Indian Journal of Experimental Biology
Vol. 27, June 1989, pp. 497-501

# Assessment of immunological parameters during tumour development in a murine model

K Suresh, K Nirmala, Kezia Kuruvilla & D M Vasudevan*
Amala Cancer Research Centre, Trichur 680 553, India
Received 2 November 1987; revised 24 November 1988

Natural Killer activity assessed by $^{51}Cr$ release assay from K-562 cells showed detectable activity from 5th day after tumour transplantation, reaching a peak on 12th day and thereafter showing a gradual decline in the activity. Antibody dependent cell mediated cytotoxicity estimated by $^{51}Cr$ labelled sheep red blood cells anti SRBC system demonstrated a peak activity on 5th day. Cytotoxic T lymphocyte activity detected by $^{51}Cr$ release of Dalton's lymphoma ascites target cells showed a peak on 10th day. Antibody complement mediated cytotoxicity revealed a similar pattern as natural killer cell activity.

The tumour antigens, generally expressed during malignant transformation of cells, may elicit an humoral response (ACC, antibody complement mediated cytotoxicity) as well as cell mediated immune responses (ADCC, antibody dependent cell mediated cytotoxicity and NK, natural killer cell mediated cytotoxicity and cytotoxic T lymphocyte (CTL) mediated lysis[1]). ADCC represents a potentially powerful cytotoxic mechanism in which humoral and cellular immune effectors co-operate[2]. Natural killer (NK) cell is yet another effector cell type that occur in non-immune individuals and is supposed to be the first level of defense against tumour growth *in vivo*[3]. NK cell is able to kill syngenic, allogenic and xenogenic tumour cells with no evidence of prior sensitization[4]. CTL are normally generated as an host immune response to cell associated antigens (viral infections or malignant transformations) and are involved in allograft rejection. These effector cells can destroy relevant tumour target cells *in vitro* and protect host *in vivo*[5,6]. Humoral immune response involves complement mediated lysis of cells bearing antibody to cell surface antigen. The present study was undertaken to evaluate these immunological parameters during tumour development in a murine tumour model.

## Materials and Methods

Dalton's lymphoma ascites cells were collected from mice, washed and injected (ip) into Balb/c mice ($1 \times 10^7$ cells/ml/mouse) and the tumour grown in the ascites form in peritoneal cavity. On different days after tumour transplantation the animals were sacrificed and blood collected by heart puncture, the separated serum was used for the study of antibody complement mediated lysis. RPMI-1640 containing 10% heat inactivated new born calf serum (Hysel, India) was used as complete medium.

*Preparation of effector cells for NK, ADCC and CTL-activity*—Spleen from immunized Balb/c mice was removed asceptically into a petridish containing cold RPMI-1640 + 10% new born calf serum (Hysel, India). The spleen was gently teased with forceps to remove capsule and connective tissue and then very gently pressed through a seive with plastic plunger of a disposable syringe in the presence of cold medium. The cell suspension was centrifuged at 400 *g* for 5 min, the sediment resuspended and left standing at 4°C for 45 min. The supernatant cells were taken, RBC lysed by treating with cold Tris-Ammonium Chloride buffer (*p*H 7.2) at 4°C for 10 min. The cells were washed in the medium containing serum and viability checked by trypan blue exclusion method.

*Assay of NK activity*—In this assay K-562 (myelogenous leukemic) cells susceptible to NK cell lysis but resistant to T cell cytotoxicity were used. Approximately $1 \times 10^6$ cells/ml K-562 were incubated with 100 $\mu$Ci of $^{51}Cr$ for 2 hr at 37°C. The cells were washed thrice in RPMI-1640 containing 10% new born calf serum and resuspended in the medium to a final concentration of $1 \times 10^5$ cells/ml.

The above mentioned single cell suspension of spleen (effector) and labelled K-562 (target) cells at different effector target ratio of 10:1, 30:1 and 100:1 were incubated at 37°C for 4 hr in RPMI-1640 with

*Correspondent author

10% new born calf serum. All tests were done in triplicate. After incubation, supernatant was removed and radio-activity was measured in a gamma ray spectrometer. Percent specific cytolysis was calculated as:

$$\frac{\text{Mean experimental release-mean spontaneous release}}{\text{Mean total release - mean spontaneous release}} \times 100$$

Spontaneous release was determined by incubating labelled target cells in the absence of effector cells. Total release was obtained by adding 1 *N* HCl into the labelled target cells.

*Generation of CTL in in vitro mixed lymphocyte tumour culture*—In murine system, CTL production in culture can be accomplished with spleen cells as responder cells from animals immunised *in vivo* with tumour cells. Secondary *in vitro* stimulation gives a more rapid and stronger CTL response in mixed lymphocyte tumour culture (MLTC). Dalton's lymphoma ascites cells aspirated from the Balb/c mice were washed thrice in normal saline and the cell pellet was treated with Mitomycin C (30-100 $\mu$g/2-3 $\times$ $10^6$ cells) for 30 min at 37°C to serve as stimulator cells in MLTC. Spleen cells at different concentration ($10^7$-$2 \times 10^7$) were co-cultured with a fixed concentration of irradiated Mitomycin-C treated stimulator cells ($10^4$ cells) in complete medium at 37°C in 5% $CO_2$ atmosphere for different days. The CTL response peak was evaluated. The co-cultured spleen cells were tested for their cytotoxic potential by $^{51}Cr$ release assay against radiolabelled Dalton's Lymphoma Ascites cells from day-1 to day 7 of incubation.

*Cytotoxicity assay for CTL*—The cultures at the end of 4th day were centrifuged, cell pellet washed and resuspended in medium and viability checked by trypan blue exclusion method. These effector cells were incubated with radio labelled Dalton's lymphoma ascites target cells at different effector target ratio of 10:1, 30:1 and 100:1 at 37°C for 4 hr. All the tests were done in triplicate. After incubation tubes were centrifuged, supernatant was removed and radioactivity was measured in a gamma ray spectrometer. Percent specific cytolysis was calculated as before.

*Antibody dependent cellular cytotoxicity (ADCC)*—Target cells (sheep red blood cells) were collected in Alsever's solution kept at 4°C, washed in normal saline and incubated $1 \times 10^6$ cells/ml with 100 $\mu$Ci $^{51}Cr$ for 45 min, washed twice and resuspended in saline. The minimum hemolytic dose (MHD) was determined by incubating Sheep red blood cells (SRBC) with serial dilution of anti-SRBC antibody (raised in New Zealand rabbit by immunizing with SRBC) in saline (1:10 to 1:60000), and further incubating with fresh rabbit serum (1:1 diluted) at 37°C for 30 min. The MHD was calculated as the dilution of anti-SRBC antibody demonstrating 50% lysis of sensitized SRBC. SRBC sensitized with 2 MHD were used in further cytotoxic studies.

The effector cells obtained from single cell suspension of spleen were assayed for cytotoxic studies with anti-SRBC antibody coated SRBC target cells as described earlier at different effector:target ratios of 10:1, 30:1 and 100:1. The percent specific cytolysis was determined as in the case of NK cell assay.

*Antibody complement mediated cytotoxicity*—Blood from the tumour bearing animal was taken, serum separated and heat inactivated at 56°C for 30 min to eliminate intrinsic complement activity. The specificity of tumour specific antibodies in the tumour serum used was confirmed by testing normal mouse serum which did not show any cytotoxicity against Dalton's lymphoma ascites cells in the presence of complement. Dalton's lymphoma ascites cells were washed thrice in RPMI, viability checked and were finally suspended to a concentration of $1 \times 10^5$ cells/ml in the medium. Antiserum was serially diluted in culture medium so as to get 1:1, 1:2, 1:4 and 1:8 dilutions of antibody. 0.1 ml of the diluted antibody was added to each tube. $1 \times 10^4$ Dalton's lymphoma ascites cells in 0.1 ml RPMI + 10% heat inactivated serum was then added to the tubes. 50 $\mu$l 1:10 dilution fresh rabbit serum was added as complement source. Final volume in all tubes was adjusted to 2 ml. Controls were kept without antibody and complement; with antibody and without complement; with complement and without antibody. All tests were done in triplicate. The tubes were incubated at 37°C for 2 hr and cytotoxicity assessed by trypan blue dye exclusion method.

## Results

The immunological status in tumour bearing animals reflected in the NK, ADCC, ACC and CTL activities were assessed on different days (from day 0 to death of animal) after tumour transplantation. NK cell activity in normal mouse (as noted on day 0) was found to be 1.4% $\pm$ 0.4. The activity showed a gradual increase from day 3 (2.1% $\pm$ 0.29) and reached maximum on 12th day of tumour inoculation (59.67% $\pm$ 0.415) (Table 1). Thereafter the activity was found to decline and animals were dead by 20th day. Effector:target ratio of 100:1 was found to give maximum percent specific cytolysis as shown in the Fig. 1.

Antibody dependent cellular cytotoxicity assayed using SRBC target cells at different attacker:target

ratios displayed detectable activity on the day -0- (normal) (12.48% ± 0.37) peaked on 5th day (43% ± 1.6) (Table 2). Thereafter the activity was found to decline abruptly (Fig. 2). High amount of % specific lysis was observed in effector target ratio of 100:1 (Fig. 3).

In our studies peak CTL response was found on 4th day of co-culturing. Hence this was used as a standard in further experiments. CTL activity estimated by release of $^{51}Cr$ from labelled Dalton's lymphoma ascites tumour cells showed detectable activity on the day 10 after immunisation (after 4 days of co-culture) (3.85% ± 0.153) and then the activity decreased gradually (Table 3). Again high activity was observed in the effector target ratio of 100:1.

Humoral immune responses was demonstrated by using Dalton's lymphoma ascites tumour cells treated with mouse antibody and fresh rabbit complement. The activity was observed on the day 9 (10.5% ± 0.415), with a peak on the 13th day (43.3% ± 0.8) which gradually declined. (Table 4). By the 19th day no activity was detectable (Fig. 2).

## Discussion

The growth of tumour is a multistep process characterised by progressively increased malignant potential of the tumour cell in the face of host resistance. Immunological changes during tumour progression in a mice tumour model as assessed by NK cell, T cell, ADCC and ACC activities have been

Table 1—Natural killer cell mediated cytolysis of radiolabelled K-562 target on different days after tumour inoculation at an effector:target ratio 100:1

[Values are mean ± SE from 3 observations in each group]

| Days after tumour inoculation | % specific cytolysis |
|---|---|
| 0 (Normal) | 1.4 ± 0.4 |
| 3 | 2.1 ± 0.29 |
| 5 | 7.8 ± 0.560 |
| 7 | 10.1 ± 0.24 |
| 9 | 12.59 ± 1.8 |
| 11 | 25.4 ± 0.73 |
| 12 | 59.67 ± 0.415 |
| 14 | 18.58 ± 0.63 |
| 17 | 7.85 ± 0.370 |

Table 2—Antibody dependent cellular cytotoxicity of $^{51}Cr$ labelled sensitized SRBC at effector:target ratio 100:1

[Values are mean ± SE from 3 observations in each group]

| Days after tumour inoculation | % specific cytolysis |
|---|---|
| 0 (Normal) | 12.48 ± 0.37 |
| 3 | 22.6 ± 0.83 |
| 5 | 43 ± 1.6 |
| 7 | N.D |
| 9 | N.D |
| 11 | N.D |
| 13 | N.D |
| 15 | N.D |
| 17 | N.D |
| 19 | N.D |

N.D = Not detected

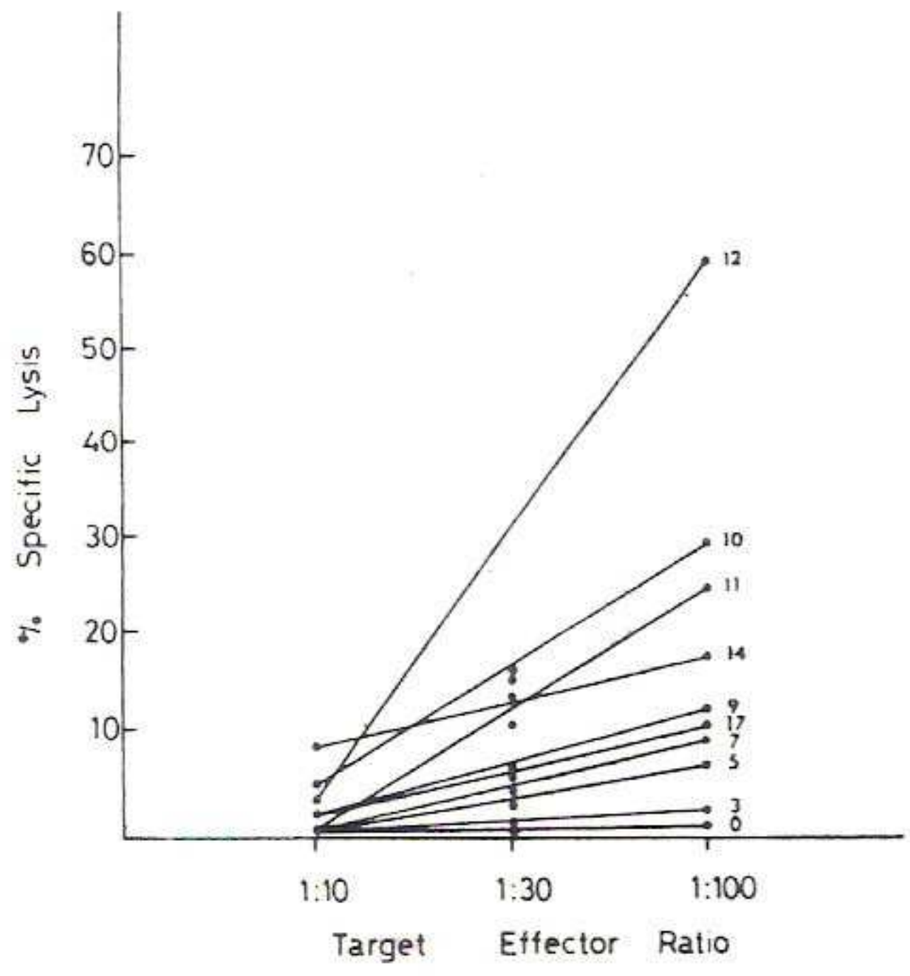


Fig. 1—Natural killer cell activity against target K-562, assessed by $^{51}Cr$ release assay on different days after tumour inoculation at target:effector ratios of 1:10, 1:30 and 1:100. Numbers indicate days after tumour inoculation

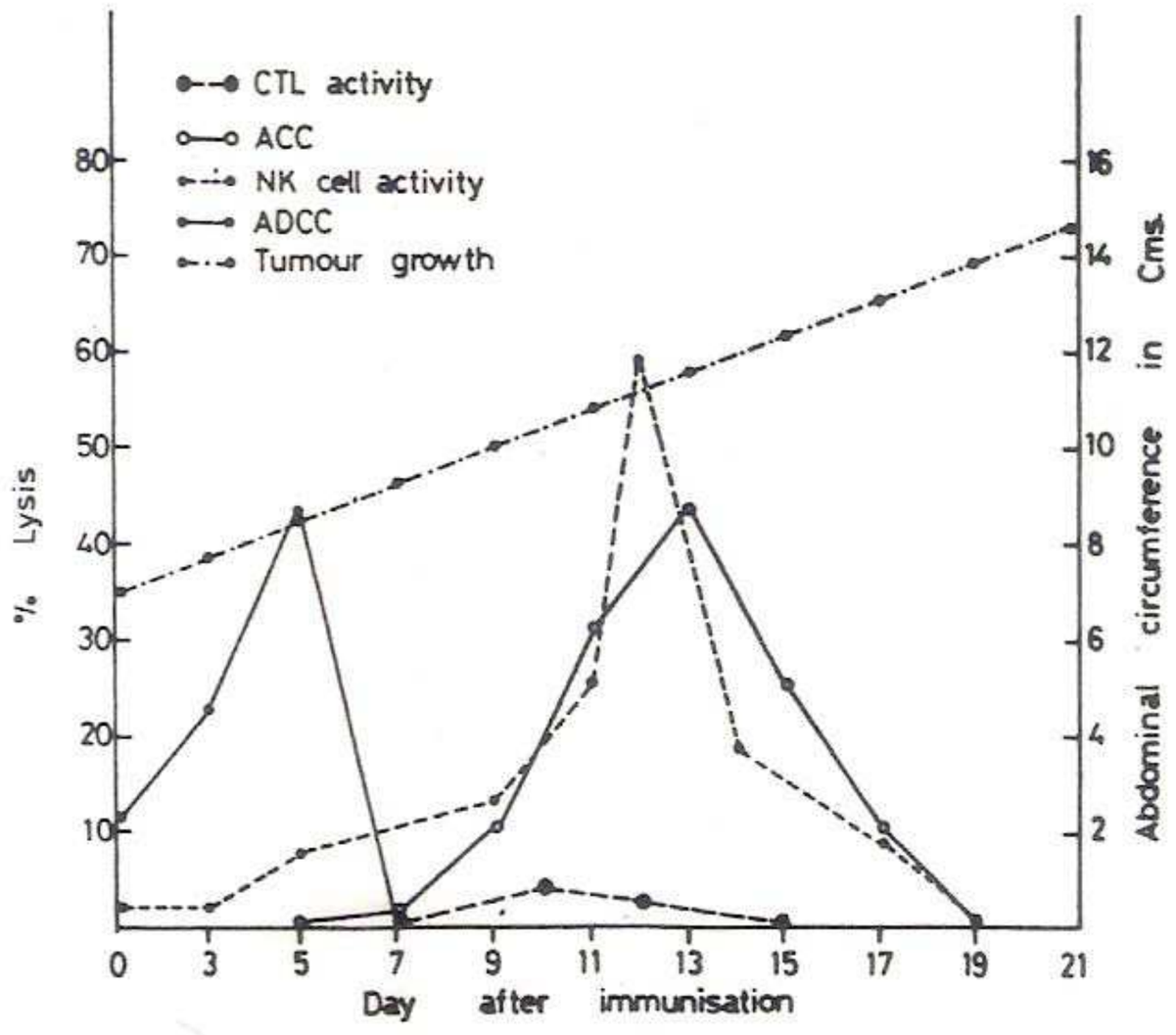


Fig. 2—Profiles of natural killer cell activity, antibody dependent cell mediated cytotoxicity, CTL activity and antibody complement mediated cytotoxicity during tumour growth

Table 3—CTL mediated lysis of $^{51}Cr$ labelled DLA cells at effector:target ratio 100:1

[Values are mean ± SE from 3 observations in each group]

| Days after tumour inoculation | % specific lysis |
|---|---|
| 0 (Normal) | N.D |
| 3 | N.D |
| 5 | N.D |
| 7 | N.D |
| 9 | N.D |
| 10 | 3.85 ± 0.153 |
| 12 | 2.23 ± 0.1203 |
| 14 | N.D |
| 17 | N.D |
| 19 | N.D |

N.D = Not detectable

Table 4—Antibody - complement mediated lysis of DLA cells assessed by trypan blue exclusion method

[Values are mean ± SE from 3 observations in each group]

| Days after tumour inoculation | Cytotoxicity |
|---|---|
| 0 (Normal) | N.D. |
| 3 | N.D. |
| 5 | N.D. |
| 7 | 2.1 ± 0.117 |
| 9 | 10.5 ± 0.415 |
| 11 | 31.5 ± 1.5 |
| 13 | 43.3 ± 0.8 |
| 15 | 25.05 ± 0.94 |
| 17 | 10.7 ± 1.1 |
| 19 | N.D. |

N.D. = Not detectable

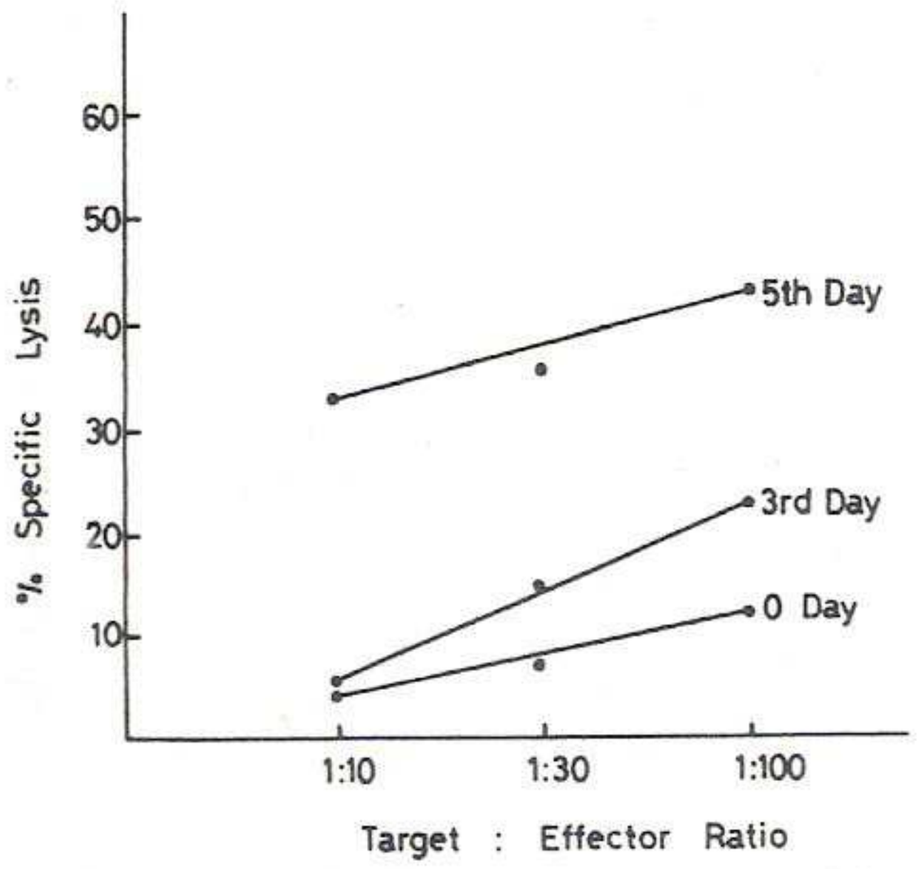


Fig. 3—Antibody dependent cell mediated cytotoxicity against sensitized SRBC assessed by $^{51}Cr$ release assay on different days after tumour inoculation at effector:target ratios of 10:1, 30:1 and 100:1. Numbers indicate days after tumour inoculation

studied. NK cell activity is absent in new born spleen and matures to adult level in 4-8 weeks followed by a period of decline[7]. In normal controls NK activity against K-562 target cells has been reported to be very low[8]. In our studies normal NK cell activity was found to be very low but significant activity was detected on day 5 after immunisation which peaked on 12th day and then declined gradually to the basal level. NK cells provide a transient suppression to growth of tumour[9]. However if the tumour bypasses the rejection mechanism for some reason, reaching critical size NK cells would no longer be operative. Therefore NK cells may not provide absolute protection against tumour growth. In our experiments, CTL activity was detectable only on 10th and 12th day of immunisation. It has been reported that when T cell activity falls, NK cell activity is enhanced. One possible explanation for these findings is that the mouse NK cells are pre-T cells and in nude mice considerable number of pre-T cells with low density of antigen has been demonstrated[10].

Evaluation of ADCC by K cells in relation to tumour growth is a prerequisite for understanding its contribution in host tumour interaction. In the present study detectable ADCC was observed on the day 0 and peaked on day 5, post tumour inoculation. Thereafter abrupt fall of activity was recorded. Decline of ADCC activity by the K cells, granulocytes and macrophages in advanced growth of tumour might be due to inhibitory factors released by growing tumours[11].

The decline in cell mediated immunity could be attributed to immunosuppressive factors. Cell mediated immuno suppression causing facilitation of tumour growth has been described as being mediated by a specific class of cells called suppressor cells[12]. Immuno suppressive activity has been reported as being associated with T lymphocytes and B lymphocytes. Specific humoral blocking agent like anti-tumour antibody and tumour antigen may be accounted for the fall in immune response in the later stage of tumour growth. Tumour-associated antigen-antibody complexes have been implicated as potentially effective immunosuppressive molecule in tumour host. In large tumour bearing hosts (advanced stage) humoral and cellular effector activities are possibly eliminated by plasma blocking factors and suppressor cells.

**Acknowledgement**

Financial support by DST (Govt. of Kerala) and

assistance by Dr. R. Krishnakumar in the preparation of manuscript are acknowledged.

## References

1 Herberman R B, Holden H T, West W H, Bonnard G D, Santon A, Nunn M E, Kay H D & Ortaldo J R, in *Tumour associated antigens and their specific immune response*, edited by F F Spreafico and R Arnon (Academic Press, New York) 1974, 129.

2 Render Dolhuis, in *Natural cell mediated immunity against tumours*, edited by R B Herberman (Academic Press, New York) 1980, 289.

3 Herberman R B & Ortaldo J R, *Science*, 214 (1981) 24.

4 Nunn M E, Herberman R B, Holden H T, *Int J Cancer*, 20 (1977) 381-91.

5 Cerottini J C & Brunner K T, *Adv Immunol*, 18 (1974) 67.

6 Herberman R B, in *Advances in immunology*, Vol 41, edited by Frank J Dixon (Academic Press, New York) 1987, 271.

7 Herberman R B & Oldham R K, *J Natn Cancer Inst*, 55 (1975) 749.

8 Emanuala Lanza & Julie Y L, in *NK cells and other natural effector cells*, edited by R B Herberman (Academic Press, New York) 1982, 335.

9 Urban J & Hanshreeber, in *NK cells and other natural effector cells*, edited by Herberman R B (Academic Press, New York) 1982, 1393

10 Loor F & Roelants G E, *Nature*, 251 (1974) 229.

11 Chattopadhyay U, Bhattacharya S & Chakroborty N G, *Neoplasma*, 33 (1986) 157.

12 Ray P K, in *IC & human cancer*, Vol 15 of Compt Topics in Immunobiology, edited by F A Salinas & M G Hanna, Jr (Plenum Press, New York and London) 1985, 151.