Augmentation of the Development of Immune Responses of Mice against Allogeneic Tumor Cells after Adriamycin Treatment

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ABSTRACT

In C57BL/6J mice, depending on the dose of P815 cells used for immunization, Adriamycin exerted different effects on the cell-mediated lytic response and complement-dependent cytotoxicity. At the dose of $3 \times 10^7$ P815 cells, Adriamycin treatment had no apparent effect on cell-mediated lytic response regardless of timing of drug treatment. At lower doses of antigen ($10^7$ or $5 \times 10^6$ cells), the response was augmented in Adriamycin-pretreated mice. Similarly, under conditions which led to a suboptimal complement-dependent humoral response of untreated control, Adriamycin pretreatment resulted in an augmented response; under conditions of maximal response, Adriamycin was suppressive. Suppression was maximal if the drug was injected at either the same time or shortly before or after antigen. The cell-mediated lytic response was proportional to the dose of antigen used, while the complement-dependent humoral lytic response was inversely proportional to dose of antigen in the range used in these experiments. Secondary cell-mediated lytic response in culture was also augmented if mice had been pretreated with Adriamycin 5 days before the primary immunization.

The cell-mediated lytic response of spleen and peritoneal exudate cells from mice immunized with relatively low doses of P815 cells 5 days after treatment with Adriamycin was increased 12 to 15 days after immunization. The cytotoxic effects of either spleen or peritoneal cell populations. All these effector cells were found to be anti-Thy 1.2 sensitive. The phagocytic activity of spleen cells was increased after immunization, but no drug effect was observed; following 24 hr of culture, however, cells from drug-treated immunized donors had increased phagocytic activity as compared to that of controls. Increased phagocytosis also developed in cells non-adherent to plastic.

INTRODUCTION

The great majority of the anticancer drugs available to date can cause immunosuppression in animals and in humans (13, 14, 20, 21). This action, however, may be relatively selective, depending on the pharmacological characteristics of the drug, the regimen of drug administration, and the immunological status of the host. Thus, under certain circumstances, an anticancer drug may not induce such a suppressive effect or may even cause immunoaugmentation.

Selective effects on host defense mechanisms have been reported for AM. The first indication that the greater anticancer efficacy of AM with respect to daunorubicin may be related to its effects on host defense mechanisms was reported by Schwartz and Grindey (30). They showed that the antitumor effects of AM were greater than those of daunorubicin in immunologically competent hosts but not in immunodepressed animals. Orsini et al. (26) reported that pretreatment of spleen donor mice with AM resulted in an augmented immune response to alloantigen in culture. In parallel histological studies, it was shown that spleens from AM-treated mice had an increased number of large monocyte-macrophage-like cells (26). Subsequent studies in this laboratory have confirmed and extended these findings and have indicated that all effector cells in the CML response developed in culture were of T-cell origin (5, 31). Other selective effects on cells of the monocyte-macrophage series by AM have been reported in both the mouse (17, 18) and the test tube (8). Studies in this laboratory have shown that the addition of AM directly to the primary stimulation cultures (33), depending on the time of addition and AM concentration, resulted in suppression or augmentation of the cytolistic response. Under culture conditions similar to those which resulted in augmentation of the CML response to allogeneic tumor cells, a reduction in the homolal immune response to SRBC was observed (3).

In this report, conditions under which AM can induce an augmented CML response and modulate a humoral response in mice are described. Some of the characteristics of the CML effector cells were determined for comparison with those which have been reported already for the effector cell induced in culture (6, 8, 19). Also, the effects of AM on secondary response in culture are described.

MATERIALS AND METHODS

Mice. Pathogen-free female C57BL/6 mice, 7 to 10 weeks old, were obtained from Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute. The DBA/2Cr mice used for maintaining the P815 tumor were obtained from the same source.

Antigen. P815 mastocytoma tumor, syngeneic to DBA/2Cr mice, was used as antigen. It was maintained in ascitic form by weekly passage of $5 \times 10^6$ washed tumor cells/mouse.

Tumor cells used for immunization were harvested from the peritoneal cavity of DBA/2Cr mice 4 to 5 days after the passage. Washed cells were resuspended in sterile 0.9% NaCl solutions, and $3 \times 10^7$, $10^7$, or $5 \times 10^6$ viable P815 cells were injected i.p. to each mouse. In some experiments, mice were immunized with X-irradiated P815 tumor cells. In this case, tumor cells were irradiated with 4000 rads delivered...
with a General Electric Maxitron 300 machine, and the mice received daily i.p. injections of $3 \times 10^7$ X-irradiated tumor cells for 3 days.

Tumor cells used as antigen in cultures were harvested from the peritoneal cavity of DBA/2Cr mice 3 days after passage. Washed cells were resuspended in RPMI Medium 1640 and X-irradiated with 4000 rads delivered with a General Electric Maxitron 300 machine.

**Agents.** AM was obtained from Division of Cancer Treatment, National Cancer Institute. It was dissolved in 0.9% NaCl solution, 0.5 mg/ml, immediately before use. A single dose of 5 mg/kg was injected into the tail vein at different times before or after immunization of mice with tumor cells.

The Na$_2$^{51}CrO$_4$ used for assay was purchased from New England Nuclear, Boston, Mass.

**Media.** RPMI Medium 1640 was used for washing spleen and tumor cells. For the assay, the medium was enriched with 5% FCS (Grand Island Biological Co., Grand Island, N. Y.). The medium used for cell culture contained 10% FCS with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (Grand Island Biological Co.), and gentamicin (0.1 mg/ml) (Schering Corp., Kenilworth, N. J.). 2-Mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.) was added to the medium immediately before use in a final concentration of 50 µM.

**Cell Suspension Preparation.** Spleens were excised and forced through coarse (50 mesh) and fine (200 mesh) stainless steel gauze to make a single-cell suspension. Spleen cells were washed 3 times with medium. Viability was determined by trypan blue exclusion, and the desired numbers of viable cells were used in the assay. The cells which were to be used for tissue culture were obtained under aseptic conditions. PEC were harvested by washing the mouse peritoneum with 5 ml cold 0.9% NaCl solution. PEC were washed twice with cold 0.9% NaCl solution and resuspended in RPMI Medium 1640 with 5% FCS. Viability was determined by trypan blue exclusion, and the desired numbers of spleen or peritoneal cells were used in assays.

**Culture Conditions.** The stimulation cultures of spleen cells were performed according to the method of Mishell and Dutton (22) with some modifications (33). The responder cells (5 x 10$^6$) were placed in 35- x 10-mm multiwell tissue culture plates (Linbro Chemical Co., New Haven, Conn.) in a total volume of 2 ml. Then, 5 x 10$^5$ X-irradiated P815 tumor cells were added to these cultures to obtain responder cell:stimulator cell ratios of 10:1. The cells were then incubated for 1 to 3 days at 37° in a humidified 5% CO$_2$ atmosphere.

**CML Assay.** The CML activities of the cells harvested from cultures or obtained from immunized mice were determined, unless otherwise indicated, in a standard 4-hr $^{51}$Cr release assay as described previously (33). The specificity of the CML has been reported previously (5, 26).

**CDCC Assay.** The CDCC assay, which measures antibody response (19), was performed as follows. Following incubation of effector cells with $^{51}$Cr-labeled target cells for 0.5 hr, a 20-fold dilution of rabbit complement was added to the tubes, and incubation continued for an additional one-half hr. Cells were assayed in duplicate at 5 different E:T ratios. The results are expressed as percentage of specific release which is the value obtained with immune effectors minus the value obtained with nonstimulated cells, the latter ranging between 8 and 12%. The duplicate values, in general, did not vary by more than ±2%, and those which varied by ±5% or more were not considered.

**Phagocytic Activity Assay.** The phagocytic activities of spleen cell populations were assessed by the macrophage effector function assay of Hersey (12), which was slightly modified in this laboratory (8). Briefly, SRBC were labeled with $^{51}$Cr and coated with mouse anti-SRBC antibodies. Spleen cells prepared as described above were incubated with coated or uncoated $^{51}$Cr-labeled SRBC for 4 hr. Nonphagocytized $^{51}$Cr-labeled SRBC were then lysed by hypotonic shock. The percentage of $^{51}$Cr released was calculated as given for the CML assay. The percentage of protection was calculated using the formula:

\[
\% \text{ of protection} = 100 - \% \text{ of } ^{51}\text{Cr release}
\]

The percentage of specific protection was given by: percentage of protection of antibody-coated $^{51}$Cr-labeled SRBC minus percentage of protection of $^{51}$Cr-labeled SRBC without antibodies. The duplicate values, in general, did not vary by more than ±2%, and those which varied by ±5% or more were not considered.

**Anti-Thy 1.2 Treatment.** The monoclonal anti-Thy 1.2 antibody (New England Nuclear; Lot LK114) was used for the identification of T-effector cells. Various numbers of adherent, nonadherent, or unfractionated effector cells were mixed with anti-Thy 1.2 antiserum (1:500 dilution) and kept on ice for 20 min. After addition of complement, cells were incubated for 45 min at 37°. Control sets of cells received antisera, complement, or medium alone. After incubation, cells were washed with warm medium, and 2 x $10^7$ $^{51}$Cr-labeled P815 target cells were added for the standard 4- or 18-hr cytotoxicity assay.

**Separation of Plastic-adherent and -nonadherent Cells.** PEC and spleen cells were placed in 60-mm Falcon Petri dishes (Falcon Plastics, Oxnard, Calif.) and incubated for either a 90-min or a 24-hr period. Cells nonadherent to plastic were removed by decanting supernatant following swirling of the plates for 15 sec. This procedure was repeated 2 more times with the addition of warm medium. After 3 ml of cold medium were added, the adherent cells were dislodged with a rubber policeman and collected.

**Data Base.** All experiments were repeated at least 3 times, and, unless otherwise stated, one of the 3 reproducible experiments was presented. In general, the results which were obtained at only one of the 5 tested E:T ratios are presented; however, these results are representative of what was seen at all E:T ratios tested.

### RESULTS

In preliminary experiments, the effects of AM preimmunization treatment on the humoral (CDCC) and cellular (CML) immune response of mice to $3 \times 10^7$ P815 tumor cells were studied under the same conditions (5 mg/kg, i.v., Day -5) as those which had been used to demonstrate augmentation of CML in culture (see Ref. 26). It was found that, under these conditions, there was no effect on CML responses of either spleen or lymph node cells, while there was an increase of the CDCC response of spleen cells on Day 4 after immunization followed by a small inhibition on Day 5, but no such effects were seen with lymph node cells (data not shown). In view of this discrepancy between these negative results and the reported augmentation of primary CML response in culture with responder cells from similarly treated donor mice (26), the influence that various experimental conditions might have in possibly visualizing an augmenting effect of AM on primary immune responses in mice was studied.

**CML and CDCC Response of Mice Treated with AM at Different Times before and after Immunization.** The importance of timing of drug administration in relation to antigenic stimulation was studied first.

Ten groups of C57BL/6 mice were immunized with $3 \times 10^7$ viable P815 tumor cells on Day 0. Each group received a single i.v. injection of AM on one of the selected days either before or after immunization. Three mice from each group were sacri-
fied on Day 5 after immunization for the CDCC response assay, while another 3 mice from each group were sacrificed on Day 11 after immunization for the CML response assay. The days chosen for assays were close to the peak of the respective responses (15). The results are presented as a percentage of the control response observed in untreated immunized mice normalized to 100. Control values in 3 experiments were: CML response, 45 to 65% specific ¹¹Cr release; CDCC response, 15 to 30% specific ⁵¹Cr release; CDCC response; CML response.

The CML response of spleen cells from mice treated with AM before or after immunization with this high dose of P815 (3 x 10⁷ cells) was not significantly affected, except perhaps for a minor suppressive effect in mice given injections of AM either 1 or 2 days after immunization. The CDCC response was, however, strongly suppressed by AM in mice given injections of the drug 1 day before immunization, on Day 0, or on Day 1 after immunization. Injection of AM 2 days after immunization had a less suppressive effect, and injection 4 days after immunization had little or no effect. Injection of AM 5 days before immunization again resulted in a slight reduction of the CDCC response assayed on Day 5 as seen in the preliminary experiment. These results indicated differences in the sensitivity of cellular and humoral immune responses to AM treatment.

Importance of the Antigen Dose as a Parameter Determining the Effect of AM Pretreatment on CML and CDCC Responses in Mice. C57BL/6 mice were treated with AM and 5 days later immunized with P815 cells. Mice were sacrificed 12 days later, and their spleen cells were assayed for CML activity. As observed in previous experiments, mice receiving 3 x 10⁷ cells developed a high CML response; in those mice, AM treatment had no discernible effect (Chart 2, top). The lower doses of antigen (10⁶ and 5 x 10⁶ cells) induced lower CML responses, and in these cases, AM pretreatment had an augmenting effect. The highest augmentation was observed with spleen cells from mice immunized with 5 x 10⁶ cells, where the response of untreated controls was the lowest (Chart 2, top).

In another group of experiments, AM-pretreated mice were immunized with the same 3 doses of P815 tumor cells, and the CDCC response was measured. Since (in the preliminary studies of the kinetics of the CDCC response in mice mentioned above) an AM-induced augmentation had been observed on Day 4 after immunization (data not shown), that day was chosen for assay. The lowest dose of antigen (5 x 10⁶ cells) induced the strongest CDCC response, while higher doses resulted in lower CDCC responses (Chart 2, bottom). In this case, the augmenting effect of AM was observed in mice immunized with 3 x 10⁷ cells, and only a slight effect was noted in mice immunized with 10⁷ cells. In mice immunized with 5 x 10⁶ P815 cells, pretreatment with AM had a suppressive effect.

Therefore, the antigen dose-response relationships in CML and CDCC responses have different patterns, and when the antigen dose was 5 x 10⁶ cells, AM caused CML augmentation and CDCC suppression. Augmentation of both responses by AM was observed, however, in groups where the response of untreated controls was low.

Effect of AM Pretreatment on the Kinetics of the CML Response in Mice Immunized with a Low Dose of P815 Tumor Cells. Based on these observations, the kinetics of CML response after immunization with allogeneic P815 tumor cells was studied. C57BL/6 mice, either untreated or AM treated (5 mg/kg, Day −5), were immunized (Day 0) with 5 x 10⁶ viable P815 cells. The CML response of PEC and spleen cells was assayed on the indicated days in order to detect the presence of effector cells in an organ where they are formed and also at the site of antigen injection. Under these conditions, the highest response for both PEC and spleen cells was observed on Day 10 (Chart 3). It was still at the same level on Day 11 but after that dropped sharply. The CML response of spleen cells from AM-treated mice was the highest on Day 11 and remained high, so that significant augmentation above the control response was observed from Days 11 to 15. The response of PEC from AM-treated mice was maximal on Day 10, the same day as that of the controls, but it remained significantly augmented above control level on all days assayed (Chart 3). The ability of AM to induce an augmented CML response was also demonstrated when X-irradiated P815 tumor cells were used (data not shown).

Effect of AM Pretreatment on the Development of Second-

\[ \text{CML RESPONSE} \]

\[ \text{CDCC RESPONSE} \]

\[ 3 \times 10^6 \quad 1 \times 10^6 \quad 5 \times 10^5 \]

Number of Cells Used for Immunization

Chart 2. Top, AM effects on the CML response of mice immunized with different doses of P815 tumor cells. CML activity of spleen cells was measured 12 days after immunization (Day 0) of untreated (■) and AM- (Day −5) treated (▲) mice. The results were obtained at E:T ratio of 25:1. Bottom, AM effects on the CDCC response of mice immunized with different doses of P815 tumor cells. CDCC activity of spleen cells was measured 4 days after immunization (Day 0) of untreated (■) and AM- (Day −5) treated (▲) mice. The results shown were obtained at E:T ratio of 100:1.
Augmentation of Immune Responses in Mice by AM

Chart 3. Kinetics of the CML response developed in C57BL/6 mice immunized with 5 x 10⁶ viable P815 tumor cells. The CML activity of PEC and spleen cells from untreated (■) and AM- (Day -5) treated (□) mice was assayed on the indicated days after immunization (Day 0). The results shown were obtained at E:T ratio of 6:1.

ary CML Response to P815 Tumor Cells in Culture. The C57BL/6 mice were randomly divided into 6 groups which were treated as follows: (a) AM given 5 days before primary immunization and 5 days before secondary stimulation; (b) AM given 5 days before primary immunization; (c) primary immunization and AM given 5 days before secondary stimulation; (d) primary immunization with no AM given; (e) no immunization with AM given 5 days before stimulation in culture; and (f) no AM treatment and no immunization. The primary immunization dose was 10⁷ viable P815 cells, a dose which had been found suitable for the detection of the augmenting effect of AM. Eight weeks after immunization, the spleen cells were taken for secondary stimulation in culture. The CML response of these cells was assayed on Days 1 to 3 after the initiation of cultures, namely, before the time a primary response would have reached peak levels (Chart 4).

The cells from mice which received an AM injection before primary immunization developed a higher response than did those which received AM treatment only 5 days before secondary stimulation or received no AM at all. It appears from these results that treatment with AM before immunization with 10⁷ P815 cells caused an augmenting effect on a secondary response to the same antigen in culture. However, AM treatment only before the secondary stimulation in culture had no effect on the secondary CML response as measured on Days 2 and 3 after stimulation.

Characterization of the CML Effector Cells Developed in Mice. During the studies of AM effects on the CML response in culture (9, 31), it had been found that AM induced changes which resulted in cell populations developing increased phagocytic activity during a 4-day culture. Increased phagocytic activity was also found to develop during culture when only cells which were nonadherent to plastic before culture were used. In order to compare the results obtained in culture with that found in the mouse, the phagocytic activity of spleen cells

Chart 4. Effect of AM treatment of C57BL/6 mice on the development of secondary response to P815 tumor cells in culture. The immunization dose was 10⁷ viable P815 cells, and the AM dose was 5 mg/kg. The groups were as follows: AM treated 5 days before primary immunization and 5 days before secondary stimulation in culture (■—■); AM treated 5 days before primary immunization (■—■); primary immunized and AM treated 5 days before secondary stimulation (□); not AM treated but primary immunized (□); not immunized but AM treated 5 days before stimulation in culture (□—□); not AM treated and not immunized (□—□). Responder cell:stimulator cell ratio in secondary cultures was 10:1. The results shown were obtained at E:T ratio of 12:1.

Chart 5. Phagocytic activity of spleen cells from untreated (■) or AM-treated (□) immunized C57BL/6 mice in comparison to untreated unimmunized controls (□). Spleen cells were taken before immunization and 11 days after immunization with 5 x 10⁶ P815 tumor cells. The activity of the whole spleen cell populations and those nonadherent to plastic after a 90-min or a 24-hr incubation period was assessed in the standard 4-hr assay. The results shown were obtained at E:T ratio of 12:1.
from AM-treated and -untreated mice, before and after immunization, was determined (Chart 5). As reported already (4), the phagocytic activity of the spleen cells assayed 5 days after AM treatment, but before immunization, was the same as that of the controls. When tested 11 days after immunization, (i.e., 16 days after AM treatment), the phagocytic activity of spleen cells from AM-treated immunized mice and immunized controls was the same, although slightly elevated above that of unimmunized controls. This difference was greater when only the nonadherent fraction of the effector cell populations was assayed. However, if the spleen cells were incubated for as little as 24 hr, a marked difference in the phagocytic activity between AM-treated cells and controls was observed. The phagocytic activity of spleen cells from untreated immunized mice was almost unchanged. In contrast, the phagocytic activity of spleen cells from AM-treated immunized mice was significantly increased from the values obtained before incubation and from those of the 2 controls. As had been shown with effector cells developed in culture (4, 5, 31), the increase in phagocytic activity in this system was also observed in the nonadherent fraction of spleen effector cells.

To investigate the possible relationship of this effect to the development of cytotoxic cells, the CML activity in plastic-adherent and -nonadherent fractions of immune effectors was also determined. C57BL/6 mice were immunized with $5 \times 10^6$ viable P815 tumor cells 5 days after AM treatment. PEC and spleen cells were harvested 11 days later and separated by plastic adherence. The CML activity of the whole PEC and spleen cell populations and their fractions was assessed (Chart 6). The cytolytic activity of the spleen cells from untreated donors was mainly associated with the nonadherent fraction, and only low lytic activity was detected in the adherent fraction. When PEC were assayed, the CML activity was observed in both adherent and nonadherent fractions, although the latter was higher. Furthermore, the augmentation of CML response induced by AM treatment was apparent in both adherent and nonadherent fractions when either PEC or spleen cells were assayed.

The presence of CML effector cells in both fractions raised questions about their nature. As shown in Chart 7, a complete abrogation of the CML activity of both spleen and peritoneal cells was observed, as the result of treatment of the effecter cell population with anti-Thy 1.2 plus complement. Similar results were obtained with the adherent and nonadherent fractions (data not shown). Nevertheless, questions about the possible presence of cytotoxic macrophages in the peritoneal cavity remained, since the lysis of labeled target cells by macrophages is a much slower process than is lysis by cytotoxic lymphocytes (11). As shown in Chart 8, when PEC were treated with anti-Thy 1.2 plus complement and assayed by both a 4- and an 18-hr $^{51}$Cr release assay, all the CML activity of the effector cells present in peritoneal exudate was abrogated by anti-Thy 1.2 treatment regardless of the duration of the assay.
DISCUSSION

Several investigations of the action of AM on the host defense system have been reported in recent years. The early studies described the suppressive effects of the drug on cellular and humoral immune responses (8, 35). However, it was demonstrated subsequently that AM actually has immunomodulating activity (3, 9, 10, 26, 33). In fact, depending on conditions, AM could selectively affect particular immune functions, this ultimately resulting in inhibition or augmentation of the response (3, 10, 33).

This paper reports the effects of AM on both cellular and humoral immune responses to alloantigens in mice under different conditions. Particular attention has been focused on the effects of timing of drug administration and dose of antigen used for immunization. The nature of the cell function affected by the drug was also explored.

AM induced augmentation of the CML response developed in culture with both spleen (26, 33) and lymph node cells, but the degree of augmentation was dependent on culture conditions. Under conditions where the response of cells from untreated control mice was lower, the degree of augmentation was higher. The induction of suboptimal alloageneic responses by spleen and lymph node cells in culture was obtained by varying the responder cell:stimulator cell ratio.

It had been suggested previously that the augmenting effect of AM on the CML response to alloantigens represents an immunorestorative rather than a true augmenting effect (33). Consistent with this hypothesis is the observation that AM had no augmenting effect on the CML response of mice immunized with $3 \times 10^7$ P815 cells, namely when a high CML response was induced in untreated control mice. However, when the antigen dose was lowered, augmentation was seen. Indeed, when the effects of AM on the CML and CDCC responses in mice were studied in relation to the dose of antigen used for immunization, it was found that the augmenting effect of the drug was apparent under conditions in which untreated mice developed a suboptimal response, regardless of whether such a response was elicited by low or high antigen dose. As the augmented responses did not exceed the highest control response obtained, the data again suggested a normalization of the response by AM. That AM induced suppression of the CDCC under the same conditions as it induced augmentation of the CML should be noted. Others have also reported the dissociation of drug effects on cellular versus humoral responses (1, 2, 34), demonstrating the selectivity by varying the drug dose or the time of drug administration.

Despite the multiplicity of studies of the effects of AM on immune responses (3, 5, 9, 26, 32, 33), the mechanisms of action involved are not yet clear. The early observations that pretreatment of mice with AM induced an increase in the number of large mononuclear cells (26) have been confirmed recently. Five days after treatment, an increased number of such cells are detected in the spleen (5). They are not mature macrophages as indicated by the fact that there is no detectable change in phagocytic activity at that time; however, there is a significant increase of phagocytic activity of spleen cells from AM-treated mice after a period of culture (Refs. 4, 9, and 31 and this paper). Because phagocytic activity and accessory function of macrophages are not necessarily in parallel (23), it may be proposed that the accessory function appears earlier in the ontogeny of macrophages. Because it has been demonstrated that the CML effector cells that develop in either mice (this paper) or culture (31–33), whether augmented by AM or not, are of T-cell origin, it may be proposed that the increase in activity of T-effector cells is a consequence of an increased accessory function in AM-pretreated mice.

Accessory function is actually defined as a polyclonal lymphocyte activation (6); therefore, a possible involvement of other types of cells during different stages of development of immune responses should not be ruled out. Thus, one of the possible mechanisms of AM action, as has been implied for some other immunoaugmenting drugs (1, 7, 25, 34), could be elimination or decrease of suppressor cells. This does not appear to be the case with AM, however, since recent reports indicated no inhibitory effect of the drug on the generation of either "specific" or "nonspecific" suppressor cells in culture (27, 28). Another function that might be considered as a possible target of AM action would be a T-helper cell function. The finding that the effect of AM on the development of the PEC response in culture against T-dependent and T-independent antigen was the same suggested that its effect was T-helper independent (3), while more recent findings using heat-treated antigen indicated that spleen cells from AM-treated mice can develop a CML response without cooperation of T-helper cells (9). Furthermore, the studies of the effects of AM pretreatment on the development of the secondary response in culture reported here also support this hypothesis. The augmenting effect of AM pretreatment on secondary CML response in culture was observed only with cells from mice treated with AM before priming. AM treatment of mice before secondary stimulation in culture had no effect. Several recent studies (24, 29, 36) have indicated that Lyt 1 + cells play an important role in secondary as well as primary cytotoxic response. Thus, the finding that AM treatment of primed mice 5 days before secondary stimulation in culture had no augmenting effect is consistent with the conclusion that AM affects cells other than T-helper cells. While a concurrent direct effect on precursor T-killer cells has not been ruled out, the one AM action that has been ascertained, as indicated in the preceding discussion, is the augmenting effect on macrophage precursors cells (Refs. 3, 9, 26, and 31 and this paper) with the concomitant expansion of T-cell effectors. In fact, the injection of AM, 5 mg/kg, induces necrosis of lymphoid tissue (26), while mature macrophages seem to be selectively resistant to toxic effects of AM (18). It may be postulated, therefore, that macrophage involvement in the recovery of the damaged lymphoid tissue is a trigger for the generation of an increased pool of precursor cells of the same line, which was indeed observed 5 days after AM treatment (4, 26). Although the results presented in this paper demonstrated that macrophages from AM-treated mice are not directly involved in the killing of the P815 target cells, a main role of those cells seems to be in the initiation phase of the immune response. Such a hypothesis is supported by the findings that silica treatment, either in vitro (33) or in vivo (18), could abrogate the development of cytototoxic effectors only if applied shortly before or very close to the time of exposure to antigen.

Because macrophage-associated accessory functions seem to be essential for the polyclonal expansion of precursors of...
committed T-cells (16) and based on the preceding discussion, it may be postulated that the augmented cytotoxic response observed in these experiments resulted from the presence of increased accessory cell function. This possible mechanism implicates a direct interaction of those cells with the precursors of T-killer cells, since (as discussed above) T-helper function and T-suppressor function do not appear to be necessarily involved in the AM-induced effects.

In conclusion, the results presented here demonstrate, for the first time, that the augmenting effect of AM repeatedly shown in culture can also be observed in mice under the conditions of suboptimal response. This was found for both cellular and humoral immune responses. A key role for cells of the monocytic-macrophage type with accessory function has been proposed in determining the AM-induced augmentation of the responses. This proposal is consistent with the data reported in this and preceding communications (3, 9, 26, 31, 33).

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