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Circulating Antibodies in Rats Bearing Grafted Colon Carcinoma[1]

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SUMMARY

Sera from rats bearing primary or grafted colon carcinoma may contain antibodies that can react with antigenic determinants at the surface of cultivated colon cancer cells. Assays with various target cells and absorption experiments suggest that antigens recognized by circulating antibodies are common to independent lines of cultivated colon cancer cells. They are therefore cross-reacting, tumor-type-specific antigens. They could be embryonic or fetal antigens, because some sera from multiparous animals react with colon cancer cells. However, blocking experiments suggest that these antigens differ from the carcinofetal antigen previously demonstrated on the surface of intestinal cancer cells by xenoantiserum.

INTRODUCTION

Antibodies cytotoxic to established cell lines of intestinal carcinoma have recently been detected in patients with colorectal cancer (7). However, blood group antigens and, potentially, HLA antigens may interfere with tumor-associated antigens. This interference does not exist in a syngeneic experimental model. We have therefore looked for circulating antibodies directed against cultivated cancer cells in inbred rats bearing syngeneic, grafted colorectal tumors. A carcinofetal antigen detectable by heteroimmunization had previously been demonstrated on the membrane of cells cultivated from these tumors (4).

MATERIALS AND METHODS

Animals and Tumors. Syngeneic BD IX rats have been maintained by brother-sister mating in our laboratory since 1971. Their inbreeding was confirmed by permanent acceptance of skin graft. Induction of intestinal carcinomas by 1,2-dimethylhydrazine and the obtaining of transplantable lines of these tumors have been reported previously (5, 6). Lines DHA, DHB, DHC, DHD, and DHE obtained from individual colorectal carcinomas were used in this work. In addition, graftable tumors were obtained by s.c. injection of cultured syngeneic astrocytoma cells (see below).

Cell Culture. Established lines of colon cancer cells DHB, DHD, and DHE have been initiated by trypsinization of the grafted tumors (4). They grew as epithelial-like monolayers in Ham's F-10 medium, supplemented with 10% fetal bovine serum and antibiotics, and were serially passaged after 0.25% trypsin dissociation. Cultivated cells injected s.c. grew as adenocarcinomas in syngeneic rats. Tests for *Mycoplasma* contamination by orcein staining (2) or by cultivation on *Mycoplasma* culture medium (Baltimore Biological Laboratories, Cockeysville, Md.) were negative. In addition, other culture lines have been obtained from the Department of Biochemistry, University of Dijon, Dijon, France (normal hepatocytes from Wistar rats) and from the Department of Tumor Immunology, Karolinska Institutet, Stockholm, Sweden (neurinoma GE 3 and astrocytoma 290 T, both lines obtained from BD IX rats treated with ethylnitrosourea).

Rat Sera. Sera were obtained from BD IX rats by cardiac puncture or by cutting the end of the tail. They were collected from normal rats (males or virgin females), normal multiparous females (having had more than 2 pregnancies), rats bearing s.c. grafted tumors, or rats in which primary intestinal tumors had been induced by s.c. administrations of 1,2-dimethylhydrazine (6). In addition, sera were obtained from rats immunized by 3 i.p. injections at 2-week intervals of 2×10^6, 4×10^6, and 2×10^6 DHD cells treated by mitomycin and neuraminidase and from rats grafted 3 times without success with colon tumor DHE.

Immunofluorescence. Target cells were prepared by trypsinization of cell cultures. In most of this work, the DHD cell line was used because it was the easiest to obtain in culture conditions. Living cells (10^5) were incubated for 20 min at room temperature, with 50 μl undiluted rat serum containing 16% fetal bovine serum. Cells were washed 3 times in Ham's F-10 medium and then incubated for 20 min in fluorescein isothiocyanate-conjugated goat antiserum to rat *7S* γ-globulin (Hyland, Costa Mesa, Calif.) diluted 1/10 in Ham's medium containing 25% fetal bovine serum. The cells were washed 3 times, suspended in 20 μl buffered glycerol/Ham's medium, 1/1 (v/v), and examined for membrane fluorescence $\times$ 400 using an Orthoplan fluorescence microscope (Leitz, Wetzlar, Germany). The ratio of cells demonstrating membrane fluorescence to total living cells was determined on coded samples, according to the criteria recommended by Gunvén and Klein (3). At least 100 cells were counted for each test. The dead cells (usually less than 5%) showing intracellular fluorescence were not included in the count.

Standardization of Results. In preliminary experiments, results were evaluated by the percentage of living, mem-

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brane fluorescence-positive cells. However, it was later found that there was considerable variation among the results obtained when the same serum was tested on different preparations of the same line of target cells. These discrepancies seemed mostly to depend on variation in antigenic expression at the cell surface. Although they are influenced by culture conditions, they could not be adequately controlled to yield reproducible results when duplicate experiments were performed on different days. A standard serum was therefore pooled from several rats hyperimmunized against DHD tumor cells. Aliquots from this standard at 5 different dilutions (0.50, 0.25, 0.12, 0.06, and 0.03) in pooled normal rat serum were reacted with target cells in each assay under the same conditions as those of the unknown tested sera. The unknown sera could be characterized by a FI[2] equal to the dilution of the hyperimmune standard serum staining the same percentage of target cells.

RESULTS

Reactivity of Various Rat Sera on DHD Target Cells. Of 40 sera of normal BD IX strain rats (males or virgin females), 2 had a FI of 0.04. The other 38 sera had a FI less than 0.03 (*i.e.,* they stained a lower percentage of DHD cells than did hyperimmune standard serum at 3% dilution). FI values less than 0.03 were therefore considered as not specific.

Sera from 4 of 8 rats hyperimmunized against DHD cell line gave a FI greater than 0.50. FI of the other 4 sera were, respectively, 0.03, 0.16, 0.24, and 0.25. FI was also found to be elevated in sera from 5 rats that rejected 3 consecutive grafts of tumor line DHE (FI: 0.04, 0.08, 0.12, 0.13, and 0.47).

FI was found to be above 0.03 in 25 of 35 sera from rats bearing the grafted tumor line DHD from which the target cells used in the test were derived. FI exceeded 0.10 in 12 cases and 0.25 in 3 sera (Chart 1). There was no significant relationship between FI and tumor size, although sera from rats with very small or very large tumors were often negative. Six rats were bled out 3 months after excision of the DHD tumor and were found to be free of any detectable local or metastatic recurrence. FI was found below 0.03 in 2 cases and did not exceed 0.05 in the other 4 cases.

In most of the rats grafted with the other colon cancer lines, sera from the tumor bearer did not react with DHD target cells (Chart 1). However, FI greater than 0.03 was found with sera from 10 animals bearing colon cancer lines DHB, DHC, or DHE; in 4 of these sera, FI exceeded 0.10. Reactivity with DHD target cells was also checked in serum from 13 rats in which colon carcinomas had been induced by repeated injections of 1,2-dimethylhydrazine. FI was found elevated in only 3 of 13 sera (FI: 0.04, 0.06, and 0.08).

Immunofluorescence tests were performed on 9 sera from rats bearing the syngeneic astrocytoma 290 T; 8 sera were negative and 1 had a FI of 0.04.

Elevated FI was found in sera from 5 of 14 multiparous females. It exceeded 0.10 in 2 animals (0.15 and 0.21). Whether the females were pregnant at the time of serum collection apparently did not influence the incidence of positive results (multiparous, pregnant: 2/7; multiparous, not pregnant: 3/7).

[2] The abbreviation used is: FI, fluorescence index.

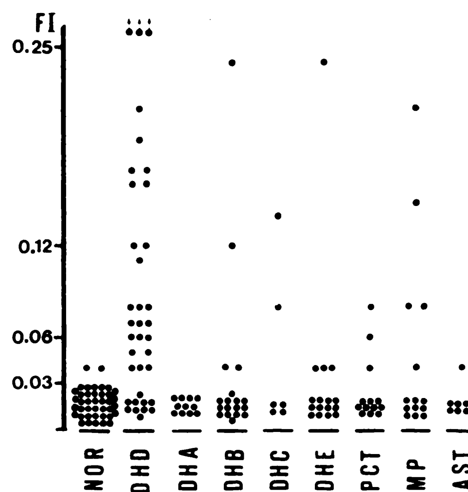

Chart 1. FI of various rat sera on DHD colon cancer target cells. *NOR*, normal rats; *DHD, DHA, DHB, DHC, DHE*, rats grafted with homologous colon cancer lines; *PCT*, rats bearing chemically induced primary colon tumors; *MP*, multiparous rats; *AST*, rats grafted with syngeneic astrocytoma.

Table 1
Membrane fluorescence of colon tumor bearer sera on various target cells

	Tested serum			
Target cells	373 (DHD-bearer) (%)	285 (DHD-bearer) (%)	267 (DHB-bearer) (%)	604 (normal) (%)
Colon cancer DHD	44[a]	62	38	0
Normal colon	0	0	0	0
Normal fibroblasts	0	NT[b]	0	0
Normal hepatocytes	0	NT	0	0
Astrocytoma	2	0	2	2
Neurinoma	2	NT	0	0

[a] Percentages of stained cells are reported.
[b] NT, not tested.

Reactivity with Other Target Cells. Sera reacting with DHD target cells did not yield positive fluorescence results when tested on a variety of other normal or malignant cells: freshly dissociated normal colonic cells, cultivated lines of normal fibroblasts or hepatocytes, and 2 cell culture lines of syngeneic nervous system tumors (Table 1). Similarly, the sera did not react with freshly dissociated intestinal cells from 16- and 19-day-old fetuses or from whole fetal tissue from 11-day-old embryos. Unfortunately, it was not possible to isolate the intestine from embryos at this age.

On the other hand, sera reacting with DHD target cells could also stain cells from the 2 other cultivable colon cancer lines, DHB and DHE. Absorption experiments summarized in Table 2 show that it was possible to remove the reactivity of these sera completely by absorption with DHD or DHE cancer cells.

Blocking with Rabbit Serum against Rat Colon Carcinofetal Antigen. We have previously reported that cultivated DHB, DHD, and DHE colon cancer lines shared a common

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carcinofetal antigen located on the cell membrane. This antigen was demonstrated by the immunofluorescence method, using a rabbit immune serum (4). In order to determine the relationship between this carcinofetal antigen and antigen(s) reactive with sera from tumor-bearing rats, attempts were made to block each immune system by antibodies from the other system. Each immune system was recognized by its specific fluorescein-conjugated anti-Ig serum diluted in serum of the other species to avoid a cross-reactivity between rat and rabbit Ig. As reported in Table 3, these blocking attempts failed, suggesting that sera of tumor-bearing rats do not react with the same antigen(s) on DHD cells as the serum from rabbits immunized against colon cancer lines.

Table 2

Absorption of colon tumor bearer sera by different lines of colon cancer cells

Tested sera	Absorption[a]	Target cells[b] (%)		
		DHD	DHB	DHE
285 (DHD bearer)	None	59	92	43
285 (DHD bearer)	DHE	0	2	0
312 (DHE bearer)	None	48	60	26
312 (DHE bearer)	DHD	0	0	0
312 (DHE bearer)	DHE	0	0	0
373 (DHD bearer)	None	50	68	32
373 (DHD bearer)	DHE	2	2	0
267 (DHB bearer)	None	38	67	29
267 (DHB bearer)	DHD	0	0	0
249 (Multiparous)	None	42	62	28
249 (Multiparous)	DHD	0	2	0

[a] Absorption was performed by incubating 5×10^7 living colon cancer cells/ml of serum.

[b] Percentages of stained target cells are reported.

DISCUSSION

A large variety of antigenic determinants may be expressed on the surface of cancer cells and evoke an immune response in the tumor-bearing host (1, 9, 11): (*a*) tumor-associated private antigens, the specificity of which is restricted to 1 individual tumor; (*b*) tissue-type specific antigens, cross-reacting with tumors originating from identical tissue but absent from normal fetal or adult tissues; (*c*) tissue-type-specific embryonal antigens common to embryonic and cancerous tissues, with organ specificity; and (*d*) widespread embryonic or fetal antigens, shared by various tumors and fetal tissues. In addition, carcinofetal components, although unable to evoke an immune response in the syngeneic host, may be detected by heteroimmunization.

All these varieties of antigens have been recognized by Steele and Sjögren (8, 10) on rat colon cancer cells, using lymphocyte-mediated cytotoxicity and serum blocking activity as detection techniques. The circulating antibodies, demonstrated by immunofluorescence in the present work, could have been directed against 1 or several of these tumor-associated antigens. Reactions of sera with various target cells and absorption experiments suggest that at least some sera react predominantly with cross-reacting, tissue-specific antigens. Whether these antigens are or are not fetal or embryonic cannot clearly be established at the present time. The lack of reactivity of sera with intestinal cells from 16- and 19-day-old fetuses cannot exclude a reactivity with intestinal cells from earlier embryos, if such could have been isolated. Reactivity of multiparous rat sera with colon cancer cells confirms that these cells contain embryonic or fetal antigenic determinants but do not preclude their identity with antigens recognized by tumor-bearer sera. As absorption experiments have only been performed on a few sera, it cannot be excluded that antibodies detected in other tumor-bearer sera are not directed

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Table 3

Reciprocal blocking of membrane immunofluorescence on DHD target cells by syngeneic or xenogeneic antisera

Reaction of DHD cells with a syngeneic, positive antiserum was not decreased by 3 preliminary incubations of DHD cells in a xenogeneic (rabbit) anti-DHD antiserum. In the same way, 3 incubations in syngeneic anti-DHD antiserum did not decrease the reaction of xenogeneic antiserum with DHD cells.

Target cells	Blocking serum	Tested serum	Fluorescein-conjugated serum	Positive cells (%)
DHD	None	285 (rat, DHD bearer)	Anti-rat Ig[a]	91
DHD	Normal rabbit[b]	285 (rat, DHD bearer)	Anti-rat Ig[a]	89
DHD	Rabbit, anti-DHD[b]	285 (rat, DHD bearer)	Anti-rat Ig[a]	88
DHD	None	Rabbit anti-DHD[b]	Anti-rabbit Ig[c]	100
DHD	Normal rat	Rabbit anti-DHD[b]	Anti-rabbit Ig[c]	98
DHD	285 (rat, DHD bearer)	Rabbit anti-DHD[b]	Anti-rabbit Ig[c]	100

[a] At 10% dilution in Ham's F-10 medium containing 10% normal rabbit serum.

[b] Normal rabbit serum and anti-DHD rabbit serum were absorbed *in vivo* in BD IX rats before the assay (4).

[c] At 10% dilution in Ham's F-10 medium containing 10% normal rat serum.

against private antigens, specific for 1 individual tumor cell line. This could explain the higher incidence of reactive sera in animals bearing the DHD tumor line, which is homologous to the DHD target cells. Furthermore, since the positive sera from animals bearing primary intestinal tumors had only a marginal degree of reactivity and cells from these tumors were not tested for absorption or immunofluorescence staining, the presence of common antigen(s) on primary tumor cells is still open to question.

The complexity of antigenic systems detected by membrane immunofluorescence probably explains the lack of relationship between serum reactivity and tumor size. During the growth of a given tumor, antibodies characterizing each antigenic determinant could or could not be evoked by this antigen and could or could not be masked by interacting with antigen released from the tumor (10).

Further experiments are therefore necessary to analyze more extensively the complexity of humoral immune reactions against rat colon carcinoma. Such a study would be useful for better interpretation of circulating antibodies associated with human colorectal cancer (7).

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