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Research Article

The present study explores the interactions between lymphocytes and monocytes that are required for expression of procoagulant activity (PCA) by monocytes in response to purified protein derivative of the tubercle bacillus (PPD) or tularemia antigen. The PCA response was antigen specific: peripheral blood mononuclear cells (PBM) from donors sensitive to PPD or tularemia showed an increase in PCA only in response to the sensitizing antigen. The PCA was tissue factorlike in that Factors VII and X were required for expression of the activity, whereas Factor VIII was not. Maximum PCA developed only after 36 to 72 h. Fractionation of PBM into lymphocytes and monocytes after antigenic stimulation demonstrated that greater than 90% of the PCA was associated with monocytes. Isolated monocytes or lymphocytes incubated with sensitizing antigen had the same PCA as control cells. Purified lymphocytes that had been pulsed with antigen were unable to elicit a PCA response from monocytes to which they were added. However, adherent monocytes incubated with antigen, then washed free of unbound protein, were able to trigger lymphocytes to become stimulatory for PCA toward responding monocytes. The development of antigen-specific PCA in PBM could be blocked by including a monoclonal antibody to HLA-DR antigen in the incubation. The antibody had no effect on the clotting assay, on preformed PCA, cell viability, or on stimulatory antigen […]

Antigen-induced Monocyte Procoagulant Activity

Requirement for Antigen Presentation and Histocompatibility Leukocyte Antigen-DR Molecules

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Abstract

The present study explores the interactions between lymphocytes and monocytes that are required for expression of procoagulant activity (PCA) by monocytes in response to purified protein derivative of the tubercle bacillus (PPD) or tularemia antigen. The PCA response was antigen specific: peripheral blood mononuclear cells (PBM) from donors sensitive to PPD or tularemia showed an increase in PCA only in response to the sensitizing antigen. The PCA was tissue factorlike in that Factors VII and X were required for expression of the activity, whereas Factor VIII was not. Maximum PCA developed only after 36 to 72 h. Fractionation of PBM into lymphocytes and monocytes after antigenic stimulation demonstrated that >90% of the PCA was associated with monocytes. Isolated monocytes or lymphocytes incubated with sensitizing antigen had the same PCA as control cells. Purified lymphocytes that had been pulsed with antigen were unable to elicit a PCA response from monocytes to which they were added. However, adherent monocytes incubated with antigen, then washed free of unbound protein, were able to trigger lymphocytes to become stimulatory for PCA toward responding monocytes. The development of antigen-specific PCA in PBM could be blocked by including a monoclonal antibody to HLA-DR antigen in the incubation. The antibody had no effect on the clotting assay, on preformed PCA, cell viability, or on stimulatory antigen itself. These results indicate that elaboration of PCA by mononuclear cells may be an intrinsic part of the classical immune response to antigen, and may explain the presence of fibrin in immune lesions, as well as the occurrence of thrombotic complications in many immune disorders.

Introduction

Cell-mediated immunity is typified by the delayed type hypersensitivity (DTH)[1] reaction. A hallmark of the lesion is the deposition of fibrin which accounts for the characteristic induration distinguishing positive from negative reactions (1, 2). It seems unlikely that nonspecific tissue damage is the initiator of this coagulation. In the model of experimental allergic encephalomyelitis, one of the earliest identifiable abnormalities is fibrin deposition (3). This argues for coagulation occurring relatively early in the course of immune lymphocyte activation. Such lymphocyte activation in response to protein antigen requires antigen presentation by monocyte/macrophages (4) or dendritic cells (5), although other cells (6, 7) can also perform this function. Antigen presenting cells share the feature of expressing Ia-like antigens on their surface (6–8). The Ia protein provides the context for T cells to recognize foreign antigens, and it is here that the specificity of the immune response may be controlled (8).

A likely mediator of coagulation in immune-mediated reactions is the procoagulant activity (PCA) that can be induced to appear on the surface of cells of the monocyte/macrophage lineage (9). Peripheral blood mononuclear cells (PBM) can be induced to produce large amounts of PCA in response to mitogenic lectins (10), bacterial lipopolysaccharide (LPS) (11), antigen/antibody complexes (12), proteolytic products of complement activation (13), sensitizing protein antigens (14), and allogeneic stimuli (15). The PCA is expressed and contained by monocytes, not lymphocytes (16–18), and is cell bound, not released into the medium (18, 19). To date, cellular interactions between lymphocytes and monocytes that culminate in monocyte PCA production have been described in systems with polyclonal stimuli that do not require specific recognition (16–18). Neither LPS or antigen/antibody complexes have been shown to have a role in DTH; and the time course of monocyte PCA production in response to these stimuli (6 h) is not at all in line with the time course of DTH reactions (24–48 h). The present studies, therefore, addressed the cellular events required for expression of monocyte PCA in response to a sensitizing protein antigen. The cellular pathways were found to differ markedly from those previously described in LPS or immune complex induced PCA. In addition, the time course of the process correlated nicely with the appearance of fibrin in in vivo cell-mediated immune tissue lesions.

Methods

Cell isolation and culture. Mononuclear cells were isolated from the peripheral blood of fasting healthy donors by centrifugation over Ficoll-Hypaque at 1,400 *g*, 22°C, for 12 min, as previously described (18). The PBM contained an average of 78% lymphocytes, 22% monocytes, and <1% polymorphonuclear leukocytes as assessed by Wright's stain and nonspecific esterase stains (20). Platelet contamination of PBM could be reduced to <0.01 platelets/mononuclear cell by centrifuging whole blood at 120 *g* for 15 min, removing the platelet-rich plasma before Ficoll-Hypaque centrifugation, and subsequent centrifugation of the isolated PBM at 120 *g* for 5 min through a 1-ml cushion of fetal calf serum (FCS) (Sterile Systems, Logan, UT). The mononuclear cells were washed twice in RPMI-1640 (Gibco Laboratories, Grand Island, NY) containing 100 U/ml penicillin, 50 μg/ml streptomycin, 2 mM Hepes (Gibco Laboratories), pH 7.4, and resuspended in the above medium containing 10% heat-activated FCS. All media were prepared with pyrogen-free water and stored in acid washed, high temperature baked glassware. Monocytes were separated from lymphocytes by incubating PBM at 1×10^6/ml in

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1. *Abbreviations used in this paper:* DTH, delayed type hypersensitivity; FCS, fetal calf serum; LPS, lipopolysaccharide; PBM, peripheral blood mononuclear cells; PCA, procoagulant activity; PPD, purified protein derivative of the tubercle bacillus; [^{3}H]TdR, tritiated thymidine.

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RPMI/10% FCS in 16-mm wells of plastic tissue culture plates (Costar 24, Cambridge, MA). 1 ml of cells was added to each well at 37°C and 5% CO_2 for 24–48 h. Nonadherent cells (lymphocytes) were removed by vigorously washing with RPMI-1640, and the adherent cells (monocytes) were detached by incubating wells at 4°C for 45 min in Puck's saline A containing 3 mM EDTA and 3% (wt/vol) bovine serum albumin. Lymphocyte recovery was 67±7% and monocyte recovery was 66±8%. All monocyte populations were >98% nonspecific esterase positive, with no identifiable platelets by light microscopy. Lymphocyte fractions contained <2% cells staining positive for nonspecific esterase. PBM at 1×10^6/ml, isolated lymphocytes at 8×10^5/ml, or isolated monocytes at 2×10^5/ml were incubated in 12 × 75-mm polypropylene tubes (Falcon Labware, Oxnard, CA) in RPMI/10% FCS with indicated stimuli at 37°C, 5% CO_2. After incubation for the times indicated in the text, the cells were washed twice with RPMI-1640 and resuspended in 0.5 ml of RPMI-1640. Survival of PBM as assessed by eosin exclusion was 99% at 0 h, 96% at 24 h, 88% at 48 h, 74% at 72 h, 69% at 96 h, and was not affected by purified protein derivative of the tubercle bacillus (PPD) or tularemia except at high concentrations used in the dose-response experiments.

Tritiated thymidine ([^{3}H]TdR) uptake as a measure of antigen recognition by T cells was determined by incubating 2×10^5 PBM/0.2 ml RPMI-1640 plus 15% FCS with indicated stimulus in 96-well round-bottom plates for 5 d at 37°C, 5% CO_2. 1 μCi of [^{3}H]TdR (Amersham Corp., Arlington Heights, IL) was added to each well and 18 h later the cells harvested on a MASH device and assayed by liquid scintillation counting for [^{3}H]TdR uptake.

Proteins. PPD in its lyophilized form was obtained from the National Institute of Allergy and Infectious Diseases, Bethesda, MD. It was reconstituted with pyrogen-free water and added to cell culture in concentrations indicated in the text. Tularemia skin test antigen (Foshay-type) phenol-merthiolate inactivated, lyophilized lot 1-lyo 1, was a generous gift of Dr. Michael S. Ascher of the University of California, Irvine, CA. Bacterial LPS from *Escherichia coli* B4:0111 was obtained from Dr. Richard Proctor, Departments of Medical Microbiology and Medicine, University of Wisconsin.

Potential LPS content of proteins was assessed by the heat inactivation assay (21). Tularemia and PPD preparations heated to 99°C for 30 min lost their ability to induce lymphocyte mitogenesis and monocyte PCA. LPS, either alone or added to stimulatory antigen, did not lose its mitogenic or PCA-inducing capability when similarly treated. Stimulatory antigens were found to contain <5 ng LPS/mg protein by this method.

Monoclonal antibody to human HLA-DR antigen (22) was obtained from the American Tissue Culture Collection (No. HB55) and added to cell cultures at a final concentration of 50 μg/ml. To ensure that any inhibition of the procoagulant response by this antibody was not due to antibody interaction with stimulating antigen, absorption of PPD with immobilized anti-HLA-DR was carried out. Rabbit anti-mouse IgG (Cappel Laboratories, Cochranville, PA) was coupled to a 10% suspension of protein-A armed staph (Enzyme Center, Boston, MA) and agitated overnight at 20°C with 200 μg/ml monoclonal anti-HLA-DR antibody in 0.12 M NaCl, 0.03 M Tris acetate, pH 8.5. The staph-bound antibody was centrifuged, combined with 80 μg/ml PPD in RPMI-1640, 25 mM Hepes, and incubated overnight at 20°C. The immunosorbant was centrifuged, and the supernate used as absorbed PPD.

Assay of procoagulant activity. PCA was quantitated in a one-stage recalcification assay (18). The cells were assayed in their viable state for expression of procoagulants on their surface. Cell sample, 0.05 ml, was combined at 37°C with 0.05 ml of pooled normal human platelet poor plasma. Addition of 0.05 ml of 25 mM $CaCl_2$ with manual rocking in a 37°C water bath marked the beginning of the coagulation reaction; the initial appearance of an insoluble fibrin clot marked the endpoint. Normal plasma was obtained as follows. Blood from 25–30 normal donors was collected into 0.1 vol citric acid/sodium citrate buffer, pH 5.0, and centrifuged at 5,000 *g* for 30 min. The plasmas were pooled, centrifuged at 5,000 *g* for 30 min to completely remove platelets, aliquoted, and frozen at −70°C. Rabbit brain thromboplastin (DADE Div., American Hospital Corp., Miami, FL) was used as a PCA standard and assigned a value of 100,000 mU. The clotting times of sequential 10-fold dilutions of this standard were carried out and a log-log standard curve of clotting times vs. milliunits PCA was constructed. The curve was linear from 10 mU (180 s) to 100,000 mU (16.5 s). RPMI-1640, with or without fetal calf serum, PPD, Tularemia antigen, LPS, or monoclonal antibody to HLA-DR consistently demonstrated <7 mU (>200 s) PCA/ml.

Characterization of the PCA was carried out substituting plasma from individuals congenitally deficient in a single coagulation factor for normal pooled plasma in the PCA assay. Factor VIII-deficient plasma was obtained from a local severe hemophiliac with <1% Factor VIII activity. Plasmas deficient in Factors VII and X were obtained commercially (General Diagnostics, Morris Plains, NJ).

Results

To establish immunologic specificity of the PCA response to soluble protein antigens, PBM from eight individuals skin test positive to PPD (>10 mm induration in response to 5 U PPD) but without exposure to tularemia, and three individuals sensitized to tularemia (immunized with single intradermal injection of *Francisella tularensis* LVS, reference 23) but not skin test positive to PPD, were incubated with each antigen for 48 h and the level of PCA expressed on the surface of viable cells determined. Table I demonstrates a 5–6-fold increase in PCA of PBM exposed to sensitizing antigen compared with cells exposed to nonsensitizing, or to no antigen. Comparison of PCA to the proliferative response was used to confirm antigen specificity (Table I). PBM incorporated high levels of [^{3}H]thymidine only upon exposure to relevant antigen.

Time course of PCA expression. To determine the kinetics of the PCA response, PBM were cultured in the presence or absence of the appropriate sensitizing antigen for increasing time intervals, then washed and assayed for PCA (Fig. 1). Cells incubated without antigen showed a very slight increase in PCA at the early time points as compared with PBM assayed immediately after isolation from freshly drawn blood. PBM incubated with sensitizing antigen showed little or no PCA over controls for the first 12 h. Thereafter, there was a steady increase

Table I. Immunologic Specificity of Procoagulant Response of PBM from Individuals Sensitized to Protein Antigens

Cells of subject sensitive to:	Stimulus*	PCA/10^6 cells‡ Clotting time		[^{3}H]Thymidine uptake§
		s	*mU*	*cpm*
Tularemia	None	80	40±4	244±19
Tularemia	Tularemia	53	230±21	17,166±1,124
Tularemia	PPD	78	43±5	866±185
PPD	None	90	25±4	256±43
PPD	Tularemia	87	30±1	276±92
PPD	PPD	61	135±2	12,479±338

* PBM incubated at 37°C with RPMI-1640 plus 10% FCS with 0.154 M NaCl, 10 μg/ml tularemia, or 40 μg/ml PPD.
‡ PBM, 1×10^6/ml cultured 48 h, washed with and resuspended in 0.5 ml RPMI-1640 for assay by one-stage plasma recalcification time. Conversion to milliunits as in Methods. Results are mean milliunits ±SD of triplicate samples from a representative experiment.
§ PBM, 2×10^5/0.2 ml, culture 6 d, pulsed with 1 μCi [^{3}H]thymidine 18 h before harvesting and scintillation counting. Results are mean cpm ±SD of triplicate samples from a representative experiment done in parallel with the procoagulant experiment included in the table.

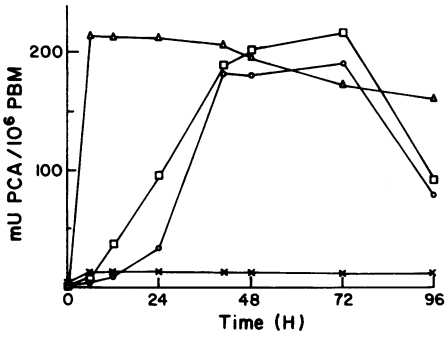

Figure 1. Time course of procoagulant response. PBM, 1 × 10^6, from a donor sensitive to PPD were incubated at 37°C, 5% CO_2, in 1.0 ml of RPMI-1640 plus 10% FCS with 20 μg PPD, 0.15 M NaCl, or 10 μg LPS; PBM, 1 × 10^6, from a donor sensitive to tularemia, were incubated in 1.0 ml of above medium with 10 μg tularemia, NaCl, or LPS as above. At times indicated, duplicate cultures were washed with, and resuspended in RPMI-1640 for PCA assay as in Methods. ×, Saline control; △, LPS; □, PPD; ○, tularemia. The data are from two experiments using the same donors, and are representative of data from three other PPD-sensitive donors, and two other tularemia-sensitive donors.

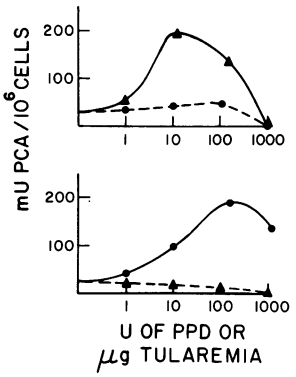

Figure 2. Procoagulant response of immune PBM varies with concentration of antigen. PBM, 1 × 10^6, were incubated in 1 ml RPMI-1640 plus 10% FCS, with increasing concentrations of PPD (10 U/μg) or tularemia antigen for 48 h at 37°C, 5% CO_2. Cells were then washed twice and resuspended in RPMI-1640 for assay of PCA as in Methods. (*Top*) PBM from donor immunized to tularemia, skin test negative to PPD. (▲——▲) Tularemia antigen; (●- - -●) PPD. (*Bottom*) PBM from donor skin test positive to PPD, and without exposure to tularemia. (●——●) PPD; (▲- - -▲) tularemia antigen. Data are from two experiments with duplicate samples for tularemia-sensitive donor, and three experiments with duplicate samples for PPD-sensitive donor.

in PCA, with maximum activity expressed between 36 and 72 h. PCA of antigen-stimulated cells decreased between 72 and 96 h. This was not due to decreased cell viability, as activity was expressed per 10^6 viable cells as assessed by eosin exclusion.

There was a consistent difference in the initial kinetics of PCA expression induced by PPD or tularemia among individuals sensitive to the respective antigens. Differential cell counts showed no significant difference in numbers of monocytes between donors in these studies. To ensure that PBM from various donors did not differ in their ability to express PCA, 10 μg/ml LPS was used as stimulus. PBM from all donors demonstrated rapid induction of high levels of PCA with similar maxima and time courses (Fig. 1). This makes it unlikely that differences in PCA were due to differences in capabilities of PBM from different donors to express procoagulant. The amount of PCA induced by LPS was generally higher than that induced by antigen. There was not, however, any additive effect on resultant PCA of PBM incubated simultaneously with LPS and antigen (data not shown).

Dose dependence of PCA response. To determine the threshold level of antigen necessary for initiating the PCA response, increasing concentrations of appropriate antigen were incubated with immune PBM for 48 h. The intact PBM were then assayed for expression of PCA on the cell surface. Fig. 2 demonstrates a dose-dependent increase in PCA of immune PBM exposed to the sensitizing antigen. Tularemia-sensitive PBM expressed maximum PCA of 200 mU/10^6 cells on exposure to 10 μg/ml of tularemia. Very high levels of this antigen seemed to be toxic, as 25% of the PBM (either immune or nonimmune) exposed to 1,000 μg/ml tularemia for 48 h were nonviable by eosin exclusion. Many of the viable cells became vacuolated as compared with very little vacuolization and 87% viability of PBM exposed to 10 μg/ml tularemia for 48 h. Tularemia antigen as low as 1 μg/ml gave slight but reproducible increases in PCA over control cells. The dose-response relationship shown in Fig. 2 is for cells from a single tularemia-sensitive donor. The dose-response curves of three tularemia-sensitive donors showed differing magnitudes of maximum PCA expressed, but maximum PCA was always elicited at 10 μg/ml tularemia antigen. Tularemia immune PBM exposed to increasing concentrations of PPD expressed only base-line levels of PCA.

PBM from a PPD-sensitive donor also showed a dose-dependent increase in PCA in response to increasing concentrations of that sensitizing antigen, with no PCA expression after exposure to an irrelevant antigen, tularemia. The dose-response curve shown in the lower panel of Fig. 2 is for cells from a single PPD-sensitive donor. PBM from all eight PPD-sensitive donors tested showed positive dose-response curves, but the maximum PCA was elaborated at different concentrations of PPD for each donor.

Cellular source of antigen-induced PCA. To determine the cellular source of PCA expressed by PBM after antigen stimulation, PBM from an individual sensitized to tularemia were incubated 48 h with or without antigen. PBM assayed directly demonstrated the expected fivefold increase in PCA of antigen stimulated vs. control cells. Parallel cultures that had been exposed to antigen were separated via differential adherence to plastic into lymphocytes and monocytes. Table II clearly shows almost all basal PCA to be derived from monocytes, and all incremental PCA in response to antigen to segregate with the monocyte population. In no experiment were lymphocytes seen to increase their PCA above the very low basal levels. Identical experiments with cells from PPD-sensitive donors, and PPD as antigen likewise showed monocytes to be the source of PCA (data not shown).

Cellular interactions in the antigen-specific PCA response. Since monocytes derived from either control or antigen-pulsed PBM were shown to express the vast majority of PCA of the PBM, we tested whether monocytes alone could manifest the PCA response. Monocytes were first isolated from tularemia or PPD-sensitive donors, exposed to sensitizing antigen in vitro, and the resultant PCA monitored (Table III, data from Tularemia-sensitive donors shown). After a 48-h incubation, monocytes exposed to antigen showed no more PCA than monocytes exposed to medium alone. However, monocytes incubated in the presence of lymphocytes and antigen showed a 5–6-fold increment in PCA over monocytes and lymphocytes without antigen (Table II). Thus, it seemed that monocytes required lymphocytes to manifest an increase in PCA in response to sensitizing antigen. We next determined whether the interaction was lymphocyte to monocyte, as in other PCA responses (17, 18), or monocyte to lymphocyte to monocyte, as in other antigen driven,

Table II. Procoagulant Content of Cell Populations from Antigen-pulsed and Unstimulated Human PBM

Cell population*	Stimulus*	PCA/10^6 cells§
		mU
Lymphocytes and monocytes‖	None	45±5
Lymphocytes and monocytes‖	Tularemia	225±16
Lymphocytes¶	None	9±1
Lymphocytes¶	Tularemia	11±0
Monocytes**	None	415±22
Monocytes**	Tularemia	2,650±173

* Indicated cell populations derived from 1×10^6 PBM incubated with indicated stimulus for 48 h at 37°C 5% CO_2.
‡ Tularemia, 10 μg/ml, or an equal volume of RPMI-1640.
§ 10^6 cells of the indicated population, washed twice with and resuspended in 0.5 ml RPMI-1640, then assayed for PCA as in Methods. Results are mean milliunits ±SD from two experiments using cells from the same donor.
‖ Lymphocytes 99% esterase negative, and monocytes 98% esterase positive, combined in 9:1 ratio then assayed for PCA.
¶ Lymphocyte population 99% esterase negative prepared by two sequential plastic adherence steps to deplete monocytes.
** Monocyte population 98% esterase positive prepared by adherence to plastic and detachment with Puck's saline A-bovine serum albumin-EDTA as in Methods.

Table III. Monocyte-Lymphocyte Collaboration is Required for Generation of the Antigen-specific Procoagulant Response of Human PBM

Cell population	Stimulus*	PCA/10^5 Monocytes‡
		mU
Monocytes§	Saline	35±16
Monocytes§	Tularemia	27±7
Lymphocytes‖ + stimulus → monocytes¶	Saline	25±3
Lymphocytes‖ + stimulus → monocytes¶	Tularemia	31±5
Lymphocytes, 5% monocytes** + stimulus → monocytes¶	Saline	33±11
Lymphocytes, 5% monocytes** + stimulus → monocytes¶	Tularemia	215±18

* Tularemia, 10 μg/ml, or equal volume of 0.154 M NaCl.
‡ 1×10^5 responding monocytes washed with and resuspended in 0.5 ml RPMI-1640 for assay of PCA as in Methods. Results are mean milliunits ±SD of duplicate samples from two experiments using two different donors.
§ 1×10^5 monocytes 98% esterase positive incubated for 48 h in the presence or absence of Ag.
‖ 1×10^6 lymphocytes with 1% esterase-positive cells in 1 ml RPMI-1640/10% FCS incubated with or without Ag for 24 h, washed, and added to 1×10^5 autologous responding monocytes in a final volume of 1 ml RPMI-1640/10% FCS for a further 24 h.
¶ 1×10^5 autologous responding monocytes in a final volume of 1 ml RPMI-1640/10% FCS for a further 24 h.
** 1×10^6 lymphocytes plus 5×10^4 adherent monocytes incubated with or without Ag in 1 ml RPMI-1640/10% FCS for 24 h. The lymphocytes were removed from the adherent monocytes, washed, and added to 1×10^5 autologous responding monocytes in a final volume of 1 ml RPMI-1640/10% FCS for a further 24 h.

non-PCA processes (24). Lymphocytes pulsed with antigen and washed free of nonbound protein were unable to induce PCA in autologous monocytes to which they were added (Table III). However, lymphocytes in the presence of autologous adherent monocytes (20:1 ratio) pulsed for 24 h with antigen, washed free of unbound protein, and removed from esterase-positive cells, were able to induce a fresh population of autologous monocytes to express a sevenfold increase in PCA (Table III).

These data implied antigen presentation by monocytes to lymphocytes. To confirm this, monocytes from a PPD-sensitive individual were allowed to adhere to plastic and the lymphocytes vigorously washed away. The monocytes were incubated 24 h with or without 20 μg/ml PPD, washed, and autologous lymphocytes that had been depleted of monocytes were added. After another 24 h, the lymphocytes were removed, washed, and added for 24 h to responding monocytes which, after 24 h, were then assayed for PCA. Table IV demonstrates that antigen-pulsed monocytes were able to induce in lymphocytes the ability to stimulate responding monocytes to increase expression of PCA. Such antigen-pulsed monocytes did not require that free antigen be present with lymphocytes, as antigen pulsed, washed monocytes were fully competent to initiate the PCA response. Tables III and IV show data for tularemia or PPD-sensitive cells, respectively; identical cellular interactions were necessary for the PCA response to each antigen.

As the development of increased PCA was antigen specific and required antigen presentation, we reasoned that Ia-like protein present on the surface of reacting cells would be important for the elaboration of a response. PBM from a PPD-sensitive individual were incubated with either saline, PPD, saline and anti–HLA-DR antibody or PPD and anti–HLA-DR antibody. This monoclonal antibody, ATCC No. HB55, was raised in mice and shown to recognize HLA-DR antigen (22). Table V gives data demonstrating that the presence of this antibody prevents the increase in PCA of PBM seen with sensitizing antigen. Addition of increasing concentrations of the antibody (25, 50, 100, and 200 μg/ml) directly to the clotting assay had no effect (data not shown). To demonstrate that inhibition was at the cellular level, and not due to interference with soluble antigen, anti–HLA-DR was immobilized on Staph protein A-rabbit anti-mouse IgG. The immobilized anti–HLA-DR was incubated overnight with PPD and then removed by centrifugation. The "absorbed PPD" was used as stimulus for PBM cultures and induced a full measure of PCA. Addition of nonbound HLA-DR antibody to PBM and absorbed PPD again prevented the development of increased levels of PCA. Cell counts and viability, as determined by eosin exclusion, showed no difference between antibody treated and nonantibody-treated cultures (93±4, and 91±2% viability, respectively). Inclusion of an isotype-matched monoclonal antibody to HLA-class I antigens present on both lymphocytes and monocytes (25) did not impede the ability of PBM to develop a PCA response to PPD. What's more, identical experiments using tularemia antigen and cells from a tularemia-sensitive donor gave similar results (Table VI).

Coagulation factor requirements of antigen-induced PCA. The PCA assay was repeated on control cultures of PBM, cultures that had been incubated with sensitizing antigen, or with LPS.

Table IV. Requirement for Antigen Presentation in the Procoagulant Response to Specific Antigen

Cell population	PCA/10^5 monocytes (*mU*) Control*	PPD‡
Monocytes§ → Lymphocytes‖	15±3	18±4
Monocytes§ + Ag → Lymphocytes‖	16±2	165±33
Plastic¶ → Lymphocytes	9±4	13±6
Plastic¶ + Ag → Lymphocytes	7±2	7±3

*, ‡ Adherent monocytes, washed free of lymphocytes, were incubated with 0.154 M NaCl (control) or 20 μg/ml PPD for 24 h, washed, and combined with lymphocytes and medium for 24 h. Lymphocytes were then withdrawn, washed, and added to fresh (responding) monocytes for 24 h. Responding monocytes were then assayed for PCA as in Methods. Results are mean milliunits ±SD from three experiments using three different donors.
§ 2×10^5 monocytes were adhered to plastic by incubating 1.0–1.2 $\times 10^6$ PBM in 1 ml RPMI-1640/10% FCS in each well of a 24-well plate for 2 h. Lymphocytes were vigorously washed off, and the remaining monocytes incubated with or without PPD for initiating the process outlined above.
‖ Lymphocytes (1×10^6) in 1 ml RPMI-1640/10% FCS without stimulus were added to saline or PPD-pulsed monocytes.
¶ Plastic culture wells, without monocytes, were incubated with 0.15 mM NaCl or 20 μg/ml PPD for 24 h, washed, and treated identically to the monocyte-containing wells.

Table V. Monoclonal Antibody that Recognizes HLA-DR Antigen Inhibits the Development of PPD-induced PCA in PPD-sensitive PBM

Monocytes of PPD-sensitive donor incubated with:*	Antibody to HLA-DR antigen‡	PCA
		mU/10^6 PBM
Saline	0	14±4
Saline	+	11±3
PPD§	0	215±12
PPD§	+	18±4
PPD preabsorbed with antibody to HLA-DR‖	0	163±13
PPD preabsorbed with antibody to HLA-DR‖	+	19±1
PPD + monoclonal antibody to HLA-class I¶	0	302±60

* 1×10^6 PBM from PPD-sensitive donor, indicated stimulus, with or without monoclonal anti-HLA-DR antibody, and was incubated at 37°C, 5% CO_2 for 48 h, then washed and assayed for PCA. Mean±SD of three experiments done in duplicate using two different donors.
‡ ATCC No. HB55, previously shown to recognize HLA-DR antigen (22), had a final concentration in culture of 50 μg/ml.
§ PPD at 10 μg/ml final concentration.
‖ PPD, 80 μg/ml, incubated overnight with monoclonal anti-HLA-DR antibody which had been immobilized on Staph Protein A-rabbit anti-mouse IgG. The immunoabsorbant was centrifuged and the supernate used as "absorbed PPD" to demonstrate presence or absence of effect by anti-HLA-DR on the soluble protein Ag as opposed to the responding cells. Results are from two experiments done in duplicate.
¶ Monoclonal anti-human HLA-class I added at final concentration of 50 μg/ml.

Table VI. Monoclonal Antibody that Recognizes HLA-DR Antigen Inhibits the Development of Tularemia-induced PCA in Tularemia-sensitive PBM

PBM of tularemia-sensitive donor incubated with:*	Antibody to HLA-DR antigen‡	PCA
		mU/10^6 PBM
Saline	0	7±2
Saline	+	9±2
Tularemia§	0	206±74
Tularemia§	+	13±1
Tularemia + monoclonal antibody to HLA-class I‖	0	166±1

* 1×10^6 PBM from Tularemia-sensitive donor were incubated with indicated stimulus, with or without monoclonal anti-HLA-DR antibody, at 37°C, 5% CO_2, for 48 h, then washed and assayed for PCA. Results are mean milliunits ±SD from a representative experiment done in duplicate.
‡ ATCC No. HB55, previously shown to recognize HLA-DR antigen, had a final concentration in culture of 50 μg/ml.
§ Tularemia, 10 μg/ml.
‖ Monoclonal anti-human HLA-class I added at final concentration of 50 μg/ml.

However, plasmas from individuals congenitally deficient in a single coagulation factor were substituted for normal plasma to determine which factors were necessary for the induced PCA to have its full effect. As shown in Table VII, antigen-induced PCA required the presence of Factors VII and X, whereas full activity was retained in the absence of Factor VIII. This is the same activity profile demonstrated by LPS-induced PCA.

Discussion

The deposition of fibrin is a prominent feature of lesions of delayed type hypersensitivity, a prototype of cell-mediated immunity. Edwards and Rickles (14), and Geczy and Meyer (26), have presented compelling evidence that this fibrin deposition is the culmination of a series of reactions initiated by the expression of procoagulant molecules on the surface of monocytes. The cellular events necessary to express procoagulant molecules on the surface of monocytes and macrophages can be initiated

Table VII. Factor Dependence of Antigen-induced PCA

Stimulus*	PCA (mU/10^6 PBM)‡ Plasma factor deficiency:	Normal	VII	X	VIII
None		30	25	<5	20
LPS		260	30	10	240
Tularemia		220	20	<5	230

* PBM from a tularemia-sensitive donor were incubated at 1×10^6/ml in RPMI plus 10% FCS with either RPMI-1640, 10 μg/ml LPS, or 10 μg/ml tularemia, for 48 h at 37°C, 5% CO_2. The cells were then washed and resuspended in 0.5 ml RPMI-1640 for assay.
‡ Assay for PCA carried out as in Methods. To determine what coagulation factors were required for the PCA-induced coagulation to take place, plasmas from individuals congenitally deficient in a single coagulation factor were substituted for normal plasma.

by a variety of stimuli (10–15). Among the more extensively studied of these are bacterial LPS (10, 11, 17, 19, 27) and antigen-antibody complexes (12, 18, 28). These stimuli are often used to elicit the Shwartzmann reaction in experimental animals, and have been found to initiate unidirectional communication from lymphocytes to monocytes, which then express PCA (17, 18). The thrombohemorrhagic disorder of the Shwartzmann reaction may be due to monocyte PCA. A different set of cell interactions may be involved in the allogeneic induction of monocyte PCA as Helin and Edgington (29, 30) have recently shown. Currently available experimental data do not adequately address the mechanism by which monocyte PCA can be elicited as a response to a specific sensitizing antigen with no such expression after exposure to a nonsensitizing antigen. The present study demonstrates that human PBM can express potent PCA on their surface as a result of interactions with lymphocytes that have been presented in a specific manner with antigen to which they have been previously sensitized.

All donors for these experiments were sensitive by testing to either PPD or tularemia, but no one was sensitive to both. The degree of increase of PCA above base-line levels varied somewhat from donor to donor, but was consistent over time for each donor. Among the eight donors sensitive to PPD that were tested, none demonstrated an increase in PBM PCA on exposure to tularemia. Also, [^{3}H]TdR uptake of these PBM upon exposure to tularemia did not increase above baseline, which is in agreement with previous studies (23, 31).

Geczy and Meyer (26) demonstrated PBM PCA correlated quite well with skin reactivity as a measure of previous sensitization to an antigen. We did not attempt such correlations, as our goal was to define the cellular interactions required for the elaboration of this activity.

Increases in PBM PCA upon exposure to sensitizing antigen reached a maximum between 36 and 72 h. The kinetics of the response varied from individual to individual, with cells from PPD sensitive donors generally showing increased PCA at earlier times than cells from tularemia-sensitive donors. We did not, however, observe the very early increase with subsequent decrease of PCA in exposed cells to PPD reported by Lyberg et al. (32). It is possible that that study and the present one used different stimuli, as preparations of PPD are a mix of multiple proteins (33).

The cellular source of the PCA of antigen-stimulated PBM was the monocyte. Using populations > 98% positive for nonspecific esterase staining, we demonstrated that all the PCA resided in the monocyte population, with a negligible amount of activity in the nonspecific esterase-negative lymphocyte population. This is in agreement with a large number of other studies that have looked at the induction of PBM PCA by a number of stimuli (16–18, 29, 34).

The cellular interactions required for this specific response to sensitizing antigen are demonstrated in this report. There was an absolute requirement for antigen presentation to lymphocytes. Lymphocytes that were free of monocytes that encountered soluble protein antigen were subsequently unable to induce an increase in PCA in responding monocytes. On the other hand, lymphocytes that were presented with protein antigen by monocytes were able, when separated from those monocytes and washed, to induce significant increases in activity among responding monocytes. These data point to a scenario of monocyte uptake of soluble antigen, presentation of bound antigen to immune lymphocytes, and subsequent communication from lymphocyte to monocyte of instructions to express PCA.

Specific immune reactions to soluble protein antigens demonstrate a specificity of cellular recognition at the antigen presentation step. The cell that presents antigen must possess an Ia-like antigen that is identical to the Ia determinant of the sensitized T cell. This genetically determined recognition seems essential for T lymphocytes and monocytes to interact at this step (24). To demonstrate the presence or absence of such restrictions of cellular interactions in the antigen-induced procoagulant response, we employed a monoclonal antibody to the human HLA-DR antigen (22). We have used the anti–HLA-DR antibody as a means of establishing specificity of cellular recognition due to the impracticality of mixing lymphocytes and monocytes from different donors. Alloantigens are stimulators of the monocyte procoagulant response (29), and very high background in our experiments combining lymphocytes and monocytes from different donors bear this our (data not shown). Monoclonal antibodies to HLA-DR molecules, therefore, seemed an unambiguous approach. The use of such antibodies has been employed successfully in the past in several different systems to define the necessity of specific recognition events between cell populations in the elaboration of an immune response (35).

Addition of antibody HB-55 to cultures of PBM and sensitizing antigen resulted in abbrogation of the procoagulant response. An isotype matched monoclonal antibody to HLA-class I antigens had no effect on the system. Bergholtz and Thorsby (36), employed polyclonal antisera to HLA-DR antigens to demonstrate inhibition [^{3}H]thymidine response to PPD by human peripheral blood T lymphocytes. Given the dissimilar immunologic reagents, it is difficult to know whether there is similarity in recognition for the proliferative and PCA responses. Other antigen-driven systems have been found to depend on non-DR molecules on cells that are HLA-DR positive (37, 38). It is therefore interesting that anti–HLA-DR should block the PCA response. The disparity cannot be completely explained at present, but it is worth noting that the previous studies were looking at a different response ([^{3}H]thymidine uptake) to antigen than is the present study (PCA expression). Given cell surface molecules may well have different roles in each system. Since we have not employed antibodies to other cell surface histocompatibility proteins, we cannot say what effect they might have on the PCA system.

Current studies are underway to elucidate the step at which anti–HLA-DR antibody inhibits the elaboration of specific antigen-induced PCA. In preliminary experiments with purified populations of monocytes and lymphocytes, we have found the monocyte to be the site of inhibition of the procoagulant response by this monoclonal anti–HLA-DR antibody. The use of [^{3}H]thymidine uptake by immune T cells has shown that the antigen presentation step is interfered with by anti–HLA-DR antisera (36). We have recently described a PPD reactive human T cell clone that mediates the procoagulant response to PPD presented by autologous monocytes (39). This should prove invaluable in exploring the question of HLA-DR dependence of the reaction, and in defining the point at which HLA-DR is required.

The PCA demonstrated by monocytes in these studies is consistent with tissue factorlike activity. Previous studies described tissue factorlike activity expressed on monocytes after exposure of PBM to specific antigen as well as many other stimuli

(13, 15, 18, 29, 40, 41). Neither of the antigens used as stimuli in this study demonstrated any PCA of their own. The activity was dependent upon the presence of Factors VII and X, and independent of the presence of Factor VIII. However, serial dilution of the monocyte PCA demonstrated a Factor VIII dependency with more dilute procoagulant, in parallel with the Factor VIII dependency of dilute preparations of brain thromboplastin (data not shown) (42, 43). What's more, the addition of concanavallin A to PCA-positive mononuclear cells effectively inhibited the expression of the procoagulant activity (data not shown). These data are consistent with, however not conclusive for, identity of the PCA as tissue factor. Such definitive proof must await the purification of the apoprotein of human tissue factor and development of appropriate antibodies.

This two step (monocyte-lymphocyte-monocyte) process contrasts with studies of the induction of human peripheral blood mononuclear cell PCA by LPS and immune complexes (17, 18). Both LPS and antigen/antibody-induced PCA involve an initial interaction of the stimulus with a lymphocyte with no requirement for stimulus presentation by monocytes. This lymphocyte is then triggered and able to interact with monocytes, inducing increased PCA. A second, slower induction of monocyte PCA has been recently described. Allogeneic stimuli induce monocyte PCA via T cell instructive mechanisms that differ from LPS or immune complex driven systems (29, 30). The system described in this report then constitutes a third pathway of monocyte procoagulant induction.

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