Cure of B16F10 Melanoma Lung Metastasis in Mice by Chronic Indomethacin Therapy Combined with Repeated Rounds of Interleukin 2: Characteristics of Killer Cells Generated in Situ

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ABSTRACT

We had earlier shown that a single round of interleukin 2 (IL-2) in chronically indomethacin treated mice can totally or nearly totally ameliorate established, experimental lung metastases and reactivate natural killer (NK) and lymphokine activated killer-like cells in the spleen. The present study examined whether a lasting cure of metastasis is obtainable by chronic indomethacin therapy (CIT) combined with single or multiple rounds of IL-2, and if so, what are the morphological, phenotypic, and functional properties of tumoricidal cells generated in situ. Experimental lung metastasis was produced in B6 mice by an i.v. injection of 10^6 B16F10 melanoma cells to compare therapeutic effects of six protocols: (a) CIT (14 mg/ml in drinking water) starting on day 5; (b) two rounds of IL-2 given on day 5; (c) two rounds of IL-2 alone; (d) two rounds of IL-2 alone; (e) two rounds of IL-2 plus indomethacin given only during the IL-2 therapy; and (f) which was similar to (c), but in addition, followed by repeated injections of IL-2 (25,000 units twice a day on Mondays and Fridays for 8 consecutive weeks). Results revealed that chronic indomethacin therapy alone or two rounds of IL-2 alone, or two rounds of IL-2 plus discontinuous indomethacin therapy reduced the lung metastases (examined at 21—25 days) by about two-thirds. In contrast, both single and multiple rounds of IL-2 in chronically indomethacin treated mice totally or nearly totally eradicated the lung metastases. However, longer-term disease-free survival (>13—16 months) resulted only with multiple rounds of IL-2. With chronic indomethacin therapy alone, NK-like (AGM-1^+ ^, Thy-1^− ^, Lyt-2^+ ^) killer lymphocytes (capable of killing NK sensitive YAC-1 lymphoma and B16F10 melanoma targets) appeared in the spleens, but not lungs; no killer activity was generated in macrophages at either site. Addition of a single round of IL-2 generated lymphokine activated killer-like killer lymphocytes (also capable of killing an NK resistant target) of the same phenotype, but of higher activity in the spleen; some lymphokine activated killer-like killer function was generated among pulmonary lymphocytes which were AGM-1^+, Thy-1^− ^, Lyt-2^+ ^, as well as among splenic but not pulmonary macrophages. A repetition of IL-2 therapy in these indomethacin treated animals generated the highest tumoricidal activity for all the targets in splenic and pulmonary lymphocytes which expressed AGM-1 as well as Thy-1 but not Lyt-2. Furthermore, significant killer function was now seen among splenic as well as pulmonary macrophages. Injection of AGM-1 antibody into these mice totally annulled the therapeutic effects on lung metastasis as well as killer cell reactivation. These results reveal that chronic indomethacin therapy combined with multiple rounds of IL-2 activates killer cells of the broadest spectrum and geographic distribution including the tumor site to result in a lasting cure of experimental metastasis, AGM-1 bearing cells playing a central role in this cure.

INTRODUCTION

We have recently shown that NK^2 lineage cells appear in high numbers in mice bearing spontaneous or transplanted tumors (1), but remain inactivated in situ due to PGs, primarily of the E_{2} series, secreted by host macrophages appearing in the lymphoid organs as well as the tumor site (2). Another possible source of PGE_{2} is tumor cells themselves. Some tumor lines grown in vitro (3) and tumors growing in vivo (4) have been reported to secrete PGE_{2}. PGE_{2} mediated immunosuppression has also been reported in human cancer patients (5—7). We have further shown that PGE_{2} released by macrophages isolated from tumor bearing hosts can equally suppress the polyclonal activation of T-cells, generation of CTL as well as generation of LAK cells in vitro (8). Finally, macrophage-secreted PGE_{2} has been found to down regulate the expression of cytolytic activity within the same macrophage population (9). Taken together, these observations suggest that release of PGE_{2} in vivo in the tumor bearing host may have pansuppressor effects against functional activation of numerous effector lineages. We have identified at least two mechanisms by which PGE_{2} prevents T-cell activation: a down regulation of IL-2R and an inhibition of IL-2 production (10); the latter mechanism has also been reported by others (11, 12). PGE_{2} action against NK and LAK cell activation may also be explained via similar mechanisms because of their IL-2 dependence; however, the mode of PGE_{2} action against macrophage activation remains to be elucidated.

Based on the above knowledge, we have recently initiated a series of studies in murine tumor models in order to devise therapeutic protocols aimed at reactivating killer lineage cells in vivo for the prevention and treatment of metastatic disease, which remains as the most serious obstacle in cancer cure. In the first series, we have recently shown that CIT designed to block PG synthesis in vivo, reduced the growth of s.c. mammary carcinomas in C3H mice and prevented their spontaneous metastases to the lungs; it caused a revival of host NK activity and a loss of NK suppressor function of tumor infiltrating macrophages (13). However, lung metastases, once well advanced, could not be completely cured with this therapy. In the second series, we used an experimental lung metastasis model with an i.v. inoculation of 10^6 highly metastatic B16F10 melanoma cells into C57BL/6 mice to compare the effects of CIT, therapy with a single round of IL-2, and a combination of the two on the progression of metastatic disease and on killer cell activation in the spleen (14). The rationale for this combination therapy was based on the expectation that a relief from PGE_{2}-mediated suppression by the chronic indomethacin treatment would allow a regeneration of IL-2R on the killer lineage lymphocytes; a supply of exogenous IL-2 over and above the endogenous production which may remain inadequate would then allow a maximal activation of tumoricidal cells in vivo. This study revealed that CIT alone (starting on day 0 or 5 after tumor inoculation) or a single round of IL-2 alone (for 5 days on days 10—14) reduced the number of lung nodules by two-thirds when examined on day 21. CIT (either protocol) supplemented with a single round of IL-2 as above completely or

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: NK, natural killer; AGM-1, asialo GM-1; CIT, chronic indomethacin therapy; IL-2, interleukin 2; rIL-2, recombinant interleukin 2; IL-2R, interleukin 2 receptor; LAK, lymphokine activated killer; PG, prostaglandin; CTL, cytotoxic T-lymphocyte.

4 R. S. Parhar, J. Nelson, and P. K. Lala, unpublished data.
nearly completely ameliorated the lung metastasis. Tumoricidal activity for NK sensitive targets inclusive of B16F10 cells was generated in splenocytes (examined on day 21) with all protocols, the magnitude increasing in the following order: indomethacin alone, IL-2 alone, and a combination therapy. Activity against NK resistant targets was only generated by IL-2 treatment, most notably with the combination therapy. Phenotypes of killer splenocytes were asialo GM-1", Thy-1", and Lyt-2" in all cases. Long-term survival of the host or the properties of potentially tumoricidal cells appearing at the tumor site were not examined in this study.

Present experiments were designed as a follow-up of the above studies using the B16F10 melanoma model of experimental lung metastasis in C57BL/6 mice to test whether a lasting cure of metastatic disease can be achieved by chronic indomethacin therapy supplemented with one or multiple rounds of IL-2, and if so, what are the characteristics of killer cells generated in situ responsible for such cure. We also examined whether repeated IL-2 therapy alone or an intermittent administration of indomethacin during the IL-2 therapy was as efficacious as chronic indomethacin administration combined with repeated rounds of IL-2.

MATERIALS AND METHODS

Mice. C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) at 8–10 weeks of age were used as hosts for the production of experimental metastases.

Tumor. B16F10 melanoma line cells (15, 16) with a strong lung metastasizing ability were injected i.v. at a high inoculum dose of 10⁹ cells/mouse to produce experimental metastases, the natural history of which at the microscopic and macroscopic levels has been reported earlier (14). This tumor line does not produce any significant level of PGE₂ in vitro.

Experimental Protocols for Therapy. Groups of six, randomly assigned, tumor inoculated animals were subjected to the following therapeutic regimens: group 1, indomethacin (Sigma, St. Louis, MO), 14 µg/ml in drinking water starting on day 5 after tumor inoculation (when lungs exhibit profuse micrometastases) and continued throughout the experimental period, as reported earlier (14); group 2, indomethacin, as in group 1, plus recombinant human IL-2 (17, 18) (r-IL-2, produced in Escherichia coli), Lots LP9C and LP315, kindly supplied by The Cetus Corp., Emeryville, CA, 25,000 units/inoculation given every 8 hours i.p., for 5 days on days 10 through 14, as reported earlier (14); group 3, same group 2 plus a second round of r-IL-2 for 5 days on days 20 through 24; group 4, two 5-day rounds of IL-2 alone on days 10 through 14 and 20 through 24 at dose levels similar to that in group 3; group 5, same as group 4, but in addition, given indomethacin in the drinking water only during the two 5-day rounds of IL-2. Control animals in the foregoing groups received either 0.2% ethanol in the drinking water (the vehicle for dissolving indomethacin) or injections of excipient buffer used for dissolving IL-2 or both, as required; group 6, same as group 3 but in addition receiving an i.v. injection of 60 µl of an anti-asialo GM-1 antibody (Wako Chemicals, Dallas, TX) on days -1, 4, 9, and 12, to test whether this treatment abrogated therapeutic effects observed in group 3. Control animals for group 4 received injections of a similar volume of normal rabbit serum instead of the antiserum which was raised in rabbit. This regimen for anti-asialo GM-1 treatment was based on our pilot studies designed to ablate NK activity in the spleen; similar or lower dosages have been reported to abolish NK (19) as well as CTL (20) activities in the murine spleen.

All groups (experimental and control) were killed at an appropriate time, as detailed below. Duplicates of groups 2 and 3 were also left for survival and autopsy at death. Experimental mice in these groups continued to receive indomethacin, and the control mice received 0.2% ethanol in drinking water until death. Survival only was recorded in a seventh group of five animals identical to group 3 which, in addition, received biweekly injections of IL-2 (Mondays and Fridays, 25,000 units, i.p., twice daily) for 8 consecutive weeks after the second round of IL-2.

Killing of Animals. Experimental and control mice in groups 1 and 2 were killed on day 21; those in groups 3–6 on day 25. All internal organs were examined for visible melanotic foci. Both lungs were removed for melanoma colony counts on fresh lungs under a dissecting microscope. Following this count, one lung of each pair was fixed in Bouin's fixative (14) for a duplication of the colony count and a subsequent histological preparation, and the second lung was used to isolate mononuclear cells for testing tumoricidal activity. Splenectomy were also collected for killer cell assays.

Isolation of Effector Cells for Killer Cell Assays. Unfixed lungs from each group were pooled, washed in ice-cold minimal essential medium (GIBCO, Grand Island, NY) to remove contaminating blood, and were finely minced with iris scissors. The fragments were then gently pressed in a siliconized glass homogenizer and rinsed with ice-cold minimal essential medium to release the mononuclear cells; the floating lung fragments were discarded subsequently. The resulting single cell suspension was then washed on a column of Ficoll-paque (Pharmacia Fine Chemicals, Dorval, Quebec) to remove erythrocytes, cellular debris, and most tumor cells, if any. The lymphoid cells (>95% pure) were recovered from the medium-gradient interface. Splenectomy pooled from each group were mechanically dispersed into a single cell suspension and fractionated as above to collect macrophages and lymphoid cells as separate populations. Enrichment of macrophages and lymphoid cells in these respective fractions was >95%.

Killer Cell Assay. A 4-h 51Cr release assay, as reported earlier (13, 14) was used to measure the percentage of specific cytotoxicity of effector lymphocytes at various effector:target ratios, using 51Cr-labeled YAC-1 lymphomas and B16F10 melanoma targets. When macrophages were used as effector cells, the 51Cr release assay was carried out for 18 h, using the same targets.

Phenotyping of Killer Cells. Killer cells were phenotyped for Thy-1, Lyt-2, and AGM-1 using a complement mediated cytotoxicity assay in the presence of appropriate antibodies as detailed earlier (14). The efficacy of the complement mediated cytotoxicity assay at appropriate dilutions of antibody and complement had been demonstrated in pilot experiments (14).

Statistical Evaluation. The significance of differences in the number of lung metastases between any two groups was determined by the Wilcoxon rank sum tests, using two-sided P values (21).

RESULTS

Effects of Therapy on Experimental Lung Metastasis. Results presented in Table 1 are based on melanoma nodule counts prepared on day 21 for groups 1 and 2 and day 25 for groups 3–6. Since scores on the same lung before and after fixation showed less than 2% variation, data derived from one fresh lung and the other fixed lung were pooled for presentation.

The difference between control and experimental mice in all groups (Table 1) were highly significant (P < 0.01), whereas the controls for various therapeutic protocols in groups 1–5 exhibited similar nodule counts (P ≧ 0.1 between any two of the controls). CIT alone (group 1) led to a marked (60%) reduction in the number of melanoma nodules. This therapy supplemented with a single round of IL-2 (group 2) nearly completely (99%) reduced the median number of foci; one of the mice showed node-free lungs on inspection. Addition of a second round of IL-2 (group 3) in chronically indomethacin treated mice resulted in 100% reduction of the median number of metastases. The difference between control and experimental mice in all groups (Table 1) were highly significant (P < 0.01), whereas the controls for various therapeutic protocols in groups 1–5 exhibited similar nodule counts (P ≧ 0.1 between any two of the controls). CIT alone (group 1) led to a marked (60%) reduction in the number of melanoma nodules. This therapy supplemented with a single round of IL-2 (group 2) nearly completely (99%) reduced the median number of foci; one of the mice showed node-free lungs on inspection. Addition of a second round of IL-2 (group 3) in chronically indomethacin treated mice resulted in 100% reduction of the median number of metastases. The difference between control and experimental mice in all groups (Table 1) were highly significant (P < 0.01), whereas the controls for various therapeutic protocols in groups 1–5 exhibited similar nodule counts (P ≧ 0.1 between any two of the controls). CIT alone (group 1) led to a marked (60%) reduction in the number of melanoma nodules. This therapy supplemented with a single round of IL-2 (group 2) nearly completely (99%) reduced the median number of foci; one of the mice showed node-free lungs on inspection. Addition of a second round of IL-2 (group 3) in chronically indomethacin treated mice resulted in 100% reduction of the median number of metastases. The difference between control and experimental mice in all groups (Table 1) were highly significant (P < 0.01), whereas the controls for various therapeutic protocols in groups 1–5 exhibited similar nodule counts (P ≧ 0.1 between any two of the controls). CIT alone (group 1) led to a marked (60%) reduction in the number of melanoma nodules. This therapy supplemented with a single round of IL-2 (group 2) nearly completely (99%) reduced the median number of foci; one of the mice showed node-free lungs on inspection. Addition of a second round of IL-2 (group 3) in chronically indomethacin treated mice resulted in 100% reduction of the median number of metastases. The difference between control and experimental mice in all groups (Table 1) were highly significant (P < 0.01), whereas the controls for various therapeutic protocols in groups 1–5 exhibited similar nodule counts (P ≧ 0.1 between any two of the controls). CIT alone (group 1) led to a marked (60%) reduction in the number of melanoma nodules. This therapy supplemented with a single round of IL-2 (group 2) nearly completely (99%) reduced the median number of foci; one of the mice showed node-free lungs on inspection. Addition of a second round of IL-2 (group 3) in chronically indomethacin treated mice resulted in 100% reduction of the median number of metastases. The difference between control and experimental mice in all groups (Table 1) were highly significant (P < 0.01), whereas the controls for various therapeutic protocols in groups 1–5 exhibited similar nodule counts (P ≧ 0.1 between any two of the controls). CIT alone (group 1) led to a marked (60%) reduction in the number of melanoma nodules. This therapy supplemented with a single round of IL-2 (group 2) nearly completely (99%) reduced the median number of foci; one of the mice showed node-free lungs on inspection. Addition of a second round of IL-2 (group 3) in chronically indomethacin treated mice resulted in 100% reduction of the median number of metastases. The difference between control and experimental mice in all groups (Table 1) were highly significant (P < 0.01), whereas the controls for various therapeutic protocols in groups 1–5 exhibited similar nodule counts (P ≧ 0.1 between any two of the controls). CIT alone (group 1) led to a marked (60%) reduction in the number of melanoma nodules. This therapy supplemented with a single round of IL-2 (group 2) nearly completely (99%) reduced the median number of foci; one of the mice showed node-free lungs on inspection. Addition of a second round of IL-2 (group 3) in chronically indomethacin treated mice resulted in 100% reduction of the median number of metastases.
showed histological features which depended on the time of death. One mouse dying on day 34 had features similar to those of group 2b (indomethacin plus one round of IL-2). In the rest (which survived much longer), microscopic melanoma was seen only when macroscopic melanoma was also visible. Melanoma-free areas of the lungs in these cases showed only very small mononuclear cell infiltration.

**Survival Time and Health of Animals.** Survival studies were only conducted for groups 2 and 3, specified above, since some of the experimental animals in these groups exhibited nodule-free lungs at sacrifice as noted in Table 1. Results presented in Table 2 and Fig. 1 show that control mice in both groups died between 22 and 28 days. CIT supplemented with a single round of IL-2, resulted in no long-term survivor, although there was an appreciable prolongation of the survival time (32–39 days). Mice at autopsy showed 20–43 lung nodules, some of which were large, indicating that a paucity of nodules noted on day 25 (Table 1) in group 2b was not permanent. Administration of a second round of IL-2 (group 3b) resulted in long-term survival (480+ days) in one-third of the animals. Five of six animals developed a local melanotic skin nodule at the injection site, first noticeable between 50 and 63 days. Of these mice, two that died on days 63 and 82, respectively, were left alone. At autopsy, they showed 9 and 11 lung nodules, respectively. The local tail tumor was removed by amputation of the tail in the remaining three survivors on day 93 when the tumor sizes were 8–10 mm in diameter associated with an ulceration of the skin. Of these mice, one died at 132 days, but the lungs and other organs were melanoma free, so that the reason for death

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effects of various treatment protocols on lung metastases</th>
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<tbody>
<tr>
<td>Group</td>
<td>No. of surface melanoma nodules in the two lungs*</td>
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<tr>
<td></td>
<td>Range</td>
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<tr>
<td>1a</td>
<td>Control</td>
</tr>
<tr>
<td>1b</td>
<td>Chronic indomethacin</td>
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<tr>
<td>2a</td>
<td>Control</td>
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<tr>
<td>2b</td>
<td>Chronic indomethacin + one round IL-2</td>
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<tr>
<td>3a</td>
<td>Control</td>
</tr>
<tr>
<td>3b</td>
<td>Chronic indomethacin + two rounds IL-2</td>
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<tr>
<td>4a</td>
<td>Control</td>
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<tr>
<td>4b</td>
<td>Two rounds IL-2</td>
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<tr>
<td>5a</td>
<td>Control</td>
</tr>
<tr>
<td>5b</td>
<td>Two rounds Indomethacin + IL-2</td>
</tr>
<tr>
<td>6a</td>
<td>Normal rabbit serum treated 3b</td>
</tr>
<tr>
<td>6b</td>
<td>Anti-AGM-1 treated 3b</td>
</tr>
<tr>
<td></td>
<td>* Differences between a and b were highly significant (P &lt; 0.01) for all groups.</td>
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<td>* Confluent; maximum countable number short of confluencc was approximately 600.</td>
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<td>* Number of nodule-free mice (out of 6) was one in group 2b, four in group 3b.</td>
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<td>* Compared to controls in group 3a.</td>
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5b), similar to the results obtained with CIT alone (group 1b). There was no significant difference (P > 0.1) among these three groups. Compared to these groups, the therapeutic success with CIT supplemented with one or two rounds of IL-2 (groups 2b and 3b) was distinctly superior (P < 0.01), whereas there was no significant difference (P > 0.1) between the two IL-2 treated groups receiving CIT (groups 2b and 3b) when nodule counts alone were considered. It is evident that with the combination therapy, a continuous use of indomethacin initiated prior to IL-2 therapy (group 3b) was distinctly superior to the administration of indomethacin only during the IL-2 therapy (group 5b).

Therapeutic benefit of indomethacin supplemented with 2 rounds of IL-2 (group 3b) was completely abrogated by repeated injections of an AGM-1 antibody as noted in group 6b; colony counts in this group was, in fact, slightly higher than but not significantly different (P > 0.1) from that in the control animals in group 3a which did not receive the indomethacin and IL-2 therapy. Injection of normal rabbit serum to similar mice (group 6a) also showed some significant (P < 0.01) deleterious effects, although not as marked as that caused by asialo GM-1 antibody. The difference between groups 6a and 6b was still highly significant (P < 0.01).

A small number of liver nodules were noted in control groups 1a–5a and experimental group 6b, but no liver nodules were seen in experimental groups 1b–5b or group 6a listed in Table 1.

Histology of the lungs recovered at sacrifice revealed features similar to those as reported earlier (14). A strong mononuclear cell infiltration in the lungs was seen only after IL-2 therapy under various conditions listed in Table 1 most marked in group 3b. Lungs free of visible nodules did not reveal microscopic melanoma. Lungs recovered at autopsy of animals dying after a therapy with indomethacin plus a single round of IL-2 all showed a small number of macroscopic as well as microscopic melanoma foci in the lungs, which also exhibited considerable mononuclear cell infiltration. At autopsy, lungs from mice in group 3b (indomethacin plus two rounds of IL-2)
remains uncertain. The remaining two mice have survived past 480 days and are in good health. They are being maintained on CIT.

Another group of 5 mice (group 7, Table 2) received the same treatment as in group 3b, followed by repeated IL-2 therapy, 25,000 units twice daily on Mondays and Fridays for 8 consecutive weeks. Three of these animals have so far remained in good health, beyond 400 days. Two mice died during the IL-2 therapy from unknown reasons which might include IL-2 toxicity. Their internal organs were melanoma free; lungs showed strong mononuclear cell infiltration.

Tumoricidal Activity and Characteristics of Killer Cells Appearing in Spleen and Lungs. Two targets were used in the evaluation of cytotoxicity. As shown in Fig. 2, normal C57BL/6 splenocytes showed good cytotoxicity for YAC-1 lymphoma and fair cytotoxicity for B16F10 melanoma cells. In both cases complement mediated abrogation of killer function was only noted after treatment of effector cells with anti-AGM-1 antibody but not anti-Thy-1 or Lyt-2 antibodies, indicating that the natural killer cell phenotype was AGM-1⁺, Thy-1⁻, and Lyt-2⁻. Splenocytes of tumor inoculated mice (group 2 controls) exhibited little or no cytotoxicity against either of the targets.

Fig. 3 shows that CIT alone caused a significant restoration of killer function of splenic lymphocytes against both tumor targets, mediated by AGM-1⁺, Thy-1⁻, and Lyt-2⁻ effector cells. No significant killer activity was shown by pulmonary lymphocytes in these mice.

CIT combined with a single round of IL-2 generated strong killer function in splenic lymphocytes for both tumor targets (Fig. 4), higher than that resulting from CIT alone (cf. Fig. 3), again mediated by AGM-1⁺, Thy-1⁻, and Lyt-2⁻ effector cells. In addition, significant killer activity was generated in pulmo-
KILLER CELLS IN METASTASIS CURE WITH INDOMETHACIN PLUS IL-2

Fig. 5. Cytotoxicity of splenic (left) and pulmonary (right) lymphocytes (subjected to various treatments) against YAC-1 targets in tumor-injected mice subjected to CIT and two rounds of IL-2. Animals at the top received this therapy alone (group 3b in Table I); those at the bottom also received repeated injections of anti-AGM-1 antibody (group 4b in Table I); those in the middle also received repeated injections of normal rabbit serum (NRS, group 4a in Table I). Effector cells were pooled from six mice. Data represent means of triplicate wells with <5% variation. •, untreated effector cells; ○, plus C'; △, Anti-Thy-1 plus C'; □, Anti-AGM-1 plus C'; △, Anti-Lyt-2 plus C'.

Macrophages isolated from the spleens and the lungs of control tumor-free and tumor-injected mice subjected to various protocols were tested for cytotoxic function against YAC-1 and B16F10 targets (Fig. 7). Little or no killer function was exhibited by macrophages isolated from normal tumor-free mice or control tumor inoculated mice or tumor inoculated mice treated

Fig. 6. Data as in Fig. 5, except that cytotoxicity was measured against B16F10 targets. NRS, normal rabbit serum.
with indomethacin alone. CIT combined with a single round of IL-2 generated a small amount of macrophage killer function in the spleen for both tumor targets, but higher for the YAC-1 target. This killer function was stimulated further with two rounds of IL-2. In this case, a small amount of killer activity was also generated in pulmonary macrophages. A prior treatment of effector cells with anti-AGM-i antibody reduced this killer function by splenic adherent cells to 60–70% of the original level (results not presented), indicating detectable levels of AGM-i expression by killer macrophages.

DISCUSSION

Experimental lung (and liver) metastases produced in C57BL/6 mice by an i.v. inoculation of an overdose of B16F10 melanoma cells could be totally eradicated by chronic indomethacin therapy combined with repeated rounds of IL-2, leading to healthy, long-term survivors. CIT alone (in confirmation of our earlier report, Ref. 14), or two rounds of IL-2 alone or two rounds of IL-2 plus indomethacin given during these rounds, all resulted in a substantial but not a complete amelioration of metastases. Although a single round of IL-2 given to mice placed on CIT resulted in nodule-free lungs when examined at short term, as noted in the present as well as the earlier reported series (14), none of the animals showed long-term, disease-free survival, indicating the need for a repetition of the IL-2 therapy. Excellent results have also been reported in experimental models with repeated therapy combining LAK cells and IL-2 (22–29); this combination as well as IL-2 alone have recently been applied to human trials, leading to promising results (30–33), in which indomethacin has occasionally been used intermittently to combat side effects of IL-2. Present study in mice reveals that intermittent use of indomethacin during IL-2 therapy is not as efficacious as chronic, uninterrupted indomethacin therapy combined with multiple rounds of IL-2. Whether the latter therapeutic protocol can ameliorate human metastatic disease remains to be tested. With this objective in mind, we have tested a lower chronic dose of indomethacin plus identical doses of IL-2 in the present tumor model for possible extrapolation to the human. We noted that a dose of 7 μg/ml given in the drinking water is slightly inferior to the dose of 14 μg/ml; however, the difference is not statistically significant (P > 0.1). The major advantage of the presently described mode of combination therapy would be its simplicity, permitting its practice in conventional cancer centers. Prostaglandin mediated immunosuppression, also reported in human cancer patients (5–7), makes these patients logical candidates for this therapeutic protocol.

It was interesting to note that despite a cure of the metastatic disease, local melanoma nodules in the skin at the i.v. injection site appeared at later intervals in a large proportion of mice, evidently resulting from extravasation of tumor cells during the injection. Similar tumors have also been reported by Mule et al. (26) in i.v. tumor inoculated mice undergoing LAK cell and IL-2 therapy. In our series, these mice were curable by amputation of the tail. It is highly likely that there was a poor migration of the activated mononuclear cells to the tail skin, as suggested by a preliminary histological examination of the tumor site in amputated tails. Whether this condition is peculiar to the tail skin or skin in general is being investigated by percutaneous implantation of melanoma cells in the trunk of mice undergoing similar therapy.

Characterization of tumoricidal effector cells in mice subjected to different therapeutic protocols reveals several features: (a) CIT alone reactivated NK-like (AGM-1*, Thy-1*, Lyt-2*) killer lymphocytes effective against NK sensitive YAC-1 as well as B16F10 targets but not NK resistant thymic lymphoma 9705 target (data not shown) in the spleen as reported earlier (14), but not the lungs. No killer macrophages were generated at either site with this therapy; (b) CIT combined with one round of IL-2 generated a higher killer activity among splenic lymphocytes, which were AGM-1*, Thy-1*, and Lyt-2*, consistent with our earlier findings (14). These cells had LAK-like activity since they also killed an NK resistant target thymic lymphoma 9705 (data not presented). Some LAK-like killer function was also generated in pulmonary lymphocytes which were AGM-1* as well as Thy-1*. Finally, some killer activity was also generated in splenic macrophages; (c) a repetition of IL-2 therapy in mice maintained on indomethacin generated the highest LAK-like killer activity in splenic as well as pulmonary lymphocytes which expressed AGM-1 as well as Thy-1. Furthermore, significant killer function in macrophages was now seen in the spleen as well as the lungs. These results reveal that highly active killer cells of the broadest spectrum and wide geographic distribution in the body inclusive of the tumor site are generated in mice subjected to CIT combined with repeated rounds of IL-2. While AGM-1 remained as a common marker for all killer lymphocytes generated by the different therapeutic protocols, an expression of Thy-1 was noted only when IL-2 was used, the highest expression going hand-in-hand with the highest degree of activation. Thus a lower level of expression of Thy-1 in splenic effector lymphocytes in indomethacin treated mice receiving a single round of IL-2 may be explained by the fact that the mice were killed 7 days after the last IL-2 injection, whereas mice receiving two rounds of IL-2 were killed 1 day after the last IL-2 injection. This may also explain the presence of Thy-1* effector lymphocytes in the lungs of the latter but not the former group; these cells may have either a short life span or an activation span in the lungs, and Thy-1 may represent a terminal activation marker on these cells.

It is likely that killer lymphocytes as well as killer macro-
phages contribute to the therapeutic success in the combination therapy using repeated rounds of IL-2. Cell lineages bearing AGM-1 were possibly most important, since in vivo injection of the AGM-1 antibody totally negated the therapeutic benefits. Most likely AGM-1 is a universal marker for most or all murine killer lineage cells at some stage of their life in vivo. Although the effector stages of NK cells (AGM-1, Thy-1, and Lyt-2) (34), CTL (AGM-1, Thy-1, and Lyt-2) (20, 34), and in vitro generated LAK cells (AGM-1, Thy-1, Lyt-2) (27, 28, 35–37) are distinguishable to some extent by surface markers and target cell selectivity, precursor stages of these lineages remain less clearly distinguishable (38). Precursors of NK (34, 38, 39) and LAK cells (36, 39) as well as CTL (40) have been reported to express AGM-1. Although in vivo treatment with anti-AGM-1 antibody may not effectively eliminate the ability of spleen cells to generate LAK cells in vitro (38), it has recently been shown that LAK cells generated in vivo with IL-2 therapy express AGM-1 (41). Finally, we noted some AGM-1 expression on the killer macrophages generated in vivo.

We have recently shown that PGE2 or macrophages isolated from tumor bearing host can suppress the activation of numerous effector lineage cells in vitro, the macrophage mediated suppression being relieved by indomethacin (8). Activation of NK cells, polyclonal activation of T-cells, and generation of CTL and LAK cells (which are all IL-2 dependent) are all vulnerable to this suppressor mechanism, which blocks the generation of IL-2R as well as the production of IL-2 (10). Thus, it is not surprising that CIT combined with repeated IL-2 therapy reactivates the broadest spectrum of killer lymphocytes in situ in the metastasis bearing mice. However, the need of exogenous IL-2 for an activation of tumoricidal macrophages in vivo in indomethacin treated hosts was an unexpected finding. Although an indirect mechanism such as the production of unidentified macrophage stimulating growth factors by the same or another subset of macrophages in short-term cultures in the presence of γ interferon unmasked the role of indomethacin enhancing for tumoricidal function by PGE2 produced by the same or another subset of macrophages in short-term cultures in the presence of γ interferon, the IL-2 mediated activation of phagocytes by PGE2 produced by the same or another subset of macrophages in short-term cultures in the presence of γ interferon remains to be established.

Although an indirect mechanism such as the production of unidentified macrophage stimulating growth factors by the same or another subset of macrophages in short-term cultures in the presence of γ interferon, the IL-2 mediated activation of phagocytes by PGE2 produced by the same or another subset of macrophages in short-term cultures in the presence of γ interferon remains to be established.

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