

Sera from patients with colon, breast and lung cancer induce resistance to lysis mediated by NK cytotoxic factors (NKCF)

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Summary Natural killer (NK) cells are involved in the antitumoral immunologic mechanism. These cells act through the release of cytotoxic molecules defined as NK cytotoxic factors (NKCF). Inhibitory factors of NK and NKCF mediated lysis have been described in *in vitro* assays. This study evaluates the induction of resistance to NKCF cytotoxicity by sera from 27 patients with colon, breast and lung cancer. Addition of these sera to the cytolytic assay where K562 cells and concentrated NKCF were used, induced resistance to NKCF mediated cytotoxicity in 21 cases (77%). The sera from the group with metastasis blocked NKCF lysis more markedly than the group with local tumours. However, no differences were observed when the groups with colon, breast and lung cancers were compared. This blocking effect was not found to be related to gamma interferon (IFN) levels. In a previous study, we described a tumour factor (NK-RIF) produced by human cell lines derived from metastatic adenocarcinomas. This factor blocked lysis of tumour target cells by NK cells. Consequently, it is proposed that the release of similar tumour factors with a capacity to induce resistance to NKCF may be involved in tumour growth and metastatic spreading *in vivo*.

Natural killer (NK) cells have been implicated in the control of distant metastatic diseases (Herberman & Holden, 1978; Trinchieri & Perussia, 1984). They have been defined as a subset of peripheral blood lymphocytes with distinctive morphological, phenotypic and functional characteristics. NK cells display the structural features of large granular lymphocytes (LGL) (Grossi *et al.*, 1978, 1982; Saksela *et al.*, 1979; Timonen *et al.*, 1981) and the ability to lyse a variety of neoplastic and non-neoplastic target cells, including virus-infected ones and hematopoietic precursors (Herberman *et al.*, 1982, 1986; Trinchieri & Perussia, 1984). NK cells from human, rat or mouse, release natural killer cytotoxic factors (NKCF), functionally defined as lymphokines selectively cytotoxic for NK target cells (Herberman *et al.*, 1979, 1986; Savary & Lotzova, 1986).

The mechanisms involved in the resistance or susceptibility of tumour cells to NK cells are poorly understood (Stern *et al.*, 1980; Powel *et al.*, 1987; Harris *et al.*, 1987; Peña *et al.*, 1990a,b), but studies have demonstrated that natural killer cell activity is diminished in colon, breast and lung cancer, especially in advanced stages or in those with metastatic processes (Lin *et al.*, 1987; Tartter *et al.*, 1987; Hisamatus *et al.*, 1986). Thus, the deficient activity of NK cells results in increased numbers of metastatic deposits and tumoral escape from immunologic vigilance (Schantz *et al.*, 1986).

We have recently described a factor (NK-RIF) released by cell lines with adenocarcinoma metastasis and which induces resistance of K562 cells to NK mediated lysis by blockage of NKCF activity without affecting target and effector conjugate formation (Serrano *et al.*, 1989; Solana *et al.*, 1990a,b). It does not interfere with macrophage, lymphokine activated killer (LAK) or T cytotoxic cell mediated lysis. Considering the possibility that tumour cells release this factor or others with similar activity *in vivo*, this paper studies the presence of NK-RIF like activity in sera from patients with colon, breast and lung adenocarcinomas. The capacity of these sera to induce resistance of K562 cells to lysis by NKCF obtained from normal donors was tested. This can be of biological interest and useful in clinical diagnosis and prognosis.

Materials and methods

Patient sera

Sera were obtained from 27 patients from the Oncology Service-General Hospital- aliquotes and stored at -70°C until further use. Primary disease site was colon in ten patients, breast in 12 and lung in five. At the time of sera collection, five patients had no clinical or pathological evidence of metastatic dissemination, 11 had clinical metastasis and 11 had undergone surgical extirpation of the primary tumour. The stage of the disease and other relevant characteristics are shown in Table I. Control sera were obtained from age and sex matched normal blood donors.

Table I Patient profile study

Patient	Primary tumour	Tumour stage	Tumour location
1	Breast	IV	Lung and liver metastasis
2	Breast	IV	Lung and liver metastasis
3	Colon	B – Dukes	Local
4	Colon	D – Dukes	Lung and liver metastasis
5	Breast	III	Local
6	Breast	IV	Bone metastasis
7	Lung	IV	Kidney metastasis
8	Breast	IV	Bone metastasis
9	Colon	D – Dukes	Liver metastasis
10	Lung	III	Local
11	Colon	D – Dukes	Lung and liver metastasis
12	Colon	D – Dukes	Liver metastasis
13	Lung	III	Local
14	Breast	III	Extirpated
15	Colon	B – Dukes	Extirpated
16	Breast	III	Extirpated
17	Breast	II	Extirpated
18	Breast	II	Extirpated
19	Colon	A – Dukes	Extirpated
20	Colon	D – Dukes	Extirpated
21	Colon	C – Dukes	Extirpated
22	Breast	III	Extirpated
23	Lung	IV	Brain metastasis
24	Breast	II	Extirpated
25	Colon	D – Dukes	Liver metastasis
26	Lung	I	Local
27	Breast	II	Extirpated

Cell line

Cell line K562 were grown in RPMI 1640 supplemented with 10% heat inactivated foetal calf serum, 2 mM L-glutamine, 100 U ml⁻¹ penicillin and 50 µg ml⁻¹ gentamycin at 37°C in 5% CO₂.

Production of NKCF

NKCF was prepared as previously described (Solana *et al.*, 1990a). Briefly, cell free supernatants were generated by incubating NK effector cells obtained from normal volunteers 2 × 10⁶ with K562 (effector-target ratio 50:1) in serum free medium. After 48 h of incubation at 37°C in 5% CO₂ air atmosphere, the cultures were centrifuged at 400 g for 10 min and the supernatants collected and filtered through a 0.22 µm Millipore filter. The supernatants were concentrated ten times with a Minicon B15 microconcentrator and stored at -70°C until further use.

NKCF mediated chromium release assay

K562 target cells (10⁶ ml⁻¹) were incubated with 1 mCi of sodium ⁵¹CrO₄ for 1 h. After washing three times, 10⁴ labelled cells in 50 µl were used as targets in a cytotoxicity assay by adding 50 µl of patient serum and 50 µl of NKCF. The cytotoxicity assay was performed in triplicate in 96 U-round-bottomed well plates. For spontaneous and total release, samples of target cells were resuspended in culture medium and 10% triton X-100 (final volume 150 µl), respectively. Controls with target cells, NKCF and culture medium and controls with target cells, NKCF and serum from age and sex matched healthy donors were always included. Cells were incubated for 24 h at 37°C in 5% CO₂. Supernatants (100 µl) were then collected and counted in a gamma counter (Ultra-gamma LKB). Specific lysis was determined with the following equation:

$$\% \text{ lysis} = \frac{(\text{problem c.p.m.} - \text{spontaneous c.p.m.})}{(\text{total c.p.m.} - \text{spontaneous c.p.m.})} \times 100$$

Specific lysis was not significantly affected by the addition of control sera. The specific lysis of controls without serum or with control sera ranged from 10% to 17% in the different experiments (mean ± s.e.m.: 14% ± 1%). Individual inhibition percentages were calculated as follows:

$$\% \text{ blockage} = \left(1 - \frac{\% \text{ lysis with patient serum}}{\% \text{ lysis with control serum}}\right) \times 100$$

Each sample was tested in three independent experiments and variations never exceeded 15% inhibition.

Determination of gamma interferon levels

The IFN levels were determined by a two-site immunoradiometric assay - IRMA (Sucrose Boots-celltech diagnostic limited, Berkshire, UK). Concentrations were quantified directly by incubation with an ¹²⁵I-labelled antibody complex which was then immobilised by incubation with a sheep anti-σIFN antibody coupled to the solid phase. In our experimental conditions the assay had a minimum sensitivity of

Table II Relationship between the stage of the disease and the number of patient's sera presenting blockage or non-blockage of NKCF cytotoxicity

Tumour	Blockage ^a			Total
	Colon	Breast	Lung	
Without metastasis	2/2	5/3	1/2	8/7
With metastasis	5/1	4/0	2/0	11/1
Total	7/3	9/3	3/2	

^aBlockage was defined as more than 90% inhibition of NKCF cytotoxicity.

4 U ml⁻¹ and values below this concentration were considered not detectable.

Statistical methods

Comparisons between groups were based on Student *t*-test.

Results

Sera from patients with metastasis block NKCF cytotoxicity significantly more than sera from those with local growth tumours

We divided neoplasm patients according to the degree of tumour invasion and results show that 11 out of 12 with metastasis (stage IV/D) had a blocked capacity of NKCF lysis while only half of the patients without metastasis (stages I/A, II/B and III/C) presented the same capacity (Table II).

Moreover, when the intensity of the inhibitory activity was analysed, we found that this was different in the group with metastasis when compared to the non-metastasis one. Sera from patients without metastasis slightly blocked NKCF mediated lysis (mean ± s.e.m.: 26% ± 16% blockage) whereas sera from patients with metastasis markedly inhibited NKCF lysis (mean ± s.e.m.: 65% ± 9%) (Figure 1).

The group with extirpated tumours showed heterogeneous results with respect to the blockage of NKCF cytotoxicity which cannot be either attributed to the time past since surgery (Table III) or to possible microneoplasm remaining in these patients once we analysed their current clinical situation.

No differences were observed in tumour origin

NKCF cytotoxicity was blocked in seven out of 10, nine out of 12 and three out of five colon, breast and lung cancers,

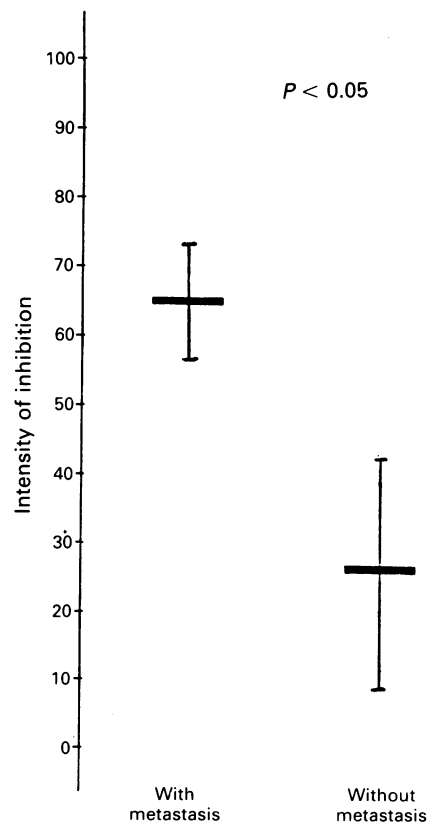


Figure 1 Mean ± s.e.m. of the inhibitory intensity of NKCF mediated cytotoxicity by sera from patients with (*n* = 11) or without metastasis (*n* = 5). The difference between the two groups is significant according to Student *t*-test.

Table III Relationship between percentage of blockage in extirpated breast and colon cancer and the time since surgery

Site of disease	Months since surgery	% Blockage
Colon	1	-
Breast	1	100
Colon	2	45
Colon	3	100
Colon	5	-
Breast	8	100
Breast	9	67
Breast	9	35
Breast	10	41
Breast	13	-
Breast	15	-

(-) non-blockage.

respectively, demonstrating that the site of the primary disease is not the main factor determining the capacity of these sera to inhibit NKCF cytotoxicity (Table II).

The blockage effect to NKCF cannot be attributed to σ IFN

As previously reported, treatment with σ IFN can also induce resistance to NKCF lysis, thus, we also measured σ IFN levels in sera from these patients. Most of them (24/27) had detectable levels of interferon while it was not detectable in controls (Table IV). We suggest that the high levels of serum interferon in these subjects is a consequence of the immunologic response of the host organism to neoplasm. However, NKCF blockage cannot be attributed to the presence of IFN, as it appears in all the stages of the disease regardless of the induction of resistance to NKCF cytotoxicity.

Discussion

Several hypothesis explaining NK resistance of different tumour cells have been suggested. These include, for example, a defect in the recognition of target cells with a relationship between HLA antigen expression and tumour cell performance (Stern *et al.*, 1980; Karre *et al.*, 1986; Powell *et al.*, 1987; Rodger *et al.*, 1988; Alonso *et al.*, 1989; Ljnggren & Karre, 1990; Solana *et al.*, 1990b; Peña *et al.*, 1990a,b); a resistance of target cells to lytic factors such as TNF or NKCF produced by immunocompetent cells (Roozmond *et al.*, 1987); or an inhibited NK activity owing to tumour suppressor factors or peptides such as prostaglandin E2 and NK-RIF (Harris *et al.*, 1987; Serrano *et al.*, 1989, 1990; Solana *et al.*, 1990a). However, the exact mechanisms by which NK cells interact with target cells remains unclear. The different roles that the molecules play on the target cell surface such as recognition elements by NK effectors have been postulated (Vodilenich *et al.*, 1983; Hercend *et al.*, 1984; Natuk & Welsh, 1987; Johnson *et al.*, 1987).

Many authors have reported diminished NK activity in different experimental models and in patients with tumours with different stages of the disease (Hisamatsu *et al.*, 1986; Schantz *et al.*, 1986; Wei & Heppner, 1987; Lin *et al.*, 1987; Tartter *et al.*, 1987). Cells from freshly explanted solid tumours are resistant to NK mediated lysis and the causes determining NK resistance are not clearly understood.

The presence of factors in sera from tumours patients inhibiting NK cytotoxicity has been described, although results are not conclusive (our unpublished results; Brenner *et al.*, 1986). This is probably due to the presence in this sera of a variety of factors such as σ IFN which has a dual effect on NK lysis, either activating NK cells (Herberman *et al.*, 1986) or inducing NK resistance in several target cells (Ramirez *et al.*, 1990). Thus, in order to find an experimental system where only the induction of resistance to lysis is evaluated, we studied the capacity of sera from tumour patients to block lysis of K562 cells by concentrated NKCF.

The analysis of 27 sera from patients with colon, breast and lung cancer demonstrated that 10/11 from metastatic and 2/5 from local disease had the capacity to block NKCF mediated cytotoxicity of NK target cell line K562. This capacity was not related to the stage of the disease, but only to the presence of the metastasis.

Although a number of tumour factors have been defined to inhibit NK cell activity, the induction of NK resistance has been related mainly to the activity σ IFN and a new tumour growth factor, released *in vitro* by tumour cell lines from metastatic adenocarcinomas, defined as NK-RIF (NK-resistance inducing factor). Both make K562 cells resistant to NK and NKCF lysis. Moreover, NK-RIF does not affect LAK, macrophage and T cells mediated cytotoxicity nor conjugate formation between the NK effector and target cells (Serrano *et al.*, 1989, 1990; Solana *et al.*, 1990a,b).

Other proteins such as α 2-macroglobulin or non-specific proteases may also participate in the blockage observed by neutralising with NK cytotoxic factors before they can lyse the target cells as they interact with other lymphokines (James, 1990).

When we studied the presence of σ IFN in these patients, our results showed high levels of σ IFN in most of them. No significant relationship between σ IFN levels and the degree of NKCF lysis blockage were found, suggesting that it is not the main agent responsible for the induction of NKCF resistance.

In conclusion, although further studies on the molecular definition of this activity are required, the data presented show that sera from patients with metastatic cancers induce resistance to K562 to NKCF lysis and suggest that molecules that induce NK resistance can be produced *in vivo* by metastatic neoplasm cells. These results prompt the possibility that the release of NK-RIF like factors facilitate tumour growth and the spread of neoplasm, allowing tumour cells to evade the host resistance mechanisms. Investigations leading to the understanding of the mechanisms involved in NK resistance induced by tumours factors will lead to a better

Table IV Relationship between the stage of the disease and σ IFN level

Patient	Colon		Patient	Breast		Patient	Lung	
	Disease stage	IFN level		Disease stage	IFN level		Disease stage	IFN level
19	A	14	17	II	-	26	I	70
3	B	37	18	II	52	10	III	60
15	B	44	24	II	38	13	III	40
21	C	38	27	II	40	7	IV	53
4	D	60	5	III	65	23	IV	49
9	D	64	14	III	47			
11	D	43	16	III	60			
12	D	44	22	III	38			
20	D	52	1	IV	61			
25	D	39	2	IV	-			
			6	IV	47			
			8	IV	-			

(-) non-detectable level. Measured in U ml⁻¹.

knowledge of the NK-tumour cell interaction and will contribute to a better evaluation of the diagnosis and prognosis of cancer patients.

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