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Brief Definitive Report

INTERFERON γ AND LIPOPOLYSACCHARIDE PROMOTE MACROPHAGE ADHERENCE TO BASEMENT MEMBRANE GLYCOPROTEINS

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Although the ability of mononuclear phagocytes to interact with basement membranes and other extracellular matrices is well documented, the mechanisms involved and their possible modes of regulation are poorly understood (1). Monocytes must penetrate the basement membranes of vascular endothelia during their emigration from blood to tissue (1) and they can encounter basement membranes again after they have differentiated into macrophages (e.g., see reference 2). Mononuclear phagocyte–basement membrane interactions are of considerable importance, for example, in chronic inflammatory disorders and the pathogenesis of atherosclerosis (1). However, the physiological and pathological factors that control mononuclear phagocyte interactions with the basement membrane have not been adequately defined, and the mechanism(s) used by these cells for diapedesis have not been rigorously explored. Macrophages do possess specific laminin-binding proteins (3, 4) and it seems likely that they also express collagen-binding proteins. How such surface proteins contribute to basement membrane interactions or how they might be regulated by factors that control the state of macrophage differentiation and activation are problems that have not been addressed.

In this study, we analyzed the ability of thioglycollate (TG)-elicited mouse peritoneal macrophages, a population of recently extravasated monocytes, to interact with the basement membrane glycoproteins laminin and type IV collagen, as well as fibronectin, and we examined the effects of macrophage activation on these interactions.

Materials and Methods

Mice. Female C57/BL6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 7 wk of age and were used within 4 wk after their arrival.

Macrophages. TG-elicited macrophages were obtained as described previously (5) from mice that had been injected 3–4 d before being killed with 1.5 ml of a 4% (wt/vol) solution of TG broth (Difco Laboratories, Detroit, MI). This procedure yielded 2–3 × 10^7 peritoneal cells, of which >90% were macrophages, as determined by Wright-Giemsa staining.

Adherence Assays. Multiwell tissue culture plates (11.3 mm, Costar, Cambridge, MA) were coated overnight at 4°C with PBS (0.2 ml) containing 20 μg/ml of either murine laminin (Gibco Laboratories, Grand Island, NY), murine type IV collagen (Collaborative Research,

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Inc., Lexington, MA), or bovine plasma fibronectin (Gibco Laboratories). This protein concentration was chosen because it resulted in the saturation of all plastic binding sites. The laminin and fibronectin solutions were filtered through a 0.45-μM Uniflo filter (Schleicher & Schuell, Keene, NH) before addition to the wells. The amount of protein lost by filtration was <10% of the initial concentration, as determined by the Lowry protein assay. Type IV collagen solutions were centrifuged at 13,000 *g* for 5 min before use. The coated wells were washed twice with PBS to remove excess protein and MEM containing 10% FCS (0.1 ml) was added to the coated wells and uncoated plastic wells. Macrophages, obtained as described above, were resuspended in MEM containing 10% FCS and 5×10^4 cells were added to all of the wells. Rat rINF-γ (Amgen Biologicals, Thousand Oaks, CA) and LPS (*Salmonella typhosa*; Difco Laboratories) were added to some wells either alone or in combination at the concentrations indicated in the appropriate figure legends. The plates were then incubated at 37°C for either 4, 8, or 24 h. Nonadherent cells were removed by washing the wells twice with warm HBSS. Adherent cells were fixed in methanol and stained with a 10% solution of Giemsa stain in PBS. The cells were examined using brightfield optics with a Nikon Diaphot microscope equipped with a 16-square reticle. Using a ×10 objective, the surface area of this grid was measured with a stage micrometer and determined to be 0.5 mm^2. For each experiment, the number of cells in this area was determined twice.

Results

IFN-γ (0.1, 5, and 20 U/ml) and LPS (0.1 and 2.0 ng/ml) were added, either alone or in combination, to TG-elicited macrophages that had been plated in protein-coated wells. These stimuli by themselves did not induce macrophage activation, as assessed by their ability to lyse P815 tumor cells spontaneously. However, the addition of IFN-γ at a concentration ≥5 U/ml together with LPS at a concentration of 2.0 ng/ml, but not 0.1 ng/ml, did induce activation (data not shown).

TG-elicited macrophages adhered avidly to fibronectin-coated surfaces, as well as to tissue culture plastic (Fig. 1, *A* and *B*). Within 4 h of incubation, approximately all of the cells plated on these substrata had adhered. IFN-γ and LPS, when added

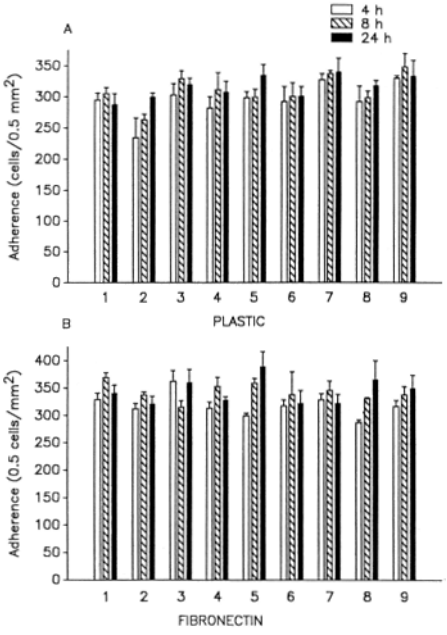

FIGURE 1. Macrophage adherence to tissue culture plastic and fibronectin-coated surfaces. Adherence assays were performed in multiwell tissue culture plates (11.3-mm diameter) as described in Materials and Methods. Briefly, the plastic wells were either left uncoated (*A*) or coated overnight at 4°C with 0.2 ml of a 20 μg/ml solution of bovine fibronectin (*B*). After washing the fibronectin-coated wells with PBS to remove excess protein, 5×10^4 TG-elicited macrophages were added to all of the wells. Rat rINF-γ and LPS were added to some wells at the following concentrations: (*1*) Control; (*2*) 0.1 ng/ml LPS; (*3*) 2 ng/ml LPS; (*4*) 0.1 U/ml INF-γ; (*5*) 5 U/ml INF-γ; (*6*) 20 U/ml INF-γ; (*7*) 0.1 ng/ml LPS and 0.1 U/ml INF-γ; (*8*) 0.1 ng/ml LPS and 5 U/ml INF-γ; (*9*) 0.1 ng/ml LPS and 20 U/ml INF-γ; (*10*) 2 ng/ml LPS and 0.1 U/ml INF-γ; (*11*) 2 ng/ml LPS and 5 U/ml INF-γ; (*12*) 2 ng/ml LPS and 20 U/ml INF-γ. The plates were incubated at 37°C for either 4, 8, or 24 h and then washed with warm HBSS to remove nonadherent cells. The adherent macrophages were fixed, stained, and counted. The numbers shown represent the mean (± SEM) number of adherent cells observed in two (*A*) or three (*B*) independent experiments.

separately or in combination, did not increase the amount of adherence on these substrata.

In striking contrast to their ability to adhere on fibronectin and tissue culture plastic, TG-elicited macrophages exhibited minimal adherence on laminin-coated surfaces even after 24 h of incubation (Fig. 2 *A*). Macrophages plated on laminin remained in suspension and were viable for at least 24 h. LPS at a concentration of 2.0 ng/ml, and IFN-γ at both 5 U/ml and 20 U/ml, promoted an increase in laminin adherence that was evident at 4 h and maximal at 8 h of incubation (Figs. 2 *A* and 3). This induction of adherence was concentration dependent. Increased adherence was not observed with either 0.1 ng/ml of LPS or with 0.1 U/ml of INF-γ. IFN-γ at both 5 U/ml and 20 U/ml, when added together with 2.0 ng/ml of LPS, induced a more marked increase in adherence than when added alone. However, no synergistic effects were observed when 0.1 U/ml INF-γ was added together with any concentration of LPS.

Adherence to laminin in the presence of INF-γ and LPS was greatest after 8 h of incubation (Fig. 2 *A*). Decreased adherence was observed with longer periods of incubation. After 24 h, the amount of adherence was, at most, similar to that observed at 4 h (Fig. 2 *A*). Those cells that did detach from the laminin substratum after 24 h in the presence of IFN-γ and LPS were still viable. In fact, these cells could be re-induced to adhere to laminin rapidly by the addition of 50 ng/ml PMA (reference 3; data not shown). Also, when removed and plated on type IV collagen-coated wells they could be re-induced to adhere by the addition of INF-γ and LPS. Macrophages that were pre-incubated in suspension in the presence of IFN-γ and LPS for periods of 4 h or less and then washed did not adhere to laminin-coated wells.

TG-elicited macrophages did not adhere well to type IV collagen, but IFN-γ and

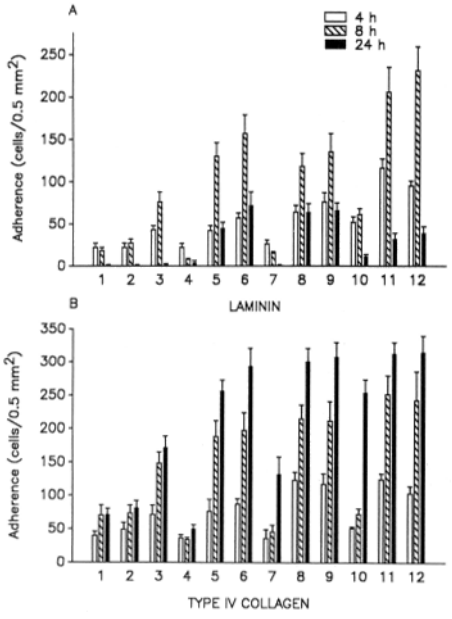

FIGURE 2. Macrophages adherence to laminin and type IV collagen-coated surfaces. The plastic wells of multiwell tissue culture plates (11.3-mm diameter) were coated overnight at 4°C with 0.2 ml of a 20 μg/ml solution of either murine laminin or murine type IV collagen in PBS. Adherence assays were carried out as described in Materials and Methods. INF-γ and LPS were added along with 5×10^4 TG-elicited macrophages to the laminin (*A*) or type IV collagen (*B*) coated wells as follows: (*1*) Control; (*2*) 0.1 ng/ml LPS; (*3*) 2 ng/ml LPS; (*4*) 0.1 U/ml INF-γ; (*5*) 5 U/ml INF-γ; (*6*) 20 U/ml INF-γ; (*7*) 0.1 ng/ml LPS and 0.1 U/ml INF-γ; (*8*) 0.1 ng/ml LPS and 5 U/ml INF-γ; (*9*) 0.1 ng/ml LPS and 20 U/ml INF-γ; (*10*) 2 ng/ml LPS and 0.1 U/ml INF-γ; (*11*) 2 ng/ml LPS and 5 U/ml INF-γ; (*12*) 2 ng/ml LPS and 20 U/ml INF-γ. The plates were incubated at 37°C for either 4, 8, or 24 h and then washed with warm HBSS to remove nonadherent cells. The adherent macrophages were fixed, stained, and counted. The numbers shown represent the mean (± SEM) number of adherent cells observed in six (*A*) or four (*B*) independent experiments.

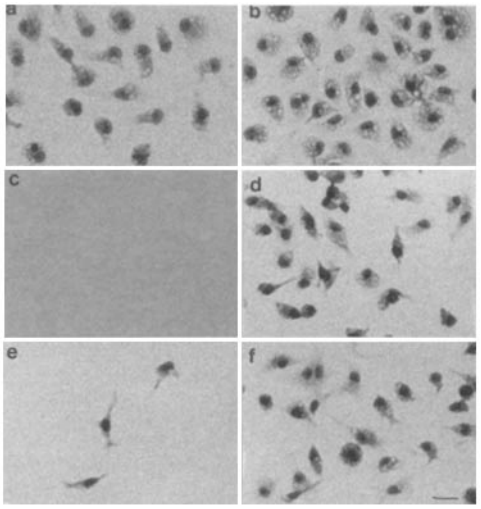

FIGURE 3. Photomicrographs of adherent macrophages. TG-elicited macrophages were allowed to adhere to either fibronectin (*a, b*), laminin (*c, d*), or type IV collagen (*e, f*) wells for 8 h in either control medium (*a, c, e*) or in medium containing IFN-γ (20 U/ml) and LPS (2 ng/ml) (*b, d, f*). After washing, adherent cells were fixed and stained as described in Materials and Methods. Stained cells were photographed using brightfield optics. Bar, 50 μM.

LPS treatment also promoted their adherence on this substratum (Figs. 2 *B* and 3). The concentrations of INF-γ and LPS required for increased type IV collagen adherence were similar to those required for laminin adherence (cf. Fig. 2, *A* and *B*). Adherence to type IV collagen in the presence of IFN-γ and LPS did not diminish with longer incubation times, an observation that is very distinct from their behavior on laminin. Maximal adherence to type IV collagen was observed at 24 h of incubation, and at this time point, approximately all of the cells plated in the presence of both IFN-γ and LPS had adhered. The number of macrophages that did adhere to type IV collagen in the presence of IFN-γ and LPS was five- to sixfold greater than the number of cells that adhered at 24 h in the absence of these stimuli (Fig. 2 *B*). It is also interesting to note that the maximum number of cells that adhered to Type IV collagen was approximately one-third greater than the maximum number that adhered to laminin at the same protein concentration used to coat the wells.

Although IFN-γ and LPS did not promote adherence on fibronectin or plastic, they did increase macrophage spreading on these substrata (Fig. 3). By comparison, macrophages that were induced to adhere to laminin and type IV collagen with INF-γ and LPS spread less than cells on fibronectin and plastic (Fig. 3).

Discussion

The mechanisms used by mononuclear phagocytes to adhere to and penetrate through basement membranes have not been elucidated (1, 3). Within this context, the key finding reported here is that the ability of recently extravasated monocytes (TG-elicited macrophages) to adhere to basement membrane glycoproteins is not a constitutive function of these cells but that it can be induced by stimuli that control the state of macrophage activation. In contrast, these cells do adhere constitutively to fibronectin, as well as to tissue culture plastic. Macrophage activation does not augment these interactions.

Although activation induces the adherence of macrophages on both laminin and type IV collagen, important differences do exist in their behavior on these two sub-

strata. Adherence to laminin is a transient event that is maximal at 8 h after the addition of IFN-γ and LPS, and diminishes with longer periods of incubation. The kinetics of adherence to type IV collagen, however, are quite different. Maximal adherence is observed at 24 h of incubation in the presence of these stimuli and this adherence does not appear to be transient. These observations may provide insight into possible mechanisms used by mononuclear phagocytes to interact with basement membranes. Laminin is a basement membrane component that is involved in cell attachment and anchoring (6). Type IV collagen, the major structural component of basement membranes, while possibly being involved in cell attachment (7), serves as a barrier that cells must either migrate through or degrade in order to traverse the basement membrane (8). Thus, the initial attachment of mononuclear phagocytes to basement membranes may involve a transient attachment to laminin that is followed by a more prolonged association with type IV collagen.

Macrophage activation, induced by IFN-γ and LPS, is associated with many cellular alterations, including the modulation of specific cell surface receptors (9). The data we present here suggest that it also enhances the ability of macrophages to interact with basement membrane components, probably by increasing the expression or otherwise activating receptors for these components on the macrophage surface (3). Induction of mononuclear phagocyte adherence to basement membranes is likely to be an initial, requisite step for diapedesis. Subsequently, adherence may trigger the secretion of specific proteases (e.g., collagenases [10, 11] or other secretory products [12]) that could facilitate their passage across the basement membrane.

Summary

The ability of thioglycollate (TG)-elicited peritoneal macrophages, a population of recently recruited monocytes, to adhere to the basement membrane glycoproteins laminin and type IV collagen is not a constitutive function of these cells. Adherence can be induced, however, by treatment with IFN-γ and LPS. In general, IFN-γ is more potent than LPS in promoting this adherence. Maximal adherence, however, is observed when IFN-γ (≥5 U/ml) is used together with LPS (2.0 ng/ml). These requirements parallel the conditions needed to obtain tumoricidal activation of TG-elicited macrophages. Adherence to laminin, in the presence of these stimuli, is transient, being maximal at 8 h after their addition and diminishing with longer periods of incubation. In contrast, adherence to type IV collagen does not appear to be transient and IFN-γ and LPS induce a more prolonged association of macrophages with this substratum.

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