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# Long-term immune and cytogenetic effects of high level natural radiation on Ramsar inhabitants in Iran

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**Abstract**

Ramsar, a northern coastal city of Iran, overlooking the Caspian Sea, has some high level natural radiation areas (HLNRAs) as well as over 50 hot springs with low and high radium contents used as spas by the public and vacationers. The average whole body dose received by population in these areas is about 5 times higher than the normal background radiation level. Studies on the long-term effects of high level natural radioactivity on some immunological and cytogenetical parameters, in the Ramsar inhabitants are summarized in this paper. Our results showed a significant increase of CD69 expression on TCD4+ stimulated cells ($P < 0.004$) and a significant increase of total serum IgE ($P < 0.05$), and also higher incidence of stable and unstable chromosomal aberrations in the HLNRA group compared to the control group with normal background radiation ($P < 0.05$).Other humoral immune parameters, did not show significant differences between the two groups.
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## 1. Introduction

There are many high level natural radiation areas (HLNRAs) throughout the world such as in Brazil, China, India and Iran. The sulfurous hot springs in

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Ramsar, Iran, contain high $^{226}Ra$ concentrations (from 1 to 146 KBq/m$^3$), which flows in to the surrounding areas, leading to the creation of an HLNRA and population exposure. In the HLNRAs of Ramsar, Talesh Mahalle has the highest radiation levels. The potential annual effective doses of the public in these elevated level natural radiation areas, range from 0.7–131 mSv with a mean value of 6 mSv, (Sohrabi and Esmaili, 2002). Accordingly, low level natural radiation area have potential doses comparable to that of the world average of 2.4 mSv/y reported by UNSCEAR (2000). The mean effective dose resulting from $^{226}Ra$ due to consumption of vegetables in this area is reported to be 12 times greater than the average effective dose resulting from this radionucleide due to foods and drinking water in normal area (Ghiassi-Nejad et al., 2003).

Immune system constitute one of the most important defense mechanisms against the establishment and growth of cancer induced by various environmental agents, including ionizing radiation. It is known that high dose ionizing radiation depresses immunity and dysfunction of this system will therefore yield increase in infectious diseases, and cancers. However, the nature of the health effects of low-level ionizing radiation has been the subject of considerable controversy. It has been observed in human populations of high background radiation areas in China, that low dose radiation could stimulate the immunological responses and suggest there might be a stimulatory effect of low-level radiation on the defense mechanisms of the human body (Liu, 1997). Another studies on the immune status of the inhabitants of the HLNRA revealed that there was a definite increase in reactivity of T lymphocytes to phytohemagglutinin(PHA), a slight increase in the percentage of B lymphocytes and a tendency toward enhancement of unscheduled DNA synthesis in peripheral blood lymphocytes (Liu et al., 1987). Also, the study of chromosome aberration frequency in peripheral blood lymphocytes is a sensitive assay for detecting exposure to radiation and increased frequencies of stable and unstable aberrations has been reported in the areas of high background radiation in China (Chen and Wei, 1991).

More recently, it has been suggested that chromosome aberration frequency is itself an indicator of cancer risk rather than just a reflection of exposure (Hagmar et al., 1994; Bonassi et al., 2000).These observations have prompted a series of experimental studies in our laboratory on the effects of high level natural radiation on some basic immunological and cytogenetical parameters in the inhabitants of HLNRAs of Ramsar.Considering the known health risks from ionizing radiation, the main objective of this study was focused on the potential impact of this exposure on the health of local residents as well as to detect any changes that could be attributed to the effects of high level natural radiation.This report is part of these studies.

## 2. Materials and methods

### *2.1. Study population and dose estimates*

The survey group consisted of 50 inhabitants of the HLNRA who have lived there for generations, with a mean age of 40 $\pm$ 16 years. The radiation levels and dose assessments were based on the terms of effective dose. Their total internal and

external exposure levels, which were calculated as annual effective dose equivalent, ranged from 1.6–42 mSv/y with a mean value of 13 ± 12 mSv/y. 30 inhabitants of a nearby control area with similarity to the HLNRA in terms of social and economic status, including life style and living standards considered as control group, with a mean age of 38 ± 13 years, and normal background radiation level comparable to that of the world average of 2.4 mSv/y reported by UNSCEAR (2000). An annual effective dose equivalent of 2.3 ± 0.09 mSv/y was determined for controls. All persons in the group surveyed, were carefully screened for: illness, smoking habits,medical treatment, X-ray examination, viral infections, medications, allergic diseases, parasites.

### 2.2. Immunological parameters

For detection of humoral factors, samples of 5 ml of the whole blood were obtained and serums were separated. We investigated the concentration of different serum immunoglobulins of IgM, IgG, IgA, by Single Radial Immuno Diffusion (SRID) of Mansoni, and concentration of total serum IgE by ELISA, concentration of different components of the complement system (C3, C4, C1-inactivator) by SRID, rheumatoid factor (RF) and C-Reactive Protein (CRP) by serologic methods. The kits purchased from Boehringer Mannheim, and the processing of samples was done based on their instruction.

For CD69 expression, heparinized peripheral blood from each donor was taken under aseptic condition and processed in 2–4 hrs. All samples were diluted 1 in 3 in complete culture medium containing RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamin, 100 μg streptomycin and 100 IU penicillin in the presence or absence of phytohemagglutinin (final concentration of 1.5% in the culture medium). The samples were then incubated for 6 hours in humidified condition, 37 °C plus 5% $CO_2$. Fast immune Bundle Kit for three colors staining of lymphocytes was used (Becton Dickinson, USA). This kit contains anti CD3-PerCP, anti CD4-FITC, anti CD5-FITC, anti CD69-PE, gamma1-FITC/gamma2-PE control monoclonal antibodies. The samples were analyzed by Flowcytometer, FACS canm LYSIS II software (Becton Dickinson, USA). The lymphocytes were gated based on ssc/CD3 expression and the percentage of either CD4+CD69+ or CD8+CD69+ was determined for unstimulated and stimulated T cells. The processing of samples was done based on manufacturer's instruction. Statistical analyses were carried out using Student's t-test.

### 2.3. Cytogenetics

Cytogenetic analyses were performed by the conventional method and also by G-banding technique. 0.5 ml of heparinized blood was added to 4.5 ml RPMI 1640 medium (Gibco BRL), supplemented with 20% fetal calf serum (Gibco Laboratories), The medium was supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin and either 10 or 7.2 μM bromodeoxyuridine. The cells were stimulated

with 1% phytohemagglutinin (PHA) was added in to the whole blood microcultures at the beginning of culture period and cultures were incubated at 37 °C for 48–54 h and colcemid was added at a final concentration of 1 μg/ml for the last 4 h and harvested by exposure to a hypotonic solution of 75 mM KCl, followed by fixation with methanol and acetic acid in the ratio 3:1 and samples were stored at −20 °C until required (IAEA, STI/PUB/10/260, 1986). Fluorescence plus Giemsa staining of a proportion of the samples from each group for determination of cell cycle kinetics indicated that the frequency of cells in their first division was over 94%. At least 200 metaphases were scored for each individual for detection of unstable chromosomal aberrations.

Fixed cells were placed on glass slides and air-dried. Several days later banding was performed by the G-method. Chromosome aberration analysis was undertaken on slides G banded with trypsin. Karyotyping was performed using a standard light microscope. A total of 100 cells were analysed from each individual in the HLNRA and control groups. Translocations, inversions and insertions were each classified as single symmetrical aberrations, (dicentrics and centric rings as asymmetrical aberrations) and deletions, both terminal and interstitial (irrespective of whether an acentric fragment was observed), were combined and classed as deletions. All types of structural chromosome aberrations were analyzed from coded preparations. Statistical analysis were carried out using Student's t-test and linear regression.

## 3. Results

The concentarations of serum immunoglobulins and componenets of complement system are presented in Table 1.

Concentrations of IgM and IgA and IgG in the HLNRA and control groups were in the normal ranges, however, HLNRA group had slightly higher serum IgG than the control group but this difference was not significant. But total serum IgE was significantly increased in HLNRA group. Allergies and helminthic infections are characterized by elevated serum IgE, so in order to rule out one of these, we checked the prevalence of helminthic infections, and the results showed that it was the same low prevalence of intestinal parasites in both groups. The concentration of components of complement system are also in the normal ranges and did not show significant difference with control group.

Table 1
The average concentration of different classes of serum immunoglobulins and components of complement system in the HLNRA and control groups

| Humoral factors | IgM (mg%) | IgG (mg%) | IgA (mg%) | IgE (IU/L) | C3 (mg%) | C4 (mg%) | C1-inact. (mg%) |
|---|---|---|---|---|---|---|---|
| HLNRA group | 136 | 1326 | 265 | 112* | 99 | 21 | 19 |
| Control group | 129 | 1107 | 255 | 72 | 105 | 21 | 17 |
| Normal range | 80–320 | 70–2100 | 100–430 | <100 | 70–170 | 15–55 | 10–30 |

*The average concentration of IgE was significantly higher in the HLNRA group ($P < 0.05$).

Table 2
The average number of CD4+/CD69+ and CD8+/CD69+ Tcells in 10,000 CD3+ cells, in stimulated and unstimulated lymphocytes of HLNRA and control groups

| Samples | Unstimulated | | Stimulated | |
|---|---|---|---|---|
| | CD4+/CD69+ | CD8+/CD69+ | CD4+/CD69+ | CD8+/CD69+ |
| HLNRA group | 230 ± 97 | 300 ± 170 | 3500 ± 480* | 2200 ± 870 |
| Control group | 530 ± 630 | 300 ± 160 | 2100 ± 900 | 1700 ± 1100 |

*The average expression of CD69 on CD4+ stimulated Tcells in the HLNRA group was significantly higher than in the control group ($P < 0.004$).

Our results show a significant increase of CD69 expression on CD4+ stimulated T cells in the HLNRA group compared to the control group (3500 ± 480 vs 2100 ± 900 in 10000 CD3+/CD4+/CD69+ cells, $P < 0.004$). However, there was not any significant increase of CD69 expression in stimulated CD8+ Tcells. In addition there was no difference of CD69 expression on unstimulated cells of either groups. The results are shown in Table 2.

Rheumatoid factor (RF) and C-Reactive Protein , were negative for the HLNRA and control groups. Results of chromosomal aberration analysis by conventional method and chromosome banding are presented in Table 3.

Our results in this study showed a significant increase in the frequency of chromosomal aberrations, detected by G-banding method, in HLNRA group compared with the control group (6.0 ± 2.1 vs 1.5 ± 0.95 aberrations per 100 cells, $P < 0.05$). In the HLNRA group, 307 aberrations were found in 5122 banded metaphases, while in the control group only 46 aberrations were observed in 3054 metaphases. The frequency in the HLNRA group was about four times higher than in the control group. On analysis of the types of aberrations, translocations and deletions accounted for approximately 75% of the total aberrations. Breaks found by G-banding in the HLNRA group were higher than those in the control. A statistical comparison of observed and expected values showed that the distributions of chromosome breaks in both groups were nonrandom. When individual chromosomes were compared seperately, it was found that the observed values of the breaks of chromosomes 2, 9 and 8 were higher than the expected. A higher frequency of aberrations were found in the older age group and although this was not statistically significant, the possibility of an age effect is suggested.

The frequency of unstable chromosome aberrations detected by the conventional method were also significantly higher in the HLNRA than control group (4.6 ± 2.0 vs 1.6 ± 1.2 per 100 cells), which mostly consisted of breaks. We did not observe dicentrics or centric rings.

## 4. Discussion and conclusion

In the HLNRAs of Ramsar studies on the immunologic and cytogenetic factors of inhabitants have been performed. Concentrations of serum IgM and IgA were in the normal ranges for the HLNRA and control groups and in spite of the fact

Table 3
Frequency of stable and unstable chromosomal aberrations in the lymphocytes of HLNRA and control groups

| Subjects | Age X ± SD | No. of cases | Annual effective dose (mSv/y) | No. of cells analysed for st.ab. | Total st.ab | (%) of St.ab.s | Types of st.ab.s | | | | No. of cells analysed for unst.ab. | (%) of unst.ab.s (ace) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | t | inv | del | others | | |
| HLNRA group | 40± 16 | 50 | 13 ± 12 | 5122 | 307 | 6.0±2.1* | 208 | 40 | 28 | 31 | 10800 | 4.6± 2.0* |
| Control group | 38 ± 13 | 30 | 2.3 ± 0.09 | 3054 | 46 | 1.5±0.95 | 24 | 11 | 8 | 3 | 7400 | 1.6 ±1.2 |

st.ab.: stable aberrations. unst.ab: unstable aberrations. ace: acentric fragment. t: translocation. del: deletion. inv: inversion. others: duplication, insertions and complex rearrangements.
*% of stable and unstable aberrations in the HLNRA group were significantly higher than in the control group ($P < 0.05$).

that the increase in serum IgG in HLNRA group was not so marked to show probable enhanced immunologic capability, it can be concluded that relatively high level natural radiation could not affect these factors.

Total serum IgE is significantly increased in HLNRA group (112.15 vs. 71.93 IU/L, $P < 0.05$). It was not due to the helminthic infections, because of the same low prevalence of it in both groups. The possible mechanisms may be due to stimulatory effects of low dose radiation on immunity, such as stimulation of Th2 responses. This is probably caused by an up-regulation of Th2 cells or may represent a shift from Th1 to Th2 cytokine profile due to high level natural radiation exposure. Significant increase of serum total IgE in the HLNRA group ,is consistent with reports by (Tada et al., 1971), showing enhanced and sustained production of IgE in rats irradiated with 400 R, X-ray and (Yamashita, 1987) who have reported the increase of IgE response in genetically low IgE responder SJL/J mice, after irradiation. Romagnani (1990) reported that the Th2 lymphocytes are more radioresistant than other lymphocytes and IL-4 produced by these cells, enhance IgE production. Marcelletti and Katz (1989) concluded that low-dose irradiation causes prolonged high levels of IgE synthesis in mice because radiation disturbs the regulatory function of certain Ly-1-expressing CD4+ T cells which normally counterbalance those CD8+ T cells that play an important role in the IgE regulatory network.

However, De Kruyff et al. (1995) indicated that although some functions of CD4+ T cells are resistant to radiation, other funtions, particularly those that depend on the production of IL-4 and IL-5, are greatly diminished by ionizing radiation. These observations appear to be counter to previous reports indicating that mice treated with low doses irradiation have an enhanced capacity to produce IgE (Chiorazzi et al., 1976), or to others indicating that total lymphoid irradiation enhances the later production of IL-4 and IL-5. However, these experimental systems are quite distint and can not be directly compared. The radiation doses used in the in vitro studies of De Kruyff ,were considerably higher (500–2500 rad) than the invivo studies using low-dose irradiation(<200 rad), which was thought to diminish suppressor cell activity against IgE synthesis. Also, it is not comparable with natural radiation doses in our study. The studies involving total lymphoid irradiation examined T cell function which developed weeks after treatment rather than helper function immediately after irradiation.Thus, the use of irradiated populations of T lymphocytes in experimental models must take in to consideration the relative sensitivities/insensitivities to irradiation of different T cell functions and of the production by T cells of different cytokines (De Krueff et al., 1995).

In a preliminary study, aimed at investigating the impact of high natural radioactivity on the respiratory system on the students living in HLNRAs of Ramsar compared with students in normal background radiation areas, the existence of higher incidence of some allergic symptoms has been shown in HLNRA students. Significantly higher frequencies of allergic symptoms such as rhinitis and weakness and fatigue, shedding tear and number of times of catching cold during a year,

was reported in HLNRA, while there were no difference in bacterial respiratory diseases between the childrens of both groups (Emem Verdizadeh, 2000).

Our results show a significant increase of CD69 expression on CD4+ stimulated T cells in the HLNRA group compared to the control group ($P < 0.004$). However, we did not observe any significant increase of CD69 expression in stimulated CD8+ Tcells. In addition there was no difference of CD69 expression on unstimulated cells of either groups.It has previously been shown that in vitro gamma irradiation of lymphocytes increases the expression of CD69 on PHA stimulated cells (Chen et al., 1997). They showed CD69 functions as a marker for response to radiation, but unlike antigen or mitogen, radiation-induced CD69 expression does not lead to proliferation. CD69 is early activation marker of lymphocytes, involving in activating several proto-oncogenes. Therefore, our findings would suggest that individuals living in the area with high natural radiation may be the group at risk for higher incidence of late radiation effects.

Our results in this study show that there are significantly higher frequencies of stable and unstable chromosome aberrations, in inhabitants of HLNRA compared to control group, which may be interpreted as an effect of elevated natural radiation present in the area, because an attempt was made to approximately match for age and smoking habits. Linear regression analysis showed the correlation between the incidences of the total structural chromosome-type aberrations and accumulated doses, either in HLNRA or in control group. It is evident that if anything meaningful is to be seen in people exposed to low levels of radiation, a large number of cells are needed to be examined (Jiang et al., 1999).

The relationship between the chromosome aberration and increasing age was similar to that between the chromosome aberration and accumulated dose. This may suggest that the increase is due to an increase in accumulated exposure to environmental natural background radiation as reported by Tawn and Whitehouse (2001) and Tonomura et al. (1986). It was previously demonstrated in China that the frequency of chromosome aberration of peripheral lymphocytes detected by G-banding technique in inhabitants of a high background radiation area , was higher than that in people of a control area (Yao et al., 1985).

An increase in chromosomal aberrations in peripheral blood lymphocytes of persons living in an environment with elevated natural background radiation level has also been observed in India (Kochupillai et al., 1976; Cheriyan et al., 1999), Brazil (Marcello, 1975; Franca, 1997), China (Chen and Wei, 1991; Chen et al., 1985) as well as in our previous investigations in Iran,detected by conventional method (Fazeli et al., 1990) .

The fact is that the increase of frequency of chromosome aberrations were observed in some high background radiation areas (HBRAs) and the possibility of effects induced by ionizing radiation can not be excluded. Nevertheless, on the contrary, cell-mediated immunity examination revealed that there is a tendency of strengthening of immune functions among the HBRA inhabitants (Liu et al., 1987). Many reports of epidemiology and radiobiology and the results of investigation in HBRA of Yangjiang denoted that in case of low dose exposure the defense and repair system might be superior to the disadvantageous effects and the

individuals exposed to low level radiation several times above the average would not increase the relative risk of cancer as shown in the long-time observations in areas of high background radiation. On the contrary, the mortality of all cancers in HBRA was generally lower than that in control area, but not significant statistically (Tao et al., 2000).

In the HLNRAs of Ramsar, at the present there are no reliable radio-epidemiological data regarding the incidence of cancer. Further studies are needed to clarify stimulative effects of radiation or more susceptibility of people to the late effects of radiation exposure and allergic diseases. Health surveillance of exposed human groups is the main source of data for assessing the level of risk associated with exposure to radiation, so long-term monitoring of health status of residents, establishment of a cancer registry system, aerobiological studies and cytokine assays are recommended in these areas.

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