Treatment of a Moloney Lymphoma with Cyclophosphamide and $H$-2-incompatible Spleen Cells

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SUMMARY

This study was performed to determine whether, as an adjunct to cyclophosphamide (CY), a number of $H$-2-incompatible lymphoid cells too small to induce clinically evident graft-versus-host disease would exert a significant antitumor effect and whether clinically significant antitumor specificity might be imparted to a subclinical graft-versus-host reaction by preimmunization of the spleen cell donors. Adult BALB/c mice bearing a disseminated transplantable BALB/c Moloney virus-induced lymphoma (LSTRA) with tumor-associated antigens were treated with sublethal CY plus spleen cells from C57BL/6 mice that either were nonimmune or had been immunized with normal BALB/c tissue, a BALB/c chemical sarcoma, an antigenically related murine sarcoma virus (Moloney), or LSTRA cells. The results, represented by long-term survivors, showed that, in the absence of detectable graft-versus-host disease and in conjunction with CY, nonimmune spleen cells exerted a significantly greater antitumor effect than was observed with CY alone; that spleen cells immune to host isoantigens were no more effective than nonimmune cells; but that preimmunization of the spleen cell donors with murine sarcoma virus (Moloney), or LSTRA rendered their cells significantly more immunotherapeutically effective in the CY-treated tumor-bearing hosts.

INTRODUCTION

Clinically detectable tumors, are, with rare exceptions (1, 10), not susceptible to eradication by immune lymphocytes alone. However, in several tumor models, lymphoid cells were quite effective in inhibiting or eradicating disseminated antigenic tumors if used as an adjunct to sublethal noncurative chemotherapy, provided that the drug had a potent antitumor effect and that the lymphoid cells were viable and immune to TAA$^3$ (6-8, 12, 17, 18, 23, 27).

The most extensively studied model involved the treatment of a BALB/c Moloney lymphoma with sublethal CY plus spleen cells from syngeneic (11), hemisyngeneic F$_1$ hybrid (17), or allogeneic but $H$-2-compatible mice (11, 18) preimmunized against TAA by exposure either to a Moloney lymphoma of C57BL/6 origin or to an antigenically-related MSV. All of the studies highlighted the critical importance of lymphoid cells specifically immune to TAA.

Application of this adoptive chemoimmunotherapy approach to $H$-2-incompatible donor-host combinations can be potentially complicated by GVH reactions which, when severe enough to be fatal, have an antitumor effect detectable by bioassay or autopsy (2-5, 21, 25, 26). The aim of this study was to determine whether, as an adjunct to CY, $H$-2-incompatible lymphoid cells in numbers that would not induce clinically evident GVH disease would exert a significant antitumor effect and whether clinically significant antitumor specificity might be imparted to any subclinical GVH reaction by the preimmunization of spleen cell donors against normal host tissue antigens or TAA.

MATERIALS AND METHODS

Mice. Adult BALB/c ($H$-2$^d$) and C57BL/6 ($H$-2$^b$) mice were obtained from the production colonies of Texas Inbred Mice, Houston, Texas.

Virus. A pool of MSV, designated RP 114, was kindly provided by Dr. John B. Moloney (National Cancer Institute, Bethesda, Md.). Tumors were induced with this pool in adult BALB/c mice and were removed on Day 10 when they contained abundant MSV (14). Virus was extracted by differential ultracentrifugation (1 ml of extract per g tumor) (24). The resultant pool, designated RP-114-S-1, was stored at $-70^\circ$ in 0.3-ml aliquots in heat-sealed vials and was used for all experiments.

Immunization with MSV. Adult mice were inoculated with 0.05 ml of a 10$^{-1}$ dilution of MSV i.m. into the right hind limb. As previously reported with the same MSV-M pool (9), tumors developed within 1 week and regressed within 2 to 3 weeks. One to 3 weeks after regression, the mice were reinoculated i.m. with the same amount of MSV and did not develop tumors. Most donors were immunized with a 3rd dose of MSV 2 to 4 weeks after the 2nd one, and some received a 4th dose. Spleens were obtained and used 2 to 3 weeks after the last MSV inoculation. The immunization scheme induces resistance to histocompatible Moloney lymphoma cells (13) and renders donor lymphoid cells effective for chemoimmunotherapy (11).

Tumor. The target for chemoimmunotherapy was an ascitic lymphoma, designated LSTRA, which was induced in a newborn BALB/c mouse with MLV and which has been serially transplanted in adult BALB/c mice for >300 genera-

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tions. It contains virus (22) and possesses tumor-specific cellular and/or virion antigens that cross-react with other leukemias induced by MLV (19), as well as with tumors induced by MSV (13). Accordingly, mice immunized with MSV became resistant to LSTRA or other Moloney lymphomas (13). The LSTRA, when inoculated i.p. or s.c. in a dose of >100 cells, kills all normal adult BALB/c mice within 16 days (17). For chemoimmunotherapy, mice were inoculated s.c. with 10⁶ LSTRA cells on Day 0 and were treated on Day 4. By Day 3, the tumors were grossly palpable and demonstrably (by bioassay) disseminated to spleen, lymph nodes, liver, and lungs. No palpable LSTRA ever regressed spontaneously.

Drug. CY was dissolved in distilled water, and the appropriate concentration was injected i.p. as a single dose at 0.01 ml/g body weight. The dose was always less than the 10% lethal dose. The drug was kindly provided by Mead Johnson and Company, Evansville, Ind.

Preparation of Spleen Cells. Spleen cell suspensions were prepared in Hanks' balanced salt solution, as previously described (9). For immunotherapy, 4 to 5 X 10⁷ trypan blue-unstained nucleated cells were injected i.p. (0.2 ml/mouse).

Immunization with Spleen Cells or LSTRA. Cell suspensions were prepared as described above and were inoculated i.p. (5 to 10 X 10⁶ cells/recipient in 0.1 ml) every 2 to 3 weeks. Spleen cells from the immunized mice were obtained and used 2 to 3 weeks after the 3rd or 4th immunization.

Preparation of Antibody against Donor γ-Globulin. (20) were tested against serially diluted serum from C57BL/6 mice by end point precipitation in a double diffusion system in an agarose gel, as previously described (16). The center well contained BALB/c anti-C57BL/6 serum. C57BL/6 sera could always be diluted 1:32 or 1:64 and still yield precipitin lines with a specific antiglobulin. Sera of BALB/c mice given injections of C57BL/6 spleen cells were similarly tested for the presence of C57BL/6 γ-globulin.

RESULTS

BALB/c mice were inoculated with LSTRA on Day 0. On Day 4, they received CY, 180 mg/kg, a sublethal but immunosuppressive dose (16). Six hr later, some of the mice received spleen cells from C57BL/6 donors that were either nonimmune or that had been preimmunized with normal BALB/c spleen cells, a methylcholanthrene-induced BALB/c tumor, MSV, or LSTRA. In 2 experiments, the same pools of spleen cells were concurrently injected into non-tumor-bearing BALB/c controls pretreated with CY. No clinically grossly detectable GVH disease was observed in any of the control groups or in the tumor-bearing chemoimmunotherapy hosts, in contrast to the hunched posture, ruffled fur, lethargy, dermatitis, and wasting exhibited by mice undergoing ultimately fatal GVH disease (15).

The results of the 4 experiments performed were reproducible and are pooled and summarized in Chart 1. Treatment with immune spleen cells but no CY had no effect, so that all 48 mice thus treated or left totally untreated were dead by Day 13. CY alone prolonged the median survival time to Day 25, with 1 mouse surviving inexplicably beyond Day 100. (Indeed, this was the only mouse, of several hundred treated, that survived after it was given CY alone; however, only the concurrent data are presented.) Treatment with CY plus nonimmune cells increased the number of long-term survivors significantly (p < 0.01), with 26% surviving >100 days. Similar results were obtained with CY plus cells immunized only against host isoantigens, i.e., with BALB/c spleens or a BALB/c methylcholanthrene sarcoma. Treatment with CY plus cells from mice immunized with MSV was even more effective, as reflected not so much by the 40% long-term survivors as by the marked prolongation of the MST from Day 25 to Day 80. Finally, in 3 experiments, mice received CY plus cells from C57BL/6 mice immunized with LSTRA, and 52% of the tumor-bearing mice survived >100 days.

The results show that, in the absence of detectable GVH disease, the administration of normal H-2-incompatible spleen cells in conjunction with CY was associated with a greater antitumor effect than that observed with CY alone (p < 0.01); that immunization of the spleen cell donors against host isoantigens did not render them any more effective; and that preimmunization of the spleen cell donors against TAA rendered their cells far more immunotherapeutically effective in the CY-treated tumor-bearing host. Cumulatively, the number of long-term survivors after administration of CY plus cells immune to MSV or to LSTRA (32 of 72) was significantly (p < 0.01) larger than that observed after treatment with CY plus cells that were nonimmune or immune to BALB/c isoantigens (21 of 92).

Tumor-bearing and non-tumor-bearing BALB/c mice that received CY plus spleen cells from any type (immune or nonimmune) of C57BL/6 donor were bled, and their sera were tested for the presence of C57BL/6 γ-globulin. Almost all of the mice were moderately positive (1:4 to 1:32) on Day 15 (i.e., Day 11 after donor spleen cell infusion), and all were negative by Day 28. The presence or absence or specific titer of the allotype was not correlated with the survival of any mouse or any group of mice.

One other experiment was performed to determine whether immune C57BL/6 cells without CY could cope with a relatively small tumor load. BALB/c mice received 1 X 10⁶ (not 1 X 10⁹) LSTRA cells i.p. One day (rather than 4 days) later they received 1 X 10⁸ (not 5 X 10⁷) spleen cells i.p. from C57BL/6 mice immunized with MSV or LSTRA. All 14 mice thus treated died on Days 9 to 12, as did untreated controls.

DISCUSSION

The application of adoptive chemoimmunotherapy to grossly histoincompatible donor-host combinations would be fraught with and complicated by the problem of a GVH reaction, which, in turn, may have a role in tumor therapy. Unfortunately, the GVH reaction as an antitumor reaction usually has been studied under conditions of fatal GVH disease, so that its antitumor effect has been demonstrated by bioassay or by postmortem examination rather than by long-term tumor-free survivors (2—5, 21, 25, 26). Such studies have shown that fatal GVH disease induced by nonimmune H-2-incompatible lymphoid cells did, in fact, inhibit or eradicate lymphoid tumors (2—5, 21, 25) but exerted little effect on primary Moloney sarcomas (9) or on mammary...
compatible spleen cells were used, but significant GVH disease was avoided by the use of H-2-compatible DBA/2 donors. The results showed that treatment with CY plus spleen cells from DBA/2 mice that were nonimmune, or immune only to host isoantigens, was no more effective than was treatment with CY alone, whereas treatment with CY plus DBA spleen cells immune to MSV was far more effective. In the present study, H-2-incompatible spleen cells were used, but significant GVH disease was avoided by the use of fewer cells than would be required to cause fatal GVH disease under comparable conditions (15). The results of these studies, in contrast to those obtained with H-2-compatible cells, showed that administration of nonimmune H-2-incompatible spleen cells in conjunction with CY was indeed associated with a greater antitumor effect than that observed with CY alone, as reflected by significantly more long-term survivors. With CY, spleen cells from donors preimmunized against host isoantigens were also effective, but no more so than nonimmune cells. However, as in all previous studies, adoptive chemoimmunotherapy with cells preimmunized to TAA was most effective.

Although none of the pools of the C57BL/6 spleen cells induced grossly detectable GVH disease in tumor-bearing or non-tumor-bearing CY-treated BALB/c controls, it is likely that a subclinical GVH reaction did occur and was by itself immunotherapeutically effective even when induced by nonimmune cells or cells immune to normal host isoantigens. However, it is probable that the postulated GVH reaction was immunotherapeutically even more effective against the tumor cells when the GVH reaction was induced by cells specifically immune to TAA.

The specificity of immunization must be qualified by the fact that immunization with LSTRA cells would of course immunize against host isoantigens, Moloney antigens, and any non-Moloney antigens that the tumor might have acquired in the course of its long transplantation history, and that, although the critical immunogen in the MSV is presumed to be the cellular and/or virion tumor-specific antigens present on the primary tumors induced by MSV in the C57BL/6 mice, the MSV used may certainly have carried with it some normal BALB/c isoantigens. However, the therapeutic efficacy of the cells preimmunized with MSV cannot be attributed totally to possible immunization with BALB/c isoantigens because of the relatively lesser efficacy of cells immune only to host isoantigens present on normal BALB/c cells or on an unrelated BALB/c tumor.

The results of the γ-globulin allotype studies presented suggest that a permanent chimerism was not necessary, since no correlation was observed between the titer or duration of donor allotype in host serum and the fate of the mouse with regard to the tumor. The possibility remains, however, as suggested in other studies (9), that the donor cells must persist for some indeterminate period of time, perhaps 2 weeks or more, in order to be immunotherapeutically effective against the tumors.

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**REFERENCES**

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