Natural killer cell activity in workers exposed to benzidine and β -naphthylamine

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Abstract

To investigate the effects of benzidine (BZ) and β -naphthylamine (BNA) on the immune system in man, the activity of natural killer (NK) cells as well as the relative number (percentage) of Leu 11a positive cells in peripheral blood lymphocytes were measured in 63 dyestuff workers exposed to BZ and BNA (aromatic amines, AA). The cytotoxic potential per NK cell (unit NK cell activity) was approximated by dividing the NK activity per fixed numbers of unseparated mononuclear cells by the percentage of Leu 11a positive lymphocytes that mediated NK activity. Thirty one of these workers had previously been treated for bladder cancer and then cured (ex-cancer AA workers) whereas the remaining 32 had not been diagnosed as having bladder cancer (non-cancer AA workers). There was no significant difference in the gross NK activity per unseparated peripheral mononuclear cells among ex-cancer AA workers, non-cancer AA workers, and the control group ($p > 0.05$). The relative number of Leu 11a positive cells, on the other hand, was significantly higher in AA workers than in the control group ($p < 0.01$). The unit NK cell activity, as a result, was significantly more reduced in both ex-cancer and non-cancer AA workers than in the control group ($p < 0.05$ and $p < 0.01$, respectively). Between ex-cancer and non-cancer AA workers, no significant difference was observed in terms of unit NK cell activity. These results indicated that the function of NK cells per se was impaired in AA workers

whereas the number of circulating NK cells was relatively increased.

It has been established that workers exposed to benzidine (BZ) and β -naphthylamine (BNA) have a greatly increased risk of developing bladder cancer.^{1,2} The number of people who are at high risk for bladder cancer due to occupational exposure to aromatic amines (AA) amounts to more than 6000 in the United States,³ 3000 in Japan (Ad hoc committee on industrial hygiene of the Japan Dyestuff Industry Association), and 2500 in China.⁴ Therefore, management of asymptomatic workers at high risk for bladder cancer is still important.

In 1956 Crabbe *et al* reported the usefulness of the Papanicolaou test that since then has been widely used to detect occupational bladder cancer.⁵ The Papanicolaou test, however, is effective only after cancer has fully developed since only then are abnormal cells observed by microscopy. In 1979 Kumar *et al* reported a new test, in which the killing activity of lymphocytes against a bladder cancer cell was examined in workers at risk.^{6,7}

In recent years many findings indicate that natural killer (NK) cells have an important role in inhibiting tumourigenesis. NK cells are a population of lymphocytes with the capability to lyse a variety of aberrant cells independent of the major histocompatibility complex.^{8,9} In patients with bladder cancer the function of NK cells has been reported to be impaired but the occupational background of the patients was not described¹⁰ (and T Morita *et al*, unpublished data). At present, there is no systemic report on NK function in workers at a high risk for bladder cancer. In the present study we measured the activity and relative number of NK cells in workers exposed to BZ and BNA.

Subjects

The 63 male subjects were retired or currently employed factory workers; all had been exposed to BZ or BNA (aromatic amines, AA) before 1972. Thirty one, aged 54 to 70 (mean 62), had been previously treated for bladder cancer and cured (ex-cancer AA workers). The remaining 32 aged 43 to 69 (mean 58) had not been diagnosed as having bladder cancer (non-cancer AA workers). The control group consisted of 33 "healthy" male volunteers aged 42 to

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64 (mean 54) who were working at the same factory but without a history of occupational exposure to BZ and BNA.

No subject had signs and symptoms indicative of infection at the time of the study; none had used drugs that could affect immunological analysis. There were nine, 16, and 15 smokers in the ex-cancer, non-cancer, and control groups respectively; the proportion of smokers did not statistically differ among the three groups. According to several investigators, there is no significant relation between NK cell activity and the age of these ranges in human adults.^{11,12} Therefore, the difference in age among the three groups was not considered to be a confounding variable.

Methods

The procedure was explained to all subjects and this study was conducted with their informed consent.

Mononuclear cells were separated from heparinised peripheral blood by Ficoll-Conray density gradient centrifugation, washed twice with phosphate buffered saline, and resuspended in PRMI 1640 medium supplemented with 10% fetal calf serum (FCS).

NK cell activity was determined by ⁵¹Cr release assay,¹³ using mononuclear cells as effector cells and the human chronic myeloid leukaemia derived cell line, K562, as target cells. Target cells (2×10^6) were labelled with 3.7 MBq of sodium chromate ($\text{Na}_2^{51}\text{CrO}_4$) (New England Nuclear, Boston, Massachusetts) for two hours at 37°C in humidified 5% CO_2 in air. The cells were then washed three times and resuspended at $1 \times 10^5/\text{ml}$ in 10% FCS-RPMI1640 medium. Aliquots (100 μl) of target cells were mixed with 100 μl of effector cell suspensions in round bottomed 96 well microculture plates to give effector:target (E:T) ratios at 5:1, 10:1, 20:1, and 40:1 in triplicate. The plates were incubated for four hours. From each well 100 μl of supernatant were removed and counted on a gamma counter. The spontaneous ⁵¹Cr release, determined by incubating labelled target cells in the medium alone, did not exceed 15% of the maximal release that was determined by adding 1% Triton X-100 (Sigma Chemical Co, St Louis, MO). The per cent specific cytotoxicity was calculated as follows: per cent specific cytotoxicity = (experimental ⁵¹Cr release - spontaneous ⁵¹Cr release)/(maximal ⁵¹Cr release - spontaneous ⁵¹Cr release) $\times$ 100. The mean per cent specific cytotoxicity obtained at each E:T ratio was plotted against the logarithm of the E:T ratio and a linear dose response curve was obtained with a least squares fit method.¹⁴ We defined one lytic unit (LU) as the number of effector cells needed to achieve 33.3% cytotoxicity of 10^4 target cells which was obtained from the dose-response curve. To evaluate the NK activity per whole mononuclear cells, the number of lytic units in

10^7 effector cells (LUs/ 10^7 cells) was calculated.

For direct immunofluorescence staining, 2×10^6 mononuclear cells were incubated with 15 μl of FITC conjugated monoclonal anti-Leu 11a antibody (Becton Dickinson, Mountain View, California) on ice for 30 minutes, washed twice with RPMI 1640, and resuspended in 10% FCS-RPMI 1640. All samples were then analysed for immunofluorescence on a flowcytometer (EPICS CS, Coulter Electronics, Hialeah, FL). Non-lymphocytic cells contaminating the preparations were excluded from analysis using scatter gates set on the 90° and straight light scatter profile. Ten thousand lymphocytes were analysed for each sample and the number of fluorescing cells were expressed as a percentage of the total lymphocytes. Anti-Leu 11a antibody is thought to detect those cells that have a high degree of NK cell activity.^{15,16}

To approximate the cytotoxic activity per one NK cell, we defined the "unit NK cell activity" by dividing the LUs/ 10^7 cells by percentage of Leu 11a positive lymphocytes.

Owing to the non-Gaussian distribution, all data were analysed statistically by the Wilcoxon rank sum test.

Results

The results of NK activity per whole peripheral mononuclear cells are summarised among ex-cancer AA workers, non-cancer AA workers, and the control group (table). There was no statistically significant difference among the three groups. It was noted, however, that the degree of individual variability was high in the AA workers as compared with the control group. Thus the control group had a range of NK activity per whole peripheral mononuclear cells between 3.7 and 104 LUs/ 10^7 , whereas six ex-cancer and seven non-cancer AA workers were outside this range. We then analysed the relative proportions of Leu 11a positive lymphocytes that actually mediated cytotoxic activity in the same groups. The proportions of Leu 11a positive cells in peripheral lymphocytes in AA workers were significantly higher than that in the control group (fig 1). Between ex-cancer and non-cancer AA workers, however, no

Natural killer (NK) cell activity per whole mononuclear cells

	No of subjects	NK cell activity (LUs/ 10^7 cells)	
		Mean	Range
Ex-cancer			
AA workers	31	43.7	0.03 ~ 177
Non-cancer			
AA workers	32	20.1	0.5 ~ 269
Control group	33	47.1	3.7 ~ 104

LU = Lytic unit. Mean = Median value. AA = Aromatic amines.

Figure 1 Relative number of natural killer (NK) cells in lymphocytes identified by monoclonal antibody Leu 11a. Each point represents one subject. Transverse line indicates median.

difference was observed at all. Based on these results, unit NK cell activity was calculated in every individual. As is evident, both ex-cancer and non-cancer AA workers showed significantly reduced unit NK cell activity as compared with the control group (fig 2).

Discussion

No significant difference was found in the NK cell activity per whole mononuclear cells among ex-cancer AA workers, non-cancer AA workers, and the control group. The proportion of Leu 11a positive cells in the lymphocytes, on the other hand, was significantly higher in both ex-cancer and non-cancer AA workers than in the control group. These results suggested that both ex-cancer and non-cancer AA workers had significantly reduced cytotoxic activity per one NK cell. It remains to be discovered whether the increased proportion of peripheral Leu 11a positive cells in AA workers is due to the increase in absolute number of Leu 11a positive cells or to the decrease in other lymphocyte subsets such as T lymphocytes. Interestingly, it was reported by Jennings *et al* that the number of Leu 11a positive cells was also increased in workers exposed to dioxin 17 years before the study, although NK activity was not studied in the report.¹⁶ In either case it seems

Figure 2 Unit natural killer (NK) cell activity. Each point represents one subject. Transverse line indicates median.

conceivable that the reduced cytolytic capacity of each NK cell is somehow compensated by the relative increase in the number of circulating NK cells, thus maintaining the NK activity as a whole peripheral pool in AA workers. It was particularly noted that there was no significant difference in the unit NK cell activity between ex-cancer and non-cancer group of AA workers, suggesting that the reduction of the unit NK cell activity was primarily due to the exposure to AA per se rather than to the history of cancer. In patients with overt bladder cancer it had already been reported that the NK cell activity was greatly reduced¹⁰ despite the relative increase in Leu 11a positive cells (T Morita *et al.*) Thus the present observation in cancer free AA workers—namely, the reduced unit NK cell activity with apparently normal

range of NK cell activity per whole mononuclear cells—might be considered as a characteristic feature of the high risk population.

Kumar *et al* reported that the lymphocytes in workers exposed to α -naphthylamine, β -naphthylamine, and benzidine had an increased killing activity to bladder cancer cell line.^{7,8} The assay conditions in their study, however, differed from ours in the cytotoxicity assay time (four *v* 40 hours) and target cells (K562 *v* bladder cancer cell). Our present assay system primarily detects NK activity whereas theirs may well include cytotoxic activity of other effector cells than NK cells such as macrophages and T lymphocytes.

The mechanism for the altered lytic activity and proportion of NK cells in AA workers are totally unknown. It seems rather mysterious that such immunological alterations persist in workers who ceased to be exposed to carcinogenic chemicals nearly 20 years ago. This may imply either that the trace chemicals persisted in the body for years or that the effect was on the progenitor lymphoid cells with a self renewal potential. In a practical sense the present results show that the proportion of Leu 11a positive cells rather than gross NK activity provided a much more sensitive index to reflect immunological alterations in AA workers. This assay also has an advantage in that the proportion may be quantitatively and reproducibly determined by a flow-cytometric analysis as compared with sensitive bio-assay such as NK assay. We hope that the measurement of Leu 11a positive cells may turn out to be a helpful marker for the follow up of workers exposed to carcinogenic chemicals such as AA and dioxin.

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Requests for reprints to Professor S Araki.

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