Fibrin and Fibrinogen Degradation Products With an Intact D-Domain C-Terminal γ Chain Inhibit an Early Step in Accessory Cell-Dependent Lymphocyte Mitogenesis

By S.C. Robson, R. Saunders, L.R. Purves, C. de Jager, A. Corrigall, and R.E. Kirsch

Although the low molecular weight degradation products of fibrinogen (FgDP) and fibrin (FbDP) are known to inhibit lymphocyte blastogenesis, the effect of purified macromolecular FgDP and FbDP (molecular weight, 90 to 200 Kd) is unclear. We have examined the effect of these latter FgDP and FbDP and find that products that contain the D domain inhibit lymphocyte proliferation in response to T-cell mitogens, allogeneic mononuclear leukocytes, and anti-CD3 in vitro. Plasmic digestion of D1 in the absence of calcium with removal of the C-terminal end of the γ chain or disruption of the γ-γ C-terminal cross-link site of D-dimer (DD) by puffadder venom (PAV-D) abrogates their inhibitory potential. Prior incubation of monocytes with DD or D1 inhibits subsequent lymphocyte transformation. Binding studies with radiolabeled DD and PAV-D confirm that monocytes interact only with DD. This specific binding may be competitively inhibited by monoclonal antibodies to CD11b/CD18 or by peptide analogues of the C-terminal γ chain of fibrinogen that mimic the adhesion recognition site of integrins. We postulate that DD and D1 bind to CD11b/CD18 on adherent monocytes and modulate lymphocyte activation. These products are typically present in the plasma of patients with disseminated intravascular coagulation with sepsis and could therefore influence inflammatory processes in vivo.

ALTERATIONS in coagulation and/or fibrinolysis are recognized features of acute and chronic inflammatory states, delayed hypersensitivity response, and neoplasia. Coagulation pathways may be activated by interleukin-1 (IL-1), tumor necrosis factor-α (TNF), immune complexes, and endotoxin, all of which are capable of stimulating the synthesis and release of tissue factor and apoprotein III of thromboplastin. Subsequent thrombin generation may then lead to deposition of fibrin at sites of immune reaction. Fibrinolysis in inflammatory states is thought to be initiated and regulated in a similar fashion. Cytokines released by activated T lymphocytes and accessory cells induce the secretion of plasminogen activators and activator inhibitors, which result in the balanced generation of plasmin.

PROTEOLYTIC cleavage of fibrinogen by plasmin produces a set of terminal degradation products (FgDP) that includes fragments D and E. Plasmic digestion of cross-linked fibrin (FbDP) leads to the formation of fragment D-dimer (DD), which is composed of two D moieties with cross-linked C-terminal γ chains, and fragment E.

FbDP and FgDP have diverse biologic activities that include the modulation of immune responses. Catabolism of fibrinogen and fibrin may result in negative feedback on effector limbs of the immune system.

The original observation of Girmann et al. that micro-molecular plasmic degradation products of fibrinogen are able to suppress lymphocyte blastogenesis in vitro and immune responses in vivo have been confirmed by other studies. The role of purified individual macromolecular degradation fragments of fibrinogen and fibrin has not been clarified.

The mechanism by which the FgDP and FbDP suppress lymphocyte blastogenesis is not known. The fragments may act directly on T lymphocytes or may inhibit monocyte or accessory T-cell interaction, which is required for lymphocyte proliferation. This monocyt or accessory cell–T-cell contact modulated by FgDP and FbDP may be mediated by integrins and other adhesive protein receptors.

We present data suggesting that the inhibition of mitogen-induced lymphocyte blastogenesis by FgDP and FbDP is also mediated by macromolecular (molecular weight [Mr] 90 to 200 Kd) species and, where studied, appears to be dependent on the intact C-terminal γ chain of the D-domain. We postulate that, after binding to adherent monocytes, intact D domains are able to inhibit an early step of lymphocyte activation in vitro.

MATERIALS AND METHODS

Reagents

The following monoclonal antibodies (MoAbs) were from Ortho Diagnostics (Kantam, NJ): OKM1 (IgG2b), directed against the α chain of Mac 1 (CD11b); OKIa1 (IgG2a), directed against human DR framework; and OKT3 (IgG3) directed against CD3. OKM10 (IgG), directed against a different epitope on CD11b, was a gift from S. Silverstein (Columbia University, New York, NY). HM23 (IgG) monoclonal directed at the β2 integrin (CD18) subunit was a gift from Dr J.E.K. Hildreth (Johns Hopkins Medical School, Baltimore, MD). Anti-intercellular adhesion molecule-1 (anti-ICAM-1) (84H10) was a kind gift from W. Makgoba (Hammersmith Hospital, London, UK). Anti–IL-2 receptor (anti–IL-2R) (IgG2a) directed against Tac (IL-2 receptor, CD25) was purchased from Biotest Diagnostics (Dreieich, Germany). Recombinant IL-2 was a gift of Dr S. Ress (University of Cape Town Medical School, Cape Town, South Africa). Peptide G15 (Gly-Gln-Gln-His-His-Leu-Gly-Gly-Ala-Gly-Asp-Val) corresponding to residues 397-411 of the γ chain of fibrinogen (g15), Arg-Gly-Asp-Val (RGOV), and Gly-Pro-Arg-Asp (GPRD) were from Sigma Chemical Co (St Louis, MO). Peptide L10 (Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val) was synthesized by Dr D. Botes (Department of Biochemistry, University of Cape Town) and was a gift from Dr Botes. RPMI-1640 with glutamine and fetal calf serum were from Highveld Biologicals (Johannesburg, South Af-

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Preparation of Fibrinogen, Fibrin, and Derivatives

Fibrinogen was purified from fresh normal human plasma by repeated ammonium sulphate precipitation. The final precipitate was dissolved in 0.005 mol/L sodium citrate and dialyzed exhaustively against 0.025 mol/L imidazole, pH 8.0. If necessary, the preparation was depleted of fibrinogen by passage through a gelatin sepharose column. Plasminogen was purified from normal human plasma on a lysine sepharose column, and was subsequently activated by urasinase (500 Ploug Units/mg plasminogen) at 37°C, pH 8. Plasmin was added to fibrinogen (3.2 mg plasmin/100 mg fibrinogen) in the presence or absence of 0.01 mol/L EDTA, and digestion was timed to give fragments D1, E, and D3 and low molecular weight fragments (LMWT-FgDP). In parallel experiments, 0.1 mL of thrombin and 0.1 mL of 1 mol/L CaCl2 was added to 40 mg fibrinogen in 0.05 mol/L Tris HCl, pH 7.4, with 0.15 mol/L NaCl at 4°C to produce a fibrin clot. Digestion of the clot with plasmin was timed to give fragments DD and E with LMWT-FbDP. Digestion was terminated by the addition of 1,000 Kallikrein inactivator units of aprotonin in 0.025 mol/L EACA.

After digestion, D (DD) and E were separated by chromatofocusing using a PBE 94 K9:30 column (Pharmacia) equilibrated with 0.025 mol/L imidazole, pH 8.26. One hundred milligrams of fibrinogen digest in 0.025 mol/L imidazole was applied to the column, and proteins were eluted with polybuffer 74 (1:8) adjusted to pH 3.5. Protein concentrations were determined using the BioRad protein assay (BioRad, Richmond, CA). Samples were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (gradient 8% to 16%) under nonreducing conditions. Polybuffer (and LMWT-FDP) were removed from the samples using a PM30 membrane in an Amicon Diaphragm (Amicon Corp, Danvers, MA). Fibrinogen-related antigen (FRA) levels were confirmed in an enzyme-linked immunosorbent assay (ELISA).

After chromatofocusing, aliquots free of E were subjected to gel filtration. A pre-equilibrated ACA 34 (LKB, Bromma, Sweden) column (100 x 2.5 cm) was used to separate DD from D1, D2, and D3. Proteins were eluted with phosphate-buffered saline (PBS) and their concentrations determined as above. Puffadder (Biss arietis) venom proteases were used to lyse C-terminal long cross-links of DD to prepare PAV-D. All samples were concentrated by centrifugal microconcentrators (Centricon; Amicon) or by dialysis against polyethylene glycol and were then dialyzed against RPMI and filtered sterilized before use in lymphocyte transformations. PAGE was performed to confirm the purity of the FgDP and FbDP derivatives.

Radiolabeling DD and PAV-D

Radiolabeling of DD and PAV-D was performed by the iodogen method as described.

Cell Isolation

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood from normal adult volunteers. Purification of T Cells and Monocytes

Adherent monocytes were enriched by adherence on tissue culture plastic dishes in the presence of 15% autologous serum. Nonadherent cells were removed after 2 hours of incubation, passed through a nylon wool column, and then subjected to antibody-mediated lysis with OKM1 and OKA1 in the presence of fresh rabbit complement. The purity of the population of T cells was confirmed by immunofluorescent labeling with anti-CD3 to be in excess of 98% and the cells were then used in lectin-stimulated cultures on their own or were added back to monocyte cultures.

Lectin-Stimulated Culture of T Cells

Thymidine incorporation of mitogen-driven lymphocyte transformation was assessed by an in vitro microculture method. This was correlated to blast counts as an indication of transformation of lymphocyte populations. In brief, 50 mL of cells (either PBMC or purified T lymphocytes, at 2 x 10^6 cells/mL LD was mixed with 100 mL RPMI + 10% AB serum in 96-well tissue culture trays. Fibrinogen, purified FgDP, or FbDP (FDP) preparations (25 mL at varying concentrations) and 25 mL of lectin preparation (either PHA-P at final concentrations of 0 to 100 mg/mL or Con-A at final concentrations of 0 to 50 mL/mL) were added at various time points, depending on the particular experiment. At 72 hours, cells were pulsed with 20 mL of thymidine (specific activity, 0.24 Ci/mmol; 0.2 gCi radiolabeled thymidine with 0.2 g cold thymidine per culture) per well and harvested 18 hours later on glass fiber filters with a standard automated harvester. The glass fiber filters were counted in Instagel (Packard, Downers Grove, IL) in a Packard beta counter. Efficiency of counting for [3H]-thymidine was estimated at 72%. The stimulation index was expressed as the ratio of stimulated to background counts.

Activation and Kinetic Studies

Purified DDW as added to lymphocytes at either 6 hours or 1 hour before mitogen stimulation, or at 1, 2, 6, or 12 hours after mitogen stimulation. 2H-thymidine was added on day 3, and cells were harvested 18 hours later. In separate studies, DD was added to lymphocytes 2 hours before optimal mitogenic lectin stimulation, and lymphocyte proliferation was then assessed daily for 5 days following commencement of cultures.

In all studies, cell viability was monitored using exclusion of trypan blue staining.

Phenotype Determination

CD54, CD25, and OKIa1 expression on T lymphocytes was determined by flow cytometry using a Coulter TPS/1 (Coulter, New York) and confirmed by immunofluorescent microscopy. The total T-cell numbers were determined by CD3 expression.

Priming of Monocytes and Lymphocytes With FDP

Monocytes were allowed to adhere to 96-well flat-bottomed tissue culture trays and washes to remove contaminating nonadherent cells. They were then incubated for 3 hours in the presence of various FgDP and FbDP and extensively washed before the addition of T cells in the presence of mitogen as described above. Similarly, purified T cells were exposed to various FgDP and FbDP for 3 hours, and then added to untrreated monocytes. Lymphocyte proliferation was assessed as previously described.
Allogeneic-Cell–Stimulated Mixed Lymphocyte Reactions

Normal lymphocytes to be used as stimulator cells were purified and irradiated with an experimental cobalt source (20 Gy). They were used at concentrations of 10^4, 5 x 10^4, and 10^5 cells per 200-μL flat-bottomed well in the absence or presence of varying concentrations of DD. Responder cells at 10^4 cells/well were added to the stimulator cells, cultured for 6 days, and then pulsed with 3H-thymidine as described.35 Control wells contained no responder cells.

CD3-Mediated Mitogenesis

Immobilized anti CD3 induced T-cell activation was performed according to Samstag et al36 Cells were cultured on coated wells for 5 days in the presence or absence of varying concentrations of DD, after which they were pulsed with 3H-thymidine and harvested.

Cell Binding Studies

The interaction of FbDP and adherent monocytes was determined by radioligand binding assays. Typically, adherent monocytes were incubated for 48 hours in RPMI 1640, 5% autologous serum, and then washed and exposed to RPMI 1640 containing HEPES (30 mmol/L), human serum albumin (0.5 wt/vol), and azide (0.02%) plus 125I-DD either in the presence or absence of excess (>100-fold) cold DD in a final volume of 500 μL. 125I-DD concentrations ranged from 2.5 to 100 nmol/L. After 60 minutes of incubation at 37°C, the supernatant fluid was removed and the cells were washed extensively before lysis by the addition of 500 μL of NP-40 2% in 0.1 mol/L Tris HCl, 0.45 mol/L NaCl. Total bound (cell lysates) and free (supernatant fluids) counts were determined by counting aliquots separately in a Beckman gamma counter. Determination of OKM1, OKM10, and MHM23 competitive inhibition of binding of 125I-DD to monocytes was performed using 10 to 20 μg/mL of the monoclonal.38 Cells were preincubated with MoAbs for 60 minutes at 37°C, washed, and then incubated with various concentrations of 125I-labeled DD in the presence and absence of unlabeled DD in 100-fold excess as described above. Experiments to examine binding of PAV-D to monocyte-macrophages were as described for DD. 125I-labeled PAV-D was used at concentrations of 2.5 nmol/L to 200 nmol/L and unlabeled PAV-D was added in a greater than 100-fold excess. Adherent monocytes were preincubated with the peptide analogues at concentrations of 0.5, 1, and 2 mg/mL to competitively inhibit DD binding to monocytes. Results were analyzed and plotted by Enzfitter (Elsevier, Cambridge, UK) using Scatchard plots with validation of ligand saturation binding.37 Identical experiments were performed in parallel with PAV-D in the same nanomolar range. Results were expressed as Fg DD bound per monocyte and inhibition of DD binding was expressed as a percentage of the total control DD bound. Time course and reversibility studies were performed and confirmed that ligand binding was at equilibrium under the experimental conditions used.37

Statistical Analysis and Study Design

Significant differences were determined by nonparametric comparison of continuous variables by the Mann-Whitney U test.

The study necessitated regular blood donations from healthy volunteers and was approved by the Ethics and Research Committee of the University of Cape Town.

RESULTS

Preparation of FgDP and FbDP

PAGE of purified derivatives of plasmic digests of fibrinogen and fibrin confirmed the purity of the FgDP and FbDP used for later cell culture experimentation (Fig 1).

Effects of FDP on Mitogenic Lymphocyte Proliferation

The effects of DD, D, and E on Con-A mitogenic lymphocyte proliferation are shown in Table I. 3H-thymidine incorporation in control lymphocyte experiments ranged from 10,000 to 28,000 cpm for PHA-P (0 to 100 μg/mL) and from 10,000 to 35,000 cpm for Con-A (0 to 100 μg/mL)–stimulated cultures. Intra-assay variation of triplicates at individual points of reported experiments was less than 15%. Inter-assay variation arose from differing responses of donor control lymphocytes. Hence, reported data are from representative experiments reproduced on at least three occasions with differing donor control lymphocytes.

Both DD and D inhibited Con-A–stimulated responses. By equivalent titration it was determined that DD was notably more potent than D. For 50% suppressive activity, 58 μg/

![Fig 1. Purification of fibrinogen and derivatives. SDS-polyacrylamide gradient gel electrophoresis (5% to 20%) of nonreduced fibrinogen and fragments DD, PAV-D, D1, and D3. A kit containing prestained protein molecular mass markers (Amersham International, Amersham, UK) for molecular mass values of 14 to 200 Kd was used for calibration standards.](attachment:image.png)
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Table 1. Effect of Purified Fibrinogen DD, D, and E on Lymphocyte Proliferation in Response to 20 pg/mL Con-A

<table>
<thead>
<tr>
<th>FDP</th>
<th>12.5 µg/mL</th>
<th>12.5 µg/mL</th>
<th>1 µg/mL</th>
<th>0.25 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen (254)</td>
<td>20,659</td>
<td>17,706</td>
<td>15,438</td>
<td>14,912</td>
</tr>
<tr>
<td>DD (663)</td>
<td>870</td>
<td>3,667</td>
<td>9,695</td>
<td>12,687</td>
</tr>
<tr>
<td>D (347)</td>
<td>4,190</td>
<td>10,775</td>
<td>12,706</td>
<td>14,010</td>
</tr>
<tr>
<td>E (167)</td>
<td>13,327</td>
<td>11,007</td>
<td>13,627</td>
<td>17,574</td>
</tr>
</tbody>
</table>

Results are median counts per minute, ³H-thymidine incorporation, from individual experiments representative of three separate experiments. Background unstimulated counts for various FRA (1,000 pg/mL) are in parentheses. Mitogen and serum concentrations were used to give in the order of 50% of maximum potential cpm of stimulated lymphocytes. The Cmax is thus the median count of control wells (17,428). All cpm lower than th Cmax indicate true inhibitory effects.

mL of D and 6.3 µg/mL of DD were required (Fig 2). Fragment E in low concentrations enhanced stimulation indices of lymphocyte responses. Native fibrinogen had no effect on lymphocyte proliferative responses to Con-A. Results for PHA-P were similar (data not shown).

Activation and Kinetic Data

DD at concentrations predicted to give 50% suppressive activity (10 µg/mL) had maximal inhibitory potential when added before Con-A mitogenic stimulation, suggesting that effects on lymphocyte proliferation were those of "anti-activation" (Fig 3). Assessment of lymphocyte viability confirmed that DD was not cytotoxic.

Experiments in which DD (50 µg/mL) was added to lymphocytes for 2 hours before lectin stimulation, followed by early or delayed addition of ³H-thymidine to cultures, suggest that the major defects underlying lymphocyte hyporesponsiveness appear to be a delay in the onset of cellular activation. In control lymphocytes, peak proliferation occurred on day 4 or 5, depending on the mitogen concentration. However, in DD-treated lymphocytes, peak proliferation was usually delayed to day 5 or 6, i.e., 24 to 48 hours later than that of control cells (Table 2).

Further Effects of DD on Lymphocyte Proliferative Responses

Mixed lymphocyte reaction (one way). Lymphocyte proliferation in response to allogeneic irradiated cells was inhibited by DD at a concentration 50 µg/mL (Table 3). Anti-CD3–mediated mitogenesis. Anti-CD3–mediated triggering of T lymphocytes in the presence of monocyte-macrophages was inhibited by DD at a final concentration of 50 µg/mL at varying concentrations of anti-CD3 (solid phase) (Table 4).

Phenotype of Resting and Con-A–Stimulated PBMC in the Absence and Presence of DD

Resting PBMC had low levels of immediate (ICAM-1, CD54), early (IL-2R, CD25), and late activation (human DR framework, OKIa1) molecules as anticipated (Table 5). After Con-A stimulation, the cell phenotype was modified in a predictable fashion with enhanced expression of all markers within 48 hours. After exposure to DD (50 µg/mL), we ob-

Fig 2. Equivalent titrations of FDP. The graph depicts the percentage of inhibition of proliferation of lymphocyte cultures in response to 20 µg/mL Con-A noted at varying concentrations of (O) DD, (A) D, or (C) LMWT-FdDP (FRA-DD). Results are expressed as median cpm of triplicates of an experiment representative of three separate experiments.

Table 2. Kinetics of Inhibition of Con-A (20 µg/mL) Mitogenic Lymphocyte Proliferation by DD

<table>
<thead>
<tr>
<th></th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16,232</td>
<td>25,776</td>
<td>27,695</td>
<td>8,846</td>
</tr>
<tr>
<td>+DD (50 µg/mL)</td>
<td>1,264*</td>
<td>6,543*</td>
<td>22,764</td>
<td>26,094</td>
</tr>
</tbody>
</table>

Results are median counts per minute, ³H-thymidine incorporation, from individual representative experiments. Variation of triplicates was within 15%. DD incubation with lymphocytes delayed peak proliferation to day 6.

* P < .05 Mann Whitney U test (n = 9 for each group).
Table 3. Effect of DD on One-Way Mixed Lymphocyte Reaction (1-MLR)

<table>
<thead>
<tr>
<th>Irradiated Stimulator Cell Number</th>
<th>0</th>
<th>10⁵</th>
<th>5 x 10⁵</th>
<th>10⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁶ Responder lymphocytes</td>
<td>538</td>
<td>10,481</td>
<td>20,946</td>
<td>27,766</td>
</tr>
<tr>
<td>10⁵ Responder lymphocytes + DD (50 µg/mL)</td>
<td>845</td>
<td>1,329*</td>
<td>5,910*</td>
<td>11,017*</td>
</tr>
</tbody>
</table>

Results are median counts per minute, ³H-thymidine incorporation, from individual typical experiments. Variation of triplicates was within 15%.

* DD significantly inhibited lymphocyte proliferation (Mann Whitney U test, P < .05; three experiments with n = 9 for each group).

Table 4. Effect of DD on Anti-CD3-Triggered Mitogenesis

<table>
<thead>
<tr>
<th>Anti-CD3 (µg/mL)</th>
<th>0</th>
<th>165</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control lymphocytes</td>
<td>420</td>
<td>14,447</td>
<td>34,961</td>
</tr>
<tr>
<td>Control lymphocytes + DD (50 µg/mL)</td>
<td>757</td>
<td>2,962*</td>
<td>7,214*</td>
</tr>
</tbody>
</table>

Results are median counts per minute, ³H-thymidine incorporation, from individual typical experiments. Variation of triplicates was within 15%.

* DD significantly inhibited lymphocyte proliferation (Mann Whitney U test, P < .05; three experiments with n = 9 for each group).

Monocyte, 20:1) significantly inhibited proliferation of T cells (P < .05 Mann Whitney U test) (Fig 5). Prior incubation of DD with the T cells had little effect on the subsequent response to Con-A (20 µg/mL).

Table 5. DD Delays the Expression of Early and Late Activation Markers of Mononuclear Cells After Mitogenic Stimulation With Con-A (20 µg/mL)

<table>
<thead>
<tr>
<th>Con-A Stimulated PBMC</th>
<th>Controls</th>
<th>DD (50 µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 h</td>
<td>90 h</td>
<td>48 h</td>
</tr>
<tr>
<td>CD54</td>
<td>4</td>
<td>88</td>
</tr>
<tr>
<td>CD25</td>
<td>5</td>
<td>52</td>
</tr>
<tr>
<td>DR (OKm1)</td>
<td>17</td>
<td>64</td>
</tr>
</tbody>
</table>

Results are the percentage of CD-labeled cells and are expressed as medians of an experiment representative of three (*Mann Whitney U test, P < .05 for comparison with controls).

Cell Binding Studies With ¹²⁵I-DD

Radiolabeled DD bound to monocytes with a kd of 8.53 x 10⁻⁸ mol/L with a binding capacity of 40 fg DD/cell (or 120,000 binding sites/cell) (Fig 4). This binding was specific and could be inhibited by 72% after preincubation of the cells with 10 µg/mL OKM1, by 76% with OKM10, and by 72% with MHH23 (median values of five experiments). Because the kd remained unchanged, the inhibition appeared competitive. PAV-D did not bind to monocyte-macrophages, suggesting that the intact y-y cross-link site of the C-terminal chains was important in binding to the CD11b/CD18 complex on monocytes. Lymphocytes, even after activation, did not bind detectable amounts of DD (data not shown).

Effect of Peptides on Interaction of Monocyte-Macrophages With ¹²⁵I-DD

Peptide GPRD had no effect on the interaction of ¹²⁵I-DD with adherent monocytes. Maximal inhibition of binding was observed with G15 and L10 at 2 mg/mL, which could reduce cell binding of ¹²⁵I-DD to 6.4 fg/cell (median levels of five experiments). Peak levels of inhibition with RGDV peptide at 2 mg/mL resulted in 13.8 fg DD/cell specific binding.

Involvement of Monocyte-Macrophages in the Effects of DD and D on Lymphocyte Proliferation

Incubation of DD with monocytes before the addition of purified T cells (98% CD3⁺) at optimal cell ratios (T cell to monocyte, 20:1) significantly inhibited proliferation of T cells (P < .05 Mann Whitney U test) (Fig 5). Prior incubation of DD with the T cells had little effect on the subsequent response to Con-A (20 µg/mL).

Dependence of the Effects of DD and D on the C-Terminal y Chain

Removal of the C-terminal y chain by prolonged digestion of D1 to D3 in vitro abrogated the inhibitory potential of D1 at 100 µg/mL in a lymphocyte transformation assay in response to Con-A (0 to 20 µg/mL) (Fig 6A). Similarly, disruption of the C-terminal y-y cross-link site by PAV digestion of DD to PAV-D removed all inhibitory potential at 100 µg/
mL and, in fact, resulted in stimulation of proliferation relative to controls (Fig 6B) \((P < .05, \text{Mann Whitney U test})\).

**DISCUSSION**

In contrast to previous studies that have examined the effects of mixtures of high and/or low molecular weight FgDP and FbDP on lymphocyte proliferation,13-16 our study has evaluated the effects of purified high molecular weight products of fibrinogen and fibrin on lymphocyte proliferation in response to T-cell mitogenic lectins, allogeneic cells, and anti-CD3.

The finding that intact fibrinogen had no effect on lymphocyte mitogenesis is not surprising because most of the functions of this protein, apart from clot formation, appear to be mediated by its degradation products. We found that the higher molecular weight digestion products, only those containing the intact D-domain inhibited lymphocyte proliferation.

Fragment E has the propensity of binding noncovalently to DD and D, forming DD or D-E complexes.26 Because the amino-terminal end of the \(\alpha\) chain of fibrinogen is known to be responsible for the uptake of fibrin monomers by monocyte-macrophage,38 it is possible that noncovalently bound fragment E could result in absorptive endocytosis of these complexes. In FDP preparations, this phenomenon could influence the inhibitory effects of DD or D, which appear to bind to the surface of monocyte-macrophages (see below). Although circulating DD and D are initially complexed to E, fibronectin, and fibrin monomers in vivo,39-41 free circulating DD and D are described during disseminated intravascular coagulation42 after proteolytic removal of fragment E.43

Fragments containing the D domain are known to mediate a variety of effects including calcium binding,44 the regulation of fibrinogen synthesis,45 the ability to inhibit fibrin polymerization,46 and platelet aggregation.47 Indeed, all of these functions appear to be mediated by an intact C-terminal end of the \(\gamma\) chain of the D-domain. In vivo, plasminolysis of fibrinogen results in fragment D1, which possesses an intact C-terminal \(\gamma\) chain.44 In vitro, prolonged digestion of fibrinogen, in the absence of calcium, results in further degradation of D1 to D3, which differs from D1 only by the loss of a 10-Kd peptide containing the Lys-Gln-Ala-Gly-Asp-Val (KQAGDV) sequence from the C-terminal end of the \(\gamma\) chain.45 Our finding that D3 was not inhibitory suggested that fragment D1 might mediate its inhibitory effect via the 10-Kd fragment lost during the conversion of D1 to D3.

The finding that DD also inhibits lymphocyte mitogenesis is in keeping with this hypothesis because DD consists of two molecules of D linked covalently at the C-terminal end of their \(\gamma\) chains. This antiparallel covalent linkage does not involve loss of the KQAGDV sequences46 and does not appear to inhibit binding of DD to a human promonocytic leukemia cell line47 or to monocyte-macrophages in the present study. Puffadder venom specifically cleaves the \(\gamma\) chains of cross-linked DD between the cross-link sites, yielding two identical monomers. The C-terminal ends of the cleaved \(\gamma\) chains differ from that of intact D1 in that the terminal post-cross-link site amino acids are retained through an isopeptide bridge. Presumably, replacement of the colinear Lys-Gln-Ala-Gly-Asp-Val (KQAGDV) sequence of the \(\gamma\) chain by the isopeptide cross-linked KQAGDV sequence results in conformational changes that do not permit binding.

Our findings that purified DD inhibits lymphocyte transformation when added to cultures before or at the time of addition of mitogen agree with those obtained by Edgington et al13 for a mixture of LMWT-FDP. Both studies suggest that FDP inhibit the early stages of blastogenesis and act as relative "antiactivators." We did not routinely attempt to deplete lymphocyte cultures of monocytes before mitogenic stimulation. Monocytes influence lymphocyte responsiveness to mitogens17 and, because variable stringency of depletion may result in different experimental findings, we have chosen to use methods similar to those used by others studying the effect of fibrinopeptides on lymphocyte proliferation.13

Our results raised the possibility that the inhibitory effects of FgDP and FbDP are mediated via their interaction with monocytes and led us to study the effect of preincubating either monocytes or T cells with D or DD and reconstituting

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**Fig 5.** The effects of DD on lymphocyte proliferation appear to be mediated through adherent PMBC. (A) T cells (98% pure, CD3+) did not proliferate in the absence of adherent cells. The addition of \(10^8\) T cells to varying proportions of adherent cells restored the mitogenic capacity of the T cells to baseline values. Peak proliferative responses were noted at T cell to adherent cell ratios of the range from 5 to 20. Peak stimulation indices were found to be at ratios of 20:1 for Con-A 20 \(\mu\)g/mL. (B) T cells and adherent cells were incubated separately in medium with 100 \(\mu\)g/mL DD (final concentration) for 6 hours at 37°C. Thereafter, cells were washed three times and mixed in microculture wells with untreated T cells or adherent cells at a T cell to adherent cell ratio of 20:1. The cells were then stimulated with 20 \(\mu\)g/mL Con-A to give approximately half-maximal stimulation in controls at day 3. Results presented graphically are median cpm \(^3\)H-thymidine incorporation of an experiment representative of three separate experiments. (\(\bullet\)) \(^3\)H-thymidine cell population; (\(\square\)) stimulation index.
the mixture of both cell groups, after extensive washing and immediately before stimulation. Data from these studies strongly suggested that DD interacts with monocytes that in turn influences the proliferative response of T cells to various stimulants. We have shown that DD binds to monocyte-macrophages with saturable kinetics. Specific binding can be inhibited by both anti-CR3 (CD11b) MoAbs (OKM1 and OKM10) directed at the α chain of CR3 (Mac-1) and MHM23 directed at the β-2 integrin chain of CR3 (CD18). Altieri et al. found similar receptors for labeled intact fibrinogen on human monocyte-macrophages. DD does not bind to resting or mitogen-activated T cells, although we have noted the presence of fibronectin-binding proteins on these cells. These fibronectin-binding proteins have been purified by affinity chromatography (unpublished data), as described by others.2,22

PAV-D did not bind to monocyte-macrophages, suggesting that CR3 recognizes the intact C-terminal γ chain and, by inference, probably the colinear sequence Lys-Glu-Ala-Gly-Asp-Val (KQAGDV). This sequence is also recognized as being responsible for fibrinogen, fibrin, and D1 binding to CD11b/CD18 on polymorphonuclear leukocytes stimulated with phorbol esters.21

Circulating FbDP and FgDP, at concentrations often comparable to those used in our study, have been noted in many clinical conditions.40,42,52,53 FbDP and FgDP are present at higher concentrations at sites of tissue damage or inflammation.13

Their potential interactions with CR3 may alter Fc receptor expression and function in a comparable manner to those established for MoAbs directed against CR3.24 Increased release of IL-1 and IL-6 by monocyte-macrophages after incubation with FDP24,55 would tend to augment lymphocyte proliferation.56,57 However, we have shown that the early expression of IL-2R by mitogen-stimulated lymphocytes is diminished in cultures in which blastogenesis is inhibited by DD. This suggests that the inhibition of lymphocyte responses may be mediated by early changes in IL-2R kinetics or even by IL-2-independent pathways.56

We conclude that binding of FDP DD or D by monocytes may modify the early monocyte-lymphocyte interactions essential for the induction of lymphocyte proliferation.

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Fig 6. The effects of D1 and DD on lymphocyte proliferation appear to be mediated through an intact C-terminal γ chain. Proliferation of lymphocytes was assessed in the presence of 0 to 20 µg/mL Con-A and (A) 100 µg/mL D1 or D3 or (B) 100 µg/mL DD or PAV-D. Results are expressed as median cpm [3H]-thymidine of an experiment representative of three separate experiments.
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Fibrin and fibrinogen degradation products with an intact D-domain C-terminal gamma chain inhibit an early step in accessory cell-dependent lymphocyte mitogenesis

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