Transglutaminase Levels and Immunologic Functions of BCG-Elicited Mouse Peritoneal Macrophages Isolated by Centrifugal Elutriation

Vimlarani Khera and Kapil Mehta
Department of Clinical Immunology and Biological Therapy, The University of Texas, M.D. Anderson Cancer Center, Houston

BCG-elicited mouse peritoneal macrophages were separated into three subpopulations by counterflow centrifugal elutriation. The three subpopulations were characterized on the basis of the level of a protein cross-linking enzyme, tissue transglutaminase. Subpopulation-3 consisted of large cells (>95% esterase positive and >90% viable) and had at least a fivefold higher transglutaminase activity (35 ± 6 nmol/hr/mg) as compared to macrophages in subpopulation-1 (6 ± 2 nmol/hr/mg) and at least a threefold higher enzyme activity as compared to subpopulation-2 (11 ± 2 nmol/hr/mg). Subpopulation-3 also showed sevenfold higher phagocytosis of IgG-coated sheep red blood cells. The three subpopulations showed no difference in their ability to kill Listeria monocytogenes as determined by [3H]-thymidine release. Subpopulations-2 and -3 caused 90% inhibition of murine adenocarcinoma (EMT-6) tumor cell growth in the presence or absence of lipopolysaccharide. Subpopulation-1 had a poor ability to inhibit EMT-6 cell growth (29 ± 12%). However, in the presence of lipopolysaccharide, this activity increased by at least threefold (92 ± 7%). The three subpopulations showed no significant difference in their cytolytic activity against murine mastocytoma (P815) target cells in the presence or absence of lipopolysaccharide. These results suggest that tissue transglutaminase may have no significant role in bactericidal, tumoricidal, or tumoralistic function of macrophages; however, it might have some role in promoting the Fc-receptor-mediated phagocytic function of the macrophages.

Key words: bactericidal, tumoricidal, phagocytosis, cytostasis

INTRODUCTION

Tissue transglutaminase (TGase) is an 80,000 dalton Ca\(^{2+}\)-dependent intracellular thiol enzyme that catalyzes the formation of \(\xi\) (\(\gamma\)-glutamyl)-lysine cross-links between proteins and covalent conjugation of polyamines to proteins [18,19]. It is a monomeric protein and is found in a variety of cell types including macrophages [14,28,32], fibroblasts [31], mast cells [15], and organs such as whole lung [6] and brain [35]. In the macrophages, tissue TGase is thought to play a role in several cellular functions, including activation [23] and receptor-mediated endocytosis [9,12]. Recent evidence indicates that tissue TGase from macrophages may also function in the modulation of extracellular matrix, perhaps via cross-linking of matrix macromolecules [4,13]. Similarly high levels of tissue TGase have been reported in inflammatory macrophages elicited with thioglycolate, protease-peptone, and mineral oil [23,28,33]. Differentiation of human peripheral blood monocytes to mature macrophages and terminal differentiation of human monocytic leukemia (THP-1) cells [27] and human promyelocytic (HL-60) leukemia cells are associated with induction of tissue TGase [8,30]. Human monocytes, treated with agents such as lipopolysaccharide and interferon-\(\gamma\), accumulate much higher levels of this enzyme as compared to nonactivated monocytes [25,29]. However, there is no information available on the exact role the tissue TGase plays in macrophage functions.

Inflammatory reactions involving bacterial infections such as Mycobacteria and Salmonella generally lead to the production of systemically activated macrophages, having increased secretory capacity and ability to destroy tumor cells or engulfed bacteria [5,21]. Because inflammatory macrophages also accumulate large amounts of

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Vimlarani Khera’s present address is Department of Microbiology, The University of Texas Medical Branch, Galveston, TX 77550.

Reprint requests: Kapil Mehta, Department of Clinical Immunology, P.O. Box 41, UT M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

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tissue TGase [23,28,33], we separated the *Bacillus Calmette Guerin* (BCG)-elicited mouse peritoneal macrophages by centrifugal elutriation into three subpopulations and then evaluated the differences in their functions in terms of tissue TGase levels and activity.

The results reported here suggest a heterogeneity in BCG-elicited mouse peritoneal macrophages in terms of tissue TGase levels and that tissue TGase may be involved in augmenting the phagocytosis of IgG-coated particles by macrophages.

**MATERIALS AND METHODS**

**Materials**

Swiss CD-1 male mice, 4–8 wk old, were purchased from The University of Texas Science Park (Bastrop, TX). BCG was from Connaught Laboratories (Willowdale, Canada). RPMI-1640 and fetal calf serum (FCS) were from Hazleton Biologicals (Denver, CO). Human albumin was obtained as a 10% solution from Travenol Laboratories (Glenzdale, CA). Detoxified *Salmonella typhosa* lipopolysaccharide (LPS) was obtained from Ribi, Inc. (Hamilton, MT). *N*-N'-dimethyl casein was from Calbiochem (LaJolla, CA). Monoclonal antibodies to guinea pig liver tissue TGase was kindly provided by Dr. Paul Birkbeckler from the Samuel Robert Nobel Foundation (Ardmore, OK). Rabbit antisera to human factor XIII subunit was purchased from Behring Diagnostics (San Diego, CA). Horseradish peroxidase–mouse IgG conjugate was from BioRad Laboratories (Irvine, CA). Anti-sRBC IgG was from Cordis Laboratory (Miami, FL). [*H]*-putrescine (sp. activity = 29.8 Ci/mmole) was from New England Nuclear (Boston, MA) and methyl [*H]*-thymidine (sp. activity = 19 Ci/mM) was from Amersham (Arlington Heights, IL). All the culture media and sera were screened for endotoxin by using a Limulus amebocyte lysate test (M.D. Bioproducts, Waleksville, MD) and were used only when they contained less than 0.25 ng/ml endotoxin.

**Immunization of Mice**

Lyophilized preparations of BCG were resuspended in 5 ml of physiological saline, and 0.2 ml of this suspension (1 × 10⁹ bacilli) was injected intraperitoneally into each animal. Animals were boosted with 10⁹ bacilli 22 d after the primary injection. The peritoneal cells were harvested 4 d after the secondary injection in RPMI containing 100 U/ml heparin, 10 mM HEPES, 100 μg/ml streptomycin, and 100 U/ml penicillin.

**Subfractionation of BCG-Elicited Macrophages**

Pooled peritoneal exudate cells from ten mice were washed once with RPMI-1640 by centrifugation and resuspended in elutriation media consisting of phosphate-buffered saline (PBS) without Ca⁺² and Mg⁺² and supplemented with 0.5% human serum albumin, 0.22% sodium bicarbonate, and 20 ng/ml gentamicin. Peritoneal exudate cells (250 × 10⁶) in 20 ml of cold elution media were loaded onto a Beckman JE-6B elutriator rotor. The cells were loaded at a medium flow rate of 30 ml/min at a rotor speed of 3,500 ± 5 rpm and at a temperature of 4°C. Elutriation of cells was achieved by step-wise increments in the flow rate, while the rotor speed was maintained at a constant rate as described previously [37,38]. Fractions exiting the chamber were continuously monitored by cell size analysis by using a model ZBI Coulter counter interfaced to a channelizer. Calibration of cells was achieved by using 2 μm-, 5 μm-, and 10 μm-diameter polystyrene microspheres. Cells collected in the first 75 ml of medium at a flow rate of 30 ml/min predominantly contained red blood cells (80 ± 10%). At this flow rate, an additional 200 ml fraction was collected, and the cells in this fraction were referred to as subpopulation-1. The flow rate was then increased to 40 ml/min, and a 150 ml fraction was collected. Cells in this fraction were referred to as subpopulation-2. After collection of cells at a flow rate of 40 ml/min, the centrifuge was switched off, and the flow rate was increased to maximum. The cells collected in this fraction were termed subpopulation-3. Each subpopulation was washed immediately twice in RPMI-1640 medium supplemented with 10% FCS. Cytospin preparations were made from each subpopulation, and these were stained with Wright's stain for differential counts. Also, the cells from each subpopulation were stained with α-naphthyl butyrate for nonspecific esterase, as described by Tucker et al. [36], and with trypan blue to determine the viability.

**Culture of Macrophage Subpopulations**

Unfractionated pooled BCG-elicited peritoneal cells and the three subpopulations separated by centrifugal elutriation were cultured at 37°C in 5% CO₂ for 1 hr at a cell density of 2.5 × 10⁶ cells/well in six-well plates for tissue TGase assay and at a density of 2.5 × 10⁵ cells/well in 96-well microtiter plates to study the bactericidal, cytolytic, and cytostatic functions. Cells at a density of 5 × 10⁵ cells/well were cultured in four Chamber slides in duplicates at 37°C in 5% CO₂ for 1 hr to study the phagocytic function. After 1 hr incubation the nonadherent cells were removed by washing two times with RPMI-1640, and the cells were further processed for individual assays as described.

**Assay of Tissue TGase Activity**

Tissue TGase activity in cell extracts was measured as a Ca⁺²-dependent incorporation of [*H]*-putrescine into dimethyl casein [24]. Macrophage monolayers were
washed three times in 20 mM Tris-buffered saline (pH 7.6) and were scraped into a minimal volume of the same buffer containing 150 mM NaCl, 1 mM EDTA, and 15 mM β-mercaptoethanol. The cells were then lysed by sonication for 30 sec. Tissue TGase activity in cell lysates was determined at 37°C in a final volume of 100 μl reaction mixture containing 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mg/ml N,N'-dimethyl casein, 5 mM CaCl2, 15 mM β-mercaptoethanol, and 0.4 mM [3H]-putrescine. The enzyme activity was expressed as nanomoles of putrescine covalently incorporated into dimethyl casein per hr per mg of cell protein. Protein content of cell extracts was determined by using Biorad's Bradford dye reagent.

**Immunoochemical Identification of Tissue TGase**

The cell lysates were solubilized for detection of tissue TGase in 20 mM Tris-buffered saline (pH 7.6) with 1% sodium dodecyl sulfate (SDS), 0.75 mM β-mercaptoethanol, 2.5% sucrose, and 0.001% bromophenol blue, and they were boiled for 3 min. Solubilized cell extracts were fractionated by electrophoresis on a 10% polyacrylamide slab gel and then immunoblotted onto nitrocellulose paper. The nitrocellulose paper was neutralized with 3% gelatin, and tissue TGase was detected by using mouse monoclonal antibody produced against guinea pig liver tissue TGase [3] in 1% gelatin Tris buffer. The nitrocellulose paper was washed after incubation for 2 hr with TGase-specific monoclonal antibody and treated with horseradish peroxidase–mouse IgG conjugate secondary antibody. The tissue TGase was identified by treating the paper with horseradish peroxidase substrate.

In one series of experiment, shown in Figure 1B, we increased the sensitivity of the assay by using 125I-labeled antitissue TGase antibody. Cell extracts were fractionated by electrophoresis and electroblotted to nitrocellulose in a usual manner. The filter was neutralized and incubated with 125I-labeled antibody to tissue TGase for 2 hr at 29°C. The filter was then washed extensively, dried, and subjected to autoradiography as described previously [28]; 125I-labeled antibody against factor XIII was also used to detect plasma TGase for Western blot analysis of the unfractionated cells and the subpopulations.

**Bactericidal Activity**

Bactericidal activity of macrophages in unfractionated and separated subpopulations was determined according to a previously described method [2]. In brief, heat-killed [3H]-thymidine-labeled *Listeria monocytogenes* were added to macrophage monolayers to obtain a macrophage:bacilli ratio of 1:20. The plates were centrifuged at 400g for 5 min to overlay bacilli on macrophages, and they were then incubated for 45 min at 37°C in 5% CO2. The cultures were then washed two times with RPMI containing 10 mM EDTA, followed by four washes with plain medium to remove unphagocytosed bacilli, and they were resuspended in fresh medium. After 1-hr incubation at 37°C, the spent medium was examined for the released radioactivity. Residual counts were also determined in infected macrophage monolayers by solubilizing the cells in 1% SDS. Percent bactericidal activity was calculated as follow:

\[
\text{% bactericidal activity} = \frac{\text{cpm in supernatant}}{\text{cpm in supernatant} + \text{residual cpm in macrophages}} \times 100
\]

**Phagocytosis of IgG-Coated Sheep RBCs**

Phagocytosis of macrophages in unfractionated and three subpopulations was determined according to a previously described method [34]. Briefly, IgG-coated sheep RBCs (sRBCs) were added to macrophage monolayers to obtain a macrophage:sRBC ratio of 1:20. The chamber slides containing macrophages and IgG-coated sRBCs were further incubated for 1 hr at 37°C. Unphagocytosed sRBCs were removed by washing the slides four times with RPMI, and sRBCs attached to the surface of macrophage were lysed by brief exposure to hypotonic shock with distilled water. The slides were stained with Wright's stain and examined under a light microscope. Macrophages with three or more sRBCs were considered positive for phagocytosis. Phagocytic index was calculated as the product of percentage of cells ingesting more than three sRBCs and the average number of particles ingested per cell. A minimum of 100 cells were counted in unfractionated and separated subpopulations to calculate phagocytic index.

**Cytotoxicity Against P815 Cells**

Macrophage monolayers of BCG-elicted macrophages and the three subpopulations thereof were incubated with and without LPS (50 ng/well) for 5 hr. Mouse mastocytoma cells (P815) were prelabeled with [3H]-thymidine and were overlaid on macrophage monolayers at an effector to target cell ratio of 2:1. After 16-hr co-culture, the plates were spun at 400g for 5 min, and a 0.1 ml aliquot of the supernatant was aspirated and placed in a scintillation cocktail. The radioactivity (A) released by the target cells was determined in supernatants from cultures incubated in the presence of macrophage monolayers. The total radioactivity (B) of labeled target cells was determined by lysing the cells, and the spontaneous release of radioactivity (C) was determined on supernatants from target cells incubated with medium
RESULTS

Transglutaminase Activity in Thioglycollate and BCG-Elicited Macrophages

A significantly high level of tissue TGase activity was observed in vivo BCG-elicited (activated) mouse macrophages as compared to unstimulated (resident) peritoneal macrophages. Similarly, inflammatory macrophages elicited by sterile thioglycollate injection had much higher levels of tissue TGase enzyme activity compared to unstimulated resident macrophages (Fig. 1A). Thioglycollate-elicited macrophages had at least fourfold higher activity (80 nmol/hr/mg cell protein), and BCG-induced macrophages had at least 13-fold higher activity (270 nmol/hr/mg cell protein) as compared to resident peritoneal macrophages (20 nmol/hr/mg cell protein). As shown in Figure 1B, the increase in enzyme activity was proportional to an increase in enzyme amounts as determined by Western blot analysis with an iodinated-antibody specific for tissue TGase. The preparation and characterization of this antibody have been described previously [3]. A single immunoreactive band was detected at 80,000 daltons in resident, thioglycollate, and BCG-induced peritoneal macrophage extracts. Quantitation of band intensity with a densitometer revealed that thioglycollate-elicited macrophages contained four times more immunoreactive bands than resident cells and BCG-elicited macrophages contained about 12 times more immunoreactive tissue TGase than those of resident cells.

The large increase in tissue TGase in activated and inflammatory peritoneal macrophages could be due to the induction of the enzyme in resident peritoneal macrophages or due to infiltration of young macrophages from the circulating pool with an inordinately high amount of tissue TGase at the focus of inflammation. BCG-elicited mouse peritoneal macrophages were fractionated into three subpopulations by centrifugal elutriation on the basis of size and density. The three subpopulations were then identified on the basis of tissue TGase activity/levels and studied individually for immunological functions. Experiments were carried out to study the role of tissue TGase in bactericidal, cytostatic, tumoricidal, and phagocytic functions.

Characterization of Three Subpopulations

Total cell recovery was 90 ± 6% (mean ± SD) of the number of unfractionated cells loaded onto the elutriation chamber. Only 10% of the mononuclear cells eluted at a flow rate of 30 ml/min constituted subpopulation-1 (Table 1). The cells in subpopulation-1 were 95 ± 7% viable, and 8 ± 2% of the total cells were recovered in this subpopulation. Cells in this subpopulation were shown by Wright’s stain to be predominantly lympho-

Cytostatic Activity Against EMT-6 Cells

Quadruplicate samples of macrophage monolayers were incubated in the presence and absence of LPS (50 ng/well) in 96-well microtiter plates. After 5-hr incubation with and without LPS, the macrophage monolayers were washed once with complete medium. The monolayers were overlaid with mouse adenocarcinoma cells (EMT-6) suspended in 0.2 ml of medium with an effector to target ratio of 10:1. The cultures were incubated for a further 16-hr period and pulsed with [3H]-thymidine (0.1 μCi/well). The cells were harvested by trypsinization of adherent cells and were processed for liquid scintillation counting by an automatic cell harvester (Cambridge Tech., Inc., Cambridge, MA). The cytostatic activity was determined as the percent inhibition of [3H]-thymidine incorporation in EMT-6 cells caused by macrophages and was calculated by comparison with [3H]-thymidine incorporation in EMT-6 cells in the absence of macrophages.

![Graph of Tissue TGase activity](image)
cytes (48 ± 6%), and 50% of the total cell population stained positive for nonspecific esterase (52 ± 8%). As shown in Table 1, about 20% of the cells eluted at a flow rate of 40 ml/min of the total unfractionated peritoneal exudate cells and constituted subpopulation-2. The majority (95%) of cells eluted in this subpopulation appeared to be macrophages by light microscopy and on staining with α-naphthyl butyrate (Table 2). Very few cells were lymphocytes (10 ± 8%) and granulocytes (8 ± 2%) and had high viability (97 ± 8% Trypan blue negative). The rotor/off fraction, i.e., subpopulation-3, constituted about 70% of the eluted cells and was composed mostly of large granular macrophages (95 ± 2%); the rest (5%) of the cells were lymphocytes or granulocytes. This subpopulation had highly viable cells (99 ± 9%) and was enriched in macrophages. The size of cells collected in subpopulation-1 was about 7 μm, that of subpopulation-2 was between 7 μm and 10 μm, and cells in subpopulation-3 were 8-12 μm, as characterized by using standard size microspheres (Fig. 2). This suggests that these subpopulations consisted of cells with different sizes, and they could be separated consistently in ten experiments from pooled peritoneal cells of ten mice in each experiment.

**TGase Activity in Three Subpopulations of BCG-Elicited Macrophages**

Tissue TGase activity in cell extracts of unfractionated and three subpopulations of BCG-elicited macrophages was measured by studying the incorporation of [3H]-putrescine into dimethyl casein as shown in Figure 3A. Macrophages in subpopulation-1 showed low TGase activity (6 ± 1 nmol/hr/mg) as compared to unfractionated macrophages (20 ± 7 nmol/hr/mg). Subpopulation-2 had higher tissue TGase activity (12 ± 5 nmol/hr/mg) as compared to subpopulation-1 but was still less than the unfractionated cells (20 ± 7 nmol/hr/mg). Macrophages from subpopulation-3 were large, granular, and had at least fivefold higher tissue TGase activity (32 ± 2 nmol/hr/mg) as compared to small macrophages in subpopulation-1. Tissue TGase activity in subpopulation-3 was relatively higher as compared to macrophages in subpopulations-1 and -2 and to macrophages obtained after primary immunization without the secondary booster dose (15 ± 2 nmol/hr/mg). The increase in tissue TGase activity after booster was associated with an increase in the number of esterase-positive cells from 50% (prebooster) to 95% following the booster (data not shown). Cell extracts from unfractionated macrophages and the three subpopulations were also fractionated on 10% SDS polyacrylamide gel electrophoresis, and TGase band was identified by Western blot technique by using a tissue TGase-specific monoclonal antibody. As shown in Figure 3B, the enzyme activity was proportional to the enzyme levels in three subpopulations. Thus, increased enzyme activity in subpopulation-3 was due to increased accumulation of enzyme peptide rather than activation of preexisting enzyme. The immunoreac-

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**TABLE 1. Separation of BCG-Elicited Mouse Macrophages into Three Subpopulations by Centrifugal Elutriation**

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Flow rate (ml/min)</th>
<th>Cells recovered (x 10^6)</th>
<th>Recovery (%)</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>10-20</td>
<td>8 ± 2</td>
<td>95 ± 7</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>30-40</td>
<td>16 ± 5</td>
<td>97 ± 8</td>
</tr>
<tr>
<td>3</td>
<td>Max.*</td>
<td>100-140</td>
<td>66 ± 10</td>
<td>99 ± 9</td>
</tr>
</tbody>
</table>

*The centrifuge was switched off, and the buffer flow through the chamber was increased to maximum.

---

**TABLE 2. Differential Counts in Three Subpopulations of BCG-Elicited Mouse Peritoneal Macrophages**

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Wright’s stain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>10 ± 6</td>
</tr>
<tr>
<td>1</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>2</td>
<td>10 ± 8</td>
</tr>
<tr>
<td>3</td>
<td>6 ± 3</td>
</tr>
</tbody>
</table>

---

**Fig. 2. Size distribution of subpopulations obtained from BCG-elicited mouse peritoneal exudate cells, separated by counterflow centrifugal elutriation.** Representative size distribution of unfractionated (---), subpopulation-1 (Δ), subpopulation-2 (●), and subpopulation-3 (○) was done by using a Coulter ZBI counter (amp 2, current 1) coupled to a C-1000 channelizer with an X-Y recorder (Coulter Electronics, Hialeah, FL). Abscissa represents relative cell number, ordinate the size distribution. Arrows indicate the size distribution of 2, 5, and 10 μm standard reference beads.
Phagocytic Function

Adherent macrophage monolayers of BCG-elicited peritoneal cells or the three subpopulations were tested for their ability to phagocytose the IgG-coated sRBCs and the bacterial cells as shown in Figure 4. Unfractionated macrophages had a phagocytic index of 500, and each macrophage on an average had phagocyctosed more than three sRBCs. Subpopulations-1 and -2 had relatively low phagocytic index (<300), and the cells had very few phagocyctosed sRBCs (n = <3). The macrophages in subpopulation-3 were most active in phagocytosing the IgG-coated sRBCs and showed at least fourfold higher phagocytic index (>1, 400) as compared to cells in subpopulations-1 and -2 (<300). Also, the number of sRBCs phagocyctosed per macrophage was higher (six to eight sRBCs) in the cells of subpopulation-3.

We also tested the ability of macrophages in the three subpopulations to lyse the prelabeled L. monocytogenes. The results are shown in Figure 4. There was no significant difference in the degradation of the bacilli by macrophages in the three subpopulations. Unfractionated pooled cells showed about 50% killing, whereas the cells in subpopulation-1 induced 58% killing; subpopulation-2
showed 62% killing, and subpopulation-3 showed 55% killing. These results suggested that there is no correlation between the amount of enzyme level present in three subpopulations and the killing of bacilli by the macrophages measured as release of radioactivity.

**Cytolytic and Cytostatic Functions**

The three subpopulations were also studied for the spontaneous and LPS-induced cytolytic and cytostatic functions of macrophages. The cytolytic activity of macrophages was studied against murine mastocytoma (P815) target cells. The results are shown in Figure 5. Unfractionated macrophages from BCG-induced mice did not induce a marked release of radioactivity from prelabeled target cells as compared to the macrophages in subpopulation-1, subpopulation-2, or subpopulation-3. On an average, the percent cytolysis induced by unfractionated macrophages or macrophages in three subpopulations was around 20%. Addition of LPS (50 ng/well) to the macrophage cultures from unfractionated cells or the three subpopulations did not further augment their ability to lyse the P815 target cells, suggesting that the enzyme tissue TGase is not required for the tumor-cell-killing function by macrophages. Thus, macrophages in subpopulation-1 with TGase levels at least sixfold less were as active in lysing P815 cells as the macrophages in subpopulation-3.

The cytostatic function of macrophages in unfractionated and three subpopulations was determined by using mouse adenocarcinoma (EMT-6) as target cells by measuring the inhibition of DNA synthesis. As shown in Figure 5, the macrophages in subpopulation-1 were poor in inducing the growth inhibition of EMT-6 cells. Macrophages in subpopulation-2 and subpopulation-3 exerted a much stronger inhibitory effect against the EMT-6 cells. However, treatment of macrophages in subpopulation-1 with LPS caused a significant increase in the cytostatic activity against the tumor cells. No further increase in cytostatic activity of macrophages in subpopulation-2 and subpopulation-3 was observed following their treatment with LPS. These results may suggest that young macrophages which are low in TGase activity require a trigger signal in order to become activated, whereas large macrophages, which have much higher tissue TGase levels, do not need such a signal to express antitumor activity.

**DISCUSSION**

Recently we reported that serum retinoids exert a strong inhibitory effect on macrophage-mediated tumor-cell-killing functions [26]. This inhibitory effect of retinoids was associated with the induction of large amounts of tissue TGase in macrophages. Also, presence of dansyl cadaverine, a competitive inhibitor of TGase, during the activation period abrogated the retinoid-induced inhibition of LPS, and interferon-γ triggered activation of murine macrophages. It has also been reported that in vivo-activated macrophages accumulate large amounts of tissue TGase and are highly active in tumor-cell-killing functions [9,23,28]. We, therefore, initiated these studies to investigate whether the tumor cell killing and other functions of macrophages could be attributed to different subpopulations containing different levels of tissue TGase activity.

We decided to work with BCG-elicited macrophages as they have at least 200–300-fold higher TGase activity...
than the resident unstimulated macrophages and are also highly activated for tumor-cell-killing functions. Therefore, BCG-elicited macrophages were separated into three fractions that had very low to high activity/level of tissue TGase by centrifugal elutriation. The three subpopulations were consistently and reproducibly separated on the basis of TGase activity, esterase staining, and morphological criteria. Subpopulation-3 yielded maximum cell recovery and had large and dense macrophages with about sevenfold higher TGase activity and levels as compared to macrophages in subpopulation-1. The cells in subpopulation-1 were relatively smaller in size and showed less spreading on the plastic surfaces. Subpopulation-2 represented an intermediate stage with about twofold higher TGase activity and levels as compared to subpopulation-1 but had threefold less enzyme activity as compared to macrophages in subpopulation-3. The enzyme activity in each subpopulation was proportional to the accumulation of enzyme peptide rather than activation of preexisting enzyme as determined by immunoblot, using a tissue TGase-specific antibody. The presence of a single immunoreactive band at the 80 kD position also suggested that the enzyme activity was specifically due to tissue TGase rather than factor XIII. Since Adany et al. [1] recently reported the presence of factor XIII in human peritoneal macrophages, we further evaluated the participation and presence of factor XIII in the TGase activity of three macrophage subpopulations by polyacrylamide gel electrophoresis and Western blot analysis of lysates of these cells by using rabbit antibody against subunit-a of factor XIII. In contrast to differences in tissue TGase levels seen in three macrophage subpopulations (Fig. 3), there was negligible difference in factor XIII levels detected with the antiafactor XIII antibodies in the unfractionated cells and the three subpopulations. These data suggested that the difference in enzyme activity in three subpopulations was due to differences in the tissue TGase levels only, because antiafactor XIII antibody failed to detect any cross-reactive bands.

Macrophages in subpopulation-3, which have the highest tissue TGase levels of all the three subpopulations, were also most active in phagocytosing the IgG-coated particles (sRBCs). Indeed, the significance of TGase in receptor-mediated phagocytosis and endocytosis had been suggested in the past [7,12,13]. Thus, agents that inhibit TGase activity in rat peritoneal macrophages block Fc-receptor-mediated phagocytosis [14]. Conversely, the endocytoses of immune-complexes via Fc-receptor produces an activation of TGase and stimulation of amine incorporation into membrane proteins of intact cells [11]. Similar results were later reported with guinea pig [23] and murine [33] macrophages. This might have some biological significance, as monocytes with high TGase activity have been isolated from patients with systemic lupus erythematosus and Hodgkin's disease [16,22].

Unlike the phagocytic function, the bactericidal activity did not vary with varying levels of tissue TGase in macrophages from three subpopulations and from the unfractionated cells. Subpopulation-3, which had the highest tissue TGase level, was not different from either subpopulation-1 or subpopulation-2 as far as the uptake and killing of bacterial cells was concerned. Uptake of bacilli by macrophages is also thought to be a receptor-mediated phenomenon, as alteration in the adhesion of bacilli to surface results in poor uptake of bacteria. The results obtained, therefore, suggest that the enzyme TGase may not have a role in all kinds of receptor-mediated phagocytosis of particles, because no significant difference in the ability to lyse the phagocytosed bacilli by macrophages in three subpopulations was observed, despite the fact that they had significantly different levels of the enzyme.

We were interested also in studying a correlation between high TGase activity and tumoridal and cytostatic activities of macrophages. Mouse mastocytoma (P815) cells were used for studying the cytolytic function and a mouse adenocarcinoma (EMT-6) cell line was used to study the cytostatic function. The three macrophage subpopulations did not show any noticeable difference in the cytolytic function against P815 target cells. Similarly, cytostatic activity of macrophages in three subpopulations was not much different, as determined by their ability to inhibit DNA synthesis of EMT-6 tumor cells following their coculture. Also Chapes and Haskill [5] found varying cytolytic and cytostatic activity in the activated macrophage subpopulations separated by velocity gradient fractionations with HS-3 target cells. The reason for the discrepancy between our present and earlier reported results [26] that inhibition of macrophage cytostatic activation was associated with induction of TGase and also from the observation of Chapes and Haskill [5] that cytolytic activity was associated with small macrophages could be due to the nature of the target cells used. The mechanism of macrophage–tumor cell killing vary with heterogenous populations of macrophages and with varying other enzyme contents besides tissue TGase [17]. Both P815 and EMT-6 cells are resistant to tumor necrosis factor-mediated killing, the major cytolytic/cytostatic mediator released by activated macrophages. Thus, anti-TNF antibodies could not neutralize the macrophage-induced killing of P815 cells [10]. Our recent studies suggest that TGase is responsible for inhibition of TNF release from activated macrophages (K. Mehta, in preparation). Similarly, the results reported by Hopper and Cahill [20] suggest that small macrophages, comparable to subpopulation-1 with low TGase levels in the present study, are more active in
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lysing the fibrosarcoma (L-4) target cells, a cell line susceptible to TNF. Thus, based on our present studies, we strongly feel that induction of TGase in macrophages is associated with inhibition of TNF-mediated functions and that this enzyme might be responsible for augmenting at least the Fc-receptor-mediated functions of macrophages. Further studies on the mechanisms by which tissue TGase modulates the TNF release in macrophages and also studies on potential substrates of the enzyme in macrophages are in progress.

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Transglutaminase Levels and MΦ Functions