High-Affinity Binding of Interferon-γ to a Basement Membrane Complex (Matrigel)

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Abstract

Recently it was demonstrated that growth factors are bound to the extracellular matrix, and can regulate cell behavior. Using three different types of binding assays, we have examined the interaction of interferon-γ with a basement membrane produced by the Engelbreth-Holm-Swarm tumor. Basement membrane was found to bind interferon-γ in both a time- and concentration-dependent manner. Equilibrium binding analysis revealed a high-affinity site with a dissociation constant of 1.5 10^{-9} M and a maximum binding capacity of 1.6 10^9 sites/mm^2 of basement membrane. Competition studies show that the binding is inhibited by heparan sulfate, suggesting that basement membrane-heparan sulfate proteoglycan could be the binding site. This interaction was clearly confirmed by native polyacrylamide gel electrophoresis and dot-blot analysis with purified basement membrane molecules. Furthermore, the carboxy-terminal part of the interferon-γ molecule contains an amino acid cluster, very closely related to a consensus sequence, present in more than 20 proteins known to bind sulfated glycosaminoglycans such as heparin. These data demonstrate a possible role of extracellular matrix components in storing cytokines and in modulating the cellular response to such factors. (*J. Clin. Invest.* 1991. 87:878–883.). Key words: binding site • extracellular matrix • local concentration • heparan sulfate proteoglycan

Introduction

Increasing evidence now demonstrates that cellular activities depend on multifactorial events involving soluble-phase factors such as cytokines (1, 2), and/or solid-phase factors such as extracellular matrix components (3, 4). These soluble- or solid-phase factors bind to specific cell surface receptors, some of which have been characterized (5, 6). Basement membranes are specialized extracellular structures which underlie epithelia and are composed of collagen IV, laminin, nidogen/entactin, and proteoglycans. These matrices affect a variety of biological activities including cell adhesion (7, 8), migration (8, 9), growth and differentiation, neurite outgrowth (10), regulation of cell morphology (11), tumor metastases (6, 12–14), and regulation of morphogenetic processes during development (15). The cellular response depends on the cell type and amount and form of the basement membrane. Recently, it has been shown that all four components both alone and in combination are able to exert biological activity (8, 16–21). Laminin appears to be the most active in this regard (10, 17, 20). The large heparan sulfate proteoglycan termed pearlecan has recently been cloned and partially sequenced and contains some 40% homology to the amino-terminal end of the laminin chains (21). It has been shown to promote hepatocyte adhesion and a specific receptor has been isolated (22). This, however, does not appear to be the only function of the heparan sulfate which also serves to regulate the passage of macromolecules through the basement membrane owing to its strong negative charge (23).

Cytokines are soluble factors, with multiple overlapping cell regulatory actions (1). The response of a cell to a given cytokine is dependent on the local concentration of the cytokine, the cell type, and other cell regulators to which it is concomitantly exposed (1). For example, interferon-γ (IFN-γ) is a pleiotropic factor that has many effects on different cells (24). It induces the antiviral state in uninfected cells, can regulate cell growth and differentiation, regulates the immune response, and inhibits tumor cell growth (24–27). Although cytokines such as IFN-γ are generally thought of as soluble factors, it is possible that these proteins may be in a bound form, and thus, either be stabilized to prevent breakdown and/or immobilized to allow continued and localized stimulation of cells. Recently, growth factors such as fibroblast growth factor (FGF),[1] have been found to be incorporated into the extracellular matrix where they are stored. In the case of FGF, the thin extracellular matrix of the basement membrane was found to be a storage depot and the major ligand was heparan sulfate proteoglycan (28).

Here we have investigated whether IFN-γ can bind to the basement membrane. Since intact basement membrane from tissues is difficult to isolate reproducibly and in purity, we have used a reconstitued basement membrane from the Engelbreth-Holm-Swarm (EHS) tumor to study the interaction of IFN-γ. This basement membrane matrix, termed matrigel, contains all of the components of authentic basement membrane and in the electron microscope has similar lamina densa–like segments to those observed in tissue-derived basement membranes (29–31).

Methods

^{125}I iodination of IFN-γ. Recombinant human IFN-γ with a specific activity of 2.10^7 U/mg was a generous gift of Roussel Uclaf, Paris, France. 10 μg of IFN-γ was reacted in 50 μl of 0.2 M phosphate buffer, pH 7.4, containing 0.8 M NaCl, 0.02% Tween 20, 500 μCi of Na^{125}I (New England Nuclear-DuPont, Boston, MA), and 2.5 μg of chloramine T. After 1 min at ambient temperature, the reaction was stopped by addition of 10 μl of sodium metabisulfite at 0.3 mg/ml. Proteins were then separated from free iodine by molecular sieving on a prepacked Sephadex G 25 PD 10 column (Pharmacia-LKB, Uppsala, Sweden), equilibrated with 0.1 M phosphate buffer, pH 7.4, containing

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1. *Abbreviations used in this paper:* FGF, fibroblast growth factor; GM-CSF, granulocyte/macrophage colony-stimulating factor.

0.5 M NaCl, 0.02% Tween 20, and 10% of fetal calf serum. ^{125}I-labeled IFN-γ, of which the specific radioactivity was 20 μCi/μg, was aliquoted and stored at −20°C. The high ionic strength buffer used here was found to be necessary to prevent aggregation of IFN-γ. If labeled in more physiological conditions (e.g., PBS), IFN-γ eluted in the void volume of a G75 Sephadex column, as reported by others (32).

Preparation of matrigel-coated dish. Matrigel was prepared as described (29). This mixture of matrix proteins is in solution at 4°C but forms a gel when warmed at 24–37°C. Matrigel was thawed at 4°C and diluted to 5 mg/ml with ice-cold 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, and 30 μl per well was allowed to gel overnight at 35°C in a 96-flat botton well assay plate (Pro Bind 3915, Becton, Dickinson & Co., Lincoln Park, NJ). Wells were then saturated for 1 h at 37°C with 200 μl of 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 20 mg/ml BSA.

Binding studies. In general, 100 μl of unlabeled IFN-γ (12.5 ng/ml to 5 μg/ml), containing 25 nCi of radiolabeled IFN-γ employed as a tracer, was incubated in matrigel-coated wells, at room temperature. After the time necessary to reach equilibrium (or at different times for kinetic studies), the supernatant fraction was removed and the matrigel was rapidly washed with 200 μl of PBS/BSA 0.1%/Tween 20 0.02%. Bound radioactivity was then removed by dissolving the matrigel with 4 M NaOH. The supernatant fraction plus the wash and dissolved matrigel were counted in a gamma counter (1260 Multigamma, Pharmacia-LKB) to determine free and bound radioactivity. Experiments were performed twice, with triplicates for each data point. The quantity of IFN-γ bound in each well was calculated from the specific radioactivity of IFN-γ in each concentration. Nonspecific binding was determined according to Chamness and McGuire (33) using a lim $(B/F) = 0.2$. To estimate K_d and B_{max} (maximum binding capacity), data were represented as specific bound ligand plotted against the logarithm of free ligand concentration, according to the equation $B_s = M_s \cdot K_d \cdot F/(1 + K_d \cdot F)$, in which B_s and M_s are "surface concentrations" of bound IFN-γ and coated matrigel, respectively, as described (34).

Enzymatic digestion of heparan sulfate. Matrigel was prepared as described above and incubated overnight with 50 μl of $CaCl_2$ (1 μM), Tris-HCl (50 mM) containing either 10 U of heparitinase (Miles Scientific Div., Naperville, IL) or 2.5 units of heparinase (Sigma Chemical Co., St. Louis, MO). After washing, 100 μl of ^{125}I-IFN-γ (25 nCi) was incubated for 4 h with the matrigel. Bound radioactivity was determined. Nonspecific binding was calculated with a 200 M excess of cold IFN.

Competition study. Matrigel-coated wells were incubated (4 h at room temperature) with 100 μl of radiolabeled IFN-γ (25 nCi/ml), in the presence of increasing concentrations of competitors (heparin from Serva, Heidelberg, FRG; heparan sulfate from Sigma Chemical Co.; and rabbit anti-human IFN-γ from Genzyme Co., Boston, MA). Nonspecific binding was determined with a 200 M excess of cold IFN. Bound radioactivity was then determined as described above and the percentage of binding inhibition was calculated.

Native polyacrylamide gel electrophoresis (PAGE). This electrophoretic system was developed to monitor interactions between proteins that have different isoelectric points. The 5% acrylamide/0.5% bis acrylamide gel, in 50 mM Tris/40 mM boric acid, pH 8.5, was allowed to polymerize as usual under vacuum in a horizontal gel mold. The comb was inserted in the middle of the gel.

Samples consisted of IFN-γ (5 μg) alone or in mixture with different matrix components such as laminin (5 μg, Gibco Laboratories, Grand Island, NY), collagen type IV (5 μg, Gibco Laboratories), heparan sulfate (2.5 μg), and matrigel (12.5 μg). Before electrophoresis, samples were incubated 4 h at 4°C. Tank buffer was Tris/borate buffer, pH 8.5. Running conditions were constant voltage (200 V) for 1 h at 4°C. Proteins were revealed by Coomassie Blue R-250 staining as usual.

Dot blots. A described procedure was followed to prevent glycosaminoglycans loss (35). Briefly, 1 μl of glycosaminoglycan (from 1 to 10 mg/ml in PBS) was applied onto a nitrocellulose sheet and air-dried. The nitrocellulose was then fixed in 5% paraformaldehyde + 0.5% cetylpyridinium chloride for 30 min at room temperature and washed in buffer. Purified basement membrane proteins (10 μg) were applied onto nitrocellulose by suction as usual. The nonoccupied binding sites were blocked with PBS/BSA 3% for 30 min at 37°C. The nitrocellulose strips were incubated overnight at 4°C with interferon-γ (1 μM in PBS/BSA 1%), washed in PBS, and incubated with rabbit anti-IFN-γ followed by peroxidase-labeled anti-rabbit IgG. Alternatively radiolabeled IFN was used, and after washing, the nitrocellulose filters were directly counted.

Domain mapping with monoclonal antibodies. MAbs B22, B27, 32, and 35 which define different epitopes on the IFN-γ molecule are described elsewhere (36). Radiolabeled IFN-γ was allowed to react, in the presence of different MAbs, with heparan sulfate (1 μg), using the dot-blot assay. After washing, each nitrocellulose strip was counted to determine the bound radioactivity.

Interferon assay. Antiviral activity was determined with a microtiter inhibition of cytopathic effect assay against vesicular stomatitis virus (VSV) on a monolayer of Wish cells. Cells (5×10^4/well) were grown in 100 μl of DME supplemented with 10% fetal calf serum on plastic or on basement membrane (30 μg/well). Before cell culture, basement membranes were incubated 6 h at 4°C with IFN-γ and extensively washed. Then, 24 h after the addition of the virus, cell viability was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described (37). In parallel experiments, IFN-γ was used to determine the exact quantity of IFN remaining on the basement membrane just before cell culture.

Results

Binding of IFN-γ to whole basement membrane. When added to a layer of gelled basement membrane, radiolabeled IFN-γ binds in a time-dependent manner (Fig. 1). At room temperature, the steady state is reached in 3.5 h. Under the same conditions, binding of IFN to plastic (noncoated wells) was found to be nonexistent.

Scatchard analysis of IFN-γ binding to basement membrane was carried out (Fig. 2 *a*). The biphasic curve indicated a nonspecific binding (horizontal part of the plot) and a single class of binding sites. The data were corrected for specific binding as described (33), and plotted with the semilogarithmic representation (38) (Fig. 2 *b*). From the s-shape curve, representing the binding site, the dissociation constant K_d = 1.5 nM was determined. A high affinity between IFN-γ and basement membrane was demonstrated. The number of binding sites was calculated at 1.6 10^9/mm^2 of coated basement membrane.

Binding inhibition assay. We next studied the nature of the interaction between IFN-γ and basement membrane (Fig. 3). IFN-γ was incubated on matrigel in the presence of different components, and the inhibition of specific binding was measured. NaCl (3 M), but not Tween 20 (0.5%), inhibited 100% of the binding, suggesting that the type of interaction is more ionic than hydrophobic. As a control, polyclonal antibody

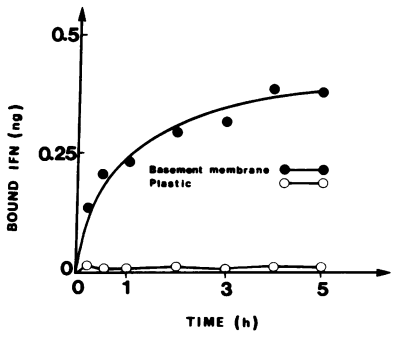

Figure 1. Effect of time on the binding of IFN-γ to basement membrane. Basement membrane–coated (●) or noncoated wells (○) were incubated at room temperature with 1.25 ng of radiolabeled IFN-γ. After the time indicated, bound IFN-γ was determined.

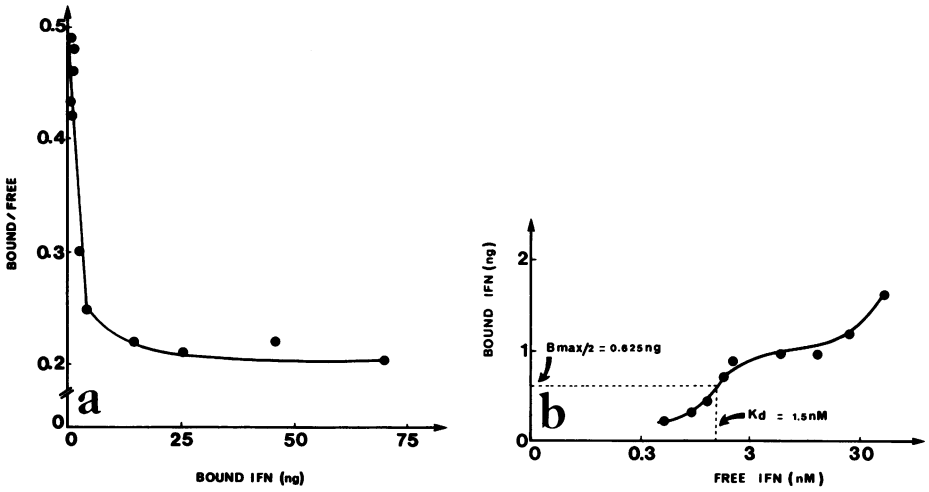

Figure 2. Effect of IFN-γ concentration on the binding of IFN-γ to basement membrane. 25 nCi/ml of radiolabeled IFN-γ was incubated for 4 h at room temperature, with 150 μg of coated basement membrane complex and with increasing concentrations of cold IFN-γ (from 12.5 ng/ml to 5 μg/ml). Bound and free IFN-γ were then measured as described in Methods. (*a*) Scatchard plot of the raw binding data. (*b*) Semilogarithmic plot of the data corrected for nonsaturable binding, using 0.2 as the limit of bound/free.

against IFN-γ (diluted 1/300) was found to fully prevent the specific binding. Glycosaminoglycans such as heparan sulfate or heparin (20 μg/ml) inhibited the binding to the same extent. Binding of IFN-γ to basement membrane may therefore be due to the component heparan sulfate proteoglycans.

Native PAGE analysis of the binding. Electrophoresis under native conditions was carried out to further investigate this binding, (Fig. 4). In this system, IFN-γ, of which the pI = 8.7, is slightly positive and will migrate toward the cathode (lane *1*). In contrast, BSA (pI = 5.2) is negatively charged and migrated toward the anode (lane *2*). When IFN-γ and BSA are mixed, their migration is delayed (because of opposites, charges which may result in some interaction), but each protein migrates to its side (lane *3*). Heparan sulfate glycosaminoglycan, which is highly negative, should migrate (lane *6a*) toward the anode but cannot be visualized because it is not stained by Coomassie Blue. When IFN-γ is electrophoresed in the presence of heparan sulfate (lane *6b*), it migrates toward the anode instead of the cathode, demonstrating that there is a strong interaction between IFN-γ and heparan sulfate. 2.5 μg of heparan sulfate was able to completely bind IFN-γ (5 μg) and to permit its migration to the glycosaminoglycan side.

The same experiment was performed with laminin (lane *4a*) and laminin plus IFN (lane *4b*) or collagen type IV (lane *5a*) and collagen type IV plus IFN (lane *5b*). In all cases, the migration of IFN-γ is not significatively affected by the presence of either laminin or collagen type IV, showing that there are no interactions between these components. Whole basement membrane complex (matrigel) and matrigel plus IFN-γ were also tested with two different preparations of matrigel (lanes *7* and *8*). Only a small part of the IFN-γ migrates on its side (cathode side) confirming that matrigel binds IFN (compare cathode side of lane 1 with cathode side of lane *7b* and *8b*). As control, ribonuclease A (Sigma Chemical Co.), which is also basic—pI = 8.9 (lane *9a*)—and ribonuclease A plus heparan sulfate (lane *9b*) were tested, and ribonuclease migration was not affected by the presence of heparan sulfate. Thus, the interferon–heparan sulfate interaction appears specific.

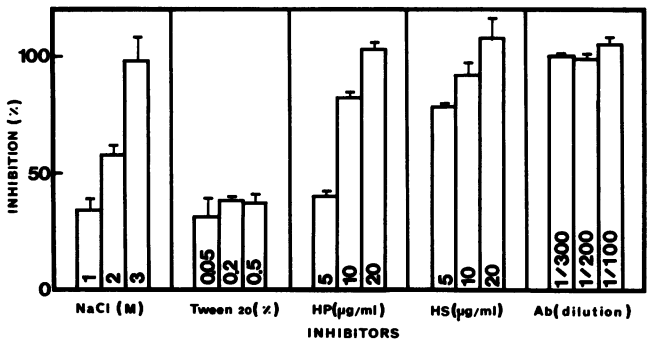

Figure 3. Effect of various inhibitors on the binding of IFN-γ to basement membrane. IFN-γ was coincubated with NaCl, Tween 20, heparin (*HP*), heparan sulfate (*HS*), or polyclonal antibody against IFN-γ diluted in PBS/BSA 1% (*Ab*), 4 h at room temperature. Nonspecific binding was determined as the bound radioactivity in the presence of a 200 M excess of cold IFN-γ.

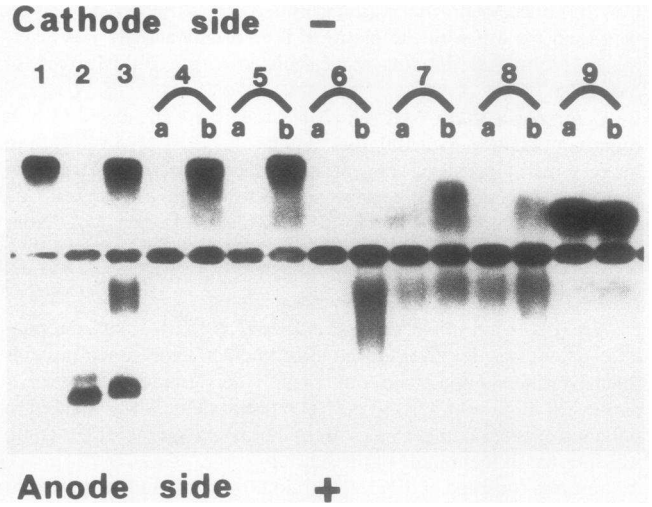

Figure 4. Effect of isolated proteins of the basement membrane, and whole basement membrane complex, on the IFN-γ migration in a native PAGE system at pH = 8.5. Lane *1*, IFN (5 μg). Lane *2*, BSA (5 μg). Lane *3*, IFN (5 μg) + BSA (5 μg). Lane *4a*, laminin (5 μg); *4b*, laminin (5 μg) + IFN (5 μg). Lane *5a*, collagen type IV (5 μg); *5b*, collagen type IV (5 μg) + IFN (5 μg). Lane *6a*, heparan sulfate (2.5 μg); *6b*, heparan sulfate (2.5 μg) + IFN (5 μg). Lane *7a* and *8a*, matrigel (12.5 μg), *7b* and *8b*, Matrigel (12.5 μg) + IFN (5 μg). Lane *9a*, ribonuclease A (5 μg), *9b*, ribonuclease A (5 μg) + heparan sulfate (2.5 μg).

Enzymatic degradation of basement membrane-glycosaminoglycan. Matrigel was incubated with either heparinase or heparitinase. Radiolabeled IFN-γ was allowed to react as described above with enzyme-treated matrigel or with untreated matrigel (to determine the 100% of binding). Digestion with heparinase completely abolished the binding, while digestion with heparitinase reduced it to 50% only. These data suggest that IFN binds to heparan sulfate heparin-like sequences, but we cannot rule out the fact that heparinase digestion may be more complete than that of the heparitinase.

Binding of IFN-γ to purified basement membrane components. In order to measure the direct binding of IFN-γ to purified basement membrane components, ligand dot-blot experiments were performed. As shown in Fig. 5, only heparan sulfate binds IFN-γ, confirming the binding activity found in the Scatchard analysis. Whole basement membrane complex (10 μg) dot-blotted onto nitrocellulose filters also binds IFN-γ, but weakly, presumably because of the low content of heparan sulfate (30). The experiment was also carried out with other extracellular matrix components including collagen type I, III, and fibronectin. None of these components bound IFN-γ (data not shown).

In order to assess the specificity between heparan sulfate and IFN-γ, radiolabeled IFN was also allowed to interact with different glycosaminoglycans. As shown in Table I, keratan sulfate, dermatan sulfate, and chondroitin sulfate do not significantly bind IFN-γ. This binding seems to be restricted to heparin and to heparan sulfate (the latter containing heparin-like sequences). The exact polysaccharide residues involved in the binding remain to be determined.

Domain mapping. IFN-γ was allowed to react with heparan sulfate, in the presence of MAbs to IFN-γ. Among the four MAbs tested, only B22 prevent the binding between IFN and heparan sulfate (Table II). This MAb recognizes the last 15 amino-acids (carboxy-terminal side) of the IFN-γ molecule, whereas B27, 32, and 35 define other domains (36). These data demonstrate that the carboxy-terminal part of IFN-γ is involved in its binding to heparan sulfate.

Antiviral activity of basement membrane-bound interferon. Basement membrane-coated wells were incubated with various concentrations of IFN-γ, washed, and tested for antiviral activity. As shown (Table III), cells grown on basement membrane-bound IFN were protected against the cytopathic effect of the virus in a dose-dependent manner, whereas cells grown on basement membrane alone were not. Thus, the IFN-γ bound to the basement membrane is biologically active.

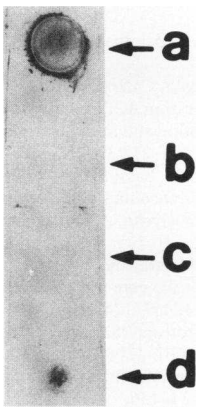

Figure 5. Dot-blot analysis of the binding of IFN-γ. IFN-γ (1 μM) was incubated with nitrocellulose strips dot-blotted with 10 μg of (*a*) heparan sulfate, (*b*) collagen type IV, (*c*) laminin, and (*d*) whole basement membrane complex. After washing, IFN-γ was stained with rabbit anti-human IFN-γ followed by peroxidase labeled anti-rabbit IgG.

Table I. Interactions of IFN-γ and Glycosaminoglycans

Glycosaminoglycan dot-blotted onto nitrocellulose filter	Nitrocellulose bound radioactivity
	cpm
Keratan sulfate	117.1
Dermatan sulfate	1,254.0
Chondroitin sulfate	2,140.6
Heparan sulfate	13,438.5
Heparin	28,224.2

Radiolabeled IFN-γ was incubated overnight at 4°C with the indicated glycosaminoglycans (1 μg) dot-blotted onto nitrocellulose. After washing in PBS, the nitrocellulose strips were counted.

Discussion

Previous data reported that extracellular matrix components may interact with growth factors such as FGF (28) or with cytokines such as IL-3 or granulocyte/macrophage colony-stimulating factor (GM-CSF) (39). We present, for the first time, the binding of human interferon-γ to basement membrane, as determined in several different and specific binding assays. Binding data reveal a high-affinity site for IFN-γ with a K_d of 1.5×10^{-9} M and a maximum binding capacity of 1.6×10^{-9} IFN molecules/mm^2 of basement membrane.

A relatively long time is needed for IFN-γ binding. This could be explained by the three-dimensional structure of the basement membrane which could slow diffusion of molecules. It should be also noted that the heparan sulfate proteoglycan represents < 5% of the total protein present in matrigel (30). This also explains the importance of nonspecific binding (B/F = 0.2 in the Scatchard plot) which corresponds to IFN-γ trapped, but not bound, in the basement membrane layers. The number of basement membrane binding sites is very high compared to IFN-γ cellular receptor number (from 100 to 10,000 per cell). This is well in excess of the number of molecules needed to saturate cellular IFN-γ receptors (40). Furthermore, the affinity of IFN-γ for basement membrane is close to the reported affinity of this molecule for its cellular receptor: 10^{-9} to 10^{-11} M (40). Therefore, we speculate that in vivo, basement membrane likely interacts with this cytokine and that this cytokine could be in an active state.

Inhibition experiments, performed to identify basement membrane components involved in IFN-γ binding, showed

Table II. Domain Mapping of IFN-γ

MAb	Bound radioactivity
	%
—	100
B27	92.1
B22	14.6
32	87.5
35	112.1

Radiolabeled IFN-γ was incubated overnight at 4°C with heparan sulfate (1 μg) dot-blotted onto nitrocellulose, in the presence of the indicated MAbs. After washing, the nitrocellulose strips were counted.

Table III. Basement Membrane-bound IFN Antiviral Activity

Basement membrane-bound IFN	Viable cells
ng	%
0.379	64.0
0.193	57.7
0.055	38.8
0.016	13.0
0.004	11.3
0.001	5.7
0	5.1

Wish cells ($5 \cdot 10^4$/well) were cultured overnight on matrigel preincubated with various quantities of IFN-γ, and then challenged for 24 h with vesicular stomatitis virus. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Matrigel-bound IFN was estimated in parallel experiments with radiolabeled IFN.

that heparan sulfate was an effective competitor. Electrophoretic and dot-blot analysis confirmed the specificity of the binding. Heparan sulfate proteoglycan is the binding site for IFN-γ, as in the case of basic FGF, IL-3, and GM-CSF (28, 39). This is also supported by the fact that heparinase or heparitinase digestion of basement membrane-glycosaminoglycan significantly reduced the binding of IFN-γ.

The affinity of IFN-γ for heparan sulfate proteoglycan found here, 1.5 nM, is higher than the affinity of basic FGF, 610 nM (28), although basic FGF (pI = 9.8) is more basic than IFN-γ (pI = 8.7). This suggests that the binding of IFN to basement membrane is not based on a simple electrostatic interaction, but may involve a defined region of the molecule. Moreover, heparan sulfate cannot significantly fix ribonuclease A (another basic protein pI = 8.9) in our electrophoretic system, showing that the presence of a net positive charge on the molecule is not sufficient for binding. On the other side, IFN-γ does not bind to other sulfated glycosaminoglycans such as dermatan, keratan, or chondroitin sulfate, in spite of their negative charge. Thus, this interaction appears to be specific.

The carboxy-terminal part of the IFN-γ molecule contains a cluster of nine amino acids: Ala^{124}–Ser^{132} (41) very closely related to a consensus sequence present in more than 20 known heparin-binding proteins (42). Binding inhibition experiments have been done with four MAbs directed against different parts of the IFN-γ molecule to determine the binding site on IFN-γ. Only antibodies directed against the carboxy-terminal part of the molecule prevent the binding, whereas the others do not.

Our data and that of other investigators (28, 43) suggest that extracellular matrix components could provide a local concentration of a given cytokine, direct the range of action of such soluble factors, and act as a physiological storage depot around cells. In the case of basic FGF, it was suggested that the regulation of capillary growth and the neovascular response may result from displacement of growth factors from their storage sites within the basement membrane and surrounding extracellular matrix (28, 44). Extracellular matrix-heparan sulfate also binds IL-3 and GM-CSF. Once bound, these cytokines can be presented in an active form to hemopoietic cells, thus providing a mechanistic explanation for the observed absolute dependence of haemopoietic cells on intimate stromal cell contact (43). Interferon-γ has an extremely broad range of biological activities on a great number of different cells (1, 24). Once bound to basement membranes, this molecule is still active.

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