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Natural cytotoxicity in lymphatic metastasis

I. In vitro studies using the rat tumor BSp73 and its variants

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Summary. *Serial transplantation of primary BSp73 ascites cells to a subcutaneous (SC) site gave rise to the appearance of two solid variants differing in their capacity to metastasize via the lymphatics. Tissue cultures derived from variant AS (nonmetastasizing) showed epithelioid morphology, while cultures derived from variant ASML (metastatic) showed spherical morphology. Upon cloning, both variants proved to be operationally homogeneous. Susceptibility of cultured BSp73 cells to NK and macrophage-mediated cytotoxicity was closely correlated to morphology, inasmuch as epithelioid cells were susceptible, while spherical cells were resistant, to lysis. With stimulated effector cells a general increase in cytotoxicity was observed, but epithelioid cells still showed a higher susceptibility level. Resistance of ASML-type cells to natural cytotoxicity was not due to the lack of recognition structures or to a general increase in the mechanical stability of spherical cells. This was concluded from cold target inhibition and from hypotonic shock treatment experiments, respectively.*

Introduction

Systemic metastatic spread has been postulated to be under the surveillance of natural immunity, especially by NK cells (for review see [10]). By analysis in murine systems it was established that metastatic spread could indeed by increased by depletion of NK-effector cells in the tumor recipient [11–14, 33, 36]. It was also observed, however, that clones derived from single metastatic foci in the lung were not generally resistant to NK lysis [14, 28]. This indicated that susceptibility to NK lysis may not be sufficient to prevent a tumor cell from colonizing distant tissues; i.e., surveillance by natural cytotoxicity may not be stringent enough to prevent extravasation and outgrowth of sensitive tumor cells.

With respect to lymphatic metastasis it has to be considered that the level of natural cytotoxicity in the lymphatic compartment is basically low [30, 34, 44, 46], but can be markedly increased by topical or systemic stimuli [16, 20]. Tumor-derived products may constitute one source of stimuli [4, 5]. There is no straightforward way to test for a putative influence of natural cytotoxicity on lymphatic metastasis, since selective elimination of this effector mechanism is achieved only in very special circumstances (e.g., in beige mice, [36]). But indirect evidence can be provided by analyzing the susceptibility of established tumor lines to natural cytotoxicity and correlation of this parameter to the metastatic capacity. If an inverse correlation can indeed be demonstrated (as is the case with some tumor lines showing hematogenous metastasis [10, 13]), it can further be questioned whether the two phenomena are causally linked or whether they are independent consequences of a common cause.

The BSp73 model consisting of parental tumor lines and variants differing in lymphatic metastasis [25] promised to be highly suitable for the approach outlined above. Like most other spontaneous rat tumors [26, 27, 43] BSp73 is unable to induce functional immunity in syngeneic recipients, irrespective of the immunization protocol used (M. Zöller, unpublished findings).

Host response to this type of tumor is mediated essentially by NK cells and macrophages [3, 19, 42, 44]. Hence, natural cytotoxicity is the dominant feature in the system. Furthermore, metastasis proceeds spontaneously via the lymphatics, unless tumor cells are injected directly into the bloodstream [25].

Materials and methods

Animals. Inbred BDX rats (6–10 weeks old; specific pathogen-free) were obtained from Thomae GmbH, Biberach, Germany. *Corynebacterium parvum* (Cp; Burroughs Wellcome) treatment involved IP administration of 3.5 mg dry weight per animal on day −7.

Tumor lines. The appearance of BSp73 and the establishment and maintenance of tumor lines derived from it have been described in detail previously [25]. Line A was derived from autochtonous ascites by continuous IP passage; line S was derived from the primary solid tumor by continuous passage of solid tissue; variant AS was obtained by SC implantation of ascites cells followed by continuous SC passage of solid tissue; variant ASML was derived from lung nodules occurring during the 2nd passage of AS and was maintained by serial SC implantation of metastatic nodules.

Tissue culture cell lines. Enzymatic dispersion of solid tissue, media, and culture conditions have been described in detail elsewhere [43]. Detachment of adherent cells was carried out according to Fidler [9] by short-term incubation with a film of 0.25% trypsin and 3 m*M* EDTA in Ca- and Mg-free Hank's media. Cells were kept as suspension cultures under standard conditions.

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Cultured cells were found to be free of Mycoplasma (analysis kindly performed by Dr W. Niklas, DKFZ) and the following virus species: Kilham, PVM, murine corona, Sendai, adenovirus, Reo-3, Theiler, and H-1 (analysis kindly performed by Dr V. Kraft, Zentralinstitut für Versuchstiere, Hannover).

Cloning by minimal dilution techniques. Single-cell suspensions were diluted to 0.3–50 cells per 0.2 ml and distributed to flat-bottomed Microtest II plates (96 wells, Falcon). After incubation at 37° C, 5% CO_2 for 2–3 weeks, cell colonies in < 30% of the wells were detached by trypsin/EDTA and were expanded. The metastatic capacity of clones (Table 1) was found to be very similar to that of uncloned AS and ASML variants [25].

Nonadherent effector cells (NK). Peritoneal exudate cells (PEC) were collected by using 10 ml HEPES-buffered Eagle's medium supplemented with 1,000 IU heparin. Spleens were suspended by mincing and pressing through several layers of sterile cotton gauze. Ficoll-Metrizoate (Nyegaard) centrifugation was carried out for 20 min at 2,000 rpm. Cells floating on top of the lower phase were collected, washed and placed for 2 × 1 h in a 75-cm^2 tissue culture flask (1–4 × 10^7 cells per flask). Nonadherent cells were collected. In one experiment, HY 3-Ag3 cells [15] were used as effectors. The growth medium used was Iscove's (GIBCO) supplemented with 10% Con A supernatants from rat spleen cells.

Adherent effector cells (macrophages). PECs from untreated or Cp-pretreated rats were washed and seeded into 35- × 10-mm petri dishes. After 2 h, nonadherent cells were removed by rinsing the dishes with medium (3 cycles). Cell densities were adjusted according to the estimate that 40% of resting and 80% of stimulated PEC would adhere to plastic. Bone marrow-derived macrophages were obtained after culturing bone marrow cells for 6–7 days in the presence of colony-stimulating activity [22]. These macrophages were interacted with target cells either directly or after incubation for 18 h with macrophage-activating lymphokines [22].

Table 1. Metastasizing capacity of cloned BSp73 AS and ASML variants

Clone[a]	Local takes	Metastases[b]	
		Lymph nodes[c]	Lung[d]
10AS-2	4/6	1/6	–
10AS-6	6/6	1/6	–
10AS-7	6/6	2/6	–
12AS-3	6/6	–	–
12AS-9	6/6	2/6	1/6
17AS-1	6/6	–	–
17AS-4	6/6	1/6	–
14ASML-1	6/6	6/6	6/6
14ASML-3[e]	5/6	1/6	1/6
14ASML-4	6/6	6/6	6/6
14ASML-7	5/6	5/6	5/6
14ASML-10	6/6	4/6	4/6
14ASML-12	4/6	3/6	1/6

[a] Standard inoculum 2 × 10^4 viable cells SC into the flank
[b] Animals bearing AS-derived tumors were killed and necropsied after 4–6 weeks when tumor diameters exceeded 5 cm. Animals bearing ASML-derived tumors were kept until death (6–8 weeks) and necropsied thereafter
[c] In animals with AS clones, only ipsilateral lympg nodes were harboring metastases. In animals with ASML clones, the ascending lymph tree, including mediastinal nodes, also contained metastases. In roughly half these animals, metastatic growth was also observed in nondraining (contralateral) nodes
[d] Macroscopic evidence confirmed by histology
[e] With the 14ASML-3 clone a high incidence of metastases (5/6 in lymph nodes, 5/6 in the lung) was observed following an inoculum of 10^5 cells

^{51}Cr-release assay. The test was carried out essentially as already described [44]. In short, effector cells were dispensed into U-shaped wells of microtiter plates at concentrations of 6 × 10^4 to 100 × 10^4 cells per well (triplicates). Labeled target cells (50–250 μCi per 2–10 × 10^6 cells) were added (10^4 per well) and the volume was adjusted to 0.25 ml. After centrifugation (4 min, 50 *g*), plates were incubated for 4 h at 37° C, 5% CO_2. Total lysis was determined by incubating target cells in a solution of 5% SDS. Incubation was terminated by centrifugation (5 min, 450 *g*) and radioactivity contained in the supernatant was counted.

Calculation:

$$\% \text{ spec. cytotoxicity} = 100 \times \frac{\text{Cts supern, test wells} - \text{cts supern, medium control}}{\text{Cts supern, SDS control} - \text{cts supern, medium control}}.$$

The release with medium was 2%–4%/h. In experiments with four to six replicates it was established that a specific cytotoxicity > 5% was statistically significant according to the Mann-Whitney U-test.

^{14}C-TdR release assay. This test for direct macrophage-mediated cytotoxicity was carried out as described elsewhere [21]. The test period was 36 h.

Cold target inhibition assay. Unlabeled tumor cells were added to 4-h test cultures so that the ratios of 'cold' target cells to labeled target cells (10^4 per well) were 1–10 : 1. Relative inhibition was calculated as:

$$\% \text{ Inhibition} = 1 - \frac{\text{spec. cytotoxicity in presence of cold targets}}{\text{spec. cytotoxicity in absence of cold targets}} \times 100 .$$

Results

Susceptibility of cloned and uncloned cells towards cytolysis by NK cells

The susceptibility of uncloned BSp73 variants was compared in a series of experiments, examples of which are shown in Figs. 1 and 2. As can be seen, AS cells were highly susceptible irrespective of the adaptation to and the duration of tissue culture conditions. A different effect was observed with tumor line S, which was heterogeneous with respect to morphology in culture. Freshly explanted preparations with a predominance of spherical cells (e.g., S/2 in Fig. 1) were resistant. Cultures obtained after prolonged selection for epithelioid cells (e.g., S/21) were susceptible. Ascites cells, when tested immediately after withdrawal from the animal, were resistant. After a long

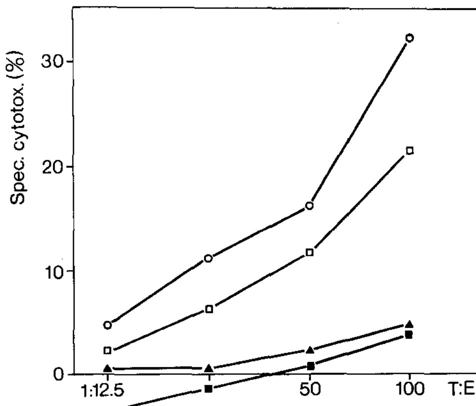


Fig. 1. Susceptibility of in vitro-cultured cells derived from BSp73 variants S, AS, and ASML to cytotoxicity by NK cells. Cr-release assay, 4 h. Effector cells, BDX spleen cells, 45%/54% Percoll interlayer: target cells, BSp73 AS/17 (17th passage in vitro; ○——○); S/2 (■——■); S/21 (□——□); ASML/6 (▲——▲)

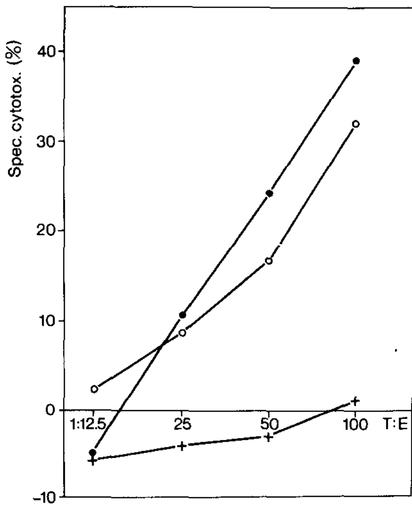
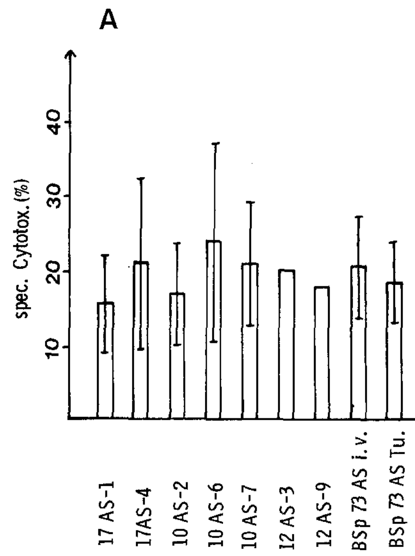


Fig. 2. Susceptibility of in vitro- and in vivo-grown BSp73 cells to cytotoxicity by NK cells. Cr-release assay, 4 h. Effector cells. BDX spleen cells, 45%/54% Percoll interlayer, target cells, BSp73 AS in vivo (○——○); AS/16 (16th passage in vitro; ●——●); BSp73 A (ascites, in vivo; +——+)



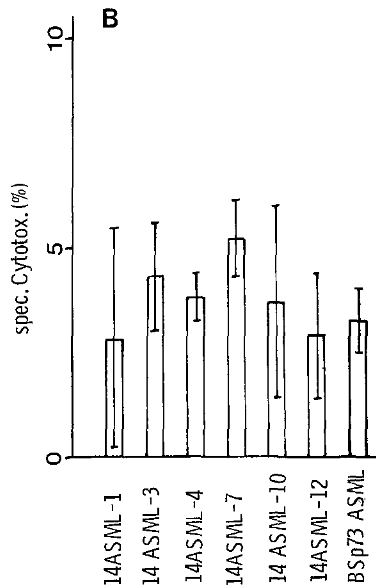


Fig. 3A, B. Susceptibility of cloned AS (**A**) and ASML (**B**) cells to cytotoxicity by NK cells. (NB different scale). Cr-release assay, 4 h. Effector cells BDX spleen cells, 45%/54% Percoll interlayer; effector : target ratio, 50 : 1. Target cells as indicated. AS IV, uncloned BSp73 AS cells in vitro; BSp73 AS Tu, uncloned AS cells in vivo. BSp73 ASML, uncloned ASML cells in vitro. *Bars* and standard deviations refer to three or more experiments (except for 12AS-3 and 12AS-9; one experiment each)

period of adaptation to culture conditions, susceptibility was observed as soon as epithelioid cells emerged and overgrew the otherwise spherical population (data not included). ASML-derived cells, which became established as an apparently homogeneous, continuously growing population of spherical cells only after prolonged tissue culturing (> 4 weeks), were resistant to lysis anyway (Fig. 1).

The next series of experiments was concerned with cloned cell lines. A summary of several experiments is given in Fig. 3 (note the scales of the ordinates). As can be seen, there was no marked difference between uncloned AS cells and AS clones, nor could we identify differences in susceptibility among the clones. With cloned and uncloned ASML cells, cytotoxicity was mostly below the significance level of the release assay (Fig. 3B). Apparent differences within the groups of AS clones or ASML clones were insignificant according to the Mann-Whitney U-test.

The most clear-cut discrimination between AS and ASML was achieved with HY 3-Ag3 effector cells, a permanent mouse T-cell line with endogenous NK activity [15]. While AS clones were killed even at E : T ratios of 1 : 1, ASML clones proved to be resistant throughout the E : T range tested, as is shown in Fig. 4.

So far, a clear difference in cytotoxicity to all types of epithelioid (AS-like) cells compared with all types of spherical (ASML-like) cells has been observed. The question emerged as to whether resistance of ASML cells could be overcome by using activated effector cells. This was indeed the case. After

treatment of effector cell donors with Cp, cytotoxicity was markedly increased for both AS and ASML cells (Fig. 5). Nonetheless, the overall difference between the two types of cells persisted. Similar results were obtained with effector cells after activation with polyinosinic : polycytidylic acid for 24 h in vitro (data not shown).

Expression of recognition structures for NK cells on AS and ASML targets

The above observation raised the question of the extent to which recognition structures for NK cells were actually present on ASML cells. The experimental approach consisted in adding unlabeled competitor cells to the ^{51}Cr-release assay at different cold-to-hot target ratios. Only tests with a basic cytotoxic rate >20% were used for calculating relative inhibition. Nevertheless, considerable variability had to be accepted in these experiments, despite strict standardization of the test procedure. With PEC from untreated BDX rats no reproducible difference in the inhibitory capacity of AS and ASML competitors was observed (Fig. 6). Concordant results were obtained in two other series of competition experiments (data not shown) in which we used either HY 3-Ag3 effector cells or YAC target cells or both types of cells together.

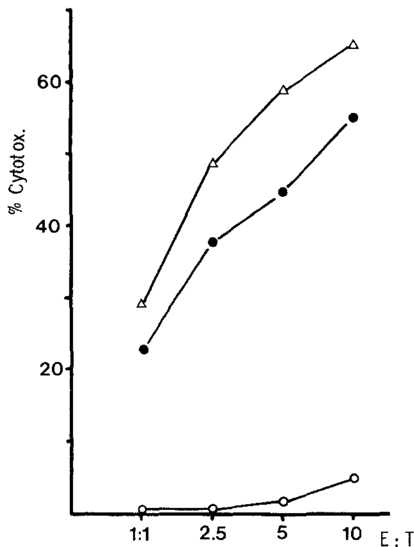


Fig. 4. NK-type cytotoxicity by the mouse T-cell clone HY3-Ag3. Cr-release assay, 4 h. Target cells, YAC (△——△); 17AS-4 (●——●); 14ASML-1 (○——○)

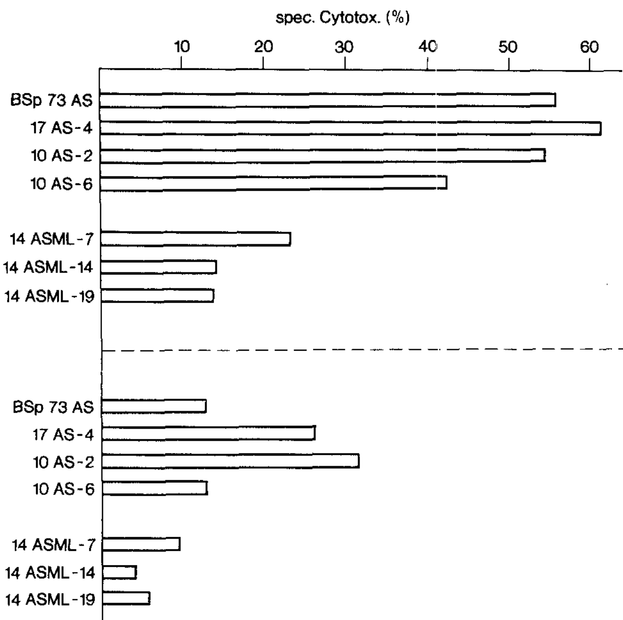


Fig. 5. Susceptibility of cloned BSp73 cells to *C. parvum*-stimulated rat spleen cells. Cr-release assay, 4 h. Effector cells were derived from animals pretreated with 3.5 mg Cp IP on day −7 (*bars* above *broken line*) or from untreated animals (*bars* below *broken line*). Spleen cells, 45%/54% Percoll interlayer, E : T = 50 : 1. Target cells as indicated; BSp73 AS, uncloned AS cells

Differential susceptibility of AS and ASML cells to other mechanisms of cytolysis

The nature of the resistance of ASML cells was further elucidated in different types of cytotoxic systems. With peritoneal macrophages from BDX rats, AS cells proved to be as susceptible to lysis as classic macrophage targets (i.e., P815), while ASML cells showed pronounced resistance (Table 2). This could be overcome to some extent by activation of effector cells. To assure that the cytolytic activity was mediated by macrophages rather than by a contaminant

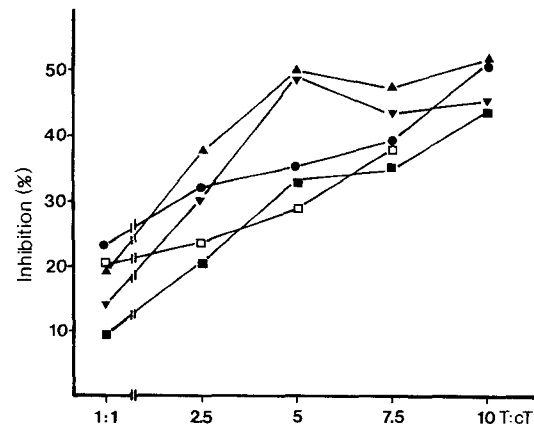


Fig. 6. Cold target competition of AS and ASML clones. Cr-release assay, 4 h. Effector cells were PEC after Ficoll Metrizoate purification and depletion of plastic-adherent cells. Hot target cells were uncloned BSp73 AS, E : T = 50 : 1; Ct : T, cold target to hot target ratio. Cold targets: uncloned BSp73 AS (●——●); 17AS-4 (▲——▲); 10AS-7 (■——■); 14ASML-1 (□——□); 10AS-6 (▼——▼)

Table 2. Cytotoxicity by resting and activated macrophages

Macrophage source	Pretreatment	^{14}C-TdR release from[a]		
		P815	17AS-4	14ASML-1
Peritoneal cavity	None	25 (± 2)	18 (± 3)	0
	Corynebacterium parvum IP	75 (± 2)	76 (± 3)	16 (± 2)
Bone marrow	None	3 (± 4)	4 (± 4)	1 (± 4)
	Lymphokine activated in vitro[c]	42 (± 6)	38 (± 8)	2 (± 7)

[a] Effector target ratio 5: 1
[b] Mean of four determinations (± standard deviation)
[c] Incubation with colony-stimulating activity 18 h prior to test

effector cell type, similar experiments were performed with pure macrophages derived from bone marrow precursors [22]. These effector cells manifested very low spontaneous cytotoxicity against the various targets, but acquired marked cytolytic activity against susceptible targets during interaction with macrophage-activating lymphokines (Table 2).

When exposed to a DA anti-BDX allo-antiserum, both As and ASML cells were able to absorb alloantibodies. However, complement-dependent cytotoxicity was observed only with AS cells (54.6% specific cytotoxicity with antiserum diluted 1 : 40), and not with ASML cells (0.2% specific cytotoxicity).

It has been claimed [3] that lysability by NK cells is paralleled by the osmotic fragility of the target cell. This was not observed with BSp73 cells. After treatment of ^{51}Cr-labeled clones with hypotonic medium (dilution of medium in water = 1 + 3) for 4 h at 37° C, label release was 45.4% ± 4.4% and 43.4% ± 4.3%, respectively, for ASML and AS cells (mean values from 5 clones of each type). Hence, the resistance of ASML cells to cytolysis seems to be no general phenomenon but rather restricted to immune mechanisms.

Discussion

Among the postulated roles of natural cytotoxicity in vivo, restriction of tumor metastases takes a prominent place [2, 10]. In this context, three major issues have been addressed, namely the consequences of in vivo versus in vitro growth of tumor cells with regard to susceptibility to lysis by NK cells, the consequences of effector cell activation on differential susceptibility, and the correlation of metastasizing capacity and resistance to natural cytotoxicity on a clonal level.

In the BSp73 system, the four in vivo variants gave rise to two distinct morphotypes in culture [25]. With respect to NK-mediated cytolysis, in vitro-cultured BSp73 cells showed two stable constellations of properties: cells of the epithelioid morphotype, which are nonmetastatic, were highly susceptible; while cells of the spherical morphotype, which are highly metastatic, were resistant. Fluctuations observed with cloned cell lines were within the range of reproducibility of NK assays. Among the in vivo-grown BSp73 variants, only A and AS were directly amenable to cytotoxicity testing. Freshly prepared suspensions from solid AS tissues proved to be highly susceptible. No cells with epithelioid morphology were ever isolated from the ASML variant, which was resistant irrespective of the duration of tissue culturing. From these results it is reasonable to conclude that susceptibility is an inherent property of the epithelioid morphotype. Furthermore, since AS cells were highly susceptible prior to any culturing in vitro, and since ASML cells did not gain susceptibility despite definite adaptation to tissue culture, it can be concluded that susceptibility of BSp73 cells is not an in vitro artefact.

Susceptibility of tumor cells to the tumoricidal activity of macrophages is not necessarily correlated with the susceptibility to lysis by NK cells [40], although a parallelism in the susceptibility pattern was observed in some rat [3, 45] and mouse tumor systems [29]. BSp73 variants clearly represent a further example of condordant susceptibility towards both NK cells and (activated) macrophages. This has important implications for the interpretation of phenomena observed in vivo (see companion paper).

According to recently published evidence, resistant tumor lines may become susceptible when exposed to highly activated NK cells [8, 10, 35, 38]. This is not the case with target cells devoid of recognition structures [6]. In the BSp73 system, pretreatment of effector cell donors with interferon-inducing agents resulted in a general increase of lytic activity. Although ASML cells now showed significant lysis, AS clones were lysed even better, so that there was still a marked difference between the two morphotypes. The killer cell line HY 3-Ag3 was shown to exhibit a cytotoxic 'pattern' rather similar to that of mouse NK cells [1]. When they were tested on BSp73 targets, high kill rates were obtained with AS cells, while kill rates with ASML cells were not significant.

These findings led to the question of expression of recognition structures on AS vs ASML cells. Appropriate expression of recognition structures on target cells [37] is manifested in binding to the NK effector cells [31], which is a necessary but not a sufficient condition for target cell lysis, i.e., positive target cell binding does not necessarily correlate with susceptibility to lysis [29], but a partial or total loss of recognition structures (e.g., by target cell differentiation [7]) leads to both reduced target cell binding and resistance to lysis [6, 39]. In the BSp73 system, both susceptible and resistant morphotypes were able to act as competitors in the cytotoxic assay. Furthermore, both types of cells were able to absorb alloantibodies, but only AS cells were lysed in the presence of complement. Accordingly, both cell types were able to induce allospecific killer cells, which could only kill AS cells and not ASML cells in vitro (D. Bellgrau, unpublished findings). From these data it can be concluded that ASML-type cells are resistant to the different mechanisms of immune cytolysis despite the presence of the relevant recognition structures. Similar observations were made with variants of the human K562 line [23]. With murine tumor variants a slightly different picture emerged. Selective resistance to NK-mediated cytotoxicity, but not to other mechanisms of immune cytolysis, was observed both in the presence [41] and in the absence [6] of recognition structures.

The actual mechanism(s) of resistance is presently not known. Besides reduced osmotic fragility [3], which was observed neither with ASML cells nor with K562 variants [23], an active involvement of the target cell membrane during the lethal hit phase has to be considered [17, 31]. It is conceivable that particular features or functions of the membrane (e.g., receptors for cytolytic signals or repair capacities) may render a cell resistant to immune cytolysis without affecting its overall stability [8, 24, 41].

The question as to whether resistance of ASML cells to natural cytotoxicity may be mechanistically linked to metastatic spread can only be addressed by in vivo studies. The obvious next step towards this goal is to monitor the survival of BSp73 cells with defined susceptibility in the syngeneic animal. This is the issue dealt with in the companion paper.

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