DISCUSSION

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Relevance of NK cells in control of malignancy has already been documented by several authors, works of several of them have been mentioned in Introduction. NK cells also have important functions in tumour surveillance *in vivo*. Haller *et al.*,(1977) and Riccardi *et al.*,(1980) showed the role of NK cells in resistance against syngeneic tumour cells *in vivo*. Hanna and Burton (1981) observed inhibition of tumour metastasis by NK cells *in vivo*. Kiessling *et al.*,(1975) and Sendo *et al.*,(1975) indicated restrictive role of NK cells against the development of transplanted tumours. NK cells are capable of eliminating established tissue metastases (Whiteside and Herberman, 1995). Cytotoxic activity of NK cells against tumour target cells has also been studied *in vitro* (Kiessling *et al.*,1975, Herberman *et al.*, 1975).

NUMBER OF NK CELLS IN NORMAL AND TUMOUR BEARING MICE

To begin with, the number of NK^N cells and NK^T cells were determined. The number of NK^T cells obtained from spleen of tumour bearing mice was noticeably more in comparison to that of normal mice. Tumour bearing mice contained four times more NK cells than normal mice (Fig.1). Kurosawa and coworkers (1993) also found an increase in the number of NK^T cells in early stages of tumour development. The increase in the number of NK cells appears to have some correlation with the development of the tumour . This could be

inhibitory in nature as because NK cells have a prominent role in immunity against malignancy

Mitogenic or antigenic induction is necessary for the functional differentiation of immunocompetent cells like B and T cells. Con A mediated stimulus cause T cells to become cytotoxic in a polyspecific manner against allogeneic and tumour target cells *in vitro* (Stobo and Paul, 1973; Waterfield *et al.*, 1975; Heininger, 1976; Chakravarty and Clark 1977; Green *et al.*,. 1978; Chakravarty, 1980) and also *in vivo* (Anaclerio *et al.*, 1974; Waterfield and Waterfield, 1976; Chaudhuri and Chakravarty, 1981; Chakravarty and Chaudhuri, 1983). Similarly, Pokeweed mitogen (PWM) can activate B cells polyclonally (Coutintio *et al.*, 1975; Charmot *et al.*, 1975; Anderson *et al.*, 1972; Parkhouse *et al.*, 1972; Shortman *et al.*, 1973). Although NK cells are known to act spontaneously (Herberman *et al.*, 1975; Kiessling *et al.*, 1975; Talmadge *et al.*, 1980; Gorelik *et al.*, 1982), Henney and coworkers (1981) have shown that IL-2 treatment can activate the NK cells for heightened performance. Activation of NK cells with different doses of IL-2 has been tested in our study in reference to blastogenesis and DNA synthesis.

BLASTOGENESIS AND DNA SYNTHESIS WITH IL-2 TREATMENT OF NK CELLS

Maximum number of blasts in NK cell population from normal mice were observed at 24 hours after activation with 100U of rIL-2. These blasts also incorporated a significant amount of ^{3}H-TdR (Fig.3). This is interesting to note as because such correlation was not always observed with mitogenic stimulation of murine T cells (Chakravarty and Chaudhuri, 1983). Furthermore, the blastogenesis

and DNA synthesis data presented in Fig.2, Fig.3 and Fig.4 indicates the dose of 50 μl of gIL-2 effectively equivalent to that of 100U of rIL-2. The dose of 50 μl gIL-2 has been used routinely in our experiments considering it equivalent to 100U of rIL-2.

The cytotoxic ability of NK cells activated with IL-2 has been tested next in ^{51}Cr release and in ADCC assay.

NK CELLS IN ^{51}CR RELEASE ASSAY

The spontaneous cytotoxicity of NK^N cells increased four times after 24 hours activation with IL-2. Treatment beyond 24 hours did not improve the result further (Table5). NK^T cells are spontaneously lot more cytotoxic than the NK^N cells(Fig.5). However, IL-2 treatment did not boost this response any further.

NK^T cells being in continuous exposure to the tumour cells are likely to be sensitized and do not get boosted further by IL-2 treatment. Some authors observed that T lymphocytes from alcoholic patients are in an activated state and could not be stimulated further with mitogen like phytohaemagglutinin (Deviere *et al.*, 1988; Roy Chowdhury 1995; Chakrabarti *et al.*, 1977).

It is customary to consider the spontaneous cytotoxic response of NK cells as natural immunity rather than as acquired one. Here we find that NK^N cells respond maximally within 24 hours of IL-2 treatment; a time span necessary for sensitized cells to become cytotoxic. This raise the possibility of NK cells being equivalent to sensitized cells.

NK CELLS IN ADCC AND SUPPRESSOR FACTORS

NK cells are quite formidable in immune surveillance. Not only are they spontaneously cytotoxic, they are also the bearers of Fc receptors (Kay *et al.,* 1977; Herberman *et al.,* 1977; Oehler *et al.,* 1978; Lanier *et al.,* 1988; Ravetch and Kinet 1991); which suggests their possible participation in ADCC type of reaction against tumour target cells. Herberman and coworkers (1977) in fact demonstrated the ADCC activity by NK cells in mice whereas Oehler and coworkers (1978) showed ADCC activity of NK cells in rats; others (Peter *et al.,* 1975; Trinchieri *et al.,* 1975; West *et al.,* 1976; Kay *et al.,* 1977) demonstrated ADCC of these cells in humans.

In our experiments, the NK cells were incubated with anti TAA serum for half an hour allowing the antibodies to bind with the Fc receptors of the NK cells. The ^{51}Cr labeled tumour target cells were then added in the assay tubes. In such ADCC experiments, we observed 56 to 62 % cytotoxicity with NK^N cells (Fig. 5, Table 3). This index of cytotoxicity was about six times more than the spontaneous cytotoxicity of the NK cells in absence of anti TAA antibody treatment (Table 2 and Fig. 5).

Spontaneous cytotoxicity of NK^T cells are almost double to the cytotoxicity of NK^N cells (Table 2 and Fig.5); this response only slightly improve in ADCC assay. Furthermore, IL-2 treatment does not cause much improvement in ADCC response of NK^N cells (Table 3) and slightly better response was seen with NK^T cells. The ADCC response goes down by 48 hrs of IL-2 activation (Table 5).

The significant depression in ADCC of NK^T cells compared to spontaneous ADCC shown by NK^N cells (Fig.5) demands some

discussion. The possibility of suppressor factors operative in tumour bearing hosts are known. These are serum gangliosides (Bergelson *et al.,* 1989), Prostaglandin E2 (PGE2) produced by tumour cells (Fulton *et al.,*1985; Alleva *et al.,* 1994) and macrophages from tumour bearing mice (Pelus and Bockman 1979; Watson *et al.,* 1991;), transforming growth factor β (TGF-β) produced by a variety of normal and transformed cells (Sulitzeanu, 1993; Alleva *et al.,* 1994) and serum antibodies (Hellstrom *et al.,*1970; 1973; Hellstrom and Hellstrom 1969; 1970; 1974; 1979) which may act as blocking factors for the cell mediated arm of immunity.

These factors cannot be considered in total operation in case of NK^T cells as because these cells perform well in spontaneous cytotoxicity and in response to IL-2 treatment. To account for depression in ADCC response by NK^T cells, one may argue for loss of Fc receptors of the cells. Fc receptors are the key molecules to initiate ADCC; further studies need to be carried to resolve this point. Interestingly, Paul and Chakravarty (1988) reported the lesser density of mitogen receptors on the T cells as the basis for delayed immune response in Indian frugivorous bat, *Pteropus giganteus.*

Here, presence of anti TAA antibodies in the serum of tumour bearing mice as detected in this study (Table 7) needs to be focused in proper perspective. This antibody might very well play a role in ADCC type of reaction *in vivo*. Then, antibodies to the tumour cell antigen might play a significant anti tumour role in ADCC type of reaction *in vivo,* rather than being only blocking factor. At present it is difficult to design some experiments to prove this point *in vivo.*

HOMING OF IL-2 ACTIVATED NK CELLS

The cytotoxic activity of NK cells *in vitro* and its boosting with IL-2 tempt one to use these cells for adoptive transfer to curb the tumour growth. To begin with, we were interested to know whether the NK cells find their was to the tumour site. ^{3}H radiolabelled NK cells were used for the purpose.

High radioactive count of the tissue piece from the tumour gives a fair indication of radiolabelled NK cells seeking the tumour site (Fig.6). The pieces taken from the centre of the tumour showed highest radioactive count at 12 hours. Considering this highest count as 100%, the mean radioactive counts from other tissues were compared. The radioactive count from the central zone of tumour stayed relatively high upto 48 hours. Counts for the peripheral tissue of tumour are also reasonably high at different hours, but at slightly lower level. The highest count at tumour site seems to indicate it as preferential site for homing of IL-2 activated radiolabelled NK cells.

Reasonable incorporation of radioactivity was observed in other tissues studied, with least incorporation at the skin site. That the NK cells have a preference for organs that harbour them is possibly reflected from significant level of count from the spleen and mesenteric lymph nodes. The liver showed 69.6% infiltration at 12 hours. This high count in the liver could be that the NK cells are also residents of the liver (Itoh *et al.*, 1988; Hata *et al.*, 1990) and, or the cells are broken down by the liver after completion of its life cycle. The released radioactivity might find its way to the kidney in the process of excretion and may be the reason for relatively high count from kidney.

NK cells constitute an appreciable proportion of lymphocytes in the skin (Whiteside and Herberman, 1995), possibly that is why the radioactive incorporation in skin is reasonable, although least in comparison to the other organs studied.

Here, NK cells seem to behave like Con A activated syngeneic T cells having preference for tumour site as shown by Chakravarty and Jha (1997). In their findings, the radioactive count were more with tumour tissue at periphery to begin with at 12 hours and the maximum count from central zone at 24 hours.

THERAPEUTIC USE OF NK CELLS

The cytotoxic ability of NK cells and their ability of seeking tumour site suggest some definite role for them in tumour surveillance *in vivo* and also the possibility of their therapeutic use. Therapeutic use of NK cells have been done here in two ways. First was by stimulating the host residential NK cells *in situ* by IL-2 injections and second, by adoptive transfer of NK cells from syngeneic mice after IL-2 activation.

IL-2 ACTIVATION *in vivo* : IL-2 infusions in tumour bearing mice were carried out via two routes, intravenously and at the tumour site. Intravenous injections of IL-2 seems to be better in restricting the tumour growth and thereby increase longevity of the host. This is evident from the slope of the curves present in Fig.7 and the data in Fig.8. This tumour growth restriction however, cannot be attributed to host residential NK cells only as the participation of T lymphocytes cannot be ruled out. This protocol could very well activate some T cells in the host and make them reactive to the malignant cells. Several workers documented activation of T

lymphocytes with IL-2 *in vivo* and their effectiveness against malignant cells (Mills *et al.*,1980; Cheever *et al.*, 1982; Eberlein *et al.*,1982; Hefeneider *et al.*,1983; Majumdar *et al.*, 1984; Maitra and Chakravarty, 1990). Thus IL-2 activation *in vivo* seems to be operative against malignancy in mice.

The use of IL-2 for treating cancer patients is already in practice (Lotze *et al.*, 1985; Rosenberg *et al.*,1993), however toxic side effects are produced by high doses in use (Rosenstein *et al.*,1986; Schwartzentruber *et al.*,1988; Belldegrun *et al.*,1987; Kragel *et al.*, 1990; Siegel *et al.*, 1991).To counter this toxicity one can think of adoptive transfer of NK^T cells which are formidably cytotoxic even without IL-2 activation (Table 4).

ADOPTIVE IMMUNOTHERAPY

The NK^T cells indeed restricted the tumour growth in adoptive immunotherapy even when $0.5x10^6$ cells were injected (Fig.15). IL-2 treated NK^T cells perform better than simple IL-2 injections. We may recall that IL-2 treatment does not improve much the spontaneous cytotoxicity of NK^T cells but improved their ADCC response (Table 4). This finding suggests use of NK^T cells obtained from surgically removed tumours and expanded and activated *in vitro* for therapy of the patients.It has also been noted that ADCC response of NK^N cells is also good with or without IL-2 treatment (Table 3 & Fig. 5).

NK^T cells' potential in therapeutic use naturally ushered the exploration of potential of NK^N cells in this count. NK^N cells were found to be effective against tumour growth (Fig 9). However, treatment with IL-2 increased their effectiveness (Fig.11); increase in

cell number from $0.5x10^6$ to 10^6 in a dose improved the result further (Fig.13 and 14). Adoptive transfer of IL-2 activated NK cells have been found to be effective in patients with metastatic gastric and renal carcinomas (Hercend *et al.*, 1990; Yasumura *et al.*, 1994).

Interestingly, we found that IL-2 perform better when injected intravenously than at tumour site. Conversely NK^N or NK^T cells injected at tumour site do better job than being injected intravenously. The cells injected at the tumour site possibly cut short their journey through vascular system in the process of homing at tumour site.

THERAPY AFTER SURGICAL REMOVAL OF TUMOUR

Chakravarty and Jha (1997) have shown that, polyclonally activated syngeneic T cells after adoptive transfer can prevent successfully recurrence of tumours in about 67% of the experimental animals, after surgical removal of solid fibrosarcomas. Following similar protocol, adoptively transferred $0.5x10^6$ IL-2 activated NK^N cells, then with weekly IL-2 injections could inhibit tumour recurrence in 92.3% cases (Figs.17 and 18). Such adoptive transfer experiments involving activated T or NK cells were found to be very effective, possibly because they had to respond only against the residual malignant cells but not against the total tumour load.

CONCLUSION

Findings in the present investigations can be concluded as follows. NK^N cells can mount cytotoxic response to the tumour target cells and the cytotoxicity can further be increased by IL-2 treatment.

NK^T cells are four times more in number than the NK cells from normal mice.Their spontaneous cytotoxicity is at least two times more than the NK^N cells and the response does not improve much with IL-2 treatment. They can mount better ADCC response compared to spontaneous cytotoxicity of NK^N cells and also not much dependent on IL-2 treatment. Thus NK^T cells seem to be hopeful for adoptive transfer immunotherapy for human patients. NK^T cells can be obtained from the surgically removed tumour and draining lymph nodes which usually harbour NK cells (Trinchieri, 1989; Whiteside and Herberman, 1990; Lorenzen *et al.*, 1991). Short term culture of NK^T cells is likely to enrich the population in absence of any inhibitory or suppressor factor likely to be present in tumour bearing hosts. Furthermore, not much dependency of NK^T cells in IL-2 treatment for their spontaneous cytotoxicity and ADCC response becomes a plus point for treating the patient with autologous NK^T cells without boosting injections of IL-2 which often produce serious side effects.

As because NK^N cells mount significant response with IL-2 treatment, IL-2 therapy in low dose possibly may be introduced as a prophylactic measure for the people in the risk group of developing tumours, such as old and immunosuppressed persons.

We have shown that IL-2 treated NK^N cells could preferentially home at tumour site and injections of the cells followed by weekly injections of IL-2 for five weeks could succesfully inhibit recurrence of tumours in about 92% cases where the tumours had been surgically removed prior to the adoptive transfer. This type of therapy to eliminate the few residual tumour cells after surgery may be adapted for human patients.

It occurs with anybody working with NK cells why they are spontaneously cytotoxic, when usually other lymphocytes need prior activation to express their function; even macrophages do better job after antigenic activation. One way to resolve this question will be to consider the NK cells as sensitized by unknown stimulus, so the NK cells will be technically of status of memory cells. In a future study this may be resolved following some criteria used in other studies. Different workers found the virgin and sensitized T cells differ in their sensitivity to Hydroxyurea and Bromodeoxyurea in course of cytotoxic differentiation (Cantor and Jandinski, 1974; Nedrud *et al.*, 1975; Chakravarty and Clark, 1977). Recently, Das and Chakravarty (1997) showed elegantly the differential sensitivity of the virgin and memory T cells and also tumour infiltrating lymphocytes to DNaseI treatment. The virgin or memory status of NK cells especially NK^T cells might have a lot to do with stimulatability and dose requirement of IL-2 and to establish how far tumour associated antigen might stimulate the cells, if at all.