AMELIORATION OF B16F10 MELANOMA LUNG METASTASIS IN MICE BY A COMBINATION THERAPY WITH INDOMETHACIN AND INTERLEUKIN 2

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Metastasis remains the most formidable obstacle in cancer therapy. While there is reasonable evidence to suggest the existence of a natural cellular surveillance against the development of certain neoplasia (1), as well as the metastatic spread of tumor cells via circulation (2), such a surveillance often fails to contain the growth of most primary tumors or their metastatic dissemination. We have recently shown (3) in a number of murine tumor models, that the host NK cell activity declines rapidly during the development of transplanted or spontaneous tumors. This decline results from neither the disappearance of NK lineage cells (3) nor their maturation arrest (4), but from their rapid inactivation by the host-derived NK suppressor cells appearing in the lymphoid organs, as well as at the tumor site (4). They have been characterized as cells of the monocyte/macrophage class with phagocytic ability and bearing surface Mac-1. Their suppressor function is mediated by PG, and is abrogated in the presence of indomethacin, an inhibitor of PG synthesis (4). We have further noted that tumor-derived macrophages, or chemically pure PGE_2 can block a variety of lymphocyte responses in vitro: functional activation of NK cells, lectin (Con A)-induced lymphocyte proliferation and generation of killer cells, alloreactive proliferation of T cells and their subsequent differentiation into CTL, as well as the generation of lymphokine (rIL-2)-activated killer (LAK)[1] cells, the macrophage-mediated suppression being preventable in the presence of indomethacin (Lala, P. K., and R. S. Parhar, unpublished observations). These results suggest that the production of PG, particularly of the E series (PGE), may have pansuppressor effects against the activation of various classes of effector cells with tumoricidal potential in vivo in the tumor-bearing host. While the source of PGE has been identified as host macrophages in numerous tumor models in our study (4), certain tumors may also be capable of producing PGE (5). Furthermore, we have identified two important mechanisms of PGE_2-mediated blockade of T cell activation in the presence of alloantigens or a polyclonal mitogen Con A: a down regulation of IL-2-R development on the stimulated lymphocytes, and an inhibition of production of IL-2 by these cells; we saw no effect on the interaction between IL-2-R and IL-2, nor on the lytic function of CTL once generated (6).

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[1] *Abbreviations used in this paper:* AGM1, asialo-GM-1; CCDF, cytotoxic cell differentiation factor; LAK, lymphokine-activated killer cell; LICC, lymphokine-induced cytotoxic cells.

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A down regulation of transferrin receptor development on T cells has also been documented (7). While the precise mechanism of PGE_2 action on NK lineage cells remains to be fully understood, a suppression of their activation (4) as well as their killer function (8, 9) has been documented.

We have recently examined the in vivo relevance of the PG-mediated suppression of natural host defence against cancer cells to their metastatic spread from the primary site (9). Using three C3H mammary carcinoma lines derived from a single spontaneous tumor, but varying in their lung metastasizing ability, we have observed that chronic indomethacin therapy shortly after a subcutaneous tumor transplantation produces the following effects: (*a*) a reduction in the growth of the primary tumor, (*b*) a prevention of lung metastasis, (*c*) a restoration of host NK activity, and (*d*) an abrogation of NK suppressor function of tumor-infiltrating macrophages (9). These results suggest that the PG-mediated NK suppression promotes tumor metastasis, which can be contained by indomethacin therapy. However, we find that lung metastasis from the primary tumors, once well established and advanced, can not be completely cured with indomethacin therapy when the therapy is provided at later intervals (Parhar, R. S., J. Nelson, and P. K. Lala, unpublished observations). Encouraged by the recent success of a combination therapy with IL-2 and LAK cells of established tumors and their metastases in mice (10–18) and man (18, 19), the present study was designed to test whether a combination therapy with indomethacin and IL-2 is superior to indomethacin alone or IL-2 alone in ameliorating established experimental metastasis of B16F10 melanoma cells. The rationale of this study was based on the contention that a relief of the endogenous suppression with indomethacin would allow a generation of sufficient levels of IL-2-R on the various effector lineage lymphocytes, which then can be stimulated further by the exogenous IL-2 provided over and above the endogenous IL-2 levels achieved by such therapy. Furthermore, we examined the characteristics of the splenic effector cells generated by such therapeutic measures in vivo, as revealed by their surface phenotype and cytocidal spectrum.

Materials and Methods

Mice. Young adult (8–10 wk old) C57BL/6 (henceforth called B6) mice (The Jackson Laboratory, Bar Harbor, ME) were used as hosts for the production of experimental metastasis.

Tumor. B16F10, a melanoma line with high lung metastasizing ability, produced initially by I. J. Fidler (20, 21), was obtained through the courtesy of A. Chambers of the Department of Radiation Oncology, The University of Western Ontario. This line, in our hands, does not produce any significant level of PGE_2 in vitro. Cells grown in tissue culture were injected intravenously at a high inoculum dose of 10^6 cells per mouse to produce experimental lung metastasis. Mice killed on day 5 all showed large numbers of well-established foci of microscopic metastases (micrometastases) in both lungs. Mice killed on day 10 all exhibited high numbers of visible (macro) metastases. Hosts usually died during week 4 after injection. Those killed on day 21 or autopsied at death showed confluent lung metastases of various sizes, and occasional melanotic foci in the liver. The experimental protocol was designed on the basis of this background information.

Experimental Protocol for Therapy. A total of 35 animals were injected intravenously via the tail vein with 10^6 melanoma cells. They were randomly assigned to seven groups of five animals per group. Five groups of experimental animals were subjected to the following therapeutic protocols: (Group 1) Indomethacin (Sigma Chemical Co., St. Louis, MO), at a concentration 14 μg/ml in drinking water, provided throughout the experi-

mental period from day 0 to day 21. Indomethacin was initially dissolved in absolute ethanol and then diluted in drinking water, resulting in a concentration of 0.2% ethanol, as reported earlier (9). Bottles were changed twice a week with freshly prepared materials. (Group 2) Indomethacin at a similar concentration provided from day 5 to day 21. (Group 3) Human rIL-2 (22, 23) (produced in *Escherichia coli*, lots 9A and LP9C, kindly supplied by the Cetus Corp., Emeryville, CA), 25,000 U per inoculum, injected intraperitoneally every 8 h for 5 d on days 10–14. (Group 4) Indomethacin, as given to group 1, plus rIL-2 as given to group 3. (Group 5) Indomethacin as given to group 2 plus rIL-2 as given to group 3. Two groups of control animals were used: the first group received vehicle alone used for dissolving indomethacin, i. e., 0.2% ethanol in drinking water throughout the experimental period (day 0–21); and the second group received the same drinking vehicle as above (day 0–21), plus intraperitoneal injections of the excipient control (solvent buffer for IL-2) every 8 h for 5 d (days 10–14).

Experiments were repeated for a second time to include group 1 as above in the control series, and groups 2 and 5 in the experimental series, using six mice per group. Since these essentially reproduced the results of the first series, the detailed results of the second series will not be presented.

Killing of Animals. All animals (both experimental and control groups) were killed on day 21, followed by a thorough visual examination of all internal organs for visible melanotic nodules, identifiable on the basis of the black melanin pigment. Lungs were removed and fixed in Bouin's Fixative for melanoma colony counts (24) and subsequent histological preparation. With this fixation procedure, black melanotic nodules stood out prominently on the light yellow background. Freshly isolated spleens were used to make single cell suspensions, free from red cells and debris (3), for use as effector cells in killer-cell assays against numerous tumor targets. For this purpose, cells were pooled from the animals in each group.

Killer-Cell Assay. A 4-h ^{51}Cr-release assay, as described earlier (3), was used to measure the percent specific cytotoxicity of spleen cells at various E/T ratios (12.5:1–100:1). The cytocidal spectrum was assessed by using the following tumor targets, all obtained from exponential phase growth in tissue culture and exhibiting >98% viability: YAC-1 lymphoma (a highly NK-sensitive line); B16F10 melanoma (a moderately NK-sensitive line in our hands), and thymic lymphoma 9705 (an NK-resistant line, kindly provided by Dr. F. P. H. Chan of the Department of Anatomy, The University of Western Ontario).

Phenotyping of Killer Cells. Killer cells were phenotyped for the presence of the surface markers Thy-1, Lyt-2, and asialo-GM-1 (AGM1) by using a complement-mediated cytotoxicity assay in the presence of appropriate antibodies. Monospecific anti-Thy-1.2 antibody (E Thy-1.2 of the National Institutes of Health [Bethesda, MD] catalogue, courtesy of Dr. J. G. Ray, NIH) and anti-Lyt-2 mAb (Becton Dickinson & Co., Sunnyvale, CA) were used at 1:20 dilution. Rabbit anti-AGM1 (Wako Chemicals, Dallas, TX) was used at 1:200 dilution. Rabbit complement (low tox, Cedarlane Laboratories, Hornby, Canada) was used at 1:20 dilution. These dilutions were based on control studies. Untreated spleen cells, or spleen cells treated with complement alone (controls), or with appropriate antibody (at 4°C for 45 min, followed by three washes), followed by complement (at 37°C for 45 min, followed by three washes) were used as effector populations. The E/T ratio was computed on the basis of the effector cell number before antibody or complement treatment so that the results could not be biased by an enrichment of a particular subset in the surviving effector population (25). All the treatment and the washes were carried out in the same tube to avoid any cell loss by transfer.

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 (26).

Results

Effects of Various Therapeutic Protocol on Experimental Lung Metastasis. Visible melanoma nodules detectable on day 21 after an intravenous inoculation of 10^6 melanoma cells reached a confluence in many of the control animals, the

TABLE I

Effects of Various Therapeutic Measures on Lung Colony Number

Protocol	Number of macroscopic melanoma nodules in the two lungs (day 21)			
	Range	Mean	Median	Percent reduction (median)
Control*	221–600+‡	418	473	0
	(297–600+)§	(471)	(473)	(0)
Indomethacin alone (days 0–21)	97–192	163	176	63
Indomethacin alone (days 5–21)	157–201	175	173	64
	(193–278)	(224)	(213)	(55)
IL-2 alone	102–174	146	152	68
Indomethacin (days 0–21) + IL-2	0–5‖	1	0	100
Indomethacin (days 5–21) + IL-2	0–9¶	2	0	100
	(0–17)	(9.5)	(9)	(98)

* Data in the two control groups were very similar and thus were pooled.
‡ The maximum number of countable nodules short of confluence was ~600.
‖ Only one animal showed five nodules.
¶ Only one animal showed nine nodules.
§ Data in parentheses represent results from the second series of experiments.

maximum scorable number in a pair of lungs being ~600. Both control groups provided very similar data and are thus pooled in Table I. Two of five animals in each control group also showed a small number of nodules on the surface of the liver, but on no other organs such as kidneys, spleen, intestine, mesentery, or reproductive organs. The two protocols of indomethacin administration alone (starting on day 0 or day 5) and the protocol of IL-2 therapy alone led to a substantial (all significant at $p \ll 0.01$) and very similar (63–68% median) reduction in the lung nodules as compared with the control animals. There was no significant ($p \gg 0.1$) difference between any two of these three experimental groups. A combination therapy with indomethacin plus IL-2, irrespective of the onset of indomethacin therapy (i. e., day 0 or 5) led to a complete freedom from visible melanotic foci on the lung surface in all animals, except one animal in each group that showed a small number of residual nodules also heterogeneous in size (Table I). The reduction of metastases with either protocol of combination therapy was highly significant ($p \ll 0.01$) compared with the control or either of the indomethacin groups, or the group receiving IL-2 alone. There was no significant difference ($p \gg 0.1$) in the results provided by the two protocols of combination therapy. None of the experimental animals exhibited any liver nodules. Representative photographs of the lungs are presented in Fig. 1.

A repetition of some of these experiments (second series) closely reproduced the results of the first series, as indicated by the data in parentheses in Table I.

A histological examination of the lungs that exhibited visible surface nodules also showed the presence of tumor cell foci in sections. Sections of nodule-free lungs of mice that had received a combination therapy were also free from microscopic foci of tumor cells. However, whether these lungs were totally devoid of tumor cells can only be answered on the basis of serial sections, which have not yet been performed. All experimental lungs exhibited visible levels of mononuclear cell infiltration. The infiltration was higher with IL-2 therapy than

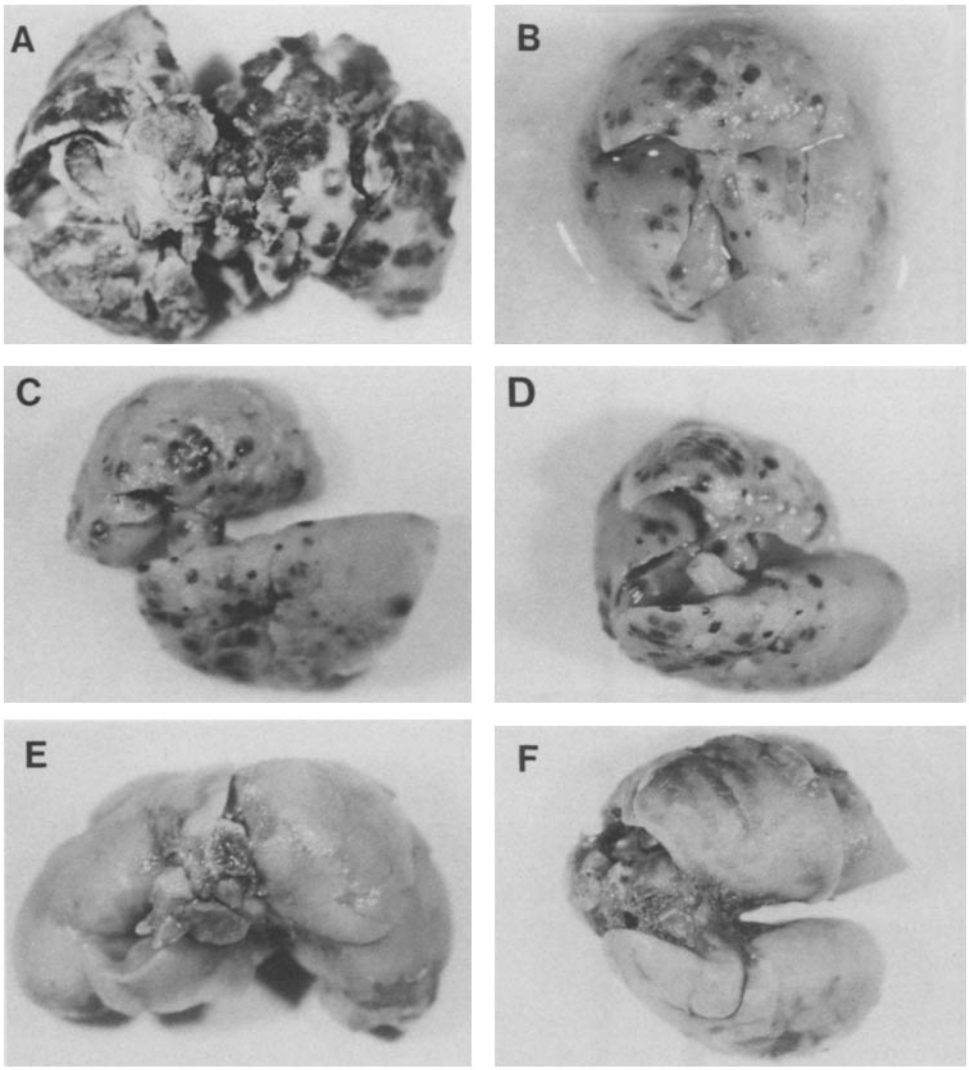

FIGURE 1. Representative photographs of the lungs from melanoma-inoculated mice. (*A*) Control mouse receiving 0.2% ethanol in drinking water from day 0 and excipient buffer (for dissolving IL-2) intraperitoneally on days 10–14. (*B*) Mouse receiving indomethacin alone from day 0. (*C*) Mouse receiving indomethacin alone from day 5. (*D*) Mouse receiving IL-2 alone on days 10–14. (*E*) Mouse receiving indomethacin from day 0 plus IL-2 on days 10–14. (*F*) Mouse receiving indomethacin from day 5 plus IL-2 on days 10–14.

with indomethacin therapy and was most marked with the combination therapy. In the latter case, alveoli in some areas of the lungs were totally replaced by young granulation tissue containing mononuclear cell exudate, possibly representing the sites of clearance of the original tumor foci. Representative histological pictures are presented in Fig. 2.

Cytocidal Spectrum and the Phenotype of Splenic Killer Cells Generated In Vivo. Results are only presented for the first series of experiments, since the second series closely reproduced the data as presented in the first series.

YAC-1 Target. Figs. 3 and 4 present the results of percent specific cytotoxicity

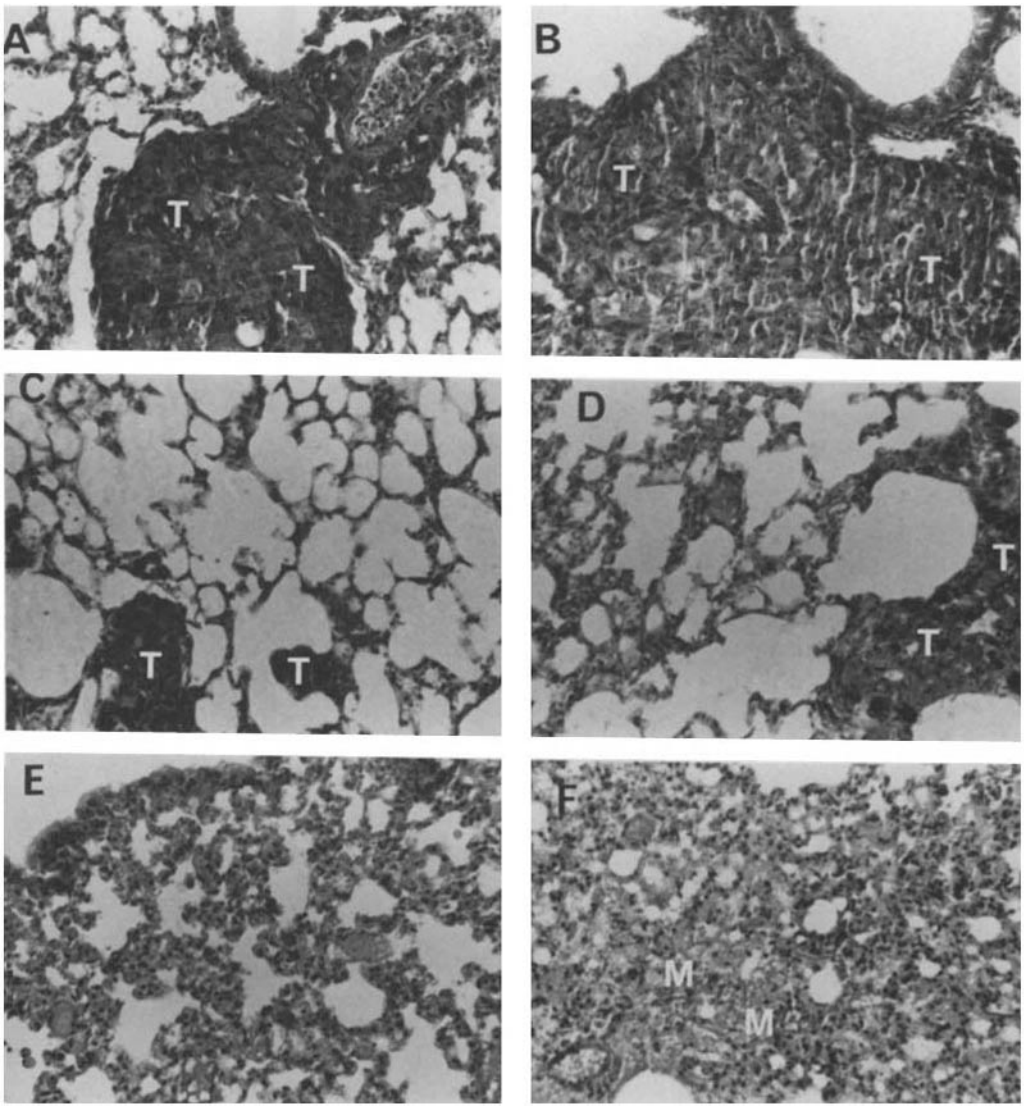

FIGURE 2. Representative photomicrographs (× 160) of H and E–stained sections of paraffin-embedded lungs of (*A*) mouse killed on day 5 after intravenous inoculation of 10^6 cells; (*B*) control mouse receiving 0.2% ethanol in drinking water from day 0 and excipient buffer (for dissolving IL-2) intraperitoneally on days 10–14; (*C*) mouse receiving IL-2 alone; (*D*) mouse receiving indomethacin from day 0 plus IL-2 on days 10–14; (*E*) mouse receiving indomethacin from day 0 plus IL-2 on days 10–14; (*F*) mouse receiving indomethacin from day 5 plus IL-2 on days 10–14. *T*, melanoma tumor nodules in *A–D*. Note variable degrees of interstitial mononuclear cell infiltration in *C–F*, more marked in *D* than in *C* and most marked in *E* and *F*. A large area of young granulation tissue represented by mononuclear cell exudate (indicated by *M*), replacing alveoli is shown in *F*. Such areas are encountered in both groups *E* and *F*, although not shown in *E*. They possibly represent the replacement sites of original melanoma nodules.

of normal spleen, spleen of control tumor–inoculated mice, or experimental tumor inoculated mice subjected to different therapeutic protocols. As expected, YAC-1 target was sensitive to lysis by normal B6 splenic effector cells that

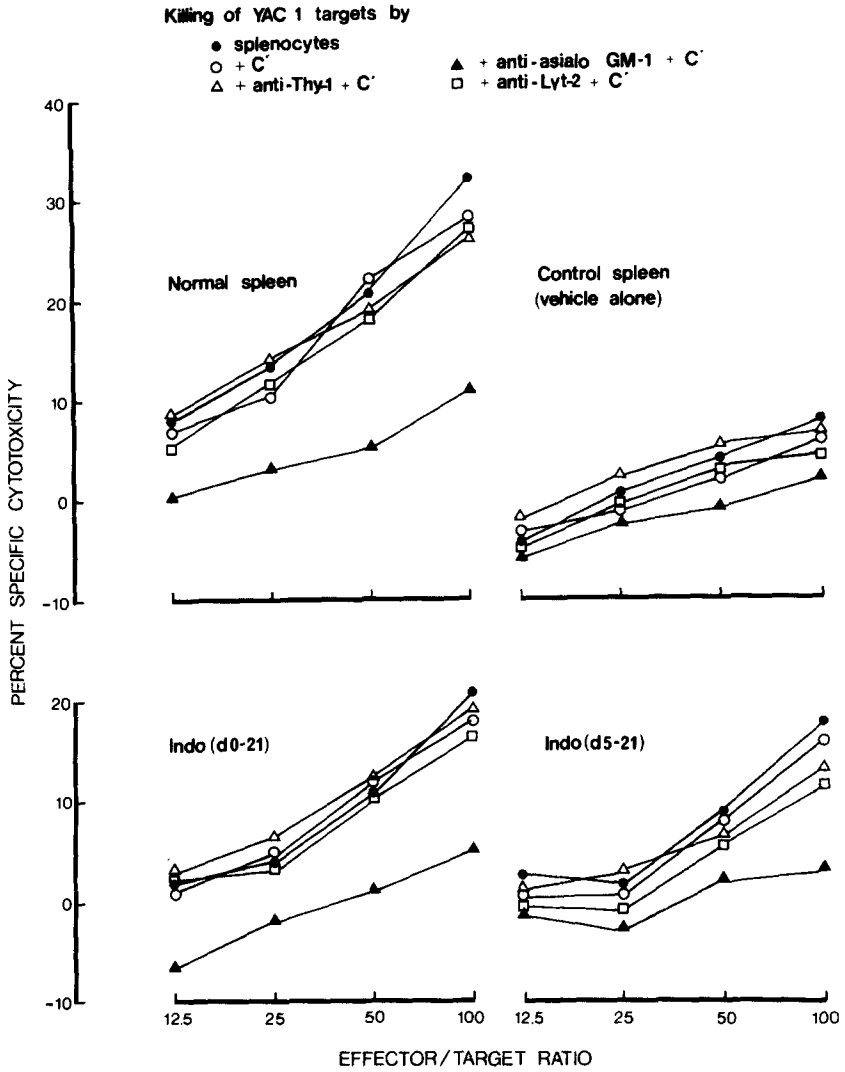

FIGURE 3. Anti YAC-1 cytotoxicity of splenic effector cells (subjected to various treatments) from normal healthy mice and tumor-inoculated mice treated with vehicle alone or indomethacin. Control (vehicle alone) group received 0.2% ethanol in drinking water from day 0. Control animals receiving the same treatment plus excipient buffer intraperitoneally (days 10–14) provided very similar results and are not presented. Effector cells were pooled from five mice in each group. Data represent means from triplicate wells with <5% variation. Indo, indomethacin.

expressed surface AGM1 but not Thy-1 or Lyt-2. Killer activity was reduced to near background levels in the spleen of control tumor inoculated groups. Treatment with indomethacin alone beginning on day 0 caused a substantial restoration of the killer activity measured on day 21, again $AGM1^+$, $Thy\text{-}1^-$ and $Lyt\text{-}2^-$ cells. Similar but somewhat less marked effects were produced by indomethacin therapy beginning on day 5 (Fig. 3). IL-2 therapy alone (Fig. 4) restored the killer function to above normal levels, and the effector cells were $AGM1^+$, $Thy\text{-}1^-$, and $Lyt\text{-}2^-$. Combination therapy with IL-2 plus indomethacin starting

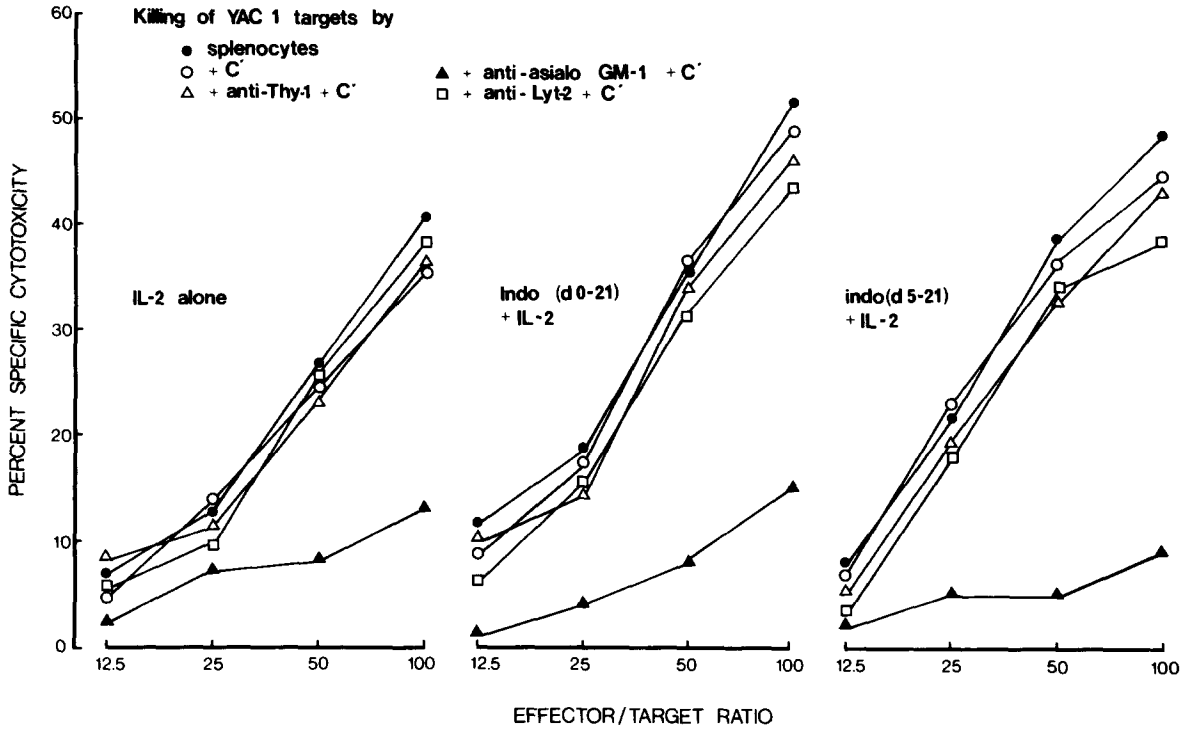

FIGURE 4. Anti YAC-1 cytotoxicity of splenocytes (subjected to various treatments) from tumor-inoculated animals treated with IL-2 alone or indomethacin plus IL-2. Effector cells were pooled from five mice in each group. Data represent means from triplicate wells with <5% variation. Indo, indomethacin.

either on day 0 or on day 5 stimulated the killer activity further and the killer cells were of the identical phenotype (Fig. 4).

B16F10 Target. B16F10 was found to be moderately sensitive to lysis by normal splenic effector cells, which also proved to be of the classical NK phenotype, i. e., AGM1$^+$, Thy-1$^-$, and Lyt-2$^-$ (Fig. 5). Cytotoxic responses of this target to spleen cells from control tumor–inoculated mice or tumor–inoculated mice subjected to various therapeutic protocols were nearly identical to that of YAC-1 target, except for the fact that with indomethacin alone starting on day 0 or day 5, there was a complete restoration of killer function (Figs. 5 and 6). The killer cell phenotype, on all occasions, was again AGM1$^+$, Thy 1$^-$, Lyt 2$^-$.

Thymic Lymphoma 9705 Target. As shown by the poor cytolytic ability of normal splenic effector cells (Fig. 7), this tumor line is relatively NK resistant. Tumor bearing caused a further reduction of the splenic effector function, which was restored to control levels by indomethacin therapy starting on day 0 or day 5. IL-2 alone or IL-2 combined with indomethacin in either protocol caused an appreciable stimulation of killer activity above normal levels, the highest stimulation being provided with the combination therapy of IL-2 plus indomethacin beginning on day 0. In all cases, a nearly complete loss of effector function was only achieved with anti AGM1 + C′, indicating that the effector cells were again AGM1$^+$, Thy-1$^-$, and Lyt-2$^-$.

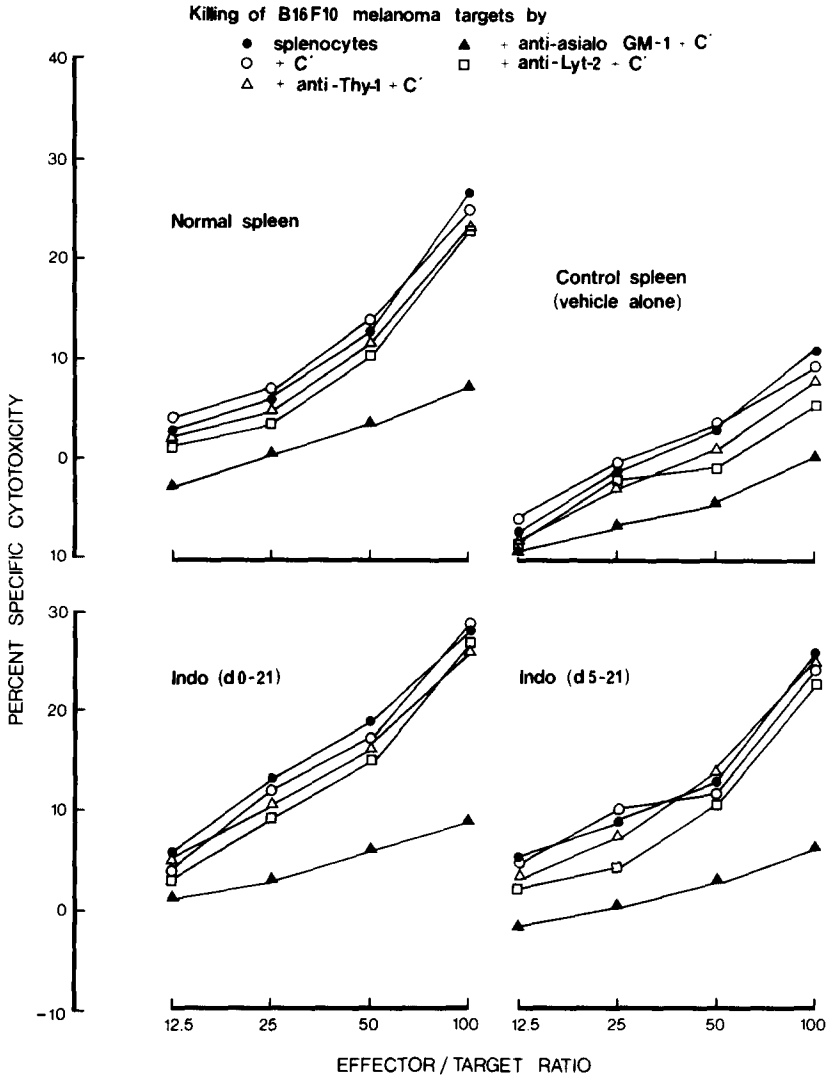

FIGURE 5. Anti-B16F10 cytotoxicity of splenocytes (subjected to various treatments) from normal healthy mice and tumor-inoculated mice treated with vehicle alone or indomethacin. Control (vehicle alone) group is the same as in Fig. 3. Those receiving, in addition, the excipient buffer intraperitoneally, provided nearly identical data (not presented). Effector cells were pooled from five mice in each group. Data represent means from triplicate wells with <5% variation. Indo, indomethacin.

Discussion

Chronic administration of indomethacin by the oral route (14 μg/ml of drinking water) or a 5-d regimen of intraperitoneal administration of 25,000 U IL-2 every 8 h led to a similar and substantial, but incomplete, containment of experimental lung metastasis produced by an intravenous injection of 10^6 B16F10 melanoma cells in B6 mice. Lack of any appreciable difference between the effects of the indomethacin regimen initiated on day 0 and day 5 can be explained by our earlier observations that tumor transplantation in mice causes

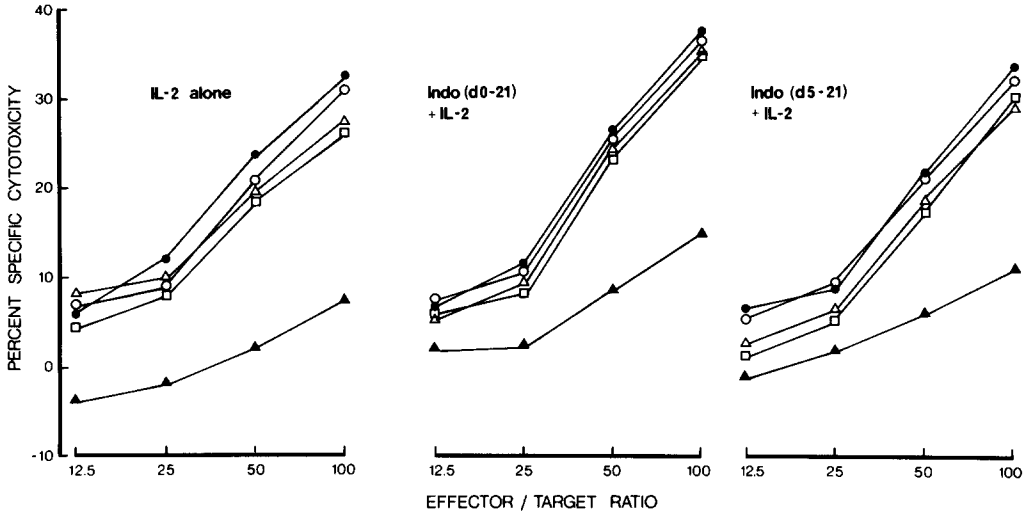

FIGURE 6. Anti-B16F10 cytotoxicity of splenocytes (subjected to various treatments) from tumor inoculated mice treated with IL-2 alone or indomethacin plus IL-2. Effector cells were pooled from five mice in each group. Data represent means from triplicate wells with <5% variation. Indo, indomethacin. Killing of B16F10 melanoma targets by: ●, splenocytes; ○, + C′; △, + anti-Thy-1 + C′; ▲, + anti-asialo GM-1 + C′; □, + anti-Lyt-2 + C′.

a transient and abortive stimulation of host NK activity, followed by a lasting suppression of this activity beginning on days 6–7 (3). Present results of indomethacin therapy alone are in agreement with reports from our own (9) as well as other laboratories (27–30) of the containing effects of this agent on tumor growth, and more importantly on the natural metastasis from the primary site. Similarly, the present results of IL-2 therapy alone, in spite of some differences in the protocol, are in essential agreement with those reported by Rosenberg's group in several murine models of metastasis, including other lines of B16 melanoma (10, 12–16, 18, 31). The inadequacy of indomethacin alone or IL-2 alone to affect a complete regression of established metastasis during the experimental period is explicable on the following grounds: (*a*) a relief of the endogenous PG-mediated suppression may permit a regeneration of IL-2 receptors on the effector lymphocytes as well as an improved synthesis of endogenous IL-2 (6), but the latter may still be inadequate for a maximal and sustained activation of the effector cells; (*b*) exogenous IL-2 alone may fail to cause an optimal stimulation, because of an inadequate generation of IL-2 receptors (6) on the effector lineage cells, if the endogenous suppression is not relieved. Such PG mediated suppression may, in addition, inhibit the production of other lymphokines such as IL-1 (32, 33) and cytotoxic cell differentiation factor (CCDF) (34–36), which are believed to be required for the lymphocyte activation/differentiation pathway. These possibilities are supported by our findings that chronic indomethacin treatment combined with a single round of IL-2 therapy led to a complete or nearly complete amelioration of experimental lung metastasis generated by a very high inoculum of B16F10 melanoma cells in a highly reproducible manner. The beneficial effects of this combination therapy seem to be comparable to the effects of combined IL-2 and LAK cell therapy of metastasis in the murine system (10–18). Furthermore, we have recently noted

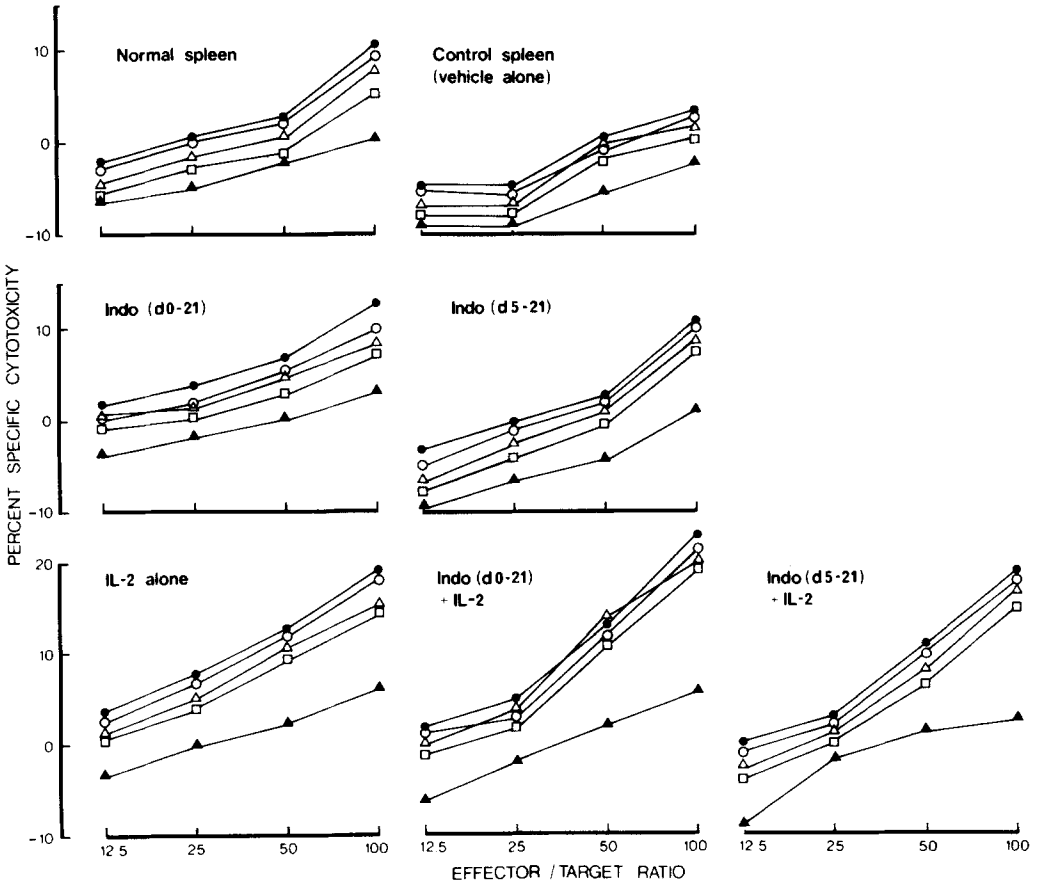

FIGURE 7. Cytotoxicity of splenocytes (subjected to various treatments) from normal healthy mice, and tumor-inoculated control and experimental mice (subjected to various therapeutic protocols) against the thymic lymphoma 9750. Control (vehicle alone) group is the same as in Fig. 3. Those receiving, in addition, the excipient buffer intraperitoneally, provided very similar results (not presented). Effector cells were pooled from five mice in each group. Data represent means from triplicate wells with <5% variation. Indo, indomethacin. Killing of thymoma targets by: ●, splenocytes; ○, + C′; △, + anti-Thy-1 + C′; ▲, anti-asialo GM1 + C′; □, anti-Lyt-2 + C′.

(Parhar, R. S., J. Nelson, and P. K. Lala, unpublished observations) that a combination of indomethacin and IL-2 can also cause a marked regression of spontaneous lung metastasis developing from a primary murine adenocarcinoma.

The effector cells generated in the host spleen by indomethacin alone, IL-2 alone, or a combination of indomethacin plus IL-2 in vivo, all expressed AGM1. This by itself cannot be considered as an exclusive NK cell marker, since AGM1 expression has also been reported (37) for cytotoxic T cells. However, an absence of Thy-1 or Lyt-2 on these cells indicates an NK-like phenotype. A superior functional activation of these effector cells was always achieved with the combination therapy, irrespective of the tumor target tested. With indomethacin alone, the cytolytic activity was essentially confined to the NK-sensitive targets YAC-1 and B16F10. Administration of IL-2 generated a broader spectrum of killer function extending also to the NK-resistant target thymic lymphoma 9705, the

killer activity being more marked with the combination therapy. Although this would suggest the generation of LAK-like cells in vivo, typical LAK cells generated in in vitro exhibit a different phenotype. They are Thy-1$^+$ and AGM1$^-$ and Lyt-2$^\pm$ (17, 18, 35; our unpublished observations). Another closely related effector cell class with a broad cytocidal spectrum generated in vitro from NK-like precursor cells requires the presence of IL-2 and a newly described lymphokine, CCDF, produced by macrophages in the presence of indomethacin (34–36). This cell class has been named lymphokine-induced cytotoxic cells (LICC), which are Thy-1$^+$, Lyt-2$^-$, and AGM1$^-$ (35). With the present combination therapy, one would expect that LICC and LAK cells should have been generated in vivo. Several possible explanations may be offered for the observed phenotypic discrepancy: (*a*) the effector cell phenotype may depend on its activation and differentiation status (36, 38) and may have been influenced in our case by a termination of the IL-2 therapy one week before mice were killed. In indomethacin-treated animals killed shortly after IL-2 therapy, splenic effector cells are found to be AGM1$^+$ and Thy-1$^\pm$ (Parhar, R. S., and P. K. Lala, unpublished observations); (*b*) an expression of Lyt-2 on the anti–tumor effector cells generated by IL-2 therapy in vivo may depend on the immunogenicity of the tumor concerned. The effector cells are Lyt-2$^+$ when tumors have detectable immunogenicity, but are Lyt-2$^-$ in the case of nonimmunogenic tumors (39), suggesting that IL-2 therapy may recruit both NK and T lineage cells; and (*c*) in the present study, the effector cells were sampled from the spleen and not from the tumor site i. e., the lungs, where a different subset may accumulate preferentially. Our preliminary (unpublished) studies reveal that AGM1$^+$, Thy-1$^+$ effector cells with high anti-B16F10 killer activity can be recovered from the lungs of the animals subjected to the combination therapy. The cytocidal spectrum and the phenotype of the various mononuclear cells infiltrating in situ, as well as the long term survival of mice placed under combination therapy, are currently under investigation.

Summary

Our earlier work revealed that PGE-mediated inactivation of NK cells in tumor-bearing mice by host macrophages promoted spontaneous lung metastasis that could be prevented or ameliorated by chronic indomethacin therapy. Since PGE was found to suppress the in vitro development and/or activation of a family of tumoricidal lymphocytes such as CTL, NK, and LAK cells by one or both of two mechanisms, that is to say, a down regulation of IL-2-R and an inhibition of IL-2 production, the present study tested whether a combined therapy with indomethacin and IL-2 was more effective than one with indomethacin or IL-2 alone in ameliorating established experimental lung metastasis. B6 mice injected intravenously with 10^6 highly metastatic B16F10 melanoma cells showed profuse micrometastases in the lungs by day 5, and macrometastases by day 10 which were confluent on day 21. Chronic indomethacin therapy by the oral route (14 μg/ml in drinking water) starting on day 0 or day 5, or a single round of IL-2 therapy (25,000 U rIL-2, every 8 h for 5 d on days 10–14) reduced the number of metastatic nodules by two-thirds (from a median of 473 in control mice receiving vehicles alone) by day 21. A single round of IL-2 as above, combined with either protocol of indomethacin therapy, completely or

nearly completely irradicated the lung metastases, corroborated by a histological examination. An evaluation of splenic killer cell activity measured with a 4-h ^{51}Cr-release assay against NK-sensitive YAC-1 lymphoma and B16F10 melanoma or NK-resistant thymic lymphoma 9705 targets revealed negligible activity in control tumor-bearing mice, and a good restoration of activity against NK-sensitive targets with either protocols of indomethacin therapy. IL-2 alone or a combination of IL-2 and indomethacin given by either protocol generated strong killer activity against all these targets, most marked with the combination therapy. Splenic killer cell phenotype in normal as well as all treated animals was ASGM1$^+$, Thy-1$^-$, and Lyt-2$^-$. The combination therapy resulted in the strongest mononuclear cell infiltration in the lungs, with areas of young granulation tissue suggestive of repair sites of original metastases.

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