Communications

Autoimmune reactions in uveal melanoma

A. H. S. RAHI

Department of Pathology, Institute of Ophthalmology, University of London

Choroidal melanomas may remain localized for more than 30 years (Newton, 1965); malignant melanomas of the iris may not metastasize at all (Ashton, 1964), and some of these intraocular neoplasms may undergo spontaneous regression (Fuchs, 1910). From time to time several hypotheses have been advanced in an attempt to explain these puzzling features of the behaviour of uveal melanomas. Furth (1953) first proposed that melanomas are tumours conditioned by changes in host environment. Dunphy (1957) then elaborated the theory of "defensive substances" present in the body which prevented a uveal melanoma from disseminating into the general circulation. Anderson and O'Neill (1957) referred to these substances as "biochemical barriers". This concept was supported by Reese (1963), who stated that certain "unknown factors", probably immunological in nature, kept the tumour dormant for a long time or inhibited their growth completely, metastases developing only when there was a breakdown in the immunity of the host. Jensen (1963), in his thesis on uveal melanomas, upheld the views of earlier workers and argued that variations in metastases, such as their frequency, temporal relationship, latency, and in certain cases spontaneous necrosis, suggested the existence of such factors as immunization and resistance. Duke-Elder and Perkins (1966) appeared to favour this hypothesis, thus explaining infrequent and delayed metastases in spite of the early dissemination of tumour cells in the blood stream. Such a latency is not peculiar to ocular melanomas and in fact several instances of dormant cancers of the breast, kidney, etc., are on record (Gordon-Taylor, 1959). To explain dormancy and spontaneous regression, Burnet (1964, 1967) suggested that the homograft rejection phenomenon was essentially a homeostatic mechanism to eliminate cells of any clone undergoing malignancy, and since a neoplastic cell carried a new surface antigen it might be destroyed by a similar mechanism to graft rejection. It is now believed that cancers probably develop and proliferate as a result of a failure in the equilibrium between the spontaneous proliferation of neoplastic cells and the ability of the body's immune mechanism to control this process (Turk, 1969). The role of immunological processes in the control and development of malignant neoplasms has been appreciated only in the last few years, although it has been suspected since the work of Simon and Thomas (1908). Foley (1953) is considered to be the first to have demonstrated specific antigenicity of chemically induced tumours. This was most closely anticipated by the work of Gross (1943) who demonstrated active immunization in C₃H mice to transplants of early methyl-cholanthrene-induced tumours. Reports of tumour immunity before this time could not be accepted, as these earlier experiments had been conducted either in animals of unproven homogeneity or with tumours that had been transplanted for

long periods (Hauschka, 1952). Malignant cells do not appear to be recognized as self either because of loss of normal tissue-specific antigens, as seen in thyroid carcinomas, or because of the acquisition of new (tumour-specific) antigens as found in nearly all experimental tumours (Alexander and Hall, 1970). The new antigen in chemically induced tumours is so individually specific that it will not cross-react with tumour antigens from other animals, in which the neoplasm was induced by the same chemical (Prehn and Main, 1957; Old, Boyse, Clarke, and Carswell, 1962). Thus, there is no correlation between the antigenic nature of the tumour and the chemical which induces the neoplasm, even in two tumours induced by the same agent in the same animal. On the contrary, tumours induced by viruses (*i.e.* RNA viruses—Rous Sarcoma virus; oncogenic DNA viruses—polyoma viruses, SV40 virus, and adenoviruses—type 3, 7, 12, 18, 31) in a wide selection of animals (chicken, mice, monkeys, hamsters) possess specific antigens for the causative virus, irrespective of the species or strain of animal used (Defendi, 1963; Trentin and Bryan, 1964; Huebner, Rowe, Turner, and Lane, 1963). The only exception to the rule are hybrids formed by adenotype 7 with SV40 (Rowe and Baum, 1964). This general pattern is so constant as to suggest that certain human tumours, like Burkitt's lymphoma and leukaemia, may have a viral origin, and in fact EB virus has been demonstrated in the former (Epstein, 1970). Tumour antigens appear very early in the process of carcinogenesis and may occur even in the precancerous stage (Turk, 1969), as evidenced by suppressed cellular immunity in leukoplakia (Lehner, 1970). How these antigens appear is a matter of conjecture. Either there occurs a spontaneous mutation or, in the wake of neoplastic differentiation, there is apparently a sudden expression of certain suppressed antigens present in all normal cells. The findings that tumour-specific antigens cross-react with antigens present in embryonic tissue, but normally absent in the fully mature animal, is consistent with the latter view that these antigens appear by a process of de-differentiation rather than by mutation (Gold and Freedman, 1965). If the tumour contains antigens which are not recognized by a central immune mechanism as being part of self, the body will react against them, either by elaborating antibodies which neutralize or destroy, with or without complement, the antigens circulating free or fixed to cell membranes; or by a cell-mediated immune response, in which case sensitized lymphocytes developed in thymus-dependent areas of lymph node and spleen, invade the tumour to produce an inflammatory response similar to that seen in failed corneal graft or any other allogeneic tissue rejection. Gorer (1961) has divided the effector mechanism of immunological reactions against tumour tissue into three categories:

- (1) The humoral antibody which, together with complement, can destroy tumour cells;
- (2) The macrophages which are sometimes capable of destroying tumour cells (ascitic tumours) with the help of opsonizing antibodies;
- (3) The cytotoxic lymphoid cells acting in the same way as in the rejection of homografts.

It is possible that *in vivo* all these factors work in combination, the first two predominating in lymphomas, leukaemia, ascitic tumours, and haematogenous spread of solid tumours, and the third predominating in the rejection of solid tumours. Concrete evidence that immunology plays an important role in the control of tumours, including uveal melanomas, can be found in the study of experimental tumours and also in their human counterparts.

The evidence of tumour immunity in animals may be summarized as follows:

- (1) Mice immunized against chemically induced tumours from another syngeneic mouse by

repeated injections of irradiated, but otherwise viable, tumour cells resist transplantation of the same tumour at a later date (Revesz, 1960; Old and others, 1962).

(2) In the case of virus-induced tumours, animals actively immunized with the virus become resistant to subsequent challenge with tumours induced by the same virus (Sjögren, Hellström, and Klein, 1961; Sachs, 1962).

(3) Animals showing spontaneous regression of tumours become resistant to further transplants of the same tumour.

(4) Actively immunized animals contain antibodies in their serum which are cytotoxic to tumour cells *in vitro*. Intravenous injection of lymphocytes from the thoracic duct of rats, specifically immunized against a tumour, can protect other rats from the growth of that tumour transplanted into them (Delorme and Alexander, 1964). Similarly, when tumour cells mixed with immune cells in a 1 : 1 ratio are injected subcutaneously in a histocompatible normal recipient, there is complete suppression of the grafted tumour (Klein and Sjögren, 1960; Old and others, 1962).

(5) Mice with a depressed immune response or a poor immune surveillance mechanism, *i.e.* those subjected to antilymphocytic serum or neonatal thymectomy, show a significant increase in neoplastic lesions (Law, 1965). Similarly, mice suffering from "graft versus host" disease invariably develop lymphoid tissue tumours resembling Hodgkin's disease.

(6) Polycyclic hydrocarbons (3-methylcholanthrene; 1, 2-5, 6-benzanthracene) are potent carcinogens as well as immunosuppressive agents. These compounds produce leukaemia only in those strains of mice in which they can induce immunosuppression.

(7) Rats with chemically induced fibrosarcoma do not show blood-borne metastasis until antibody has been removed from the circulation (Alexander and Hall, 1970).

There is varied and abundant evidence of similar immunological control in man and the vast literature on the subject may be summarized as follows:

(1) The incidence of neoplasms increases with age, whereas ability to produce cell-mediated immune responses (delayed hypersensitivity) to antigens like tuberculin decreases with age. This diminished activity has been well documented in a recent survey on skin test reactivity of patients with uveitis (Weber and Schlaegel, 1969).

(2) There is an impaired capacity of cancer patients with advanced disease to reject homografts and to develop skin reactions of the delayed type (Southam, 1967). Anergy in Hodgkins disease and diminished humoral antibody response in lymphomas is well documented (Miller, 1965).

(3) Immunization of tumour patients with their own tumour tissue, with or without systemic adjuvant, has been found to increase specific antibody response and their survival rate (Mathé, 1969).

(4) Convalescent sera from cases of Burkitt's tumour, when transfused into other patients, induce tumour regression lasting for several weeks. Similarly, sera from patients showing spontaneous regression of malignant melanoma can induce regression in other melanoma patients (Sumner and Foraker, 1960; Teimourian and McCune, 1963; Nathanson, Hall, and Farber, 1967).

(5) Patients with hypogammaglobulinaemia often develop lymphoreticular malignancy (Seligmann, Fudenberg, and Good, 1968).

(6) Immune suppression with antilymphocytic serum increases the incidence of lymphoma (Sell, 1969).

(7) Recently, a case has been reported in which a reticulum cell sarcoma developed at the site of injection of antilymphocytic serum (Deodhar, Zuklinca, Vidt, Robertson, and Hazard, 1969).

(8) Antibodies to skin melanomas (Lewis, Ikonopisov, Nairn, Phillips, Fairley, Bodenham, and Alexander, 1969), gynaecological tumours (Graham and Graham, 1955), and breast cancers (Taylor and Odili, 1970), have been successfully demonstrated in several cases.

(9) Spontaneous regression of some human tumours like melanomas, hypernephromas, neuroblastomas, choriocarcinomas, and Burkitt's tumours, are on record (Brunschwig, 1963; Everson, 1964; Everson and Cole, 1966; Boyd, 1966).

(10) Lymphocytic infiltration of tumours is a good prognostic sign and correlates well with survival data available for breast cancer (Black, 1965; Hamlin, 1968; Bell, Friedell, and Goldenberg, 1969), gynaecological tumours (Graham, 1957), and neuroblastomas (Martin and Beckwith, 1968) and neoplasms of the digestive tract (Black and Speer, 1958).

(11) Very late recurrence in the case of some tumours (uveal melanomas) and the so-called dormant cancers (Hadfield, 1954) are well-recognized phenomena.

(12) Patients with cancer show a slight increase in the incidence of a second cancer as compared to the normal population. People suffering from leukaemia, for instance, often develop malignancy of other organs.

(13) Removal of a primary tumour sometimes helps in the regression of secondaries (Walter and Israel, 1970), perhaps because of the release of immunoblasts which were in an immobilized state in the presence of the primary tumour (Alexander and Hall, 1970).

(14) Leucocytes from a patient immunized against a tumour (*i.e.* melanoma) help remission when injected into the donor against whose tumour the sensitized leucocytes were produced (Nadler and Moore, 1966).

(15) Large numbers of tumour cells may be found in the peripheral circulation without the development of metastases (Moore, Sandberg, and Schubarg, 1957).

(16) Cellular immunity against tumours of urinary bladder, nasopharynx and other organs has been demonstrated by several independent workers (Hellström and Hellström, 1967; Bubenik, Perlmann, Helmstein, and Moberger, 1970; Chu, Stjernswärd, Clifford, and Klein, 1967).

The above experimental and clinical studies, therefore, suggest that the association between the tumour growth and the immune response of the host is more than casual. Although several suggestions have been made in the past to explain delayed metastases, spontaneous necrosis, and regression in the case of uveal melanomas, no specific immunological reactions have so far been demonstrated in such patients. The present study was, therefore, undertaken to determine whether specific humoral immune responses are involved in such patients. The effect of patients' own sera on the growth and metabolism of their tumour cells was studied *in vitro* and the site of immune reaction was localized by immunofluorescence.

Experimental methods

Immediately after enucleation the eyes were placed in sterile culture medium 199 containing penicillin (200 units/ml.) and streptomycin (100 µg./ml.) as preservative. 20 ml. blood were also collected at the time of operation. The specimens were then transported at ambient temperatures to the laboratory on the same day and in most cases within 2 hours. Sera so obtained were inactivated at 56°C. for 30 minutes and stored at -30°C. As source of complement, serum from a donor with AB group was obtained and similarly stored in 2-ml. aliquots at -30°C. Eyes were orientated and opened by either vertical or horizontal section in the manner described by Ashton (1967). One half of the globe was processed for routine paraffin and celloidin sections and the other half was used for preparing cell suspensions. The tumour tissue was dispersed in T.C. 199 mechanically by scissors and scalpel. Trypsin was not used as its effect on surface antigen is not known, and, moreover, it has been found to expose otherwise hidden antigenic sites (Irvine, 1962) perhaps by digesting away some of the surface glycoproteins (Burger and Noonan, 1970). The viability of tumour cells was analysed by dye-exclusion tests, using 0.2 per cent. aqueous trypan blue. The cell count was finally adjusted to 1 million cells per ml. in T.C. 199 with 20 per cent. inactivated foetal bovine

serum. Blood samples from 21 cases of uveal melanoma were tested for antibodies against their own tumours by a culture technique and, where possible, by immunofluorescence and RNA synthesis inhibition tests.

(A) COMPLEMENT-DEPENDENT CYTOTOXICITY TEST

The method adopted was essentially that of Pulvertaft, Doniach, Roitt, and Hudson (1959) for demonstrating the autoaggressive nature of thyroid autoantibodies in Hashimoto's disease. Cells dispersed in nutrient medium containing normal human serum rapidly become attached to the glass wall of the culture chamber. In the presence of specific antibodies and complement, a cytotoxic effect is obtained and the cells fail to attach to the glass surface. Essentially, cytotoxicity is recognized by failure of tumour cells to continue to adhere and grow on the cover glass, which seals the top of a special culture chamber made of a Teflon ring stuck to a microscope slide by silicone grease. With each tumour three tests were set up. To each chamber 1 ml. cell suspension was added and then topped up with:

- (i) 1 ml. normal serum containing complement;
- (ii) 1 ml. inactivated patient's serum;
- (iii) 0.5 ml. patient's serum + 0.5 ml. complement.

The chambers were then sealed by coverslips, avoiding any air bubbles, and were incubated at 37°C., inverted (*i.e.* coverslip down) for the first 48 hours and then right side up for the next 2 to 5 days. The chambers were examined under contrast phase microscopy, and a serum was recorded as being cytotoxic when chamber (iii) showed very little or no growth as compared to chambers (i) and (ii). In the absence of growth an estimate was made of the relative number of cells adhering to the cover glass in all the three chambers, and this was later verified after fixation in alcohol-ether mixture and staining with haematoxylin and eosin. Degrees of cytotoxicity were then assessed in terms of the cytological changes in fixed cells.

(B) COMPLEMENT-DEPENDENT INHIBITION OF RNA

The effect of patients' serum (in the presence of complement) on ribonucleic acid (RNA) synthesis was assessed by the capacity of tumour cells to incorporate radioactive uridine (Lewis and others, 1969). RNA synthesis is greatly reduced when the cell is damaged by cytotoxic antibodies in the presence of complement. 1 ml. cell suspension containing 5 million cells in T.C. 199 was put into each of the three tubes and the following were then added:

- Tube 1—1 ml. normal serum with complement;
- Tube 2—1 ml. patient's inactivated serum;
- Tube 3—0.5 ml. patient's serum + 0.5 ml. complement.

The mixture was incubated at 37°C. in a water bath for 1 hour and then 2 μ Ci (3 Ci/mM) tritiated uridine was added to each tube and incubated for a further period of 1 hour. At the end of incubation the tubes were chilled and then centrifuged. To the deposit 5 per cent. trichloroacetic acid was added to precipitate proteins. After extraction with absolute alcohol, the proteins were digested in normal potassium hydroxide and then mixed in a scintillator NE 233 with solubilizer NE 520, according to the direction of the manufacturers (Nuclear Enterprises, Edinburgh). The radioactivity was then assayed in a Packard tricarb liquid scintillation counter. The count was expressed in terms of net ^3H uridine uptake in counts per 5 minutes.

The test was regarded as positive if the uptake in Tube 3 was at least 20 per cent. smaller than that in Tubes 1 and 2. A difference of ± 10 per cent. in Tubes 1 and 2 was ignored when interpreting the result.

(C) IMMUNOFLOUORESCENCE STUDIES

Immunofluorescent staining of tumour cells was carried out by the sandwich technique (Nairn, 1969). For membrane fluorescence 1 ml. of original cell suspension in T.C. 199 was centrifuged and

washed with phosphate buffered saline. The patient's own serum was then added and after incubation at 37°C. for 30 minutes the cells were again centrifuged and washed and fluorescent anti-human immunoglobulin (IgG/IgM) serum was then added. After further incubation for 30 minutes the cells were centrifuged and washed twice in buffered saline. A drop of cell suspension was placed on a clean microscope slide and mounted in buffered glycerol saline. A Vickers fluorescent unit was used for the present study which has an HBO 200 mercury vapour lamp as a light source. UG 2 excitor filter was used with bright field condenser and GG 4 + Wratten E as barrier filter.

For cytoplasmic fluorescence, tumour tissues processes at 4°C. were blocked (in paraffin), sectioned, and stained according to the technique of Sainte-Marie (1962).

In both membrane and cytoplasmic fluorescence, normal sera were used as control; sera from uveal melanoma patients were also tested for cross-reacting antibodies.

Results

(a) Cytotoxicity tests

Of the 21 tumours examined, seven showed the presence of cytotoxic autoantibodies which were active only in the presence of complement (Figs 1, 2, 3, and 4, opposite and overleaf). Three cases reported here as negative showed poor growth in all the chambers.

FIG. 1a Phase-contrast photomicrograph of growing melanoma cells in normal serum. $\times 132$

(b) RNA synthesis inhibition tests

These were performed in four cases. Positive results were obtained in two cases which had also shown the presence of cytotoxic antibodies by culture technique. In one case positive by the culture technique, the result was inconclusive. One case was negative.

(c) Immunofluorescence tests

Three cytotoxic sera tested for membrane fluorescence against their respective tumours gave positive results; some of the cells, however, in the same preparation also showed

FIG. 1b Phase-contrast photomicrograph of growing melanoma cells in patient's own decomplemented serum. $\times 285$

cytoplasmic fluorescence (Fig. 5, overleaf). This was perhaps due to the fact that the cell suspension used in the present test was obtained by mechanical means and, therefore, was a mixture of dead, damaged, and viable cells. During the test a curious phenomenon was noted. Some of the control slides treated with normal serum at times showed fluorescence of a few of the cells. This was attributed either to cross-reacting antibodies in normal serum, or to *in vivo* fixation of autoantibodies on to the tumour cells. Cytoplasmic fluorescence was not observed in histological preparations tested for cross-reacting antibodies, which are believed to be present in skin melanomas (Lewis and others, 1969). The clinical, pathological, and serological data are summarized in the Table (overleaf).

Discussion

It seems certain that at least some human neoplasms possess antigens which are not present in other tissues of the individual bearing the tumour (Taylor and Odili, 1970). These neoantigens are called tumour-specific transplantation antigens (TSTA). It may be recalled that the human body reacts to antigenic stimuli by elaborating specific antibodies where antigen is available in soluble or macromolecular form in the circulation, and by producing aggressive sensitized lymphocytes where the antigen is fixed at a distant site. It is, therefore, natural to find both humoral and cellular responses in human cancers (Klein and Oettgen, 1969), the first produced against circulating tumour components and the second against the fixed tumour antigens, as in the case of solid tumours. Both these effector mechanisms are important for the control of tumour growth. A solid tumour may be infiltrated by lymphocytes, which limit its growth by reacting directly with tumour-specific antigens or perhaps by the local secretion of certain toxic substances which are collectively called lymphokines (Dumonde, 1970). The circulating tumour cells, on the

FIG. 2(a and b) *Phase-contrast photomicrograph showing complement-dependent cytotoxicity of autologous serum; only a few cells and cellular debris remain attached to the cover glass. a $\times 132$; b $\times 285$*

other hand, are probably eliminated by the humoral antibodies. Thus, a constant battle goes on between the tumour and host defences and a quantitative factor perhaps controls the ultimate outcome (Hellström and Hellström, 1967).

Although cellular immunity appears to be deficient in advanced cancers, perhaps

FIG. 3 Cultured melanoma cells in normal serum from Case MM II stained with haematoxylin and eosin. $\times 132$

FIG. 4 Melanoma cells from the same case in patient's own serum. Although cells are attached to the cover glass they show extensive nuclear and cytoplasmic degeneration. Haematoxylin and eosin. $\times 285$

because of general debility and immune exhaustion by constant bombardment with tumour antigens (Green, Anthony, and Baldwin, 1967), it plays an important role in host resistance to tumours (Law, 1969). Hughes and Lytton (1964) and Stewart (1969)

FIG. 5 Membrane and cytoplasmic fluorescence of melanoma cells treated with autologous serum. ×380

Table I Clinical, pathological, and serological data of uveal melanomas studied

Tumours	MM 1	MM 2	MM 3	MM 4	MM 5	MM 6	MM 7	MM 8	MM 9	MM 10	MM 11	MM 12	MM 13	MM 14	MM 15	MM 16	MM 17	MM 18	MM 19	MM 20	MM 21
Age (yrs)	59	61	68	55	35	60	55	71	59	64	37	60	61	66	62	65	53	48	73	46	65
Sex	F	M	F	M	F	M	F	F	M	F	M	F	F	F	M	M	M	M	M	F	M
Site	C	C	C	C	C	C	C	C	C	C	C	C	C	C	I	C.B	C	C	C	C	C
Cell type	S	E	S	S	S	S	S	S	S	S	S	S	E	M	E	S	M	M	S	S	S
Reticulin content	M	H	M	H	H	H	L	M	L	L	M	H	M	H	H	L	H	H	M	M	M
Pigmentation	L	M	H	L	M	N	N	L	N	L	N	L	M	H	H	M	M	H	M	L	N
Necrosis	-	+	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-	-
Cellular infiltration	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	+	+	-	-
Cytotoxic test	-?	-?	-?	+	-	-	+	+	-	-	+	-	+	-	-	+	+	-	-	-	-
RNA inhibition	o	o	o	(-x)	o	o	o	o	o	o	o	o	+	o	o	o	+	-	o	o	o
Immunofluorescence	AS	o	o	-?	o	o	-	+	+	-	-	o	o	+	o	o	o	-	-	-	-
	HS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- = Negative/Absent
+ = Positive/Present
o = not tested
-? = poor growth in all chambers
+? = shown to be positive when culture was stained

(-x) = quenching made the result unreliable so recorded as negative
AS = tested against patient's own serum
HS = tested against serum of other uveal melanoma patients

Site
C = choroid
I = iris
CB = ciliary body
Cell type
S = spindle
M = mixed
E = epithelioid

Reticulin pigment L = light M = moderate H = heavy N = none

found that a quarter of the patients with carcinomas and sarcomas gave delayed skin reactions in response to intradermal inoculation of autologous tumour cell extracts. A similar response has been recently demonstrated in about 40 per cent. cases of skin melanomas (Fass, Herberman, Ziegler, and Kiryabwire, 1970). A positive *in vitro* test for

cellular immunity in melanoma has been reported by Hellström, Hellström, Pierce, and Yang (1968). Humoral antibody response to human and animal melanomas has been described in the past (Finney, Byers, and Wilson, 1960; Coon and Fain, 1960). But more conclusive evidence has been provided by Lewis and others (1969), who have not only confirmed the findings of Morton, Malmgren, Holmes, and Ketcham (1968) and Muna, Marcus, and Smart (1969), but have also shown that circulating antitumour antibodies are directly related to the clinical state, antibodies being absent in metastasizing melanomas.

One-third of the melanoma patients tested in our present series had in their sera cytotoxic antibodies which reacted with the cells of their own tumour in tissue culture. Immuno-fluorescence investigations have shown that antigen is found both on the surface and inside the tumour cells. Melanin is perhaps not one of the antigens because sera from patients with vitiligo containing antimelanin antibodies do not react with melanoma cells (Langhof, Feuerstein, and Schabinski, 1965). Although Lewis and others (1969) have demonstrated that antibodies reactive against cytoplasmic constituents of skin melanomas cross-react with melanoma cells of other individuals, we did not find similar cross-reactivity among the uveal melanomas and we are, therefore, in agreement with Mansell and Verrier-Jones (1971) that the antibodies in melanoma are perhaps individual-specific. Three of our cases, in which the tumour was treated with normal serum, showed fluorescence in a few cells. This was due either to the non-specific fixation of some of the isoantibodies present in the normal serum (Morton and others, 1968) or to *in vivo* fixation of autoantibodies on some of the tumour cells. Since uveal melanomas contain blood sinuses devoid of endothelium in which blood circulates quite freely among tumour cells (François, 1963), it is possible that some of the antibodies become fixed to the tumour cells. In fact this sort of fixation has been found in a large number of cases of skin melanomas (Mansell and Verrier-Jones, 1971) and the role of such antibodies has been the subject of a recent survey (Hellström, Sjögren, Warner, and Hellström, 1971). The cytotoxicity test used in the present study not only takes into account the capacity of the cells to stick to the glass surface as an indication of viability, but also their growth potential as modified by pre-treatment with sera containing specific complement-fixing cytotoxic antibodies. When in doubt, the cells sticking to the glass surface were fixed and stained and the cytological details studied for evidence of cell damage. In one case cells in the antiserum chamber did not fall off the cover glass and, therefore, according to Lewis's criteria, the test would have been reported as negative. Staining of the cells, however, indicated that all the cells in that chamber had undergone a sort of coagulative necrosis, nuclear details were absent and the cytoplasm was deeply eosinophilic. Complement-dependent inhibition of the synthesis of RNA is a sensitive test which measures cytotoxicity. A reliable result requires a fairly high viability index of the original cell suspension. Heavily pigmented and necrotic tissues often present difficulty since they are likely to produce quenching, which makes the result unreliable.

Reese, Archila, Jones, and Cooper (1970) have recently published an analysis of 100 cases of uveal melanoma and have shown that 22 per cent. of cases had cellular and lytic necrosis of the tumour and 5 per cent. had coagulative necrosis. Flat malignant choroidal melanomas are particularly inclined to show necrosis and inflammatory changes, and Font, Spaulding, and Zimmerman (1968) found post-necrotic scarring in 40 per cent. of their cases. Uveitis is not uncommon in uveal melanomas and is generally believed to be the result of necrosis and release of cytotoxic agents. Uveitis, can, however develop in the absence of necrosis (Kirk and Petty, 1956) and, conversely, tumour necrosis from photo-

coagulation may not lead to inflammation (Hepler, Allen, and Straatsma, 1968), so that it is necessary to revise our ideas of the genesis of uveitis in intraocular melanoma. This may have an immunological basis such as has been postulated for the cellular infiltrates found in other malignant conditions. Even if tumour necrosis controls the ultimate severity of the inflammatory reaction, it cannot be explained, at least in some cases, only on the basis of hypotension in vascular sinusoids of the tumour and passive congestion, since the size of the tumour appears to be unrelated to the incidence of necrosis (Reese and others, 1970). These authors, therefore, argue that inflammation and necrosis may represent an immunological response which causes the tumour to regress. Since we have now demonstrated cytotoxic autoantibodies in one-third of our cases, it is possible that these predictions may be true. In fact we found necrosis in about half of our positive cases, which might have some significance, since only 20 per cent. cases among non-reactors showed necrosis. Autoimmune serological reactions to ocular melanoma were studied by Howard and Spalter (1966). Although fifteen sera were examined, they looked for antibodies against autologous tumour in only three cases. No antigen-antibody reactions were detected by gel diffusion, haemagglutination, and complement-fixation techniques. These authors were, however, successful in detecting, by complement-fixation tests, specific antibody in rabbits immunized against uveal melanoma. Thus, in spite of their failure in a very small series to detect tumour antibody, they seem to conform to the idea that uveal melanomas are antigenic. They might have been successful if they had used immunofluorescence and tissue culture techniques and, therefore, they rightly conclude that "it would be of interest in future investigations of this type to include laboratory techniques for study of autoimmune phenomena on a cellular level".

Cellular immunity to uveal melanoma was studied in six female patients by exposing cell cultures to autochthonous lymphocytes and a negative result was reported (Hart, Reznikov, and Hughes, 1968). The authors mentioned various reasons why their findings should not be accepted as proof that no such response exists. Apart from their own remarks on failure to detect a cell-mediated immune response, there are a few more points worth consideration. Since these authors used trypsin to prepare their cell suspensions, it is likely that some of the antigens on the cell membrane (against which lymphocytes react) were removed during this process. After primary cultures were established, cells were subcultured, again using trypsin. Cytotoxicity tests were performed with cells obtained from such sub-cultures. It may be recalled that *in vitro* or *in vivo* cytotoxicity depends upon antigen density on the tumour cell membrane (Kaliss, 1962). In sub-cultures such density is likely to be low, because mitosis leads to dilution of antigen in descendant cells while mutant cells may not contain antigen at all, the total effect being loss of tissue specific antigen in culture (Weiler, 1959). We, therefore, believe that the reasons for their failure to detect cell immunity are twofold: first, they studied a very limited number of cases (6), and secondly they were dealing with cell suspensions of low antigenicity because of trypsinization and subsequent loss in sub-culture. Furthermore, it is also possible that their cases lacked cellular immunity either because of immune paralysis by direct inhibition and exhaustion (Dresser and Mitchison, 1968) or because of immune deviation (Asherson and Stone, 1965) or feed-back inhibition of the response by humoral antibodies (Axelrad and Rowley, 1968). It is also possible that sensitized cells migrated into the tumour so that very few were left in the general circulation (Lehner, 1970). It has recently been stated that cellular sensitization on the whole is quite uncommon in skin melanomas (Currie, Lejeune, and Fairley, 1971) and may be absent when the tumour is apparent (Nagel, Piessens, Stilmant, and Lejeune, 1971).

It is difficult to assign any definite role to the complement-dependent cytotoxic antibodies found in intraocular melanomas. As Lewis and others (1969) have suggested, their presence in skin melanomas might determine the clinical status of the patients, *i.e.* the cytotoxic antibody, being able to destroy blood-borne melanoma cells, may limit dissemination of the tumour and subsequent development of secondaries. This might be true with uveal melanoma also, wherein secondaries may be long deferred even in cases in which embolization into the general circulation has occurred early (Goder and Velhagen, 1970). A similar situation has been encountered in rats with primary fibrosarcomas which do not give rise to blood-borne metastases until antibody has been removed (Alexander and Hall, 1970). It must, however, be emphasized that more work is required to discover the role of tumour-specific antibodies in human uveal melanomas; a close follow-up of cases showing such response might provide some clue for further studies. Patients showing specific antibody response are being followed up and the result will be communicated in due course.

Summary

Tumour tissue and sera were obtained from 21 cases (10 females and 11 males) of uveal melanoma. Autoantibodies reactive against the patients' own melanoma cells (autochthonous antigen) were demonstrated in seven cases by complement-dependent cytotoxicity in short-term culture, complement-dependent inhibition of RNA synthesis, and immunofluorescence staining of viable and fixed tumour cells in histological preparations. Although the presence of antibodies in serum could not be correlated with cell type, reticulin content, and pigment distribution, tumour necrosis was evident in half of the positive cases. No cross-reacting antibodies were demonstrated. The relevant literature on the nature and role of immune reactions in human neoplasms in general and in melanomas in particular has been reviewed and the possible role of the autoantibodies demonstrated in the present study has been suggested. Further study and close follow-up are necessary before any definite conclusions can be reached.

The author cannot adequately thank Prof. N. Ashton, F.R.S., who not only introduced him to the subject but also provided guidance at every stage of this project. This work has been made possible only by the wholehearted co-operation, in providing materials, of the consultant ophthalmologist and the residents at Moorfields Eye Hospital (City Road and High Holborn), St. Bartholomew's Hospital, St. Thomas' Hospital, Royal Northern Hospital, Western Ophthalmic Hospital, Royal Eye Hospital, and Lambeth Hospital, London; South Middlesex Hospital, Isleworth; St. George's Hospital, Tooting; Royal Eye Hospital, Surbiton; and Royal Berkshire Hospital, Reading. Thanks are also due to Miss C. G. Munn and Mr. A. McNeil for technical assistance and to Miss K. O'Malley for secretarial help.

References

- ALEXANDER, P., and HALL, J. G. (1970) *Advanc. Cancer Res.*, **13**, 1
 ANDERSON, B., and O'NEILL, J. (1957) *A.M.A. Arch. Ophthalm.*, **58**, 337
 ASHERSON, J. L., and STONE, S. H. (1965) *Immunology*, **9**, 205.
 ASHTON, N. (1964) *Brit. J. Ophthalm.*, **48**, 650
 ——— (1967) "Techniques for the Histological Examination of the Eye". Association of Clinical Pathologists, Broadsheet No. 59. B.M.A., London
 AXELRAD, M., and ROWLEY, D. A. (1968) *Science*, **160**, 1465
 BELL, J. R., FRIEDEL, G. H., and GOLDENBERG, I. S. (1969) *Surg. Gynec. Obstet.*, **129**, 258
 BLACK, M. M. (1965) In "Progress in Clinical Cancer", ed. I. M. Ariel, vol. 1, p. 26. Grune and Stratton, New York

- BLACK, M. M. and SPEER, F. D. (1958) *Surg. Gynec. Obstet.*, **106**, 163
- BOYD, W. (1966) "The Spontaneous Regression of Cancer". Thomas, Springfield, Ill.
- BRUNSCHWIG, A. (1963) *Surgery*, **53**, 423
- BUBENÍK, J., PERLMANN, P., HELMSTEIN, K., and MOBERGER, G. (1970) *Int. J. Cancer*, **5**, 39
- BURGER, M. M., and NOONAN, K. D. (1970) *Nature (Lond.)*, **228**, 512
- BURNET, M. (1964) *Brit. med. Bull.*, **20**, 154
- (1967) *Lancet*, **1**, 1172
- CHU, E. H. Y., STJERNSWÄRD, J., CLIFFORD, P., and KLEIN, G. (1967) *J. nat. Canc. Inst.*, **39**, 595
- COON, J. H., and FAIN, W. R. (1960) *Surg. Forum*, **10**, 1
- CURRIE, G. A., LEJEUNE, F., and FAIRLEY, G. H. (1971) *Brit. med. J.*, **2**, 305
- DEFENDI, V. (1963) *Proc. Soc. exp. biol. (N.Y.)*, **113**, 12
- DELORME, E. J., and ALEXANDER, P. (1964) *Lancet*, **2**, 117
- DEODHAR, S. D., ZUKLINCA, A. G., VIDT, D. G., ROBERTSON, A. L., and HAZARD, J. B. (1969) *New Engl. J. Med.*, **280**, 1104
- DRESSER, D. W., and MITCHISON, N. A. (1968) *Advanc. Immunol.*, **8**, 129
- DUKE-ELDER, S., and PERKINS, E. S. (1966) "System of Ophthalmology", vol. 9, p. 871. Kimpton, London.
- DUMONDE, D. C. (1970) *Proc. roy. Soc. Med.*, **63**, 899
- DUNPHY, E. B. (1957) *Amer. J. Ophthalm.*, **44**, 313
- EPSTEIN, M. A. (1970) *Advanc. Cancer Res.*, **13**, 383
- EVERSON, T. C. (1964) *Ann. N.Y. Acad. Sci.*, **114**, 721
- and COLE, W. H. (1966) "Spontaneous Regression of Cancer". Saunders, Philadelphia
- FASS, L., HERBERMAN, R. B., ZIEGLER, J. L., and KIRYABWIRE, J. W. M. (1970) *Lancet*, **1**, 116
- FINNEY, J. W., BYERS, E. H., and WILSON, R. H. (1960) *Cancer Res.*, **20**, 351
- FOLEY, E. J. (1953) *Ibid.*, **13**, 835
- FONT, R. L., SPAULDING, A. G., and ZIMMERMAN, L. E. (1968) *Trans. Amer. Acad. Ophthalm. Otolaryngol.*, **72**, 877
- FRANÇOIS, J. (1963) *Brit. J. Ophthalm.*, **47**, 736
- FUCHS, E. (1910) *v. Graefes Arch. Ophthalm.*, **77**, 304
- FURTH, J. (1953) *Cancer Res.*, **13**, 477
- GODER, G., and VELHAGEN, K. H. (1970) *Ophthalmologica (Basel)*, **161**, 372
- GOLD, P., and FREEDMAN, S. O. (1965) *J. exp. Med.*, **121**, 439
- GORDON-TAYLOR, G. (1959) *Brit. med. J.*, **1**, 455
- GORER, P. A. (1961) *Advanc. Immunol.*, **1**, 345
- GRAHAM, J. B., and GRAHAM, R. M. (1955) *Cancer*, **8**, 409
- GRAHAM, R. R. (1957) "Proc. III nat. Cancer Conf., Detroit, 1956", pp. 659-663
- GREEN, H. N., ANTHONY, H. M., and BALDWIN, R. W. (1967) "An Immunological Approach to Cancer." Butterworth, London
- GROSS, L. (1943) *Cancer Res.*, **3**, 326
- HADFIELD, G. (1954) *Brit. med. J.*, **2**, 607
- HAMLIN, I. M. E. (1968) *Brit. J. Cancer*, **22**, 383
- HART, D. R. L., REZNIKOV, M., and HUGHES, L. E. (1968) *Arch. Ophthalm. (Chicago)*, **79**, 748
- HAUSCHKA, T. S. (1952) *Cancer Res.*, **12**, 615
- HELLSTRÖM, I., and HELLSTRÖM, K. E. (1967) *Prog. exp. Tum. Res.*, **9**, 40
- , ———, PIERCE, G. E., and YANG, J. P. S. (1968) *Nature (Lond.)*, **220**, 1352
- , SJÖGREN, H. O., WARNER, G., and HELLSTRÖM, K. E. (1971) *Int. J. Cancer*, **7**, 226
- HEPLER, R. S., ALLEN, R. A., and STRAATSMA, B. R. (1968) *Arch. Ophthalm. (Chicago)*, **79**, 177
- HOWARD, G. M., and SPALTER, H. F. (1966) *Ibid.*, **76**, 399
- HUEBNER, R. J., ROWE, W. P., TURNER, H. C., and LANE, W. T. (1963) *Proc. nat. Acad. Sci. (Wash.)*, **50**, 379
- HUGHES, L. E., and LYTTON, B. (1964) *Brit. med. J.*, **1**, 209
- IRVINE, W. J. (1962) *Ibid.*, **1**, 1444

- JENSEN, O. A. (1963) *Acta ophthalm. (Kbh.)*, Suppl. 75
- KALISS, N. (1962) *Ann. N.Y. Acad. Sci.*, **101**, 64
- KIRK, H. Q., and PETTY, R. W. (1956) *A.M.A. Arch Ophthalm.*, **56**, 843
- KLEIN, E., and SJÖGREN, H. O. (1960) *Cancer Res.*, **20**, 452
- KLEIN, G., and OETTGEN, H. F. (1969) *Ibid.*, **29**, 1741
- LANGHOF, H., FEUERSTEIN, M., and SCHABINSKI, G. (1965) *Hautarzt*, **16**, 290
- LAW, L. W. (1965) *Science*, **147**, 164
- (1969) *Cancer Res.*, **29**, 1
- LEHNER, T. (1970) *Brit. J. Cancer* **24**, 442
- LEWIS, M. G., IKONOPISOV, R. L., NAIRN, R. C., PHILLIPS, T. M., FAIRLEY, G. H., BODENHAM, D. C., and ALEXANDER, P. (1969) *Brit. med. J.*, **3**, 547
- MANSSELL, P. W. A., and VERRIER-JONES, J. (1971) Proc. 122nd. Meeting Path. Soc. Gt. Britain and Ireland
- MARTIN, R. F., and BECKWITH, J. B. (1968) *J. Pediat. Surg.*, **3**, 161
- MATHÉ, G. (1969) *Brit. med. J.*, **4**, 7
- MILLER, D. G. (1965) In "Immunological Diseases", ed. M. Samter, p. 372. Little Brown, Boston
- MOORE, G. E., SANDBERG, A., and SCHUBARG, J. R. (1957) *Ann. Surg.*, **146**, 580
- MORTON, D. L., MALMGREN, R. A., HOLMES, E. C., and KETCHAM, A. S. (1968) *Surgery*, **64**, 233
- MUNA, N. M., MARCUS, S., and SMART, C. (1969) *Cancer*, **23**, 88
- NADLER, S. H., and MOORE, G. E. (1966) *Ann. Surg.*, **164**, 482
- NAGEL, G., PIESSENS, W. F., STILMANT, M. M., and LEJEUNE, F. (1971) *Europ. J. Cancer.*, **7**, 41
- NAIRN, R. C. (1969) "Fluorescent Protein Tracing", 3rd. ed. Livingstone, Edinburgh
- NATHANSON, L., HALL, T. C., and FARBER, S. (1967) *Cancer (Philad.)*, **20**, 650
- NEWTON, F. H. (1965) *Arch. Ophthalm. (Chicago)*, **73**, 198
- OLD, L. J., BOYSE, E. A., CLARKE, D. A., and CARSWELL, E. A. (1962) *Ann. N.Y. Acad. Sci.*, **101**, 80
- PREHN, R. T., and MAIN, J. M. (1957) *J. nat. Cancer. Inst.*, **18**, 769
- PULVERTAFT, R. J. V., DONIACH, D., ROITT, I. M., and HUDSON, R. V. (1959) *Lancet*, **2**, 214
- REESE, A. B. (1963) "Tumors of the Eye", 2nd. ed., p. 241. New York, Hoeber
- , ARCHILA, E. A., JONES, I. S., and COOPER, W. C. (1970) *Amer. J. Ophthalm.*, **69**, 91
- REVESZ, L. (1960) *Cancer Res.*, **20**, 443
- ROWE, W. P., and BAUM, S. G. (1964) *Proc. nat. Acad. Sci. (Wash.)*, **52**, 1340
- SACHS, L. (1962) *J. nat. Cancer Inst.*, **29**, 759
- SAINTE-MARIE, G. (1962) *J. Histochem. Cytochem.*, **10**, 250
- SELL, S. (1969) *Ann. intern. Med.*, **71**, 177
- SELIGMANN, M., FUDENBERG, H. H., and GOOD, R. A. (1968) *Amer. J. Med.*, **45**, 817
- SIMON, C. E., and THOMAS, W. S. (1908) *J. exp. Med.*, **10**, 673
- SJÖGREN, H. O., HELLSTRÖM, I., and KLEIN, G. (1961) *Cancer Res.*, **21**, 329
- SOUTHAM, C. M. (1967) *Prog. exp. Tumour Res.*, **9**, 1
- STEWART, T. H. M. (1969) *Cancer (Philad.)*, **23**, 1368
- SUMNER, W. C., and FORAKER, A. G. (1960) *Ibid.*, **13**, 79
- TAYLOR, G., and ODILI, J. L. (1970) *Brit. J. Cancer*, **24**, 447
- TEIMOURIAN, B., and McCUNE, W. S. (1963) *Amer. Surg.*, **29**, 515
- TRENTIN, J. J., and BRYAN, E. (1964) *Proc. Amer. Ass. Cancer Res.*, **5**, 64
- TURK, J. L. (1969) "Immunology in Clinical Medicine", p. 204. Heinemann, London
- WALTER, J. B., and ISRAEL, M. S. (1970) "General Pathology", 3rd ed., p. 516. Churchill, London
- WEBER, J. C., and SCHLAEGEL, T. F. (1969) *Amer. J. Ophthalm.*, **67**, 732
- WEILER, E. (1959) *Exp. Cell Res.*, Suppl. 7, p. 244