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Mechanisms involved in natural killer cell mediated target cell death leading to spontaneous tumour regression

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Abstract. We have studied the mechanisms involved in the spontaneous regression of a rat histiocytoma in syngeneic hosts and tumour cell death processes. In addition to the natural killer (NK) cells which act through antibody dependent cellular cytotoxicity (ADCC), TNF-α also participates in the induction of necrosis in tumours. We have shown that the tumour cell is killed by necrosis which is perforce mediated, and apoptosis leading to target cell DNA fragmentation. A prior activation of the effector cells is essential before it can kill the target cell, as naive effector cells are ineffective. Activation of effector cells is mediated by Thl type of cytokines *in viro* and *in vivo*. IFN-γ seems to play an important role in tumour regression as injection of antibodies to IFN- γ *in vivo* inhibited tumour rejection.

Keywords. NK cell cytotoxicity; tumour regression; cytokines.

1. Introduction

Most of the tumours are immunogenic but the immunity they evoke is either too weak to reject a rapidly growing tumour or the tumour induces a suppressor effect on the host immune system (Old *et al* 1962; Naor 1979). The role of lymphocytes in tumour rejection is well established (Flood *et al* 1980; North 1985). The presence of *p1849X cytotoxic lymphocytes and/or natural killer (NK) cells has been demonstrated in the tumour masses undergoing rejection (Herberman *et al* 1980; Evans 1986). NK cells from tumour regressing hosts (immune NK cells) have been shown to possess cytotoxic activity against tumour cells through antibody dependent cellular cytotoxicity (Herberman and Holden 1978).

We have been studying the mechanism of regression of a rat histiocytic tumour, AK-5 (Khar 1986). The AK-5 tumour grows as ascites and as solid tumour depending on the transplantation site. Intraperitoneal injection of AK-5 cells kills all the animals, however, tumour cells when injected subcutaneously grow as solid tumours but 60–70% animals reject the tumour spontaneously. We have studied the mechanism of tumour rejection and have demonstrated the participation of $CD8^+$ NK cells and antitumour antibody in inducing tumour cell death (Khar 1993). In addition, animals which regress the tumour have high levels of TNF-α in circulation, which is also cytotoxic to AK-5 cells *in vitro* (Khar *et al* 1993).

The mechanism of cell mediated cytolytic process encompasses distinct sequences of events which include recognition and binding of effector to target, effector activation and degranulation and target cell death (Herberman *et al* 1986). The preliminary step of conjugate formation is facilitated by the expression of FcRγ on the effector cell

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surface which in turn enables the recognition and killing of tumour targets through ADCC (Trinchieri 1989; Young 1989). Studies so far have clearly indicated that the binding of cytotoxic cells to targets is a prerequisite for activating lytic process (Herberman *et al* 1986).

In the present study, we report the participation of immune cells in killing the tumour targets by performing a variety of functions leading to the complete regression of the tumour *in vivo.* We have also studied the activation of the effector cell function by different cytokines *in vitro.*

2. Materials and methods

2.1 *Animals and tumour*

Six week old Wistar rats from the inbred colony of this laboratory were used in the present study. AK-5 tumour was maintained as ascites and the solid tumour was developed by injecting AK-5 cells (5×10^6) subcutaneously in rats (Khar 1986).

2.2 *Antibodies*

Anti-IL-2 (S4B6), (R4-6A2), anti-IL-4 (11B11), anti-IL-10 (SXC1), anti-IL-12 (4D6) and anti-TNF-a were used in this study. HRPO conjugated sheep anti-rat IgG and anti-mouse IgG were from Amersham, UK.

The anti-AK-5 antiserum was collected from the rats which had previously rejected subcutaneous AK-5 tumour. The antiserum was subjected to 18% and 12% sodium sulphate precipitation and dialyzed against phosphate buffered saline (PBS). The anti-AK-5 antibody levels were checked by complement mediated lysis assay. The positive samples were pooled, filter sterilized and stored frozen at —20°C in NaN_3.

Splenocytes were stained with monoclonal 3·2·3 which recognizes RNK.P1 marker on rat NK cells. After washing with PBS the cells were treated with FITC-conjugated antimouse Ig for 30 min. The cells were washed and analysed by flow cytometry.

2.3 *Enzyme-linked immunosorbent assay*

The serum cytokine levels were estimated by enzyme-linked immunosorbent assay (ELISA) according to Schneegans *et al* (1989) with some modifications using specific monoclonals against different cytokines. Briefly, 50 µl (1:100 in PBS) aliquots were added to 96-well microtiter plates (Flow Labs, VA, USA) and incubated at 4°C overnight. The wells were blocked with 2% bovine serum albumin (BSA) in PBS. The cytokines were detected with specific anticytokine antibodies by incubating at 37°C for 1 h. The microtiter plates were rinsed with PBS-Tween-20 (0·05% Tween-20)- and HRPO-conjugated sheep anti-rat IgG (1: 5000) was added. After 40 min incubation at 37°C, the plates were rinsed with PBS-Tween-20, and developed with substrate OPD and H_2O_2. The colour intensity was measured at 490 nm using an ELISA reader. The IL-12 levels in serum were assayed by the enzyme-linked immunofiltration assay (ELIFA) technique (Pierce Chemical Co., USA), which works on the same principle as ELISA.

2.3a *Formation of effector: target conjugates:* Conjugate formation assay was carried out as described earlier (Kausalya *et al* 1994). Briefly, splenocytes from normal and tumour bearing animals (Ficoll-Hypaque fraction) were mixed with target cells at a ratio of 5 :1 in the presence or absence of anti-AK-5 antiserum (40 μg/ml). The cell mixture was incubated at 25°C for 1 h, vortexed gently before flow cytometric analysis or microscopic study.

2.3b *Preparation of tumour-infiltrating lymphocytes:* Solid AK-5 tumours of different sizes and ages were harvested aseptically and minced into pieces. The minced tumour tissue was stirred in 50 Al RPMI 1640 medium containing 10 mg deoxyribonuclease, 100 mg collagenase, and 250 U hyaluronidase (Sigma, St. Louis, USA) for 1 h at 37°C (Topalian *et al* 1987). The single cell suspension was washed and the lymphocyte fraction was separated on a Ficoll-Hypaque gradient. The slight contamination of AK-5 cells in the lymphocyte fraction was removed by specific lysis of AK-5 cells with anti-AK-5 antibody and normal rabbit serum as the source of complement. The TILs obtained were washed, tested for viability by trypan blue dye exclusion method, and used in the cytotoxicity assays.

2.3c *Preparation of enriched NK cells and cytotoxicity assay:* Splenocytes from tumour-bearing animals were subjected to a two-step (50% and 62%) discontinuous Percoll density gradient centrifugation. The fraction obtained at the interface served as enriched NK cells, since it comprized 40–60% mAb 3·2·3-positive NK cells as determined by flow cytometry. Cytotoxicity assay was performed as described previously (Bright *et al* 1994). Briefly, enriched NK cells were incubated with ^{51}Cr-labelled AK-5 cells in the presence of anti-AK-5 antibody at an E:T ratio of 100:1 for 4 h. Chromium released in the supernatant was counted in a Packard gamma counter, and the percentage cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release-spontaneous release}}{\text{total release-spontaneous release}} \times 100.$$

2.3d *Extraction and electrophoresis of DNA:* Fixed tumour cells were washed, suspended in citrate-phosphate buffer and the fragmented DNA was extracted following the previously published procedure (Gong *et al* 1994).Fragmented DNA with loading buffer was electrophoresed on 0·8% agarose gel at 2 V/cm for 16 h.The DNA in the gels was visualized under UV after staining with 5 tg/ml ethidium bromide.

AK-5 cells (1 x 107) were injected i.p. into naive or immune animals (which had previously rejected the tumour) After 3 h the tumour cells were harvested by peritoneal lavage and the cells were fixed and processed for DNA fragmentation analysis as described above.

3. Results and discussion

AK-5 tumour is highly immunogenic and elicits a strong antibody response when injected through i.p. or s.c. routes. These antibodies are cytotoxic to AK-5 cells in the presence of complement (Khar *et al* 1993). Several virally or chemically induced tumours have been shown to be immunogenic and elicited antibodies to membrane

Table 1. NK mediated killing of AK-5 cells through ADCC*.

Group	Cytotoxicity (%)
AK-5 cells	4·2
AK-5 + anti-AK-5	2·6
AK-5 + NK	6·8
AK-5 + NK + anti-AK-5	92.6
Meth A + NK + anti-AK-5	1·4
ZAH + NK + anti-AK-5	2·5

*Cytotoxicity was calculated with respect to NP-40 treated 100 % release and after subtraction of spontaneous release values. The effector to target cell ratio was 50 :1 and the ^{51}Cr release was studied for 4h; $CD8^+$ NK cells were sorted on a FACS using mAb 3·2·3. Meth A and ZAH tumours were used as non-specific target cells.

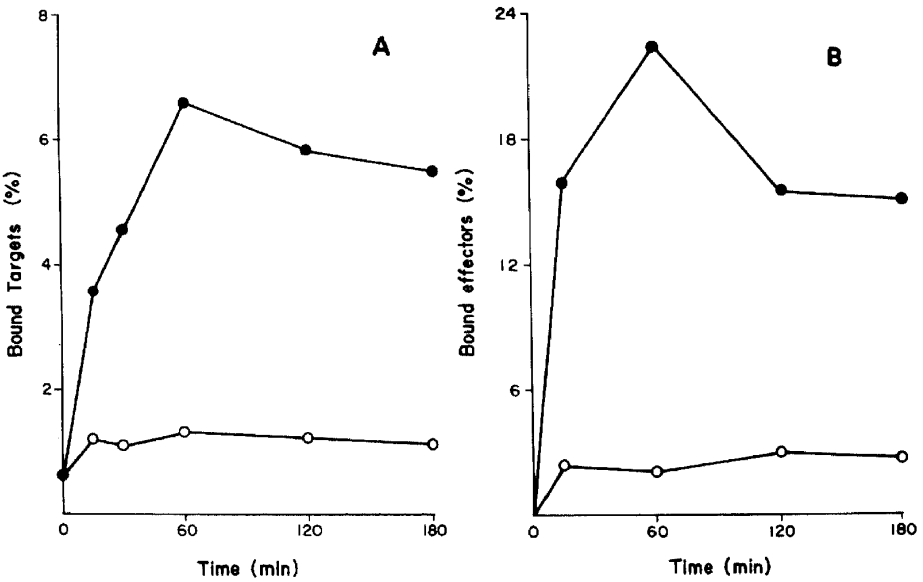

Figure 1. Per cent bound targets (A) and bound effectors **(B)** in an effector: target co-culture in the presence of antitumor antibody. Effect of different incubation periods on the binding of naive NK (O) and immune NK (●) to AK-5 target cells. These results are representative of 3 similar experiments.

associated antigens (Moore *et al* 1973; Wood and Barth 1974). Animals which had rejected the AK-5 tumour were immune to subsequent challenges with this tumour transplanted through either route. We have been able to demonstrate that the cytotoxic acitivity against AK-5 resides with $CD8^+$ NK cells. Use of fractionated splenocytes as well as 3·2·3 positive NK cells prepared by sorting on a flow cytometer in cytotoxicity assays suggested NK cells to be the effector cells in our system (table 1). Non-specific targets like Meth A and Zajdela ascitic hepatoma (ZAH) were not killed by NK cells obtained from AK-5 tumour bearing animals. NK cells are able to induce killing in AK-5 targets through ADCC (Khar 1993). NK cells have been shown to possess antitumour activity against a large variety of animal and human tumours

(Trinchieri 1989).Cytotoxic activity of NK cells has been shown to restrict colonization of tumours to form metastasis (Trinchieri 1989).

In our system, the action of the NK cells is mediated with the help of antitumour antibody. That is probably how the recognition of the target cells takes place as has been demonstrated earlier (Woodruff 1986). The implication of conjugate formation as the preliminary step in the mechanism of cell mediated lysis has been well documented (Herberman *et al* 1986). Investigations on the mechanism of cytolysis indicated an augmented surface FcR (CD 16) expression in immune NK cells resulting in recognition and binding. Similar results were obtained by us when effector and target cells were co-incubated in the presence of antitumour antibody (figure 1). We have also observed binding of several effectors to the same target cell upon co-culture for 90 min or more (figure 2B, C). Incubation up to 3 h leads to complete disintegration of the target cells (figure 2D). These observations suggest killing of the target cell through necrosis (table 1, figure 2) and apoptosis (figure 2D). In addition, a quantitative increase in CD25 positive NK cells following a brief exposure to antibody tagged AK-5 cells highlighted the requirement of NK cell activation in AK-5 tumour killing (Kausalya *et al* 1994). Further, the ability of immune splenocytes to form conjugates and kill YAC-1 (data not shown), a NK-insensitive cell line and not Meth A or ZAH, NK-insensitive tumour targets, confirmed the specificity of the system. The failure of naive NK cells to form specific conjugates with AK-5 cells suggested the requirement of

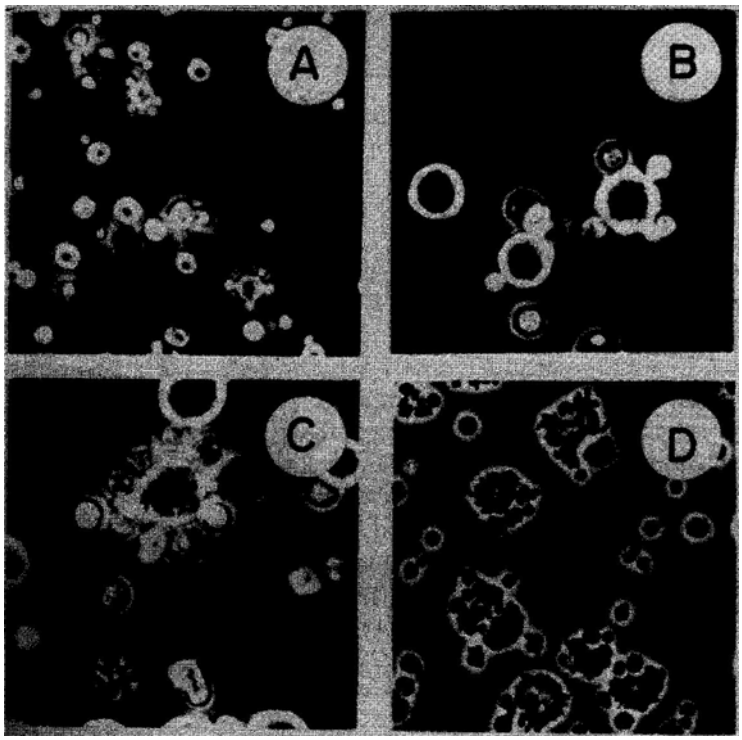

Figure 2. Formation of effector:target conjugates between AK-5 and NK cells in the presence of anti-AK-5 antiserum. **A, B** and **C** show multiple effectors bound to the target cell and **D** shows complete disintegration of the target cell after 3 h co-culture..

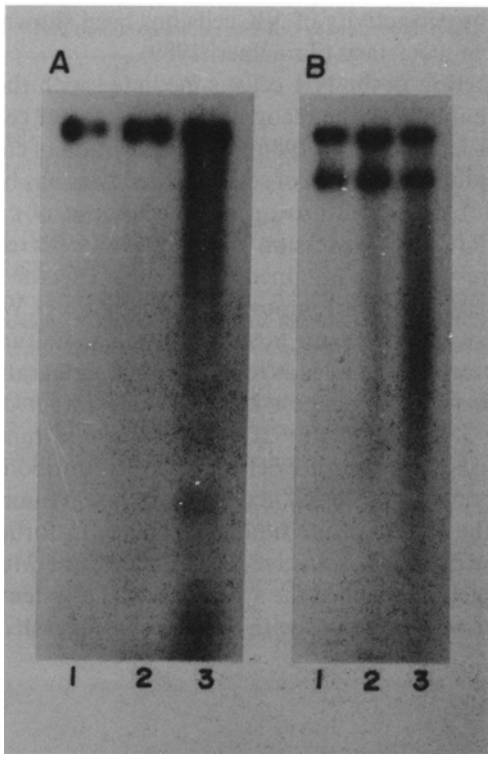

Figure 3. DNA fragmentation in AK-5 cells *in vivo* (A) and *in vitro* (B). Lanes: 1, control; 2, 1 h incubation; 3, 3 h incubation. This is a representative picture of 10 similar experiments.

specific activation/priming for the effector cells to form conjugates with the target before lysis.

The AK-5 tumour cell is killed by necrosis and apoptosis (Kausalya *et al* 1994). We have previously shown that necrosis is mediated by over expression of a pore-forming protein, perforin, when stable conjugates are formed between the AK-5 and the NK cells with the help of anti-AK-5 antibody. Similarly, apoptosis has been recognized as an important mode of tumour cell death and tumour immunologists have recognized the importance of understanding the mechanism of induction of apoptotic programme in a tumour cell because of its tremendous potential in cancer therapy. We have studied the DNA fragmentation in AK-5 cells *in vitro* after its interaction with the effector cell (Bright *et al* 1995). AK-5 cells undergo DNA fragmentation both in *vitro* as well as *in vivo* (figure 3). NK mediated fragmentation *in vitro* is observed after 1 h of co-culture between the target and the effector cells in the presence of antitumour antibody. These observations suggest apoptosis as an imporant mode of tumour cell killing.

In addition to the splenocytes, we also tested the cytotoxic potential of tumour infiltrating lymphocytes (TILs). The ADCC activity of TILs isolated from AK-5 tumours during the early growth phase was comparable to that of the splenocytes. In the absence of antitumour antibody, TILs did not show any cytotoxic activity against AK-5, suggesting non-participation of cytotoxic T lymphocytes in killing the AK-5 tumour cells (figure 4). In contrast, TILs isolated from rejecting or healing tumours

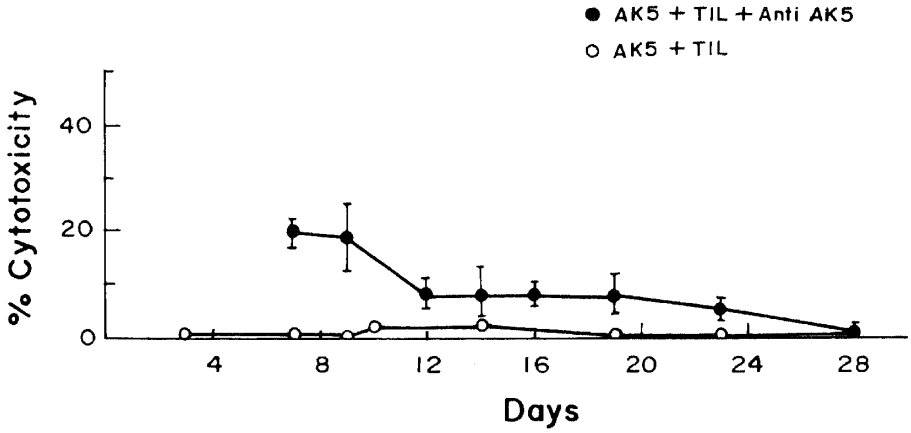

Figure 4. Cytotoxic activity of tumour infiltrating lymphocytes against-AK-5 cells in the absence (O) and presence (●) of anti-AK-5 antiserum on different days after tumour transplantation..Values shown are mean ± SEM..These experiments have been conducted 3 times and this figure represents one such experiment.

failed to express any induction of ADCC activity; rather there was decrease in ADCC from 10th day onwards. However, TTLs when cultured either in the absence or presence of rIL-2 for 18 h, regained their cytotoxic activity (Bright *et al* 1994). These observations suggest that the inactive nature of TTLs in AK-5 tumour could be due to a target-directed exocytosis of NK lytic factors, resulting in the inactivation of NK cell function which is restored upon overnight culture (Bright *et al* 1994).

Since naive NK cells were not effective in killing the tumour targets in different types of assays and the NK cells required some sort of priming/activation prior to their interaction with AK-5 cells, we studied the role of different cytokines. AK-5 tumour regressing animals had high levels of IL-2, IL-12 and IFN-γ in circulation during the rejection phase of the tumour (Hegde *et al* 1995; Kausalya *et al* 1995). Our present observations confirm our earlier results and show higher levels IL-2, IL-4, IL-12, TFN- γ and TNF-α in tumour regressing animals, whereas IL-10 levels are not significantly altered (figure 5). These observations suggest a predominant Th1 type of activation which is probably involved in the induction of cell mediated immune response. IL-4 expression may be involved with the antitumour antibody response which is ultimately involved in the specificity of interaction between the effector and the target cells. Our studies demonstrate different responses that mediate spontaneous regression of AK-5 histiocytic tumour. These studies show the working together of different immune cells to achieve complete regression of the tumour and point towards greater need for multipronged attacks in activating the immune system against cancers.

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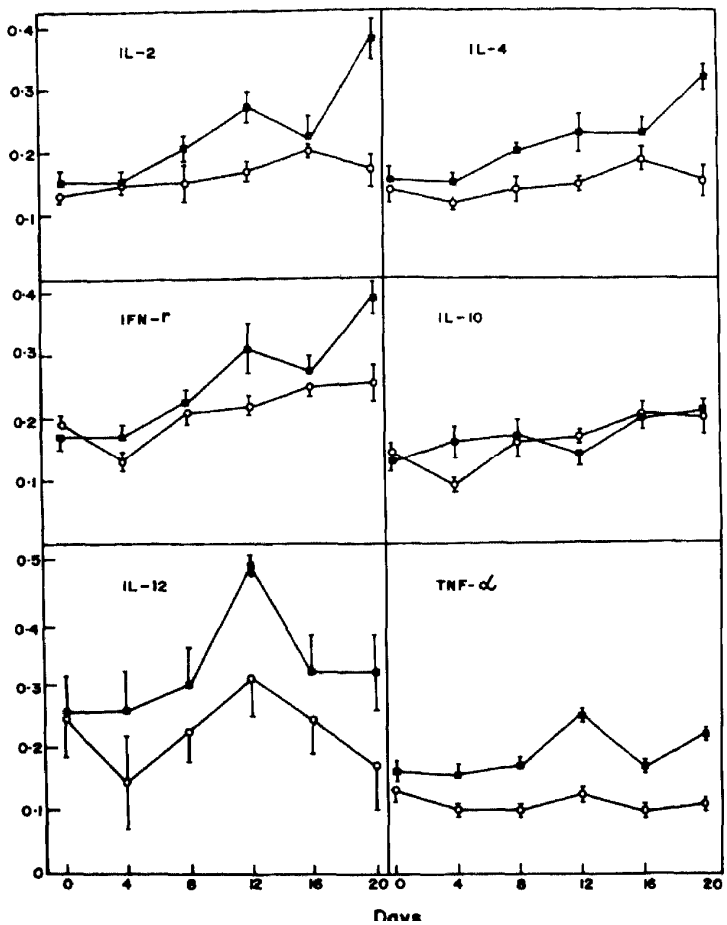

Figure 5. Cytokine levels in the sera of AK-5 tumour rejecting animals on different days after the tumour transplantation. Each cytokine was estimated in 10 µl (1:50 diluted) serum by ELISA using specific monoclonal antibodies. The values represent mean ± SEM of 10 animals..The results are shown as early rejectors (●), animals which rejected the tumour between day 10– 15 and late rejectors (0), where the rejection was observed after day 20.

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References

Bright J J, Hegde S P, Begum Z and Khar A 1994 Modulation of natural killer cell function by AK-5, a spontaneously regressing rat histiocytoma; *Cell Immunol.* **154** 54-65

Bright J J, Kausalya S and Khar A 1995 Natural killer cell activation-associated induction of target cell DNA fragmentation in a spontaneously regressing rat histiocytoma; *Immunology* **85** 638-644

Evans R 1986 The immunological network at the site of tumor rejection; *Biochim Biophys Acta* **865** 1-11

Flood P M, Kripke M L, Rowley D A and Schreiber H 1980 Suppression of tumour rejection by autologous anti-idotypic immunity (T-lymphocytes/immune regulation/immunological unresponsiveness); *Proc Natl. Acad. Sci. USA* **77** 2209-2213

Gong J, Traganos F and Darzynkiewiez Z 1994 A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry; *Anal. Biochem.* **218** 314-319

Hegde S P, Bright J J, Kausalya S and Khar A 1995 AK-5 tumor induced expression of IL-12: role of IL-12 in AK-5 regression; *Cell Immunol.***162** 241-247

Herberman RB and Holden HT 1978 Natural cell-mediated immunity; *Adv. Cancer Res.* **27** 305-377

Herberman R B, Holden H T, Varesio L, Tanimaya T, Pucetti P, Kirchner H, Gerson J, White S, Keisari Y and Haskill J S 1980 Immunological reactivity of lymphoid cells in tumours; *Contemp.Top Immunobiol.* **10** 61-78

Herberman R B, Reynolds C W and Ortaldo J R 1986 Mechanism of cytotoxicity of natural killer cells; *Annu Rev Immunol.***4** 651– 680

Kausalya S, Hegde S P, Bright J J and Khar A 1994 Mechanism of antibody dependent natural killer mediated AK-5 tumour cell death; *Exp. Cell Res.***212** 285-290

Kausalya S, Varalakshmi C and Khar A 1995 Correlation of *in vitro* and *in vivo* effects of interferon-γ with the spontaneous regression of a rat histiocytoma; *J. Int Cytokine Res.***15** 647-654

Khar A 1986 Development and characterization of a rat histiocyte-macrophage tumor line; *J Natl.Cancer Inst* **76** 871-878

Khar A 1993 Characterization of spontaneous immunological regression in a histiocytoma.I.Role of NK cells and antitumor antibodies; *Int J.Oncol* **2** 393-398

Khar A, Leela Kumari A and Mubarak Ali A 1993 Characterization of spontaneous immunological regression in histiocytoma.II**.**Participation of tumour necrosis factor; *Int.J.Oncol* **2** 399-402

Moore M, Witherow P J, Price C H G and Clough S A 1973 Detection by immunofluorescence of intracytoplasmic antigens in cell lines derived from human sarcomas; *Int.J.Cancer* **12** 428-437

Naor D 1979 Suppressor cells; permitters and promoter of malignancy? ; *Adv. Cancer Res.***29** 45-125

North R J 1985 Down regulation of antitumor immune response; *Adv. Cancer Res.***451** – 43

Old L J, Boyse E A, Clarke D A and Carswell E A 1962 Antigenic properties of chemically induced tumours; *Ann. NY Acad Sci* **101** 80-106

Schneegans A S K, Gaveriaux C, Fonteneau P and Loor F 1989 Indirect double sandwich ELISA for the specific and quantitative measurement of mouse IgM, IgA and IgG subclasses; J. Immunol. Methods **119** 117-125

Topalian S L, Muul L M, Solomon D and Rosenberg S A 1987 Expansion of human tumour infiltrating lymphocytes for use in immunotherapy trials; *J. Immunol. Methods* **102** 127-141

Trinchieri G 1989 Biology of natural killer cells; *Adv.Immunol.* **47** 187-376

Wood G W and Barth R F 1974 Immunofluorescent studies of the serologic reactivity of patients with malignant melanoma against tumour-associated cytoplasmic antigens; *J. Natl Cancer Inst* **53** 309-316

Woodruff MFA 1986 The cytotoxic and regulatory role of natural killer cells in experimental neoplasia; *Biochim. Biophys. Acta* **865** 43-57

Young J D E 1989 Killing of target cells of lymphocytes: a mechanistic view; *Physiol.Rev.***69** 250-257