MECHANISMS OF TUMOR CELL CAPTURE BY ACTIVATED MACROPHAGES: EVIDENCE FOR INVOLVEMENT OF LYMPHOCYTE FUNCTION-ASSOCIATED (LFA)-1 ANTIGEN

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The lymphocyte function-associated (LFA)-1 molecule is expressed on certain populations of macrophages that have an augmented capacity to capture tumor cells. Accordingly, we analyzed the role of LFA-1 in the establishment of such cell-cell interactions. Fab'2 fragments of the M17/4, anti-LFA-1 monoclonal antibody (MAb) inhibited the interaction between activated macrophages and tumor cells by up to 80% in a dose-dependent manner. The anti-LFA-1 MAb reduced (between 55 to 79%) the number of P815, LSTRA, or EL-4 tumor cells bound to trypsin-sensitive structures on bacillus Calmette Guerin activated macrophages. The inhibition appeared selective, because a Fab'2 fragment of anti-Mac-1 did not inhibit such binding. Inhibition of tumor cell capture could be observed as soon as 15 min after the onset of the cell-cell interaction between activated macrophages and tumor cells. Optimal inhibition occurred when both tumor targets and macrophages were precoated with the MAb. Although P815, LSTRA, EL-4, and BW5147 tumor cells all expressed LFA-1, only the first three but not BW5147 cells were bound by activated macrophages. Furthermore, endotoxin-pulsed macrophages elicited by thioglycollate broth expressed the LFA-1 antigen but did not exhibit selective tumor cell capture. Finally, anti-LFA-1 inhibited the development of weak into strong binding. Taken together, the results suggest that LFA-1 molecules can participate in the interaction between activated macrophages and neoplastic cells.

Mononuclear phagocytes, when activated by various stimuli, are endowed with the ability to capture and then to destroy tumor cells selectively (1). The general cell-cell interaction that initiates this important host defense response can be divided into two distinct subtypes (2, 3). The first, termed nonselective binding, is a low-affinity, high-capacity binding that cannot be abrogated by trypsin. LFA-1 does not lead to cytolyis (2). The second, termed selective binding, is a high-affinity low-capacity binding that requires the presence of trypsin-sensitive surface structures on the macrophages, is mediated only by primed or activated macrophages, and leads to cytolyis (2, 3). Initially, macrophages bind a variety of cells in which binding can rapidly be disrupted by application of <16 dynes force per cell (4). If the targets are neoplastic, this nonselective and weak binding can be converted by primed and activated macrophages over 60 to 90 min at 37°C to binding requiring >200 dynes per cell to disrupt (4).

The lymphocyte function-associated (LFA)-1 antigen is a member of a glycoprotein family that is important to a variety of cellular adherence reactions (5, 6). Specifically, LFA-1 molecules participate in the adhesion between target cells and cytolytic T lymphocytes (CTL) or natural killer (NK) cells, neutrophils and antibody-coated targets, and homotypic reactions between B lymphocytes (7-17). Evidence for this assertion lies in the observation that monoclonal antibodies (MAb) directed against LFA-1 block adhesion between leukocytes and targets. Interestingly, neutrophils from patients deficient in LFA-1 do not adhere well to glass (18). LFA-1 comprises an α-chain of Mr η 180,000 and a β-chain of Mr η 95,000 (5, 6, 19). The Mac-1 antigen, which is the receptor for C3bi and mediates adhesion between monocytes or granulocytes and C3bi-coated particles, shares a common β-chain with LFA-1 (5, 19-21). The α-chain of LFA-1 however, is distinct from the α-chain of Mac-1 (19). Deficiencies in the Mac-1/LFA-1 glycoprotein family are associated with profound defects in host defense mechanisms (15, 17, 19, 22-24).

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2 Abbreviations used in this paper: LFA, lymphocyte function associated; TG, thioglycollate broth; MAb, monoclonal antibody; RCF, relative centrifugal force.
Participation of LFA-1 in Tumor Cell Binding by Macrophages

**MATERIALS AND METHODS**

**Mice.** Inbred female, specific-pathogen-free C57BL/6J mice were obtained from The Jackson Laboratory, Inc., Bar Harbor, ME. Mice of 8 to 16 wk of age were used. The animals were kept in the Animal Care and Use Committee at Duke University.

**Reagents.** Bacillus Calmette Guerin (BCG), Phipps strain 1029 was purchased from The American Type Culture Collection (ATCC), Rockville, MD. The BCG strain was grown in 10 ml of cold HBSS supplemented with 10% FBS containing trypsin. Macrophage monolayers after exposure to BCG were used for 20 min at room temperature with 50% of Na25CrO4 as described (33). In brief, BCG-activated macrophages were prepared in monolayers at 3 x 106 macrophages/cm² as described in 6 min. After nonadherent cells were removed with vigorous washing, EMEM + 10% FBS containing trypsin were preincubated for 20 min at room temperature and the plates were incubated at 37°C in 5% CO₂ for 18 hr. After trypsinization, macrophage monolayers at 37°C were incubated with 5 x 106 cells/well, and 0.1 ml containing 5 x 106 cells/ml were added to each microtiter well. The content of each well was transferred into a tube and was counted in a Packard gamma scintillation spectrometer. Triplicate wells were used for each point tested. Selective binding was determined by the formula:

\[ \text{Percent inhibition} = \frac{(X - Y) \times 5 \times 10^4}{(X - Y) \times 5 \times 10^4} \]

Where:

- \( X \) = cpm bound to EBSS treated macrophages
- \( Y \) = cpm bound to trypsinized macrophages

**Quantification of Strength of Binding.** To determine the strength of binding, we made use of a modification of the method of McClay et al. (4, 34). In brief, BCG-activated macrophages were prepared in monolayers at 3 x 106 macrophages/cm² as described in 6 min. After nonadherent cells were removed with vigorous washing, EMEM + 10% FBS containing 5% FBS. Aliquots of 2 x 106 macrophages in 100 μl of medium were plated in flat bottomed microtiter plates (Limbro 7604605). The plates were incubated at 37°C in 5% CO₂ for 2 hr and the monolayers were washed (×3) to remove nonadherent cells.

**Treatment of Macrophages in Vitro.** Macrophage monolayers were incubated at 37°C with 0.15 ml of EMEM + 10% FBS containing LPS for 18 hr. After this exposure period, macrophage monolayers were subjected to radiololution assay and were assayed for their ability to bind tumor targets.

**Monoclonal Antibodies (MAb).** The MAb used in this study were described (8, 19, 32). The MAbs used in this study were described (8, 19, 32). The MAb used in this study were described (8, 19, 32). The MAb used in this study were described (8, 19, 32).

**Trypsinization.** Macrophage monolayers were treated with 1 mg/ml trypsin in Earl's balanced salt solution (EBSS) for 20 min at 37°C (2).
various doses of m17/5 (aLFA-1 MAb) F(ab')n fragments capture. TG broth, which binds targets principally in the nonselective manner (2), their overall binding capacity was reduced with trypsin in incubation. The results are expressed as number of targets selectively bound to macrophages (bars) and as percent inhibition of binding (graph) (see Materials and Methods). This experiment was repeated twice, and similar results were obtained.

Figure 1. Dose response course of targets binding inhibition. BCG-activated macrophages (2 × 10^6) treated or untreated with trypsin were incubated with various doses F(ab')2 of anti LFA-1 MAb for 20 min. Target cells (P815) were added, and binding was measured after a 1-hr incubation. The results are expressed as number of targets selectively bound to macrophages (bars) and as percent inhibition of binding (graph) (see Materials and Methods). This experiment was repeated twice, and similar results were obtained.

Figure 2. Anti-Mac-1 F(ab')2 fragments do not inhibit selective target capture. BCG macrophages (2 × 10^6) were treated with 50 μl of various doses of m1/70 (a-Mac-1 MAb) F(ab')2 fragments (dashed line) or with various doses of m17/5 (aLFA-1 MAb) F(ab')2 fragments (solid line). The results are expressed as the number of P815 targets selectively bound (see Materials and Methods). This experiment was repeated twice, and similar results were obtained.

Input of F(ab')2 -m17/5 (ng/wll)

No of P815 Cells Selectively Bound (X10^-7)

100 60 30 0

Input of F(ab')2 (ng/wll)

12 10 8 4 0

No of P815 Cells Selectively Bound (X10^-7)

100 60 30 0

Effect of anti-LFA-1 F(ab')2 fragments on development of strong target binding. Previous experiments have demonstrated that the number of tumor targets bound by BCG-activated macrophages over a range of applied RCF yields a predictable pattern (4). Basically, the great majority of added targets (i.e., 90%) remain bound to the macrophage monolayer from 1 to 100 × G of applied RCF. The number of targets then gradually declines with increasing RCF until only 20 to 35% of the targets remain bound at >1200 × G of RCF. Targets remaining bound at RCF less than 100 × G includes targets that are strongly bound. Thus weak binding may be calculated by subtracting the number of strongly bound targets from the total number of targets bound at 100 × G (4). Additional experiments have demonstrated that weakly bound targets can be converted to strongly bound if the targets are tumor cells and if the macrophages are activated (4).

When we preincubated macrophages with the M17/4 anti-LFA-1 MAb and then added P815 tumor cells, the F(ab')2 fragments of the M17/70 did not significantly affect binding of any of the three targets (see legend, Fig. 4).

Localization of cellular site of inhibition. In the experiments described above, binding was assessed after preincubation of the macrophages for 20 min with MAb, followed by addition of targets without removal of unbound antibody. To determine whether inhibition required precoating of LFA-1 on targets, as well as on macrophages, the macrophages or targets were treated separately with antibody, were washed extensively, and were then mixed. Effective inhibition of binding by anti-LFA-1 could be obtained only when both targets and activated macrophages were treated with F(ab')2 fragments of αLFA-1 (Table II).

Relationship between expression of LFA-1 on tumor cells and macrophages and their potential for macr

ophase-target interaction. Various tumor lines were then analyzed for their expression of LFA-1 and the ability to be captured by activated macrophages. Tumor cells of the P815, BW5147, EL-4, and LSTRA lines expressed comparable levels of LFA-1 (Fig. 5A). A MAb of the same isotype as the M17/4 (e.g., the M3/38 αMac-2 MAb) failed to bind to tumor cells significantly but could bind to TG-elicited macrophages (data not shown). When we examined binding of these targets, the BW5147 cells were not efficiently captured by BCG-activated macrophages as compared with P815 EL-4, and LSTRA targets (Fig. 5B). The BW5147 cells could not be effectively lysed by the same macrophages (data not shown).

TG-elicited macrophages can be induced to express the LFA-1 antigen after overnight incubation with endotoxin (28), conditions that do not lead to induction of competence for selective binding (1). When we analyzed the ability of such macrophages to bind tumor cells, either 2 ng/ml or 10 ng/ml of lipopolysaccharide (LPS) induced LFA-1 expression on TG-elicited macrophages but not augmented capacity for P815 capture (Fig. 6). The low level of trypsin-sensitive binding mediated by LPS-treated TG macrophages could not be significantly inhibited by F(ab')2 fragments of the M17/5 MAb (Fig. 6). In the same experiment, the interaction mediated by BCG macrophages was inhibited by 52% by the anti-LFA-1 MAb.

Effect of anti-LFA-1 F(ab')2 fragments on development of strong target binding. Previous experiments have demonstrated that the number of tumor targets bound by BCG-activated macrophages over a range of applied RCF yields a predictable pattern (4). Basically, the great majority of added targets (i.e., 90%) remain bound to the macrophage monolayer from 1 to 100 × G of applied RCF. The number of targets then gradually declines with increasing RCF until only 20 to 35% of the targets remain bound at >1200 × G of RCF. Targets remaining bound at RCF less than 100 × G includes targets that are strongly bound. Thus weak binding may be calculated by subtracting the number of strongly bound targets from the total number of targets bound at 100 × G (4). Additional experiments have demonstrated that weakly bound targets can be converted to strongly bound if the targets are tumor cells and if the macrophages are activated (4).

When we preincubated macrophages with the M17/4 anti-LFA-1 MAb and then added P815 tumor cells, the
We present evidence here that LFA-1 molecules participate in the cell-cell interaction between activated macrophages and neoplastic targets. F(ab')₂ fragments of the M17/5 anti-LFA-1 MAb reduced the number of three neoplastic cells that were captured by BCG-activated or pyran-primed macrophages (Table I and Fig. 4). The extent of inhibition, which varied from 55 to 79%, depended upon the dose of antibody (Fig. 1) and the target cells (EL-4, LSTRA, and P815) that were strongly bound (Table I). The overall number of targets bound at 100 × G, however, decreased; this decrement could be attributed to the loss in the number of targets that were strongly bound (Table III).

**DISCUSSION**

The estimated number of weakly bound targets was not significantly different between treated and untreated groups (Table III). The number of strongly bound targets (i.e., targets remaining adherent at 1300 × G) was significantly inhibited (Table III). The overall number of targets bound at 100 × G, however, decreased; this decrement could be attributed to the loss in the number of targets that were strongly bound (Table III).

**TABLE I**

<table>
<thead>
<tr>
<th>Macrophage population</th>
<th>Treatment</th>
<th>No. of Targets Bound (x 10⁷ ± SD)</th>
<th>Contribution to Inhibition</th>
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<tr>
<td>BCG-activated macrophages</td>
<td>Medium only</td>
<td>4.11 ± 0.8</td>
<td>21%</td>
</tr>
<tr>
<td></td>
<td>M17/5</td>
<td>2.59 ± 0.5</td>
<td>31%</td>
</tr>
<tr>
<td>Pyran-primed macrophages</td>
<td>Medium only</td>
<td>3.50 ± 0.4</td>
<td>13%</td>
</tr>
<tr>
<td></td>
<td>M17/5</td>
<td>2.35 ± 0.5</td>
<td>47%</td>
</tr>
</tbody>
</table>

* Pyran-primed or BCG-activated macrophages (2 × 10⁶) were plated per well.
* Macrophage monolayers were pretreated with 50 μl/well of medium or 444 ng/well of F(ab')₂ of M17/5 (αLFA-1).
* Data are given as the total amount of P815 targets bound per culture.
* Percentage of the total degree of inhibition when decrease in total number of targets bound is 100%.
* The results are given as the difference between the number of targets bound to trypsinized macrophages (see Materials and Methods).
* Similar data obtained in two experiments.

**TABLE II**

<table>
<thead>
<tr>
<th>Group</th>
<th>F(ab')₂ Added (ng/well)</th>
<th>Pretreatment of Macrophages</th>
<th>Pretreatment of P815</th>
<th>Number of Selectively Bound Targets (x 10⁷)</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13.7</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>444</td>
<td>-</td>
<td>-</td>
<td>6.6</td>
<td>52</td>
</tr>
<tr>
<td>3</td>
<td>148</td>
<td>-</td>
<td>-</td>
<td>8.4</td>
<td>39</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>444</td>
<td>-</td>
<td>12.7</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>148</td>
<td>-</td>
<td>13.1</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>125</td>
<td>13.6</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>444</td>
<td>125</td>
<td>8.2</td>
<td>40</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>148</td>
<td>125</td>
<td>9.8</td>
<td>28</td>
</tr>
</tbody>
</table>

* BCG-activated macrophages (2 × 10⁶) were treated with F(ab')₂ of anti LFA-1 MAb and selective binding radiolabeled P815 cells was determined (see Materials and Methods).
* Macrophages were treated with 50 μl containing 444 ng or 148 ng antibody for 20 min room temperature. Antibody excess was not removed.
* Macrophages were treated in group C. Antibody was aspirated, and the cultures were washed three times with medium.
* Radiolabeled P815 cells (4 × 10⁶) were treated with 10 μg of F(ab')₂ fragments in Table: 125/ng/5 × 10⁶ targets added per well for 20 min on ice, were washed three times, and were added to untreated macrophages.
* Targets were treated as in group D and were added to macrophages treated as in group C.
* Groups 1 through 5 were repeated four times, and groups 6 through 8 were repeated twice. Similar results were obtained in these additional experiments.
tumor binding (Fig. 2). Selective rather than nonselective binding was inhibited (Table I and Fig. 6B). The presence of LFA-1 on both the tumor cells and activated macrophages appeared to be important in the interaction (Table II). This conclusion does not imply, however, that the interaction of LFA-1 molecules is necessarily homologous (i.e., LFA-1 binding to LFA-1); the heterologous binding of LFA-1 to other molecules on both macrophages and tumor cells could also participate.

The mere presence of LFA-1 on macrophages or tumor cells does not appear sufficient to initiate selective binding. The BW5147 tumor cells, which express levels of LFA-1 similar to those expressed by other tumor cells (e.g., P815, LSTRA, and EL-4) (Fig. 5A), are not captured (Fig. 5B) or lysed (data not shown) by BCG-activated macrophages. Conversely, inflammatory macrophages induced to express LFA-1 antigen by culture with LPS (28) do not acquire augmented capacity for selective capture of tumor cells (29, 30). These results suggest that the presence of LFA-1 molecules on macrophages or tumor cells is in itself not sufficient to produce competence for extensive capture of tumor cells by macrophages. At present, we have no evidence as to whether LFA-1 is necessary for the establishment of selective binding. We are unaware of the existence of either LFA-1 negative macrophage populations endowed with selective-binding capacity or of LFA-1 negative, nonadherent tumor cells that can be selectively bound to activated macrophages, although we have searched extensively for such cells.

LFA-1 has been suggested to participate in the establishment of firm cell-cell adherence between various leukocytes and targets once recognition has occurred (7, 9–12, 15). Many cell pairs involving macrophages, including macrophages that do not express surface LFA-1 (25, 28), establish weak cell-cell attachments (4). When a specific recognition system is put into play, the weak binding can be actively converted into strong (4, 34); such weak and strong binding can be readily distinguished from one another by quantifying the strength of cell-cell attachments (4). We have shown that MAb directed against LFA-1 do not block the establishment of either nonselective binding (Table I) or weak binding (Table III). We additionally show that the M17/5 MAb does inhibit the development of selective binding (Table I) and of strong binding (Table III). Finally, certain macrophages that express LFA-1 can establish only weak, nonselective binding (see Figure 6) (2, 4, 33). Taken together, these data suggest that LFA-1 molecules may participate in the conversion of weak, nonselective binding into strong, selective binding by macrophages.

The precise molecule to molecule interactions that occur in this and in other systems of cell-cell attachment remain to be established (35). For example, one direct route of examining this question (i.e., specific reconstitution LFA-1 negative cells of cells with LFA-1) awaits molecular cloning of the LFA-1 molecule. By use of MAb, LFA-1 has been postulated to stabilize binding after rec-

**TABLE III**

Effect of Fab'2 fragments of anti-LFA-1 on the development of strong tumor cell binding by activated macrophages

<table>
<thead>
<tr>
<th>Targets Bound (x 10⁶) ± SD</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of targets bound at 100 × G of RCF</td>
<td>Medium</td>
</tr>
<tr>
<td>Total number of targets bound at 1300 × G of RCF (number of targets strongly bound)</td>
<td>12.0 ± 1.3</td>
</tr>
<tr>
<td>Estimated number of targets weakly bound</td>
<td>39.3 ± 1.7</td>
</tr>
</tbody>
</table>

*Monolayers of BCG-activated macrophages were prepared and were treated with 444 ng/well of M17/5 Fab'2 as before. The details of the reverse centrifugation assay are described in Materials and Methods. Each experimental data point was obtained with four replicates per determination. The results shown are representative of three separate experiments.
ognition of other structures by the T cell receptor (7, 9–11). The presence of LFA-1 on B cells, T cells, myeloid cells, NK cells (6), and primed/activated macrophages (25, 28) suggests that this molecule may well play a general role in adhesion reactions. LFA-1 has been found to function in T cell-dependent kill, natural killing, and antibody-dependent cellular cytoxity by neutrophils (11, 15). We present evidence in this report that LFA-1 plays a role in the adhesion between activated macrophages and tumor cells. The data presented here further are consistent with the possibility that LFA-1 may function as a stabilizing molecule after an initial recognition step has taken place. In any case, LFA-1 now appears to participate in one important cell-cell interaction of activated macrophages; it will be of interest to determine whether this molecule participates in other recognition functions mediated by activated macrophages as well.

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