EVALUATION OF ANTICANCER DRUGS BY LYMPHOCYTE ELECTROPHORESIS

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(Received February 10, 1986)

The spleen cells in tumor-bearing and normal mice treated with Krestin (PSK), mitomycin C (MMC) or adriamycin (ADM) were analyzed by cell electrophoresis and flow microcytometry. In normal mice, the splenocyte electrophoretic mobility histogram was observed as a bimodal pattern, and low and high mobility cells (LMC and HMC) corresponded with B and T cells, respectively. In sarcoma-180-bearing mice, an intermediate mobility peak (IMC) appeared between the low and high peaks. Although every anticancer drug depressed the IMC when the tumor was cured, MMC reduced the absolute number of splenic Ig* and Thy-1* cells, and ADM injured Ig* cells in normal as well as in tumor-bearing mice. PSK, however, depressed splenomegaly by tumor-burden in spite of a slight increase in splenocytes of normal mice. In a previous paper, it was reported that the thymocyte mobility histogram was restored to a normal pattern by treatment with PSK in tumor-bearers, while it was made more abnormal by treatment with MMC because of injury to cortical thymocytes. From these results, it may be considered that an anticancer drug which restored the splenocyte mobility histogram to a normal pattern without damages to thymocytes is preferable for cancer therapy.

Keywords — lymphocyte electrophoresis; surface marker; tumor-bearing; PSK; mitomycin C; adriamycin; drug evaluation

INTRODUCTION

In a previous paper,1) it was reported that PSK, an anticancer protein-bound polysaccharide, restored the histogram of thymocyte electrophoretic mobility in sarcoma-180-bearers to a normal pattern while mitomycin C (MMC) caused a more abnormal pattern than that in the non-treated group, even though both drugs cured the tumors 21 d after tumor inoculation. The population of low mobility cells containing immature and cortical thymocytes (Lyt-1+·2+)2) decreased markedly in the MMC-treated mice. On the other hand, it was reported that splenocytes in normal mice showed a skewed distribution in cell electrophoresis due to the presence of two electrophoretically different populations of T and B cells.3,4)

In the present study, we determined electrophoretic changes of splenocytes in the course of tumor development and administration of the anticancer drugs, PSK, MMC or adriamycin (ADM). We also confirmed that restoration of the splenocyte electrophoretic pattern was associated with the anticancer effect of the drugs whose mode of action was different from others.

MATERIALS AND METHODS

Animal and Tumor — ICR strain female mice (obtained commercially from CLEA Japan Inc., Tokyo) 5–6 weeks old were used throughout this study. Sarcoma-180 was maintained in ICR mice by a weekly intraperitoneal transfer of 1 × 10⁶ tumor cells.

Chemicals — Hanks' balanced salt solution (HBSS) and Eagle's minimum essential medium (MEM Eagle) were purchased from Gibco, U.S.A. MMC and ADM were commercially obtained from Kyowa Hakko Kogyo Co., Ltd., Tokyo and PSK was obtained from Kureha Chem. Ind., Tokyo. Fluorescein-isothiocyanate (FITC)-anti Thy-1, 2, FITC-anti Lyt-1 and FITC-anti Lyt-2; rabbit anti aGM; and FITC-anti rabbit IgG and FITC-anti mouse IgG for flow microcytometric analysis were purchased from Becton Dickinson & Co. (U.S.A.), Wako Pure Chem. (Japan) and Miles Lab. (U.S.A.), respectively.

Administration of Drugs — Sixty mice were inoculated subcutaneously in the right flank with sarcoma-180 cells (1 × 10⁶). The sixty mice were divided into 4 groups; a non-treated con-
trol group, a PSK treated group, a MMC treated group and an ADM treated group. PSK (10 mg/kg), MMC (1 mg/kg) or ADM (2 mg/kg) was intraperitoneally injected, every other day, for a maximum of 20 d from the day after tumor inoculation. On the measuring day, three mice were sacrificed and each spleen was removed for measurement. At the same time, the weight of each tumor was measured. The same measurements were made with control mice.

Preparation of Splenocytes — Spleen cells were prepared as described by Veit et al. The cells were finally suspended in a medium required for subsequent manipulations. The viability of the cells, which was assessed by 0.5% trypan blue dye exclusion, was 90—95%. For removal of phagocytic cells, splenocytes, $1 \times 10^8$ in 10 ml of nutrient medium supplemented with 10% fetal bovine serum, were incubated at 37 °C for 30—45 min with 50 mg of carbonyl iron particles (for lymphocyte preparation: Wako Pure Chem. Japan) in a plastic bottle. The cells containing the carbonyl iron particles were magnetically removed from those not adhering according to the procedure described by Golstein et al. 6)

Separation of T Cells and B Cells from Splenocytes — T cells and B cells were separated by treatment with a nylon wool column (Nylon Fiber for separation of T cells; Wako Pure Chem. Japan), described by Julius et al. The effluent cells (T cells) were eluted with the medium and the adherent cells (B cells) were released by washing the nylon wool.

Cell Electrophoretic Measuremet — The electrophoretic mobility of lymphocytes was determined with a fully automated cell electrophoretic apparatus (Parmoquant-L; produced by Kureha Chem. Inc. Co., Ltd., Tokyo). As the details of the apparatus and method of measurement were described previously, only a brief description is given here. Cells were suspended in MEM Eagle at an approximate concentration of $5 \times 10^5$ cells/ml and placed in the rectangular quartz chamber in 2 ml amounts. Electrophoresis was performed at 13 mA for 3 s at 24 °C. Ten to twenty migrating cells, in view, were traced by image processing and the position of each cell was stored instantaneously. After two hundred cells were measured, the data and conditions of electrophoresis were printed out.

Surface Marker Analysis of Splenocytes — One hundred μl aliquots of single cell suspensions ($1 \times 10^7$ cells/ml) were incubated with an appropriate FITC-labeled monoclonal antibody for 30 min at 4 °C and then washed twice. In the case of the anti-aGM1 antibody, cells were incubated with antibody followed by washing, and then incubated with FITC-labeled anti-rabbit IgG antibody for 30 min at 4 °C. The percentage of cells expressing Ig+, Thy-1+, Lyt-1+, Lyt-2+, aGM1+, phenotypes were then determined by a fluorescence-activated cell sorter (FACS IV; Becton-Dickinson, Mountain View, CA).

RESULTS
Changes in the Tumor Growth and the Spleen Weight in Sarcoma-180-Bearing Mice Treated with PSK, MMC or ADM

Seven to 28 d after subcutaneous inoculation of sarcoma-180, tumor growth was evaluated by weighing the tumor mass after sacrificing the mice (Fig. 1). The percentages of the inhibition of tumor growth 28 d after tumor inoculation were 100% in the PSK-treated group, 91% in the MMC-treated group and 95% in the ADM-treated group. The hosts were almost free from tumors after 4 weeks.

In the tumor-bearing mice, PSK suppressed spleen enlargement in comparison with untreated tumor bearers (Fig. 2 (b)). ADM made the spleen smaller than normal after 3 weeks, but it

![Graph showing tumor weight over time](image-url)

**Fig. 1. Tumor Weight of Tumor-Bearing Mice Treated with PSK, MMC or ADM**

- ×, — ×, non-treated; ○ — ○, PSK-treated;
- Δ — Δ, MMC-treated; □ — □, ADM-treated.
Anticancer Drugs and Lymphocyte Mobility

![Graph](image)

**FIG. 2.** *Spleen Weight in Normal or Tumor-Bearing Mice Treated with Antitumor Drugs*

- × — ×, non-treated;
- ○ — ○, PSK-treated;
- △ — △, MMC-treated;
- □ — □, ADM-treated.

returned to normal weight after 4 weeks. MMC was inclined to decrease the spleen weight.

When the antitumor agents were injected into normal mice, the spleen weight of PSK-treated mice slightly increased. On the contrary, the spleen weight of MMC- or ADM-treated mice was inclined to decrease compared with those in non-treated mice (Fig. 2 (a)).

**Changes in the Electrophoretic Mobility Histogram of Splenocytes during Tumor Growth**

The histogram of splenocyte electrophoresis in normal mice shows a bimodal pattern, a major peak with approximate mobility of 0.7 μm/s/V/cm, and a minor peak of 1.1 μm/s/V/cm (Fig. 3). In tumor-bearing mice, cells with an intermediate mobility between the lower and the higher mobility peaks increased as the tumor developed. Fourteen days after tumor inoculation, the electrophoretic mobility pattern showed a strong peak with a mobility of approximately 0.9 μm/s/V/cm. After 21 d this strong peak disappeared and a new peak with a mobility of 1.0 μm/s/V/cm appeared which was smaller than that of the normal peak.

The borderlines between the low and intermediate mobility cells and intermediate and high mobility cells (LMC, IMC, HMC) were tentatively placed at mobility values of 0.8 and 1.0 μm/s/V/cm, respectively.

**The Electrophoretic Mobility Pattern of Splenocytes Fractioned by a Nylon Wool Column**

From normal mice, unfractinated sple-
nocytes exhibited a bimodal mobility pattern, where as each subpopulation of cells that either adhered to or did not adhere to the nylon wool exhibited a unimodal pattern (Fig. 4). The cells which adhered to the column had a mobility of 0.6 to 0.8 μm/s/V/cm which corresponded to the slower peak in the pattern for unfractionated splenocytes. The non-adherent cells had a mobility of 1.0 to 1.2 μm/s/V/cm which corresponded to the faster peak in the pattern for unFractionated splenocytes.

In tumor bearing mice, the bimodal pattern of the unFractionated splenocytes was not very clear and the subpopulations, fractionated by nylon wool, showed broader patterns; that is, the adherent cells contained faster cells (0.8 to 1.0 μm/s/V/cm) and the non-adherent cells contained slower cells (0.7 to 1.0 μm/s/V/cm).

Changes in the IMC and LMC of Splenocytes during Treatment with Antitumor Drugs

Figure 5 shows the percentages of IMC in the spleen. IMC increased with tumor growth in the non-treated group. In contrast, in the PSK-or chemotherapeutic agent-treated groups, the increase of IMC was inhibited, and the percentages of IMC dropped to normal levels after a complete cure. When anticancer drugs were injected into normal mice, the IMC of the splenocytes tended to increase in the PSK-treated group 7 to 14 d after drug administration and to decrease in the MMC-treated group but there were no changes in any of the groups 28 d later.

Figure 6 shows the percentages of LMC in the spleen. LMC tended to decrease with tumor growth. PSK and MMC restored the LMC to a normal level, but ADM decreased the level below that of the untreated control group. These

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**FIG. 4.** Electrophoretic Mobility Pattern of Splenocytes in Normal or Tumor-Bearing Mice (35 d after Inoculation) Fractionated by the Nylon Wool Column
(a), (d) unFractionated cells; (b), (e) adherent cells to nylon wool; (c), (f) non-adherent cells to nylon wool.

**FIG. 5.** Incidence of Intermediate Mobility Cells (0.80 < Mobility < 1.00 μm/s/V/cm) of Splenocytes in Normal or Tumor-Bearing Mice Treated with Antitumor Drugs
- × - ×, non-treated; ○ - ○, PSK-treated; Δ - Δ, MMC-treated; □ - □, ADM-treated.
FIG. 6. Incidence of Low Mobility Cells (Mobility ≤ 0.80 μm/s/V/cm) of Splenocytes in Normal or Tumor-Bearing Mice Treated with Antitumor Drugs

- × - ×, non-treated; ○ - ○, PSK-treated; △ - △, MMC-treated; □ - □, ADM-treated.

phenomena were similar in normal mice. The percentages of LMC in the spleen of normal mice treated with ADM decreased markedly during the 4 weeks.

Figure 7 shows the electrophoretic mobility pattern of splenocytes in tumor-bearing mice treated with various antitumor drugs 28 d after tumor inoculation. In the PSK-treated group, the development of IMC was suppressed in comparison with untreated tumor bearers. Although IMC was suppressed in the group treated with these chemotherapeutic agents, ADM was found to injure LMC considerably.

**Flow Microcytometry of Surface Markers of Splenocytes**

While lymphocyte subsets showed only minor changes during tumor growth, Ig+ cells levels tended to decrease, following increase of the cell population without markers of Ig, Thy-1 nor αGM1.

Table 1 shows the incidence of lymphocyte subsets in normal and tumor-bearing mice treated with antitumor drugs 28 d after the beginning of drug administration. The incidence (%) of Ig+ cells markedly decreased in normal or tumor-bearing mice treated with ADM. The number of Ig+ or Thy-1+ cells in the spleen was reduced by the administration of MMC or ADM, as shown in Fig. 8.

**DISCUSSION**

Tumor-transplanted mice showed spleno-
TABLE I. Incidence of Lymphocyte Subsets in the Spleen of Normal and Tumor-Bearing Mice 28 d after the Beginning of Drug Administration

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment</th>
<th>Incidence (%) of lymphocyte subsets</th>
<th>Spleen cell No. (× 10^7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ig⁺</td>
<td>Thy-1⁺</td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td>60</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>PSK</td>
<td>59</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>MMC</td>
<td>47</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>ADM</td>
<td>51</td>
<td>40</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>None</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>PSK</td>
<td>62</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>MMC</td>
<td>57</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>ADM</td>
<td>42</td>
<td>35</td>
</tr>
</tbody>
</table>

FIG. 8. The Absolute Number of Ig⁺ Cells or Thy-1⁺ Cells of Splenocytes in Normal or Tumor-Bearing Mice 28 d after the Beginning of Drug Administration

megaly which was in part due to an increment in the lymphoid elements. The rest of the increase in splenic cellularity was primarily due to erythroid elements and to a minor extent to other cells such as granulocytes, monocytes and macrophages. The weight and the absolute number of lymphocytes in the spleen increased two or three-fold normal level at 1–4 weeks of the tumor’s age in the tumor-bearing host. Lala et al. 9) showed that in tumor-bearing mice there was a rise in the levels of null (S-Ig⁻, Thy-1⁻) small lymphocytes in the spleen and suggested that the generation of anti-tumor effector T cells was suppressed by an appearance of the suppressor lineage lymphocytes.

In splenocyte electrophoretic mobility, the pattern of the mobility histogram changed markedly in the course of tumor development (Fig. 3). In normal mice, it was clear from the experiment with nylon wool fractionation that LMC consisted mainly of B cells and HMC consisted of T cells. In tumor-bearing mice, both the adherent and nonadherent cells included the IMC, which increased as the tumor grew (Fig. 4). Many reports showed the appearance of suppressor cells in the spleen of tumor bearers when assayed by inhibition of the in vitro reactivity of normal lymphocytes to T and B mitogens10) or macrophage-colony forming cells.11) From these facts, it is of interest whether the IMC functions as an immunosuppressor. For the moment, however, what the IMC are remains unclear.

PSK is a purified protein-bound polysaccharide, extracted from cultured mycelium of Coriolus versicolor (Fr.) Quel in Basidiomycetes. PSK has not only been known to inhibit the growth of a variety of experimental tumors12,13) but is also orally administered for the clinical treatment of cancer patients in Japan.14) It has been reported that PSK can restore lymphocyte and macrophage functions to a normal level, which have been depressed by tumor burden.15,16)
Anticancer Drugs and Lymphocyte Mobility

When PSK was injected into normal mice, the spleen weight and the IMC in the spleen increased slightly 1 to 2 weeks after the administration of PSK (Figs. 2, 5). Ohno et al. \textsuperscript{17} showed that the administration of PSK enhanced the spleen weight and \textsuperscript{3}H\textsuperscript{t}hymidine uptake by the spleen cells in normal mice and promoted the growth of small round cells in the region of the periarteriolar lymphoid sheath of the spleen and the volume of lymph follicle (B cells region) of the spleen, but PSK administration had no effect on the ratio of cell numbers of T to B cells.

When PSK was administered to tumor-bearing mice, splenomegaly due to tumor-burden was depressed and the IMC, as well as the LMC, were restored to their normal levels. These phenomena coincided with the results of flow micrcytoctmetry. Namely, PSK recovered the incidence (%) and the absolute numbers of lymphocyte subsets.

There are many papers concerning augmentation of cytotoxic activity,\textsuperscript{18,19} restoration of natural killer (NK) activity\textsuperscript{20} and inhibition of suppressor activity\textsuperscript{21} in tumor bearers treated with PSK. In the MMC or ADM-treated group, as well as the PSK-treated group the splenic IMC decreased toward a normal level in accordance with the inhibition of tumor growth. However, ADM is considered to injure B cells from the analyses of mobility histograms (Fig. 6) and the flow micrcytoctmetry (Table I). Although MMC also suppressed the splenic IMC, there were marked changes in the pattern of thymocyte mobility histogram as a result of damage of immature thymocytes. These phenomena coincided with the suppression of antifnisfectious activity in the tumor-bearing hosts. From these facts it can be said that an anticancer drug which restores the pattern of splenocyte mobility histogram to normal without damage to thymocytes is preferable for cancer therapy.

REFERENCES


