HEPATOCYTE-TUMOR CELL INTERACTION IN VITRO

I. Conditions for Rosette Formation and Inhibition by Anti-H-2 Antibody*

BY VOLKER SCHIRRMACHER,‡ RACHANEE CHEINGSONG-POPOV,‡ AND HEINZ ARNHEITER§

From the Institut für Immunologie und Genetik am Deutschen Krebsforschungszentrum, D-6900 Heidelberg, Federal Republic of Germany; and the Institut für Medizinische Mikrobiologie der Universität Zürich, CH-8028 Zürich, Switzerland

In many tumor systems, the location and extent of metastases are nonrandom, suggesting that unique cell properties are important in tumor arrest and formation of secondary tumor colonies. The lung and the liver are among the most frequently involved organs. These organs are also the predominant site of metastases by tumor cells of a model system of chemically induced lymphomas in DBA/2 mice that we have been investigating (1-5). The system consists of a low-metastatic parental tumor (Eb), a spontaneous high-metastatic variant thereof (ESb), and a high-metastatic tumor line of independent origin (MDAY-D2).

We previously described that ESb and MDAY-D2, but not Eb, tumor cells had the unique properties of adhesion to and invasion of normal lung tissue in vitro (1, 6). Now we describe another unique characteristic of the two metastatic tumor lines, namely, a selective binding to isolated hepatocytes in vitro.

Recently Kolb et al. (7) demonstrated a lectin-like receptor on rat liver cells that has specificity for galactosyl residues and may play a role in the trapping of recirculating desialylated lymphocytes in the liver (8). In our study, we investigated whether a similar interaction might be responsible for the trapping of circulating metastatic tumor cells in the liver.

Materials and Methods

Tumor Cells. Eb is a Heidelberg subline of the methylcholanthrene-induced DBA/2 lymphoma L5178Y, and ESb is a spontaneous variant thereof with highly metastatic potential (1, 5). The etiology of the metastatic DBA/2 tumor MDAY-D2 has been described (9). The tumor cells were passaged in tissue culture using RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) with 10% fetal calf serum (FCS) and 2×10^{-5} M 2-mercaptoethanol.

Murine Hepatocytes. Liver cell suspensions were prepared as previously described (10). An 8-12-wk-old mouse was injected with 0.3 ml of 3.6% chloral hydrate in phosphated-buffered saline and 0.1 ml of heparin (500 U/ml). The hepatic portal vein and inferior vena cava were cannulated with a 21-gauge needle and a 16-gauge plastic catheter, respectively. The liver was perfused *in situ*, first with perfusion buffer (10) that was saturated with O_2 at 36°C for 2-3 min,

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‡ Institut für Immunologie und Genetik am Deutschen Krebsforschungszentrum, Heidelberg, Federal Republic of Germany.

§ Institut für Medizinische Mikrobiologie der Universität Zürich, Zürich, Switzerland.

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and then with 0.05% collagenase in the same buffer for 8–9 min. The liver was then removed, cut into pieces, filtered, and then suspended in Williams medium E supplemented with insulin and 15% FCS. Cells were washed three times for 15 min at 30 *g*. 2×10^5 cells were plated into wells of Falcon multiwell tissue culture plates (No. 3008; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) in a total vol of 400 µl supplemented Williams medium E. The cells were incubated at 37°C in 5% CO_2 for 2–3 h and washed once before use.

Neuraminidase Treatment of Tumor Cells and Rosette Assay. Tumor cells were washed three times with Hanks' balanced salt solution. 20 µl of Neuraminidase test (1 U/ml from *Vibrio comma* [*Vibrio cholerae*] Behringwerke AG, Marburg/Lahn, Federal Republic of Germany), was added to 2×10^7 tumor cells in 1 ml and incubated at 37°C for 30 min. After three washes, the tumor cells were resuspended in 2 ml of Williams medium E. For the rosette assay, 250 µl of ascites tumor-cell suspension (1×10^7/ml) was added to the hepatocyte culture and incubated at 37°C in 5% CO_2 for 1 h. The cultured hepatocytes were carefully washed three times before the number of rosette-forming cells was determined. Hepatocytes with three or more tumor cells bound were counted as rosette-forming cells (RFC).

Antibody-inhibition Test. Various alloantisera known to react with the tumor cells were used to test for possible blocking of rosette formation. 250 µl of tumor cells (10^7/ml) were preincubated with 25 µl of appropriately diluted antisera at 37°C for 30 min. After two washes, they were then tested for their binding to the hepatocytes.

Results and Discussion

Freshly isolated hepatocytes from DBA/2 mice were incubated for 3 h in tissue culture medium in Petri dishes in order to attach to the dishes. They were then incubated for 1 h at 37°C with either of the three syngeneic tumor-cell lines, Eb, ESb, and MDAY-D2, and carefully washed. Examination of the cultures by an inverted microscope (Diavert; E. Leitz, Stuttgart, Federal Republic of Germany) revealed that the two metastatic tumor lines ESb and MDAY-D2 had attached to the liver cells whereas the low-metastatic parental tumor Eb had not (Fig. 1 A and B). About 40% of the hepatocytes formed spontaneous rosettes with the metastatic tumor line ESb. The percentage of RFC and the average number of tumor cells bound per liver cell was greatly increased when the tumor cells were pretreated with neuraminidase (Fig. 1 C and D). Under these conditions, even the low-metastatic tumor Eb was bound by the hepatocytes (Fig. 1 C). Freshly isolated liver cells without preculture showed a decreased tendency to bind the tumor cells, so that no rosettes were observed with untreated tumor cells, and a smaller percentage bound neuraminidase-pretreated Eb or ESb tumor cells. This observation could be explained by adaptation of the liver cells to the tissue culture conditions, attachment to the plastic, and recovery from the collagenase pretreatment. That the tumor-cell binding was stable or even increased after recovery from enzyme treatment and was similarly observed after a prolonged culture period (24–72 h) indicates that the receptor responsible for the binding is not an artifact induced by the collagenase treatment but may be real and may play a physiological role in vivo.

The results from our syngeneic tumor model system suggest that hepatocytes bind metastasizing tumor cells better than nonmetastasizing ones. It remains to be investigated, however, with different tumor lines, whether this is a general phenomenon. Selectivity of the tumor-cell binding to hepatocytes was shown by the fact that other normal cells, such as Kupffer cells, peritoneal macrophages, lymphoid cells, and kidney cells (cell line BHK-21) did not form rosettes.

The dependency of tumor-hepatocyte-rosette formation on the temperature of incubation is illustrated in Fig. 2. Virtually no rosettes were formed at 0°C and only

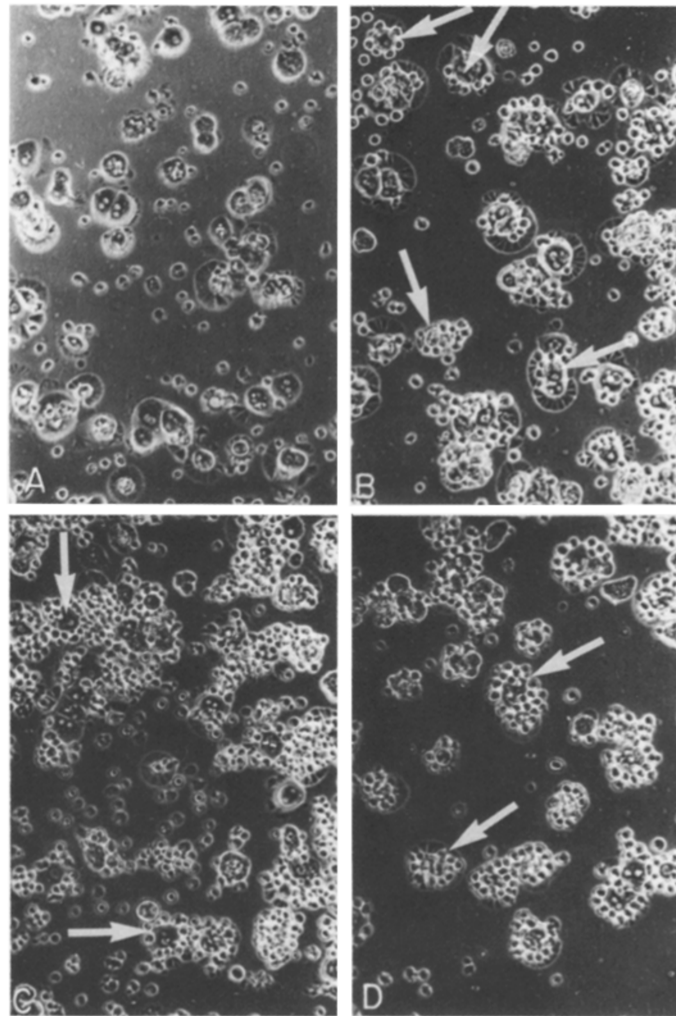

FIG. 1. (A) and (B) spontaneous rosette formation between cultivated hepatocytes from DBA/2 mice and the metastatic DBA/2 tumor variant ESb (B). No rosettes were observed with the parental line Eb (A). (C) and (D) Rosette formation of the same hepatocytes with neuraminidase-pretreated parental (Eb-N) or variant (ESb-N) tumor cell lines. The hepatocytes were isolated by a collagenase-perfusion technique and precultivated for 3 h in vitro. The adherent liver cells were then coincubated for 1 h at 37°C with the tumor cells from suspension tissue culture and then carefully washed. Arrows indicate typical rosettes.

a few at 4°C, although at 22°C about as many rosettes were formed with the tumor cells—whether untreated or neuraminidase pretreated—as at 37°C. These results suggest that membrane fluidity and possibly free movement of receptors may be required for the binding of metastatic tumor cells by the hepatocytes. Pretreatment of the liver cells with cytochalasin B (5 μg/ml) for 30 min at 37°C did not interfere with the cells' ability to form rosettes, which indicates that intact microtubules may not be required for binding.

To characterize the tumor cell molecules that are possibly involved in the binding to the hepatocytes, antibody inhibition experiments were performed. Three types of

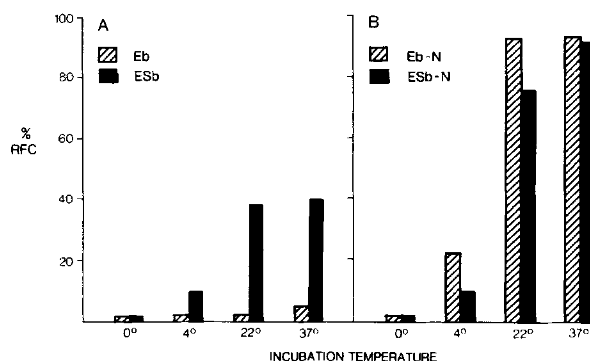

Fig. 2. Temperature dependence of the rosette formation between hepatocytes and untreated (A) or neuraminidase-pretreated (B) tumor-cell lines. Hepatocytes were prepared and precultivated as in Fig. 1. They were then adjusted to the indicated temperature and coincubated with the tumor cells at this temperature for 1 h.

antibody were used: alloantibodies directed (*a*) against H-2, (*b*) against non-H-2, or (*c*) against Thy-1 antigens from DBA/2 mice. Normal or neuraminidase-treated DBA/2 tumor cells were pretreated with various amounts of these respective antisera, washed, and then tested for binding to the hepatocytes. Some representative results of such blocking experiments are given in Table I. With the metastatic tumor line ESb, anti-H-2 sera produced quite strong inhibition, whereas the other two alloantibodies had either no effect (anti-non-H-2) or a comparatively weak inhibitory effect (anti-Thy-1.2). In contrast, when using the parental line Eb, which only bound to the liver cells after neuraminidase treatment, all three sera produced quite a good blocking effect. Compared to the cytotoxic titer assayed against the untreated tumor cells, anti-non-H-2 serum was the strongest inhibitor of rosettes formed with Eb-N, whereas the anti-H-2 serum was the strongest inhibitor of rosettes formed with ESb or ESb-N.

Little is known about the mechanism of the tumor-cell-hepatocyte binding and its inhibition by specific antibody. Preliminary results indicate that the previously described lectin-like hepatic membrane receptor (7, 11) may mediate the spontaneous binding of the tumor cells. Exposure of hepatocytes to neuraminidase abolished their ability to specifically bind desialylated glycoproteins (12), and also abolished their ability to bind the metastatic and the neuraminidase-treated Eb and ESb tumor cells (V. Schirrmacher, R. Cheingsong-Popov, and H. Arnheiter. Unpublished results.). The blocking by anti-H-2 serum was only observed when the tumor cells were pretreated, and not when the hepatocytes were pretreated with the antiserum. Allogeneic hepatocytes from C57BL/6 (H-2^b) formed rosettes equally well with ESb (H-2^d) as did the syngeneic hepatocytes from DBA/2 (H-2^d), which suggests that alloantigenic compatibility is not required for the rosette formation. However, anti-H-2^d serum inhibited ESb rosettes with allogeneic as well as with syngeneic hepatocytes (V. Schirrmacher, R. Cheingsong-Popov, and H. Arnheiter. Unpublished Results.). These results may indicate that H-2 molecules play a role in the spontaneous interaction of the metastatic tumor cells with the hepatocytes. Binding of anti-H-2 antibody to the H-2 molecules possibly blocks by steric hindrance rather than by covering up the determinants recognized by the hepatocyte receptor, because alloantigenic determinants do not seem to be involved.

A number of other cell-interaction phenomena have been described to be under

TABLE I

Effect of Preincubation of Tumor Cells with Defined Alloantisera on Their Ability to Form Rosettes with Isolated Syngeneic Hepatocytes

Experiment number	Preincubation with	Dilution	Tumor cell-hepatocyte-rosette formation						
			ESb		ESb-N		Eb	Eb-N	
			RFC	Inhibition	RFC	Inhibition	RFC	RFC	Inhibition
						%			
I	—		80		86		<1	92	
	Anti-H-2^{d}*	1:16	13	84	6	93	—	6	93
	Anti-H-2^{d}	1:64	27	66	26	70	—	47	50
	Anti-H-2^{d}	1:256	50	38	60	30	—	88	7
	Anti-H-2^{d}	1:1,024	80	0	84	2	—	87	5
II	—		92		94		<1	84	
	Anti-non-H-2‡	1:4	88	4	92	2	—	13	85
	Anti-non-H-2	1:16	89	3	92	2	—	24	71
	Anti-non-H-2	1:64	90	2	94	0	—	47	44
	Anti-non-H-2	1:256	92	0	94	0	—	84	0
	Anti-Thy-1.2§	1:4	44	52	73	22	—	47	44
	Anti-Thy-1.2	1:16	69	25	89	5	—	50	40
	Anti-Thy-1.2	1:64	92	0	90	4	—	77	8
	Anti-Thy-1.2	1:256	93	0	91	3	—	92	0

* This CBA/J anti-BALB/c alloantiserum (No. 456) had a 50% cytotoxicity titer of 1:500 against Eb and one of 1:175 against ESb.

‡ This B10.D2 anti-DBA/2 alloantiserum obtained after skin graft rejection had a 50% cytotoxicity titer of 1:20 against Eb and one of 1:4 against ESb. The cytotoxicity titer against Eb-N could not be determined because the majority of cells were lysed by complement in the absence of antibody.

§ The cytotoxicity titer of this monoclonal antibody (OLAC Ltd., Shaw's Farm, Blackthorn, Bicester, Oxon, England) was 1:250 against Eb and 1:10 against ESb.

H-2 control: they include lymphocyte-lymphocyte and lymphocyte-macrophage interactions (13) and also nonimmunological reactions such as the adhesion of mouse fibroblasts (14) or the contact inhibition of kidney epithelial cells (15).

Further studies are aimed at a better characterization of the molecules involved in the interaction of normal hepatocytes with neoplastic cells. This may help to elucidate, at a molecular level, the liver tropism of many metastatic tumor cells and possibly to find selective competitive inhibitors for this process.

Summary

Murine hepatocytes, isolated by an *in situ* collagenase-perfusion technique and cultured in Petri dishes, were shown to form rosettes with liver-metastasizing syngeneic tumor cells. Pretreatment of the tumor cells with neuraminidase generally increased the binding, whereas pretreatment of the liver cells with neuraminidase abolished the binding completely. The tumor-cell binding may be mediated by the previously described lectin-like receptor of hepatocytes that also was sensitive to neuraminidase treatment and that bound desialylated cells better than normal cells.

Anti-H-2 sera could efficiently inhibit the rosette formation of metastatic tumor cells with the hepatocytes, which points to a possible role of H-2 molecules in this interaction of neoplastic and normal cells.

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