Analysis of Peripheral Blood Lymphocytes of Atomic Bomb Survivors Using Monoclonal Antibodies

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(Received December 13, 1985)
(Revised version, accepted March 18, 1986)

Indirect immunofluorescence assay/Monoclonal antibody/Lymphocyte subsets/Atomic bomb survivors

In order to study the effects of exposure to atomic bomb radiation on the immune competence of man, the proportions of peripheral blood lymphocyte subsets (subpopulations) were determined by an indirect immunofluorescence antibody assay using monoclonal antibodies and fluorescence microscopy. The study was based on a total of 104 Adult Health Study participants in Hiroshima, including 29 individuals exposed to 100+ rad, 46 exposed to 1-99 rad, and 29 0 rad controls.

No change in the proportion of Leu-1 positive cells (total T cells) and Leu-2a positive cells (cytotoxic/suppressor T cells) and the ratio of Leu-3a/Leu-2a was observed with age, while Leu-3a positive cells (helper/inducer T cells) decreased with age and HLA-DR positive cells (B cells and monocytes) increased with age, with the differences occurring predominantly in the oldest age group (age > 75). The proportion of HLA-DR positive cells was higher in males, but there was no significant sex difference in the proportions of other cell types and the ratio of Leu-3a/Leu-2a. Radiation exposure did not significantly affect the proportions of Leu-1, Leu-2a, Leu-3a, and HLA-DR positive cells and the ratio of Leu-3a/Leu-2a. No interaction between the effects of age and radiation exposure was demonstrated.

INTRODUCTION

Human immunologic competence is maintained by complex intercellular interactions between a variety of lymphocyte subsets, macrophages, etc. Disturbances of the regulation of these interactions can lead to autoimmune disease and immunodeficiency with the development

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of various clinical symptoms. Accordingly, the study of lymphocyte subsets is an important first step in order to understand the mechanism of immunologic homeostasis.

Lymphocytes are highly sensitive to radiation relative to other mammalian tissues and cells, and it has been reported that lymphocyte counts and lymphocyte subsets change following radiation therapy for malignant tumors of various organs. Evaluation of lymphocyte subsets of A-bomb survivors may be useful for the elucidation of the association of carcinogenesis, aging, and susceptibility to infection with radiation exposure. Up until 1980, changes in peripheral blood lymphocyte of A-bomb survivors were studied by using their ability to bind sheep red blood cells to mark T cells as well as complement receptors as markers for B cells. However, the use of monoclonal antibodies specific to lymphocyte subsets, developed from the recently devised hybridoma technology has made it possible to analyze specific and detailed changes in T-cell subsets as well as in total T-cells. Accordingly, using new techniques, the following parameters were studied in A-bomb survivors who received examinations at Hiroshima RERF over a six week period: the changes in the proportions of peripheral blood lymphocytes such as T cells, cytotoxic/suppressor T cells, helper/inducer T cells, and B cells and monocytes. We report the results of fluorescence microscope analysis of the relationship between the proportions of lymphocyte subsets, aging, and exposure to A-bomb radiation.

MATERIALS AND METHODS

Samples

The study participants were selected from the Adult Health Study (AHS) population. In the AHS, medical examinations have been offered biennially since 1958 to a fixed population which initially numbered approximately 20,000. Age at examination and radiation dose (T65DR total dose) were used to classify participants into three and four categories, respectively (age: 36–55, 56–75, 76+; dose: 0, 1–49, 50–99, 100+). Persons not in Hiroshima at the time of A-bomb (NIC) or for whom T65DR dose estimates can not be calculated were excluded from this study. Recently though the accuracy of T65DR has been questioned, it is anticipated that the general ordering of subjects into unexposed, and low, medium, and high doses will be preserved under any new dosimetry system.

An attempt was made to select a stratified random sample of 108 persons from among those who visited Hiroshima RERF for AHS examinations over a six week period. Due to the time constraints and to the age-dose distribution of the AHS cohort, those aged over 56 years and in the 50–99 rad dose category were few in number.

Furthermore, since some diseases are known to affect the proportions of lymphocyte subsets, the disease histories, both before and after the AHS examination, of the 108 participants were investigated. Ultimately, the analysis was based on 104 persons, excluding four with diseases that are thought to affect lymphocyte subsets: two cases, one diagnosed as having chronic thyroiditis and the other multiple myeloma at time of examination; one case who died of stomach cancer about four months after examination; and one case who died of pancreas cancer six months after examination and who was presumed to have developed cancer before...
the examination. Two cases who had cervical cancer and breast cancer resections in 1971 and 1973, respectively, were included in this study since no clinical abnormalities were observed at the time of their AHS examinations (conducted 10 years and 8 years after surgery, respectively) and no relapse has been observed four years thereafter. These two cases were considered to represent complete cures and their past cancers were assumed to impart little or no effect on the test results.

Table 1 shows the distribution of the 104 participants by age, dose, and sex. Since the number of cases is small in the 50-99 rad dose group, the cases of this group were combined with the 1-49 rad group for statistical analysis. Moreover, since sex could not be controlled at the time of selection, the proportion of males is extremely small which made it difficult to conclusively investigate possible sex effects.

Isolation of peripheral blood lymphocytes

Five milliliters of venous blood were drawn from each subject, defibrinated using glass beads, and mixed with an equal volume of phosphate-buffered saline (PBS) in 15 ml centrifuge tubes (Corning Co.). Four milliliters of Ficoll-Hypaque (gravity: 1.077) were underlaid and the tubes were centrifuged at 400 \( \times \) g for 30 minutes at room temperature. The mononuclear cells at the interface were harvested and diluted with PBS; the mixture was centrifuged once at 510 \( \times \) g for 10 minutes and twice at 240 \( \times \) g for 10 minutes, and resuspended in PBS at a final concentration of 1 \( \times \) \( 10^7 \)/ml.

Analysis of peripheral blood lymphocyte subsets using indirect immunofluorescence antibody assays

The monoclonal antibodies used were anti-Leu-1, anti-Leu-2a, anti-Leu-3a, and anti-HLA-DR produced by Becton Dickinson Co. Anti-Leu-1 is a monoclonal antibody reactive with more than 95% of peripheral blood T cells, but not with most B cells, null cells, monocytes, or granulocytes. Anti-Leu-2a reacts with cytotoxic/suppressor T cells and anti-Leu-3a to helper/inducer T cells without exception. Anti-HLA-DR reacts with B cells, monocyte/macrophages, and activated T cells.
First, to 0.1 ml of four aliquots of each patient's lymphocytes (1 x 10⁶ cells) described above, 50 µl of each monoclonal antibody (anti-Leu-1, anti-Leu-2a, anti-Leu-3a, and anti-HLA-DR) diluted to 1/10 was added, allowed to react at 4°C for 20 minutes, centrifuged and washed twice in PBS. Then, 10 µl of antimouse IgG-fluorescein isothiocynate (FITC, 1:10, Tago Co.) was added to each tube and allowed to react at 4°C for 15 minutes, after which the mixture was centrifuged and washed twice before being fixed with 1 ml of 1% paraformaldehyde. More than 200 mononuclear cells were counted under the fluorescence microscope, and the percentage/proportion of membrane fluorescence positive cells was determined.

**Method of statistical analysis**

Data employed in the analysis were sex, age, A-bomb exposure dose, and the four lymphocyte marker determinations obtained through indirect immunofluorescence antibody assays, described above.

In this report, effects of the independent variables of sex, age, and exposure dose on lymphocytes percentages and the dependent variables were studied using analysis of variance and regression analysis. Appropriate normalizing transformations for the dependent variables were selected by examination of normal probability plots. For Leu-2a, HLA-DR positive cells and Leu-3a/Leu-2a, logarithmically transformed values were used as dependent variables in regression analyses. Since the percentage of lymphocytes reactive with the other two antibodies seemed to show relatively normal distributions they were used without transformation.

**RESULTS**

Figure 1-1,2 shows the means of proportions and their 95% confidence intervals for each subgroup defined by the age and dose categories. From this figure, several trends are present but the corresponding confidence intervals are so wide that the trends or effects are not conclusive and thus the data was examined more carefully with additional statistical tests.

Table 2 shows the summary of results of analyses of variance tests relating sex, age, and radiation dose to the proportions of Leu-1, Leu-2a, Leu-3a, and HLA-DR positive cells based on all 104 cases. This table indicates that: 1) a change with age is observed in the proportion of HLA-DR positive cells; 2) a difference by sex is also observed in the proportion of HLA-DR positive cells; 3) radiation effects are not evident; and 4) there is no significant interaction between age and radiation exposure. The number of males studied was too small (see Methods and Materials) to study the interaction of sex with the other variables.

Further analyses of each lymphocyte subpopulation are described below. Since no significant interactions were observed, we focused our attention on the main effects of the individual factors.

1) Leu-1 positive cells

As shown in Table 2, changes in the proportion of Leu-1 positive cells by age, radiation dose, and sex were not observed. As shown in Figure 1-1, the 76+ age-group and the exposed groups had lower values, but the differences were not significant. The female group also showed
lower values, but the difference was not significant either in the analysis of variance (Table 2) or in the regression analysis.

2) Leu-2a positive cells

As mentioned above, since normal probability plotting suggested that logarithmically transformed values of the proportion of Leu-2a positive cells are roughly normally distributed, the proportions of Leu-2a positive cells were analyzed after log transformation.

From the results of regression analysis and analysis of variance, there is no evidence of simple sex, age, or dose effects on the proportion of Leu-2a positive cells. None of those factors
individually was significantly related to this measurement (Table 2). The four lowest values are concentrated at the extreme of the age range, and hence they strongly influence the analysis. Thus analysis excluding these four points were also made. As in the aforementioned results, no effects due to exposure dose or sex were observed. In addition no significant effects of age were evident.

Fig. 1-2. Proportions of antibody positive cells by age and radiation dose

Left figure:
Based on the entire 104 cases

Right figure:
Excluding extreme points
Leu-3a: lowest 2 cases
HLA-DR: maximum and minimum cases
Table 2. Summary analysis of variance tables (Based on entire 104 cases)

| Effects                        | Sums of Squares | df | Mean Square | F    | P     |
|-------------------------------|-----------------|----|-------------|------|-------|
| Leu-1                         |                 |    |             |      |       |
| Sex                           | 12.34           | 1  | 12.34       | 0.0531 | 0.8   |
| Age                           | 510.1           | 2  | 255.0       | 1.133 | 0.3   |
| Dose                          | 144.0           | 2  | 71.99       | 0.3199 | 0.7   |
| Error 1                       | 22054           | 98 | 225.0       |       |       |
| Age-dose interaction          | 215.4           | 4  | 53.85       | 0.2318 | 0.9   |
| Error 2                       | 21838           | 94 | 232.3       |       |       |
| Leu-2a (log-transformed)      |                 |    |             |      |       |
| Sex                           | 0.2167          | 1  | 0.2167      | 0.7482 | 0.4   |
| Age                           | 1.112           | 2  | 0.556       | 1.963 | 0.15  |
| Dose                          | 0.0546          | 2  | 0.0273      | 0.0963 | 0.9   |
| Error 1                       | 27.76           | 98 | 0.2832      |       |       |
| Age-dose interaction          | 0.5275          | 4  | 0.1319      | 0.4553 | 0.8   |
| Error 2                       | 27.23           | 94 | 0.2897      |       |       |
| Leu-3a                         |                 |    |             |      |       |
| Sex                           | 21.01           | 1  | 21.01       | 0.1317 | 0.7   |
| Age                           | 463.7           | 2  | 231.9       | 1.501 | 0.2   |
| Dose                          | 178.6           | 2  | 89.3        | 0.5781 | 0.6   |
| Error 1                       | 15138           | 98 | 154.5       |       |       |
| Age-dose interaction          | 146.3           | 4  | 36.58       | 0.2294 | 0.9   |
| Error 2                       | 14992           | 94 | 159.5       |       |       |
| HLA-DR (log-transformed)      |                 |    |             |      |       |
| Sex                           | 4.304           | 1  | 4.304       | 16.92 | <0.0001 |
| Age                           | 1.430           | 2  | 0.7150      | 2.848 | 0.06  |
| Dose                          | 0.0948          | 2  | 0.0474      | 0.1888 | 0.8   |
| Error 1                       | 24.62           | 98 | 0.2512      |       |       |
| Age-dose interaction          | 0.7006          | 4  | 0.1752      | 0.6885 | 0.6   |
| Error 2                       | 23.92           | 94 | 0.2544      |       |       |

1. Sums of squares for each effect is calculated as the difference of the residual sum of squares between the model excluding the effect and the model including it.
2. The variance estimates (mean square) corresponding to this error were used to evaluate effects of age and dose factors. Therefore F-tests for these two factors and based on (2,98) degrees of freedom.
3. The variance estimates of this error were used to test sex and age-dose interaction effect. Degrees of freedom for these tests are thus (1,94) and (4,94), respectively.
3) Leu-3a positive cells

Because the distribution of the proportion of Leu-3a positive cells is almost normal, the measured values were employed in the analysis without transformation. Results of regression analysis show that age, sex, and exposure dose did not significantly affect the proportion of Leu-3a positive cells, as expected from Figure 1-2. However, it was possible that the two lowest proportions are masking the effect of age. However, analyses excluding these two points indicate that the effects of exposure dose and sex remain non significant (p=0.9). On the other hand, although heterogeneity among the three age categories was not significant (p=0.08), the mean proportion in the 76+ age group was about 6 percentage points lower than that of the other two groups, a marginally significant decrease (p=0.04) whether or not adjustment was made for the effects of the other covariates.

4) HLA-DR positive cells

The logarithmically transformed proportion of HLA-DR positive cells was used as the dependent variable in the regression analyses.

A large effect of sex was apparent, with the proportion being higher in males (geometric means: 22.2% for males, 13.5% for females); this effect was highly significant (p<0.0001) whether or not adjustments were made for the other covariates. After adjustment for sex and age, differences between the three radiation dosage groups were not statistically significant (p=0.8). Again special attention had to be paid to the proportion of HLA-DR positive cells in relation to age, since one value at each end point of the age range seems to deviate from the general pattern, thereby possibly distorting the effect of age. On the other hand, when analyses were performed excluding these two values, there was a highly significant heterogeneity (p=0.003), with the mean for the 76+ age group significantly higher than that of the other two groups combined by a factor of 1.4 (p=0.01). The difference between the 56-75 and <55 age-groups was not statistically significant (p=0.3). The regression models including linear and/or quadratic terms in age were also studied and all showed an increasing trend with age.

Table 3.  Regression coefficients of Analysis of the ratio of Leu-3a/Leu-2a (log-transformed)

|                  | Grand Mean | Sex     | Age 56-75 | Age 75+ | Dose 1-99 | Dose 100+ | Age | Dose |
|------------------|------------|---------|-----------|---------|-----------|-----------|-----|------|
|                  | -1.099     | -0.140  | -0.007    | -0.066  | 0.0002    | 0.038     |     |      |
|                  |            |         | (0.174)   | (0.139) | (0.145)   | (0.151)   |     |      |
|                  |            |         | (0.174)   |         | (0.149)   |           |     |      |
|                  |            |         |           |         | (0.163)   |           |     |      |
|                  |            |         |           |         |           |           | -0.0003 | (0.004) |
|                  |            |         |           |         |           |           | -0.00002 | (0.0005) |
|                  |            |         |           |         |           |           | 37.33 | 36.84 |
|                  |            |         |           |         |           |           |       | 36.95 |
| Sum of Squares   |            |         |           |         |           |           |       |      |
| Degrees of Freedom| 102       | 97      |           |         |           |           |       | 99    |

( ): Standard error
5) Leu-3a/Leu-2a

Since logarithmically transformed values of the ratio of Leu-3a/Leu-2a are roughly normally distributed, the ratios of Leu-3a/Leu-2a were analyzed after log transformation. Results of regression analysis show that age, sex and exposure dose did not significantly affect the ratio of Leu-3a/Leu-2a (Table 3).

**DISCUSSION**

Whether immune competence is altered in A-bomb survivors as a late effect of exposure to ionizing radiation has not yet been fully elucidated, although currently available evidence is all negative. We analyzed lymphocyte subsets as a general measure of immune competence of A-bomb survivors.

Rosette formation techniques, methods using heterogeneous antiserum, and other methods have been employed in the past for the identification of lymphocyte subsets. However, with the development of the hybridoma production technique by Kohler and Milstein, a large number of monoclonal antibodies have now been produced. Using these antibodies, investigations of lymphocyte subsets can now be performed in greater detail and more accurately than before.

Changes in lymphocyte subsets that occur in certain diseases are being clarified. For example, a decrease in the number of cytotoxic/suppressor T cells is observed in autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjögren syndrome. An increase of cytotoxic/suppressor T cell and a decrease of helper/inducer T cell numbers are reported for infectious viral diseases such as infectious mononucleosis, cytomegalovirus infection, and hepatitis B (HB) viral hepatitis. In this regard, it is of interest that the proportions of total T cells were low in the two cases who were not under treatment for cancer at the time of examination and died of cancer within a year after examination, although these cases were excluded from the analysis in this study (Table 4).

The results of the present study revealed no effect of radiation exposure on the proportions of peripheral blood lymphocytes positive for Leu-1 (T cells), Leu-2a (cytotoxic/suppressor T cells), Leu-3a (helper/inducer T cells), or HLA-DR (B cells and monocytes) and the ratio of

| Age | Sex | Leu-1 | Leu-2a | Leu-3a | HLA-DR | Case Details |
|-----|-----|-------|--------|--------|--------|--------------|
| 81  | Female | 31.2  | 4.3    | 2.9    | 13.8   | Died of stomach cancer four months after examination |
| 75  | Female | 25.9  | 10.2   | 30.8   | 28.0   | Died of pancreas cancer six months after examination |
| 82  | Male  | 51.4  | 11.0   | 28.0   | 7.3    | Multiple myeloma (IgA-\(\lambda\)) |
| 82  | Female | 52.1  | 4.5    | 39.0   | 32.5   | Chronic thyroiditis |
Leu-3a/Leu-2a in A-bomb survivors. Concerning the lymphocyte subsets of A-bomb survivors, Akiyama et al.\(^1\) reported that the proportions of T and B cells derived by the rosette technique were not related to exposure. With regard to the function of T cell subsets, activity of suppressor T cells is reported to be more sensitive to radiation than that of helper T cells.\(^2\) On the other hand, Wasserman et al.\(^2\) reported that the subset of T cells possessing IgG-Fc receptors derived by the rosette technique decreased after X-irradiation of 1,600 rad in vitro, and that no change was observed in the proportion of either Leu-2a or Leu-3a positive cells.

Concerning the effect of age on lymphocyte subsets, many reports state that T cells generally decrease with age,\(^2\)\(^{22-25}\) but others state that they remain unchanged.\(^2\)\(^{26,27}\) Nagel et al.,\(^2\) using monoclonal antibodies, reported that although the proportion of OKT4 (helper/inducer T cells) showed no change, OKT3 (T cells) and OKT8 (cytotoxic/suppressor T cells) decreased with age. Concerning the proportion of B cells, some investigators reported no difference with aging\(^2\)\(^{25,29}\) while others reported an increase.\(^2\)\(^3\) Our results showed that there was no difference in the proportions of Leu-1 positive cells and Leu-2a positive cells and the ratio of Leu-3a/Leu-2a with age, although the proportions of Leu-3a positive cells tended to decrease and that of HLA-DR positive cells tended to increase. The effect of age is concentrated primarily in the oldest age group (76+) for the proportions of Leu-3a positive cells and HLA-DR positive cells.

Matsumoto et al.\(^3\) have reported on the sex difference in the subsets and subpopulations of lymphocytes, stating that higher values of OKT4 positive cells are observed in females and of OKT8 cells in males and no sex difference were observed in OKT11 (total T cell) and OKIa (B cell). Our results showed that the proportion of HLA-DR positive cells (B cells and monocytes) is higher in males.

In this study, lymphocyte subsets were determined using a fluorescence microscope. To minimize errors in the determination, a single trained viewer counted all lymphocytes. Identification was made without knowledge of dose, age, sex, etc. Also results obtained by the trained technician were almost identical to those obtained using Fluorescence Activated Cell Sorter. In addition, we do not know why our ratios of Leu-3a/Leu-2a are higher than those of other reports.

Except for finding a reduced phytohemagglutinin reactivity of peripheral blood lymphocytes with age in the exposed group,\(^1\) studies of cellular immunity of A-bomb survivors conducted so far have shown no effect of exposure on the proportions of lymphocyte subsets, the differentiation ability of B lymphocytes, or the function of concanavalin A-induced suppressor T lymphocytes.\(^3\) Based on the current data, there was no significant effect due to radiation exposure. A difference, if any exists, in immune competence between A-bomb survivors and the nonexposed may be too small to be detected by such immunological tests at the present time, some 40 years after the A-bombings.

However, the increased incidence of breast, lung, and thyroid cancers in A-bomb survivors is present even now. It is clear that immunocompetence is important in carcinogenesis. While the immunologic surveillance theory\(^3\) of defense against cancer is now recognized as not applicable to all cancer types, persons whose immune systems are suppressed by drugs have an increased risk of some neoplasms.\(^3\) Thus we can not rule out the possibility that A-bomb exposure may still have some effects on immunocompetence. In the continued interest of
accurately defining the late effects of the atomic bombs, the qualitative and quantitative characteristics of the A-bomb radiation exposure doses are periodically refined. If warranted by future dose assessments, the data reported here will be reanalyzed and subsequently reported.

ACKNOWLEDGMENT

The authors wish to express their sincere appreciation to Mrs. Yoshiko Watanabe and Mrs. Kyoko Ozaki of the Immunology Laboratory for the great technical support they have extended in the conduct of this study. We are indebted to Drs. Kenneth J. Kopecky and Michael A. Bean for discussion of the data and review of the manuscript.

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