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EMBRYONIC ANTIGEN EXPRESSION ON 2-ACETYLAMINOFLUORENE INDUCED AND SPONTANEOUSLY ARISING RAT TUMOURS

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Summary.—2-Acetylaminofluorene induced mammary and ear duct carcinomata and spontaneously arising mammary carcinomata and sarcomata were shown to express embryonic antigens at the cell surface by their reaction with serum from multiparous female rats. These observations with essentially non-immunogenic tumours are comparable with early findings showing that embryonic antigens are also expressed on aminoazo dye induced rat hepatomata, and sarcomata induced with 3-methylcholanthrene. Re-expression of embryonal components, therefore, may be a concomitant of neoplastic transformation.

THE CONCEPT that malignant cells re-express embryonal characteristics has gained considerable support from recent studies showing that experimental animal tumours induced by oncogenic viruses or chemical carcinogens exhibit tumour associated embryonic antigens (Alexander, 1972; Baldwin, 1973). The appearance of so-called "oncofoetal" antigens on human tumours is also exemplified by α-foetoprotein associated with hepatocellular carcinoma and carcinoembryonic antigen associated with malignancies of the gastrointestinal tract (Laurence and Neville, 1972).

These observations suggest that re-expression of embryonal components may be a feature of many, if not all, malignant cells and furthermore raises the possibility that the tumour rejection antigens demonstrable on experimental animal tumours (Deichman, 1969; Pasternak, 1969; Baldwin, 1973) may be identified as embryonic antigens. These postulates have been analysed comprehensively using a range of immunogenic rat tumours including 3-methylcholanthrene (Mc) induced sarcomata and 4-dimethylaminoazobenzene (DAB) induced hepotomata maintained by transplantation in syngeneic Wistar rats (Baldwin, Glaves and Pimm, 1971; Baldwin *et al.*, 1972*a*; Baldwin, Glaves and Vose, 1972*b*; Baldwin *et al.*, 1974*a*). From these studies it was concluded that embryonic antigen expression was a concomitant of malignant change, although it was possible to differentiate between these antigens and the tumour rejection antigens by their specificities (Baldwin *et al.*, 1972, 1974*a*; Baldwin, Glaves and Vose, 1974*b*).

All of the tumours employed in the previous studies have been immunogenic, as defined by their capacity to elicit tumour rejection responses in syngeneic hosts. Other tumours, such as mammary carcinomata induced by 2-acetylaminofluorene (AAF) or arising spontaneously within the breeding population and also spontaneously developing sarcomata, are generally deficient or demonstrably lacking in tumour rejection antigens (Baldwin and Embleton, 1969*a*, *b*; 1971; 1974). These, therefore, have been examined for tumour associated embryonic antigen to determine further whether this expression

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is a constant feature of malignant change and also whether these antigens are related to the tumour rejection antigens.

MATERIALS AND METHODS

Tumours.—The tumours studied included mammary carcinomata and one ear duct carcinoma induced in female rats fed continuously on a diet containing 0·04% w/w 2-acetylaminofluorene (Baldwin and Embleton, 1969*a*). Spontaneously arising mammary carcinomata and sarcomata developed in breeding female rats between 179 and 565 days of age (Baldwin and Embleton, 1969*b*). Tumours were passaged in syngeneic Wistar rats of the same sex as the primary host and early transplant generations were preserved in liquid nitrogen vapour. Their immunogenicity was determined by the tumour rejection response elicited in rats immunized by implantation of irradiated tumour grafts or by surgical excision of growing tumour against a challenge inoculum just sufficient to produce consistent tumour growth in controls. These characteristics have been published previously (Baldwin and Embleton, 1969*a*, *b*; 1971; 1974) and can be summarized as *Mammary carcinoma:* AAF 20—no significant resistance against challenge with 5×10^4 tumour cells; AAF 56—no protection to challenge with 5×10^4 tumour cells; AAF 57—no protection to challenge with 10^3 tumour cells; Sp11—no protection to challenge with 10^2 tumour cells; Sp15—resistance induced to challenge with 10^3 but not 10^4 tumour cells. *Ear duct carcinoma:* AAF 49—no resistance to challenge with 5×10^4 tumour cells. *Sarcoma:* Sp7—no resistance to challenge with 10^5 tumour cells; Sp24—resistance to 10^3 but not 10^4 tumour cells.

Serum donors.—Serum was taken from rats having had more than 4 pregnancies and which were pregnant at the time of assay. Virgin female rats were used for controls and were generally age matched.

Tissue cultures.—Cell culture lines were initiated from single cell suspensions of trypsinized transplanted tumours and maintained by serial subculture in Waymouth's medium supplemented with 20% foetal calf serum and antibiotics. They were used as a source of target cells for microcytotoxicity tests between the first and sixth passage.

Microcytotoxicity tests.—The complement dependent cytotoxicity of multiparous rat sera for plated target cells was assayed in Falcon Microtest plates (3034) as previously described (Baldwin *et al.*, 1972*b*). The percentage cytotoxicity was calculated from the numbers of cells present in wells treated with test serum compared with those surviving in wells treated with control virgin rat serum.

Membrane immunofluorescence tests.—The indirect membrane immunofluorescence test was performed on viable tumour cells in suspension as previously described (Baldwin and Barker, 1967). Fluorescence indices (FI) were calculated from the proportion of unstained cells in samples exposed to virgin control sera compared with the proportion of unstained cells in samples exposed to test serum. A value of 0·30 or greater is taken to represent a significant reaction.

RESULTS

Microcytotoxicity tests

Embryonic antigens were detected at the surface of AAF induced mammary and ear duct carcinomata by the complement dependent cytotoxicity of sera from multiparous rats compared with that of virgin control sera (Table I). All 4 tumours showed some significant reactivity with the sera, although the cytotoxicity was variable and generally low compared with that previously obtained with DAB induced hepatomata and Mc induced sarcomata (Baldwin *et al.*, 1972*b*). None of the sera used could be shown to have any reactivity against cells from adult liver, lung, diaphragm or kidney. Table II summarizes tests with a larger panel of multiparous rat sera against AAF induced tumours.

Similar tests with spontaneously arising sarcomata and mammary carcinomata showed comparable results (Table III). Again the percentage cytotoxicity was generally low but significant when demonstrable and only a low proportion of the multiparous rat sera showed reactivity. Table IV summarizes tests with a larger panel of multiparous rat sera against these tumours. None of the sera used

TABLE I.—*Cytotoxicity of Multiparous Rat Sera for 2-Acetylaminofluorene Induced Rat Tumours*

Multiparous serum No.	No. of cells* surviving after treatment with: Test serum	Control serum	Percentage cell reduction	$P<$
Mammary carcinoma AAF20				
4887	52±2	95±4	45	0·0005
4906	78±2	66±5	−18	—
4907	69±4	66±5	−5	—
4914	98±4	95±4	7	0·10
4929	85±4	95±4	11	0·025
5039	75±4	95±4	22	0·0005
Ear duct carcinoma AAF49				
4832	70±6	75±4	7	0·25
5039	70±9	75±4	7	0·30
5229	46±4	75±4	39	0·0005
4888	47±3	75±4	38	0·0005
Mammary carcinoma AAF56				
4832	37±4	46±7	20	0·10
4887	42±5	46±7	8	0·35
4906	52±2	62±2	16	0·0025
4907	61±3	62±2	1	0·45
4928	31±3	46±7	33	0·025
4941	39±2	62±2	36	0·0005
Mammary carcinoma AAF57				
4556	157±7	179±9	13	0·025
4571	210±6	179±9	−17	—
4577	152±6	174±8	13	0·025
4582	170±7	174±8	2	0·35
4586	175±3	179±9	2	0·30
4590	187±7	174±8	−8	—

* Mean ± S.E.

had any reactivity against cultured cells derived from adult normal tissues. In tests with both AAF induced and spontaneously arising rat tumours although the cytotoxicity when significant was low, it was reproducible so that in tests with serum 5039 against mammary carcinoma AAF20 percentage cytotoxicities obtained in separate tests were 22%, 20%, 28%, 25%.

TABLE II.—*Cytotoxicity of Multiparous Rat Sera for 2-acetylaminofluorene Induced Rat Tumours: Summary Table*

	Cytotoxic reaction with cells of: Mammary carcinoma AAF 20	AAF 56	AAF 57	Ear duct carcinoma AAF 49
Positive reactions*	9	5	2	2
Percentage cell reductions	11–61	16–64	13, 13	38, 39
Negative reactions	3	7	16	2
Percentage cell reductions	0–7	7, 7	0–23	7, 7

$P < 0·025$–$0·0005$.

Immunofluorescence tests

Indirect membrane immunofluorescence tests were performed using sera from multiparous rats against 2 AAF induced (AAF20 and AAF56) and 2 spontaneously arising mammary carcinomata (Sp15 and Sp11). In no case was a significant reaction demonstrable against any of these tumours, the maximum fluorescence index obtained being 0·16. In contrast, these sera showed a significant level of membrane staining against DAB induced hepatomata and Mc induced sarcomata, producing fluorescence indices of up to 0·86.

DISCUSSION

These studies demonstrate that AAF induced and spontaneously arising tu-

TABLE III.—*Cytotoxicity of Multiparous Rat Sera for Spontaneously Arising Rat Tumours*

Multiparous serum No.	No. of cells* surviving after treatment with: Test serum	Control serum	Percentage cell reduction	$P<$
Sarcoma Sp7				
4394	137±11	141± 6	3	0·40
4395	141± 9	141± 6	0	—
4498	100± 7	144±11	30	0·025
4499	116± 8	144±11	19	0·025
4558	132±10	141± 6	7	0·25
4707	116± 7	144±11	20	0·025
Sarcoma Sp24				
4363	145±17	146±14	1	0·49
4416	102±13	146±14	30	0·005
4577	83±4	84± 4	2	0·4
4582	86± 5	113± 6	24	0·0025
Mammary carcinoma Sp15				
4296	90± 7	115± 6	22	0·005
4331	107± 6	115± 6	7	0·2
4400	111± 5	115± 6	3	0·3
4451	81± 4	115± 6	30	0·0005
Mammary carcinoma Sp11				
4121	35± 4	32± 3	−8	—
4362	18± 3	32± 3	45	0·0025
4397	40± 1	32± 3	−26	—
4462	32± 5	32± 3	0	—

* Mean ± S.E.

TABLE IV.—*Cytotoxicity of Multiparous Rat Sera for Spontaneously Arising Rat Tumours: Summary Table*

	Cytotoxic reaction with cells of: Sarcoma Sp7	Sarcoma Sp24	Mammary carcinoma Sp11	Mammary carcinoma Sp15
Positive reactions*	4	4	1	2
Percentage cell reductions	14–30	13–30	45	22–30
Negative reactions	10	12	4	2
Percentage cell reductions	0	0–14	0	3–7

* $P<$ 0·025–0·0005

mours express embryonic antigens at the cell surface which can be detected by complement dependent cytotoxic reactions with antibody in the serum of multiparous rats. The level of reactivity of multiparous rat sera with these weakly or non-immunogenic tumours was generally lower, however, both in the proportion of sera reacting and in their cytotoxic indices than that obtained with more immunogenic rat tumours such as DAB induced hepatomata and Mc induced sarcomata (Baldwin *et al.*, 1972*a*, *b*; 1974*a*). This may account for the failure to detect embryonic antigens on AAF induced and spontaneous tumours by the membrane immunofluorescence technique which has previously been employed for typing embryonic antigens on rat hepatomata (Baldwin *et al.*, 1972*a*; 1974*a*).

Embryonic antigens have also been detected upon Mc induced rat sarcomata by delayed hypersensitivity reactions (Wang, 1968) and by serological methods using xenogeneic antisera raised against tumour and early embryo tissues (Thomson and Alexander, 1973). Sarcomata induced by polycyclic hydrocarbons in mice and guinea-pigs also express embryonic antigens demonstrable by tumour rejection tests (Le Mevel and Wells, 1973; Grant, Radisch and Wells, 1974) or *in vitro* assays of cell mediated and humoral immune responses (Ménard, Calnaghi and Della Porta, 1973; Burdick

and Wells, 1973). Although a much broader spectrum of tumours needs to be evaluated, there is already substantial evidence to suggest that embryonic antigen expression may be a relatively consistent feature of carcinogen transformed cells. In this context, Embleton and Heidelberger (personal communication) have demonstrated embryonic antigens on mouse cells transformed *in vitro* with polycyclic hydrocarbons.

Embryonic antigens on chemically induced tumours have generally proved to be cross-reactive (Baldwin *et al.*, 1974*a*; Thomson and Alexander, 1973; Ménard *et al.*, 1973). This is emphasized further by the data reported here showing that multiparous rat serum contains antibody reacting with cells of different tumour types, including sarcomata and mammary carcinomata. This does not exclude the possible expression of organ specific embryonic antigens on tumours since these serum donors may have been sensitized to a multiplicity of embryonic antigens during pregnancy. This is suggested by other studies (Baldwin and Embleton, 1974) where lymph node cells taken from rats bearing the essentially non-immunogenic mammary carcinomata employed in the present investigation were found to be cytotoxic *in vitro* for the autochthonous tumour and also other mammary carcinomata, but not histologically different tumour types. The neoantigens involved in these responses were viewed as being embryonic antigens since lymph node cell cytotoxicity could be blocked by treating target cells with multiparous rat serum. This, however, requires more direct evaluation since these conclusions are relevant to immunological studies of human malignant tissue where organ specific neoantigens have been identified (Hellström and Hellström, 1973).

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