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Rapid Communication

Glomerular Basement Membrane-Containing Immune Complexes Stimulate Tumor Necrosis Factor and Interleukin-1 Production by Human Monocytes

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The ability of human peripheral blood monocytes to produce tumor necrosis factor (TNF) and interleukin-1 (IL-1) in an* in vitro *model of immune complex-mediated glomerulonephritis was investigated. When isolated monocytes were incubated with human glomerular basement membrane (GBM) containing anti-GBM immune complexes, both TNF and IL-1 were produced and secreted into the medium. The time course of secretion differed, with IL-1 production being maximal after approximately 8 hours, whereas TNF levels continued to rise for 30 hours. The activities of the monocyte-derived TNF and IL-1 were inhibitable by specific antibodies. No effect was seen when monocytes were incubated separately with either GBM alone or anti-GBM IgG. The levels of TNF and IL-1 released were comparable with those induced by high concentrations of LPS, indicating that production was close to the maximal levels reported for these cells. High levels of TNF and IL-1 also were produced in response to soluble immune complexes. The results show that monocytes can produce significant levels of TNF and IL-1 in response to both surface-bound and soluble immune complexes and provide support for the participation of these monokines in glomerulonephritis. (Am J Pathol 1989, 134: 1–6)

Glomerulonephritis in humans is thought to be initiated by the deposition of antibodies in or near the glomerular basement membrane (GBM). This disease state can be mimicked in animal models by the injection of an antiserum that reacts with the host's GBM.[1–3] The resulting inflammation typically progresses through both heterologous and autologous phases. In the heterologous phase there is deposition of antibodies on the GBM, complement activation, infiltration of polymorphonuclear leukocytes, endothelial detachment and proteinuria.[3] The subsequent autologous phase, which is associated with the host's immune response to the injected anti-GBM antibody, is characterized by the presence of monocytes and lymphocytes, proliferation of the glomerular cells, and proteinuria.[4,5] The progression of glomerulonephritis through the autologous phase may result in irreversible injury and end-stage fibrosis of the glomeruli due to the deposition of collagen.[6,7] Whereas the mechanisms of injury in the heterologous phase have been studied widely,[3,8–10] the events that regulate the autologous phase and crescent formation are less well understood.

Recent studies have demonstrated that immune effector cells can contribute to the pathology of glomerulonephritis.[4,11–13] Specifically, the recruitment of blood mononuclear phagocytic cells to the glomerulus could result in the local production of interleukin-1 (IL-1) and tumor necrosis factor (TNF), which possess pluripotent activities on the host's immune and physiologic systems,[16–19] and may contribute to the alterations leading to crescentic nephritis. In support of this hypothesis, we have measured IL-1 and TNF-α biologic activity in glomerular culture supernatants from rabbits with crescentic nephritis.[19]

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This report investigates the ability of human GBM/anti-GBM immune complexes to serve as a stimulus for the production of both TNF and IL-1 by normal peripheral blood monocytes. These studies are unique in that the stimulus (GBM/anti-GBM immune complexes) is an etiologic agent of human immune glomerulonephritis and has been shown in experimental models to produce pathologic changes that evolve into crescentic nephritis and glomerular and interstitial fibrosis.[1,6,13,14,15]

Materials and Methods

Cell Isolation

Monocytes were isolated from the peripheral blood of healthy consenting donors under sterile conditions. Mononuclear cells from 100 ml whole blood were isolated from Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden; $P = 1.077$),[20] and washed once with a 0.2 M sodium phosphate buffer, pH 7.6, containing 138 mM NaCl, and 2.7 mM KCl (PBS). The cells were resuspended in 3.5 ml PBS and mixed with 6.7 ml Sepracell-MN (Sepratech Corporation, Oklahoma City, OK) containing 15 g silica/100 ml HEPEs-buffered saline. Separation of monocytes and lymphocytes was achieved by centrifugation at 2000*g* for 20 minutes at room temperature in a swing-out rotor. The top band of cells was removed and washed three times in ice-cold PBS containing 0.1% gelatin, to remove platelets.[21] After three further washes in PBS, the cells were finally suspended in RPMI medium, supplemented with 10 μl/ml penicillin/streptomycin. Cell populations contained 88 ± 6% monocytes and 12 ± 7% lymphocytes as assessed after staining for nonspecific esterase and with Wright's Giemsa stain. Viability, based on trypan blue exclusion, was always ≧99%.

Preparation of GBM/Anti-GBM Immune Complexes

GBM was prepared from glomeruli isolated from normal postmortem human kidneys, as described previously.[22] Cellular components were removed by lysis with water and solubilization in Triton X-100, deoxyribonuclease, and deoxycholate.[23] After washing, the GBM was lyophilized and stored at −20 C. The resulting basement membrane preparation showed large amorphous sheets of material by electron microscopy, with no contamination by cellular components. Lyophilized GBM was weighed and suspended in PBS at a known concentration.

Guinea pig anti-GBM, rabbit anti-guinea pig immunoglobulin IgG, and rabbit anti-human IgG were prepared by immunizing animals with pure antigen. The IgG fractions were extracted from one serum by DEAE ion exchange chromatography. The IgG fraction from Goodpasture's serum, containing human anti-GBM IgG, was purified by ion exchange on QAE Sephadex.[24]

Either 1 ml of IgG from Goodpasture's serum (10 mg/ml) or guinea pig anti-GBM (10 mg/ml) was added to 10 mg GBM and the suspension incubated overnight at 4 C. The GBM was then washed by centrifugation and either rabbit anti-human IgG or rabbit anti-guinea pig IgG (1 ml of 10 mg/ml) was added. After incubation at room temperature for 1 hour, the GBM/anti-GBM immune complexes were washed three times with PBS and stored at 4 C.

Soluble Immune Complexes

Guinea pig anti-GBM IgG (10 mg/ml) was added to an equal volume of rabbit anti-guinea pig IgG (10 mg/ml) and left at room temperature for 1 hr. This corresponds to an antigen-antibody ratio of 20:1 as assessed by quantitative precipitation. Prior to use, any precipitating immune complexes were removed by centrifugation at 12,000*g* for 10 min.

Incubation of Cells with GBM

Purified monocytes were suspended at 5×10^6/ml in RPMI (Gibco Labs., Chagrin Calls, OH) and added to 2 mg/ml GBM/anti-GBM immune complexes. To establish kinetic curves, cells were incubated for various time periods, pelleted by centrifugation at 400*g* for 10 minutes, and the supernatants removed and stored at −20 C before assay for TNF and IL-1. Control tubes contained either cells alone or monocytes with GBM in the absence of immune complexes.

TNF Assay

TNF activity was determined by monitoring lysis of either LM or L929 fibroblasts.[25] Fibroblasts (5×10^4 cells/0.1 ml) were cultured with serial dilutions of test samples in the presence of Actinomycin D (1 μg/well) in flat-bottom 96-well microtiter plates at 37 C in 5% CO_2, 95% air, in a humidified incubator. After 18 hours the plates were washed with PBS and the cells stained with crystal violet. The extent of cell lysis was then determined with a micro-ELISA reader. To quantify TNF activity, a comparison of cell lysis induced by test samples was made to recombinant human TNF-α (Cetus Corp., Emeryville, CA). One unit of activity corresponds to half-maximum cytolytic activity in the LM-cell assay. To determine whether lysis was due to TNF, several samples were treated with rabbit anti-

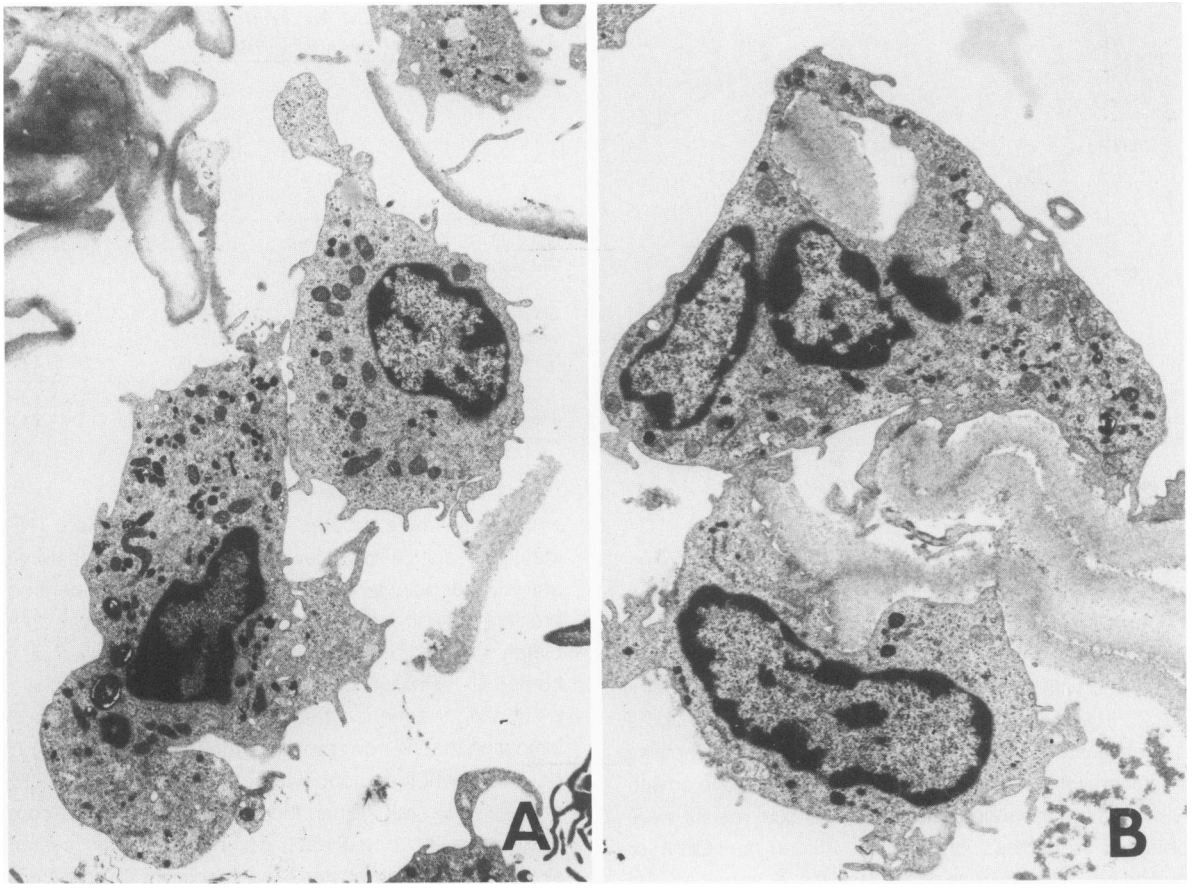

Figure 1. *Transmission electron micrograph of peripheral blood monocytes incubated with GBM alone (**A**) or containing anti-GBM immune complexes (**B**). In the presence of immune complexes, the cells adhere to and spread across the surface of the GBM. Original magnification ×9300.*

human TNF-α neutralizing antibody (Cetus, Corp.) before addition of the fibroblast targets.

IL-1 Assay

IL-1 levels were assayed by the enhancement of thymocyte proliferation to phytohemagglutinin (PHA, Burroughs Wellcome, UK) modified from the procedure of Mizel et al.[26] One unit of activity corresponds to the half-maximum thymocyte proliferation. Briefly, 5×10^5 murine thymocytes in 0.1 ml complete medium containing 2.5 μg/ml PHA was added to a 96-well culture plate. Equal volumes of $\log_2$ dilutions of supernatant from 5–6.5 $\times$ 10^5 monocytes were added to the wells in triplicate. After 55 hours incubation at 37 C in 5% CO_2, 95% air, the thymocytes were pulsed with 0.5 μCi ^{3}H-thymidine (ICN, Irvine, CA) and collected 6 hours later on glass filter strips using an automated cell harvester. ^{3}H-thymidine incorporation was determined by counting in a liquid scintillant. Results are presented as the increase in counts per minute above control monocytes at zero time, or as counts per minute of ^{3}H-thymidine incorporated by thymocytes incubated with a 1:32 dilution of monocyte supernatant. In addition, a known IL-1 standard (Cistron, Pinebrook, NJ) and a standard prepared from LPS-treated macrophages were assayed concomitantly. To determine whether activity was due to IL-1, selected samples were treated with a specific rabbit anti-human IL-1 antibody (Genzyme, Boston, MA) before the assay.

Results

In the absence of immune complexes, monocytes did not interact with GBM (Figure 1A). When incubated with GBM/anti-GBM immune complexes, however, monocytes adhered to the GBM and spread across its surface (Figure 1B). This was evident after 10-minute incubation and the cells remained adherent for up to 24 hours. Coincident with adherence to GBM, monocytes undergo a respiratory burst and release lysosomal enzymes that continues during the initial 30-minute incubation.[27]

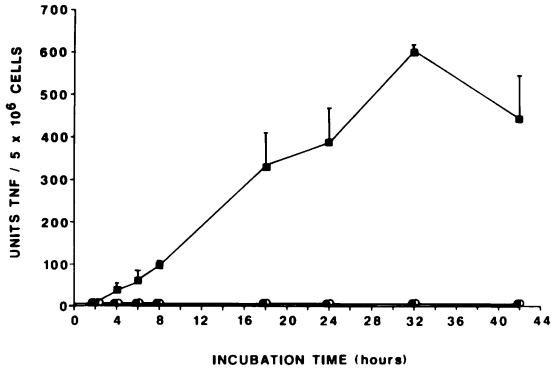

Figure 2. *TNF production by human monocytes in response to GBM/anti-GBM immune complexes. Monocytes were incubated as detailed in the Methods section, either alone (○), with GBM/anti-GBM immune complexes (■), or with GBM alone (●). The means ± SEM of 7 experiments are shown.*

The production of TNF and IL-1 monocytes in response to GBM/anti-GBM immune complexes is shown in Figures 2 and 3. After an initial lag of approximately 2 hours, TNF was observed in the medium of monocytes incubated with GBM/anti-GBM immune complexes (Figure 2). Levels increased for up to 32 hours, while some decrease was seen at longer incubation times. No activity was seen when monocytes were incubated alone or with GBM without immune complexes. Identical results were obtained regardless of whether human anti-GBM or guinea pig antibody was used. That this activity was due to TNF was shown by inactivation with anti-TNF antibody. In the presence of anti-TNF less than 5 units TNF could be measured in a sample containing 360 units TNF.

GBM/anti-GBM immune complexes induced IL-1 production from monocytes, with activity becoming measurable after 2 hours (Figure 3). The time course of IL-1 release differed from that of TNF, however, and levels did not change greatly after approximately 6–8 hours. No activity was seen in the absence of immune complexes or

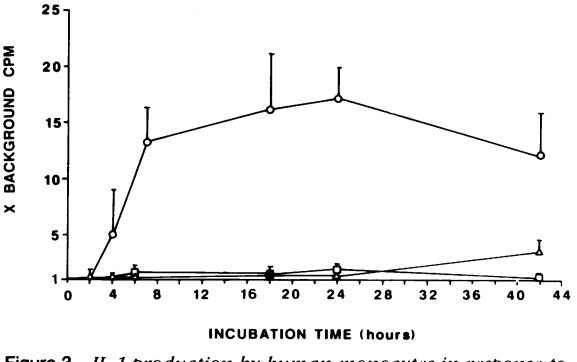

Figure 3. *IL-1 production by human monocytes in response to GBM/anti-GBM immune complexes. Cells were incubated alone (□), with GBM/anti-GBM immune complexes (○) or with GBM alone (△). The means ± SEM of 7 experiments are shown. Data is expressed as the magnitude increase above the background CPM for each sample tested.*

Table 1. *TNF and IL-1 Production by Peripheral Blood Monocytes Under Varying Conditions*

Condition of stimulation	TNF activity (units/5 × 10^6 cells)	IL-1 activity [^{3}H-thymidine uptake in thymocyte coproliferation assay (cpm)]
GBM/anti-GBM immune complexes	252 ± 126	2462 ± 551
GBM/anti-GBM immune complexes + cycloheximide	9 ± 6	60 ± 8
GBM	1.5 ± 1.0	142 ± 35
Guinea pig anti-human GBM	0.3 ± 0.1	211 ± 14
Rabbit anti-guinea pig IgG	0.4 ± 0.1	207 ± 17

Results show mean ± SD of 3 experiments. Monocytes (5 × 10^6/ml) were incubated under the conditions shown and the levels of TNF or IL-1 were measured in the supernatants after 18-hour incubation.

when cells were incubated alone (Figure 3). As with TNF, identical results were obtained either with Goodpasture's anti-GBM or guinea pig antibody. The IL-1 dependent coproliferation activity present in the supernatants was completely inhibited by pretreatment of the samples with anti-human IL-1. Production of both monokines was blocked by treating the monocytes with cycloheximide (Table 1), indicating that *de novo* protein synthesis was required. To determine whether monocyte production was due to LPS contamination rather than the presence of immune complexes, we incubated each component of the system separately with monocytes. None caused TNF or IL-1 production (Table 1). LPS levels in the low pg/ml range were measured in each of the components and in the final GBM/anti-GBM immune complexes by the limulus-lysate assay (Sigma Chemical Co., St. Louis, MO).

The amount of TNF and IL-1 produced by monocytes in the presence of GBM/anti-GBM immune complexes was compared with that induced by LPS and by soluble immune complexes. As shown in Table 2, comparable levels of both IL-1 and TNF were produced by monocytes whether LPS or GBM/anti-GBM immune complexes were the stimulus. In these studies, IL-1 production by blood monocytes in response to either 1.0 μg/ml LPS or GBM/anti-GBM immune complexes demonstrated identical kinetic curves (not shown). Soluble immune complexes were as effective a stimulus as either LPS or GBM/anti-GBM (Table 2), causing a rapid increase in both IL-1 and TNF release from monocytes.

Discussion

We have shown that IL-1 and TNF are produced *in vitro* by human peripheral blood monocytes after stimulation with GBM/anti-GBM immune complexes. The production of both monokines was dependent on the presence of immune complexes because neither GBM nor antibody

Table 2. *Comparison of TNF and IL-1 Production by Human Peripheral Blood Monocytes Stimulated with GBM/Anti-GBM Immune Complexes, LPS, or Soluble Immune Complexes*

Conditions of stimulation	TNF (units/5 × 10^6 cells)			IL-1 ^{3}H incorporation in thymocyte coproliferation assay (cpm)		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
GBM/anti-GBM immune complexes (2 mg/ml)	150	200	128	2700	11,190	2970
LPS (1.0 μg/ml)	10	168	130	ND	25,613	3298
Soluble immune complexes (1 mg/ml)	200	260	ND	3653	22,819	ND

Results shown are means from triplicate samples in individual experiments, and show direct comparisons for the same cell population after 24-hour incubation with the stimulus.
ND, not done; Exp, experiment.

alone had any effects on monokine release. The kinetics of TNF and IL-1 production differed, suggesting that each monokine may be regulated separately. Interleukin-1 was produced in a short pulse, because levels did not increase significantly beyond 6 hours, whereas TNF levels continued to increase for up to 32 hours. Lipopolysaccharide or GBM/anti-GBM immune complexes induced similar kinetics for monokine production. In addition, either a high dose of LPS or GBM/anti-GBM complexes induced similar levels of both IL-1 and TNF, suggesting that the GBM system resulted in monokine production close to the maximum levels obtainable for peripheral blood monocytes.

These results are important because they are the first direct demonstration that immune complexes deposited in or on the GBM can result in augmented IL-1 and TNF levels by human blood monocytes. This finding lends further support to the hypothesis that mediator production by monocytes that accumulate in the glomerulus in crescentic nephritis could result in the alterations seen in the autologous phase of this disease. IL-1 is an important signal for the activation of T cells,[14-16] leading to the production of interleukin-2 and γ-interferon. Deposition of fibrin in experimental glomerulonephritis have been shown previously to be monocyte/macrophage dependent,[13,28] and it is possible that these effects are mediated by IL-1 and/or TNF. In addition, both monokines have been detected in isolated glomeruli from rabbit with crescentic nephritis.[19] IL-1 and TNF have been shown to have diverse effects on a variety of cell populations, including the ability to stimulate fibroblast proliferation, enhance endothelial cell activation, augment the production of acute phase proteins, and induce the adherence of various inflammatory cells.[14-16] All of the above activation events may contribute to the derangement of glomerular structure and function associated with progressive glomerulonephritis. In addition, both IL-1 and TNF have been shown to induce active collagenase and gelatinase secretion by cultured granulation tissue.[29-31] Although these enzymes are important for tissue repair and remodeling, their excess secretion can cause extensive damage to tissue structural proteins, including basement membranes.[32]

Many previous investigators have focused on the mesangial cells as a proliferating participant in immune-mediated nephritis by their ability to produce lipid metabolites, proteases, oxygen radicals, and peptide mediators.[12,33-35] Although it is not questioned that resident mesangial cells are active during glomerulonephritis, it is now becoming clear that monocyte/macrophages and T cells play a key role in some nephritic diseases and probably contribute to glomerular fibrosis.

In conclusion, our results have shown that a stimulus that approximates the etiologic agent in certain forms of human immune glomerulonephritides is capable of activating monocytes to produce levels of TNF and IL-1 that could have pathophysiologic effects. Besides their significance in the understanding of the mechanisms of glomerulonephritis, our results may also be relevant to other diseases in which immune complex-mediated tissue injury occurs.

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