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Tumor Metastases and Cell-Mediated Immunity in a Model System in DBA/2 Mice. VIII. Expression and Shedding of Fcγ Receptors on Metastatic Tumor Cell Variants

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The expression of receptors for the Fc portion of IgG immunoglobin molecules was studied on tumor cell lines with high and low metastatic capacity. Two tumor cell lines from DBA/2 mice that had high metastatic activity, ESb and MDAY-D2, contained a high percentage of Fc receptor positive cells, as detected in a rosette assay with IgG antibody-coated erythrocytes (EA). In contrast, the low metastatic parental line Eb, from which ESb was derived, contained only a low percentage of EA-rosette-forming cells. ESb ascites tumor cells adapted to tissue culture in the presence of 2-mercaptoethanol (2ME) had a high expression of Fc receptors, whereas a cell line adapted to tissue culture in the absence of 2ME had a low expression of Fc receptors.

"Soluble" Fc receptors were detectable by their ability to bind to EA and to cause blocking of rosette formation. They were found to be present in fluids from tumor-bearing animals, such as serum and cell-free ascites. Even animals with an ascites tumor of the low-metastatic line Eb contained "soluble" Fc receptors.

The results are discussed with regard to their possible significance for tumor metastasis.

Key words: tumor metastases, Fc receptor, shedding, tumor variants

Properties of tumor cells that may be important for tumor cell dissemination and formation of metastasis are investigated in a syngeneic tumor model system in DBA/2 mice. The system consists of a methylcholanthrene-induced lymphoma (Eb) with low metastatic potential and a spontaneous variant thereof (ESb) with high metastatic capability. An unrelated methylcholanthrene-induced tumor (MDAY-D2) with pronounced metastasizing capacity is included for comparison. The major characteristics of these tumor cells have already been described [1–4] and are summarized in Table I. We showed

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TABLE I. Properties of the Tumor Cells of the Model System: Summary

	Eb	ESb	MDAY-D2
Strain of origin	DBA/2	DBA/2	DBA/2
Induction	MC,1955[a]	from Eb, 1968	see [15]
Tumorigenicity (TD_{50}, SC)	10^3-10^4	$<10^1$	$<10^1$
Metastasizing capacity	+	++++	++++
Invasiveness in vitro	±	+++	+++
Immunogenicity	++++	+	ND
Histiocytes at primary tumor	+++	±	+
Cell surface morphology: microvilli	+	++	++
Cell surface antigens			
H-2	K^d, D^d	K^d, D^d	K^d, D^d (NO K^k)
THY 1.2	+	+	-
Ig	-	-	-
Cell surface dynamics[b]			
Shedding	+	+++	ND
Modulation	+	+++	ND

[a]MC = methylcholanthrene; ND = not done
[b]Of H-2 antigens.

that ESb and MDAY-D2 but not Eb tumor cells had the capacity to adhere to and invade syngeneic normal mouse tissue in vitro [1, 5]. Furthermore, ESb tumor cells had more microvilli [1] and showed increased shedding of histocompatibility antigens [6]. The local primary tumors of the two metastatic lines were less infiltrated by host-derived macrophage-like cells than those of the tumor Eb [1].

In previous studies [4, 7] we have also defined the tumor antigens of the tumor lines Eb and ESb. The results are summarized in Table II. Tumor protection experiments revealed the presence of tumor-associated transplantation antigens (TATA) on both Eb and ESb tumor cells. TATAs of Eb and ESb were distinct and non-cross-reactive. Two antigens, one (1) characteristic for Eb and cross-reacting with a radion-induced BALB/c lymphoma, RLσ1, the other (2) characteristic for ESb and not cross-reacting with a number of unrelated tumor cells could be defined in this way. Similar antigens were characterized by means of tumor-specific syngeneic cytotoxic T lymphocytes (CTL). The similarity of the specificity patterns of protective antitumor immunity in vivo and of cytolytic T cells in vitro suggests that the latter can recognize TATAs and may thus play an important role in the establishment of protective immunity.

In the present study we report on the expression of receptors for the Fc portion of IgG immunoglobulin molecules – so-called Fcγ receptors. Fc receptors are expressed on a number of different cells such as B cells, activated T cells, macrophages, mast cells, monocytes, and various malignant cell populations. Fcγ receptors have a high affinity for the Fc portion of IgG molecules and only low affinity for Fc portions of IgM. They only weakly interact with free antibody but show a strong binding to antigen-antibody complexes, aggregated IgG, or cell-bound IgG antibody. These receptors play an essential role in antibody-dependent cell-mediated cytotoxicity. Recently a suppressive activity of Fc receptors expressed on activated T cells has also been reported [8].

We will show that both the metastatic cell lines ESb and MDAY-D2 contained a high percentage of cells with Fc receptors, as measured by the EA rosette assay, whereas the nonmetastatic line Eb had only very few of these rosetting cells. Furthermore, we will report that cell-free Fc receptors can be detected in the serum and ascites fluid from tumor-bearing animals.

TABLE II. Definition of Tumor Antigens in the Model System Eb/ESb

	Eb	ESb	MDAY-D2	P-815	SL2	RLδ1	RBL-5
I. TATA[a]							
1	+	–	–	–	–	+	–
2	–	+	–	–	–	–	–
II. T_K target[b]							
1	+	–	–	–	–	+	–
2	–	+	–	–	–	–	–

[a]TATA = tumor-associated transplantation antigen.
[b]Target antigens of syngeneic tumor-specific killer T cells.

MATERIALS AND METHODS

EA-Rosette Assay

Antibody-coated erythrocytes (EA) were prepared as follows: A 1% suspension of washed sheep erythrocytes (SRCB) was incubated for 30 min at room temperature with a subagglutinating amount of a rabbit IgG anti-sheep erythrocyte antibody (R-antiSRCB). The coated erythrocytes were washed twice in PBS. Tumor cells for the rosette assay were taken either from tissue culture as described [4] or from the ascites of tumor-bearing animals. In the latter situation the cells were preincubated at a density of 5 x 10^6/ml in Falcon flasks in a horizontal position for 30 min at 37°C to keep the tumor cells from contaminating host-derived adhering cells (macrophages and B lymphocytes). This procedure was performed twice. The rosette assay between tumor cells and the EA suspension was performed in small plastic tubes (F.A. Greiner, Nürtingen) as follows: 50 μl PBS, 50 μl of the tumor test cells (6 × 10^6/ml) and 50 μl of the 1% EA suspension was added, mixed, and centrifuged for 3 min at 150g in a WIFUG-table centrifuge. The pellet was carefully resuspended and assessed for content of rosettes in a counting chamber by assaying about 200–400 cells per sample. The tumor cells that formed rosettes were usually covered with 3–10 erythrocytes per cell. Uncoated erythrocytes were not bound by the tumor cells.

Blocking of Rosette Formation With Fc Receptor Containing Test Fluid

An EA-rosette-blocking assay was established to test for "soluble" Fc receptors in the supernatant of tumor cell cultures or in the ascites fluid or serum from tumor-bearing animals. These fluids were spun for 10 min in a Beckman airfuge and 50 μl of the EA cell pellet was incubated with 50 μl of the test solution for 45 min at room temperature. After two washings these preincubated EA cells were assayed with the tumor cells for rosette formation, as described above. The following controls were included: uncoated erythrocytes as a negative control, serum from normal animals instead of tumor-bearing animals, and EA cells that were not preincubated with test solutions (positive control). Normal DBA/2 spleen cells served as a positive control in all rosette assays.

All other materials and methods have been described elsewhere [1–4].

RESULTS

Expression of Fcγ Receptors on Metastatic and Nonmetastatic Tumor Cell Lines

The ability of the three tumor lines of our model system to form EA-rosettes with sheep red blood cells (SRBC) coated with decreasing amounts of a rabbit anti-SRBC serum was tested. The results are shown in Figures 1 and 2 and Table III. Whereas only 6–8% of

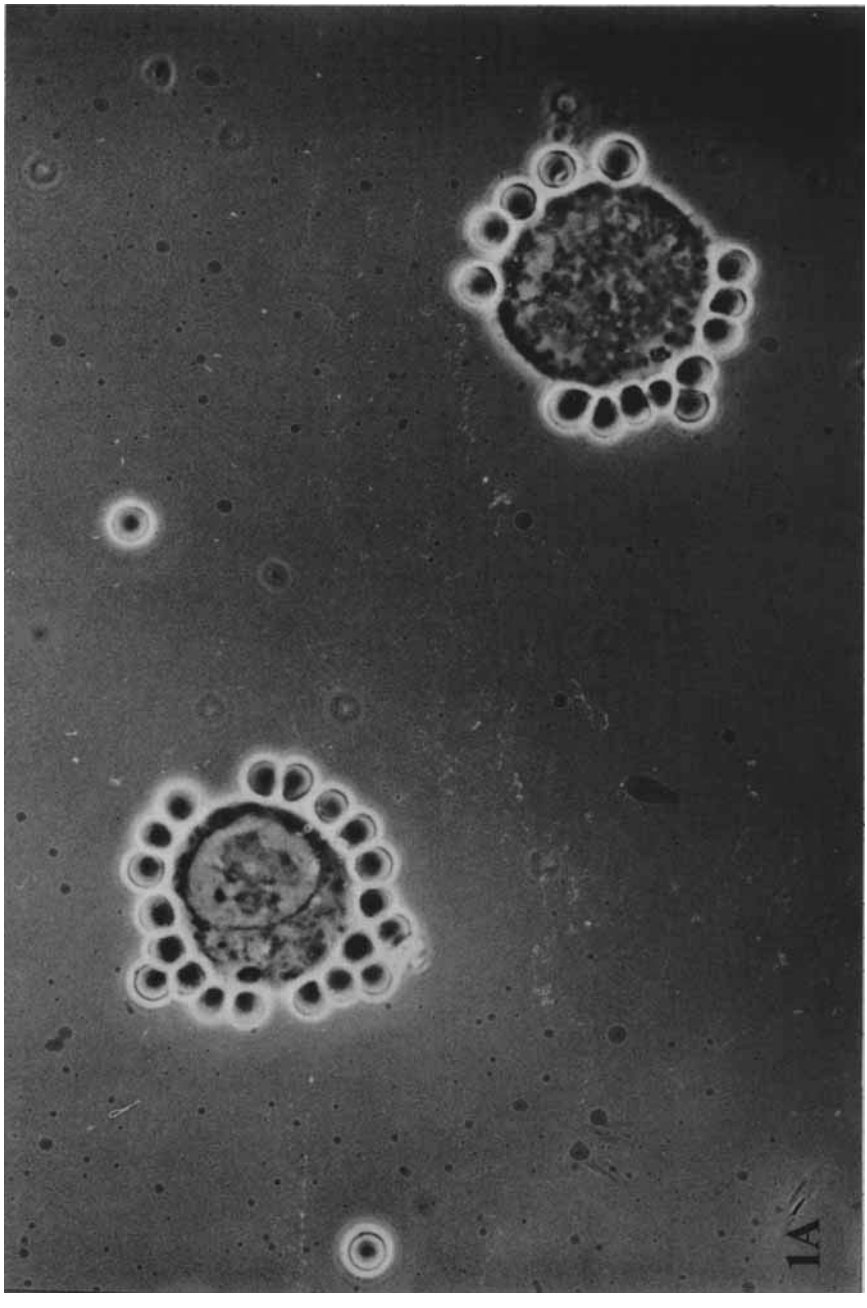

Fig. 1A. EA-rosettes formed by the tumor line MDAY-D2 [magnification, × 3,150].

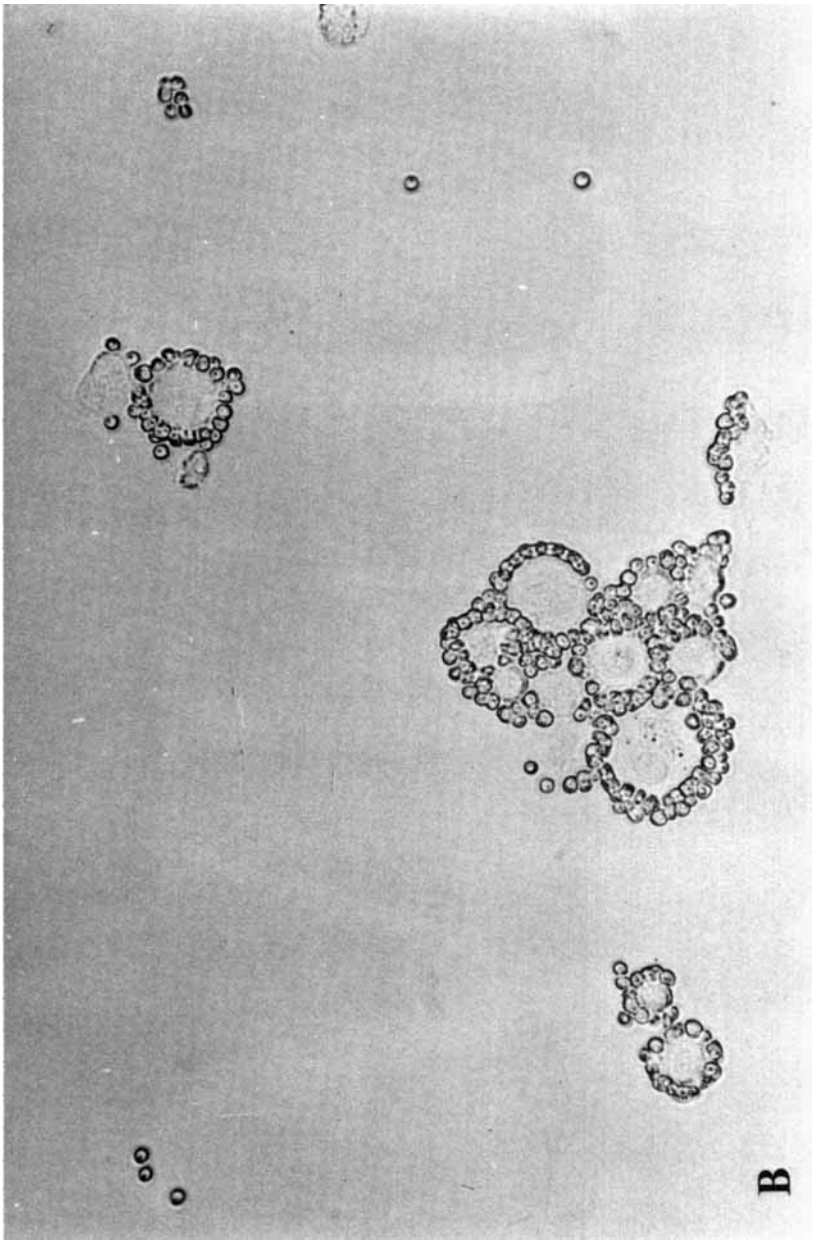

Fig. 1B. E.A-rosettes formed by the tumor line ESb [magnification, × 2,000].

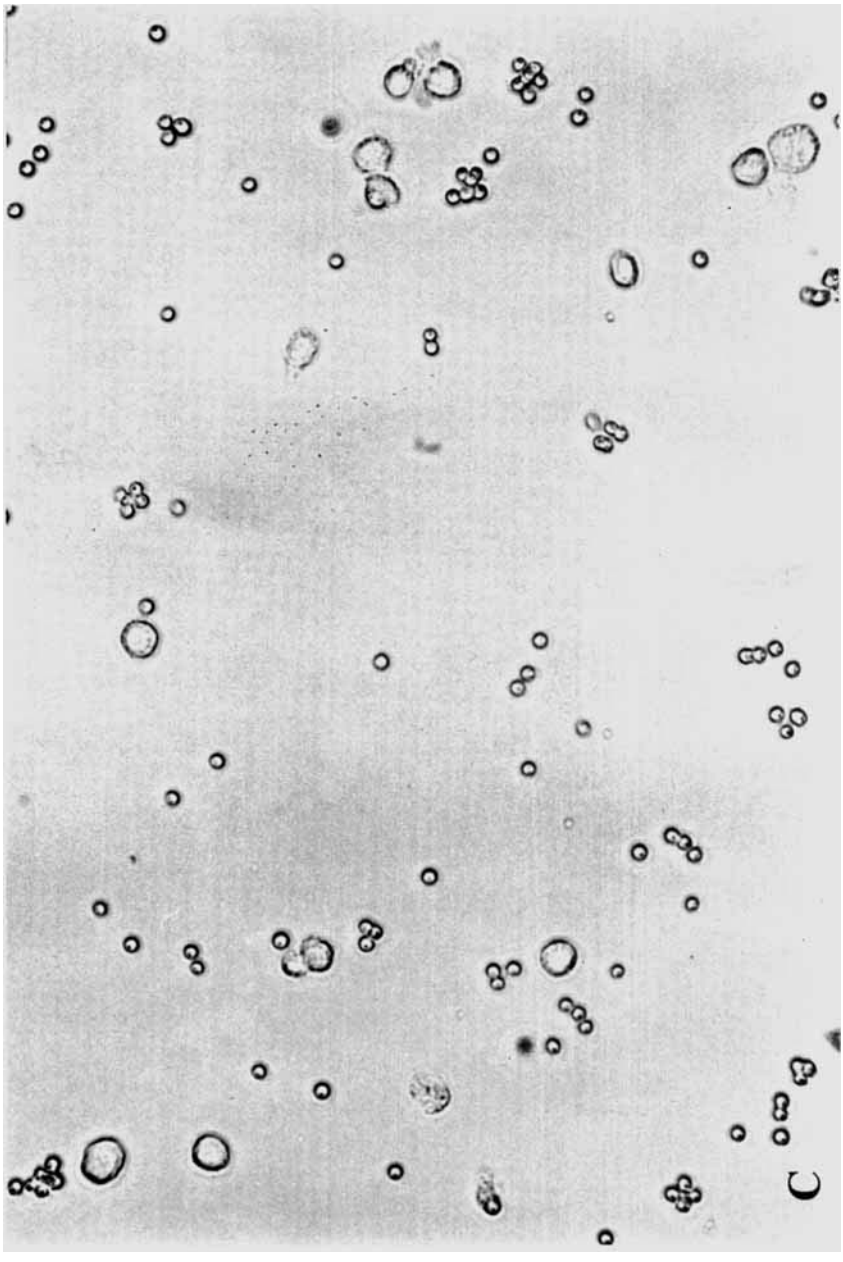

Fig. 1C. Negative results for rosette formation were obtained with the majority of cells from tumor Eb [magnification, × 1,250].

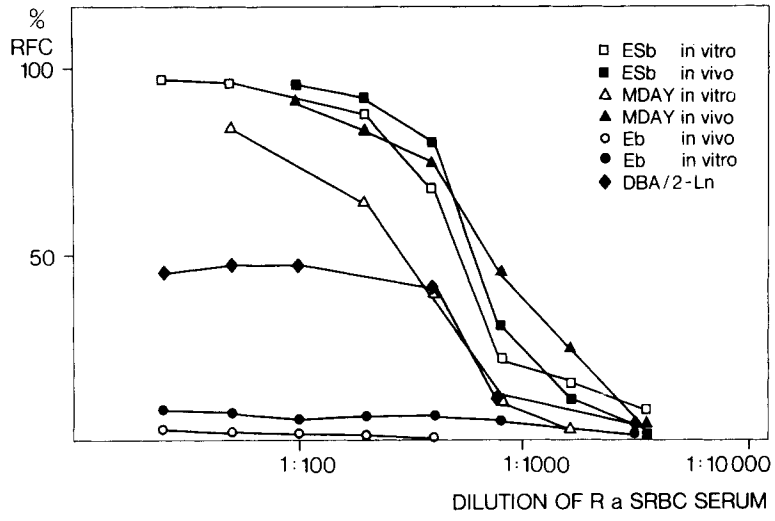

Fig. 2. Dependency of EA-rosette formation on the dilution of antiserum used for coating the red cells. The different cell types studied are indicated; MDAY = MDAY-D2; DBA/2-Ln = normal spleen cells from DBA/2.

TABLE III. RFC_{50} Titer of Tumor Cell Lines and of Normal Spleen Cells

Cell type	% EA-RFC (maximal)	RFC_{50} titer[a]	% RFC with non-coated SRBC
Eb in vitro	6	1:500	1
Eb in vivo	8	1:500	1
ESb in vitro	95	1:520	0
ESb in vivo	97	1:620	2
MDAY-D2 in vitro	87	1:360	1
MDAY-D2 in vivo	95	1:720	1
DBA/2 spleen cells	46	1:550	0

[a]Highest R anti-SRBC dilution giving 50% of the maximal % EA-RFC.

the nonmetastatic parental line Eb formed rosettes, more than 90% of the cells of the two metastatic tumor lines formed rosettes with the antibody-coated erythrocytes. Typical examples of the microscopic pictures are given in Figure 1.

The R-anti-SRBC serum used had a $\log_2$ haemagglutination titer of 10, which was mercaptoethanol-resistant and thus consisted mostly of IgG antibody. Under the conditions of the rosette assay no agglutination occurred at antiserum dilutions higher than 1:50. The highest antibody dilution at which 50% of the maximal numbers of rosettes were formed (RFC_{50} titer) was determined from Figure 1, and the results are represented in Table III. Normal spleen cells from DBA/2 mice were tested for comparison. They formed up to 46% EA-rosettes and had an RFC_{50} titer of 1:550. The RFC_{50} titer of ESb tumor lines from tissue culture or from in vivo and of MDAY-D2 from in vivo were similar or somewhat higher. The only cells with a lower RFC_{50} titer than normal cells were MDAY-D2 tumor cells from tissue culture.

Because of this difference in the expression of Fc receptors between tumor cells from in vivo and from tissue culture, we studied the stability of this marker on tumor cells passaged for various periods in tissue culture. MDAY-D2 ascites tumor cells after depletion of adherent cells formed EA rosettes up to 95%. After several passages of these cells in tissue culture this maximal number of EA-RFC decreased to lower than 50%. When ESb ascites tumor cells were treated similarly the percentage EA-RFC remained rather constant under tissue culture conditions, provided the medium contained 5×10^{-5} M 2-mercaptoethanol (2-ME). When 2-ME was omitted from the tissue culture medium, it was difficult to maintain viable ESb tumor cells in vitro. One particular tumor line, ESb_G, however, was established under these 2-ME-free tissue culture conditions. The number of EA-RFC of this line was much lower than that of the line cultured in the presence of 2-ME. In the first passage numbers it was around 20% and decreased to 10%. When the line ESb_G from tissue culture passage number 7 was inoculated back into DBA/2 mice intraperitoneally, the ascites tumor cells harvested 12 days later, after adsorption of adherent cells, were again more than 90% positive in the rosette assay (Table IV).

The ability of various other tumor lines of different histologic type and origin to form EA rosettes was also tested. The results obtained with tumor cells adapted to tissue culture are presented in Table V. The ability of various lymphoma lines to form EA-rosettes was found to vary from 3% (RBL-5) to 20% (SL2), 60% (ULMC, AKR-A) and 90% (LSTRA, SBR-1). No correlation was found with the tumor type or strain of origin.

Blocking of EA Rosette Formation by Preincubation of EA With Fc Receptor Containing Test Fluids

The possibility that the rosette-forming tumor cell lines not only express Fc receptors on the cell surface but actively shed this material into the environment similar to H-2 antigens [6] was studied. The presence of Fc receptors in ascites fluids or in the serum from tumor-bearing animals was investigated by testing these fluids for their ability to block the rosette formation between EA and the tumor cells. The antibody-coated erythrocytes were preincubated with the test fluids, then washed twice, and tested for their ability to bind to ESb tumor cells. The results from several experiments are summarized in Table VI. Pretreatment of EA with normal mouse serum had no blocking effect, whereas pretreatment with serum from ESb, MDAY-D2, or Eb tumor-bearing animals caused about 90% blocking

TABLE IV. Expression of Fc Receptors on Tumor Cells Passaged in Tissue Culture or in Vivo

	% EA-RFC						
Tumor cell	In vivo[a]	In vitro[b]					In vitro → In vivo[c]
PT		1	2	4	7	10	
ESb	97	92	91	89	84	84	84
ESb_G	97	23	23	ND	11	ND	93
MDAY-D2	95	94	94	ND	53	43	ND

[a]Washed ascites tumor cells after depletion of adherent cells.

[b]Tumor cells adapted to suspension tissue culture; the numbers of passage in tissue culture (PT) are indicated; ESb and MDAY-D2 were cultured in medium with 5×10^{-5} M 2-ME. The line ESb_G was adapted to tissue culture in medium without 2-ME: the viability at PT 1 was only 45%.

[c]Tissue culture cells from PT 7 were inoculated intraperitoneally into DBA/2♀ mice; results shown are from day 12 ascites tumor cells.

TABLE V. EA-Rosette Formation by Various Low Metastatic Tumor Cells Adapted to Tissue Culture

Tumor line	Type[a]	Strain of origin	% EA-RFC
SL2	L	DBA/2	20
P-815	M	DBA/2	80
ULMC	L	BALB/c	62
Meth. A	S	BALB/c	5
LSTRA	L	BALB/c	94
RBL-5	L	C57bl/6	3
AKR-A	L	AKR	61
SBR-1	L	B10.BR	92
Normal spleen cells	–	DBA/2	46

[a]L = lymphoma; S = sarcoma; M = mastocytoma.

TABLE VI. Blocking of Rosette-Formation Between EA and ESb Tumor Cells by Preincubation of EA With Fc Receptor Containing Test Fluids

Preincubation of EA[a]					
Serum	Ascites Fluid	Fresh	Frozen	% EA-RFC[b]	% Blocking
–	–	–	–	90	–
NMS	–	+	–	90	0
NMS	–	–	+	90	0
ESb (day 6)	–	+	–	11	88
ESb (day 5)	–	–	+	92	0
MDAY-D2 (day 9)	–	+	–	9	90
MDAY-D2 (day 8)	–	–	+	77	14
Eb (day 5)	–	+	–	10	89
Eb (day 5)	–	–	+	78	13
–	ESb (day 6)	+	–	15	83
–	ESb (day 6)	–	+	83	8
–	MDAY-D2 (day 9)	+	–	26	71
–	MDAY-D2 (day 8)	–	+	91	0
–	Eb (day 5)	+	–	40	56
–	Eb (day 5)	–	+	91	0

[a]EA were prepared by coating SRBC with 1:200 diluted RaSRBC; 50 μl EA pellet was preincubated at room temperature for 45 min with 50 μl test fluid (serum or ascites); after two washings these EA preparations were used for the test; test fluids were not heat-inactivated and were used either fresh or after storage at –20°C. (day 6) indicates day 6 after tumor inoculation.

[b]% Rosettes formed with ESb ascites tumor cells after depletion of adherent cells.

in this assay. Similar results were obtained with ascites fluids from tumor-bearing animals. Here the Eb ascites fluid was less inhibitory than the fluids from ESb or MDAY-D2 ascites. It is noteworthy that the test fluids had to be fresh. If they were frozen and thawed most of the blocking activity was destroyed.

DISCUSSION

Membranes of mammalian cells possess various recognition units that enable them to react to various environmental stimuli. A good example is the receptors for hormones and growth factors on malignant cells which can have a profound influence on tumor growth and dissemination [9, 10]. Here we describe the expression of another type of receptor on tumor cells: the Fc receptor which is specific for immunoglobulin molecules bound in an

antigen-antibody complex form. The biological significance of Fc receptors, which are present on a variety of normal and malignant cells is not clear but has been a matter of consideration in recent years [11–14].

Using an EA-rosette assay for the detection of Fc receptors, we found that the metastatic tumor variant ESb was positive, whereas the nonmetastatic parental line Eb was mostly negative. Similarly, the metastatic tumor line MDAY-D2 was found to be EA-rosette-positive, whereas the nonmetastatic original line MDAY was negative, as described recently by Kerbel et al [15]. Of course, more tumor lines with and without metastatic properties need to be investigated to test for general significance of these findings.

As shown in Table V, the ability to form EA-rosettes is not restricted to high metastatic tumor cells. A variety of low metastatic tumors, all deprived of contaminating host cells by adaptation to tissue culture, contained a high percentage of EA-RFC. The presence of Fc receptors on tumor cells is thus certainly not a property that enables them to metastasize. However, it is possible that tumor cell variants possessing Fc receptors have an advantage for metastasis formation over Fc receptor negative cells and might thus be selected out.

The EA-rosette assay is in our hands a convenient quick assay to distinguish the two tumor lines Eb and ESb. As shown from the results in Table IV, the expression of Fc receptors on ESb ascites tumor cells remained rather stable when the cells were passaged in tissue culture. Eb ascites tumor cells passaged in tissue culture also remained at the low level of 5–8% of EA-positive cells (Table III). An exception was the cell line ESb_G, which represented an ESb line adapted to tissue culture in the absence of 2-mercaptoethanol, a condition where Eb cells grow easily but where most of the ESb cells die. The observed shift in the expression of Fc receptors on the ESb_G line from high to low and back from low to high when the cells were grown again in vivo could represent a phenotypic variation influenced by the different environment. Since such shifts did not occur with the other two lines, we favor an alternative explanation; namely, that the shifts indicate the outgrowth of either Eb-like or ESb-like cells from a heterogeneous cell population under in vivo or in vitro culture conditions.

The differences observed in the EA-rosette assay between Eb and ESb tumor cells could mean either a qualitative or a quantitative difference in the expression of Fc receptors on these cells. Anderson and Grey [16] have shown that L5178 lymphoma cells, which are equivalent to Eb, carry receptors for aggregated IgG. Using an autoradiographic binding assay employing ^{125}I-labeled aggregated IgG2B, the grain counts of various cell types were as follows: 40–50 grains/cell/day for P815 mastocytoma cells and macrophages, 20–30 for allogeneically activated thymocytes, 2–3 for splenocytes, 1 for L5178 lymphoma cells, and 0.6 for positive thymocytes. Neauport-Sautes and Fridman [17] showed that an immunoglobulin-binding factor (IBF) was produced by internally labeled L1578-Y thymoma cells. After precipitation with antigen-antibody complexes the factor, a glycoprotein, was shown to consist of a major unit of 40,000 daltons and a minor unit of 20,000 daltons. Frade and Kourilsky [18] also used the T cell lymphoma L5178-Y to extract a glycoprotein having Fc receptor properties. Studies with lactoperoxidase-catalyzed surface-iodination [19] suggest that the Fc receptor from several different cell sources contains a polypeptide chain with a mol wt of about 120,000, which is highly susceptible to proteolytic degradation after cell lysis. These studies suggest that the negative results obtained with Eb cells (L5178-YE) in the EA-rosette assay are not due to an absence of Fc receptors on these cells. They rather suggest a lower sensitivity of the rosette assay as compared with the above-described Fc receptor detection assays. If this is the case, then our results would

indicate a quantitative rather than a qualitative difference in the expression of Fc receptors on Eb and ESb cells. The metastatic tumor variant has probably a higher density of Fc receptors than the parental line.

This conclusion seems to be supported by the blocking experiments performed with ascites fluid and serum from tumor-bearing animals. Here, even the fluids from Eb tumor-bearing animals had blocking activity, indicating the presence of cell-free Fc receptors. No quantitative experiments have yet been performed to test for differences in the amount of blocking activity in the fluids from animals bearing metastatic or nonmetastatic tumor cell lines. Also the exact nature of the blocking activity needs to be demonstrated. The instability of the blocking activity to freezing and thawing is in accord with the assumption that it is cell-free Fc receptor material, since this was shown before to be sensitive to this treatment [17].

In connection with metastasis the acquisition of Fc receptors by tumor cells might have selective advantage for one of several reasons: 1) it would enable the cells to bind immune complexes; these could have a stimulatory effect on tumor growth [20], cause masking of tumor-specific antigens, and/or lead to changes in the tumor cell traffic, with enhanced sequestration in the liver [21], 2) the release of "soluble" Fc receptors into body fluids might have important implications in the regulation of immune responsiveness; for instance, suppressive activity of IBF produced by L5178-Y on the humoral immune response in vitro has recently been demonstrated [17].

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