Effector Mechanisms of Human Monocyte-mediated Tumor Cytotoxicity in Vitro: Parameters of Induction of Cytotoxins from Peripheral Blood Monocytes Isolated by Counterflow Elutriation

Jim Klostergaard, Jim Turpin, and Gabriel Lopez-Berestein

Departments of Tumor Biology [J. K.] and Clinical Immunology and Biological Therapy [J. T., G. L-B.], University of Texas—M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77030

Abstract

Human peripheral blood monocytes, isolated in high purity by centrifugal counterflow elutriation from normal donors, were stimulated in vitro to release cell toxins, herein termed human monocyte toxin(s) (HMT). Bacterial lipopolysaccharide, the lipopolysaccharide 6-O-steroyl derivative of muramyl dipeptide, and 4/3-phorbol-12-myristate-13-a-acetate served as effective induction signals. Induction involved a sequence of transcription, translation, and secretion, all necessary for HMT synthesis and release into the supernatant as determined by blocking of these functions with the drugs actinomycin D, cycloheximide, and monensin, respectively; HMT levels reached a peak within 4–6 h and thereafter declined. The levels of HMT produced varied considerably from donor to donor; one parameter causing this variability appeared to be the plateletapheresis history of the donor. Monocytes from donors subjected to pheresis for the first time were responsive to induction signals immediately after adherence and could not be brought to a higher state of priming for HMT production by further in vitro culture for up to 9 days, with or without recombinant human γ-interferon. In contrast, monocytes from donors who had recently undergone pheresis (up to 1 wk earlier) were poorly responsive initially to triggering with lipopolysaccharide; however, these cells could be brought to a highly primed state for HMT production by a combination of culture in vitro for several days and a subsequent 24-h exposure to recombinant γ-interferon (0.1–1.0 units/ml). These primed cells could then be effectively triggered by lipopolysaccharide to release HMT. HMT was found to be cytotoxic (cytostatic/cytolytic) for human and murine tumor cells in vitro.

Introduction

Human and rodent cells of the monocyte/macrophage lineage can be rendered tumoricidal in vitro by exposure to a variety of combination of signals in vivo and/or in vitro (1–9). Molecular characterization of the tumoricidal mechanism itself remains enigmatic, although evidence exists for a spectrum of pathways (10–13). Among these are the release by the activated effector cell of cytotoxic macromolecules (14–23). In the human such mediators have been reported by several laboratories (24–28).

Release of these cytotoxins has been associated with achievement of the tumoricidal state. Previous investigations differed significantly in their observations of antitumor activity achieved spontaneously or requiring in vitro maturation of the monocytes.

Materials and Methods

Monocyte isolation. Monocytes from normal donors undergoing routine plateletapheresis were isolated by counterflow elutriation as previously described (Refs. 30 and 31; Footnote 3). The final monocyte populations were >95% pure as determined by morphological characteristics and nonspecific esterase staining (32). These cells were allowed to adhere to plastic for 30 min at 37°C in the presence of FCS. After washing, the monocytes were resuspended according to different protocols (see below).

Target cell lines. The EMT-6 murine adenocarcinoma (BALB/c) was provided through the courtesy of Dr. A. Kallmann, Stanford University, Palo Alto, CA. The A-375 human melanoma was provided through the courtesy of Dr. I. J. Fidler, The University of Texas-M. D. Anderson Hospital and Tumor Institute at Houston, TX. The K562 human erythroleukemia was provided through the courtesy of Dr. C. L. Reading, The University of Texas-M. D. Anderson Hospital. The murine L-929, the HeLa human adenocarcinoma, and the WI-38 human lung fibroblast were from American Type Culture Collection (Rockville, MD). Normal mouse lung fibroblast cultures were established by mincing of aseptically removed lungs of 8-day-old mice. All cell lines were negative for microbial contamination and maintained on antibiotic-free Dulbecco’s modified Eagle’s medium: F-12 medium with 10% FCS.

Reagents. Endotoxin-screened RPMI-1640 tissue culture medium and pooled human AB serum were purchased from M. A. Bioproducts (Walkersville, MD); endotoxin-screened FCS was obtained from Hyclone Co. (Logan, UT); LAH and Ca2+; Mg2+-free phosphate-buffered saline were from Grand Island Biological Co. (Grand Island, NY). FoCal was purchased from Pharmacia Laboratories (Piscataway, NJ), and Hypaque was from Winthrop Laboratories (New York, NY). Human albumin was purchased from Travelpers Laboratories (Glendale, CA) as a 10% solution. All commercial media and solutions were purchased either as pyrogen free or prescreened to contain less than 0.25 ng of endotoxin per ml.

Received 3/12/85; revised 9/10/85; accepted 10/10/85.

The abbreviations used are: FCS, fetal calf serum; HMT, human monocyte toxin; γ-HMT, human monocyte toxin, γ-class; HuTNF, human tumor necrosis factor; LPS, lipopolysaccharide; MDP, N-acetylmuramyl-L-alanyl-d-isoglutamine; SMDP, 6-O-steroyl-N-acetylmuramyl-L-alanyl-d-isoglutamine (muramyl dipeptide); PIC, polyinosinic:polycytidylic acid; PMA, 4/3-phorbol-12-myristate-13-acetate; LAH, lactalbumin hydrosol; MTI, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; IFN-γ, recombinant γ-interferon; IFN, interferon; cDNA, complementary DNA.

CANCER RESEARCH Vol. 46 February 1986 662
with the Limulus Amebocyte Lysate assay (M. A. Bioproducts). Phenol-
extracted endotoxin (Escherichia coli; serotype 0128:B12), PMA, cal-
cium ionophore A23187, tuftsin, actinomycin D, and monensin were from
Sigma (St. Louis, MO). PIC was from P. L. Biochemicals, Inc. (Milwaukee,
WI). SMDP was provided through the courtesy of Dr. Gordon Jones,
Syntex Laboratories, Palo Alto, CA. Human rIFN-γ was obtained from
Genentech (South San Francisco, CA).

**Microcytotoxicity Assays.** The actinomycin D-treated L-929 target
was used in a microcytotoxicity assay as previously described (23, 33);
this was the standard rapid assay used throughout most of these studies,
allowing evaluation of cytolytic effects after 18 h of treatment. For the
data reported for this target in Table 2 of "Results," the assay was con-
ducted identically except that actinomycin D was omitted, and cyto-
toxicity was allowed to proceed for 72 h. Similarly, the EMT-6, HeLa,
A375, K562, WI-38, and lung fibroblast targets were seeded at 2.5–10
× 10^4 cells/ml in 100–200 µl of medium. After 24 h, toxim (β-HMT; see
below) was added, and incubation was continued for an additional 18–
72 h. Cytotoxicity in these cultures was evaluated after staining with
neutral red or with MTT (34, 35).

Units/ml were calculated by linear regression as the reciprocal of the
dilution that caused reduction of absorbance for either strain, to 50% of
control untreated cultures.

**Monocyte Stimulation for Toxin Production.** For the standard prep-
paration of HMT, the following protocol was used. Washed adherent
peripheral blood monocytes, isolated as described above, were cultured
for 4–6 h (Chart 2) at 37°C in medium Dulbecco’s modified Eagle’s
medium: F-12 medium with 0.1% LAH and 90–100 ng of LPS per ml (Chart
1). Monocytes were plated at a density of 4–6 × 10^4/cm^2 (Chart
3), and release was determined by bioassay. The results from several experiments
at 0.3–100 µg/ml. Control wells included medium alone. After 4 h,
the monocytes were washed and then triggered with LPS (50 ng/ml) in a
final volume of 100 µl for 4 h. The supernatants were frozen at -20°C
until further use.

For the studies of the effects of rIFN-γ on maturation for HMT produc-
tion, monocytes were cultured in RPMI 1640 medium with 5%
human AB serum in quadruplicate microwells (1 × 10^4 cells/well) with or
without interferon (0.01–1.0 unit/ml) and for different time intervals, up to
168 h (Charts 5 to 7). After the appropriate incubation time, the
monocytes were washed and then triggered with LPS (50 ng/ml) in a
final volume of 100 µl for 4 h. The supernatants were frozen at -20°C
until further use.

To determine appropriate signals and their optimal doses for toxin
release, LPS, SMDP, tuftsin, and PIC at dose ranges from 3–1000 ng/ml
were incubated with monocytes seeded at 1 × 10^5/2-cm^2 well for 4
h, and the supernatants were frozen as above. Similarly, PMA was
incubated with monocytes at 0.03–1000 ng/ml and the LPS concentration
was reduced to 0.3–100 µg/ml. In order to induce monocytes to secrete
HMT, the following protocol was used. Washed adherent monocytes
were cultured in RPMI 1640 medium with 5% human AB serum in a
24-well plate at a density of 2 × 10^5 cells/well. The monocytes were
incubated for 4–6 h (Chart 2) at 37°C in medium Dulbecco’s modified
Eagle’s medium: F-12 medium with 0.1% LAH and 90–100 ng of LPS per
ml (Chart 1). Monocytes were plated at a density of 4–6 × 10^4/cm^2
(Chart 3), and release was determined by bioassay. The results from several experiments
at 0.3–100 µg/ml. Control wells included medium alone. After 4 h,
the monocytes were washed and then triggered with LPS (50 ng/ml) in a
final volume of 100 µl for 4 h. The supernatants were frozen at -20°C
until further use.

**Results**

**Tumor Cell Cytotoxicity of in Vitro Activated Human Monoc-
ytes Isolated by Counterflow Elutriation of Plateletapheresis Residues from Normal Donors.** The functional integrity of human
monocytes, isolated by counterflow elutriation from incident-
cally sampled donors during routine plateletapheresis of normal
donors, in tumor cell cytotoxicity has previously been established
(29, 30). The human targets used in these studies included a
kidney carcinoma (CAKI) and breast adenocarcinoma (435-S);
effector:target ratios were usually 10:1, and the duration of the
cytotoxicity assays was 48–72 h.

In initial experiments, we used a modification of the photo-
microscopic plaque assay of Fisch and Gifford (33), using these human
monocytes as effector cells. In brief, actinomycin D-treated L-
929 target cells were established as described in "Materials and
Methods." After 1 h of drug treatment, the cultures were washed,
and a range of monocytes was added (1–30 × 10^3 monocytes/
well). This procedure established a range of effector:target ratios
of approximately 1:30–1:1. To one series of cultures, LPS (100
ng/ml) was added. After 18 h of incubation, cytotoxicity was
evident microscopically, even with the lower effector:target ratios
and was augmented with LPS-treated monocytes (data not shown).
L-929 targets that had not been actinomycin D treated were
affected by LPS at 18 h but required higher effector cell
numbers.

These observations, which indicated the high sensitivity of the
actinomycin D-treated L-929 cell to the direct cytotoxic effects
of activated human monocytes in a rapid assay, provided a
significant rationale for the use of this target cell for the studies
described below.

**Attempts to Induce HMT with Various Triggering Agents in
Vitro.** Monocyte monolayers established at 1 × 10^6 cells/cm^2
well in 24-well plates were treated with various triggering agents
over a broad dose range: LPS, SMDP, tuftsin, and PIC were
used at 3–1000 ng/ml; PMA, at 0.03–1000 ng/ml; and A23187,
at 0.3–100 µM. Control wells included medium alone. After 4 h,
the supernatants were collected, and the HMT present was
determined by bioassay. The results from several experiments
were pooled; the ratio (mean ± SE) of lytic activity in test
supernatants compared to that spontaneously released (medium
alone overlaying monocytes) is expressed as a stimulation
index in Fig. 1.

**LPS, SMDP, and PMA all served as effective triggers for HMT
release.** LPS at levels as low as 3 ng/ml appeared to exceed a
threshold for triggering; this threshold was achieved only with
higher levels (>100 ng/ml) of SMDP. PMA demonstrated a
biphasic effect on HMT production; the optimum dose was in
the medium range (3–100 ng/ml). This preparation, termed β-HMT, was used in the experiments
reported in Chart 8 and Table 2.
HMT PRODUCTION BY HUMAN MONOCYTES

Fig. 1. Dose response of various triggering agents on HMT production by peripheral blood monocytes. LPS, SMDP, PMA, A23187, tuftsin, and PIC were incubated with freshly isolated monocytes, and the resulting lytic activity appearing in the supernatant 4 h later was determined following dialysis. The lytic activity in each sample was normalized to the level released spontaneously, and the ratio, or stimulation index (mean), is shown. Bars, SE.

Fig. 2. Kinetics of HMT release from triggered monocytes. Monocytes were allowed to incubate with no triggering (spontaneous, Sp) (□), or triggered with LPS or SMDP, and supernatants collected at various intervals thereafter, up to 25 h. Lytic activity in each sample is shown.

Samples harvested at various times were frozen and then assayed for HMT activity at one time on L-929 cells to eliminate variability due to fluctuations in the sensitivity of the assay. Typical results from three experiments are shown in Fig. 2.

HMT levels climbed rapidly after monocytes were exposed to the triggers, reaching a peak between 3 and 6 h. The levels declined thereafter, such that by 25 h only ~10% of the original activity remained. Only part of this decline can be attributed to slight instability to storage at 37°C (data not shown); this suggests HMT degradation occurs in the continued presence of the monocyte.

Effect of Monocyte Density on Production of HMT. Monocytes were seeded in 24-well plates at different densities, the highest being ~4 × 10⁵/cm². The wells were washed after the adherence step, and medium or medium with endotoxin was replaced; after 3 h, the supernatants were harvested and assayed for lytic activity on L-929 cells. The units (mean ± SE) compared with density are shown in Fig. 3 for two experiments. HMT production could be detected spontaneously from as few as 1 × 10⁵ cells/cm². Over the range tested, the activity recovered was virtually proportional to monocyte density. The optimal level of production was achieved with the highest level of monocytes seeded, 4 × 10⁵ cells/cm². At this level, LPS-triggered monocytes released approximately 6-fold higher levels of HMT than occurred spontaneously. These results indicated that, on the basis of surface area, optimal conditions for HMT production required essentially confluent monocyte monolayers (4–6 × 10⁵/cm²).

Role of Adherence on Monocyte Production of HMT. In a single experiment, monocytes at a density of 1 × 10⁵/ml were seeded in a 100-mm polystyrene dish or a 15-ml polypropylene tube. After 1 h the polystyrene dish was washed free of growth medium, and the medium was replaced with medium containing...
Similarly, the polypropylene tubes were subjected to centrifugation to pellet the monocytes, and the monocytes were resuspended in medium containing LAH. After triggering with LPS, HMT release was allowed to occur for 4 h, at which time the supernatants were collected from either production source, centrifuged to clarify free of cells, and subjected to bioassay. The bioassay revealed that there was no significant difference between the levels of HMT produced by monocytes adherent to polystyrene and monocytes cultured on polypropylene surfaces (data not shown). Thus the poor adherence which monocytes express on a polystyrene surface does not affect their ability to produce HMT in comparison to monocytes which are strongly adherent to polystyrene.

Requirement for Transcription, Translation, and Secretion following LPS Triggering of Monocytes for HMT Production/Release. Monocytes were seeded in 24-well plates (1 x 10^6 cells/well) and were washed after adherence. Actinomycin D, cycloheximide, or monensin was added in medium over a dose range of 0.03–10 µg/ml. After a 30-min incubation, LPS (100 ng/ml) was added to the cultures. The resulting HMT in supernatants harvested 4 h later and then dialyzed was quantitated by bioassay on L-929 cells. The units of lytic activity in test cultures, compared to a control LPS-triggered, non-drug-treated culture, were expressed as a percentage, and the results from several experiments were plotted in Fig. 4.

At the lowest level tested (30 ng/ml), actinomycin D caused ~50% inhibition of HMT production. As expected from this result, cycloheximide was also found to inhibit production (50% inhibition at ~200 ng/ml). Secretory processes perturbed by monensin also appeared to be necessary for HMT appearance in monocyte supernatants, since this drug caused 50% inhibition at around 300 ng/ml. Interestingly drug doses from 1.5–2.5 log higher than the 50% inhibitory dose still allowed HMT production/release at 10–20% of control levels.

HMT Levels Produced by LPS-triggered Monocytes from Randomly Selected Normal Donors. Monocytes obtained from randomly selected normal donors undergoing routine plateletapheresis over approximately 18 mo were used to produce HMT by the standard protocol described above using LPS as the trigger. The supernatant samples were stored frozen at −20°C (under which conditions HMT is stable) until bioassay could be conducted on all accumulated samples at one time to eliminate assay-to-assay variability. The results are shown in Table 1 as units of lytic activity per ml (mean ± SE).

The supernatants demonstrated HMT levels that varied over two orders of magnitude. No alteration in technique reasonably accounted for this striking variability. One basis for this variability was further explored (see below).

Attempts to Mature Monocytes from Initially Pheresed Normal Donors for HMT Production by Further In Vitro Culture with IFN-γ. In two separate experiments, monolayers of monocytes from normal donors with no known history of pheresis were established in microwells. Some cultures were stimulated immediately with LPS (50 ng/ml) for 4 h after adherence; other cultures were incubated in serum-containing medium with or without IFN-γ (0.01–1.0 unit/ml) for 1, 5, or 9 days. These sets of cultures were then washed, and triggering of HMT release by LPS was attempted. The supernatants obtained from all cultures were frozen at −20°C until bioassay on one set of L-929 targets. The units of lytic activity (mean ± SE) per ml for each sample are shown in Fig. 5.

Monocytes, when initially isolated, released HMT spontaneously, and even higher levels could be achieved in response to LPS triggering. However, monocytes that had been further cultured in vitro for 1, 5, or 9 days alone or with any level of IFN-γ tested no longer released significant levels of HMT either spontaneously or after exposure to LPS. Monocytes cultured under these conditions appeared to be intact morphologically and demonstrated high viability (>95%; data not shown).

Attempts to Mature Monocytes from Recently Pheresed Normal Donors for HMT Production by Further In Vitro Culture with IFN-γ. In two separate experiments, monolayers of monocytes isolated from plateletapheresis residues from normal donors who had undergone plateletapheresis up to 1 wk earlier were established in microwells. Some cultures were stimulated immediately with LPS (50 ng/ml) for 4 h after adherence; other cultures were incubated for up to 168 h in serum-containing
medium with or without rIFN-γ (0.1 or 1.0 unit/ml). IFN treatment was confined to the last 24-h interval immediately preceding washing and LPS triggering in the results shown in Fig. 6. The supernatants were harvested and frozen at −20°C until bioassay as above. The units per ml of lytic activity for each sample are shown.

In contrast to the monocytes obtained from donors who were initially pheresed, monocytes from donors with a recent pheresis history were initially incapable of HMT release, even when triggering was attempted with LPS. However, rIFN-γ (1.0 unit/ml) showed a marked ability to prime the monocytes for LPS triggering when cultured for up to 5 days; this ability was transient and declined thereafter. The levels of HMT achieved with monocytes from this type of donor and with rIFN-γ maturation were consistently higher (2500–5500 units/ml) than those observed when using monocytes from initially pheresed donors (400–1200 units/ml) (Fig. 5; Table 1).

Monocytes cultured for up to 168 h in vitro were incubated for various times with rIFN-γ up to the time of washing and LPS (50 ng/ml) triggering. Supernatants were collected after 4 h of triggering and frozen at −20°C until assay on one set of L-929 cells. The units per ml for monocyte cultures incubated in vitro for a

Fig. 6. Attempts to mature monocytes from recently pheresed normal donors by in vitro culture. Monocytes were cultured essentially as in the experiment shown in Fig. 5, except that total culture time was a maximum of 168 h, and rIFN-γ maturation was confined to the last 24-h interval immediately preceding LPS triggering in the results shown. The units per ml of lytic activity for each sample are shown.

Table 1

<table>
<thead>
<tr>
<th>Donor</th>
<th>Lytic activity (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>137 ± 15</td>
</tr>
<tr>
<td>B</td>
<td>739 ± 166</td>
</tr>
<tr>
<td>C</td>
<td>123 ± 27</td>
</tr>
<tr>
<td>D</td>
<td>97 ± 5.3</td>
</tr>
<tr>
<td>E</td>
<td>362 ± 127</td>
</tr>
<tr>
<td>F</td>
<td>394 ± 138</td>
</tr>
<tr>
<td>G</td>
<td>6277 ± 22</td>
</tr>
<tr>
<td>H</td>
<td>291 ± 108</td>
</tr>
<tr>
<td>I</td>
<td>358 ± 3</td>
</tr>
<tr>
<td>J</td>
<td>769 ± 218</td>
</tr>
<tr>
<td>K</td>
<td>890 ± 274</td>
</tr>
<tr>
<td>L</td>
<td>436 ± 2</td>
</tr>
</tbody>
</table>

*Mean ± SE determined on L-929 targets as described in "Materials and Methods."

Fig. 7. Effect of varied duration of rIFN-γ treatment on monocyte priming for HMT release. Monocytes were cultured in medium for 120 h during this incubation period, rIFN-γ (0.1 and 1.0 units/ml) was added either initially (120-h total treatment) or at 24-h intervals up to 24 h before endotoxin (E) triggering. Some cultures were not treated with rIFN-γ (0 h). HMT lytic activity (units/ml) in each culture was determined (mean). Bars, SE.

Table 2

<table>
<thead>
<tr>
<th>Target</th>
<th>Lytic activity (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-929*</td>
<td>93 ± 21</td>
</tr>
<tr>
<td>L-929D (actinomycin D)*</td>
<td>649 ± 128</td>
</tr>
<tr>
<td>EMT-6*</td>
<td>≤15</td>
</tr>
<tr>
<td>K562*</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>A375*</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>HeLa*</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>WI-38*</td>
<td>≤10</td>
</tr>
<tr>
<td>Mouse lung fibroblasts*</td>
<td>≤15</td>
</tr>
</tbody>
</table>

*Seventy-two-h assay, MTT stain.

The optimal time of rIFN-γ treatment was the 24-h interval just before LPS triggering; with somewhat longer exposure, the monocytes become less responsive to LPS, and by 120 h, the effect of rIFN-γ was completely reduced to a pretreatment state of responsiveness.

Initial Characterization of Anticellular Effects of β-HMT. The principal species of HMT, the β-class, was isolated as described (see "Materials and Methods" and Footnote 5). A single preparation of β-HMT was used in several cytotoxicity assays with a number of target cells (Table 2): L-929; L-929 with actinomycin D treatment; EMT-6; A375; K562; HeLa; WI-38, and normal lung fibroblasts. These targets showed a spectrum of sensitivities to β-HMT; the lung fibroblasts, WI-38, and EMT-6 were refractory, while the human tumor targets were intermediate in sensitivity. The effect on K562 appeared morphologically and by neutral red staining to be primarily cytostatic. The L-929 target in a 72-h assay appeared to be of highest sensitivity; when treated with actinomycin D for measurement of cytolysis in the standard 18-h assay, its sensitivity increased by about one order of magnitude.

The differential sensitivity of the A375 and L-929 targets is
HMT PRODUCTION BY HUMAN MONOCYTES

Fig. 8. Dose response of β-HMT lytic activity on tumor target cells. A single preparation of β-HMT was assayed by serial dilution on cultures of murine L-929 fibroblast-like targets and on A-375 human melanoma targets. The extent of cytotoxicity in 72–96 h was determined using the MTT assay and expressed as the percentage of untreated control cultures. The abscissa represents the activity in units/ml that the β-HMT preparation exerted on the targets. Bars, SE.

Further evident in Fig. 8. A single preparation of β-HMT was titered on these targets, and the percentage of targets remaining after 72–96 h at each dilution was determined by the MTT assay. In the single experiment shown, to achieve 50% reduction compared with controls required 4 times as much β-HMT on A375 compared to L-929 targets, as this preparation scored at 26 units/ml on the A375, and ~93 units/ml on the L-929 targets.

DISCUSSION

Macrophages are a focus of attention as an appropriate arm of host response that might be manipulated for the immunotherapy of neoplastic disease. This attention is warranted because of the ubiquitous distribution of these cells in tissues, and because of their demonstrated ability in vitro to mediate a lytic mechanism preferentially manifested on tumor cells (2, 36–39). More recently their activation by targeted agents in vivo has led to the eradication of metastatic foci in animal model systems (40–42).

Characterization of the tumoricidal mechanism has been conducted by a number of laboratories, and a striking scope of pathways has been attributed to the effector cell. Although there is widespread agreement that, under most circumstances, the lytic mechanism is facilitated by contact between the macrophage and the target cell, in many systems this has been shown not to be obligatory.

The current report describes initial studies of one of the biochemical consequences of triggering highly purified human monocytes to a tumoricidal state: the release of a cell toxin, herein termed HMT. HMT can be triggered with low levels of LPS, SMJP, or PMA (Fig. 1), which initiates a rapid cascade of events (Fig. 2) involving transcription, translation, and secretion, all necessary for the appearance of HMT in the supernatant (Fig. 4). The cytotoxic effects of HMT are manifest on allogeneic and xenogeneic anchorage-dependent and -independent tumor targets (Fig. 8; Table 2).

Our studies clearly showed that, under these conditions of isolation of highly purified, functionally intact monocytes, with strict control for endotoxin contamination, these effector cells can be rapidly promoted to demonstrate tumoricidal capacity, with release of a lytic macromolecule. We would suggest that this characteristic may reflect the responsiveness to triggers for activation of monocytes in vivo. The observation that monocytes from donors having undergone recent pheresis show poor initial LPS responsiveness, which may, however, be markedly enhanced after in vitro cultivation with rIFN-γ, is remarkable. Prior manipulation presumably causes demargination or more rapid emigration from the bone marrow of cells of the monocyte lineage. A direct interpretation is that the immature monocytes lack priming in vivo to become LPS triggered for tumoricidal capacity, which can be overcome by rIFN-γ in vitro. Indeed, the presence of subpopulations of immature monocytes defined by centrifugal elutriation has been demonstrated for oxidative burst expression. However, why a similar maturation was not observed with monocytes from donors who initially underwent pheresis is unclear, as these monocytes should have a significant immature subpopulation that may become primed in vitro. This finding raises the possibility that the mature populations are regulating the response of the immature monocytes to priming signals. Furthermore, aside from the pheresis history of the donor, there are certainly additional factors that influence the initial LPS responsiveness of the monocyte populations; their existence is suggested by the low correlation coefficient (<0.2) that we have observed between the lytic activity released by these LPS-triggered monocytes and either the time since last pheresis or the total number of prior pheresis donations (data not shown); this relationship was established for donors evaluated over 18 m.

In a subsequent report, we present biochemical and functional characterization of HMTs. Two activities in the monocyte supernatants appear to constitute the lytic species: the larger form (M, 100,000–120,000), termed α, is present in lower amounts than the predominant β-form (M, 60,000–70,000). β-HMT does not appear to be a neutral protease or arginase.

The β-HMT appears to be related to, but distinct from, HuTNF by a number of criteria. The first, which was previously mentioned, is the molecular weight as determined by molecular exclusion chromatography on Sephacryl S-200 or on HPLC TSK sieving columns; β-HMT migrates as a protein with a molecular weight of 60,000–70,000. In contrast, the recombinant HuTNF migrates as a dimer of a M, 17,000 peptide, at M, 34,000, in physiological buffers. The second molecular distinction between β-HMT and HuTNF factor is isoelectric point. In our hands, β-HMT has a pl of 5.8 as determined by chromatofocusing, whereas recombinant HuTNF has a pl of about 5.3. Additional evidence for the nonidentity for the two forms is obtained from serological characterization, using a rabbit antiserum raised against recombinant HuTNF. We have determined that, although β-HMT is neutralized by this antiserum, much higher levels of this antiserum are required to achieve full neutralization, as compared to those levels required to block biological activity of the HuTNF factor itself. One interpretation is that there are both common and distinct antigenic determinants between β-HMT and HuTNF. Another interpretation is that there are no identical determinants, but rather only slightly altered determinants for which this antiserum has lower affinity. Finally, using this antiserum against recombinant HuTNF, immunoprecipitation of HuTNF reveals a single M, 17,000 peptide, in agreement with all other evidence. In contrast, a single M, 60,000–70,000 peptide
is precipitated by this antiserum from \( \beta \)-HMT. Further isolation, purification, and characterization of \( \beta \)-HMT are under way in our laboratory.

In the human system, reports from several other laboratories have described macromolecular cytotoxins derived from human monocytes and macrophages. Reed and Lucas (24) have described a M, 45,000 toxin that arose apparently spontaneously after adherence of peripheral blood mononuclear cells; LPS triggering was not obligatory in this system, although given the very low threshold demonstrated in the current report (Fig. 1), it is not clear that a role for trace endotoxin contamination can be excluded. The kinetics of release in their system is very similar to ours, in that peak levels of toxin were achieved in 4–8 h, followed by a decline; however, they demonstrated that reinduction with LPS was possible, whereas, in our hands, it was only minimally effective (data not shown).

Matthews demonstrated that adherent peripheral blood monocytes could release a cytotoxin after 20 h of incubation with levels of endotoxin from 100 ng to 10 \( \mu \)g/ml (25). In a subsequent report (20), MDP at up to 100 \( \mu \)g/ml was found to be an ineffective inducer, whereas, in our hands, the lipophilic derivative SMDP at 30–100 ng/ml is thought to be as competent as LPS (Fig. 1); furthermore, we found PMA only at levels <1 ng/ml to be a potent trigger, and levels up to 100 ng/ml to be ineffective, whereas in the report of Matthews, levels of 10–10\(^2\) ng of this drug per ml were optimal. The appearance of this factor was delayed after triggering compared to that observed by Reed and Lucas (24) and to our findings (Fig. 2). In agreement with results shown in Chart 4, Matthews (25) demonstrated a requirement for transcription and translation after LPS triggering for production of the toxin. This toxin had an apparent molecular weight of 34,000 by molecular sieving on Ultrogel AcA 54.

In contrast to the results of Reed and Lucas (24) and Matthews (25), Hammerstrom (26) found that peripheral blood monocytes from Bacillus Calmette-Guerin-vaccinated donors required up to 3–5 days of total in vitro incubation and 24 h of incubation with crude human lymphokine preparations to release cytostatic factors. Previous studies by Hammerstrom (4–6) had shown that this protocol produced competent effector cells for direct tumor cell cytotoxicity. Two cytostatic factors were characterized and partially purified by Nissen-Meyer and Hammerstrom (43) and found to have molecular weights of 40,000 and 55,000. Whether the Bacillus Calmette-Guerin-vaccinated status of the donors in these studies of Hammerstrom was the cause of the lack of spontaneous reactivity, or whether it could be attributed to control of trace endotoxin contamination is unclear. In other respects, such as the kinetics of LPS triggering (Fig. 2), the lack of a serum requirement during triggering, and the observation that \( \beta \)-HMT appeared to be primarily cytostatic on the K562 target (Table 2), our results were similar.

Cameron reported that cytotoxic activity could be obtained spontaneously from peripheral blood monocytes cultured in vitro for 5 days (27). This factor(s) was shown to enhance host survival in animal tumor systems. Little molecular characterization was conducted, however.

Sone, et al. (28) found that human alveolar macrophages could be triggered with LPS or MDP to release a cytolytic factor. The ability of alveolar macrophages to release the factor persisted for up to 48 h, which is longer than we observed for monocytes in the current study (Fig. 5). As with the studies of Cameron (27), initial biochemical characterization of the factor is still needed.

A number of human cell lines have been exploited as models of monocytes/macrophages (44–48). Some of these have been found to release cytotoxic macromolecules following appropriate triggering. For example, Gifford, et al. (47) demonstrated that KG-1, HL-60, ML-2, and ML-3 lines could be induced with PMA to release cytotoxins; the principal species from the ML-2 line was found to have a molecular weight of \( \sim \)40,000. Armstrong, et al. (48) used the THP-1 line which, following exposure to PMA, elaborated three cytotoxic species: \( \alpha \) (M, 100,000–140,000); \( \beta \) (M, 60,000–70,000); and \( \gamma \) (M, \( \sim \)10,000). Pennica, et al. (49) reported the cloning of cDNA made from mRNA obtained from PMA-treated HL-60 cells. The cDNA sequence corresponded to a \( M, \sim 17,000 \) peptide. Northern hybridization analysis revealed only a single RNA species from either induced HL-60 cells or triggered peripheral blood monocytes. Thus it would appear that either there are additional mRNA species, not detected with this probe, which give rise to translates of the diversity of molecular weight noted for these monocyte toxins from numerous laboratories, or that all of these are derived by aggregation of a single gene product.

The possible role of HMT in human monocyte-mediated tumoricidal reactions remains to be defined. One goal is to develop specific immunoglobulin reagents reactive with both \( \alpha \) and \( \beta \)-HMT, and to use these probes in blocking of tumor cytolyis by the activated monocyte and in immunoelectron microscopy to examine the production, secretion, delivery, and actual site of action in the target, of these toxins.

Finally, because of the tumoricidal effects of HMT itself, its value as a biological response modifying agent should be considered.

REFERENCES


Effector Mechanisms of Human Monocyte-mediated Tumor Cytotoxicity *in Vitro*: Parameters of Induction of Cytotoxins from Peripheral Blood Monocytes Isolated by Counterflow Elutriation

Jim Klostergaard, Jim Turpin and Gabriel Lopez-Berestein


Updated version Access the most recent version of this article at:

http://cancerres.aacrjournals.org/content/46/2/662