Papers and Originals

Tumour-specific Antibodies in Human Malignant Melanoma and their Relationship to the Extent of the Disease

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Summary: Biopsy specimens and sera were obtained from 103 melanoma patients. Autoantibodies were demonstrated by (1) complement-dependent cytotoxicity of autologous melanoma cells in short-term culture; (2) complement-dependent inhibition of ribonucleic acid synthesis; (3) immunofluorescent staining of the cytoplasm of killed melanoma cells and of the surface membrane of viable melanoma cells. Over one-third of the sera studied had antibodies to autologous melanoma cells. Although for technical reasons all three tests could not be performed with the cells from every melanoma, whenever multiple testing was possible there was complete concordance. The autoantibodies were virtually confined to patients in whom the disease was not widely disseminated, and over 80% of such patients had positive sera. In a limited number of patients who have been followed autoantibodies disappeared as the disease progressed to become widely disseminated. Two patients with generalized disease developed autoantibodies following inoculation by their own irradiated tumour cells.

Two types of autoantibodies were recognized: one, active against antigen(s) in the cell surface membrane, was specific for each tumour—that is, only the autologous serum reacted—and was concerned in the cytotoxic activity; the other reacted with cytoplasmic antigens which appeared to be present in most or all melanoma cells.

Introduction

For many years evidence has been accumulating that there may be a host reaction to malignant melanoma and that this reaction could be immunological. Firstly, melanoma is one of the malignant diseases which occasionally undergoes spontaneous regression (Sumner, 1953; Sumner and Foraker, 1960; Smithers, 1962, 1967; Baker, 1964; Everson, 1964; Smith and Stehlin, 1965). Secondly, the disease may remain localized for a long time before it becomes disseminated (Lewis and Kiryabwire, 1968; Lewis and Johnson, 1968). Thirdly, even in patients with disseminated disease some metastatic deposits can regress at the same time as new ones appear and others continue to grow (Bodenham, 1968). Fourthly, Lewis (1967) has shown in a series of Uganda patients that those with localized tumours often had cytotoxic serum antibody active only against the patient's own melanoma cells. This suggested that there may be tumour-specific antigens in melanoma of a similar type to those found in chemically induced tumours in animals. Morton *et al.* (1968), Oettgen *et al.* (1968), and Muna *et al.* (1969), using immunofluorescence, demonstrated cross-reactivity between melanoma cell preparations and the sera from a number of different melanoma patients, but there was nothing to suggest that the antibodies studied were cytotoxic or tumour-specific. P. Mansell in collaboration with one of us (D. C. B.) (to be published) has also detected autoantibodies in some patients with malignant melanoma by several different techniques.

In experimental animals tumour-specific antigens have often been detected, and they may be of two kinds—namely, those situated on the cell surface membrane and those within the cell; frequently the sera of tumour-bearing animals contain antibodies that react with both (cf. Alexander and Fairley, 1967). Only cell surface membrane antigen would be likely to elicit a reaction which influences tumour growth, since both cell-mediated and humoral immunity can kill cells only by reaction with accessible antigens.

In the present study we have tested the reaction between serum and tumour cells from the same and different patients in two different ways: (*a*) by the ability of sera to inhibit, in the presence of complement, growth or metabolism of melanoma cells in vitro, and (*b*) by immunofluorescence to assess serum binding to cytoplasmic components of dead cells and to surface membrane of living cells. Two kinds of autoantibody have been recognized in over a third of melanoma patients—one reactive with cell surface which is also cytotoxic, the other reactive with cytoplasmic components. The occurrence of such autoantibodies was closely correlated with non-dissemination of the tumour.

Methods

Tumours and Sera

Immediately after removal the tumour was placed in a sterile container with serum-free tissue culture medium 199 (T.C. 199)

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and transported at ambient temperature to the laboratory. Samples received within 24 hours seemed as satisfactory for tissue culture as fresh material. All sera were stored at −20° C. without preservative and inactivated by heating to 56° C. for 30 minutes before testing. As a source of complement, serum from a donor with AB blood group was used and activity was assayed periodically.

Clinical Assessment

For the purpose of this study the patients were classified in three grades:

Grade 1.—Malignant melanoma confined to primary anatomical site.

Grade 2.—Invasion of regional lymph nodes with or without local satellite nodules.

Grade 3.—Tumour spread beyond the regional lymph nodes.

Procedure on Receiving Specimens

While the aim was to test the reaction of the serum with cells from the melanoma by three tests, there was sufficient material for this in only a quarter of the cases, but two out of the three tests were performed in nearly half the cases. Long-term cultures for future study were established for periods of a few weeks up to seven months in 58 of the 96 biopsies in which this was attempted. When the tumour was very small, immunofluorescence on dried cell films was often the only test that could be applied immediately and any remaining cells were cultured in the hope of obtaining sufficient proliferation for more extensive examination later. The cytotoxic effect of the serum on melanoma cells in culture was considered to be the most decisive test, and it was attempted whenever practicable (in 68 of the 103 cases).

All autoantibody detection methods required the tumour to be in the form of a cell suspension. Enzymes such as trypsin were not used, as their effect on the surface antigens is not known. Instead, the tumours were dispersed mechanically by means of scissors and scalpel—particularly scraping pieces of tumour with a straight-edged scalpel (Pulvertaft, 1959; Lewis, 1967). The resulting suspension contained melanoma and other cells, but there was seldom difficulty in distinguishing the tumour cells microscopically. The cell count was adjusted to about one million per ml. in T.C.199 with 20% foetal bovine serum. The viability of the melanoma cells in the suspensions was assayed by the dye-exclusion test with trypan blue, and ranged from 5 to 50% in different cases, with a mean of about 25%.

To permit examination of sera progressively during the course of the disease, cell suspensions were frozen in liquid nitrogen. They were dispersed in 10% dimethyl sulphoxide in T.C. 199 in 2-ml. ampoules cooled at about 1° C. per minute to −20° C. and then stored in liquid nitrogen. The viable fraction of cells which excluded vital dye was variably affected by this procedure. Cell preparations reconstituted from liquid nitrogen storage usually gave satisfactory cultures but were frequently unsuitable for membrane immunofluorescence.

Complement-dependent Cytotoxicity Test

The method, modified from Pulvertaft (1965), has been reported previously (Lewis, 1967). Essentially, cytotoxicity is recognized by failure of tumour cells to continue to adhere to the coverslip top closure of a special culture chamber formed by a ring of Teflon on a microscope slide sealed with silicone grease. Test sera were added at the beginning of incubation, which for the first 48 hours was carried out with the culture chamber inverted, coverslip down; thereafter for a further three to five days the chamber was repositioned coverslip up, to facilitate phase-contrast microscopical examination. Confusion of results by dead cells and debris was avoided because they failed to adhere to the cover glass. In a standard test 1-ml. samples of cell suspension were tested in duplicate against the following:

(1) 0·5 ml. of patient's serum + 0·5 ml. of complement.

(2) 0·5 ml. of patient's serum + 0·5 ml. of inactivated complement.

(3) 0·5 ml. of normal control serum + 0·5 ml. of complement.

(4) 0·5 ml. of serum from another melanoma patient + 0·5 ml. of complement.

(5) No serum.

A serum was recorded as being cytotoxic when the number of cells adhering to the cover glass in chamber 1 was less than 20% of the mean of chambers 2, 3, and 5, in which the values should be about the same. In nearly every case the result was obvious on inspection (Special Plate, Fig. 1).

Complement-dependent Inhibition of R.N.A.

The effect of the patient's serum plus complement on ribonucleic acid (R.N.A.) synthesis was assessed by the capacity of the cells to incorporate radioactive uridine (Ikonopisov, to be published). The rationale of the test is that R.N.A. synthesis is greatly reduced when the cell is damaged by complement-dependent cytotoxic antibodies. Some R.N.A. synthesis continues at a reduced level even after cells have sustained membrane damage, and we have found that this "cell-free" R.N.A. synthesis is inhibited by antibodies which react with cytoplasmic components. Thus the method is sensitive to antibodies which react both with the cell surface membrane and with cytoplasmic components. The procedure requires at least 20 million cells—that is, 20 ml. of the original cell suspension, which was concentrated to give a cell count of about five million per ml. All sera were dialysed to avoid introducing unlabelled uridine into the system. The concentrated cell suspension in 1-ml. aliquots was tested, in duplicate experiments, against the following:

(1) 0·5 ml. of patient's serum + 0·5 ml. of complement.

(2) 0·5 ml. of patient's serum + 0·5 ml. of inactivated complement.

(3) 0·5 ml. of normal control serum + 0·5 ml. of complement.

(4) 0·5 ml. of normal control serum + 0·5 ml. of inactivated complement.

The mixtures were incubated for one hour at 37° C. and tritiated uridine was then added at a concentration of 1·25 μCi./ml. Following a further 30 minutes' incubation the trichloroacetic acid precipitate after extraction with ethanol was dissolved in potassium hydroxide for scintillation counting. A test was considered satisfactory when the amount of uridine incorporated in tubes 3 and 4 was the same as tube 2 within ±10%. The test was regarded as positive when the uptake of uridine in tube 1 was at least 20% smaller than in tube 2.

Immunofluorescence and Serum Absorptions

Immunofluorescent staining of melanoma cells by patients' sera was carried out by the sandwich method using general procedures described in detail elsewhere (Nairn, 1969). For routine testing, films on chemically clean glass slides were made from the same original cell suspension as used for the cytotoxicity tests after first washing the cells with a standard edetic acid–bovine serum albumin buffer solution. The preparations were dried in a current of air from a fan for at least one hour and then plunged into a mixture of liquid nitrogen and isopentane at −160° C. to promote good adhesion of the films and to facilitate penetration of serum immunoglobulins into the tumour cell cytoplasm. Slides, removed from the freezing mixture after two to three minutes, were allowed to dry at room

temperature for a further hour, when they were ready for use. No chemical fixation of the cells was employed.

For staining, films were treated at room temperature with a drop of test serum for 30 minutes in a damp chamber, rinsed and washed for two 10-minute periods with standard phosphate-buffered saline (0·01 M phosphate, *p*H 7·1), drained, air-dried for a few minutes, and treated with a drop of fluorescein-labelled anti-human-globulin which had activity against human IgG and IgM, and finally again rinsed and washed with the buffered saline. Staining was normally carried out on the day of receipt of the specimen or the next day, but satisfactory results could be obtained for up to two days longer provided the films were kept dry in a sealed container with silica gel crystals at −20° C.; after this, variable antigenic deterioration occurred.

In a few experiments membrane immunofluorescent staining of living cells was obtained by the sandwich method, modified for cell suspensions, in 1-ml. polystyrene tubes. The original cell suspension was washed with the standard phosphate-buffered saline, and a drop of deposit from centrifuging at about 100 $g_{max.}$ for five minutes and containing about one million cells was gently mixed with an equal volume of test serum and left for 30 minutes at room temperature. The mixture was washed three times each in 10 ml. of the buffered saline and the cells were recovered by centrifuging at 100 *g* for five minutes on each occasion. Removal by pipette of the maximum amount of supernatant at each step helped to eliminate confusing cell debris. The fluorescein-conjugated antiglobulin was applied to the final sediment drop in the same way as the test serum and the washing was repeated. Clean technique is necessary for this test, and it is particularly important to avoid letting the cell suspension stream slowly down the side of the tube to leave tumour cells adhering to it with too few in the final sediment for satisfactory study.

The preparations mounted in buffered glycerol saline (*p*H 7·1) were examined by ultraviolet fluorescence microscopy using a darkground condenser with toric lens beneath, and a colourless barrier filter. Specificity of any immunofluorescent staining of the melanoma cells was established by a variety of tests, many of which were carried out every time a new test serum was examined. The fluorescent antiglobulin conjugate, after absorption twice each with fresh bovine liver homogenate and with well-washed human group AB red cells, gave no staining of the films or cell suspensions when used at the highest dilution which still gave maximum specific staining (about 2 mg. of globulin per ml.). Constant intensity of specific staining by the conjugate in each experiment was confirmed by including a control film treated with serum containing antinuclear factor. Sera from normal human subjects and from patients with diseases other than melanoma were also examined as negative controls. After preliminary experiments at various dilutions, all unabsorbed sera were diluted with an equal volume of the buffered saline before testing.

Tumour-specificity of any positively reacting sera from melanoma patients was assessed by staining experiments in which the activity of unabsorbed sera was compared with that of the sera absorbed twice with fresh homogenate of tumour or of skin from the immediate neighbourhood of the tumour. These homogenates were prepared from 6 μ frozen sections cut on a cryostat from blocks of tissue roughly 5 mm. square. Additional control of specificity of immunofluorescent reactions was provided by testing films of bronchial carcinoma cells or of lymphoblasts from a leukaemia patient.

Results

At least one of the tests for autoantibodies was carried out on 71 of the 103 tumour specimens available for study. Data were not obtained from 32 specimens for various technical reasons. Table I shows an analysis of results obtained by the three tests, each of which was positive in about one-third of the cases. Where more than one test was carried out on the same specimen completely concordant results were obtained (Table II).

TABLE I.—*Serum Antibody Tests Against Autologous Malignant Melanoma*

Test	Patients Tested	
	Total No.	No. Positive
Complement-dependent cytotoxicity ..	68	24 (35%)
R.N.A. synthesis inhibition	32	10 (31%)
Cytoplasmic immunofluorescence ..	47*	18 (37%)

* Three of these were examined by immunofluorescence only.

TABLE II.—*Concordance of Two or More Tests for Autoantibodies*

Patient	Complement-dependent Cytotoxicity	R.N.A. Synthesis Inhibition	Cytoplasmic Immuno-fluorescence
MU7	+	+	+
ME4	+	N.T.	+
ME10	+	+	+
ME18	+	N.T.	+
ME21	+	+	+
ME36	+	+	+
ME39	+	N.T.	+
ME49	+	+	+
ME54	+	+	+
ME58	+	+	+
ME74	+	+	+
ME79	+	N.T.	+
ME82	+	N.T.	+
ME88	+	N.T.	+
MU1	–	–	N.T.
MU4	–	–	–
MU5	–	–	–
ME1*	–	–	N.T.
ME3	–	–	N.T.
ME15	–	–	–
ME22	–	–	–
ME26	–	–	–
ME30	–	–	–
ME33	–	N.T.	–
ME35	–	N.T.	–
ME37	–	–	–
ME42	–	–	–
ME51	–	–	–
ME55	–	–	–
ME60	–	–	–
ME61	–	–	–
ME62	–	–	–
ME64	–	–	–
ME68	–	N.T.	–
ME69*	–	–	–
ME78	–	N.T.	–
ME83	–	–	–
ME86	–	N.T.	–
ME91	–	N.T.	–

N.T. = Not tested.
* Positive serum reactions were induced subsequently in these patients by autoimmunization.

Correlation between the presence of autoantibody and clinical status is summarized in Table III, which shows that of the 24 patients giving positive serum reactions only one had disseminated disease (grade 3), whereas dissemination had occurred in 42 out of 47 seronegative patients. Tumour was confined to the primary site (grade 1) in four seropositive patients but not in any who were seronegative. The clinical grade was not known at the time of testing for antibodies.

TABLE III.—*Relationship Between Clinical Extent of Malignant Melanoma and the Presence of Autoantibodies Detected by One or More of the Three Tests*

Clinical Status (Grade)	Autoantibody Positive (24 Patients)	Autoantibody Negative (47 Patients)
1	4	0
2	19	5
3	1	42

Cytotoxicity Test

Of the 68 tumours examined in this way, 24 gave a positive reaction which was directed exclusively against the patient's own tumour cells. There was no reactivity against cells from other patients with malignant melanoma. Table IV illustrates an experiment in which seven sera were tested against 13 tumours, and cytotoxicity was seen only when the serum and

tumour cells came from the same patient. Moreover, in a few cytotoxicity tests with absorbed sera neutralization of activity was observed only after absorption with autologous tumour, not with autologous skin, nor with melanomas from other patients.

R.N.A. Synthesis Inhibition

Of the 32 tumours examined in this way 10 gave a positive reaction with the patient's own melanoma cells. When sera were tested against melanoma cells from other patients a lesser degree of inhibition was sometimes observed, presumably the result of activity against " cell-free " cytoplasmic organelles from already damaged cells. Such cross-reactions with internal cytoplasmic components are described in the next section.

Cytoplasmic Immunofluorescence

The characteristic staining pattern (Special Plate, Fig. 2) was homogeneous and confined to the cytoplasm, and most of the melanoma cells present in any particular film gave a positive reaction. Only sera from melanoma patients reacted in this way. The identity of the stained cells was established by their morphology and often by their containing melanin pigment. Non-melanoma cells did not stain, except for granulocytes and macrophages, which took up the conjugate non-specifically; these were readily identified microscopically and looked the same in control and test preparations.

Table V shows that 11 out of 12 sera giving immunofluorescent staining of autologous melanoma cells also stained cells from representative tumours ME30, with which the autologous serum reacted negatively, and ME36, with which the autologous serum reaction was positive. Eight of these sera reacted with both tumours. About one-third of the sera giving autologous-negative reactions showed reactivity with cells of the two other tumours. We conclude that nearly all melanoma cells, whether from seropositive or seronegative patients, contain a common internal cytoplasmic antigen or antigens. Such antigens were not detected in human lymphoid cells or bronchial carcinoma cells (Table VI). Cross-reactivity of melanoma patient sera was unrelated to blood groups.

The serum of one seropositive patient, ME4, cross-reacted strongly with all other melanoma cells and to a lesser degree with the lymphoid and bronchial carcinoma cells. It was found to have cytoplasmic autoantibodies of low titre (1:5) to a variety of mammalian tissues, including human thyroid, rat liver, and rat gastric parietal cells. Absorption studies failed to differentiate these antibodies from any reacting against melanoma cell cytoplasm. Another autologous-positive serum, one of a series from patient ME5, was shown to have antinuclear factor of the solid staining pattern at a titre of 1:5 in addition to antibody against melanoma cell cytoplasm.

Cells from representative tumours, ME30, ME36, and ME54, were tested against sera from 31 Caucasians without melanoma comprising 12 normal individuals, 7 cases of leukaemia, 4 of glandular fever, 6 of vitiligo, and 2 of bullous pemphigoid. None gave positive cytoplasmic immunofluorescence, though the pemphigoid sera gave the typical basement membrane staining with normal skin sections (Beutner *et al.*, 1967). The sera from 26 Africans from Uganda and Kenya were also tested: they comprised 12 normal individuals, 9 cases of Burkitt's lymphoma, and 5 of carcinoma of the postnasal space. All but two of these gave immunofluorescent staining of melanoma cells, but of different pattern from that observed with the positive melanoma patient sera. It included diffuse cytoplasmic and weak nuclear staining, and six of the sera, specially examined for autoantibodies to various human and mammalian cells, showed an irregular pattern of low titre activity (1:5) against a variety of autoantigens. The nature of this serum activity was not investigated except to show that it could be neutralized by absorption with mammalian tissue such as rodent liver homogenate which had no effect on melanoma autoantibodies.

TABLE V.—*Cytoplasmic Immunofluorescent Staining of Melanoma Cells by Sera from Melanoma Patients*

Serum	No. of Sera	No. of Sera Staining Positively with Cells from Melanoma—	
		ME30*	ME36†
Positive reaction with autologous cells	12‡	10 (0·82)	9 (0·75)
Negative reaction with autologous cells	16§	4 (0·25)	6 (0·37)
Not tested on autologous cells ..	19‖	13 (0·65)	11 (0·55)

The figures in parentheses are the proportion of the totals.
* Autologous serum reaction negative.
† Autologous serum reaction positive.
‡ One of the sera failed to react with ME30 and ME36 cells; three other sera failed to react with one of the tumours only.
§ Four of the sera reacted with both tumours and two with one of the tumours only.
‖ Nine of the sera reacted with both tumours, six with one of the tumours only; four sera failed to react with either tumour.

TABLE VI.—*Cross-reactivity of Melanoma Sera in Cytoplasmic Immunofluorescence Test*

Serum	Immunofluorescent Staining of—								
	Autologous Tumour	Tumour Cells from Patients with Autologous-positive Sera			Tumour Cells from Patients with Autologous-negative Sera			Lymphoid Cells	Bronchial Carcinoma Cells
		ME36	ME62	ME54	ME26	ME30	ME61		
ME1	N.T.	+	+	+	+	+	+	−	−
ME15	−	−	−	N.T.	−	−	−	−	−
ME36	+ +	+ +	+	N.T.	−	+ +	+	−	−
ME40	N.T.	+ +	+ +	+	−	+ +	+	−	−
ME62	−	−	−	−	−	−	−	−	−
A1	+	+	+	N.T.	+	+	N.T.	−	−

N.T. = Not tested.

The cytoplasmic staining of melanoma cells by sera from melanoma patients was totally inhibited by two serum absorptions with homogenate of the autologous melanoma. In a series in which four positive sera were absorbed with each of three tumours, homologous melanoma homogenates inhibited cytoplasmic immunofluorescence by a variable amount from substantial reduction to total inhibition. In three experiments in which the serum was twice absorbed by homogenates of the patient's skin, a substantial reduction of cytoplasmic immunofluorescent staining was observed as opposed to complete inhibition when the corresponding tumour was used. In a further experiment a similar partial serum neutralization only was obtained by absorption with pigmented Negro skin and by a cell-free crude preparation of melanin obtained from a heavily pigmented melanoma. Heterologous serum absorption with various hamster melanoma tissues, including homogenates of melanoma, pigmented or non-pigmented skin, and liver, had no effect on the immunofluorescent cytoplasmic staining of human melanoma cells.

TABLE IV.—*Absence of Cross-reactivity in the Complement-dependent Cytotoxicity Test*

Sera	Tumours												
	ME4	ME38	ME39	ME47	ME49	ME52	ME58	MU9	ME36	ME60	ME61	ME62	ME64
ME4	+								−			−	
ME38		+								−	−		
ME39			+				−	−		−	−		−
ME47				+						−	−		
ME49					+		−	−		−	−		
ME52						+		−					−
ME58							+						−

Membrane Immunofluorescence

In the preceding section immunofluorescent staining was performed on cell smears which had been dried and frozen so that antibody could penetrate into the cytoplasm. Staining of live cells detects antibodies which react only with antigens on the cell surface membrane, and these must be present in sufficient amount to permit them to be seen microscopically.

The membrane staining of cells was looked for with five sera which were autologous-positive by the other tests. Staining was obtained in all five, and it took the form of an uneven ring of fluorescence at the periphery with some fine speckling of the cell surface (Special Plate, Fig. 3); the solid cytoplasmic staining of any dead cells in the suspension was ignored in reading this test. Two sera, negative by the other tests, gave negative membrane immunofluorescence with the autologous tumour cells. There appeared to be no cross-reaction by positive sera with cells from four homologous tumours examined (two of the sera were autologous-positive and two negative but they had all cross-reacted by cytoplasmic immunofluorescence). Table VII summarizes an experiment in which a tumour preparation

TABLE VII.—*Membrane Immunofluorescence Reactions Between a Melanoma and Autologous and Homologous Sera*

Sera*	ME54	ME1	ME4	ME7	ME69	A1
Membrane immunofluorescence with ME54 melanoma cells	+	–	–	–	–	–

* All sera reacted positively with autologous and homologous melanoma cells by cytoplasmic immunofluorescence.

was tested against six sera, including the autologous serum: only the autologous reaction was positive. Moreover, it could be inhibited by absorption with autologous tumour homogenates but not by absorption with any of four homologous tumours tested. In one experiment membrane immunofluorescence by autologous serum (ME69) was not inhibited by absorption with the homologous pigmented Negro skin or with the melanin preparation mentioned in the previous section. The data are consistent with the hypothesis that the autoantibody which gives membrane immunofluorescence is the same as that responsible for the complement-dependent cytotoxicity.

Changes in Serum Activity, Natural and Induced

In three patients in whom the serum was originally positive, later sera failed to inhibit growth of the culture autologous melanoma cells when the disease became widespread (Fig. A).

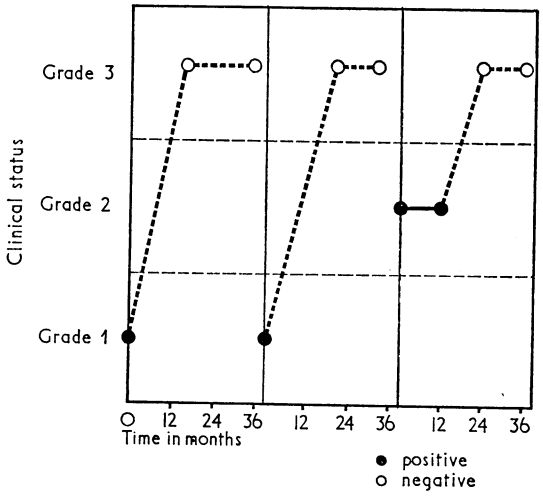

FIG. A.—Loss of autoantibody as tumour becomes disseminated (grade 3) in three melanoma patients originally seropositive when the tumour was localized (grades 1 and 2).

Whether the negative serum reactivity in these cases was due to variation of tumour antigenicity or impairment of host immunological responsiveness cannot be decided from our data.

A change from seronegative to seropositive status has been observed in only two patients (ME1 and ME69 in Table II), both of whom presented with disseminated disease without autoantibody, and, after partial excision of tumour, were injected subcutaneously at multiple sites with cells from their own tumours. The cells had been previously rendered incapable of growth by *x*-irradiation at a dose of 12,000 r delivered in vitro with a 250 kV therapy *x*-ray machine.

Discussion

Over one-third of the melanoma patients tested had in their sera antibodies which react with the cells of their own tumours. Immunofluorescence investigations indicate that at least two antibodies are concerned—one reacting with cytoplasmic constituents of the tumour cells, and the other with cell surface membrane and which in the presence of complement is cytotoxic. No evidence for cross-reaction between the different tumours has been found in the case of the surface antigen, but the internal antigen appears to be present in most if not all melanoma cells, including those from patients lacking detectable autoantibody of either type.

Only speculations are possible concerning the nature of the internal cytoplasmic antigen, and it is not clear whether it is present solely in tumour cells, since partial serum neutralization by absorption has been possible with normal pigmented or non-pigmented skin, though this was less effective than absorption with melanoma. This could arise if the specific autoantigen was present in skin at a lower concentration than in melanoma, or if it cross-reacted with a skin antigen, or if only a proportion of a number of melanoma antigens was present in the skin. The absorption studies indicate that the antigen concerned is probably not melanin, and, moreover, both melanotic and amelanotic tumour cells have been found to react with positive sera. Serum activity against melanoma cells has not been detected in patients with vitiligo, in whom autoantibodies to melanin have been reported (Langhof *et al.*, 1965).

It cannot be decided whether the cell surface antigen is present in all melanomas or only in those from patients with cytotoxic autoantibodies. This difficulty arises because the antigen is specific to each tumour, and can therefore be detected only by the autologous antibody.

Interesting biological questions are: (*a*) why the two antibodies reacting respectively with the antigens in the cytoplasm and the membrane of autologous melanoma cells occurred together in all the cases; (*b*) why there was no antibody to the internal cytoplasmic antigen in patients with disseminated disease even where the tumour cells from such patients had been shown to possess this antigen. One explanation is that the presence of an antibody to the membrane component is necessary before antibody to the internal component is produced. Immunization against the internal component might occur readily with destruction of melanoma cells by cytotoxic antibody; this might release internal antigen more effectively than gross breakdown of tissue such as in ischaemic necrosis of tumour.

Studies in experimental animals have drawn attention to the phenomenon that tumours grow and kill even though the host has not become tolerant to the tumour-specific antigens. Cytotoxic antibody has been found in experimental animals with progressive tumours (Old *et al.*, 1967). The coexistence of tumour and cytotoxic antibody can probably be explained on the basis of accessibility. The antibody, particularly if it is of the IgM type, does not readily pass vascular barriers or penetrate intercellular spaces and probably does not reach much of the tumour. We are embarking on a systematic study to deter-

mine the immunoglobulin classes of the autoantibodies to melanoma, and the first results on a few sera show that the cytotoxic activity is largely in the IgM serum fraction.

The cytotoxic antibody would be expected to destroy blood-borne melanoma cells and in this way limit dissemination of the tumour. The observation made here that circulating cytotoxic antibody was virtually present only in patients without disseminated disease could be interpreted in this way. The available facts fit the hypothesis that a host reaction mediated by circulating cytotoxic antibodies could be effective in controlling metastases but is of limited value in restricting the growth of solid tumour.

There are close similarities between the present serological reactions to human melanoma and those against chemically induced primary tumours in rodents. Such tumours were the first in which unambiguous tumour-specific antigen in the cell membrane capable of evoking a cytotoxic host reaction was demonstrated. Characteristic features of the antigens in these experimental tumours are that: (1) membrane antigen is unique to each tumour and does not cross-react (cf. Alexander and Fairley, 1967); (2) chemically induced sarcomata in rats contained an internal cell antigen which was tumour-specific but common to all the tumours (Wang, 1968); (3) in animals bearing primary tumours cell-mediated immunological host reaction against them could be detected only after they had been excised (Mikulska *et al.*, 1966); (4) a large tumour mass selectively paralyses the local lymph nodes to the tumour-specific antigens, but an immune reaction can still be induced in the animal by introducing irradiated autologous tumour into the draining areas of non-involved nodes (Alexander *et al.*, 1969).

To gain a clearer picture of the relationship between host reaction and progress of the disease the capacity of the patient to mount a cell-mediated response against his own tumour must be measured, and experiments on this further problem are in progress with the use of similar techniques.

The correlation between the presence of antibody and the clinical status of the patient raises the question whether some melanomas do not have the membrane antigen and that this favours dissemination. The tests used so far do not allow us to distinguish this possibility from loss of antibody due to some form of immune paralysis coupled perhaps with exhaustion of antibody by the tumour mass. We should eventually be able to decide the question by retesting tumours in the late stage of the disease with sera that had been obtained in the early stages, and late sera against stored or cultured cells.

Routine laboratory testing for autoantibodies in melanoma patients seems desirable, and immunofluorescence with dried melanoma cell films is probably suitable for this purpose, though not necessarily for detecting cytotoxic antibody. It might provide a useful simple method for determining whether an immunotherapeutic procedure has been effective. Sera from patients undergoing immunotherapy could also be tested for cytotoxicity against a standard preparation of tumour cells obtained before therapy and kept deep-frozen or in continued culture.

We are particularly interested in the possibility that auto-immunization with irradiated tumour cells may lead to the appearance of cytotoxic autoantibodies. This procedure has been shown to be useful in experimental animals in retarding the growth of primary sarcoma when the amount of tumour present was very small (Haddow and Alexander, 1964). On many occasions during the last half-century this form of immunotherapy has been tested clinically, but no definitive answers have been obtained. Our first two patients inoculated with irradiated autologous melanoma cells after extensive removal of disseminated tumour showed that cytotoxic serum autoantibody was produced where none could previously be detected. Whether this will prove to be of clinical benefit remains to be seen.

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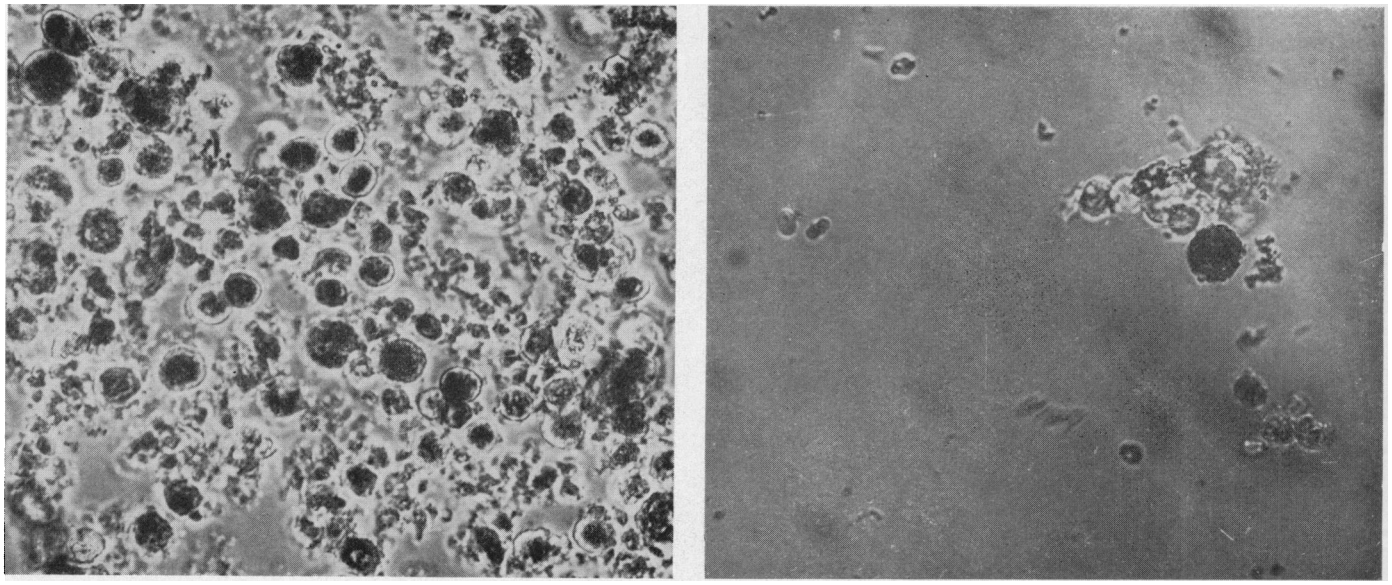

FIG. 1.—Phase-contrast photomicrographs of cultured melanoma cells from Case ME88 (Table II) showing normal growth, to left, compared with complement-dependent cytotoxic effect by autologous serum, to right, evidenced by loss of cells from coverglass. (×300.)

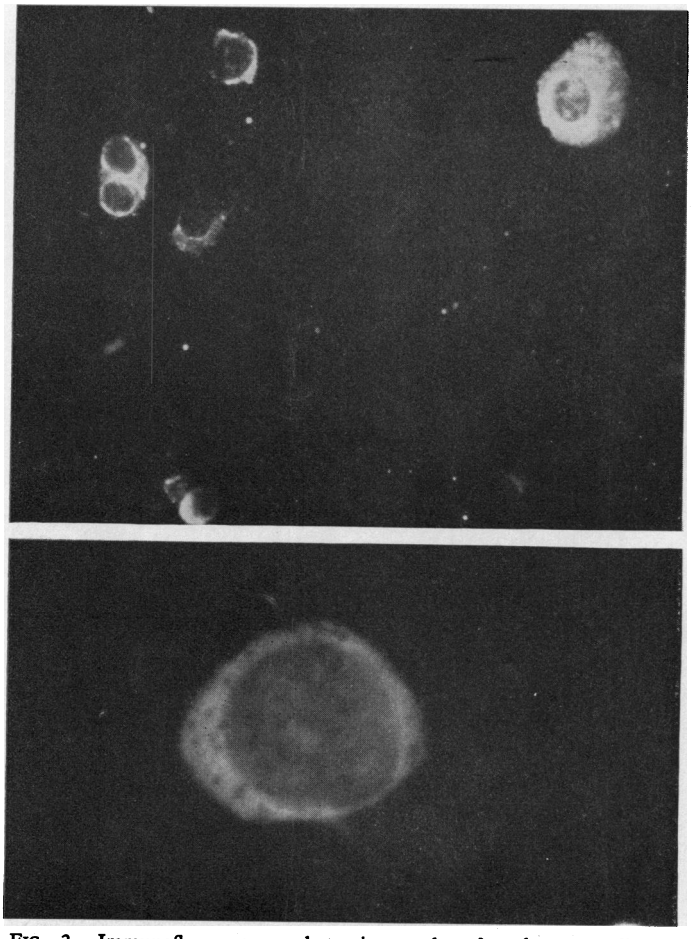

FIG. 2.—Immunofluorescence photomicrographs of melanoma cells in dried films showing cytoplasmic staining by autologous sera. Above, Case A1 (Table VI). (×250.) Below, Case ME69 (Tables II and VII) after immunization with irradiated melanoma cells; note the non-fluorescent melanin granules. (×1,000.)

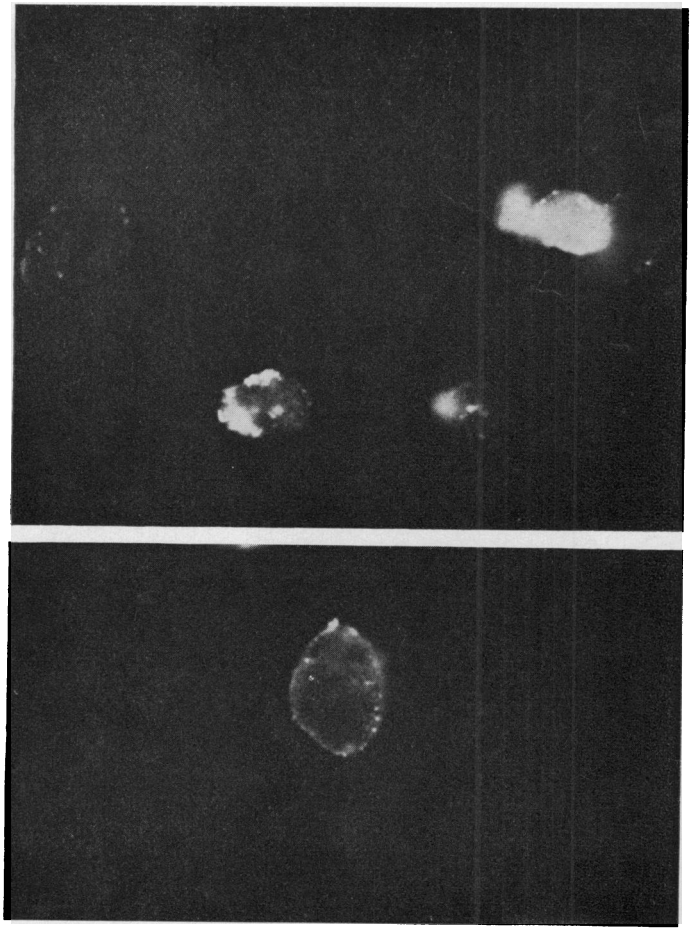

FIG. 3.—Membrane immunofluorescence of living melanoma cells in suspension, by autologous sera Case ME69, after immunization as in Fig. 2. Note speckled staining of cell surface with some aggregation of fluorescent globulin; below, it appears as a fluorescent ring where only the equator of the cell is in focus. Diffuse staining, top right, is due to reaction with dead cell cytoplasm. (×950.)