Induction and Enhancement by Monocytes of Antibody-Induced Modulation of a Variety of Human Lymphoid Cell Surface Antigens

By Robert W. Schroff, Margaret M. Farrell, Richard A. Klein, Henry C. Stevenson, and Noel L. Warner

We have previously reported that the addition of monocytes results in enhanced modulation of the T65 antigen when normal or leukemic lymphoid cells were cultured in vitro with the T101 monoclonal antibody. In the present investigation, we extend these findings to demonstrate that monocyte-enhanced modulation is a phenomenon that occurs with a variety of T and B lymphoid antigens identified by murine monoclonal antibodies. Two patterns of monocyte-enhanced modulation were observed: (1) augmentation by monocytes of existing antigen modulation by the T101 and anti-Leu-4 antibodies, and (2) induction by monocytes of previously unrecognized modulation with the anti-Leu-2 and anti-Leu-9 antibodies. Enhancement of modulation by monocytes was also detected with antibodies to surface IgM and HLA-DR antigens. Antigen modulation on lymphoid cell lines appeared to be more variable than on fresh cells, with or without monocytes. Monocyte-enhanced antigen modulation was not demonstrated with two monoclonal antibodies against solid tumors. Monocyte-enhanced modulation was shown to be dependent upon the Fc portion of the antibody, but independent of proteolytic or oxidative compounds released by monocytes. These findings indicate that the results obtained in vitro studies of antigen modulation may vary with the source of cells and the extent to which monocytic cells are present. In addition, these findings suggest an enhanced role for Fc receptor-bearing cells of monocytic origin in antigen modulation following in vivo administration of monoclonal antibodies.

THE T65 ANTIGEN is a 65,000-dalton glycoprotein present on normal T lymphocytes and chronic lymphocytic leukemia (CLL) cells which is identified by the T101 and anti-Leu-1 antibodies.1 We previously demonstrated that the presence of monocytes enhanced both the rate and extent of T65 antigen modulation in vitro.3 Exposure of lymphocytes to the T101 antibody for three hours resulted in approximately 60% decreases in T65 antigen expression in the absence of monocytes, as compared to approximately 90% decreases in the presence of autologous or allogeneic monocytes. The rapid and extensive modulation of the T65 antigen-T101 antibody complexes in the presence of monocytes was similar to that which we observed in vivo in CLL patients receiving T101 monoclonal antibody therapy.4,5

Antigen modulation is of clinical importance due to the capacity of some tumors to evade the potential effectiveness of unconjugated monoclonal antibody therapy by modulation of the target antigen.4,6 On the other hand, since antigen modulation is often accompanied by internalization of antigen-antibody complexes as we have demonstrated for the T101 antibody,7 and as documented by Pesando et al8 for the J5 antibody to the common acute lymphoblastic leukemia antigen (CALLA), antigen modulation may serve as an efficient means of delivering drugs and toxins in the form of immunoconjugates into tumor cells for therapeutic purposes. For both of these reasons, an understanding of the mechanisms involved in antigen modulation is important for the use of monoclonal antibodies in cancer therapy.

The present investigation examines the effect of monocytes upon antigen modulation of cell surface antigens present on normal and malignant lymphoid cells as well as tumor-associated antigens present on tumor cells of nonlymphoid origin. These studies represent an extension of previous studies employing a single monoclonal antibody recognizing a lymphoid antigen.1 Antigen modulation has been observed with only a limited number of antigen–antibody systems, and it is unclear what determines whether antigen modulation occurs. The present investigation was initiated to determine whether monocytes might induce modulation of a wide variety of antigens, including solid tumor cell antigens, in which modulation is rarely, if ever, seen.

MATERIALS AND METHODS

Preparation of cell suspensions. Normal human peripheral blood specimens were obtained from healthy adult volunteers by cytophoresis on a Celltrifuge II apparatus (Travenol Labs, Deerfield, Ill) as previously described.7 The resulting leukocyte preparations were then depleted of erythrocytes and granulocytes by centrifugation on Ficoll-Hypaque (Litton Bionetics, Kensington, Md) density gradients.8 The resultant mononuclear cell (MNC) preparations predominantly contained lymphocytes (mean ± SE of 81 ± 3% lymphocytes and 18 ± 2% monocytes) as assessed by Wright's staining, latex bead phagocytosis, and nonspecific esterase staining, as previously described.8 Further fractionation into lymphocyte and monocyte preparations was performed by countercurrent elutriation.12 The elutriated lymphocytes had a mean purity of 99% ± 0.3%, and the elutriated monocyte preparations were 92% ± 1.3% pure.

Lymphoid cell suspensions from three chronic lymphocytic leukemia (CLL) patients were obtained by Ficoll-Hypaque gradient centrifugation of whole peripheral blood specimens. These patients had not received chemotherapy or radiation therapy in the last month, and had not been previously treated with the T101 monoclonal antibody. All three patients demonstrated peripheral WBC counts of >69,000 per microliter and >98% malignant lymphocytes. Human cell lines of T lymphoblastoid origin (CEM, RPMI 8402, and Molt-3) were maintained in continuous culture at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Cell suspensions
were cryopreserved in liquid nitrogen before use in this study, as previously described. Preliminary experiments indicated that the degree of antigen modulation and the enhancing effect of monocytes were not affected by cryopreservation of MNC, lymphocyte, or monocyte preparations.

Modulation assay. Modulation was assessed in 1-mL cultures of cell suspensions at concentrations of 1 x 10⁶ cells per milliliter in RPMI 1640 medium plus 10% FBS, as previously described. Antibodies were diluted in RPMI 1640 with 10% FBS, and were added to cultures in 50-μL quantities to achieve the desired final concentrations, ranging from 100 μg/mL to 0.1 ng/mL. Control cultures contained no antibody.

Following incubation at 37 °C for the indicated periods of time, cultures were immediately diluted fourfold in cold phosphate-buffered saline (PBS) containing 2% bovine serum albumin and 0.02% sodium azide. Cultures were washed twice and resuspended in 100 μL of the same medium. Each specimen was then stained using an indirect immunofluorescence technique employing 2 μg of mouse monoclonal antibody per specimen, followed by 1.4 μg/mL of a fluorescein isothiocyanate (FITC)-conjugated F(ab′)₂ preparation of goat anti-mouse IgG (Tago, Burlingame, Calif) as the secondary reagent. Control cultures incubated in the absence of antibody during 37 °C incubation periods were split in half before immunofluorescence staining, with one aliquot receiving the primary antibody and the second aliquot receiving a mixture of the control murine IgG₁, and IgG₂, myeloma proteins P₃×₆3 and RPC-5 (Bethesda Research Labs, Bethesda, Md) as a negative control. Both aliquots were stained using the same FITC-conjugated goat anti-mouse IgG secondary antibody. Specimens were assessed for immunofluorescence using an Ortho Cytofluorograf 50H (Ortho Diagnostic Systems, Westwood, Mass). A minimum of 10,000 lymphoid cells were evaluated, with both monocytes and nonviable cells being gated out of the analysis. For each specimen, the mean fluorescence intensity (MFI) of all cells (both antigen-positive and antigen-negative) was calculated over a 1,000-channel scale.

Calculation of percentage of modulation. The degree of modulation was quantitated by comparing each immunofluorescent-stained specimen to the matched controls consisting of the specimen incubated in the absence of antibody and subsequently stained with the monoclonal antibody (positive control) or P₃×₆3/RPC-5 mixture (negative control). The calculation consisted of the following:

\[
\text{MFI of specimen} - \frac{\text{MFI of negative control}}{\text{MFI of positive control}} \times 100.
\]

Monoclonal antibodies. Monoclonal antibodies were purified from ascites fluid using either double-sodium sulfate precipitation, or ion exchange and affinity chromatography. All antibodies were prepared at concentrations of ≥1 mg/mL. The characteristics of the antibodies employed in this study are given in Table 1. The T101 antibody was supplied by Hybritech, Inc (San Diego); the anti-Leu-2, -Leu-4, and -Leu-9 antibodies by Becton Dickinson, Inc (Mountain View, Calif); the anti-human IgM from Bethesda Research Laboratories (Gaithersburg, Md); and the anti–HLA-DR from Dr Leslie Walker of the Scripps Clinic and Research Foundation (La Jolla, Calif). The D3 antibody, which identifies a 190-kidalton antigen present on guinea pig line 10 hepatocarcinoma, and the 9.2.27 antibody, which identifies a 250-kidalton glycoprotein present on cutaneous human melanoma as well as cultured human melanoma cell lines including FMx-Met II, were prepared in our laboratory.

Cultures of lymphocytes or lymphocytes plus monocytes 30 minutes before addition of antibody and were left in the cultures for the three-hour incubation period. Protease inhibitors and concentrations employed included phenylmethyl-sulfonylfluoride (PMSF), a serine esterase inhibitor, at 0.001%; leupeptin, an inhibitor of divalent cation-dependent proteases, at 10 μg/mL; and the general protease inhibitor, benzamidine, at a 2 μmol/L concentration, all from Sigma (St Louis). Anti-oxidants included 2-mercaptoethanol (2-ME) at 50 μmol/L (Aldrich Chemicals, Milwaukee); superoxide dismutase (SOD) at 300 μg/mL (activity, 300 U/mg; Sigma); and catalase at 300 μg/mL (activity, 10,531 U/mg; Worthington, Freehold, NJ).

Statistical analysis. Comparisons of antigen modulation in the presence and absence of monocytes were performed using two-way analysis of variance. This procedure permitted statistical evaluation of the extent of modulation with or without addition of monocytes over the range of antibody concentrations used.

RESULTS

In order to establish whether monocyte-enhanced antigen modulation was a generalized phenomenon or was restricted to only the T65 antigen which is recognized by the T101 antibody, series of anti-leukocyte antibodies were assessed for their ability to mediate antigen modulation of normal peripheral blood lymphocytes. Two classes of antibodies were selected for analysis: those which were known to mediate antigen modulation (T101 and anti–Leu-4), and those which, thus far, have not been shown to demonstrate antigen modulation (anti–Leu-2 and anti–Leu-9).

T101 and anti–Leu-4 were both capable of eliciting approximately 50% modulation of their respective antigens on normal peripheral blood lymphocytes (PBLs) in the
absence of monocytes (Fig 1). With the addition of 30% allogeneic monocytes, the degree of modulation increased to >75% with both antibodies. As expected, the anti–Leu-2 and anti–Leu-9 antibodies elicited little or no modulation on normal PBLs of their respective antigens in the absence of monocytes (Fig 2). However, in the presence of monocytes, up to 70% modulation was seen with anti–Leu-9 and >90% modulation was seen with anti–Leu-2. Statistical analysis using a paired analysis of variance procedure indicated a significant enhancement of modulation with each of these antibodies in the presence of monocytes at the 97.5% confidence level.

To determine whether modulation of a given antigen was similar on cell lines as compared to freshly isolated cells, we examined antigen modulation with the anti–Leu-9 antibody on cell lines of lymphoid origin. As shown in Fig 3, little or no modulation was seen on the three cell lines with the anti–Leu-9 antibody in the absence of monocytes. In the presence of monocytes, antigen modulation was elicited on the CEM cell line in a fashion similar to that observed with freshly isolated lymphocytes (see Fig 2). In contrast, no significant enhancement of modulation was observed under similar conditions with the Molt-3 or 8402 cell lines. As was the case with all antigens examined in this study, there was no evidence of preferential modulation within one subpopulation of antigen-positive cells as compared to others.

We wished to determine whether the ability of monocytes to induce antigen modulation with the anti–Leu-9 antibody on CEM and not the Molt-3 or 8402 cells was related to the Leu-9 antigen density on the three cell lines. To address this question, we compared the fluorescence staining intensity on the three cell lines to that obtained with freshly isolated human peripheral blood lymphocytes (Table 2). No correlation was observed between the degree of Leu-9 antigen expression and the ability to elicit modulation of that antigen in the presence of monocytes.

Since our clinical use of the T101 antibody has focused on therapy of CLL, we were interested in examining modulation of antigens present on CLL cells other than the T65 antigen, for which monocyte-enhanced modulation on CLL cells with the T101 antibody has been demonstrated in a prior publication. Two murine monoclonal antibodies were employed: an antibody against human immunoglobulin μ chain (anti-IgM), and an antibody directed against a common framework HLA-DR antigen (anti–HLA-DR). The anti-IgM antibody mediated a greater degree of antigen modulation than did the anti–HLA-DR antibody in the absence of monocytes (Fig 4). With both antibodies, the addition of human monocytes was accompanied by a statistically significant enhancement of modulation (P < .05).

We also examined antigen modulation with two monoclonal antibodies against solid tumors. The first was the D3 murine monoclonal antibody, which is specific for a 190-kilodalton glycoprotein antigen on the line 10 guinea pig hepatocarcinoma line. This animal model system has been extensively investigated by our laboratory for evaluation of monoclonal antibody therapy. No antigen modulation could be elicited using the D3 antigen in the presence or absence of human monocytes, with either a cultured line 10 hepatocarcinoma cell line or tumor cells freshly isolated from actively growing tumors (data not shown). The second murine monoclonal antibody was 9.2.27, specific for a 250-kilodalton glycoprotein–proteoglycan complex on human

**Fig 2.** Antigen modulation of normal PBLs from three donors with the anti–Leu-2 (A) and anti–Leu-9 (B) monoclonal antibodies. Culture conditions and label designations are the same as in Fig 1.
Fig 3. Antigen modulation of the Leu-9 antigen on the T lymphoid cell lines CEM (A), 8402 (B), and Molt-3 (C). Culture conditions and label designations are the same as in Fig 1, and monocytes were obtained from three different normal donors.

Previous experiments indicated that the Fc portion of the antibody molecule was required for monocyte enhancement of antigen modulation. Additional mechanisms by which monocytes could potentially be involved in the modulation of cell surface membrane antigens include protease digestion of the antigen or degradation of the antigen by reactive oxygen intermediates. To examine the potential contribution of monocyte-derived proteases, modulation cultures with normal lymphocytes and the anti-Leu-4 antibody were performed in the presence of the protease inhibitors PMSF, benzamidine, leupeptin, or a combination of all three (Fig 6). None of the protease inhibitors had an effect upon the ability of monocytes to enhance modulation in this system.

To assess the possible effects of reactive oxygen intermediates produced by the monocytes, a series of four reactive oxygen scavengers was added to modulation cultures of normal lymphocytes and anti-Leu-4 antibody (Fig 7). While the addition of 2-ME resulted in decreased modulation both in the presence and absence of monocytes, the water-soluble vitamin E derivative Trolox, superoxide dismutase, and catalase all showed no effect on the enhancing effect of monocytes.

Table 2. Comparison of Anti-Leu-9 Immunofluorescence Staining Intensity and the Ability to Elicit Leu-9 Antigen Modulation in the Presence of Monocytes

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Mean Fluorescence Intensity*</th>
<th>Percentage of Leu-9 Positive Cells</th>
<th>Antigen Modulation Elicited by Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM</td>
<td>485</td>
<td>98</td>
<td>Yes</td>
</tr>
<tr>
<td>8402</td>
<td>471</td>
<td>98</td>
<td>No</td>
</tr>
<tr>
<td>Molt-3</td>
<td>314</td>
<td>94</td>
<td>No</td>
</tr>
<tr>
<td>PBLs</td>
<td>199</td>
<td>73</td>
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*Mean channel of fluorescence staining intensity over a 1,000-channel range in the absence of monocytes.

DISCUSSION

The present investigation demonstrates that monocyte-enhanced modulation is a generalized occurrence with a variety of lymphoid antigens, and not just restricted to the T65 antigen as has been primarily described.

Of particular interest is the question of why certain antigens such as Leu-2 and Leu-9 fail to modulate in the absence of monocytes in contrast to other antigens such as Leu-4 and T101, and what parameters determine whether monocytes will induce modulation of a given antigen. Although one simple explanation would be that modulation could occur only when the density of the antigen on the cell surface was high, the present data did not support this possibility. The reported relative antigen densities of the Leu-2 and Leu-4 antigens on human T lymphocytes are very similar, at $14 \times 10^4$ and $16 \times 10^4$ antibody-binding sites per melanoma cells. This antibody has been used with a nude mouse xenograft of the human melanoma cell line FMx-Met II as a model system for monoclonal antibody therapy as well as in human therapy. Modulation was examined in the presence and absence of human monocytes with the 9.2.27 antibody, with either freshly isolated tumor cells from the xenografts or cultured FMx-Met II cells. In contrast to uncultured lymphocytes, no significant modulation was observed with this antibody-tumor system, with or without the addition of monocytes (Fig 5).

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*Mean channel of fluorescence staining intensity over a 1,000-channel range in the absence of monocytes.
diates, although it is difficult to be certain that the inhibitors were not excluded from the important determination of all three inhibitors. Presumably, the ability of each antigen to modulate is determined by the configuration (ie, integral v peripheral proteins) or fluidity of the antigen within the cell surface membrane.

Investigations into a variety of potential mechanisms have failed to identify any event other than Fc receptor-mediated binding by monocytes which contributes to the enhancement of modulation by monocytes. The enhancing effect does not appear to involve proteolytic enzymes or degradation of antigen–antibody complexes by reactive oxygen intermediates, although it is difficult to be certain that the inhibitors and anti-oxidants used were not excluded from the important areas of monocyte/lymphocyte contact. 2-ME had an inhibitory effect upon antigen modulation and its enhancement by monocytes, but was accompanied by an inhibition of antigen modulation even in the absence of monocytes. The lack of a similar effect by the other scavengers suggests that the 2-ME–mediated inhibition is through another mechanism not related to its effects on reactive oxygen species.

The results of this investigation have implications both for the clinical application of monoclonal antibodies and for the understanding of the phenomenon of antigen modulation. With respect to the latter, our results demonstrated that conclusions as to whether or not a given antigen modulates may be of particular relevance to the study of modulation of antigens associated with solid tumors of humans, since the difficulty of obtaining fresh human tissue of either normal or malignant origin often necessitates the use of cultured cells and cell lines for laboratory studies.

The receptor-mediated endocytosis of a variety of ligands, including antibodies, is believed to be mediated by internalization through coated pits. The efficiency of internalization varies greatly with different ligands; however, the reason for this variation is not well understood. The enhancing function of monocytes upon antigen modulation may be mediated through aggregation or patching of antibody–antigen complexes on the lymphoid cell surface membrane. Our studies have shown that the enhancement phenomenon is dependent on the presence of the Fc portion of antibody. Although some monocyte Fc receptors might be occupied by endogenously bound Ig, the immune-complexed antibody on the lymphoid cell surface could presumably displace the bound human Ig, allowing patching of antibody–antigen complexes within the lymphoid surface membrane and/or

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Fig 5. Lack of antigen modulation of the human melanoma cell line FMx-Met II with the 9.2.27 monoclonal antibody. Culture conditions and label designations are the same as in Fig 1, and monocytes were obtained from three different normal donors.

Fig 6. Lack of effect of protease inhibitors upon Leu-4 antigen modulation. Normal peripheral blood lymphocytes from a single donor were cultured for three hours in the presence (□) or absence (○) of 30% allogeneic monocytes. Cultures were preincubated for 30 minutes with PMSF, benzamidine (Benz), or leupeptin (Leu) before the addition of 0.1 µg/mL of antibody, as described in Materials and Methods. Combination (Comb) refers to a combination of all three inhibitors.

Fig 7. Effect of scavengers of reactive oxygen intermediates upon Leu-4 antigen modulation. Normal peripheral blood lymphocytes from a single donor were cultured for three hours in the presence (□) or absence (○) of 30% allogeneic monocytes. Cultures were preincubated for 30 minutes with Trolox (Trol), 2-ME, SOD, or catalase (Cat) prior to addition of 0.1 µg/mL of antibody, as described in Materials and Methods.
MODULATION OF CELL SURFACE ANTIGENS

lateral movement of Fc receptors within the monocyte/macrophage surface membrane. Potentially, these patches of antigen–antibody complexes could then be internalized more rapidly or efficiently than antigen–antibody complexes not crosslinked by monocyte Fc receptors. The ability of secondary antibody to augment modulation of antigens in a similar manner has been previously documented. If patching of the cell surface antigen is indeed required for this process to proceed, then modulation would be expected to be maximal at antibody concentrations yielding the greatest degree of crosslinking or lattice formation between antibody and antigen. This presumably explains why the degree of modulation seems to decrease at higher antibody concentrations with some antibodies, especially anti-Leu-9. With certain antigens, such as the T65 and CALLA antigens, antigen modulation is accompanied by internalization of the antibody–antigen complex. The ability to enhance antigen modulation in these cases may be of great importance to the therapeutic use of monoclonal antibodies in the form of drug or toxin immunoconjugates. Many of the drugs and toxins being evaluated for possible clinical use require intracellular access in order to mediate their toxic effects. Monocyte-mediated enhancement of antigen modulation may serve to augment intracellular uptake of immunoconjugates and optimize the effectiveness of this form of therapy.

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