II. BIOLOGICAL EFFECTS

Overview of Immunological Studies on A-bomb Survivors

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Immune responses PHA/MLR/Lymphocyte subsets
Among the peripheral blood lymphocytes, T-cells and B-cells significantly decreased in number with age. Radiation exposure resulted in further significant decrease of T-cell count (but not B cells) in the elderly. T-cell response to PHA and allo-antigens also decreased with dose in the elderly group. In contrast, NK cell number and function increased with age while a significant dose effect was not observed.

INTRODUCTION

More than forty years after exposure, the A-bomb survivors still exhibit increased relative risks to several kinds of solid tumors\(^1,2\). The immune system is believed to be involved in resistance to development of tumors in humans and in animals. T-cells, B-cells, NK cells and lymphokine activated killer cells have been especially implicated in protection against, or response to cancer. In addition, the immune system is a prime target of radiation injury, viz., the bone marrow and thymus, where immunocompetent cells develop, which are exquisitely sensitive to radiation. These relationships between radiation, cancer, and the immune system prompted us to undertake immunobiological studies on A-bomb survivors approximately 35–45 years after their radiation exposure.

Since immune response depends on the interactions of various types of immunocompetent cells, changes in the composition of the immune cells appeared important. However, it had been difficult to identify particular types of cells in the immune system. In recent years, with the development of a series of monoclonal antibodies (MoAbs) specific to cell surface antigens of lymphocyte subpopulations, it has become possible not only to examine changes in the composition of lymphocyte subpopulations which may be affected by both aging and radiation exposure but also to detect various rare types of cells.

A brief summary of our recent studies will be made here mainly on four subjects; lymphocyte subset frequency, lymphocyte responses to phytohemagglutinin (PHA) and alloantigen, NK activity and rare mutant T-cells. As for the earlier immunological studies, please see a review by Finch\(^3\).
MATERIALS AND METHODS

1. Lymphocyte subset measurements:
   The assay conditions have been fully described previously\(^4\). In brief, peripheral blood mononuclear cells were isolated and stained with various monoclonal antibodies to identify and enumerate lymphocyte subpopulations of the following characters using either a fluorescent microscope or flow cytometry:
   - CD5 antigen-positive mature T cells,
   - CD4\(^+\) helper/inducer T cells,
   - CD8\(^+\) suppressor/cytotoxic T cells,
   - CD19\(^+\) B cells, and
   - CD16 or 57 positive cells (which mainly consist of natural killer (NK) cells).

2. Lymphocyte response to PHA and alloantigens:
   The details of the assay have been previously described\(^5\). In brief, peripheral blood mononuclear cells were either stimulated by PHA or by X-ray inactivated allogeneic lymphocytes and their responses were measured by incorporation of \(^3\)H-thymidine into the acid insoluble fraction of DNA.

3. NK activity:
   Peripheral blood mononuclear cells were co-cultured with the K562 human tumor cell line labeled with \(^51\)Cr and release of \(^51\)Cr from target cells was taken as an index of NK activity\(^6\). Similarly, antibody dependent cell mediated cytotoxicity (ADCC) activity was measured by release of \(^51\)Cr from labeled chicken erythrocytes in the presence of rabbit anti chicken erythrocyte antibody (IgG)\(^7\).

4. Detection of rare variant T-cells:
   Mutations of the T-cell receptor genes (mainly \(\alpha\) and \(\beta\)) and the HLA-A gene were measured by flow cytometry using a pair of monoclonal antibodies, anti CD3 and anti CD4 in the former\(^8\) and anti HLA-A2 or A24 antibody and anti CD3 antibody in the latter\(^38\).

RESULTS

1. T-cell subpopulations:
   1) Effect of aging:
      A total of 850 survivors were examined in this study. It was found that the absolute number of lymphocytes bearing CD4, CD5 or CD8 antigen decreased significantly with age\(^4\) (p<0.001). When the examinees were divided into three age groups, the CD4/CD8 ratio was found to be significantly elevated in the middle age group while linear regression for the three age groups did not show any significant age-associated tendency. The ratio for females was significantly higher than that for males. This observation may be consistent with the fact that the incidence of autoimmune diseases is higher in females than in males.
In regard to the effects of aging on T cells, our results are consistent with those reported by others\textsuperscript{9–11}.

2) The effects of A-bomb radiation on the number of T cells:

The analysis of the late effects of radiation on the absolute number of CD5\textsuperscript{+} mature T cells in peripheral blood lymphocytes (PBL) showed a significant decrease (p = 0.048) in those survivors who were exposed to more than 0.5 Gy at age 30 or more (70 years or more at the time of testing) in comparison to those who were unexposed and were in the same age group\textsuperscript{4}. A similar result of decreasing numbers was also observed for CD4 antigen-positive cells in person who were more than 30 years of age at the time of bomb (ATB) and were exposed to more than 0.5 Gy (p = 0.04 for male + female). For another T cell subset, CD8\textsuperscript{+} suppressor/ cytotoxic T cells, no significant effects of radiation were observed in any age group of both male and female. A significant decrease in CD4/CD8 ratio was observed in the males who were 30 years or older ATB. This observation appears to suggest that exposure of especially older males to A-bomb radiation caused a decrease in the number of CD4\textsuperscript{+} lymphocytes among T cells.

2. T-cell functions

Lymphocyte responses to PHA and to allo-antigens were examined for 800 and 150 survivors, respectively.

Among the older and heavily exposed individuals, significantly lower responses to PHA were observed\textsuperscript{12}. Since whole mature T cells (CD5\textsuperscript{+} cells) in PBL are involved in this assay, it is consistent with the results for decreased cell number of CD5\textsuperscript{+} mature T cells as described above. The responsiveness of T cells to allo-antigens, the so-called mixed leucocyte culture response (MLR), was found to decrease significantly with increasing radiation dose\textsuperscript{5}. Since a major portion of cells responding to allo-antigens belong to the CD4\textsuperscript{+} helper/inducer subset of mature T cells, this observation can be attributed, at least partly, to the decrease in the number of CD4\textsuperscript{+} cells.

3. Detection of anomalous and rare-event (mutant) T cells

1) Detection of unusual T cells differentiated outside the thymus

Most normal T cells differentiate in the thymus and express both CD3 and CD4 or CD3 and CD8 antigens but not CD57 antigen. CD57\textsuperscript{+} T cells are considered to differentiate outside and thymus and thus to be unusual T cells\textsuperscript{13}. As mentioned previously, the number of T cells decreases with age. However, among the T cell population, certain (unusual) T cell subpopulations appear to increase with age. The frequency of CD57 positive T cells among mature T cells (CD3\textsuperscript{+} cells) has been measured for about 180 A-bomb survivors. Although CD3\textsuperscript{+}57\textsuperscript{+} to CD3\textsuperscript{+} cell ratios for control and exposed (\textgreater{} 1.5 Gy) groups were similar, both group showed an increased proportion of CD3\textsuperscript{+}57\textsuperscript{+} T cells with increasing age (unpublished data).

2) Detection of rare-event T cells, mutants of immunologically functional loci

Another approach developed for assessing somatic mutations in A-bomb survivors is the detection of unusual T cells lacking immunologically functional molecules on their surface. This
topic will be reviewed in other sections of this issue. Recently, in our department, methods were developed to detect the loss of the expression of the CD3-T cell receptor complex\(^3\) or HLA-class I antigens, such as A2 and A24\(^9\), in peripheral blood T cells. Spontaneous mutant frequencies (Mf) in these immunologically functional molecules are considerably higher (Mf order is \(\times 10^{-3} - 10^{-4}\)) than those observed for other loci frequently studied in man, such as the HPRT gene in T cells (Mf order is \(\times 10^{-6}\))\(^{14}\) and the GPA gene in erythrocytes (Mf order is \(\times 10^{-5}\))\(^{15}\), but are similar to those of the immunoglobulin gene in B cell lines\(^{16,17}\).

4. B cells

Studies concerning the B cell lineage at RERF are still in progress, so that most of the results are not yet conclusive.

Serum immunoglobulin (Ig) G, A and M levels were measured twice in the past\(^{18,19}\) and these Ig levels and immunocomplex levels\(^6\) appear to vary with age. However, at this time we have not observed an effect of radiation on their levels.

To investigate the frequency of autoimmune diseases, autoantibodies, such as rheumatoid factor, anti-nuclear antibody, anti-thyroglobulin antibody, and anti-microsomal antibody have been measured (Fujiwara et al. Rp)\(^{20}\). The frequency of these antibodies and the occurrence of autoimmune diseases as well as a recent analysis of serum IgG, A, M and E levels among survivors will be reported within a year.

A-bomb survivors are also being surveyed for serum antibody titers against EB virus antigens, (VCA, EA, EBNA). More than 90% of the population is infected with the EB virus in childhood and coexist with it opportunistically. If, however, immune functions in survivors are altered, reactivation of the EB virus may occur in vivo resulting in changes of Ab titers to the EB virus. Current results indicate that the titer of antibody to EA antigen (which is considered to increase when EB virus are reactivated in vivo) was significantly increased in exposed people compared with controls (p<0.05) but antibody-titer to EBNA (which is considered to increase when EB virus infected cells are destroyed by cytotoxic T cells) was not significantly increased among exposed people compared to the control group\(^{21}\). These findings correlate with findings by others\(^{22,23}\). These results suggest that reactivation of latent EB virus occurred more frequently in exposed people than in control people, probably due to their altered immune competence.

Several B-cell subsets have also been examined. It was found that the number of CD19 antigen positive cells which includes most of the mature B cells, decreased significantly with age but no significant dose dependent relationships were seen\(^9\).

5. NK cells

In contrast to the findings of T cells, the number CD16\(^+\) or CD57\(^+\) NK cells significantly increase with age\(^4\) among about 900 survivors. This should not be misconstrued as related in any way to the decrease of T and B cells, since the absolute number of NK cells actually increases. Furthermore, the absolute number of CD16\(^+\) cells in males was significantly higher than in females, at every age group. NK activity in PBL from about 1300 survivors was also examined and was found to significantly increase with increasing age\(^6\). In addition, Nk activity in males
was significantly higher than in females. These results correlate with findings observed for the number of CD16 and CD57 positive cells. Another functional study of the NK cell population, antibody-dependent cell-mediated cytotoxicity (ADCC) activity examined among about 800 survivors after bombing showed similar results of NK activity.7

With respect to radiation effects on the NK cell population, we have no definite conclusions. A statistically significant dose effect on the number of NK cells was observed only in CD16+ cells among male survivors 70 years or more of age at time of testing, whereas no significant dose effects on the number of CD57 positive cells was observed for any age group of both male and female. The same is true for the NK activity and ADCC activity for both sexes.

DISCUSSION

Earlier immunological studies for the atomic bomb survivors have been reviewed by Finch3. In brief, the immunological endpoints studied were as follow: anti-influenza virus antibody, hepatitis associated antigen, anti-EB virus antibody, bacteriocidal test, immunoglobulin level, leukocyte phagocytosis, chemotaxis, parietal cell antibody, auto antibody, blood group antibody, prevalence of tuberculosis, urinary infection, prevalence and incidence of rheumatoid arthritis etc. No significant dose-effects have been observed for these endpoints. Some of these measurements were conducted again using more advanced techniques.

Table 1 summarizes all the results that we have described here. Most of these studies used bulk culture assays dependent on complex cellular interactions and radiation effects might have been masked or underestimated. Nevertheless, for T-cells, these functional and numerical abnormalities could be observed in A-bomb survivors more than 40 years after their exposure. The present results can be explained as follows.

As described above, the immune system, in which T cells are involved, is affected by radiation especially in older survivors at the time of bombing. An age-related degradation of the thymus is considered to be responsible for these age-dependent radiation effects. Younger individuals possessed a fully functional thymus at the time of exposure, whereas the thymus of older individuals had been hypofunctional and in a state of atrophy at that time. Therefore we hypothesize that shortly after exposure to A-bomb radiation, mature lymphocytes had been damaged and reduced in number among both younger and older persons, while the recovery process(es) differed between the two groups. In younger survivors, the process of maturation from the precursor cells to mature T cells in the thymus continued almost normally. In contrast, in older survivors, the same process would have been incomplete and a decreased number of T cells could still be observed even more than 40 years after the radiation exposure.

This hypothesis is supported by the study of Hirokawa et al.24) in mice. After receiving lethal, whole body irradiation, followed by homologous bone marrow transplantation, adult mice exhibited a much lower T-cell functional than untreated mice of the same age. In contrast, newborn mice treated similarly exhibited to decrease in immune function.

The increase in functional and numerical abnormalities of T cell lineage can be accounted for by increases of immature and unusual or mutant T cells.

Since CD57+ cells rather than normal, CD57− T cells have been reported to be functionally
Table 1. Effects of Age and A-Bomb Radiation on the Immune System (30-40 years after exposure)

<table>
<thead>
<tr>
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<th>Age</th>
<th>Radiation</th>
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<tr>
<td>T-cell immunity</td>
<td></td>
<td></td>
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<tr>
<td>Number of mature T cells</td>
<td>↓</td>
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<tr>
<td>Number of anomalous T cells</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>Functions</td>
<td>↓</td>
<td>↓</td>
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<tr>
<td>B-cell immunity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of mature B cells</td>
<td>↓</td>
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<tr>
<td>Natural immunity</td>
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<tr>
<td>Number of NK cells</td>
<td>↑</td>
<td>N.S.</td>
</tr>
<tr>
<td>Functions</td>
<td>↑</td>
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</tbody>
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N.S.: Not significant

Studies on B-cell lineage have just started, such as detection of unusual B cells lacking expression of surface immunoglobulin.

defective\textsuperscript{25,26}, the apparent age-associated increase in CD57\textsuperscript{+} T cells may be an evidence for an abnormality, such as the age-dependent decline of T cell function. Further, it has been suggested that CD57\textsuperscript{+} T cells might develop outside the thymus\textsuperscript{13}, consistent with age-related thymic dysfunction. Therefore, one of the major causes of a decreased number and function of mature normal T cells could be attributed to the fact that T cells were forced to differentiate without the influence of the thymus resulting in greater numbers of CD57\textsuperscript{+} T cells in older A-bomb survivors after their initial radiation induced depletion.

Further, additional evidence showing an increased Mf in immunologically functional genes, TCR and HLA-class I genes in T cells may account for the abnormalities of T cell immune system in survivors.

As it is known that TCR and HLA genes belong to the immunoglobulin gene super family\textsuperscript{27}, our findings suggest that mutation rates in such genes are much higher than those in the ordinary genes. Furthermore, there are many molecules, products of the immunoglobulin gene super family\textsuperscript{27,28}, important for the functioning of T-cells. Thus, other abnormal T cells lacking immunologically functional molecules may accumulate in the body either by aging or by radiation-induced mutagenesis and may cause the altered immune function.

In order to determine age and radiation effects on the B cell immune system more precisely, studies to examine B cells positive for both CD20, mature B cell antigen, and CD5 antigen using flow cytometry are in progress. Although CD5 is mainly expressed on mature T cells, some B cells have this antigen at low density. CD5\textsuperscript{+} B cells probably participate in producing autoantibodies\textsuperscript{29–32}. Further, it is planned to examine the frequency of rare event B cells such as CD20 positive cells lacking expression of surface Ig, which are probably mutant B cells.

Although reports on the effect of age and sex on NK cells are controversial\textsuperscript{33–37}, our results
suggestion that the development of NK cells from their precursor cells are probably affected by aging in different ways than T and B cells.

Furthermore, we still are unable to conclude definitively whether A-bomb radiation affected the NK cell population. More sophisticated approaches may provide an answer or perhaps animal experiments may be required to collect more information in this area.

FUTURE PLANS

Several new approaches are under considerations or in progress, such as HLA typing, repertoire analysis of TCR and immunoglobulin genes, radiation effects on lymphoid stem cells and precursor cell frequency response to various kinds of antigens.

Quantitative measurements of lymphocytes bearing rare phenotypes or mutations are also in progress. Although the TCR mutation assay described here did not reveal a strong dose-related increase of Mf among survivors, it appears important to pursue cellular and molecular approaches for such genes involved in immune responses.

Finally, the possibility of age and/or radiation-induced changes of the immune system in relationship to carcinogenesis and susceptibility to other environmental mutagens or aging related diseases should be explored in depth in future.

REFERENCES


factor-like antibodies from CD5 (Leu-1)+ B cells are polyreactive. J. Immunol. 140: 4180-4186.