Comparison of Cellular Immunotherapies and Anti-CD3 in the Treatment of MCA-38-LD Experimental Hepatic Metastases in C57BL/6 Mice

Steven Gallinger, David W. Hoskin, J. Brendan M. Mullen, Albert H. C. Wong, and John C. Roder

Division of Molecular Immunology and Neurobiology, Mount Sinai Hospital Research Institute [S. G., D. W. H., A. H. C. W., J. C. R.], and Department of Immunology, University of Toronto, and Department of Pathology [J. B. M. M.], Mount Sinai Hospital, Toronto, Ontario, Canada M5G 1X5

ABSTRACT

An experimental model of hepatic metastases in C57BL/6 mice was used to compare the antitumor effects of lymphokine-activated killer (LAK) cells, anti-CD3-activated T-cells (ATC), and anti-CD3 alone. Liver metastases were produced by in vivo passage of MCA-38-LD adenocarcinoma via the ileocolic vein. LAK cells and ATC were generated by 3-day in vitro incubation of spleen cells in interleukin 2 and anti-CD3, respectively. Percentage of tumor volume in livers was determined with a morphometric technique. With less than therapeutic LAK cell doses (0.5-1.0 x 10^7 cells), no effect was seen in mean (+SE, -SE) percentage of tumor volume of control [23.3 (29.3, 18.5)] compared to LAK cell-treated [21.6 (29.3, 15.9)] animals. The same number of ATC significantly reduced the mean percentage of tumor volume [2.7 (4.7, 1.4)] (P < 0.005). High dose interleukin 2 also significantly decreased tumor volume. More strikingly, a single dose of anti-CD3 alone had a beneficial effect on mean percentage of tumor volume when given i.p. [1.0 (1.9, 0.4)] or i.v. [1.2 (1.7, 0.7)] (P < 0.0003). A total of 33% of anti-CD3-treated mice had no detectable liver metastases. In vitro release assays, the cytotoxicity of ATC was shown to be partially mediated by nylon wool-adherent accessory cells. The effectiveness of anti-CD3 in this immunotherapy model suggests that a similar approach may be taken to immunotherapy of human malignancies, without the requirement for in vitro-generated killer cells or exogenously administered interleukin 2.

INTRODUCTION

The adoptive transfer of LAK cells, in combination with IL-2, has shown promise in cancer immunotherapy (1, 2). However, significant shortcomings have become apparent including: (a) the toxicity of systemically administered IL-2, (b) the large number of cells required, and (c) questionable tumor targeting by the transferred cells. Major refinements are required to circumvent these problems and improve therapeutic efficacy.

Studies on the characterization of LAK cells suggest that their precursors are predominantly natural killer cells, and therefore it is this small population of peripheral lymphocytes which probably accounts for the majority of activated cytolytic effectors in LAK/IL-2 therapy (3, 4). However, T-cells comprise a greater proportion of circulating lymphocytes and represent the greatest fraction of TIL (5, 6). To date, T-cell activation and subsequent antitumor activity have not been fully exploited in cancer immunotherapy.

Reports from our laboratory as well as others have shown that activation of T-cells by mAb against the TcR/CD3 complex (7, 8) results in effectors with a broad spectrum of cytolytic activity (9-11). Moreover, administration of the mAb alone appears to be effective in mouse tumor models (12, 13).

The purpose of the present series of experiments was to compare the antitumor activity of LAK cells, ATC, and anti-CD3 antibody in a mouse model of experimental hepatic metastases. The data suggest that ATC may be more effective antitumor effectors than LAK cells and that the administration of anti-CD3 mAb alone may offset the requirements for adoptively transferred ATC.

MATERIALS AND METHODS

Mice. Adult C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were housed at the Mount Sinai Hospital Research Institute animal colony and used when 5-6 weeks old.

Tumor Cells. The murine mastocytoma P815 and lymphoma Yac 1.2 lines were maintained in complete RPMI 1640 with 10% fetal calf serum (GIBCO, Grand Island, NY). The murine MCA-38-LD (liver-derived) colon adenocarcinoma was kindly provided by Dr. M. Goldrosen (Roswell Park Memorial Institute) from in vivo passaged stock. This tumor line was passaged and expanded in vitro 3 times, following which in vivo passages by portal vein injection (via the ICV) were performed (see below). Immunotherapy studies were carried out with cells passaged in vivo every 3-4 weeks.

In vivo Passage of MCA-38-LD. Mice with well established hepatic metastases (21-28 days after ICV injection) were sacrificed by cervical dislocation and hepatectomy was performed. The liver was gently dissected into small 2-3-mm fragments and tumor cells were dissociated with trypsin (0.5%) (GIBCO). Tumor cells were washed extensively with RPMI prior to ICV injection.

Hepatic Metastasis Model. A murine model of experimental hepatic metastases, originally described by Goldrosen et al. (14), was used in the immunotherapy experiments. Briefly, C57BL/6 mice were anesthetized with ketamine and a short, lower vertical, midline incision was performed. The ileoccecal region was exteriorized and 10^3 tumor cells in 100 μl RPMI were injected, with a 30-gauge stainless steel needle (Vita Needle Co., Needham, MA), into the ICV. The operation was facilitated by the use of an operating microscope and care was taken to prevent extravasation locally around the cecum. The peritoneum was closed with 4-0 silk (American Cyanamid Co., Pearl River, NY) and the skin was closed with clips (Becton Dickinson, Parsippany, NJ). Animals tolerated the procedure well, with operative mortality less than 5%.

Generation of ATC and LAK Cells. Spleens from C57BL/6 mice were harvested and crushed aseptically. RBCs were lysed by hypotonic (0.2% NaCl) shock. In some cases, lymphocytes were then passed over pre-warmed nylon wool columns (Cellular Products, Buffalo, NY). The columns were incubated at 37°C for 1 h, following which the non-adherent cells were eluted with warm RPMI. Nylon wool-passed cells, as well as lymphocytes not nylon wool purified, were then washed extensively and cultured at a concentration of 2.5 x 10^6 cells/ml in RPMI (GIBCO) with 10% fetal calf serum. ATC were generated by the addition of a 1/500 dilution of anti-CD3 ascites mAb 145-2C11 (a gift from Dr. J. Bluestone, University of Chicago). LAK cells were generated by the addition of 1000 units/ml recombinant IL-2 (a gift from J. C. Roder, University of Chicago). LAK cells used in immunotherapy experiments and ^51Cr release assays were harvested on day 3 of culture.

Immunotherapy Studies. Four days after ICV injection of tumor cells, mice were randomized into groups for immunotherapy. Animals receiving ATC or LAK cells were given injections of 0.5-1.0 x 10^7 cells.
in 100 μl RPMI via the tail vein. Control animals received 100 μl of RPMI via the tail vein. Mice receiving LAK cells were further treated with i.p. injections of 7,500 units of IL-2 3 times a day for the next 3 days. In some cases, ATC-treated mice received IL-2 as above. An additional group of animals received high dose (40,000 units/dose) IL-2 for 3 days. Mice receiving anti-CD3 alone were given injections either i.p. or via the tail vein of a 100-μl aliquot of a 1/25 dilution (~4 μg) of 145-2C11 ascites. Animals tolerated the immunotherapies without obvious side effects.

Assessment of Tumor Volume. All animals in the immunotherapy studies were sacrificed approximately 3 weeks after tumor injection. Hepatectomies were performed and percentage of liver replacement by tumor was assessed by a pathologist (J. B. M. M.) blinded to the treatments. Because of the diffuse, nonuniform, and confluent nature of tumor growth, a morphometric technique was used. Briefly, liver volume was determined by weight displacement (15). The liver was then embedded in 2% agar and systematically sliced at a nominal thickness of 3 mm. The slices were processed for histological examination in the usual manner and 5-μm-thick sections were cut, mounted on glass slides, and stained with hematoxylin and eosin. Measurements of liver and tumor area were performed using a camera lucida and an IBM-PC computer-assisted digitizing board. The ratio of tumor area to liver area is equivalent to the percentage of tumor volume (Delesse’s Principle) (16).

**Results**

**Establishment of Hepatic Metastasis Model by ICV Injection of MCA-38-LD**

Following the third in vitro passage of MCA-38-LD cells, mice were given injections of tumor cells via the ICV. The initial take rate was 11 of 13 (85%). A mean survival of 49.3 ± 1.9 (SE) days was noted, in agreement with the study by Goldrosen et al. (14). A liver excised from a mouse with extensive tumor was used for initial in vivo passage of the tumor line. Subsequent in vivo passages by ICV injection resulted in enhanced take rates of 100%, in concordance with Goldrosen’s model. Survival following in vivo passage was 21–45 days. In a pilot experiment, 5 mice were sacrificed on day 4 after ICV injection of in vivo passed MCA-38-LD. Although gross tumor was not visible, numerous micrometastases were easily detected (Fig. 1A). A liver from an animal sacrificed on day 19 is also shown for comparison (Fig. 1B).

**Immunotherapy Experiments**

**Comparison of ATC, LAK Cells, and High Dose IL-2 in the Treatment of MCA-38-LD Hepatic Metastases.** Both LAK cells and high dose IL-2 have previously been shown to be therapeutically effective in murine models of MCA-38 metastases (18, 19). In Rosenberg’s reports, LAK cells were typically administered at a dose of 10⁸ cells. By design, we conducted experiments with a much lower dose of LAK cells (0.5–1.0 x 10⁷) plus IL-2 (7,500 U/dose) alone. Fig. 2 shows representative whole livers with targets and effectors.

**Statistical Analysis.** Percentages of tumor volumes were normalized using the log transformation, y = log (1 + x), prior to statistical analysis of means (17). Differences between means of groups were then examined by analysis of variance, followed by unpaired Student’s t tests for comparisons between two individual groups.

**Fig. 1.** Metastatic adenocarcinoma to liver at 4 (A) and 19 (B) days after ICVinjection of 10⁵ MCA-38-LD tumor cells. At 4 days, a micrometastasis was present in the center of the field (hematoxylin-eosin; × 320). At 19 days the tumor nodules (darker staining) were of various sizes and occasionally completely confluent. In this case, over 75% of liver parenchyma was replaced by tumor (hematoxylin-eosin; × 5).

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\frac{\text{experimental} - \text{spontaneous release}}{\text{maximal} - \text{spontaneous release}} \times 100
\]

where “spontaneous” is defined as the amount of ⁵¹Cr released from target cells alone, “maximal” is the total ⁵¹Cr in the targets following lysis with Triton X-100, and “experimental” is the ⁵¹Cr released in wells with targets and effectors.

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IMMUNOTHERAPY OF LIVER METASTASES WITH ACTIVATED T-CELLS

Fig. 2. Immunotherapy experiment 1. Comparison of ATC, LAK cells, and high dose IL-2 in the treatment of MCA-38-LD hepatic metastases. Mice with 4-day MCA-38-LD hepatic metastases were randomized to receive no treatment (A), 0.5–1.0 × 10⁷ LAK cells plus 7,500 units IL-2 three times a day for 3 days (B), 0.5–1.0 × 10⁷ ATC plus 7,500 units IL-2 three times a day for 3 days (C), or high dose IL-2 (40,000 units three times a day for 3 days) (D). Animals were sacrificed 21 days after tumor injection. Representative whole livers are shown.

Fig. 3. Percentage of tumor volume of livers from mice in immunotherapy experiment 1. O, percentage of tumor volume from one mouse. •, means (±SE). * statistical significance (P < 0.05) compared to control. Note log scale.

metastases in this model. We then searched for more direct methods of T-cell activation in vivo. In a preliminary experiment, IL-2 could be omitted from the treatment protocol with no effect [mean percentage of tumor volume: ATC plus IL-2, 4.5 (7.4, 2.5); ATC alone, 8.4 (16.5, 4.0)].

Effects of Anti-CD3 on MCA-38-LD Hepatic Metastases. An experiment was designed to test the effects of a single dose of anti-CD3 in the hepatic metastasis model. As shown in Fig. 4, a single dose of approximately 4 μg of anti-CD3 significantly reduced the mean percentage of tumor volume [mean percentage of tumor volume:control, 25.2 (33.6, 18.8); anti-CD3 i.p., 1.0 (1.9, 0.4) (P < 0.0003); anti-CD3 i.v., 1.2 (1.7, 0.7) (P < 0.0003)]. There was no difference between anti-CD3 given i.p. or i.v. Of note, 2 of 6 mice in the anti-CD3 i.p. group and 4 of 12 mice in the i.v. group had no detectable tumor, suggesting that these animals were ‘cured’ of their liver metastases within the time frame (3 weeks) of this experiment.

In Vitro Cytotoxicity of ATC and LAK Cells

In order to assess potential mechanisms for the in vivo effects of anti-CD3 and ATC, we analyzed the cytotoxic potential of these effector populations in vitro.

In short term (4-h) cytolytic assays, P815 was highly susceptible to ATC effectors generated by culturing spleen cells in anti-CD3 for 3 days. However, ATC prepared from nylon wool-passed cells were substantially less cytotoxic than ATC prepared from spleen cells which had not been nylon wool purified. Yac 1.2 cells were not sensitive to ATC in this short term assay. P815, Yac 1.2, and MCA-38 cells were all sensitive to LAK cell killing as expected. Surprisingly, MCA-38-LD showed almost no in vitro sensitivity to ATC.

In long term (18-h) ⁵¹Cr release assays (Fig. 5), the relative contribution of nylon wool-adherent cells to ATC killing was more obvious. Significant killing of P815 was apparent by both nylon wool-passed and non-nylon wool-passed ATC, but the difference between the two effectors (20-fold in terms of lytic units) was more noticeable than in the short term assays (3-fold). The same was observed for Yac 1.2, with non-nylon wool-passed ATC substantially more cytotoxic than nylon wool-passed cells. Again, only minimal killing of MCA-38 was noted by either population of ATC cells. No differences in LAK cell killing occurred in the long term assays.
IMMUNOTHERAPY OF LIVER METASTASES WITH ACTIVATED T-CELLS

Fig. 5. In vitro cytotoxicity of LAK cells and nylon wool-fractionated ATC. Untreated C57BL/6 spleen cells were cultured with IL-2 (LAK cells) for 3 days. Spleen cells with or without passage over nylon wool columns were also cultured with anti-CD3 (ATC) for 3 days and tested for in vitro cytotoxicity against P815, Yac 1.2, and MCA-38-LD in an 18-h assay. ET, effector:target. Values represent mean cytotoxicity in triplicate wells. Similar, but less striking results, were observed in 4-h assays.

DISCUSSION

The results suggest that ATC induced in vivo or in vitro with anti-CD3 may be more effective in preventing tumor growth than LAK cells and IL-2. In the first series of experiments, a low number of LAK cells with IL-2 had no effect on the percentage of liver replaced by hepatic metastases. However, the same number of ATC, in combination with IL-2, significantly reduced the progression of metastases. These results are reminiscent of Rosenberg's original TIL studies, suggesting that ATC, like TIL, exhibit greater antitumor potency than LAK cells in this model (20, 21). In the second series of experiments, we showed that a single injection of anti-CD3 resulted in significant reduction of hepatic metastases and apparent cures in one third of animals. Comparing immunotherapy experiments 1 and 2, it appears that anti-CD3 treatment alone is at least as effective as ATC administration. Of note, the addition of IL-2 to the ATC treatment protocol did not improve upon the effects of ATC given alone, suggesting that the in vivo effects of ATC, unlike LAK cells, are not dependent on the administration of exogenous IL-2.

The MCA-38-LD, dimethylhydrazine-induced colon adenocarcinoma (22), cell line was chosen for our studies because it has been shown to be sensitive to high dose IL-2 alone, as well as low dose IL-2 in combination with LAK cells or TIL, in murine immunotherapy experiments (19, 20). The induction of hepatic metastases by ICV injection of tumor cells was used because of its high take rate and similarity to human colon cancer metastasis via the portal vein (14). Other hepatic metastasis models require splenectomy after tumor injection (23) and therefore possess theoretical disadvantages with respect to the integrity of the immune system of the animal.

Our in vivo studies confirm and extend a recently published report from Anderson et al. (24), using the MCA-106 pulmonary metastasis model. ATC were generated in their study with IL-2 and anti-CD3, although in vitro studies suggest that the cytotoxicity of ATC derives primarily from the presence of anti-CD3 in the culture. As in our study, lower doses of LAK cells (5 × 10⁶) produced no antitumor effect, while the same dose of ATC resulted in a positive result. Two other studies have demonstrated the value of a single dose of anti-CD3 in the amelioration of s.c. tumors and pulmonary metastases in murine tumor models. Ellenhorn et al. (12) demonstrated the importance of a low dose (~4 µg) of anti-CD3 in order to maximize T-cell activation and avoid modulation of the TcR with subsequent immunosuppression. Hoskin et al. (13) achieved equally beneficial results with anti-CD3 in their ras-induced tumor model. However, in none of these three studies were LAK cells or ATC compared directly to anti-CD3 in an established hepatic metastases immunotherapy model.

The mechanism whereby anti-CD3 treatment alone exerts its antitumor effect is not known, but it is reasonable to assume that ATC are generated in vivo. It has long been appreciated that anti-TcR mAb can induce T-cell activation in a manner analogous to cognate antigen (9, 25–27). Accessory cells play a role in ATC activation by anti-CD3. The results here show that, in 4- or 18-h ⁵¹Cr release assays, nylon wool-passed spleen cells, despite being enriched for T-cells, were considerably less cytotoxic than whole spleen preparations (non-nylon wool-passed). This provides indirect evidence that nylon wool-adherent cells (i.e., monocytes) contribute to T-cell activation and/or ATC killing.

T-cell activation by mAb results in induction of IL-2 receptor expression and IL-2 release in both in vitro and in vivo studies (10, 12, 28, 29). Somewhat surprisingly though, we observed that exogenous IL-2 administration was not needed for therapeutic efficacy of ATC. This is in marked contradistinction to LAK cell therapy, where there is an absolute requirement for IL-2 (19). Perhaps IL-2 released from ATC saturates IL-2 receptors in an autocrine and paracrine manner (29).

It is not clear whether the antitumor cytotoxicity of ATC either is a direct effector-to-target cellular effect or acts through a more indirect mechanism. Careful examination of the histological sections of liver metastases in our study failed to reveal a preponderance of TIL in the controls or any of the immunotherapy groups. This suggests a more indirect effect of ATC and anti-CD3 in tumor killing.

The inability of in vitro-generated ATC to manifest substantial in vitro cytotoxicity against the MCA-38 tumor line is also consistent with an indirect antitumor function for ATC. Lack of correlation between in vitro cytotoxicity and in vivo antitumor effects have also been noted with LAK cells (19).

The results of the present murine immunotherapy studies, as well as those of others (12, 13, 24, 25), suggest that activation of peripheral blood T-cells with anti-CD3 may be of potential clinical benefit in the treatment of human malignancies. A human anti-CD3, namely OKT3, has been available for a number of years and is used clinically as an immunosuppressive agent in transplantation. With much lower doses, activation of human T-cells occurs without the receptor modulation and resultant immunosuppression that occurs with the usual transplant dosages (30, 31). We have shown that, in the MCA-38-LD hepatic metastasis model, in vitro-generated ATC are more potent cytolytic effectors than LAK cells. Moreover, systemic administration of anti-CD3 alone is as efficacious as adoptively transferred ATC. This suggests that the massive leukopheresis required in LAK cell therapy may not be needed for ATC therapy. Furthermore, the use of high doses of exogenous IL-2, as is customary in LAK/IL-2 therapy, does not appear to be of major importance for ATC efficacy. Hence, the side effects of IL-2 which have compromised LAK immunotherapy may be avoided.

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