Cellular Basis for Adriamycin-induced Augmentation of Cell-mediated Cytotoxicity in Culture

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ABSTRACT

The cellular basis for the augmented cell-mediated cytotoxic (CMC) response seen when spleen cells from Adriamycin (ADM)-treated mice were stimulated in culture was investigated. Under conditions where mature macrophages were reduced (adherent or silica-sensitive cells removed) at time of alloantigen challenge, the cells from ADM-treated mice developed levels of CMC activity much higher than the low levels which were developed by similar subsets of cells from nontreated control mice. This indicates that ADM treatment enriched a subset of cells in spleen which was nonadherent, silica-insensitive, and nonphagocytic but was capable of providing accessory function. When mature macrophages were not removed, the capability to develop an augmented level of CMC was shown to be associated with a subset of cells from ADM-treated mice which was adherent to either plastic or nylon wool. In recombination experiments, it was found that the removal of Thy 1.2+ cells from the adherent subset from ADM-treated mice had little effect on the response, while their removal from the adherent subset from nontreated mice resulted in elevated levels of response. A similar effect was obtained when Lyt 2.2+ cells were eliminated but not when Lyt 1.2+ cells were removed. This indicates that a Thy 1.2+, Lyt 1-2+ cell, involved in regulation of the response, was missing from or failed to function in the ADM-treated population. Based on these findings, ADM apparently induces modifications in two cell subsets: (a) immature cells of the monocyte/macrophage lineage which can provide accessory function; and (b) adherent, Lyt 2+ T-cells which cooperate in maintaining levels of CMC activity at "normal" levels.

INTRODUCTION

The antitumor efficacy of ADM in both murine models and humans has been well established. The possible involvement of host defense mechanism in the overall therapeutic efficacy of ADM has been proposed based upon observations made in a number of murine model systems (14, 16, 23, 24). The evidence obtained in these early studies led to the suggestion that the anti-neoplastic advantage of ADM as compared to daunorubicin was due to the fact that it was less immunosuppressive (16).

and this was explained as being a result of a relative "sparing" of macrophages by ADM (14).

In studies from this laboratory, however, it was found that, under defined conditions, treatment with ADM resulted in selective restoration or augmentation of certain immune functions (8, 20, 26). On the basis of histological as well as cell separation studies, it was proposed that the cell subsets selectively affected by ADM might be cytolytic T-cell progenitors and/or macrophages (8, 20).

The initial finding that ADM treatment of mice 5 days prior to sacrifice resulted in an augmented CMC responsiveness of spleen cells from these mice to alloantigen stimulation in culture (20) has been confirmed (8) and extended to include demonstrations of augmented CMC: (a) when ADM was added to primary sensitization culture (8, 25); and (b) in alloantigen-immunized mice which had been pretreated with ADM (8, 26). In parallel experiments, it was found that spleen cells from mice treated with ADM 5 days before sacrifice include a population of immature cells which are nonadherent, nonphagocytic, and silica-insensitive at time of sacrifice, but develop into functionally mature macrophages during culture (2). This study was undertaken to determine if this population of immature macrophages supplied accessory function for the augmented CMC responses as well as to establish if other ADM-modified cell populations were involved. As shown herein, another cell type was also found to be directly involved in the ADM-induced augmentation of CMC responses; this cell is adherent to plastic or nylon wool and has a Thy 1.2+ Lyt 1-2+ phenotype. Although phenotypically it resembles a T-suppressor cell (5), functionally it appears to be different (22).

MATERIALS AND METHODS

Mice and Tumor

Specific-pathogen-free female C57BL/6 (H-2b) and DBA/2Cr (H-2b) mice were obtained at 5 weeks of age from the Goodwin Institute for Cancer Research, Plantation, FL and were used at 8 to 12 weeks of age. Mastocytoma P815 (H-2b) was maintained as an astatic tumor by weekly passage of 5 x 10^6 cells i.p. in the DBA/2Cr syngeneic host.

Reagents

Silica, with an average particle size of 5 μm, was obtained from Steinohenbergauvein, Essen, Germany. ADM was obtained from the Division of Cancer Treatment, National Cancer Institute. ADM (0.5 mg/ml) was dissolved in 0.9% NaCl solution (saline), immediately before use. A single dose of 5 mg/kg was injected in the tail vein at different times (as indicated) before sacrifice. SRBC were obtained in 50% Alsever's solution every 2 weeks from a selected sheep housed at the Roswell Park Memorial Institute animal facility at Springville, NY. The Na_2CrO_4 (specific activity, 64 to 100 μCi per μg of chromium) used in the assays was purchased from Amersham Corporation, Arlington Heights, IL.
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Cell Suspension Preparation

Mice were sacrificed by cervical dislocation, and the spleens were removed aseptically. Cell suspensions were obtained as described previously (5, 7, 25, 26).

Culture Conditions

Spleen cells were maintained in RPMI 1640 medium supplemented as described previously (5, 25). The spleen responder cell populations (5 x 10^6 or 10^7) were placed on nontreated plastic plate cluster plates (Linbro, Hamden, CT) together with the indicated numbers of P815 stimulator cells in a total 2 ml volume of culture medium. The cells were then incubated for the indicated number of days at 37° in a humidified 5% CO₂ (95% air) atmosphere. The stimulator cells were X-irradiated (4000 rads) prior to addition to culture, and test reagents (unless otherwise indicated) were added to the cells at the initiation of culture.

Preparation of Spleen Cell Populations Nonadherent and Adherent to Nylon Wool

Spleen cells (10^7) were passed through nylon wool columns according to published procedures (1, 12). Briefly, after a 45-min period at 37° to allow cells to adhere, the nonadherent cells were displaced by dropwise addition of warm media until the eluate measured 25 ml. The remaining adherent cells were removed by vigorous agitation of the nylon wool in cold RPMI 1640 medium. The populations of cells were washed, counted, and adjusted to appropriate density.

Preparation of Spleen Cell Populations Nonadherent and Adherent to Plastic

Spleen cell populations nonadherent and adherent to plastic were obtained using the following procedure. Spleen cells (10^7) in 2 ml of culture medium were dispensed into 35-mm wells of multiwell tissue culture cluster plates in a total 2 ml volume of culture medium. The cells were then incubated for 1.5 to 2 hr at 37°, and then antiserum containing the washed adherent cells. The cluster dishes were kept on ice for 45 min, and then 0.5 ml of 1:20 diluted rabbit complement was added. The plates were incubated at 37° for 45 min, and then antiserum and complement were removed from the adherent cells by 3 vigorous washings with warmed (37°) medium.

Anti-Thy 1.2 Treatment. Spleen cells in suspension were treated as described previously (5, 26). In certain experiments, only those cells which adhered to plastic dishes were treated with antibody. In this case, 1 ml of antiserum (final dilution 1:300) was added to each well containing the washed adherent cells. The cluster dishes were kept on ice for 45 min, and then 0.5 ml of 1:20 diluted rabbit complement was added. The plates were incubated at 37° for 45 min, and then antiserum and complement were removed from the adherent cells by 3 vigorous washings with warmed (37°) medium.

Anti-Lyt 1.2 Treatment. The procedure was as given above and in the procedure of Cowens et al. (5). The monoclonal anti-Lyt 1.2 (Lot FPC644) was obtained from New England Nuclear and was used at a final dilution of 1:2000.

Anti-Lyt 2.2 Treatment. The procedure was as given above and in the procedure of Cowens et al. (5). The monoclonal anti-Lyt 2.2 (Pool ID 9/29) was a gift from Dr. E. Stocken and Dr. L. Old, Sloan-Kettering Institute, New York, NY, and was used at a final dilution of 1:2000.

Target Cells

The preparation of radiolabeled P815 (5, 25) and SRBC (1, 7) target cells has been described previously.

CTL Cytotoxicity Assay

The CTL cytotoxic assays were carried out as described previously (25).

% of specific ⁶⁷Cr release = \frac{\text{Test } ^{⁶⁷}\text{Cr release} - S}{M - S} \times 100

Spontaneous ⁶⁷Cr release (S) was measured by incubating 2 x 10⁷ ⁶⁷Cr-labeled target cells alone. Maximum ⁶⁷Cr release (M) was determined by exposing 2 x 10⁴ target cells to 2.5% Triton-X-100 (Packard Instrument Co., Downers Grove, IL) during the 4-hr assay; S was 9 to 12% in 4 hr; M was 87 to 93%. Each assay was carried out using at least 5 effector:target cell ratios in duplicate, and each experiment was repeated at least 3 times with similar results seen each time.

Phagocytic Activity Assay

The phagocytic activities of the spleen cell populations after culture were assessed by the technique of Hersey (10), which was modified in this laboratory (1, 2, 7). The percentage of protection was calculated using the following:

% of protection = 100 - \% of specific ⁶⁷Cr release

The percentage of specific protection (phagocytic activity) was given as percentage of protection of antibody-coated ⁶⁷Cr-SRBC minus percentage of protection of uncoated ⁶⁷Cr-SRBC. The latter ranged between 5 to 10% protection. Each assay was carried out using at least 5 effector:target cell ratios in duplicate, and each experiment has been repeated at least 3 times with similar results seen each time.

RESULTS

Effect of Day of ADM Administration on Level of CMC and Phagocytic Activity. A correlation had been demonstrated between the timing of observable ADM-induced modulation both of the ability of the spleen cells to develop a CMC response to alloantigen (20) and of the level of phagocytic activity seen with these cells after culture without alloantigen (2). The experiment described in Table 1 was carried out, therefore, to determine if the level of phagocytic activity in the presence of alloantigen had complement-treated cells are not shown, since they did not differ from those obtained with cells plus median by more than ±3% (S.D.) in any experiment.

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a similar dependence upon time of drug administration. As has
been reported elsewhere (2, 8), when uncultured spleen cells are
assayed, there is little or no difference in the level of phagocytic
activity between those from nontreated mice and Day -1 or -5
ADM-treated mice (Table 1). After 5 days in culture with or
without alloantigen, however, the level of phagocytic activity of
the cells from Day -5 ADM-treated mice was >2 times that of
cells from nontreated mice. In contrast, following culture there
was a decrease in the phagocytic activity which was detectable
with cells from Day -1 ADM-treated mice. The levels of CMC
activity which developed from these 2 spleen cell populations
from treated mice displayed a similar pattern relative to controls;
namely, augmented CMC with cells from Day -5 treated mice
and reduced CMC with those from Day -1 treated mice. Day
-5 ADM-treated mice were used in all subsequent experiments.

**ADM-induced Augmentation of CMC: Independence from Various Culture Conditions.** Experiments were carried out to
demonstrate that the ADM-induced augmentation of the CMC
response was observable under varied culture conditions and
therefore, the results seen in subsequent studies would not be
the consequence of the culture conditions chosen for those
studies. The results shown in Chart 1 indicate that the level of
CMC obtained with spleen cells from Day -5 ADM-treated mice
was always higher than that obtained with cells from nontreated
mice, regardless of whether the number of responding spleen
cells (Chart 1, B versus C) or the number of sensitizing allogeneic
tumor cells (Chart 1, A versus B) used in the culture was varied.
The development of an augmented CMC was, also, independent
of the presence of 2-mercaptoethanol in the culture (data not
shown). Finally, as shown in Chart 2, after various periods of
culture, the levels of phagocytic (Chart 2A) or CMC (Chart 2C)
activities which were attained with spleen cells from ADM-treated
mice were greater than those observed with cells from non-
treated controls at all time points examined.

**Increased Levels of CMC and Phagocytosis Associated with Cells Nonadherent to Plastic.** The effect of removing,

**Table 1**

<table>
<thead>
<tr>
<th>Treatment of mice</th>
<th>5-day cultured spleen cells</th>
<th>Phagocytic activity&lt;sup&gt;a&lt;/sup&gt; (% of specific protection)</th>
<th>CMC response&lt;sup&gt;b&lt;/sup&gt; (% of specific 51Cr release)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncultured spleen cells</td>
<td>(-P815-X)</td>
<td>25:1</td>
<td>251</td>
</tr>
<tr>
<td>Nontreated</td>
<td>25</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>ADM&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>22</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>ADM&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>25</td>
<td>14</td>
<td>32</td>
</tr>
</tbody>
</table>

<sup>a</sup> Phagocytic activity was assayed as protection of antibody-coated 51Cr-SRBC from hypotonic shock after a 4-hr incubation. Spleen cell samples were assayed on Day 0 (uncultured) and on Day +5 after being cultured alone (−P815-X) or with alloantigen (+P815-X). Two of the effector:target cell ratios assayed are shown. Less than 10% of the SRBC were protected from hypotonic shock in the absence of antibody, and less than 5% spontaneously lysed in 4 hr. The S.E. of the mean of replicate samples was less than 2%.

<sup>b</sup> CMC was developed in 5-day primary sensitization cultures involving 10<sup>7</sup> splenic responder cells plus 10<sup>7</sup> X-irradiated (4000 rad) P815 mastocytoma-stimu-
lating cells (P815-X) and was assessed in a standard 4-hr 51Cr release assay at 5 effector:target cell ratios (2 are shown). The background 51Cr release was <10%, and the S.E. of the mean of replicate samples was less than 2%.

<sup>c</sup> ADM<sup>-1</sup>, ADM administered on Day 1; ADM<sup>-5</sup>, ADM administered on Day 5.

Chart 1. ADM-induced augmented CMC response: independent of various culture conditions. ADM (5 mg/kg) was administered i.v. to C57BL/6 mice on Day -1 or Day -5, Day 0 being the day the mice were sacrificed and the spleen cell cultures estab-

Chart 2. ADM-induced augmentation of phagocytic activities (A and B) and CMC (C and D): dependence on time of culture and association with cells nonadherent to plastic. The responder cell populations were from nontreated (○), ADM-1-treated (△) mice and other whole populations (□, ▽) or subsets obtained upon the basis of their nonadherence to plastic (□, ▽) were used (10<sup>7</sup> responder cells; responder:stimulator cell ratio, 100:1). After the indicated days in culture, the CMC and phagocytic activities were assessed in 4-hr assays (see Table 1, Footnotes a and b). S.E. of the mean of replicate samples was <3%.
phagocytic or CMC activities were detected with the nonadherent cells from the nontreated mice while, except for phagocytic activity on Day 0, the levels observed with the nonadherent cells from ADM-treated mice were essentially equal to or greater than those seen with the unseparated spleen cell population from nontreated mice (i.e., compare Chart 2, B and D, closed symbols, to Chart 2, A and C, open symbols).

Augmentation of CMC Associated with Adherent Populations. Subpopulations of spleen cells were separated on the basis of their adherence or nonadherence to either plastic or nylon wool. The ability of separated or recombined cell pools to develop CMC responses in culture was compared to that of the nonseparated spleen cell populations from nontreated and ADM-treated mice (Table 2). Again, as was shown in Chart 2 (Chart 2D), when cells nonadherent to plastic were used, those from nontreated control mice developed only very low levels of CMC (Group 3, left) while the corresponding population from ADM-treated mice (Group 4, left) developed a level of response similar to that attained with the unseparated cells from nontreated controls (Group 1, left). The same results were obtained when the cells nonadherent to nylon wool were tested (Groups 3 and 4, right). In contrast, when the adherent cells were used, a difference was observed between those adherent to plastic and those adherent to nylon wool, in that cells from ADM-treated mice which were adherent to nylon wool developed a CMC response, but those adherent to plastic did not (Group 6). The adherent populations from nontreated controls obtained by either plastic or nylon wool separation techniques, also, did not develop a marked CMC response (Group 5). When the adherent and nonadherent populations were recombined (Groups 7 to 10), it became clear that the induction of a level of response which was higher than that attained with the nonseparated cells from the nontreated controls was only observed when adherent cells from ADM-treated mice were present (Groups 9 and 10). Thus, on the basis of these observations, ADM treatment of the mice appeared to have induced modifications in both the nonadherent and adherent subpopulations, and suggests a possible dichotomy in terms of the cell types which maintain the CMC response at normal levels and those which augment the response above normal.

Effect of Silica on the Level of CMC Response and Phagocytic Activity of Spleen Cell Populations from Nontreated and ADM-treated Mice. Since modifications of cells of the monocyte/macrophage lineage could possibly account for some of the results observed, the following experiments were carried out. Cells from cultures to which silica was added at the initiation of culture (Day 0) or 1 day later (Day +1) had little or no phagocytic activity when assayed on Day 5 of culture (Table 3). In contrast, the silica-induced inhibition of CMC responsiveness was found to be dependent upon the time of exposure of the spleen cells to silica prior to alloantigen challenge. Thus, when silica and alloantigen were added together to the spleen cell cultures either on Day 0 or Day +1, there was only a slight reduction in the level of response seen. However, when silica was added on Day 0 and antigen was added on Day +1, the ability of the spleen cells from nontreated control mice to develop a CMC response was nearly abrogated, while those from ADM-treated mice developed a detectable level of response. In both cases, no phagocytic activity was detectable when assessed after 5-day exposure to silica. This suggested that, in the spleen cell population from ADM-treated mice, there may be cells capable of providing accessory function before becoming sensitive to silica.

### Table 2

<table>
<thead>
<tr>
<th>CMC responsiveness of unseparated subsets, separated subsets, or recombination of subsets of spleen cell populations from nontreated or ADM-treated (5 mg/kg, Day −5) mice</th>
<th>Plastic</th>
<th>Nylon wool</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CMC response</strong>(^a) of cells unseparated or separated by adherence</td>
<td>% of specific release</td>
<td>% of specific release</td>
</tr>
<tr>
<td><strong>Responder cell population</strong>(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N(_{tot}) (unseparated)</td>
<td>27 ± 5(^d)</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>ADM(_{tot}) (unseparated)</td>
<td>42 ± 7</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>Nad-N(_{tot})</td>
<td>10 ± 2</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Nad-ADM(_{tot})</td>
<td>25 ± 4</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>ad-N(_{tot})</td>
<td>3 ± 1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>ad-ADM(_{tot})</td>
<td>7 ± 2</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>nad-N(<em>{tot}) + ad-N(</em>{tot})</td>
<td>28 ± 5</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>nad-ADM(<em>{tot}) + ad-N(</em>{tot})</td>
<td>28 ± 5</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>nad-N(<em>{tot}) + ad-ADM(</em>{tot})</td>
<td>41 ± 4</td>
<td>49 ± 2</td>
</tr>
<tr>
<td>nad-ADM(<em>{tot}) + ad-ADM(</em>{tot})</td>
<td>48 ± 8</td>
<td>52 ± 3</td>
</tr>
</tbody>
</table>

\(^a\) The responder cell populations were from nontreated or ADM-treated mice, and whole populations were compared to separated subsets obtained upon the basis of their adherence or nonadherence to either plastic or nylon wool. The separated subsets were used either alone or in the indicated recombined.

\(^d\) The CMC response was developed using a responder:stimulator cell ratio of 100:1 (10\(^5\) responder cells) and was assayed on Day +5 in a standard 4-hr \(^{31}Crr\) release assay at 5 effector:target cell ratios (25:1). The results are expressed as percentage of specific release. Similar results have been obtained using a responder:stimulator cell ratio of 50:1 with 5 \times 10\(^{4}\) responder cells, and the results obtained in one representative nylon wool separation experiment were essentially the same as those obtained above.

### Table 3

<table>
<thead>
<tr>
<th>Spleen cells cultured (Day 0)</th>
<th>Addition to culture(^a)</th>
<th>Phagocytic activity(^b) (% of specific particles)</th>
<th>CMC response(^c) (% of specific (^{31}Crr) release)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day +1</td>
<td>10:1</td>
</tr>
<tr>
<td>N(_{tot})</td>
<td>12</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>P815-X</td>
<td>8</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>P815-X</td>
<td>10</td>
<td>27</td>
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<td>P815-X</td>
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<tr>
<td>P815-X</td>
<td>17</td>
<td>9</td>
<td>23</td>
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<tr>
<td>ADM(_{tot})</td>
<td>32</td>
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<td>0</td>
</tr>
<tr>
<td>P815-X</td>
<td>18</td>
<td>42</td>
<td>38</td>
</tr>
<tr>
<td>P815-X</td>
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<tr>
<td>s, P815-X</td>
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<td>31</td>
<td>20</td>
</tr>
<tr>
<td>s, P815-X</td>
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<td>14</td>
<td>7</td>
</tr>
<tr>
<td>s, P815-X</td>
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<td>0</td>
</tr>
<tr>
<td>s, P815-X</td>
<td>0</td>
<td>31</td>
<td>19</td>
</tr>
</tbody>
</table>

\(^a\) The alloantigen (P815-X) and/or silica (s, 1 mg/culture) was added to the spleen cell cultures at the time the cultures were established (Day 0) or 24 hr later (Day +1) as indicated.

\(^b\) Phagocytic activity was assayed on Day +5 of culture at 5 effectortarget cell ratios. The levels of activity at 2 effectortarget cell ratios are given. For other details, see Table 1, Footnote a.

\(^c\) CMC was assessed on Day +5 of cultures, and the levels of activity at 2 effectortarget cell ratios are given. For other details, see Table 1, Footnote b.

\(^d\) LU\(_{tot}\), number of effector cells required to lyse 30% of the 2 \times 10\(^3\) radiolabeled target cells; N\(_{tot}\), spleen cells from nontreated mice; ADM\(_{tot}\), spleen cells from ADM-treated mice; ad, adherence; nad, nonadherence.

\(^e\) Mean ± S.D. of 3 individual experiments.
If these cells were immature nonphagocytic macrophages, it would be predicted that they might develop detectable phagocytic activity following the removal of the silica on Day +1. As shown in Table 4, when the free and cell-bound silica were removed after 24 hr of culture by density gradient centrifugation, the cells from ADM-treated mice retained the ability to respond to alloantigen challenge and had detectable phagocytic activity after culture, while those from nontreated mice did not. In fact, the phagocytic and the CMC activities seen were essentially equal to those seen with the spleen cells from the nontreated mice which had been cultured for 1 day without silica and then subjected to density gradient centrifugation. This finding again suggested a possible dichotomy in terms of cell types capable of maintaining the response at normal levels and those involved in augmenting the response above normal.

**Evaluation of the Adherent Cell Type Contributing to the Augmented CMC.** Further evidence for the contribution of the adherent cell population to the augmentation of the CMC is illustrated in Table 5. Spleen cells from nontreated control mice recovered by the density gradient centrifugation technique following 24-hr exposure to silica did not respond to alloantigen unless a source of adherent cells was supplied. If the adherent cells were from nontreated control mice, the level of response which developed was essentially the same in the 2 experiments shown and was similar to that obtained with non-silica-exposed cells from nontreated control mice (e.g., see Chart 2 or Table 2). If the adherent cells were from ADM-treated mice, however, an augmented level of CMC response developed. The observed correlation between augmented levels of CMC response and the presence of adherent cells from ADM-treated mice, together with the fact that the cell population from ADM-treated mice which is adherent to nylon wool develops a CMC response (Table 2, Group 6), suggested that ADM may modulate an adherent subset of T-cells. To evaluate the possible contribution of such cells, the Thy 1.2+ cells were eliminated from the plastic-adherent populations with monoclonal anti-Thy 1.2 plus complement before recombination with the nonadherent cells and challenge with alloantigen (Table 6). Groups 1 and 2 serve as positive controls for the antibody treatment and indicate that Thy 1.2+ complement treatment of the whole spleen cell populations totally abrogated their ability to develop a CMC response. Controls with complement alone did not affect the ability of the cell to respond (data not shown). Interestingly, the level of augmented CMC which developed was only marginally affected by the anti-Thy 1.2 treatment of the adherent cells from ADM-treated mice which is adherent to nylon wool. The results of 2 experiments are given. The levels of activity is indicated for 2 of the 6 effectortarget ratios evaluated in each experiment. See Table 1, Footnote b, for other details. Ns, spleen cells from nontreated mice; ADMs, spleen cells from ADM-treated mice; ad, adherent subsets.

### Table 4

<table>
<thead>
<tr>
<th>Spleen cells</th>
<th>Cultured (Day 0)</th>
<th>Silica (Day 0)</th>
<th>Density gradient separation (Day +1)</th>
<th>Phagocytic activity (Day +1)</th>
<th>CMC response (Day +1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10:1 1:1 5:1</td>
<td>10:1</td>
<td>10:1</td>
<td>10:1</td>
<td>10:1</td>
</tr>
<tr>
<td>Ns</td>
<td>−</td>
<td>−</td>
<td>24 14 35 23</td>
<td>35 23</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>17 10 33</td>
<td>+</td>
<td>0 0 0 0</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>+</td>
<td>2 2 1 1</td>
<td>2 1</td>
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</tr>
<tr>
<td>ADMs</td>
<td>−</td>
<td>−</td>
<td>56 44 35</td>
<td>48 35</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>62 49 46</td>
<td>+</td>
<td>3 0 9 5</td>
<td>3 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>15 9 18</td>
<td>15 9</td>
<td></td>
</tr>
</tbody>
</table>

The results of 2 experiments are given. The levels of activity is indicated for 2 of the 6 effectortarget ratios evaluated in each experiment. See Table 1, Footnote b, for other details.

### Table 5

Effect of adherent spleen cells from nontreated and ADM-treated (5 mg/kg, Day −5) mice on silica-abrogated CMC responsiveness of spleen cells from nontreated mice

<table>
<thead>
<tr>
<th>Cultured (Day 0)</th>
<th>Addition to culture (Day +1)</th>
<th>Density gradient separation (Day +1)</th>
<th>CMC response (% of specific 3HCr release)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ns</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>+ Add-ADMs</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>− Add-Ns</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+ Add-Ns</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

### Table 6

Effect on the level of CMC response which developed caused by removing Thy 1.2+ cells from the plastic-adherent populations

<table>
<thead>
<tr>
<th>Responder cell population</th>
<th>Control</th>
<th>Anti-Thy 1.2 + complement</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5:1</td>
<td>6:1</td>
<td>12.5:1</td>
</tr>
</tbody>
</table>

The results of 2 experiments are given. See Table 1, Footnote b, for details. Thy 1.2 response was separated on the basis of adherence to plastic and were as given in Table 2, Footnote a. See Table 1, Footnote b, for other details.

### References

- Antoniadis (PBS15-X) was added to all cultures on Day +1 after the separation step described in Footnote a (above) was completed. See Table 1, Footnote b, for other details.
- Silica (1 mg/culture) was added to the indicated spleen cultures at the time the cultures were established on Day 0.
- Ns, spleen cells from nontreated mice; ADMS, spleen cells from ADM-treated mice.

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Table 7
Identification of the Lyt phenotype of the adherent regulator T-cell

<table>
<thead>
<tr>
<th>CMC response</th>
<th>Treatment of adherent population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No + comple</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Responder cell population</th>
<th>%SR</th>
<th>LU&lt;sub&gt;50&lt;/sub&gt;</th>
<th>%SR</th>
<th>LU&lt;sub&gt;50&lt;/sub&gt;</th>
<th>%SR</th>
<th>LU&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ad-N&lt;sub&gt;nat&lt;/sub&gt;</td>
<td>28</td>
<td>47 (167)</td>
<td>25</td>
<td>89</td>
<td>42</td>
<td>150</td>
</tr>
<tr>
<td>ad-ADM&lt;sub&gt;n&lt;/sub&gt;</td>
<td>18</td>
<td>16</td>
<td>16</td>
<td>43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ad-N&lt;sub&gt;nat&lt;/sub&gt;</td>
<td>38</td>
<td>48 (128)</td>
<td>40</td>
<td>105</td>
<td>50</td>
<td>132</td>
</tr>
<tr>
<td>ad-ADM&lt;sub&gt;n&lt;/sub&gt;</td>
<td>27</td>
<td>40</td>
<td>28</td>
<td>44</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a The responder cell populations were separated on the basis of adherence to plastic and were as described in the legend to Table 2. The data given were obtained at the effector:target cell ratio of 12.5:1; however, similar results were obtained with 5 other effector:target cell ratios (both higher and lower).
b The nonadherent spleen cells from nontreated mice; ad-N<sub>nat</sub>, adherent spleen cells from nontreated mice; ad-ADM<sub>n</sub>, adherent spleen cells from ADM-treated mice; LU<sub>50</sub>, number of lytic units per culture, where one lytic unit is that number of effector cells required to lyse 30% of the 2 x 10<sup>4</sup> radiolabeled target cells; %SR, percentage of specific 51Cr release.

DISCUSSION

The results of this study indicate that cells from Day -5 but not Day -1 ADM-treated mice developed augmented levels of CMC and phagocytic activity during culture and that this observed augmentation of CMC response is apparently not dependent upon specific culture conditions. The subsequent studies using spleen cells from Day -5 treated mice indicate that silica-insensitive, nonadherent, nonphagocytic, immature cells of the monocyte/macrophage lineage, which have been shown in this laboratory to be present in the spleen of ADM-treated mice (2), could contribute accessory function during primary CMC response in culture. When mature macrophages were removed, ADM-spleen cell populations could develop a CMC response against alloantigen, while control spleen cell populations could not. The level of response which was attained, however, was essentially equal to that attained by control spleen cell populations when mature macrophages were not removed. The ability of the ADM-spleen cell populations to develop elevated levels of CMC during 5-day primary sensitization cultures was found to be associated with the ADM-induced reduction in the activity of a population of regulatory T-cells which was adherent to plastic or nylon wool.

The possible involvement of cells of the macrophage type in ADM-induced augmentation of antitumor host response has been suggested by the results of several investigations (2, 7, 14, 15, 20, 26). As recently reported by this group (2), however, when spleen cell populations from nontreated mice were compared to those from ADM-treated mice 5 days after drug administration, there was no detectable difference in terms of the number of mature adherent macrophages or level of phagocytic function. However, there was an increase in the number of nonadherent, nonphagocytic, silica-insensitive, immature macrophages. The data reported herein using both silica, a toxin for mature macrophage, and physical cell separation-recombination techniques, indicate that this immature macrophage can supply the accessory function of the mature macrophage and result in near control levels of CMC responses even though it does not have other macrophage properties (e.g., phagocytic action; see Table 4). Indeed, it has been recognized for some time that certain functions usually ascribed to mature macrophages may be expressed by cells which lack other defined characteristics of differentiated macrophages (21). Thus, host immune-response modifications observed following ADM-treatment may reflect the changes in this immature, silica-insensitive, nonadherent, nonphagocytic macrophage subset more than the relative ADM insensitivity of the mature macrophages (14).

The occurrence of an anti-Thy 1.2-sensitive adherent cell subset has been reported by others, who have ascribed either suppressor (11) or helper (6) function to this subset. The data presented in Table 2, indicating that cells from ADM-treated mice which are adherent to nylon wool can develop a CMC response, confirm the observation reported previously (20) under similar conditions. Based on this finding with nylon wool, it is apparent that a modification in a subset of "sticky" PTC must have occurred. One may speculate that the modification may be either: (a) an increase, to a functionally detectable level, of a normally occurring subset; or (b) an alteration in the surface characteristics of a subset of PTC so that they have become "sticky." Indeed, there are a number of reports suggesting cellular membranes may be a relevant target of ADM (e.g., 13, 18, 19). The observations that, regardless of whether allosensitization is induced in the animal or in culture, increased levels of T-effector activity separate with the adherent cells when ADM-treated mice are the source of the responding cells (3, 25, 26) further supports the suggestion of alteration of the T-cell surface membrane. An alternative hypothesis is that a subset of PTC which adhere to nylon wool normally exists but fails to develop a CMC response.
because of the presence of T-suppressor cells or the absence of T-helper cells in nylon wool-adherent population from non-treated mice. The ADM-induced CMC responsiveness of the nylon wool-adherent population, in this case, could be explained by modifications in one of these relatively small subsets of T-cells. It should be noted, however, that ADM treatment of mice does not eliminate the cyclophosphamide-sensitive progenitors of culture-induced T-suppressor cells (22).

The association of the capability to develop an augmented CMC response with the cells adherent to plastic, which cannot themselves develop a CMC, also, would be consistent with any of the above-mentioned possibilities. Microscopic examination of the adherent populations indicated that similar numbers of cells adhered and, following differential staining, that equivalent percentages (>90%) of the adherent cells were macrophages regardless of whether the cells were from nontreated or ADM-treated mice. Further, there was no difference in the phagocytic activity of the 2 populations at the time of adherence. These findings were consistent with the proposal that equal levels of accessory function may be provided by the 2 adherent populations and, therefore, the possibility of a modification in a critical adherent T-cell subset was examined. The finding, in the recombination experiments (Tables 6 and 7), that the removal of Thy 1.2+ or Lyt 2.2+ cells from the adherent ADM-spleen cell population had little effect on the level of response which developed while their removal from the adherent nontreated spleen cell population was associated with the development of elevated levels of response, suggests that the critical cell subset, in this case, is not a PTC or a T-helper cell but is a Lyt 1-2+, ADM-sensitive, T-regulatory cell. This T-regulatory cell appears to be different from the phenotypically similar T-suppressor cells (5), not only in terms of adherence to plastic and sensitivity to ADM but also functionally. Thus, T-suppressor cells inhibit CMC responses, while this cell maintains the response at control levels; also, T-suppressor cells are less effective in inhibiting the CTL responsiveness of spleen cells from ADM-treated mice than those from control (9), but T-regulatory cells affect both equally (Table 6, Groups 3 and 5). While it is not possible, on the basis of the data presented here, to determine whether ADM reduced the number or inhibited the function of the T-regulatory cells, clearly their activity is reduced in the adherent population from ADM-treated mice.

Based on the findings reported herein, it is probable that both ADM-modified cell types (macrophage as well as T-regulatory cells) contribute to the augmented CMC. These 2 ADM-modified cell types could be critical, also, for the ability of spleen cells from ADM-treated mice to develop CMC response to heat-treated alloantigen (9). Further, as has been indicated in a preliminary report (17), an increased production of a soluble factor having IL-2-like activity can be demonstrated using cells from ADM-treated mice. These findings are consistent with a modification in T-helper function, perhaps as a consequence of decreased T-cell down-regulation and/or increase lymphocyte-activating factor-like production by the ADM-induced cells of the monocyte/macrophage lineage which has recently been observed with peritoneal exudate cells from ADM-treated mice (4). Indeed, ADM-induced effects appear to be selective since, in addition to the precursors of the T-suppressor cells, other subsets of cells including the cells involved in ADCC (7, 16) and mature macrophages (2, 14) are not modified. Furthermore, based on the studies reported here, the reactivity of the precursors of the CTL did not appear to be altered. Thus, in the cell recombination experiments (Tables 2 and 6), the 2 (nontreated and ADM-treated) nonadherent populations containing the PTC seemed to respond equally to alloantigen when other variables were the same at the time of primary stimulation.

In conclusion, on the basis of the evidence which has been presented, it is reasonable to suggest that ADM induces selective modifications in both the nonadherent, nonphagocytic, monocyte/macrophage precursor and the adherent Lyt 1-2+ T-cell subsets and that, as a consequence, augmented levels of cell-mediated cytotoxicity can develop. Therefore, ADM, in a manner similar to cyclophosphamide, may be a useful tool in studying the effects of the enrichment and depletion of various subpopulations of cells. More specifically, one can envision the use of this agent to enrich for nonphagocytic macrophages and/or to deplete [without elimination of the cyclophosphamide-sensitive T-cells (22)] the adherent Lyt 1-2+ T-cell which apparently down-regulates CTL activity in vitro.

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Cellular Basis for Adriamycin-induced Augmentation of Cell-mediated Cytotoxicity in Culture

M. Jane Ehrke, Kazuo Ryoyama and Stefan A. Cohen


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