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INTERLEUKIN 2 AND ALPHA INTERFERON INDUCED IN VITRO MODULATION OF SPONTANEOUS CELL MEDIATED CYTOTOXICITY IN PATIENTS WITH CANCER OF THE UTERINE CERVIX UNDERGOING RADIOTHERAPY

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Abstract

In vitro modulation of spontaneous cell mediated cytotoxicity by interferon and interleukin 2 was carried out using peripheral blood lymphocytes from patients with cancer of the uterine cervix before and at different intervals after commencement of radiation treatment. A total of 150 patients with various stages of the disease were included and cytotoxicity was measured using the single cell cytotoxic assay. These results indicate a beneficial effect in vitro of interleukin 2 and interferon in augmenting spontaneous cell mediated cytotoxicity, a possibly vital anti-tumour immune mechanism in patients with relatively early cervix cancer. Natural killer cell, lymphokine activated killer cell and interferon activated killer cell activity was depressed immediately following radiotherapy. The activity of these cell types later on increased above pretreatment levels in patients with stages I, II A and II B. A similar rebound above pretreatment levels was not observed in patients with stages III and IV.

Key words: Cancer of uterine cervix; radiotherapy, spontaneous lymphocyte cytotoxicity, interleukin 2, interferon, lymphokine modulation.

The role of immunosurveillance developed by Burnett attributes to the immune system of major role in the reaction of the host against tumour cells. This theory led to a fear of irradiation having a detrimental effect on the immune system. Meyer (1), in particular, claimed in 1970 that in breast cancer radiation induced immune deficiency the incidence of visceral metastasis increased. A similar negative effect of radiotherapy was also suggested by Stjernswärd (2) but has been rejected by some other authors (3, 4).

The lymphopenic effect of irradiation has been recognised for many years and the cellular depletion which mainly involves recirculating lymphocytes may possibly explain at least in part the increased incidence of infections in some patients (5, 6) and the impairement of delayed type hypersensitivity responses (7, 8). It has also been shown that the various immunological functions of peripheral blood lymphocytes measured by in vitro assays are affected to differing extent (9–11).

Cancer of the uterine cervix represents about 12% of all cancers and 26% of all female cancers recorded at the Regional Cancer Centre, Trivandrum, India (12). At this centre the disease is almost always treated with radiotherapy and little information exists on the effects of the irradiation on the immune function in these patients.

There exists a population of peripheral blood lymphocytes capable of killing in vitro a variety of tumour and viral infected cells without prior sensitization (13, 14). These so-called natural killer (NK) cells, could be of prime importance in defence against tumours and can be modulated in vitro, by interleukin 2 (IL-2) and interferon (15, 16). We have earlier reported impairement of NK cell activity in patients with cancer of the uterine cervix along with other immunological abnormalities (17). We now present a study on the effects of radiotherapy on NK cell activity, IL-2 generated lymphokine activated killer (LAK) cells and interferon activated killer (IAK) cells, before and up to 12 months after treatment.

Material and Methods

The study group included 150 patients with proven cervical cancer. They were classified according to FIGO; 32 patients had stage I or IIA, 35 had stage IIB, 68 stage

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III, and 15 stage IV. None of the patients were known to have any other systemic or immune disease.

Stages I and IIA were treated with intra-cavitary irradiation using a Selectron low-dose after-loading system delivering a total dose of 66 Gy at point A, in 2 applications. Patients with stage IIB received a mid-pelvis dose of 32.5 Gy by external irradiation in 15 fractions over 3 weeks followed by 50 Gy at point A by intra-cavitary irradiation in 2 applications. Stage III patients received external irradiation 40 Gy in 20 fractions over 4 weeks followed by 33 Gy at point A by intracavitrary irradiation. Patients with stage IV disease were either given 45 Gy with external beam in 15 fractions over 3 weeks followed by 32.5 Gy at point A with intracavitary treatment or 55 Gy in 25 fractions over 5 weeks with external irradiation only.

Preparation of peripheral blood lymphocytes. Fifteen ml blood was collected from each patient before treatment and at subsequent follow-up visits in sterile heparinized tubes. The method of Boyum (18) was used for separation of peripheral blood mononuclear cells. Briefly, blood was diluted with an equal volume of Hank's balanced salt solution (HBSS, Gibco, USA) and centrifuged over a cushion of Ficoll Hypaque gradient (Lymphoprep, Nyegaard, Norway) at 400 g for 25 min. The mononuclear cell band was harvested, washed twice in HBSS and resuspended in RPMI 1640 medium buffered with 20 μmol/l Hepes and supplemented with 10% human AB serum, 100 μg/ml of streptomycin, 100 U/ml of penicillin, 5 μg/ml of fungizone and 300 μg/ml of fresh glutamine (complete medium).

Mononuclear cells were depleted of monocytes by incubating for 1 h in plastic dishes coated with heat inactivated fetal bovine serum at 37°C. Non-adherent cells were collected by repeated washing with complete medium. More than 95% of these cells were found to be lymphocytes by peroxidase staining. Of the lymphocytes 95% were viable as checked with Trypan Blue dye exclusion test.

Interferon (IFN). Human leukocyte interferon with a specific activity of 3×10^6 IU of IFN per mg protein, was diluted in RPMI 1640 and stored at −70°C before use. The maximum NK cell augmenting dose was found to be 250 IU/ml.

Generation of IAK cells. Effector cells (1×10^6/ml) were suspended in complete RPMI 1640 medium to which interferon was added. Cultures were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2, washed twice and resuspended in medium. Control cultures were treated in the same way but without interferon. The viability of the treated lymphocytes was unaffected as assessed by Typan Blue dye exclusion. Both treated and non-treated cells were assayed for cytotoxic activity.

Interleukin 2 (IL-2). MLA 144 cell line derived from a spontaneous lymphosarcoma of a gibbon ape continuously release high levels of IL-2 which is biochemically and biophysically identical to human IL-2. MLA 144 cells (2×10^5 cells/ml) were maintained in complete medium (foetal bovine serum in place of human AB serum) containing in addition, 5×10^{-5} mol mercaptoethanol for 72 h at 37°C in a humidified 5% CO_2 atmosphere.

The supernatant containing IL-2 was collected by centrifugation of cells at 200 g. Subsequently 250 ml of the supernatant was purified by precipitation with 85% $(NH_4)_2SO_4$. The precipitate was dissolved in 10 ml of 10 mmol Hepes buffered saline (pH 7.2), dialysed against the same buffer for 20 h and chromatographed on Sephadex G100 (Pharmacia, Sweden). The post-albumin fractions containing T-cell growth promoting activity were pooled, sterilized through 0.45 μm filters (Millipore Corp. USA) and stored in aliquots at −70°C. This preparation moved as a single band in sodium dodecyl sulphate polyacrylamide electrophoresis (SDS—PAGE).

Generation of LAK cells. LAK cells were generated by culture of responder peripheral blood lymphocytes at a concentration of 1×10^6/ml in complete medium at the dilution of IL-2 previously determined to be optimal for growth of IL-2 dependent cells (1 : 50) and also titrated to be optimal for LAK activation. Cultures were incubated for 4 days at 37°C in a humidified 5% CO_2 atmosphere. Control cultures were treated in the same way but without addition of IL-2. Viability of treated lymphocytes was unaffected as assayed by Trypan Blue dye exclusion. Both treated and non-treated cells were assayed for cytotoxic activity.

Assay for natural killer cell activity. Spontaneous cytotoxicity mediated by NK cells, LAK cells and IAK cells were measured using the single cell assay described by Grimm and Bonavida with minor modifications (19, 20). This method was preferred since it could evaluate the binding capacity as well as the lytic functions of these killer cells at the single cell level which is not possible with the conventional ^{51}Cr release assay.

Tumour target cells. The human erythroleukaemia cell line K562 was used as target for NK cells. Tumour target cells were serially passaged in complete medium and used in cytotoxicity assays within 48 h after the last passage. Viability was checked as more than 98% by Trypan Blue due exclusion.

Single cell assay. Equal numbers (2×10^5) of effector cells and target cells were mixed in a total of 0.2 ml of complete medium incubated for 10 min at 30°C and then centrifuged at 100 g for 5 min followed by gentle resuspension with a pipyette. To this conjugate suspension was added 0.5 ml 1% agarose (Sigma, USA) which had been kept in liquid phase at 38°C. Immediately 100 μl of this agarose conjugate mixture was transferred to agarose precoated slides. After soldification of the agarose, the slides were placed in plastic dishes filled with warm complete medium and incubated for 4 h in a humidified 5% CO_2 atmosphere at 37°C. Following incubation, the slides were removed, stained with 0.2% Trypan Blue and fixed with 1% formalin. The percentage of target binding cells were

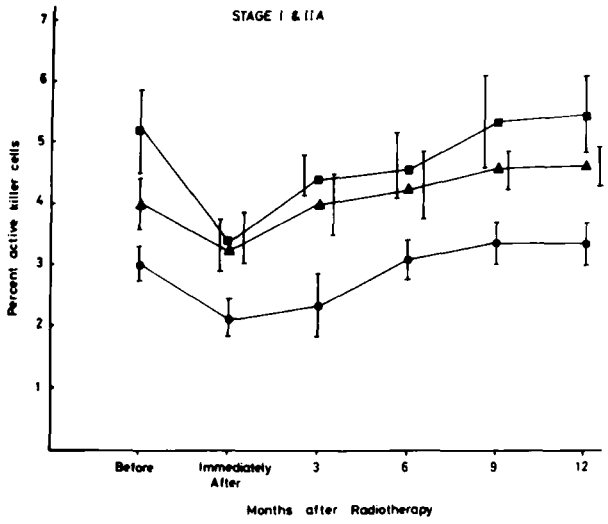

Fig. 1

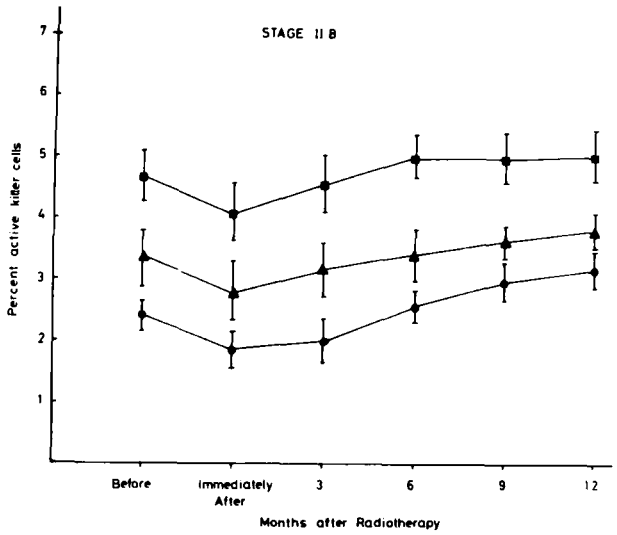

Fig. 2

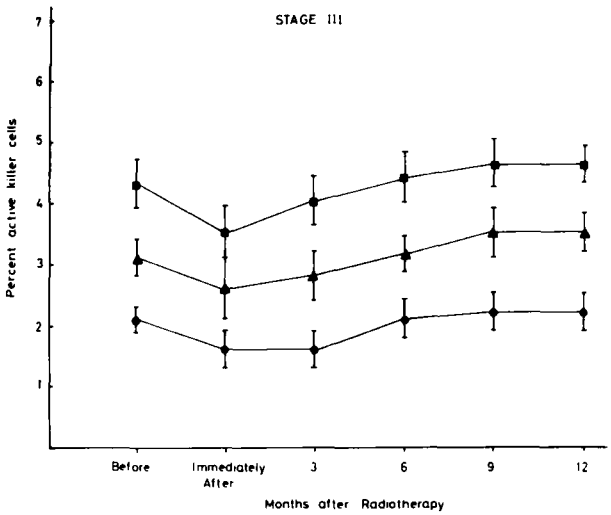

Fig. 3

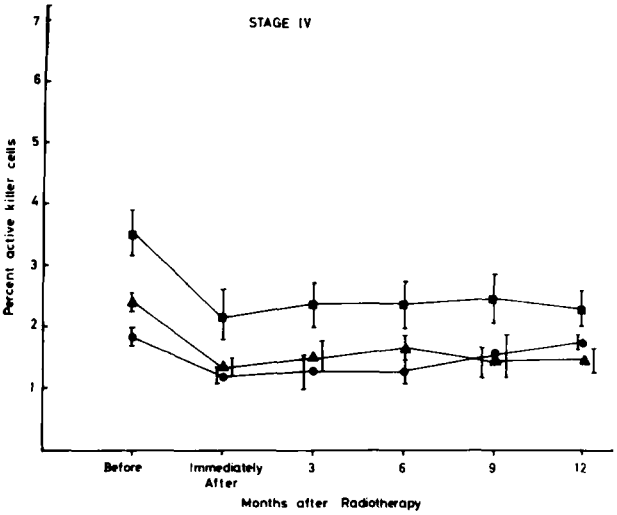

Fig. 4

Figs 1–4. Percentage active killer cells (NK, LAK and IAK) before and after radiotherapy. Mean ± SD. ●, NK cell activity, ▲, IAK cell activity, ■, LAK cell activity.

scored by counting the number of lymphocytes binding to K562 cells among 200 lymphocytes. The percentage of dead conjugated target cells was obtained by counting 100 lymphocyte K562 conjugates. Spontaneous target cell death was assessed by counting 200 target cells incubated in absence of effector cells and did not exceed 5%. The percentage of active killer cells was calculated using the formula:

$$\%\ \text{active killer cells} = (\%\ \text{target binding cells}) \times \frac{\%\ \text{dead conjugated cells}}{100} \times (1\text{-proportion of spontaneous target cell death})$$

Absolute active killer cells were calculated from the absolute lymphocyte count.

Statistical analysis was carried out using the paired Student's t-test.

Results

A total of 150 patients with squamous cell carcinoma of the uterine cervix were assayed for spontaneous cytotoxicity, lymphokine activated cytotoxicity and interferon activated cytotoxicity, before and up to 12 months following radiotherapy. Figs 1–4 and Tables 1–4 summerize the results.

Stages I and II A. These patients showed a significant decrease of the NK cell activity up to 3 months after

Table 1

NK, LAK and IAK values (Absolute cell count/mm^3) before and up to 12 months following radiotherapy for stages I & II A (n=32). NK: Natural killer cell activity, LAK: Lymphokine activated killer cell activity, IAK: Interferon activated killer cell activity

Parameter	Before radiotherapy	Immediately after radiotherapy	After 3 months	After 6 months	After 9 months	After 12 months
NK	78.0 ±11.5	37.4* ±6.8	58.2* ±11.5	85.2** ±13.5	93.8** ±16.5	94.9** ±19.1
LAK	135.0 ±23.3	61.8* ±12.1	109.1* ±17.5	127.5 NS ±30.9	151.6** ±33.4	149.8** ±27.3
IAK	107.4 ±17.2	58.1* ±12.7	102.1 NS ±19.0	119.6** ±24.6	128.6** ±24.6	128.4** ±21.0

All values represent Mean ± SD.
* Significant decrease compared to pretreatment levels ($p<0.05$).
** Significant increase compared to pretratment levels ($p<0.05$).
NS = Not significant.

Table 2

NK, LAK and IAK values (Absolute cell count/mm^3) before and up to 12 months following radiotherapy for stage II B (n=35). NK: Natural killer cell activity, LAK: Lymphokine activated killer cell activity, IAK: Interferon activated killer cell activity

Parameter	Before radiotherapy	Immediately after radiotherapy	After 3 months	After 6 months	After 9 months	After 12 months
NK	72.7 ±11.1	28.8* ±6.2	40.2* ±8.3	60.4* ±8.6	85.0** ±14.4	95.6** ±13.7
LAK	142.5 ±21.0	62.8* ±13.1	90.8* ±16.7	120.5* ±19.4	137.3 NS ±22.0	150.2** ±20.2
IAK	104.6 ±16.6	42.6* ±9.6	64.1* ±12.0	81.8* ±13.5	101.5 NS ±17.1	109.9 NS ±14.5

*, **, NS = Table 1.

Table 3

NK, LAK and IAK values (Absolute cell count/mm^3) before and up to 12 months following radiotherapy for stage III (n=68). NK: Natural killer cell activity, LAK: Lymphokine activated killer cell activity, IAK: Interferon activated killer cell activity

Parameter	Before radiotherapy	Immediately after radiotherapy	After 3 months	After 6 months	After 9 months	After 12 months
NK	65.6 ±10.9	24.1* ±7.2	30.7* ±8.0	47.4* ±9.8	53.2 ±10.7	59.4* ±11.6
LAK	132.4 ±20.9	59.9* ±13.6	75.3* ±14.8	100.0* ±17.0	112.3* ±18.9	124.8 NS ±19.9
IAK	95.4 ±15.6	39.6* ±11.7	53.1* ±12.1	78.8* ±14.2	85.6* ±16.3	95.5 NS ±15.6

*, **, NS = Table 1.

intracavitary irradiation. However, from the 6th month onwards there was a significant increase as compared to the pretreatment values. LAK cell activity remained significantly depressed till 3 months after treatment and then showed a significant increase. However, cells treated with interferon remained depressed immediately after radiotherapy and had by 3 months returned to pre-treatment levels. From then on a significant increase was noticed (Fig. 1 and Table 1).

Stage II B. These patients showed similar trends as the

Table 4

NK, LAK and IAK values (Absolute cell count/mm^3) before and up to 12 months following radiotherapy for stage IV (n=15). NK: Natural killer cell activity, LAK: Lymphokine activated killer cell activity, IAK: Interferon activated killer cell activity

Parameter	Before radiotherapy	Immediately after radiotherapy	After 3 months	After 6 months	After 9 months	After 12 months
NK	63.4 ±10.5	15.2* ±6.8	20.6* ±7.1	24.3* ±8.6	32.3* ±8.1	36.1* ±4.4
LAK	121.4 ±19.8	28.1* ±10.7	38.0* ±12.7	45.3* 15.8	51.6* ±12.7	47.6* ±10.1
IAK	81.5 ±12.6	16.1* ±4.5	24.3* ±6.0	31.1* ±8.6	30.9* ±8.1	30.7 ±5.8

*, **, NS = Table 1.

above group, however, with some differences. NK cell activity remained significantly depressed up to 6 months following radiation therapy, and then showed significant increase. LAK cell activity returned to pretreatment values 9 months after treatment and then showed significant increase. IAK cell activity returned to pretreatment values at 9 months and remained at this level without significant increase. The results are summarized in Table 2 and Fig. 2.

Stage III. The results in this group are shown in Table 3 and Fig. 3. NK cell activity did not return to pretreatment levels within 12 months after radiotherapy. LAK and IAK cell activity remained depressed up to 9 months after treatment and then attained pretreatment levels.

Stage IV. These patients showed interesting differences compared to the other 3 groups. None of the 3 parameters studied returned to pre-treatment levels within 12 months after radiotherapy, as summarized in Table 4 and Fig. 4.

Discussion

Local radiation therapy is a widely accepted treatment in various types of neoplastic diseases. Radiotherapy is associated with lymphopenia (6, 32) and also with depressed T-cell function as assessed with standard in vitro lymphoproliferative assays with mitogens and allogeneic cells (10, 11, 21). The deleterious effects of radiotherapy seem to relate directly to the volume of blood, lymph nodes or bone marrow encompassed within the irradiated volume and changes have been observed after radiotherapy to head and neck region, mediastinum, pelvis and scull (21–26). The immunosuppressive effects of irradiation tend to be prolonged and patients who are clinically in remission continue to show immune abnormalities for years after completing radiation therapy (27, 28).

Our results indicate an initial depression of NK, LAK and IAK cellular activity following radiotherapy. However, after 9 months all 3 parameters were increased above the pretreatment levels in stages I and IIA and the same was true for NK and LAK values in stage IIB. Contrarily, patients with stages III and IV never attained values higher than the pretreatment levels. In stage IV even sustained depression was observed.

Dubois & Serrou (7) reported significant impairement of NK cell activity following radiotherapy. However, a finding similar to the present study was observed by Blomgren et al. (9) who reported NK cell activity against K 562 cells on a cell for cell basis to be reduced to approximately 50% at completion of radiotherapy followed by a restoration 3–4 months later. Onsrud & Thorshy (29) observed among disease-free patients treated for stage I endometrial carcinoma, a significant increase in spontaneous cytotoxicity against K562 cells 3–5 years after pelvic irradiation compared to patients not irradiated. Rotstein et al. (30) concluded that spontaneous cytotoxicity of blood lymphocyte population on a cell for cell basis became normalized after treatment but that it took 5–6 years for this to occur.

Improvement of immune status in patients attaining remission after treatment could result from improvement in their general condition, from reduction of tumour burden or from effects of treatment on any of the various humoral or tumour derived immunosuppressive factors which have been described in malignancy. Such an improvement might be taken advantage of in clinical practice since spontaneous immunostimulation may be potentiated by immunorestorative or immunostimulating agents, thereby prolonging or augmenting the hyperimmunoactivity. It seems possible that interleukin-2 and interferon could be used as adjuvant to radiotherapy in order to maintain or potentiate the observed immunological rebound. Indeed, these biological reponse modifiers can be used effectively as immunotherapeutic agents as shown in vitro by the present study and by other authors (15, 16) and in some in vivo situations (31, 32).

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