proc. Amer. Ass. Cancer Res.* **13** (Abst.) 23 (1972) (manuscript in press, Science).
15. Kopf, A. W., Host defence against malignant melanoma. *Hosp. Prac.* **6**, 116 (1971).
16. Summer, W. C., and Foraker, A. G., Spontaneous regression of human melanoma. Clinical and experimental study. *Cancer* **13**, 79 (1960).
17. Smithers, D. W., Spontaneous regression of tumors. *Clin. Radio.* **13**, 132 (1962).
18. Smith, J. L., and Miller, T. R., Spontaneous regression of primary malignant melanoma with regional metastasis. *Cancer* **18**, 1399 (1965).
19. Little, J. H., Partial regression in primary cutaneous malignant melanoma. *Pathology* **3**, 62 (1971).
20. Bodenham, D. C., A study of 650 observed malignant melanomas in the Southwest region. *Ann. Roy. Coll. Surg. Eng.* **43**, 218 (1968).

21. Wilber, D. L., and Hartman, H. R., Malignant melanoma with delayed metastatic growth. *Ann. Intern. Med.* **5**, 201 (1971).

22. Lloyd, O. C., Regression of malignant melanoma as a manifestation of a cellular immune response. *Proc. Royal Soc. Med.* **62**, 543 (1969).

23. Lewis, M. G., Possible immunological factors in human malignant melanoma. *Lancet* **2**, 921 (1967).

24. Gray, B. K., Mehigan, J. T., and Morton, D. L., Demonstration of antibody in melanoma patients cytotoxic to human melanoma cells. *Proc. Amer. Ass. Cancer Res.* **12**, 79 (1971).

25. Jehn, U. W., Nathanson, L., and Schwartz, R. S., *In vitro* lymphocyte stimulation by a soluble antigen from malignant melanoma. *N. Engl. J. Med.* **283**, 329 (1970).

26. Oettgen, H. F., Aoki, T., Old, L. J., Boyse, E. A., DeHarven, E., and Miles, G., Suspension culture of a pigment producing cell line derived from a human malignant melanoma. *J. Nat. Cancer Inst.* **41**, 827 (1968).

27. Morton, D. L., Malmgren, R. A., Holmes, E. C., and Ketcham, A. S., Demonstration of antibodies against human malignant melanoma by immunofluorescence. *Surgery* **64**, 233 (1968).

28. Muna, N. M., Marcus, S., and Smart, C., Detection by immunofluorescence of antibodies specific for human malignant melanoma cells. *Cancer* **23**, 88 (1969).

29. Romsdahl, M. M., and Cox, I. E., Human malignant melanoma antibodies demonstrated by immunofluorescence. *Arch. Surg.* **100**, 491 (1970).

30. Lewis, M. G., Ikonopisov, R. L., Nairn, R. C., Phillips, T. M., Hamilton, F. G., Bodenham, D. C., and Alexander, P., Tumor specific antibodies to human malignant melanoma and their relationship to the extent of disease. *Brit. Med. J.* **3**, 547 (1969).

31. Nathanson, L., Jehn, U., and Schwartz, R. S., Disappearance of a tumor associated antigen in malignant melanoma after imidazole carboxamide therapy. *Cancer* **24**, 411 (1971).

32. Lewis, M. G., Immunologic studies in patients with malignant melanoma: the role of circulating antibody. *In* "Melanoma and Skin Cancer." Proceedings of the Int'l. Cancer Conf., Sydney (McCarthy, W. H., Ed.) p. 233 1972.

33. Lewis, M. G., Phillips, T. M., Cook, K. B., and B!ake, J., Possible explanation for loss of detectable antibody in patients with disseminated malignant melanoma. *Nature (London)* **232**, 52 (1971).

34. Hellstrom, I., Sjogren, H. O., Warner, G., and Hellstrom, K. E., Blocking of cell mediated tumor immunity by sera from patients with growing neoplasms. *Int. J. Cancer* **7**, 226 (1971).

35. Clark, D. A., and Nathanson, L., Cellular immunity (CMI) in malignant melanoma. *Proc. Amer. Ass. Cancer Res.* **13** (Abst) 79 (1972).

36. Old, L. J., Stockert, E., Boyse, E. A., and Kim, J. H., Antigenic modulation. Loss of TL antigen from cells exposed to TL antibody. Study of the phenomenon *in vitro. J. Exp. Med.* **127**, 53 (1968).

37. Kaliss, N., Immunological enhancement of tumor homografts in mice. A Review. *Cancer Res.* **18**, 1992 (1958).

38. Hellstrom, K. E., and Hellstrom, I., Cellular immunity against tumor antigens. *Advan. Cancer Res.* **12**, 167 (1969).

39. Hellstrom, I., Pierce, G. E., and Hellstrom, K. E., Human tumor-specific antigens. *Surgery* **65**, 984 (1969).

40. Hellstrom, I. E., A colony inhibition technique for demonstration of tumor cell destruction by lymphoid cells *in vitro. Int. J. Cancer* **2**, 65 (1967).

41. Hellstrom, I., Hellstrom, K. E., Sjogren, H. O., and Warner, G. A., Demonstration of cell mediated immunity to human neoplasms of various histologic types. *Int. J. Cancer* **7**, 1 (1971).

42. Southam, C. M., and Brunschwig, A., Quantitative studies of autotransplantation of human cancer—Preliminary Report. *Cancer* **14**, 971 (1961).

43. Brunschwig, A., Southam, C. M., and Levin, A. G., Host resistance to cancer: clinical experiments by homotransplants, autotransplants, and a mixture of autologous leukocytes. *Ann. Surg.* **162**, 102 (1965).

44. Churchill, W. H., Rapp, H., Knonman, B. S., and Boros, T., Detection of antigens of a new diethylnitrosamine induced transplantable hepatoma by delayed hypersensitivity. *J. Nat. Cancer Inst.* **41**, 13 (1968).

45. Oettgen, H. F., Old, L. J., McLean, E. P., and Carswell, E. A., Delayed hypersensitivity and transplantation immunity to soluble antigens of chemically induced tumors in inbred guinea pigs. *Nature (London)* **220**, 295 (1968).

46. Fass, L., Herberman, R. B., Ziegler, J. L., and Kiryabwire, J. W. H., Cutaneous hypersensitivity reactions to autologous extracts of malignant melanoma cells. *Lancet* **1**, 116 (1970).

47. Morton, D. L., Haskell, C. M., Pilch, Y. H., Sparks, F. C., and Winters, W. D., Recent Advances in Oncology. *Ann. Int. Med.* **77**, 431 (1972).

48. Ziegler, J. L., Lewis, M. A., Luyombya, J. M. S., and Kiryabwire, J. W. M., Immunologic studies in patients with malignant melanoma in Uganda. *Brit. J. Cancer* **23**, 729 (1969).

49. Pinsky, C. M., Oettgen, H. F., ElDomeiri, A., Old, L. J., Beattie, E. J., and Burchenal, J. H., Delayed hypersensitivity reactions in patients with cancer. *Proc. Amer. Ass. Cancer Res.* **12**, 100 (1971).

50. Savel, H., Effect of autologous tumor extracts on cultured human peripheral blood lymphocytes. *Cancer* **24**, 56 (1969).

51. Nagel, G. A., Piessens, W. F., Stilmant, M. M., and Lejeune, F., Evidence for tumor specific immunity in human melanoma. *Eur. J. Cancer* **7**, 41 (1971).

52. Cochran, A. J., Jehn, U. W., and Gothoskar, B., Cell mediated immunity in malignant melanoma. *Lancet* **1**, 1340 (1972).

53. Hellstrom, I. E., Hellstrom, K. E., Pierce, G. E., and Yang, J. P., Cellular and humoral immunity to different types of human neoplasms. *Nature (London)* **220**, 1352 (1968).

54. Clark, D. A., and Nathanson, L., Patterns of cellular immunity in malignant melanoma (in press).

55. Byrne, M., Heppner, G., Stolbach, L., Cummings, F. J., McDonough, E., and Calabresi, P., Correlation of tumor specific immunity with extent of disease in malignant melanoma. *Proc. Amer. Ass. Cancer Res.* 13 (Abst.) 46 (1972).

56. Woods, A. H., A closed system for large scale lymphocyte purification. *Blood* **35**, 39 (1970).

57. Hellstrom, I., and Hellstrom, K. E., Some aspects of humoral immunity and their possible implications for tumor prevention and therapy. *In* "Frontiers of Radiation Therapy and Oncology" (Vaeth, J. M., Ed.) Vol. 7, p. 3, 1972.

58. Hellstrom, I., Hellstrom, K. E., Sjogren, H. O., and Warner, G. A., Serum factors in tumor free patients canceling the blocking of cell mediated tumor immunity. *Int. J. Cancer* **8**, 185 (1971).

59. Currie, G. A., Lejeune, F., and Fairley, G. H., Immunization with irradiated tumor cells and specific lymphocyte cytotoxicity in malignant melanoma. *Brit. Med. j.* **2**, 305 (1971).

60. Bubenik, J., Perlman, P., Helenstein, K., and Moberger, G., Cellular and humoral immune responses to human urinary bladder carcinomas. *Int. J. Cancer* **5**, 310 (1970).

61. Vanky, F., Stjernsward, J., Klein, G., and Nilsonne, U., Serum mediated inhibition of lymphocyte stimulation by autochthonous human tumors. *J. Nat. Cancer Inst.* **47**, 95 (1971).

62. Old, L. J., Boyse, E. A., Geering, G., and Oettgen, H. F., Serologic approaches to the study of cancer in animals and in man. *Cancer Res.* **28**, 1288 (1968).

63. Oettgen, H. F., Bean, M. A., and Klein, G., Workshop in human tumor immunology. *Cancer Res.* **32**, 2845 (1972).

64. Clark, D. A., Necheles, T., Nathanson, L., and Silverman, E., Apparent HL-A5 deficiency in malignant melanoma. *Transplantation* **15**, 326 (1973).