THE INFLUENCE OF FIBRONECTIN ON HUMAN NATURAL KILLER CELL CYTOTOXICITY AND MIXED LYMPHOCYTE REACTIVITY

by

Diana Friberg

Submitted to the Faculty of the School of Graduate Studies
of the Medical College of Georgia in Partial Fulfillment
of the Requirements for the Degree of
Master of Science
July
1983

The Influence of Fibronectin on Human Natural Killer Cell Cytotoxicity and Mixed Lymphocyte Reactivity

This thesis submitted by Diana Delker Friberg has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

The signatures which appear below verify the fact that all required changes have been incorporated and that the thesis has received final approval with reference to content, form and accuracy of presentation.

This thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology.

August 26, 1983
Date

[redacted]
Advisor

[redacted]
Department Chairman

[redacted]
Dean, School of Graduate Studies

Acknowledgements

I wish to thank Dr. Jan Doran for her assistance, advice and support throughout the course of this project. Special thanks also go to Dr. Catherine Roesel for her help and understanding. I would like to thank the other members of my committee; Dr. Shyuan Hsia, Dr. Dave Lause, and Dr. Andy Reese for their guidance and cooperation. Finally, I would like to thank my husband for his love and concern throughout my graduate training.

Table of Contents

List of Figures

List of Tables

Introduction

A. Statement of the problem

The human immune system is a complex network of interactions which involve lymphoid and nonlymphoid cells as well as many different soluble factors. These various components work together in a stimulatory or suppressive manner to regulate the immune response. Recently, evidence has accumulated that a normally occurring plasma protein, fibronectin (Fn), may function as a soluble mediator of immune function.

Fn is a high molecular weight glycoprotein which is found in a soluble form in plasma and is present in an insoluble form on some cell surfaces. These Fns are known to play an important role in wound healing since they are capable of mediating cell-to-cell and cell-to-substrate adhesion. Fn can influence the morphology and proliferation of some types of tumor cells and interact with Clq and C3 complement components. Fn plays a role in nonimmune host defense mechanisms by acting as a potent opsonin for some microparticulates and by stimulating chemotaxis of fibroblasts and monocytes. Fn is capable of binding to monocytes and has been shown to enhance monocyte-macrophage mediated lysis of two cultured human tumor cell lines. In the rat, Fn suppresses the mixed lymphocyte reaction and has a cytostatic effect on carcinoma cells. Taken together, these findings implicate Fn as an immune regulatory molecule with potential clinical significance.

In studies using both humans and animals, Fn levels are depressed

as a consequence of surgical and accidental trauma, sepsis, and severe burns. In these conditions, the reticuloendothelial system (RES) shows a decreased ability to clear particulate matter from the circulation resulting in increased particle deposition in the lungs and kidneys. When Fn is repleted to normal plasma levels through the infusion of plasma cryoprecipitate, normal RES function is restored resulting in normal lung function and improved peripheral circulation. Due to the heterogeneity of Fn content in cryoprecipitate, as well as the presence of other plasma proteins, investigators are planning the use of purified Fn for replacement therapy.

Clinical trials, using purified Fn, will aid in the assessment of the in vivo effects of Fn on overall patient condition. However, to determine if Fn simultaneously affects immune function, in vitro assays provide a less complicated system for study. The research described here examines the effect of exogenous plasma Fn on in vitro natural killing (NK) and mixed lymphocyte culture (MLC) assays. These assays reflect immune events known to occur in vivo and can therefore be used to evaluate Fn's potential to affect immune function. When clinical trials do occur, it will be important to know the regulatory effect Fn may have on host immune reactivity.

B. Review of Related Literature

1. Fibronectin

a. structure

Fn is a high molecular weight plasma protein that was discovered by Morrison and coworkers in 1948 and designated cold insoluble globulin (CIG). Although this molecule has had many names related to its different functions, the currently accepted name for this glycoprotein is fibronectin (reviewed in Mossesson and Amrani 1980, Mosher and Furcht 1981, Yamada 1982). Fibronectin (fibre=fiber + nectere=to bind) exists in at least two and possibly three forms. Plasma Fn is present in blood and tissue fluids, and cellular Fn is found on cell surfaces and in the extracellular matrix. A third form of Fn has been isolated from amniotic fluid which has a slower mobility on SDS polyacrylamide gels and contains significantly more carbohydrate than plasma Fn (Ruoslahti et al. 1981). The plasma form of Fn is soluble at pH 7 and has a plasma concentration of approximately 0.3 mg/ml. Males have significantly higher plasma concentrations than females, and, in both sexes, fibronectin levels increase with age (Eriksen et al. 1982). Circulating Fn, with a molecular weight of 450,000 daltons, is composed of two nearly identical protein chains of approximately 220,000 MW, which are linked by a disulfide bridge near the COOH terminal end of the polypeptides. Each peptide chain is composed of functional domains that act as binding sites for a variety of substances including heparin, DNA, collagen, Staphylo-

coccus aureus, and cell membranes.

Cellular Fn has a nearly identical amino acid composition and tertiary polypeptide structure as plasma Fn, but instead of being a soluble dimer, it exists as insoluble multimers and aggregates. Until recently, antibodies prepared against either type of Fn have been shown to cross-react completely with the other type of the same species; recently mono clonal antibodies have been produced which react only with cellular fibronectin, indicating that there is at least some structural differences in the two types of Fn (Atherton et al. 1981). Cellular Fn may also be distinguished from the plasma form by carbohydrate composition, solubility, and in vivo and in vitro activity (Yamada and Kennedy 1979).

b. adhesive properties

Fibronectin mediates a variety of biological activities (reviewed in Pearlstein et al. 1980) which include cell-cell adhesion, cell-substrate adhesion, maintenance of normal cell morphology, cell motility and spreading, and specific binding of macromolecules. The exact mechanism by which Fn mediates cell-cell adhesiveness is unknown. When analyzed with immunoelectron microscopic techniques, chick heart fibroblasts form cell-to-cell contact regions that exhibit three types of contacts. Fn was not present in focal contact sites (10-15 nm spacing), but was found in regions of close contact (15-30 nm spacing) and extracellular matrix contacts (>30 nm spacing) (Chen and Singer 1981). This

indicates that Fn may be acting over intermediate distances to cause a general increase in cellular adhesion. Besides promoting cell-to-cell adhesion between cells of the same type, Fn can mediate the adherence of one cell type to another cell type; e.g., the adherence of blood granulocytes to vascular endothelium (Wall et al. 1982). Fn also acts as a cohesive molecular glue in cell-substrate adhesion (reviewed in Akiyama et al. 1981). It is able to promote cellular adhesion to a variety of surfaces including collagen (Pearlstein and Gold 1978), plastic culture dishes (Virtanen 1982), and glass coverslips (Rajaraman et al. 1983).

c. interaction with tumor cells

Fn added to transformed cells promotes the return of the cells to a nearly normal morphology (Chen et al. 1978). Normal cells grown in vitro display a parallel alignment of cell bodies when cultures become dense; i.e., the cells lay side by side and do not overlap or grow on top of each other. This phenomena, known as contact inhibition, is lost in many cells which become transformed by tumor viruses or carcinogens. When Fn is added to these transformed cells, they return to a nearly normal morphology (Lipkin et al. 1978). By some undetermined mechanism, Fn reconstitution alters the cell's shape and affects the cell surface architecture and cytoskeleton (Yamada et al. 1976a).

Fn may have some influence on the proliferation of tumor cells as measured by ^{3}H-thymidine uptake. Young et al. (1981) found that the addition of serum or affinity purified CIG to cultures of Walker 256 car-

cinoma cells caused a significant reduction in tumor cell proliferation when compared to controls. This finding has been confirmed in a preliminary report by Raynor and Reese (1981). Using other cell types, Bruhn and Zurborn (1983) reported that Fn concentrations of 1 mg/ml to 3 mg/ml inhibited HeLa and mouse carcinoma cell growth. In contrast, other investigators have found that the addition of Fn to transformed cells has no effect on the uptake of ^{3}HTdR (Chen et al. 1978b), glucose or 2-amino butyrate (Yamada and Pastan 1976), or on growth rate and cAMP levels (Yamada et al. 1976b).

d. levels in cancer patients

There are conflicting reports concerning plasma Fn levels in cancer patients. Pisano et al. (1972) reported low Fn levels in patients with advanced cancer. However, other reports indicate an increase in circulating Fn fragments which are the apparent result of in vivo cleavage of the Fn molecule (Parsons et al. 1979). These differences may be due to the type of Fn assay used in the study. Pisano et al. used a liver slice assay which measures gelatin binding activity of Fn, whereas Parsons et al. used an assay measuring total antigenic activity. Recently, Boughton et al. (1982) found that low plasma Fn levels correlated with relapsing episodes of acute leukemia. However, they concluded that this correlation was not related to tumor load, but rather was the result of secondary recurrent infections. In a study of patients with cancer of the breast or colon, mean antigenic Fn levels were elevated above normal

controls whether the disease was thought to be metastatic or not (Choate and Mosher 1983). Chemotherapy in patients with acute leukemia is associated with a fall in Fn levels (Choate and Mosher 1983, Brodin et al. 1983) which may contribute to lowered resistance against infection in these patients. Thus, when relating Fn levels to the status of malignancy in humans, it is probable that Fn concentrations fluctuate as a result of other clinical events (sepsis or chemotherapy), and therefore plasma Fn levels are not a suitable marker of malignancy (Choate and Mosher 1983).

It has been suggested by some that extracellular Fn bound to tumor cells can be correlated with tumorigenicity and metastatic potential of these cells. This idea is supported by experiments which show that benign tumors do not lose their extracellular fibronectin whereas metastatic tumors do (Chen et al. 1978a). Despite these findings, the correlation between tumorigenicity in vivo and fibronectin expression in vitro has been controversial. Recently, Stenman and Vaheri (1981) examined fibronectin in solid human tumors and reported that Fn is regularly detected as a pericellular matrix around individual cells of soft tissue tumors in vivo, regardless of whether the tumor is malignant or benign. Carcinomas, as well as benign epithelial tumors, were found to be distinctly Fn negative. From these observations it was concluded that expression of Fn in spontaneous solid tumors in man is a reflection of tissue differentiation rather than a characteristic distinction between malignant and benign behavior. Gibert et al. (1982) confirmed this hypothesis when they examined fibronectin in human breast tumor

cells in vivo and in vitro and found that Fn expression by these cells reflects their mesenchymal or epithelial differentiation rather than their malignant potentials.

e. chemotaxis and opsonization; implications for wound repair

Both cellular and plasma Fn may increase the rate of fibroblastic migration. As a chemoattractant, Fn promotes the directional migration of fibroblasts across a nitrocellulose filter (Gauss-Muller et al. 1981, Tsukomoto et al. 1981). This chemotactic effect may be important in directional migration of cells in the embryo (Katow et al. 1982), and in wound healing. In a study of Fn deposition at wound sites, Norris et al. (1982) found that proteolytic cleavage fragments of Fn could selectively augment the recruitment of monocytes into areas of tissue inflammation. More recently, Yonemasu et al. (1983) have demonstrated that non-fragmented Fn can stimulate both chemotactic and random locomotion of peripheral blood monocytes. This ability of Fn to affect macrophage mobility could be important in other immune phenomena requiring macrophage cooperation.

The opsonic activity of Fn has also been extensively studied (reviewed in Yamada et al. 1982). Plasma Fn promotes the binding and ingestion of certain materials by macrophages and other reticuloendothelial cells. Fn binds to S. aureus and some other gram positive organisms (Doran and Raynor 1981, Simpson et al. 1982, Myre et al. 1983), but it is apparently unable to bind gram negative organisms (Doran et al.

1982). Fibronectin's opsonic activity may be important in vivo for the control of bacterial infections. Patients with septicemia have decreased levels of circulating Fn; and studies are being conducted to determine if infusion of Fn into trauma patients will improve their clinical condition (Saba and Jaffe 1980).

The chemotactic and opsonic activities associated with Fn may be important at the site of wound repair (reviewed in Reese et al. 1983). Thus, Fn may help clear the wound site by attracting macrophages to the area and by promoting the opsonization of certain bacteria and cellular debris. In addition, Fn's adhesive abilities and its interaction with collagen and fibrin may contribute to the stability of newly repaired tissue.

f. immunoregulatory functions

The immunoregulatory properties of Fn are just beginning to be studied. Fn is produced and secreted by human macrophages (Alitalo et al. 1980) and recently has been shown to bind to human monocytes (Bevilacqua et al. 1981). There is no evidence that Fn is synthesized by or binds to lymphocytes (Bevilacqua et al. 1981, Lause et al. 1983), even though it is produced by a variety of other cells including fibroblasts and endothelial cells. Since Fn apparently does not bind to lymphocytes, it is possible that the immunoregulatory properties of Fn are due to its interaction with monocytes and macrophages. Beezhold et al. (1983) have recently reported that Fn stimulates rat macrophages to

secrete a protein that is capable of binding to mature T cells. Lause et al. (1983) have shown that Fn is capable of inducing T cell proliferation by a process dependent on a critical number of macrophages. Perri et al. (1982) studied the in vitro monocyte-macrophage mediated cytoxicity against two cultured human tumor lines, Malme melanoma and CAK-1 renal carcinoma cells and found that the addition of Fn enhanced macrophage mediated cytoxicity 2-4 fold. Taken together, these findings implicate fibronectin as a potentially important circulating molecule that may play a role in the regulation of immune function.

2. Natural killer cells

Natural killer cells were discovered only 8 years ago during efforts to study natural cell-mediated cytotoxicity. What began as an "annoying incidental observation" has expanded into a broad area of research and discovery (reviewed in Herberman, 1982c). NK functional activity was originally identified as an immediate cytotoxic response to tumor cells in vivo. Now, however, there are many recognized functions of NK cells. NK cells belong to a subclass of lymphocytes termed null cells, which are considered to be neither T nor B cells. NK cells appear morphologically to be large granular lymphocytes (LGL) with a relatively high proportion of cytoplasm that contains azurophilic granules and a kidney shaped nucleus (Timonen et al. 1981). These cells may be isolated from blood by use of a density gradient prepared with Percoll, an inert silica polymer (Timonen et al. 1980). In humans, the highest proportion of LGL's is found in the perpherial blood where they comprise 7% of all mononuclear cells (Herberman 1982a). An individual's NK activity is relatively consistent over a period of several years and a person exhibiting high NK activity will remain a high responder over time (Pross et al. 1982).

NK cells, in addition to macrophages, provide the body's first line of defense against malignant growth. In the mouse model system, depressed NK activity correlates with an inability to effectively clear tumor cells in vivo (Pollack and Hallenbeck 1982). Lack of tumor clearance can then lead to increased metastasis of various types of

tumors (Hanna 1982, Gorelik et al. 1982). However, not all tumors are susceptible to attack by NK cells. The nature of the target antigen recognized by NK cells is poorly understood, but it is thought that tumor cell differentiation plays some role in modulating its susceptibility to NK mediated lysis (Werkmeiester et al. 1982a and 1982b).

The human NK cytolytic process proceeds through a series of complex events which have recently been resolved into several clearly defineable steps (Hiserodt et al. 1982a). First, the NK cell recognizes and binds to its target cell. This binding is rapid, temperature insensitive and requires Mg++. Next, the NK cell becomes "activated" and modifies the surface of the target cell rendering it susceptible to lysis. This step is Ca++ dependent, occurs optimally at 37 C and is sensitive to the effects of various pharmacological agents including lidocaine and prostaglandin E_2 (Hiserodt et al. 1982b). Subsequent to these events, the NK cell can detach from the target and cytolysis will proceed. The detached NK cell is then recycled after a certain refractory period, and may form conjugates with and lyse other target cells. The actual lytic mechanism most likely involves the secretion of NK cytotoxic factors by the effector cell (Wright et al. 1983). NK cells are capable of mounting this cytolytic attack on a target cell immediately upon contact, without the need for prior sensitization or recognition of determinants coded by the major histocompatibility complex.

In addition to cytotoxicity against tumor cells, NK cells may also exhibit killer activity against various microbial agents: virus infected target cells (Rager-Zisman et al. 1982), parasites (Hauser 1982),

fungi and bacteria (Roder et al. 1981). It has also been suggested that NK cells may play a role in the destruction and/or control of growth and development of some normal hematopoietic and thymus cells (Roder et al. 1981). They also contribute to the rejection of bone marrow transplants and participate in graft vs host disease (Herberman 1982b).

Macrophages are important regulators of NK cell activity. Some studies indicate that human monocytes (Jondal et al. 1981) and broncho-alveolar macrophages (Bordignon et al. 1982) can suppress or inhibit NK activity. Due to the presence of suppressor monocytes, NK activity is decreased after surgical trauma in cancer patients (Uchida 1982) and in transplant patients (Ono et al. 1982, Guillou et al. 1982). Although monocytes and macrophages have been implicated as regulators of NK function, there is no published evidence that macrophage presence is required for the occurrence of cytolytic killing by LGL cells.

A positive relationship between NK cells and macrophages may be mediated by interferon in both animal model systems and humans (reviewed in Ununae 1981). The effects of interferon on NK activity have been extensively studied (Herberman ed. 1982c). NK cell activity in vivo is generally increased by interferon inducers. This increase can be impaired by the injection of agents that are toxic for macrophages. Similarly, the increase in NK activity produced by incubation of spleen cells with interferon inducers is eliminated if macrophages are depleted (Djeu et al. 1979).

3. Mixed lymphocyte reaction

In 1963, the first report appeared in the literature stating that lymphoblastic proliferation occurred in cultures containing lymphocytes from two unrelated individuals (Bain et al. 1963). Hirschhorn et al. (1963) confirmed that the mixture of lymphocytes from unrelated individuals led to cell enlargement and division, and Bach and Hirschhorn (1964) were the first to suggest that the degree of transformation in MLR might prove to be a useful quantitative measure of histocompatibility. Since that time, the MLR has become an extensively used in vitro test of genetically controlled antigenic recognition and is routinely used as a measure of donor-recipient compatibility for human allografts.

Many investigators have studied the interactions between different cell populations which are required for a proliferative response in the MLR (reviewed in Dupont et al. 1976). Macrophages are considered to be the major stimulatory cells in the MLR due to their expression of antigenic determinants coded by the Ia region of the major histocompatibility complex (MHC) (Rode and Gordon 1973, Ahmann et al. 1979). Excellent stimulation in the MLR has been achieved with highly purified populations of peritoneal and alveolar macrophages as well as spleen cells. Although B cells also bear Ia antigens, these cells were poor at stimulating and/or triggering T cells (Greineder et al. 1976). There are several reports in the literature claiming stimulation by B cells, but these studies did not eliminate phagocytes from the stimulator cell population (reviewed in Davidson 1977). Minami et al. (1980) confirmed

the lack of stimulation by B cells and pointed to macrophages as the major stimulatory cell. In their experiments, the degree of proliferation closely correlated with the number of phagocytic cells. Also, treating stimulator cells with antibodies against Ia antigens will inhibit the MLR (Albrechtsen et al. 1977). These studies and others, have confirmed that the I region of the MHC codes for the antigens responsible for the MLR. Since the MLR represents a complex set of cellular interactions known to control immune function, it provides a tool for studying the effects of immunoregulatory molecules.

There is currently no published literature concerning the effects of Fn on the human MLR. However, in the rat, Lause et al. (1982) found that Fn added to the MLR consistently caused a dose dependent inhibition of the proliferative response. Fn may be acting to suppress the MLR by inducing macrophages to produce suppressor factors which may either mask Ia-like antigens needed for stimulation or block the T cell proliferative response through some unknown mechanism.

Materials and Methods

A. Materials

1. Media:

Unless otherwise indicated, the culture media used was RPMI 1640 to which was added with 100 U/ml streptomycin, 100 U/ml penicillin, 0.25 mcg/ml fungizone, 2mM glutamine, 25mM Hepes and 2.0 gm/l tissue grade sodium bicarbonate (all reagents from Gibco). When indicated, bovine serum albumin (BSA) (Sigma), or fetal calf serum (FCS) (Flow Laboratories) was added to RPMI.

2. K562 cell line:

This myelogenous leukemia cell line, established by Lozzio et al. (1975) was kindly provided by Dr. Hsia, and was maintained in continuous culture at 37 C, 5% CO_2 in RPMI supplemented with 20% FCS. Cells were fed every two or three days and diluted sufficiently to maintain the cells at concentrations of $2\text{-}7 \times 10^5$ cells/ml.

B. Biochemical procedures

1. Isolation of Fibronectin:

Human Fn was isolated from citrated plasma by affinity chromatography on gelatin-Sepharose following a modification of the method of Rouslahti and Engvall (1978). Gelatin (ICN) was coupled to Sepharose (Pharmacia) by the cyanogen bromide treatment of Porath et al. (1973). The gelatin coated Sepharose was batch incubated with plasma for 2 hours at room temperature. The slurry was poured into a column and washed with phosphate buffered saline (PBS), pH 7.4, followed by a wash with 1 M urea in 0.05 M tris 0.15M NaCl buffer pH 7.5. The bound Fn was eluted with 4 M urea in tris buffer, pH 7.5, and concentrated by vacumn dialysis against 0.01 M tris buffer, pH 7.5. The final concentration was determined by reading the protein's optical density at 280 nm and using an extinction coefficient of 1.28 $mg^{-1}ml$. Fn was filter sterilized by passage through a 0.22 um filter. Purity of Fn was checked by running samples on 5.0% SDS polyacrylamide gels. Biological activity was determined by the ability of Fn to clump gelatin coated beads, and S. aureus Williams. When indicated, Fn was prepared in the presence of 10mM phenylmethylsulfonyl fluoride (PMSF) which was added to all buffers used in the isolation procedure.

2. Labeling of Fn with ^{125}I:

Fibronectin (1-1.5 mg in a final volume of less than 2 ml PBS) was added to an Iodogen (Pierce Chemical Co.) coated vial (0.1 mg Iodogen/vial) according to the method of Markwell and Fox (1978). To this vial was added 0.5-1.0 mCi of ^{125}I and the vial swirled gently for 10 minutes. Free iodine was seperated from protein bound ^{125}I by molecular sieve chromatography on a prepackaged Sephadex G25 column (PD-10 column, Pharmacia).

3. Preparation of Gelatin-coated beads:

Gelatin (ICN) was covalently bound to polystyrene latex beads (1.1 um in diameter, Sigma) by a modification of the carbodiimide reaction of Barker et al. (1975). Briefly, 1 ml of a 10% (w/v) suspension of beads was washed twice in 0.9% saline and resuspended in 1 ml saline followed by the addition of 120 ul of Carbodiimide (Sigma) solution (7.5 mg/10ml saline prepared in glass). Immediately, 80 ul of gelatin solution (40 mg/10ml saline) was added to the mixture. This mixture was incubated for 30 minutes at room temperature with shaking. Nonreacted active sites were blocked by the addition of 2 ml gelatin solution. After an additional 15 minute incubation the beads were washed 3 times with saline (1000xg) and resuspended in 4-5 ml of PBS, pH 7.4.

4. Plasmin treatment of Fn:

Fn was cleaved by the modification of a previously published pro-

cedure (Balian et al. 1979). Plasmin (Sigma) derived from porcine blood, was added to Fn using an approximate enzyme to substrate weight ratio of 1:100. Digestion was performed at pH 7.5 and 37 C for 3 hours. The reaction was stopped by the addition of 10mM E-aminocaproic acid (Sigma) and 10mM phenylmethyl-sulfonyl fluoride (PMSF) (Sigma). The digest was dialyzed extensively against PBS over a 24 hour period and then immediately run on SDS gels to assess the extent of cleavage.

5. Quantitative determination of human Fn:

Total antigenic Fn content of selected Fn preparations were measured using a commercial immunoassay kit from Boehringer Mannheim Co.

6. Binding assays:

^{125}I labeled Fn (0.02ml) was added to varying concentrations of target cells suspended in 0.5 ml PBS with 3.5% BSA. Cells were incubated at 37 C in 5% CO_2 for one or seven hours, washed 3x in PBS and centrifuged at 200xg for 10 minutes. Supernatants were removed and the radioactivity in the labeled cell pellet was measured in a gamma counter. To determine non-specific adherence of Fn to the plastic tubes, control tubes were set up which contained no target cells.

C. Cellular preparations

1. Isolation of peripheral blood lymphocytes:

Blood samples were drawn aseptically and defibrinated using sterile glass beads. The blood was diluted 1:3 with PBS, layered onto Ficoll-Hypaque (Pharmacia) and centrifuged at 400xg for 20 minutes following the procedure of Boyum et al. (1968). The lymphocyte band was collected with a pasteur pipette and washed 2x with RPMI media. Mononuclear cells were counted using a hemocytometer. Cell viability was determined by trypan blue dye exclusion.

2. Isolation of non-adherent cell populations:

PBL were resuspended in 5% FCS-RPMI and allowed to settle on a 100x15 mm plastic petri dish (Falcon) for 1 hour at 37 C in 5% CO_2. After 1 hour, the plate was washed by vigorous pipetting using fresh RPMI. Washings were collected and non-adherent cells were adjusted to the appropriate concentration.

3. Giemsa staining:

Cell smears or microtiter plate wells containing adherent cells were air dried and placed in May-Grunwald stain (Searle) (0.25 gm in 200 ml methly alcohol) for 3 minutes. Stain was decanted and Giemsa stain

(30 drops Giemsa in 30 ml deionized water) was added for 15 minutes. Giemsa was poured off and surfaces were rinsed in deionized water.

4. Monoesterase staining:

Cell suspensions in media plus 10% serum were spread on glass slides and allowed to air dry. Slides were fixed and stained following the procedure of Koski et al. (1976).

5. Labeling of target cells or lymphocytes:

K562 target cells, 10^6-10^7, were washed 3x in RPMI and resuspended in 0.02 ml media. One hundred uCi of $Na^{51}CrO_4$ (New England Nuclear) were added to cells and allowed to incubate at 37 C for 1 hour with occasional mixing. After 1 hour, the cells were washed 3x in RPMI and adjusted to the working concentration ($4x10^5$ or $8x10^5$ cells/well).

D. Cellular assays

1. NK Assay:

Target cells were dispensed (0.05ml/well) into 96 well round bottom microtiter plates (Cooke) at concentrations of $2x10^4$ or $4x10^4$ cells per well. Various concentrations of Fn, BSA or media were added in 0.05 ml aliquots. In certain experiments, targets were preincubated with Fn or

BSA prior to the addition of effectors; or effectors were preincubated with Fn or BSA prior to the addition of targets. When possible, 0.1 ml of effector cells was added last into the well in such a manner that adequate mixing occured. After all additions were completed, plates were centrifuged at 250xg for 5 minutes. Plates were incubated at 37 C 5% CO_2 for 4 or 18 hours. They were spun at 250xg for 5 minutes, and 0.1 ml of supernatant was removed and counted in a gamma counter. Spontaneous release (SR) of ^{51}Cr from target cells was determined by adding 0.1 ml media to 0.05 ml targets plus 0.05 ml Fn or BSA. Maximum release (MR) wells contain 0.1 ml of 5% triton X. Percent lysis was calculated as:

$$\% \text{ Target Lysis} = \frac{\text{experimental cpm} - \text{SR cpm}}{\text{MR cpm} - \text{SR cpm.}} \times 100$$

Assays are performed in triplicate or quadruplicate. Percent spontaneous release was determined as SR/MR x100.

2. MLR:

PBL were isolated and resuspended in RPMI with 10% FCS at $2x10^6$ responder cells/ml and $4x10^6$ stimulator cells/ml. Stimulator cells were irradiated for 10 minutes at 110 kVp and 3.5 mA (Faxitron Series X-ray machine). Stimulator cells were dispensed in 0.05 ml volume into flat

bottom 96 well microtiter plates (Linbro). Fn or control media was added in a 0.05 ml volume. Responder cells were added in a 0.1 ml volume in such a manner as to insure adequate mixing of cells. Plates were incubated at 37 C in 5% CO_2 and then labeled on day 5 with 0.5 uCi/well of ^{3}H-thymidine for 16-18 hours. Cultures were harvested onto glass fiber filter disks using a Brandel model 12V cell harvester. Radioactivity retained on the filter disk was measured in a scintillation counter using 5 ml of Ready Solv liquid scintillation fluid (Beckman) dispensed into vials containing the filter disks. MLR results were calculated as mean cpm of responders alone (R_x) subtracted from the mean cpm of the allogeneic response (R_xS_x) ie., mean(R_xS_x)-mean(R_x) $\pm$ SE of triplicate or quadruplicate samples.

Results and Discussion

A. Fn preparations

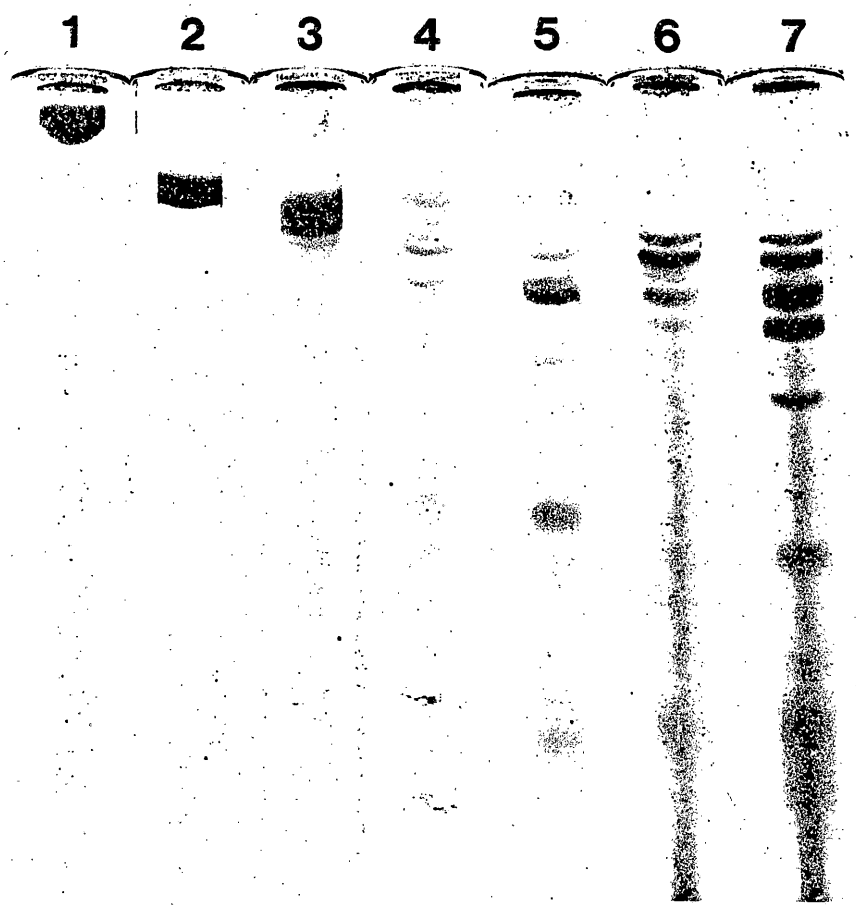
In the research reported here, sixteen different Fn preparations were isolated by gelatin-Sepharose affinity chromatography. Eight of these Fns were from individual plasma donors, while the remaining eight were made from the byproducts of factor VIII production (slime A). Ten preparations were analyzed on 5.0% SDS polyacrylamide gels under both reducing and nonreducing conditions. Five preparations were treated with plasmin and then analyzed on SDS-gels. In addition to Fns prepared by the author, two Fn preparations were obtained from outside sources. Representative gels of the Fn preparations are shown in Figure 1. Periodically, the biological activity of the preparations used in this research was determined by their ability to agglutinate gelatin coated latex beads and to bind to Staphylococcus aureus. Representative preparations were tested for antigenic activity by the use of a commercial immunoassay kit, and found to have similar or higher Fn concentrations than measured by optical density at 280 nm and using an extinction coefficient of 1.28 $mg^{-1}ml$.

B. Natural Killing Results

Initial NK assays were performed to demonstrate that the experimental procedures used resulted in adequate levels of killing and to de-

Figure 1. Polyacrylamide disc gel electrophoresis of Fn preparations.

Fn preparations were run on 5.0% SDS polyacrylamide disc gels under reducing and non reducing conditions. Beginning with lane 1, gels show non-reduced preparations of 1) JD Fn(3-3) and reduced preparations of 2) JD Fn (3-3), 3) JJM Fn, 4) slime A Fn (1-3), 5) slime A Fn (1-10), 6) JG Fn (5-20) plasmin cleaved, 7) slime A Fn (5-20) plasmin cleaved.


1
2
3
4
5
6
7

termine if either fresh or frozen cells could be used as effector cells. Adherent cell depleted mononuclear cells from seven different donors were used as effector cells in these experiments at effector:target (E:T) ratios ranging from 5:1 to 100:1. The results of two typical experiments are shown in Table 1. These experiments demostrate adequate killing over a wide range of effector:target ratios. The percentage spontaneous release, as calculated by the formula given in Materials and Methods, was consistently lower than 15%. Throughout these and subsequent experiments, some donors showed consistently high NK mediated cytotoxicity against K562 while other donors displayed low levels of killing.

Effector cells were normally isolated and used on the same day. However, in searching for a more convenient approach, effector cells were frozen in a controlled rate freezer and stored in liquid nitrogen until use, or, following isolation, cells were left in media at 4 C overnight and used the following day. Although viability of cells stored in liquid nitrogen was consistently greater then 90%, the killing ability of these cells was significantly diminished from fresh cells as shown in Table 2. Storing cells overnight for use the next day did not alter NK activity (Table 2). This same effect was seen using donor cells DF and WO (data not shown). Thus, in all further experiments, effectors were freshly isolated and used within a 24 hour period.

To determine if Fn mediated a dose-dependent effect on NK cytotoxicity, NK assays were performed using E:T ratios ranging from 5:1 to 100:1 and Fn concentrations ranging from 0 ug/ml to 500 ug/ml. Adherent cell depleted mononuclear cells isolated from four different individuals

Table 1: NK activity against K562 cells

Expt. 1		Expt. 2	
E:T ratio	% lysis	E:T ratio	% lysis
5:1	27 ± 3	10:1	21 ± 2
10:1	41 ± 2	25:1	39 ± 3
20:1	52 ± 3	35:1	49 ± 3
30:1	68 ± 5	50:1	60 ± 4
50:1	76 ± 4	70:1	65 ± 4

NK activity of two different effector cells against K562 target cells. Cytotoxicity is given as mean % lysis ± SE of triplicate cultures. Donor cells used were 1) JW, 2) DF.

Table 2: Comparison of effector cell types

effector cell type		% lysis
JD	fresh	50%
	frozen	12%
DDo	same day	55%
	next day	52%

Comparison of NK activity using either a) fresh or frozen effector cells, or b) cells used immediately after isolation or the next day. All E:T ratios shown here are 30:1.

were used as effector cells in these experiments. Bovine serum albumin (BSA) was used as a control for non-specific protein interactions. Results of a representative experiment of four experiments are shown in Table 3. At each of the E:T ratios shown, neither Fn or BSA at any concentration tested produced a significant difference in percent lysis. The Fn used in this experiment was freshly prepared immediately before use from a single plasma donor (DMo).

In order to determine if this lack of effect was restricted to this single Fn isolate, a separate experiment was performed in which two different Fn preparations were used with effector cells from one individual (DF). Revlon 34 Fn and slime A Fn (7-20) were diluted to achieve a final concentration of 264 ug/ml and added to 4 hour NK assays using E:T ratios ranging from 2.5:1 to 50:1. The results of this experiment (Figure 2) indicate that no significant effects on NK activity were observed with either Fn preparation. Using yet another cell donor (JW) in a dose-response experiment, Revlon 34 Fn at 0, 300 or 500 ug/ml showed no effect on NK activity (Figure 3). Two additional experiments, using cell donors JD and JG, also showed no Fn effect (data not shown).

In an effort to determine if Fn interacts with monocytes to play a role in NK activity, experiments were performed using both adherent depleted and whole mononuclear cell preparations. In experiments using 4 hour assay conditions, whole mononuclear effector cells showed slightly higher percent lysis of target cells than did adherent depleted cells. Fn, added at concentrations of 100 ug/ml or 300 ug/ml, did not alter NK activity in either the presence or absence of effector cells containing

Table 3: Dose-dependent effect of Fn on NK cells

Treatment		Effector:Target Ratios 5:1	15:1	30:1	50:1
0 media		-0.1 ± 3	10 ± 4	20 ± 7	27 ± 7
50 ug/ml	BSA	-0.3 ± 3	8 ± 4	21 ± 6	30 ± 7
	Fn	0.7 ± 3	10 ± 4	17 ± 6	25 ± 6
100 ug/ml	BSA	-0.5 ± 3	7 ± 4	21 ± 6	26 ± 6
	Fn	0.6 ± 3	9 ± 4	14 ± 5	25 ± 6
200 ug/ml	BSA	-0.5 ± 3	7 ± 4	17 ± 5	25 ± 6
	Fn	-0.6 ± 3	8 ± 4	14 ± 5	25 ± 6
300 ug/ml	BSA	-0.9 ± 3	7 ± 4	17 ± 5	23 ± 6
	Fn	-0.3 ± 3	8 ± 4	16 ± 5	23 ± 6

Influence of increasing Fn DMo 7-25 concentrations on NK activity using effector cells from donor JG. Values are mean % lysis ± SE. Fn and BSA treatments were not significantly different by Student's t analysis.

Figure 2. Effect of Slime A Fn and Rev 34 Fn on NK Activity.

Influence of two different Fn preparations on NK activity using DF effector cells. Proteins present at 264 ug/ml: (▲) Rev 34 Fn, (■) Slime A Fn, and (●) BSA control. Results are expressed as mean % lysis ± SE.

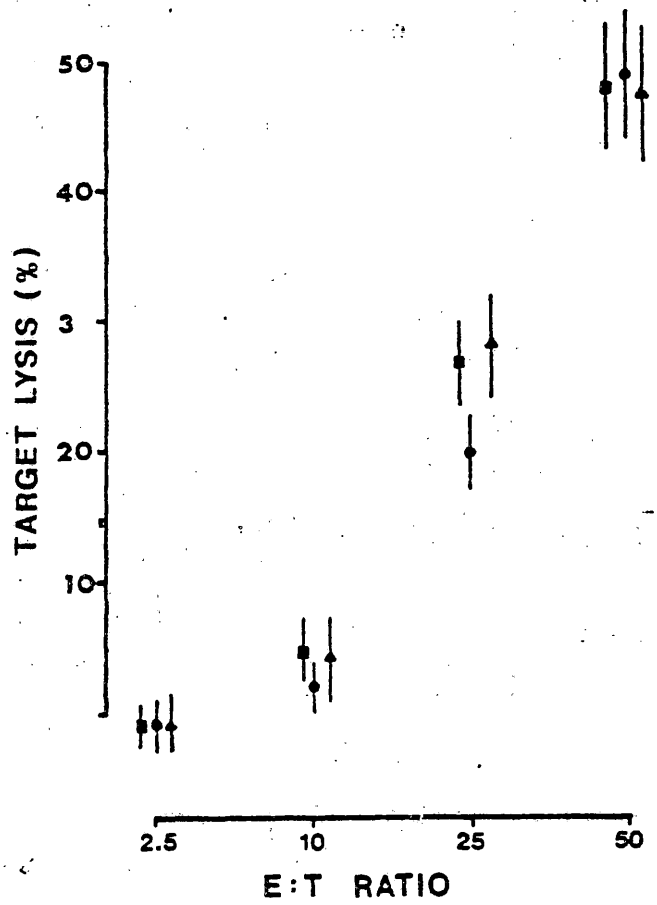
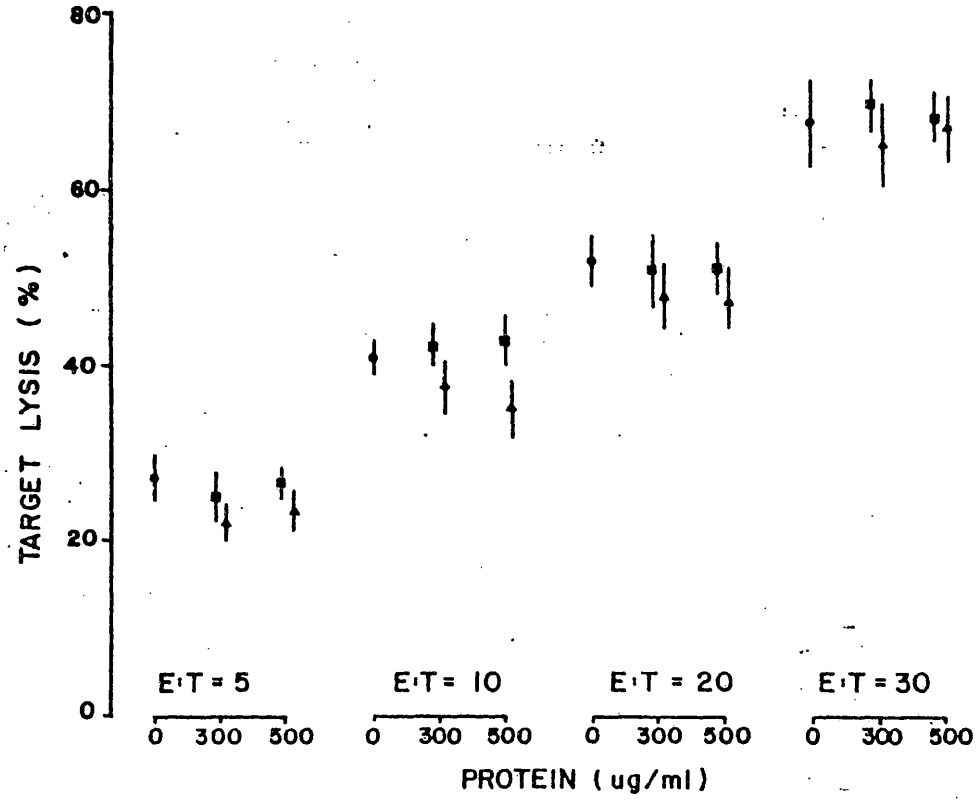

50
40
3
20
10
TARGET LYSIS (%)
2.5
10
25
50
E:T RATIO

Figure 3. NK Activity at Two Fn Concentrations.

NK activity of JW effector cells in the presence of media alone (●), BSA control (▲), or Rev 34 Fn (■). Protein concentration was either 300 or 500 ug/ml as indicated.


80
60
40
20
0
TARGET LYSIS (%)
E:T = 5
E:T = 10
E:T = 20
E:T = 30
0 300 500
0 300 500
0 300 500
0 300 500
PROTEIN (ug/ml)

monocytes. A second experiment using different effector cells, showed similar results (Figure 4). In the presence of 300 ug/ml or 500 ug/ml, Fn did not alter cytolysis of K562 by either non-adherent effector cells or effector cells containing monocytes. These experiments indicate that in a 4 hour assay system, monocytes in concentrations normally present in whole mononuclear cell preparations, do not interact with Fn to cause a change in NK cell mediated lysis.

Monocytes are known to have cytolytic activity in 18 hour assay systems. Unlike NK cells which only require 4 hours for killing, monocytes need at least 18 hours to kill target cells (Herberman 1982a). Therefore, experiments were performed using 18 hour incubation periods. One representative experiment is shown in Figure 5. Compared to the 4 hour system, higher percent lysis was achieved in the 18 hour assay; however, the presence of Fn had no effect on cytolysis either in the presence or absence of monocytes.

Next, adherent depleted cell preparations were used in 18 hour assays to determine if pre-treatment of Fn with cells would affect NK activity. Effector cells or chromium labeled target cells were preincubated with 93 ug of Fn or BSA per well, or media for a period of 4 hours. At the end of the incubation period, untreated targets or effectors were added and after 18 hours, chromium release was measured. As can be seen in Figure 6, cell lysis was not altered by the pre-incubation of Fn with either K562 targets or effectors.

Experiments were also performed to assess Fn's ability to bind to K562 cells. In the first experiment, 13 ug of radiolabeled slime A Fn

Figure 4. NK Activity using Adherent-Depleted and Whole Mononuclear Cell Preparations as Effectors.

NK activity of two different effector populations from donor JW: hatched bars represent whole mononuclear cell populations, nonhatched bars represent adherent cell depleted effectors. Clear bars are media controls, open circled bars represent assays performed in the presence of 300 ug Rev 34 Fn/ml, dotted bars with 500 ug Rev 34 Fn/ml.

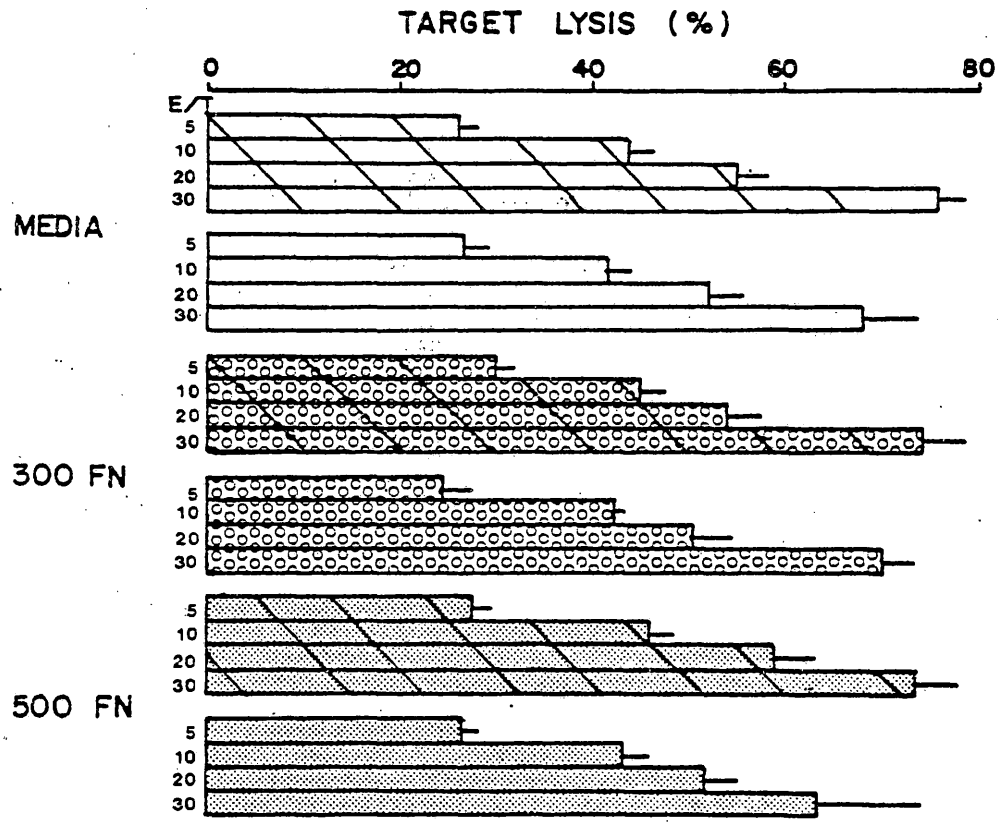

TARGET LYSIS (%)
0
20
40
60
80
E/T
5
10
20
30
MEDIA
300 FN
500 FN

Figure 5. NK Activity in 4 and 18 Hour Assays in the Presence or Absence of Monocytes.

NK activity is shown in 4 (left panel) or 18 (right panel) hour assays. Open figures (□, ○) show JD whole mononuclear cell preparations, while solid symbols (■, ●) represent JD non-adherent effector cells. Dotted lines represent Fn treatment (500 ug Rev 34/ml), solid lines represent media controls. Values are given as mean % lysis. Error bars are not shown as none of the values differed significantly from media controls using the Student's t test.

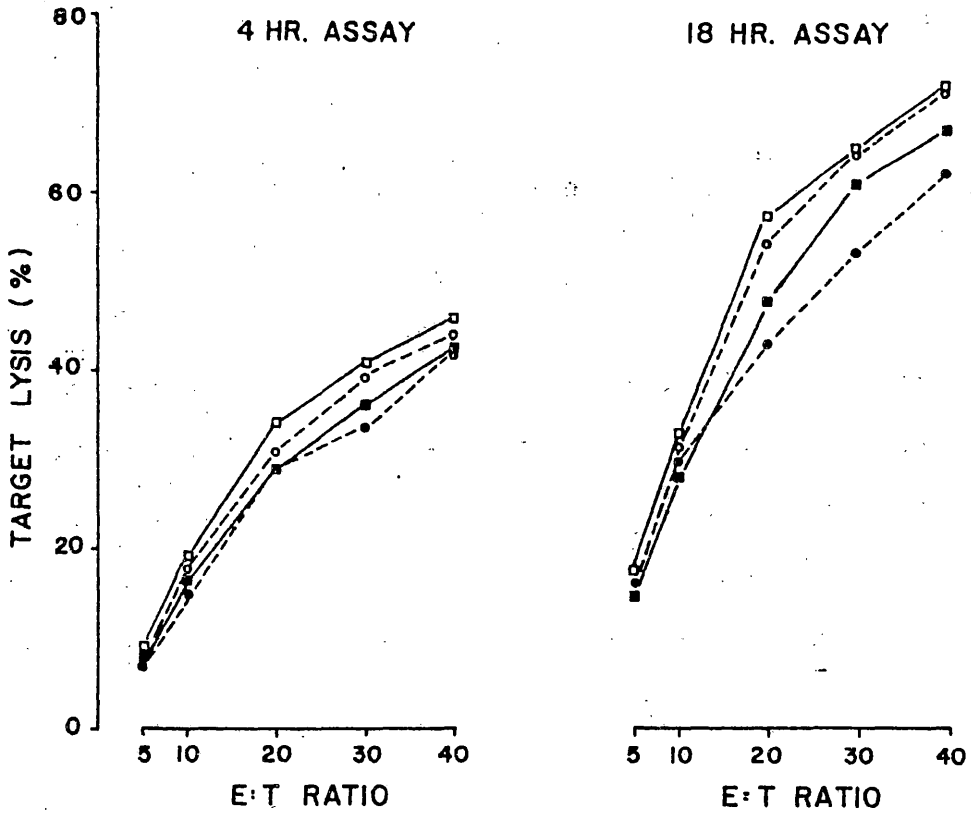
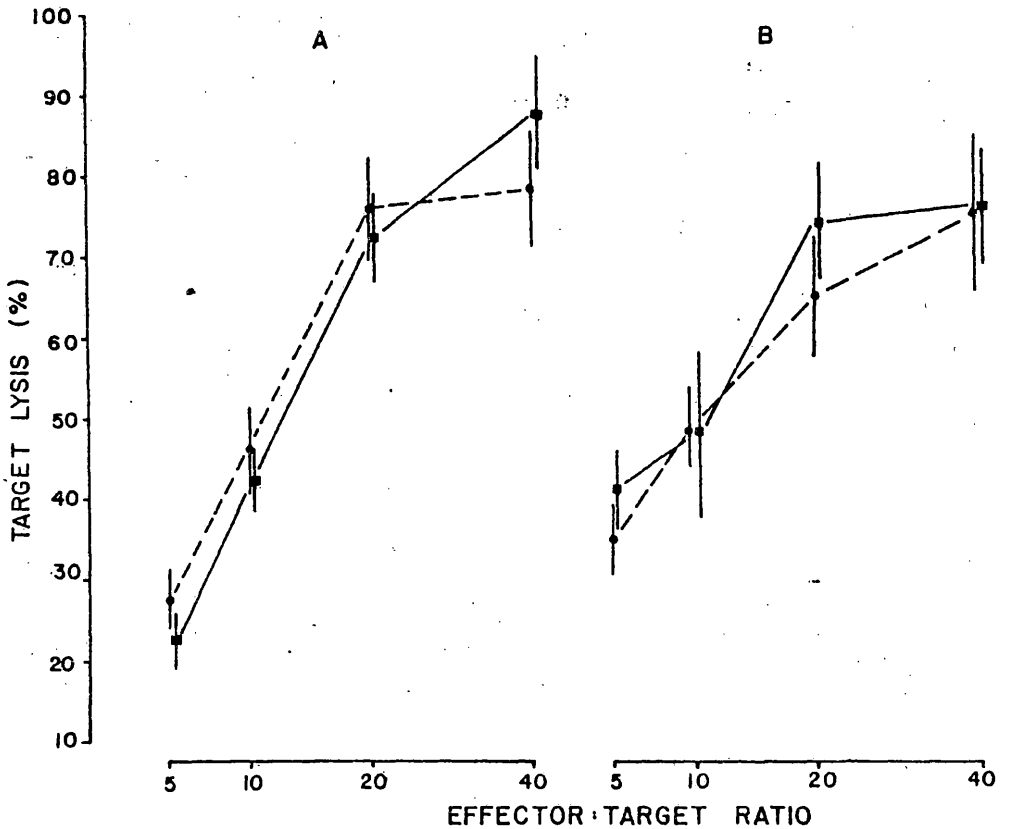

4 HR. ASSAY
18 HR. ASSAY
TARGET LYSIS (%)
0
20
40
60
80
5
10
20
30
40
E:T RATIO
E:T RATIO

Figure 6. Effect of Pretreatment of Effectors or Targets with Fn.

NK activity using DF effector cells (Panel A) or target cells (Panel B) preincubated with 465 ug/ml of DDo Fn.


A
B
100
90
80
70
60
50
40
30
20
10
TARGET LYSIS (%)
5
10
20
40
5
10
20
40
EFFECTOR : TARGET RATIO

was added to tubes containing various numbers of K562 cells. Table 4 shows the amount of ^{125}I-Fn bound to these cells at the end of 1 hour incubation. In a second experiment 12 ug of a radiolabeled single donor Fn was added to K562 cells and incubated for seven hours. Significant binding of Fn by target cells was not demonstrated in either experiment. By calculating the amount of radioactivity per molecule of Fn, one can estimate that <100 molecules of Fn were bound to each K562 cell. Surface cellular receptors known to mediate immune function, are present on cell surfaces in considerable numbers. For example, on thioglycollate induced rat macrophages, $1x10^5$ Fc receptors/cell have been detected (Ezekowitz and Gordon 1982). Thus it would appear unlikely that the small amount of Fn bound to K562 cells would be able to interfere or potentiate immune reactivity directed against these cells.

Table 4: K562 binding experiment

cell concentration	mean cpm ± SD
0	131 ± 26
$1x10^6$	387 ± 16
$2.5x10^6$	591 ± 14
$5x10^6$	822 ± 189
$8x10^6$	794 ± 115

Results of ^{125}I-Fn binding to K562 target cells after a one hour incubation. Total ^{125}I-Fn added per tube was 13 ug or 745,348 cpm.

C. NK Discussion

NK cells are thought to act as an important first line defense against tumor development because of their ability to rapidly lyse tumor cells, virally infected cells and other types of target cells. Before lysis can occur, the NK cell must effectively bind to the target cell via some type of receptor-ligand interaction (Hiserodt et al. 1982a). Because Fn is present on the surface of some types of tumor cells (Stenman and Vahaheri 1981, Gibert et al. 1982) and is also known to promote cell-to-cell (Yamada et al. 1978) and cell-to-substrate (Rajaraman 1983) adhesion, experiments were performed to examine the possible role of plasma Fn in NK cell mediated lysis.

During the course of these experiments, numerous conditions were varied in an attempt to show a Fn effect on NK cell mediated lysis. Parameters which were varied included: E:T ratios, source and concentration of Fn added, presence or absence of monocytes, length of incubation times and pre-treatment of effectors or target cells with Fn.

The finding that frozen effector cells exhibit lower killing ability than fresh cells is consistent with the published observations of other investigators (Callery et al. 1980, Strong et al. 1982). These studies, as well as the one reported here, indicate that frozen effector cells demonstrate a significant loss of NK activity against K562 targets in 4 hour assays.

In experiments reported here, three different preparations of plasma Fn were used; Fn isolated from single plasma donors, Fn obtained commer-

cially from Revlon Corp., and Fn isolated from the byproduct of factor VIII preparation (slime A). Each of the preparations showed antigenic activity as measured in a Boehringer-Mannheim kit assay and functional activity as measured by their ability to agglutinate gelatin coated gelatin beads and to bind to cells of Staphylococcus aureus. Thus, each of these Fn preparations had seemingly intact functional properties, yet failed to influence NK activity in vitro.

The fact that plasma Fn had no effect on NK cell mediated lysis does not preclude the possibility that cellular Fn might be active at the effector-target contact region. In studying cell-to-cell adhesion, Yamamada et al.(1982) reported that glutaraldehyde fixed and trypsinized human erythrocytes were readily agglutinated by cellular Fn, but only poorly by plasma Fn. Thus, even though plasma Fn can mediate cell-to-substrate adhesion and transform cells to a more normal morphology, cell-to-cell adhesion may be mediated successfully only by cellular Fn. This possibility is addressed indirectly by the action of monensin, a carboxylic ionophore, that blocks the lytic activity of NK cells (Carpen et al. 1982). Interestingly, monensin has also been shown to block the secretion of Fn by human fibroblasts such that Fn may be found in the cytoplasm of these cells but is not bound to the cell surface (Virtanen et al. 1982). One can speculate that if NK cell cytolysis depended on cellular Fn at the effector-target binding site, then monensin treatment of NK cells should destroy the NK cytolytic activity. This, however, is not the case. Although monensin does block cytolytic activity by NK cells, this blockage is not due to lack of NK cell to target cell bind-

ing. The formation of cell-to-cell contact in these experiments was normal (Carpen et al. 1982). Thus, it appears unlikely that even cellular Fn has an active role in NK cell mediated lysis.

Monocytes are known to bind Fn to their cell surfaces (Bevilacqua et al. 1981), but in experiments reported here, the presence of monocytes in the effector cell population did not alter cytolytic activity when Fn was included in the assay. Fn did not enhance a monocyte mediated killing effect in either 4 or 18 hour assays against K562 cells. However, using different assay conditions and other target cells, Perri et al. have shown that plasma Fn enhances in vitro monocyte-macrophage mediated tumoricidal activity (Perri et al. 1982). In 48 hour assays, exogenous plasma Fn, added to wells containing monocyte/macrophage monolayers and radiolabeled tumor cells, consistently caused a dose dependent enhancement of tumoricidal activity against two cultured human tumor cell lines; Malme melanoma and CAK-1 renal carcinoma. These authors suggested that this enhancement was caused by a bridging effect of soluble Fn resulting in increased monocyte/macrophage-tumor cell interaction. Such a Fn mediated increased interaction between K562 targets and NK cells is unlikely to occur since Fn apparently does not bind to K562 cells or to lymphocytes, which is the cell preparation containing NK cells during Ficoll-Hypaque isolation. To confirm the lack of Fn binding to NK effector cells, LGL cells could be isolated by Percoll density gradient and assessed for their ability to bind iodinated Fn. If no binding could be demonstrated, it would strengthen the claim that Fn has no direct effect on in vitro NK activity.

Fn might have an indirect effect on NK cells by stimulating macrophages to produce interferon or other substances known to augment NK activity. In a preliminary report, Beezhold et al. (1983) have reported that plasma Fn stimulates macrophages to produce a factor which augments lymphoproliferative responses to mitogens. However, in the 18 hour cytotoxicity reported here, Fn apparently did not stimulate monocyte production of factors which influence NK cell mediated lysis of K562 cells.

A preliminary report by Reese et al. (1983) states that Fn has the ability to act synergistically with murine NK cells to lyse MCG-T14 mouse mammary carcinoma cells in 4 hour assays. In their report, splenocyte cell populations were used as the source of effector cells. These cell preparations were not depleted of macrophages which in humans are known to interact with Fn and augment lysis of target cells in 48 hour assays (Perri et al. 1982). Since their initial publication, Reese et al. have obtained variable results when using rat Fn with murine effector spleen cells. They attribute their variable Fn effects on NK activity to macrophage secretion of stimulatory and inhibitory substances (Reese, personal communication). However, since they are using Fn isolated from a different species than their effector cells, it is difficult to interpret their data.

D. MLR Results

Human lymphocytes generally exhibit a log phase of proliferation to alloantigen from 4-6 days of a mixed lymphocyte response (Du Bois et al. 1974). To determine if Fn's effect on the MLR followed similar kinetics, a time course study was performed. Table 5 shows representative results of a 3 day time course study. In this experiment, plasma Fn was added to culture wells at concentrations of 9, 18, 45 and 90 ug/well. Cultures were labeled with ^{3}H-thymidine 18 hours prior to harvesting. The influence of Fn on the MLR was the most pronounced in cultures harvested on day 6. Thus in all future experiments, cultures were pulsed on day 5 and harvested on day 6.

Following determination of the optimum time course of proliferation, a total of 21 different Fn treatments were tested for their effect on the MLR using lymphocytes from ten different donors. Monoesterase stains were performed on final lymphocyte preparations to determine monocyte content of plated cells. Monocyte content was routinely 5-12% of total mononuclear cells; Fn's influence on the MLR appeared unrelated to monocyte concentration of either responder or stimulator cells within this range.

In general, the results of these experiments support the following claims: a) Fn made from byproducts of factor VIII preparation (slime A), which showed additional protein bands on SDS gels, caused an enhancement of the MLR, b) a lyophilized preparation showing only intact 440 Kd MW molecules on non-reducing SDS gels, showed suppression of the MLR,

Table 5: MLR time course study

Harvest day	ug/culture of Fn				
	0	9	18	45	90
5	16.2 ± 0.9	18.8 ± 0.5	18.5 ± 0.3	18.2 ± 6.2	19.9 ± 1.9
6	11.4 ± 0.5	15.2 ± 1.7	25.3 ± 5.6	28.9 ± 2.6	21.8 ± 0.3
7	14.7 ± 2.1	22.6 ± 2.7	23.6 ± 4.2	18.2 ± 1.3	17.5 ± 0.7

Time course study showing the dose-dependent effect of Fn DDo 8-23 on the allogeneic mixed lymphocyte response. Values are mean cpm ± SD x 10^{-3}. R_2S_1 donor cells were 1) DMo 2) ML.

c) freshly made Fns from individual plasma donors, showing no detectable fragmentation or contaminating proteins on SDS gels, suppress the MLR, and d) Fn made from individual donors and from slime A which was purposely cleaved with plasmin, caused no effect on the MLR.

Six different slime A Fn preparations and two Fns isolated from individual plasma donors all caused enhancement of the MLR. These preparations demonstrated good gelatin binding activity as measured by their ability to clump gelatin coated latex beads, but as can be seen in Figure 1, these preparations showed multiple protein bands on both reduced and non-reduced SDS gels.

Fn's enhancing effect on the MLR can be seen in Figure 7. In this experiment, slime A Fn 11-10 or human serum albumin (HSA) control was added to culture wells at concentrations of 10, 20, or 40 ug/well. In the presence of Fn, the proliferation showed a dose-dependent enhancement when compared to HSA and media control wells. Significant enhancement was seen at Fn concentrations as low as 10 ug/well corresponding to a physiological concentration of 50 ug/ml.

Using three different allogeneic pairs, slime A Fn 1-3 also caused a concentration dependent enhancement in all cellular combinations and their reciprocal pairings; i.e., R_1S_2 vs R_2S_1. In Table 6, the six possible responder and stimulator pairings are shown. In each case, enhancement of the response could be seen at the lowest concentration of Fn tested (15 ug/well); enhancement increased as the Fn concentration was raised to 45 ug/well.

Table 7 shows the results of an experiment in which three different

Figure 7. Enhancing Effect of Slime A Fn 11-10 on the MLR.

Effect of Slime A Fn (11-10) shown as the solid squares on the MLR using donor cells RP (responder 1), or SA (responder 2). Solid circles represent HSA controls. Results represent specific stimulation: mean cpm of $R_2S_1 - R_2 \pm SE \times 10^{-3}$.

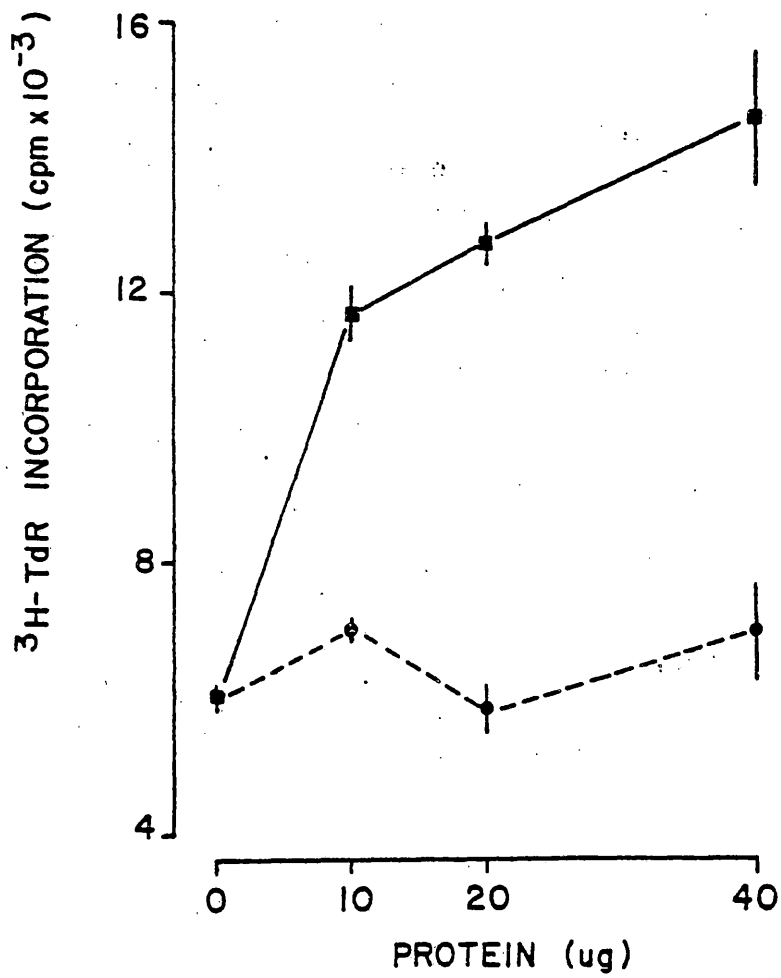

16
12
8
4
^{3}H-TdR INCORPORATION (cpm x 10^{-3})
0
10
20
40
PROTEIN (ug)

Table 6: Fn enhancement of the MLR using six allogeneic pairs

			protein concentration ug/culture		
	0 media		15	30	45
R_1S_2	6.8 ± 0.5	Fn	8.8 ± 0.1	11.1 ± 0.5	13.5 ± 0.6
		BSA	6.0 ± 0.1	6.4 ± 0.1	7.6 ± 0.3
R_2S_1	7.9 ± 0.2	Fn	11.0 ± 0.5	12.5 ± 0.5	18.1 ± 0.4
		BSA	12.3 ± 0.2	10.1 ± 0.3	11.8 ± 0.3
R_1S_3	3.3 ± 0.3	Fn	5.1 ± 0.3	5.8 ± 0.9	7.6 ± 1.0
		BSA	3.4 ± 0.5	2.7 ± 0.7	2.3 ± 0.5
R_3S_1	2.2 ± 0.5	Fn	4.2 ± 0.5	7.1 ± 0.7	8.4 ± 0.5
		BSA	1.9 ± 0.2	1.9 ± 0.2	3.2 ± 0.3
R_2S_3	6.8 ± 0.9	Fn	9.4 ± 0.9	10.0 ± 0.7	14.6 ± 0.9
		BSA	8.6 ± 0.6	6.6 ± 0.2	7.7 ± 0.8
R_3S_2	4.8 ± 0.8	Fn	8.2 ± 0.8	13.1 ± 0.5	13.9 ± 1.3
		BSA	4.0 ± 0.6	4.3 ± 0.2	5.0 ± 0.3

Slime A Fn 1-3 causes a dose-dependent enhancement of six different allogeneic MLR pairings, beginning with concentrations as low as 15 ug/culture. Values are given as mean cpm (R_xS_x-R_x) ± SE. Donor cells were 1) SM 2) RC 3) ML.

Table 7: Comparison of the effect of three slime A Fns on the MLR

	ug/culture	mean cpm x 10^{-3}
	0	6.3 ± 0.2
Fn 1-4	15	9.7 ± 0.4
	31	9.6 ± 0.1
	46	12.4 ± 0.3
Fn 1-10	23	9.9 ± 0.2
	46	10.6 ± 0.2
	69	9.0 ± 0.2
Fn 1-31	19	9.3 ± 0.2
	39	9.8 ± 0.2
	58	11.1 ± 0.3

Comparison of the effect of three different slime A Fn preparations on the MLR pairing R_1S_2. Donor cells were 1) JaG 2) DF. Values are given as mean cpm $(R_1S_2-R_1) \pm SE \times 10^{-3}$.

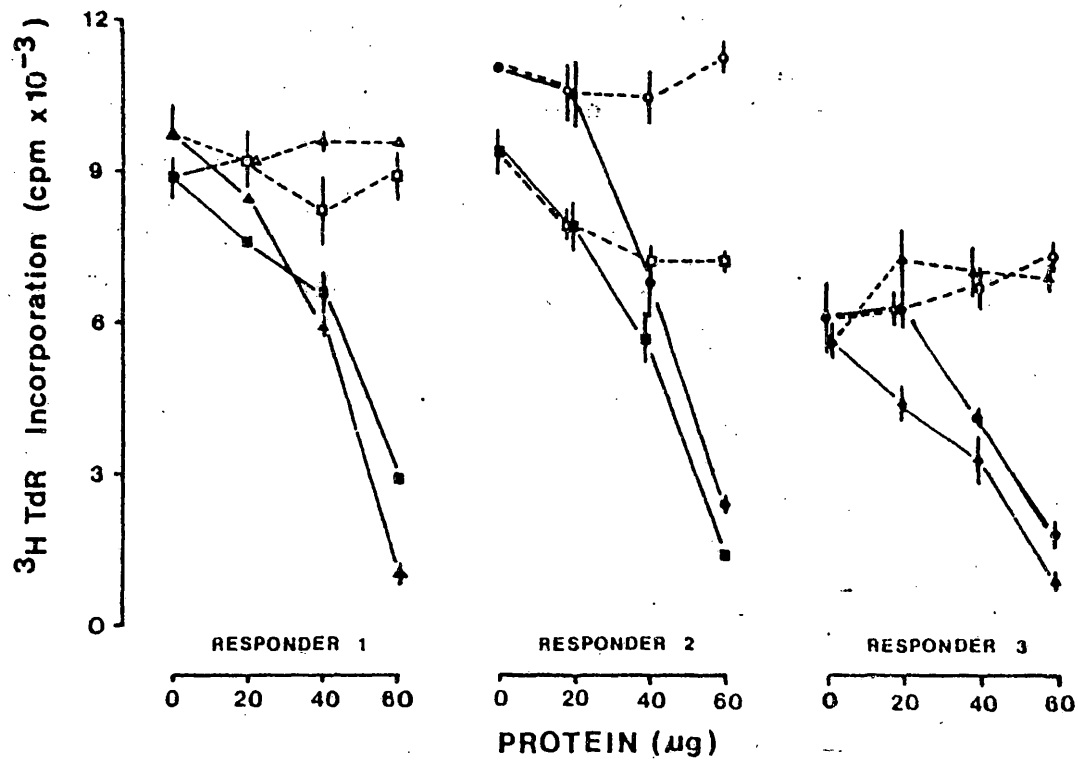
slime A Fn preparations were used with one allogeneic pair. Each of the three slime A Fn preparations resulted in significant enhancement of the MLR. Results from the experiments described thus far, led to the conclusion that Fn enhances the proliferative response between allogeneic lymphocytes. However, due to the appearance of multiple protein bands on SDS gels, it was not possible to rule out the possibility that this enhancement was caused by a fragment of Fn or a non-Fn contaminant.

Due to the variation in appearance of Fn preparations on SDS gels, a Fn preparation was tested which was obtained from Dr. Jean-Jacques Morganthaler of the Central Laboratory of the Swiss Red Cross Transfusion Service. This Fn, designated JJM, was made in the presence of the protease inhibitor phenylmethyl sulfonyl fluoride (PMSF) and sodium azide, extensively dialyzed and finally lyophilized. JJM Fn displayed the characteristic Fn pattern without secondary bands on reduced and non-reduced SDS gels stained with Coomassie Blue dye. When tested in MLRs using three separate allogeneic pairs, this Fn caused a marked suppression of all responses (Figure 8). JJM Fn not only suppressed the allogeneic response, but also caused an inhibition of ^{3}H-thymidine uptake in culture wells containing only responder cells, media and Fn. This suppression occurred at the lowest concentration of Fn present, 20 ug/well, and showed a dose-dependent response through concentrations of 60 ug/well.

The inhibition of the MLR mediated by JJM Fn was not the result of a cytotoxic effect of Fn on responding cells. Cell viability of responder cells was determined for cells treated with all Fn concentra-

Figure 8. Suppressing Effect of JJM Fn on the MLR.

Inhibition of the MLR by JJM Fn is shown using six different allogeneic pairs. Solid lines and symbols represent Fn addition, dotted lines and open symbols represent HSA controls. Each panel shows one responder cell type plated with two different stimulator cell types. Stimulator cells (●) SM, (▲)RC, (■)JW. Results are expressed as cpm specific stimulation.


^{3}H TdR Incorporation (cpm x10^{-3})
12
9
6
3
0
RESPONDER 1
RESPONDER 2
RESPONDER 3
0
20
40
60
PROTEIN (µg)

tions, viability was determined by trypan blue dye exclusion on day 6 of culture. At each concentration tested, cell number and viability remained similar to that of control wells: $7.6x10^5$ cells/well, 89% viability, $6.9x10^5$ cells/well, 83% viability, $7.5x10^5$ cells/well,85% viability, at 0, 30, and 60 ug of Fn per well respectively.

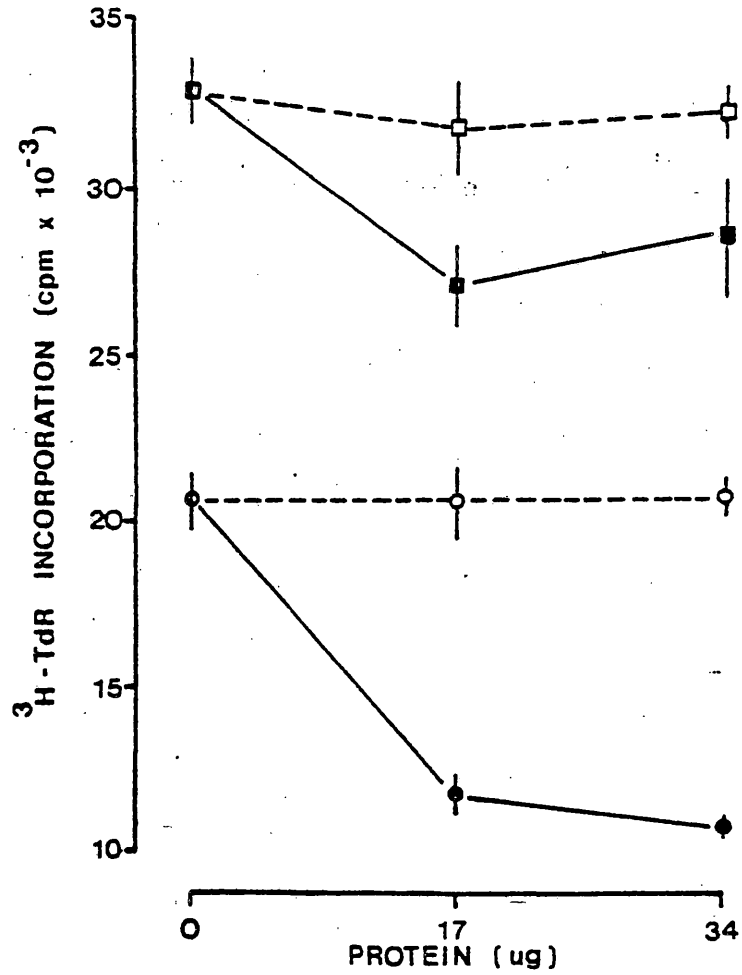
Since inhibition of the MLR was seen only when using a non-fragmented Fn preparation, efforts were made to isolate intact Fn from an individual plasma donor. The JD 3-2 Fn was isolated very carefully by a) washing the gelatin-Sepharose in batch with 4M urea prior to incubating with plasma, b) washing the gelatin-Sepharose plasma column extensively with PBS c) adjusting the flow of the column to a very slow drip rate, and d) perhaps most importantly, using the Fn immediately after isolation. Fn JD 3-2 appeared completely intact on gels run immediately after Fn preparation, and when tested in the MLR, it caused a suppression of the response. Figure 9 shows suppressed responses in experiments using different numbers of stimulator cells. Suppression occurred at $1x10^5$and $2x10^5$ stimulator cells/well. The findings in this experiment suppport the theory that intact molecules of Fn suppress the MLR.

To test the possibility that Fn fragments were responsible for enhancing effects, five different Fn preparations were made and treated with plasmin. Two additional Fn preparations were left at 37 C for 24 or 72 hours in an attempt to achieve autodegradation. Only 3 of the five attempts at plasmin cleavage resulted in good fragmentation on gels, and neither of the Fns left at 37 C revealed any fragments.

In two experiments using a single donor Fn isolated in the presence

Figure 9. Suppressive Effect of Fn JD 3-2 on the MLR Using Two Different Stimulator Concentrations.

Inhibition of the MLR using JD 3-2 Fn is shown by the solid symbols. HSA controls are shown in open symbols. Squares represent a stimulator concentration of 2×10^5 cells/well. Circles represent stimulator concentrations of 1×10^5 cells/well. Values represent cpm of specific stimulation. Donor cells were 1) DF, 2) RC.


35
30
25
20
15
10
^{3}H-TdR INCORPORATION (cpm x 10^{-3})
0
17
34
PROTEIN (ug)

of PMSF, an aliquot was left at 37 C for 72 hours and a second portion was treated with plasmin. Neither of these preparations showed cleavage products on gels. When tested in MLRs they had no significant effect on proliferative response. Table 8 shows the representative results of both of these experiments. A third experiment which was performed using a Fn made in the absence of PMSF, showed similar results (data not shown).

Up to this point, the only inhibition of the MLR had occurred using freshly made intact Fn JD 3-2 or lyophilized Fn JJM. Other Fn preparations which did not produce suppression either contained additional protein bands on gels or were manipulated for several days prior to use. Therefore, an experiment was designed to test both slime A Fn (5-20) and single donor Fn (JG 5-20) immediately after isolation, as well as fragments generated by plasmin cleavage of these preparations. Through appropriate scheduling, the Fn would be as fresh as possible when used in both experiments. The results of this experiment confirm the claim that fresh, intact Fn if used immediately after isolation will cause suppression of mixed lymphocyte proliferation (Figure 10). Decreased ^{3}H-thymidine incorporation is shown at a Fn concentration of 13 ug/well. This occurred in both pairings used. Slime A Fn which appeared intact and did not contain extra bands on SDS gels, showed no effect on the MLR. After plasmin cleavage, JG 5-20 Fn lost its suppressive influence on the MLR while slime A Fn still had no effect (Table 9).

Table 8: Effect of plasmin or 37 C treatment on the MLR

Experiment 1 R_2S_1

		protein concentration ug/culture		
		35	75	113
0 media 51.7 ± 2.7	37 C Fn	47.3 ± 3.1	57.9 ± 3.2	54.7 ± 1.3
	HSA	52.2 ± 3.2	56.1 ± 8.3	61.1 ± 2.8

Experiment 2 R_1S_2

		protein concentration ug/culture		
		13	26	39
0 media 4.2 ± 0.2	plasmin Fn	4.2 ± 0.4	4.6 ± 0.4	5.0 ± 0.8
	HSA	4.9 ± 0.4	4.3 ± 0.4	4.6 ± 0.3

Results of two experiments using Fn JD 4-1. This Fn was left at 37 C for 3 days prior to plating (expt.1) or treated with plasmin (expt. 2). Donor cells were 1) DF 2) RC. Values are given as mean cpm $(R_xS_x-R_x) \pm SE \times 10^{-3}$.

Figure 10. Dose Dependent Inhibition of the MLR by Fn JG 5-20.

MLRs performed in the presence of varying concentrations of Fn JG 5-20 (solid squares), HSA (solid circles) and Slime A Fn 5-20 (solid triangles). Results are expressed in terms of cpm specific stimulation.

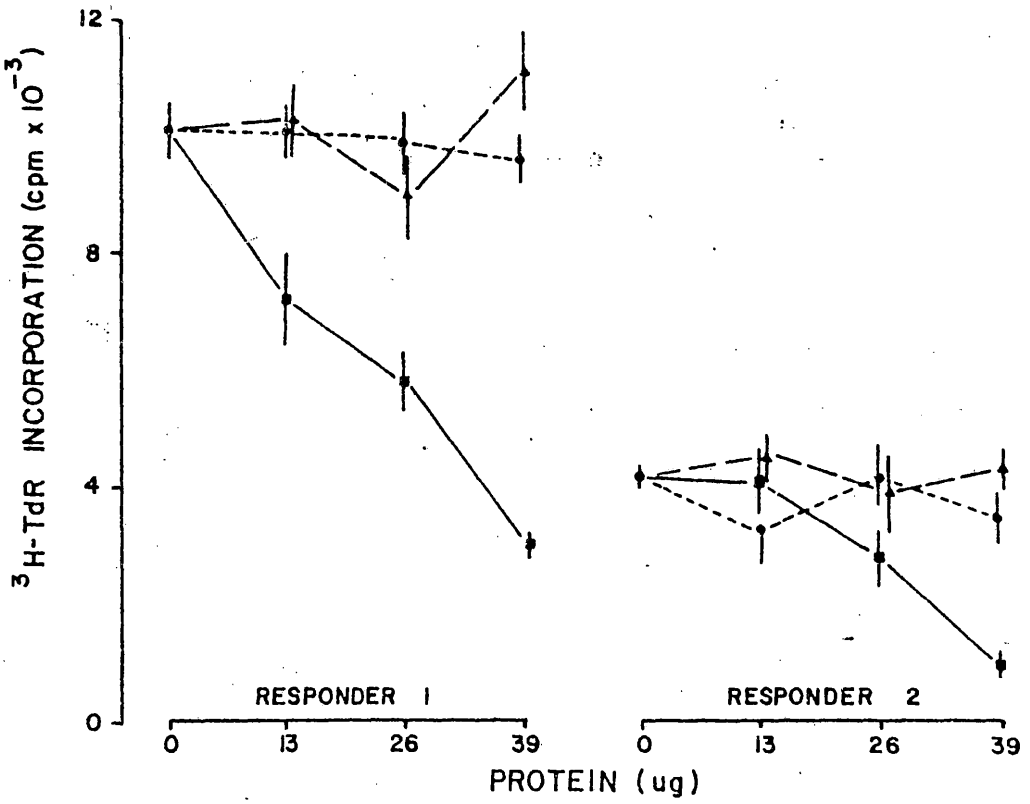

12
8
4
0
^{3}H-TdR INCORPORATION (cpm x 10^{-3})
RESPONDER 1
RESPONDER 2
0
13
26
39
PROTEIN (ug)

Table 9: Effect of plasmin cleaved Fns on the MLR

	ug/culture	mean cpm x 10^{-3} ± SE	
	0	19.1 ± 1.0	
JG Fn 5-20	5	20.2 ± 2.6	NS
	10	16.7 ± 2.4	NS
	15	16.9 ± 1.7	NS
slime A Fn 5-20	8	19.7 ± 1.1	NS
	16	21.3 ± 0.5	NS
	24	16.1 ± 1.2	NS
HSA	13	20.5 ± 1.1	NS
	16	20.6 ± 1.0	NS
	39	18.5 ± 1.0	NS

Effect of plasmin cleaved Fns on the MLR. Values are mean cpm $(R_2S_1-R_2) \times 10^{-3}$. None of the values are significantly different from the 0 control as evaluated by the Student t test. Donor cells were 1) JW 2) DF.

E. MLR Discussion

In results presented here, the heterogeneity of Fn preparations caused a marked difference in the effect of Fn on the MLR. Intact Fns obtained from individual donors and used immediately after isolation and one lyophilized preparation used immediately after reconstitution, had a suppressive effect on the MLR. This suppression was not due to a cytotoxic effect of Fn since both cell numbers and viabilities were similar in controls without Fn and in wells having a maximum Fn concentration of 60 ug/well. The same three Fn preparations which caused inhibition of the MLR, also caused a dose-dependent decrease in the uptake of ^{3}H-thymidine by responder cells cultured alone. These cells showed proliferative capacity during the 5 day incubation period, but their ability to incorporate ^{3}H-thymidine was diminished.

In contrast, seven Fn preparations caused enhancement of the MLR. These Fns were shown to contain protein bands of molecular weights other than 440,000 daltons. Most of these additional bands displayed molecular weights between the 200 Kd and 92 Kd protein standards. Even though these Fn preparations caused enhancement of the MLR, they did not cause an increased uptake of ^{3}H-thymidine by responder cells alone. Although Fn was isolated using standard procedures and appropriate precautions, one can not rule out the possibility that these additional bands represent non-Fn contaminants. Among the proteins known to influence the immune response, proteases (Bretz 1978) generally have small molecular weights (eg., elastase 34 Kd, trypsin 30 Kd). Since most of

the additional bands were in the 92-200 Kd MW range, it is unlikely that these proteins were proteases. It is also important to remember that these proteins were isolated by affinity chromatography on gelatin Sepharose and therefore should have some ability to bind gelatin.

In addition, if these proteins were non-Fn contaminants, the Fn antigenic immunoassay would have shown lower concentrations of Fn as compared with protein measurements determined by optical density at 280 nm. The immunoassay kit, which measures Fn concentration by antigenic activity, revealed Fn concentrations either similar to or higher than that measured by optical density. It is most likely, therefore, that these secondary protein bands were gelatin binding Fn fragments.

Considering the two opposite effects of Fn on the MLR, it seemed logical to conclude that intact Fn molecules mediate suppression of the MLR whereas, fragments of Fn might cause enhancement. However, when Fn was treated with plasmin and successful cleavage occurred, these plasmin derived fragments had no effect on the MLR.

Cleavage of Fn with plasmin is known to produce two large fragments with molecular weights of approximately 190 Kd and 200 Kd and several small molecular weight fragments with the most predominant fragment having a molecular weight of 30 Kd (Balian et al. 1979). The Fns cleaved here yielded fragments of similar molecular weight; however, none of these fragments had an effect on the MLR. The biological activities of various fragments of Fn have been mapped to particular regions of the Fn molecule by cleavage with a number of proteases; cathepsin D and plasmin (Balian et al. 1979), trypsin (Hayashi and Yamada 1982), alpha

thrombin (Furie and Rifkin 1980). Since different Fn fragments have not been tested individually for their effect on the MLR, it is impossible to say which portion of the molecule is responsible for the observed effects. It is important to note that the fragments present in the plasmin digests did not show the same patterns on SDS gels as the fragments present in the Fns that caused enhancement of the MLR; thus, one would not necessarily expect to find similar effects of these two different fragmented Fns on the MLR.

Besides using Fn preparations that were freshly isolated, other Fn preparations were left at 4 C, 37 C or extensively manipulated (i.e., frozen, thawed, dialyzed, left at room temperature). These Fns showed no effect on the mixed lymphocyte responses. Thus, these results emphasize the importance of using freshly prepared, intact Fn to achieve suppression of the MLR.

This is the first report that fresh intact Fn causes suppression of the MLR in humans, but a similar discovery has been reported in studies using inbred rats (Lause et al. 1982). These authors saw some inconsistency between different Fn preparations at low concentrations (1-5 ug/culture) required to achieve inhibition of the MLR, but in general they observed consistent inhibition at concentrations of 10-50 ug/culture. In experiments reported here, suppression occurred at the lowest concentrations tested which was 10 ug/culture.

In addition to showing a suppressive effect on the MLR, Fn has been implicated in other immune regulatory functions. Fn has been reported to inhibit mitogen-induced proliferation in the rat (Lause et al. in

press) and to stimulate macrophage secretion of a protein that binds to mature T cells (Beezhold et al. 1983).

The mechanism by which plasma Fn mediates immunoregulation is unknown, but it probably occurs through Fn's interaction with macrophages since Fn is known to be produced by (Alitalo et al. 1980) and bind to macrophages (Bevilacqua et al. 1981). Other proteins known to have immunoregulatory activity have been studied to determine the mechanism by which they suppress the MLR. In mice, alpha fetoprotein (AFP) induces the formation of suppressor T cells which can effectively interfere with the co-operative interaction between T cells, B cells and macrophages (Murgita et al. 1977). Alpha-2-macroglobulin (A2M) suppresses the human MLR by a non-cytotoxic mechanism that interferes with the collaboration of monocytes/macrophages with lymphocytes (Hubbard et al. 1981).

The stimulating molecules in the MLR have been shown to be products of the MHC I region referred to as Ia antigens. These are glycoproteins found on the surface of macrophages, B cells and activated T cells. Fn is thought to interact with sugar moieties on some glycoproteins (Julkunen et al. 1983); therefore, it is possible that Fn suppresses the MLR by binding to and masking the Ia antigens needed for stimulation.

Summary

The role of plasma fibronectin (Fn) as an immunoregulatory molecule was investigated using two in vitro assay systems: natural killing (NK) against K562 target cells and the mixed lymphocyte response (MLR). In NK assays, Fn showed no effect on the cytolysis of target cells despite variation of such parameters as effector cell donors, effector to target ratios, source and concentration of Fn added, presence or absence of monocytes, length of incubation, and pre-treatment of effectors or target cells with Fn. In binding studies, K562 cells did not demonstrate the ability to bind Fn to their cell surfaces. In the MLR, the effect of Fn depended on the nature of the preparation used. Freshly made Fn from two individuals and a lyophilized preparation made from multiple donor outdated plasma which showed no fragmentation on SDS gels, all mediated a suppression of the MLR. Fn prepared from byproducts of factor VIII production (slime A) which displayed additional protein bands on SDS gels, caused an enhancement of the MLR. Plasmin cleaved Fn preparations showed no effect on the MLR. Since Fn has opposite effects on the MLR which are seemingly dependent on the nature of its fragmentation, these results suggest that Fn may act to regulate immune function at sites of cellular cooperation.

References

Ahmann,G., Nadler,P., Birnkrant,A., Hodes,R. T cell recognition in the MLC response I. Non-T, radiation-resistant splenic adherent cells are the predominant stimulators in the murine MLR. J. Immunol. 123:903, 1979.

Akiyama,S., Yamada,K., Hayashi,M. The structure of Fn and its role in cellular adhesion. J. of Supramolecular Structure and Cellular Biochemistry 16:345, 1981.

Albrechtsen,D., Solheim,B., Thorsby,E. Antiserun inhibition of the MLC interaction: inhibitory effect of antibodies reactive with HLA-D associated determinants. Cellular Immunol. 28:258, 1977.

Alitalo,K., Hovi,T., Vaheri,A. Fibronectin is produced by human macrophages. J. Exp. Med. 151:613, 1980.

Atherton,B., Taylor,D., Hynes,R. Structural analysis of fibronectin with monoclonal antibodies. J. of Supramolecular Structure and and Cellular Biochemistry 17:153, 1981.

Bach,F., Hirschhorn,K. Lymphocyte interaction: A potential histocompatibility test in vitro. Science 143:813, 1964.

Bain,B., Vas,M., Lowenstein,L. A reaction between leukocytes in mixed peripheral blood cultures. Fed. Proc. 22:428, 1963.

Balian G., Click E., Crouch,E., Davidson,M., Bornstein,P. Isolation of a collagen-binding fragment from fibronectin and cold-insoluble globulin. J. Biol. Chem. 254:1429, 1979.

Barker,C.R., Worman,C.P., Smith,J.L. Purification and quantitation of T and B lymphocytes by an affinity method. Immunol. 29:765, 1975.

Beezhold,D., Lause,D., Kestler,D., Conrad,D. Regulation of macrophage secretory activity by fibronectin. J. Reticuloendothel. Soc. in press (abstract) 1983.

Bevilacqua,M., Mosesson,M., Bianco,M. Fibronectin receptors on mononuclear phagocytes. J. Exp. Med. 153:42, 1981.

Bordignon,C., Villa,F., Allavena,P., Introna,M., Biondi,A., Avallone,R., Mantovani,A. Inhibition of NK activity by human broncho-alveolar macrophages. J. Immunol. 129:587, 1982.

Boughton,B., Simpson,A. Plasma fibronection in acute leukemia. British J. of Haematology 51:487, 1982.

Boyum,A. Isolation of mononuclear cells and granulocytes from human

blood. Scand. J. Immunol. 3:697, 1974.

Bretz,U. Stimulation of lymphocytes by polymorphonuclear leukocyte elastase and cathepsin G, in vitro. Haveman,K., Janoff,A. eds. Neutral Proteases of Human Polymorphonuclear Leukocytes. Urban & Schwarzenberg. 323, 1978.

Brodin,B., Lieden,G., Malm,C., Vikrot,O. Plasma fibronectin deficiency during chemotherapy of acute myeloid leukemia. Scand. J. Haematol. 30:247, 1983.

Bruhn,H.D., Zurborn,K.H. Influence of clotting factors (thrombin, factor XIII) and of fibronectin on the growth of tumor cells and leukemic cells in vitro. Blut 46:85, 1983.

Callery,C., Golightly,M., Sidell,N., Goloub,S. Lymphocyte surface markers and cytotoxicity following cryopreservation. J. Immunol. Methods. 35:213, 1980.

Carpen,O., Virtanen,I., Saksela,E. Ultrastructure of human natural killer cells: Nature of the cytolytic contacts in relation to cellular secretion. J.Immunol. 128:2691, 1982.

Chen,L., Burridge,K., Murray,A. et al. Modulation of cell surface glycocalyx - studies on large external transformation sensitive protein. Ann. N.Y. Acad. Sci. 312:366, 1978a.

Chen,L.B., Murray,A. Segal,R.A., Bushnell,S., Walsh,M.L. Studies on intercellular LETS glycoprotein matrices. Cell 14:377, 1978b.

Chen,W.T., Singer,S. Immunoelectron microscopic characterization of three types of attachment sites at the fibroblast surface. J. Cell Biol. 91:258a, 1981.

Choate,J., Mosher,D. Fibronectin concentration in plasma of patients with breast cancer, colon cancer, acute leukemia. Cancer 51:1142, 1983.

Davidson,W. Cellular requirements for the induction of cytotoxic T cells in vitro. Immunol. Rev. 35:263, 1977.

Djeu,J., Holden,H., Herberman,R. Role of macrophages in the augmentation of mouse NK cell activity by poly I:C and interferon. J. Immunol. 122:182, 1979.

Doran,J., Raynor,R. Fibronectin binding to protein A-containing Staphylococci. Infect. & Immunity 33:683, 1981.

Doran,J., Rissing,J., Edmondson,H.T. Lack of interaction of fibronectin with gram negative bacteria. Program and Abstracts of 22nd ICAAC pg 150, (abstract) 1982.

Dupont,B., Hansen,J.A., Yunis,E. Human mixed-lymphocyte culture reaction: genetics, specificity, and biological implications. Adv. Immunol. 23:107, 1976.

Du Bois,R., Meinesz,A., Bierhorst-Eijlander,A., Groenewoud,P., Schellekens,P., Eijsvogel,V. The use of microtiter plates in mixed lymphocyte cultures. Tissue Antigens 4:458, 1974.

Eriksen,H., Clemmensen,I., Hansen,M., Ibsen,K. Plasma fibronectin concentration in normal subjects. Scand. J. Clin. Lab. Invest. 42:291, 1982.

Ezekowitz,R., Gordon,S. Surface properties of activated macrophages: sensitized lymphocytes, specific antigen and lymphokines reduce expression of antigen F4/80 and Fc and mannose/fucosyl receptors, but induce Ia. Adv. Exp. Med. and Biol. 155:401, 1982.

Furie,M., Rifkin,D. Proteolytically derived fragments of human plasma fibronectin and their localization within the intact molecule. J. Biol. Chem. 255:3134, 1980.

Gauss-Muller,V., Kleinman,H., Martin,G., Schiffman,E. Role of attachment factors and attractants in fibroblast chemotaxis. J. Lab. Clin. Med. 96:1071, 1981.

Gibert,M., Noel,P., Faucon,M., Ceccatly,M. Comparative immunohistochemical localization of fibronectin and actin in human breast tumor cells in vivo and in vitro. Virchows Arch. Cell Pathol. 40:99, 1982.

Gorelik,E., Wiltrout,R., Okumura,K., Habu,S., Herberman,R. Role of NK cells in the control of metastatic spread and growth of tumor cells in mice. Int. J. Cancer. 30:107, 1982.

Greineder,D., Shevach,E., Rosenthal,A. Macrophage-lymphocyte interaction III. Site of alloantiserum of T lymphocyte proliferation induced by allogeneic or aldehyde-bearing cells. J. Immunol. 117:1261, 1976.

Guillou,P., Hegarty,J., Ramsden,C., Davison,A., Will,E., Giles,G. Changes in human NK activity early and late after renal transplantation using conventional immunosuppression. Transplantation 33:414, 1982.

Hanna,N. Inhibition of experimental tumor metastasis by selective activation of NK cells. Canc. Res. 42:1337, 1982.

Hauser,W. NK cells induced by acute and chronic Toxoplasma infection. Cell. Immunol. 69:330, 1982.

Hayashi,M., Yamada,K. Domain structure of the carboxyl-terminal half of human plasma fibronectin. J. Biol. Chem. 258:3332, 1983.

Herberman,R.B. Natural Killer Cells. Hospital Practice 17:93, 1982a.

Herberman,R.B. Natural killer cells and their possible revelance to transplantation biology. Transplantation 34:1, 1982b.

Herberman,R.B. editor. NK Cells and Other Natural Effector Cells. Academic Press. 1982c.

Hirschhorn,K., Bach,F., Kolodny,R., Firschein,I., Hashen,N. Immune response and mitosis of human peripheral blood lymphocytes in vitro. Science 142:1185, 1963.

Hiserodt,J.C., Britvan,L.J., Targan,S.R. Characterization of the cytolytic reaction mechanism of the human natural killer lymphocyte: resolution into binding, programming and killer cell independent stages. J. Immunol. 129:1782, 1982.

Hiserodt,J.C., Britvan,L.J., Targan,S.R. Differential effects of various pharmacologic agents on the cytolytic reaction mechanism of the human natural killer lymphocyte: further resolution of programming for lysis and KCIL into discrete steps. J. Immunol. 129:2266, 1982b.

Hubbard,W., Hess,A., Hsia,S., Amos,D.B. The effects of electrophoretically "slow" and "fast" alpha-2 macroglobulin on mixed lymphocyte cultures. J. Immunol. 126:292, 1981.

Jondal,M., Merrill,J., Ullberg,M. Monocyte-induced human NK cell suppression followed by increased cytotoxic activity during short term in vitro culture in autologous serum. Scand. J. Immunol. 14:555, 1981.

Julkunen,I., Hautanen,A., Keski-oja,J. Interaction of viral envelope glycoproteins with fibronectin. Infect. and Immunity 40:876, 1983.

Katow,H., Yamada,M., Solursh,M. Occurrence of Fn on the primary mesenchyme cell surface during migration in the sea urchin embryo. Differentiation 22:120, 1982.

Koski,I.R., Poplack,D.G., Blaese,R.M. A nonspecific esterase stain for the identification of monocytes and macrophages. Bloom,B.R., David,J.R., eds. In vitro methods in cell mediated and tumor immunity. Academic Press:359, 1976.

Lause,D., Doran,J., Houston,J. The interaction of plasma fibronectin in the in vitro allograft response. Transplantation. 34:147, 1982.

Lause,D., Beezhold,D., Doran,J. Macrophage requirements in lymphocyte activation by fibronectin. J. Reticuloendothel. Soc. in press (abstract) 1983.

Lause,D., Beezhold,D., Doran,J. Induction of lymphocyte blast transform-

ation by purified fibronectin in vitro. J. Immunol. submitted 1983.

Lause,D.B., Doran,J.E., Houston,J.A., Beezhold,D.H. Modulation of rat lymphocyte transformation by plasma fibronectin. J. Reticuloendothel. Soc. in press 1983.

Lipkin,G., Knecht,M., Rosenberg,M. Glycoprotein-containing factor that mediates contact inhibition of growth. Ann. of N.Y. Acad. Sci. 312:382, 1978.

Lozzio,C.B., Lozzio,B.B. Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. Blood 45:321, 1975.

Markwell,M., Fox,C. Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro3,6-diphenyl glycoluril. Biochemistry 17:4807, 1978.

Minami,M., Shreffler,D., Cowing,C. Characterization of the stimulator cells in the murine primary mixed leukocyte response. J. Immunol. 124:1314, 1980.

Mosesson,M., Amrani,D. The structure and biologic activities of plasma fibronectin. Blood 56:145, 1980.

Mosher,D., Furcht,L. Fibronectin: Review of its structure and possible functions. J. of Invest. Derm. 77:175, 1981.

Murgita,R., Goidl,E., Kontiainen,S., Wigzell,H. Alpha-fetoprotein induces the formation of suppressor cells in vitro. Nature 267:257 1977.

Myre,E. Kuusela,P. Binding of human fibronectin to group A, C, and G streptococci. Infect. Immunity 40:29, 1983.

Norris,D.A., Clark,R.A., Swigart,J., Huff,C., Weston,W., Howell,S. Fibronectin fragments are chemotatic for human peripheral blood monocytes. J. Immunol. 129:162, 1982.

Ono,Y., Kerman,R., Kahan,B. Natural cell-mediated cytotoxicity and suppressor activity for natural cell-mediated cytotoxicity in long-term renal transplant patients. Transplantation 34:103. 1982.

Parsons,R., Todd,H., Kowal,R. Isolation and identification of a human serum fibronectin-like protein elevated during malignant disease. Cancer Res. 34:4341, 1979.

Pearlstein,E., Gold,L.I. High molecular weight glycoprotein as a mediator of cellular adhesion. Ann. N.Y. Acad. Sci. 312:278, 1978.

Pearlstein,E., Gold,L.I., Garcia-Pardo,A. Fibronectin: A review of its structure and biological activity. Molecular and Cellular Biochem.

29:103, 1980.

Perri,R., Kay,N., McCarthy,J., Vessella,R., Jacob,H., Furcht,L. Fibronectin enhances in vitro monocyte-macrophage mediated tumoricidal activity. Blood 60:430, 1982.

Pisano,J. Jackson,J., DiLuzio,N., Ichinose,H. Dimensions of humoral recognition factor depletion in carcinomatous patients. Cancer Res. 32:11, 1972.

Pollack,S., Hallenbeck,L. In vivo reduction of NK activity with anti-NK1 serum: Direct evaluation of NK cells in tumor clearance. Int. J. Cancer 29:203, 1982.

Porath,J., Aspberg,K., Drevin,H., Axen,R. Preparation of cyanogen bromide-activated agarose gels. J. Chromatogr. 86:53, 1973.

Pross,H., Baines,M. Studies of human NK cells I. In vivo parameters affecting normal cytotoxic function. Int. J. Cancer 29:283, 1982.

Rager-Zisman,B., Bloom,B.R. NK cells in resistance to virus-infected cells. Springer Seminars in Immunopathology 4:397, 1982.

Rajaraman,R., MacSween,J., Murdock,C. Exogenous fibronectin requirement for adhesion by neoplastic human cells. Exp. Cell Biol. 51:9, 1983.

Raynor,R., Reese,A. Fibronectin enhances macrophage induced cytostasis of carcinoma cells. J.Reticuloendothel. Soc. 30:13a, 1981.

Reese,A., Doran,J., Raynor,R., Mansberger,A. Role of fibronectin in wound healing. Current Adv. Oral Maxillofacial Surgery 4:1, 1983.

Reese,A., Reece,M., Martin,D. Effect of fibronectin on mouse NK activity in vitro. Fed. Proc. Abstract 1983a.

Rode,H., Gordon,J. Macrophages in the mixed leukocyte culture reaction. Cellular Immunol 13:87, 1974.

Roder,J.C., Karre,K., Kiessling,R. Natural Killer Cells. Prog. Allergy 28:66, 1981.

Ruoslahti,E., Engvall,E. Immunochemical and collagen-binding properties of fibronectin. Ann. N.Y. Acad. Sci. 312:178, 1978.

Ruoslahti,E., Engvall,E., Hayman,E.G., Spiro,R. Studies on amniotic fluid and plasma fibronectins. Biochem J. 193:295, 1981.

Saba,T., Jaffe,E. Plasma fibronectin: Its synthesis by vascular endothelial cells and role in cardiopulmonary integrity after trauma as related to reticuloendothelia function. Amer. J. Med. 68:577, 1980.

Simpson,W., Hasty,D., Mason,J., Beachy,E. Fibronectin-mediated binding of group A Streptococci to human polymorphonuclear leukocytes. Infect. and Immunity. 37:805, 1982.

Stenman,S., Vaheri,A. Fibronectin in human solid tumors. Int. J. Cancer 27:427, 1981.

Strong,D., Ortaldo,J., Pandolfi,F., Maluish,A., Herberman,R. Cryopreservation of human mononuclear cells for quality control in clinical immunology. I. Correlations in recovery of K and NK cell functions, surface markers, and morphology. J. of Clin. Immunol. 2:214, 1982.

Timonen,T. Isolation of human NK cells by density gradient centrifugation. J. Immunol. 36:285, 1980.

Timonen,T., Ortaldo,J., Herberman,R. Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. J. Exp. Med. 153:596, 1981.

Tsukomoto,Y., Helsel,W., Wall,S. Macrophage production of fibronectin, a chemoattractant for fibroblasts. J. Immunol. 127:673, 1981.

Uchida,A. Generation of suppressor cells for NK activity in cancer patients after surgery. J. Nat. Cancer Inst. 68:735, 1982.

Unanue,E. Macrophages in antigenic stimulation. Adv. in Immunol. 31:1, 1981.

Virtanen,I., Vartio,T., Bradley,R., Lehto,V. Fibronectin in adhesion, spreading and cytoskeletal organization of cultured fibroblasts. Nature 298:660, 1982.

Wall,R., Cooper,S., Kosek,J. The influence of exogenous fibronectin on blood granulocyte adherence to vascular endothelium in vitro. Exp. Cell Res. 140:105, 1982.

Werkmeiester,J., Helfand,S., Haliotis,T., Pross,H., Roder,J. The effect of target cell differentiation on human NK cell activity: A specific defect in target cell binding and early activation events. J. Immunol. 129:413, 1982a.

Werkmeister,J., Helfand,S., Haliotis,T., Rubin,P., Pross,H., Roder,J. Tumor cell differentiation modulated susceptibility to NK cells. Cellular Immunol. 69:122, 1982b.

Wright,S., Weitzen,M., Kahle,R., Granger,G., Bonavida, B. Studies on the mechanism of natural killer cytotoxicy II. Coculture of human PBL with NK-sensitive or resistant cell lines stimulates release of natural killer cytotoxic factors (NKCF) selectively cytotoxic to NK-sensitive

target cells. J. Immunol. 130:2479, 1983.

Yamada,K.M., Ohanian,S.H., Pastan,I. Cell surface protein decreases microvilli and ruffles on transformed mouse and chick cells. Cell 9:241, 1976a.

Yamada,K.M., Yamada,S.S., Pastan,I. Cell surface protein partially restores morphology, adhesiveness, and contact inhibition of movement to transformes fibroblasts. Proc. Nat. Acad. Sci. 73:1217, 1976b.

Yamada,K., Pastan,I. The relationship between cell surface protein and glucose and aminoisobutyrate transport in transformed chick and mouse cells. J. Cell Physiol. 89:827, 1979.

Yamada,K., Kennedy,D. Fibroblast cellular and plasma fibronectins are similar but not identical. J. Cell. Biol. 80:492, 1979.

Yamada,K. Biochemistry of fibronectin. The Glycoconjugates. vol. 3 Academic Press, 1982.

Yonemasu,K., Nakanishi,A., Sasaki,T., Kashiba,S. Stimulation of locomotion of peripheral blood monocytes by human plasma fibronectin. Microbiol. Immunol. 27:283, 1983.

Young,D., Doran,J., Mansberger,A., Reese,A. Effect of cold insoluble globulin on carcinoma cell growth in vitro. Oncology 38:295, 1981.