Cytotoxicity of Cortisone-Resistant Lymphocytes from Mice Treated with a Group A Streptococcus or Freund's Complete Adjuvant against Tumor Cells

Shigeru KIGOSHI, Matomo NISHIO and Masafumi OSHITA
Department of Pharmacology, Fukui Medical School, Matsuoka, Fukui 910-11, Japan
Accepted July 6, 1985

Abstract—The cortisone-resistant lymphocytes (CR lymphocytes) of mice treated with a group A streptococcus, Su strain, or Freund’s complete adjuvant (FCA) were examined for their cytotoxicity on Ehrlich carcinoma cells and sarcoma-180 cells. Female mice of the ddY strain, 7–8 weeks of age, were injected subcutaneously with streptococci or FCA in emulsion, and they were killed 14 days later. To obtain CR lymphocytes, mice treated with and without agents were injected intraperitoneally with hydrocortisone acetate (125 mg/kg) 2 days before killing. Tumor cells and CR lymphocytes from thymus, spleen or mesenteric lymph node were suspended in Hanks balanced salt solution supplemented with 2% bovine albumin. The cytotoxicity of CR lymphocytes on tumor cells was examined by the Winn test: Tumor growth was observed in mice inoculated s.c. with the mixture of tumor cells (T) and CR lymphocytes (L) at a T/L ratio of 1/10 (10⁶ tumor cells/mouse). The mesenteric and thymic CR lymphocytes of mice treated with streptococci or FCA were more effective than the corresponding lymphocytes of untreated mice in suppressing the tumor growth in animals given the cell mixture. This suggests that the treatment of mice with streptococci or FCA results in an enhancement in the cytotoxicity of mesenteric and thymic CR lymphocytes against the tumor cells.

Many studies have established that the treatment of mice with corticosteroids results in rapid atrophy of the thymus, spleen and lymph nodes and a pronounced decrease of lymphocytes in these lymphoid tissues (1–3). The lymphocytes remaining in the lymphoid tissues of mice after treatment with hydrocortisone are known as the cortisone-resistant lymphocytes which are cytotoxic to tumor cells or allogeneic cells (4–7). However, little is known about the cytotoxicity of cortisone-resistant lymphocytes of mice treated with immunologic adjuvants against tumor cells.

A number of substances including mineral oil, several gram-negative and gram-positive bacteria, acid-fast bacilli, bacterial endotoxins and cell-wall components of bacteria are used as immunologic adjuvants (8–11). However, there are significant differences in the immunologic activities among these adjuvants. Freund’s adjuvant and lipopolysaccharide from gram-negative bacteria are powerful adjuvants of antibody production (8, 9). On the other hand, tuberculosis bacilli and group A streptococci, known as immunopotentiating agents, have the ability of stimulating the immunity of the host against tumors (10, 11). These suggest that there may be some difference in the effect on the cortisone-resistant lymphocytes of mice among immunologic adjuvants.

The present study deals with the cytotoxicity of cortisone-resistant lymphocytes of mice treated with a group A streptococcus, Freund’s complete adjuvant or lipopolysaccharide from a colibacillus against Ehrlich carcinoma cells and sarcoma-180 cells.
Materials and Methods

Treatment of mice: Female mice of the ddY strain (non-inbred), 7–8 weeks of age, were treated with the following agents: Freund's complete adjuvant (FCA) containing M. tuberculosis H37 Ra (Difco Laboratories), lipopolysaccharide (LPS) from E. coli 0111: B4 (Difco Laboratories) and a group A hemolytic streptococcus, type 3 of the Su strain. LPS was dissolved in physiological saline (100 μg/ml), and streptococcal cells (12) were suspended in saline (10 mg/ml). Ten ml of the saline containing LPS or streptococci was emulsified with an equal volume of Freund's incomplete adjuvant (Difco Laboratories), and FCA was emulsified with saline. A total of 60 mice in a group was injected subcutaneously with the emulsion (0.1 ml/mouse), and they were killed 14 days later. Mice given saline alone were used as a control (untreated mice). Each of the mice treated with and without the agents was injected intraperitoneally with hydrocortisone acetate (Schering AG, 125 mg/kg) 2 days before killing to obtain cortisone-resistant lymphocytes (CR lymphocytes) (4, 12).

Preparation of cell suspension: The lymphocyte suspension was prepared from the thymus, spleen or mesenteric lymph node of mice as described previously (12), using Hanks balanced salt solution (HBSS) containing penicillin G (100 IU/ml) and streptomycin (100 μg/ml). The lymphocyte suspension contained 93–98% of lymphocytes and 2–6% of macrophages, and 94–98% of cells in the suspension were viable according to the trypan blue test. The suspension of Ehrlich carcinoma cells or sarcoma 180 cells in HBSS was prepared from mice at 10 days after i.p. inoculation of the tumor cells (13). In the tumor cell suspension, 85–90% of cells were viable according to the trypan blue test.

Cytotoxicity test of lymphocytes: The cytotoxicity of CR lymphocytes on tumor cells was examined by the Winn test (14, 15). The CR lymphocytes suspended in HBSS supplemented with 2% bovine albumin fraction V (Armour Pharmaceutical Co.) were mixed with tumor cells and incubated at 37°C for 120 min. The ratio of lymphocytes to tumor cells in the cell mixture was 3:1, 10:1 or 30:1 (5×10⁶ lymphocytes/ml). After incubation, the cells collected by centrifugation were washed twice and suspended in HBSS. A portion of the cell mixture (0.5 ml) was inoculated subcutaneously into each of 6–8 normal mice on the right flank (10⁶ tumor cells/mouse), and the growth of solid tumors in the mice (recipient mice) was observed for 5 weeks.

Identification of T cells: To identify T cells, the activity of acid α-naphthyl acetate esterase (ANAE) in CR lymphocytes was examined histologically according to the procedure of Mueller et al. (16, 17), since the lymphocytes with the ANAE activity have been reported to be T cells. Smears of lymphocytes were made with a cytocentrifuge (Sakura Seiki, CF-12SB) and fixed in Baker's formol calcium at 4°C. To demonstrate the ANAE activity in lymphocytes, the cell smears were incubated at room temperature for 16 hr in a medium (pH 5.8) consisting of 40 ml of 0.067 M phosphate buffer, 2.4 ml of hexazotized pararosaline solution (a mixture of 4% pararosaline solution and 4% sodium nitrate solution) and 10 mg of α-naphthyl acetate (Sigma Chem. Co.) in 0.4 ml of acetone. After incubation, the smears were counterstained in a 1% toluidine blue solution. A lymphocyte containing a single reddish brown spot or a few of them in its cytoplasm was scored ANAE-positive. The proportion of ANAE-positive cells in CR lymphocytes was calculated from the ratio of ANAE-positive cells to CR lymphocytes within each lymphoid tissue.

Results

Cytotoxicity of CR lymphocytes: Table 1 shows the number of tumor-bearing mice at 5 weeks after s.c. inoculation with the mixture of tumor cells and CR lymphocytes from the thymus, spleen or mesenteric lymph node of mice (tumor cells/lymphocytes: 1/10). To compare the cytotoxicity of CR lymphocytes on tumor cells, percent of tumor takes was calculated for each CR lymphocyte: Tumor takes (%)=(number of tumor-bearing mice/total inoculated mice)×100.

There was a remarkable difference in the tumor takes among groups of recipients.
inoculated with the mixture of tumor cells and mesenteric CR lymphocytes. When Ehrlich carcinoma cells or sarcoma-180 cells were mixed with the mesenteric CR lymphocytes from mice treated with and without LPS, many recipients given the cell mixture developed solid tumors (tumor takes: about 60% for Ehrlich cells and 70–75% for sarcoma-180 cells). In contrast, no tumor growth was observed in all recipients given the mixture of Ehrlich cells and mesenteric CR lymphocytes from mice treated with streptococci or FCA (tumor takes: 0%). Solid tumors grew in a small number of recipients given the mixture of sarcoma-180 cells and mesenteric CR lymphocytes from mice treated with these agents (tumor takes: about 35%).

A significant difference was also found in the tumor takes among groups of recipients inoculated with the mixture of Ehrlich cells and thymic CR lymphocytes. When Ehrlich cells were mixed with the thymic CR lymphocytes from mice treated with and without LPS, most of the recipients given the cell mixture showed tumor growth (tumor takes: about 75%). Whereas, tumor growth was observed in a small number of recipients given the mixture of Ehrlich cells and thymic CR lymphocytes from mice treated with streptococci or FCA (tumor takes: about 30%).

On the other hand, there was only a slight difference in the tumor takes among groups of recipients inoculated with the mixture of sarcoma-180 cells and thymic CR lymphocytes (tumor takes: 64–91%) or among groups of recipients given the mixture of tumor cells and splenic CR lymphocytes (tumor takes: 81–100%). However, the tumor growth in the recipients given the mixture of sarcoma-180 cells and thymic CR lymphocytes from mice treated with streptococci or FCA was less striking than that in the recipients receiving the tumor cells admixed with the corresponding lymphocytes from

| Sources of lymphocytes | Cortisone-resistant lymphocytes | Number of tumor-bearing mice/total inoculated mice |
|------------------------|---------------------------------|--------------------------------------------------|
|                        |                                 | Ehrlich cells | Sarcoma-180 cells |
| Untreated mice         | Thymic cells                    | 18/24         | 20/22             |
| Mice treated with cocci|                                 | 7/21*         | 17/24             |
| Mice treated with FCA  |                                 | 6/21*         | 14/22             |
| Mice treated with LPS  |                                 | 17/23         | 21/23             |
| Untreated mice         | Splenic cells                   | 21/22         | 24/24             |
| Mice treated with cocci|                                 | 17/21         | 19/20             |
| Mice treated with FCA  |                                 | 18/22         | 19/21             |
| Mice treated with LPS  |                                 | 21/23         | 24/24             |
| Untreated mice         | Mesenteric cells                | 13/21         | 15/20             |
| Mice treated with cocci|                                 | 0/21*         | 8/23*             |
| Mice treated with FCA  |                                 | 0/20*         | 8/24*             |
| Mice treated with LPS  |                                 | 12/20         | 14/20             |

Mice were treated with a group A streptococcus (coccii). Freund's complete adjuvant (FCA) or lipopolysaccharide (LPS) from a colibacillus, and they were killed 14 days later. Mice without treatment of these agents were used as a control (untreated mice). Cortisone-resistant lymphocytes (CR lymphocytes) were obtained from thymus, spleen or mesenteric lymph node of mice injected i.p. with hydrocortisone acetate (125 mg/kg) 2 days before killing. CR lymphocytes and tumor cells were suspended in Hanks balanced salt solution supplemented with 2% bovine albumin. The mixture of tumor cells and CR lymphocytes (tumor cells/lymphocytes: 1/10) were incubated at 37°C for 2 h. After incubation, the cell mixture was inoculated s.c. into mice (10⁴ tumor cells/mouse). Number of tumor-bearing mice represents the number of mice bearing solid tumors at 5 weeks after inoculation. *Significantly different from the values for untreated mice (P<0.05 by the χ²-test).
Fig. 1. Tumor diameter of mice after s.c. inoculation with the mixture of tumor cells and cortisone-resistant lymphocytes from mouse thymus. Cortisone-resistant thymic lymphocytes were obtained from untreated mice (●), mice treated with streptococci (○) or animals treated with FCA (△). Ehrlich carcinoma cells or sarcoma-180 cells were mixed with the cortisone-resistant lymphocytes (tumor cells/lymphocytes: 1/10). The cell mixture was inoculated s.c. into mice (10⁶ tumor cells/mouse) after incubation at 37°C for 120 min. Tumor diameter was calculated as follows: Tumor diameter (mm) = (long diameter + short diameter)/2. Each value represents the mean±S.E. of mice developing solid tumors. Number (n) of tumor-bearing mice was shown in Table 1.

Fig. 2. Percent of mice developing solid tumors at 5 weeks after s.c. inoculation with the mixture of Ehrlich carcinoma cells and cortisone-resistant lymphocytes from thymus or mesenteric lymph node of mice. Cortisone-resistant lymphocytes (CR lymphocytes) were obtained from untreated mice (●), mice treated with streptococci (○) or animals treated with FCA (△). Ehrlich carcinoma cells were mixed with the CR lymphocytes at different cell ratio (lymphocytes/tumor cells: 3/1, 10/1 and 30/1). The cell mixture was inoculated s.c. into mice (10⁶ tumor cells/mouse) after incubation at 37°C for 120 min. Percent of mice developing solid tumors was calculated from the following ratio: Number of tumor-bearing mice/total inoculated mice.
untreated mice (Fig. 1).

Thus, the mesenteric and thymic CR lymphocytes from mice treated with streptococci or FCA are considered to be more potent than the corresponding lymphocytes from untreated mice in the cytotoxicity on the tumor cells (Fig. 2).

**Proportion of ANAE-positive cells in CR lymphocytes:** The cytotoxicity of mesenteric or thymic CR lymphocytes on tumor cells differed with the sources of lymphocytes, as described above. The proportion of T cells in these CR lymphocytes was then examined by testing the ANAE-positive cells in each CR lymphocyte.

The proportion of ANAE-positive cells in the mesenteric CR lymphocytes from mice treated with streptococci or FCA was similar to that in the corresponding lymphocytes from untreated mice (59–68%), and there was only a slight difference in the proportion of ANAE-positive cells in the thymic CR lymphocytes between mice treated with and without these agents (64–73%) (Table 2). These results strongly suggest that the T cell proportion in the mesenteric and thymic CR lymphocytes from mice treated with streptococci or FCA is about the same as that in the corresponding lymphocytes from untreated mice.

Concerning this, comparisons were made on the ratio of CR lymphocytes to total lymphocytes in the thymus or mesenteric lymph node between mice treated with and without the immunologic adjuvants, since the ratio of CR lymphocytes to total lymphocytes (CL/TL ratio) in these lymphoid tissues of mice is used to represent the proportion of CR lymphocytes in each lymphoid tissue (1–3). The total lymphocytes were obtained from the lymphoid tissues of mice without the treatment of hydrocortisone (12).

As can be seen in Table 3, the CL/TL ratio in the thymus of mice treated with

| Groups of mice | Cortisone-resistant lymphocytes | Proportion of ANAE-positive cells in cortisone-resistant lymphocytes (%) |
|----------------|-------------------------------|-------------------------------------------------|
| **Un-treated mice** | Thymic cells | 63.5±6.3 |
| Mice treated with cocci | 72.8±2.5 |
| Mice treated with FCA | 66.8±5.4 |
| Mice treated with LPS | 81.2±2.5* |
| **Un-treated mice** | Splenic cells | 49.8±4.6 |
| Mice treated with cocci | 56.0±3.0 |
| Mice treated with FCA | 57.3±1.5 |
| Mice treated with LPS | 55.0±2.9 |
| **Un-treated mice** | Mesenteric cells | 63.5±4.1 |
| Mice treated with cocci | 68.3±2.9 |
| Mice treated with FCA | 59.2±3.4 |
| Mice treated with LPS | 59.4±2.2 |

To demonstrate the activity of acid α-naphthyl acetate esterase (ANAE) in cortisone-resistant lymphocytes (CR lymphocytes), smears of lymphocytes were incubated at room temperature for 16 hr in a medium (pH 5.8) consisting of 40 ml of 0.067 M phosphate buffer, 2.4 ml of hexaazotized pararosaniline solution (a mixture of 4% pararosaniline solution and 4% sodium nitrate solution) and 10 mg of α-naphthyl acetate in 0.4 ml of acetone. After incubation, the smears were counterstained in a 1% toluidine blue solution. A lymphocyte containing a single reddish brown spot or a few of them in its cytoplasm was scored ANAE-positive. The proportion of ANAE-positive cells in CR lymphocytes was calculated from the ratio of ANAE-positive cells to CR lymphocytes within each lymphoid tissue. Each value represents the mean±S.E. of 5 experiments. *Significantly different from the values for untreated mice (P<0.01 by the t-test).
The present results clearly indicate that the mesenteric and thymic CR lymphocytes of mice treated with streptococci or FCA are more cytotoxic to Ehrlich carcinoma cells and sarcoma-180 cells than the corresponding CR lymphocytes of untreated mice, but there is no significant difference in the cytotoxicity of these CR lymphocytes on the tumor cells between mice treated with and without LPS. In addition, the splenic CR lymphocytes from mice treated with and without these agents were not cytotoxic to Ehrlich cells or sarcoma-180 cells. Therefore, it may be concluded that the treatment of mice with a group A streptococcus or Freund’s complete adjuvant results in an enhancement in the cytotoxicity of mesenteric or thymic CR lymphocytes on the tumor cells, but lipopolysaccharide from a colibacillus has little effect on the cytotoxicity of these CR lymphocytes.

Examination of the T cell proportion in the above CR lymphocytes by testing the ANAE-positive cells in the lymphocytes (16, 17) also indicates that the proportion of T cells in the mesenteric and thymic CR lymphocytes from mice treated with streptococci or FCA is about the same as that in the corresponding CR lymphocytes from untreated mice (proportion of ANAE-positive cells: 60–70% for mesenteric cells and about 70% of these CR lymphocytes on the tumor cells between mice treated with and without LPS.

### Table 3. Ratio of cortisone-resistant lymphocytes to total lymphocytes in the thymus, spleen or lymph node of mice treated with and without immunologic adjuvants

| Groups of mice      | Lymphoid tissues | Number of lymphocytes in lymphoid tissues of mice (×10⁶ cells/tissue) | Ratio of A to B (%) |
|---------------------|------------------|---------------------------------------------------------------------|---------------------|
|                     |                  | Cortisone-resistant cells (A) | Total cells (B)     |                     |
| Untreated mice      | Thymus           | 30±2                    | 393±12               | 7.6                 |
| Mice treated with cocci | 29±2             | 382±11                 | 7.6                 |
| Mice treated with FCA | 33±3             | 434±23                 | 7.6                 |
| Mice treated with LPS | 25±3             | 325±34                 | 7.7                 |
| Untreated mice      | Spleen           | 91±6                   | 369±12               | 24.7                |
| Mice treated with cocci | 177±9*           | 679±14*                | 26.1                |
| Mice treated with FCA | 155±8*           | 611±26*                | 25.4                |
| Mice treated with LPS | 217±6*           | 769±17*                | 28.2                |
| Untreated mice      | Mesenteric lymph node | 16±3              | 77±4                 | 20.8                |
| Mice treated with cocci | 36±3*           | 183±7*                 | 19.7                |
| Mice treated with FCA | 41±3*           | 221±9*                 | 18.6                |
| Mice treated with LPS | 34±2*           | 168±8*                 | 20.2                |

A total of 25 mice in each group was treated with streptococci, FCA or LPS, and they were killed 14 days later. Two days before killing, each group of mice was divided into two subgroups. One subgroup of mice (20 mice) was injected i.p. with hydrocortisone acetate (125 mg/kg) to obtain cortisone-resistant lymphocytes. Total lymphocytes were obtained from another subgroup of mice (5 mice) without treatment of hydrocortisone. Total and cortisone-resistant lymphocytes were also obtained from mice given physiological saline alone before 14 days (untreated mice). Each value represents the mean±S.E. of 5 experiments. *Significantly different from the values for untreated mice (P<0.01 by the t-test).

### Discussion

The present results clearly indicate that the mesenteric and thymic CR lymphocytes of mice treated with streptococci or FCA are more cytotoxic to Ehrlich carcinoma cells and sarcoma-180 cells than the corresponding CR lymphocytes of untreated mice, but there is no significant difference in the cytotoxicity of these CR lymphocytes on the tumor cells between mice treated with and without LPS. In addition, the splenic CR lymphocytes from mice treated with and without these agents were not cytotoxic to Ehrlich cells or sarcoma-180 cells. Therefore, it may be concluded that the treatment of mice with a group A streptococcus or Freund’s complete adjuvant results in an enhancement in the cytotoxicity of mesenteric or thymic CR lymphocytes on the tumor cells, but lipopolysaccharide from a colibacillus has little effect on the cytotoxicity of these CR lymphocytes.

Examination of the T cell proportion in the above CR lymphocytes by testing the ANAE-positive cells in the lymphocytes (16, 17) also indicates that the proportion of T cells in the mesenteric and thymic CR lymphocytes from mice treated with streptococci or FCA is about the same as that in the corresponding CR lymphocytes from untreated mice (proportion of ANAE-positive cells: 60–70% for mesenteric cells and about 70% of these CR lymphocytes on the tumor cells between mice treated with and without LPS.
for thymic cells) (1–3). Thus it appears that the difference in the cytotoxicity of mesenteric or thymic CR lymphocytes between mice treated with and without the immunologic adjuvants does not depend on the T cell proportion of these CR lymphocytes. It is well known that T lymphocytes are further divided into several subpopulations such as helper T cells, suppressor T cells or killer T cells which are cytotoxic to tumor cells or allogeneic cells (18, 19). Analysis of mouse lymphocytes with the fluorescence activated cell sorter (FACS) has indicated that the CR lymphocytes and total lymphocytes from the lymph node or thymus are separable into two subgroups, and there is a slight difference in the proportion of Lyt-2+ cells in each subgroup between the CR lymphocytes and total lymphocytes from the lymph node or between the mesenteric and thymic CR lymphocytes (20). Lyt-2+ lymphocytes have been reported to comprise both killer T cells and suppressor T cells which inhibit the cytotoxicity of killer T cells on target cells (21–23).

Concerning these, comparisons of the CR lymphocytes and total lymphocytes from mice treated with streptococci or FCA showed that the difference in the T cell proportion between the CR lymphocytes and total lymphocytes was slight within the mesenteric lymph node or thymus (proportion of ANAE-positive cells: 60–70% and 60–75% for mesenteric cells, and about 70% and 60–80% for thymic cells), but there was a significant difference in the effect on Ehrlich carcinoma cells between the CR lymphocytes and total lymphocytes from these lymphoid tissues. The total lymphocytes (L) from the lymph node and thymus of mice treated with streptococci or FCA had little or no cytotoxicity against Ehrlich cells (T) at T/L ratios of 1/10 and 1/30 (tumor takes: 90–100% for mesenteric cells and 100% for thymic cells) (15). However, the CR lymphocytes from these lymphoid tissues of mice treated with the adjuvants were cytotoxic to the tumor cells at a T/L ratio of 1/10, as described above (tumor takes: 0% for mesenteric cells and about 30% for thymic cells). The cytotoxicity of the mesenteric and thymic CR lymphocytes of mice on the tumor cells, therefore, may be related to the proportion of T cell subsets such as killer T cells or suppressor T cells in the lymphocytes, although the proportion of killer T cells and suppressor T cells in these CR lymphocytes remains to be determined.

Recently, natural killer cells (NK cells) have received much attention in relation to tumor immunity. Studies on the mouse NK cells have revealed that the activity of NK cells is high in the splenic lymphocytes and low in the lymph node cells, but the thymic lymphocytes show no activity of NK cells (24). Thus it is unlikely that the cytotoxicity of thymic CR lymphocytes of mice on tumor cells is due to NK cells, although the involvement of NK cells in the cytotoxicity of mesenteric CR lymphocytes of mice against tumor cells cannot be denied.

In association with tumor immunity, the effect of immunopotentiating agents, such as group A streptococci and tubercle bacilli, on T lymphocytes, NK cells or macrophages has been studied extensively (8–11). However, very few attempts have been made to examine the effect of immunopotentiating agents on the cortisone-resistant lymphocytes, which are very efficient in cell-mediated immune reactions such as tumor immunity or allograft rejection (1–3). Accordingly, it might be of great interest that a group A streptococcus and Freund’s complete adjuvant containing a tubercle bacillus enhance the cytotoxicity of cortisone-resistant lymphocytes from the mesenteric lymph node or thymus of mice against tumor cells.

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