LYMPHOCYTE COOPERATION IS REQUIRED FOR AMPLIFICATION OF MACROPHAGE PROCOAGULANT ACTIVITY*

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The biological diversity of the immune system is reflected in the variety of immune effector systems and mediation pathways that have emerged. Among the least recognized involve the hemostatic pathways. The possibility of cellular and molecular linkages between two host defense mechanisms, the hemostatic and immune systems, has long been suggested, but has yet to be satisfactorily delineated. To support the probability of such linkages, intradermal deposition of fibrin is recognized as a prominent and consistent feature of the delayed-cutaneous-hypersensitivity reaction (DCH)\(^1\) (1, 2). Indeed, recent studies have demonstrated that fibrin deposition is required for the phenotypic expression of DCH, i.e., induration (3). That the macrophage is also critically important to this phenomenon is suggested by observations that carageenan abrogates the development of this tissue lesion (4). Other provocative observations linking the hemostatic system to immune responses include the deposition of fibrin in the lesions of allogeneic rejection (5), the massive accumulation of intraglomerular fibrin in experimental anti-glomerular-basement-membrane-type glomerulonephritis when animals are given the fibrinolytic inhibitor \(\alpha\)-aminocaproic acid (6), the appearance of perivascular fibrinogen or fibrin as the earliest feature of experimental allergic encephalomyelitis (7), and the characteristics of the general Schwartzman phenomenon (8).

These observations have been complemented by in vitro studies demonstrating that the significant but relatively modest content of procoagulant activity (PCA) of human peripheral blood mononuclear cells is markedly increased by exposure to antigen-antibody complexes (9), allogeneic cells in mixed lymphocyte cultures (10), culture in the presence of mitogenic lectins (11), bacterial lipopolysaccharides (LPS) (12, 13) and by proteolytic products of C5 (14). The PCA of mononuclear lymphoid cells has been attributed to tissue factor, a cellular membrane lipoprotein that binds Factor VII, induces specific enzymatic activity, and, via Factor X and prothrombin in the presence of Factor V, culminates in the conversion of fibrinogen to fibrin (15, 16).

The cellular events involved in the induction of PCA activity has received limited

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\(^1\) Abbreviations used in this paper: DCH, delayed-cutaneous-hypersensitivity reaction; HMEM, HEPES-buffered minimum essential medium; LPS, lipopolysaccharide(s); PCA, procoagulant activity; RBC, erythrocytes.
attention. Indeed, though it has been suggested that the monocyte/macrophage possesses PCA activity (16), cellular localization and the requirements for collaborative cellular interactions have not been firmly established. In our study, we provide direct evidence that a small proportion of murine splenic macrophages possess basal PCA activity, and that after exposure to LPS, it is the macrophage that accounts for the profound increase in PCA. However, the amplification of both viable and total content macrophage PCA by LPS requires cellular collaboration that appears to involve a direct cellular interaction of macrophages with lymphocytes that have been first exposed to LPS, a previously undescribed scheme of cellular collaboration.

Materials and Methods

**Spleen Cells.** Spleens were removed aseptically from 6- to 8-wk-old male strain A/St or BALB/c mice (L. C. Strong Laboratory, San Diego, Calif.) and cells were suspended in HEPES-buffered minimal essential medium (HMEM) that consisted of bicarbonate-free Eagle's minimum essential medium (Flow Laboratories, Rockville, Md.) that contained 25 mM HEPES, 2.5 mM NaOH, 100 U penicillin/ml, and 50 µg streptomycin/ml (Gibco Diagnostics, Gibco Invitrogen Div., Chagrin Falls, Ohio) at pH 7.3. Mononuclear cells were isolated by centrifugation over Ficoll-Hypaque at 22°C for 12 min at 1,400 g at the interface (17). Mononuclear cells from the interface were >98% lymphocytes or monocyte/macrophages by cytologic examination of Giemsa stained smears. Cells were washed twice with HMEM before use.

Adherent cells (macrophages) were isolated by incubation of mononuclear cells in complete medium at 5 × 10⁶ cells/ml for 45 min at 1 ml in 16-mm-diameter plastic tissue culture 24-well plates (Costar, Data Packaging, Cambridge, Mass.) at 37°C and 5% CO₂ (18). Nonadherent cells (lymphocytes) were decanted, subjected to an additional cycle of adherence, decanted again, and assayed for viability (>98% by trypan blue) and freedom from macrophages (<1% by neutral-red uptake and by esterase staining). Recovery of lymphocytes was 86 ± 3%. The macrophage monolayer was washed twice with HMEM and the identity of adherent cells as viable macrophages was confirmed by vital uptake of neutral red and a positive reaction for esterase with >99% of cells positive by both criteria. Macrophages were detached by agitation at 150 rpm on a gyrotory platform (Model G2; New Brunswick Scientific Co., Inc., Edison, N. J.) for 25 min at 22°C in the presence of 3% bovine serum albumin (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), HEPES-buffered Puck's saline A (0.4 gm% KCl, 80 gm% NaCl, 20 mM HEPES, pH 7.3, 1 gm% glucose, and 0.005% phenol red) (Gibco Diagnostics, Gibco Invitrogen Div.) that contained 3 mM EDTA (Sigma Chemical Co., St. Louis, Mo.). Recovery of macrophages was 78 ± 2%.

Culture. Cells were cultured at 5 × 10⁶/ml in 1-ml volumes in 16-mm-diameter, 24-well plates in complete medium consisting of 10% heat-inactivated fetal calf serum in 25 mM HEPES, 0.2 gm% sodium bicarbonate, 2 mM glutamine, 100 U penicillin/ml, and 50 µg streptomycin/ml (Gibco Diagnostics, Gibco Invitrogen Div.) in Eagle's minimum essential medium. Cultures were stimulated with 10 µg of LPS (from Escherichia coli 0111:B4) prepared by the butanol extraction method (19) and provided by Dr. David Morrison, Research Institute of Scripps Clinic, La Jolla, Calif. Cells were cultured at 37°C in a 5% CO₂ atmosphere.

**PCA Assay.** Samples of viable cells or frozen-thawed and sonicated cells in HMEM were assayed for the capacity to shorten the spontaneous clotting time of human plasma in a one-stage clotting assay. Cells were sonicated in 0.5 ml of HMEM in 12 × 75-mm tubes with a microtip probe of a Heat Systems-Ultrasoundics, Inc. (Plainview, N. Y.) model W-140 sonicator at a setting of 3 using two bursts of 10 sec each with ice-cooling. The vol was brought to 1.0 ml with HMEM, and PCA was determined from the clotting time at 37°C in recalculated, diluted normal plasma; for greater sensitivity and linearity at low PCA, severe Factor-VIII-deficient (hemophilia A) plasma was used. This corrected the recognized deviation and Factor VIII and IX dependence of low thromboplastin concentrations (20, 21). The assay consisted of 0.1 ml of test sample (or rabbit brain thromboplastin standard) plus 0.1 ml of citrated, normal fasting human plasma in 12- × 75-mm tube to which was added 0.1 ml of 25 mM CaCl₂ to initiate the assay. The rabbit brain thromboplastin standard at 36 mg (dry mass)/ml (Dade Div., American
Hospital Supply Corp., Miami, Fla.) was assigned a value of 100,000 milliunits. There was no activity present in HMEM, complete medium, or macrophage-detaching solution. The assay was used over the range of 1–10,000 milliunits, or $10^1$ to $10^5$ cells, which was linear when using severe Factor-VIII-deficient plasma or $10^5$–$10^7$ cells with normal plasma substrate. The precision (coefficient of variation) of assays was 8.7% at the lower limit and 7.0% at the upper limit. The molecular pathways will be described in detail in a subsequent study.

**PCA Plaque Assay.** For cellular localization of PCA to single viable cells, cell suspensions were incubated on glass microscope slides at 37°C for 2 h in complete medium in 5% CO₂. The medium was removed without displacing the cells, and the cells were overlaid with 1.5% agarose (Indubiose A-37; L'Industrie Biologique, Francaise, S.A.) dissolved in a mixture of three volumes of HMEM that contained 3.67 gm% CaCl₂ and one volume of platelet-free, citrate, normal human plasma. The plasma was added to the agarose at 38–39°C and 1.0–1.5 ml was immediately poured on the slide and allowed to spread while maintained at 37°C. After a 10-min incubation period, the reaction was terminated by placing the slides in cold HMEM that contained 10 U heparin/ml and washed for 16–20 h. Cells were then fixed and stained for nonspecific esterase and counterstained with methyl green. For nonviable cell assays, the cells were centrifuged onto the slides in a cytocentrifuge, fixed for 45 min in acetone, dried before pouring the agarose-plasma as described above, and then stained for nonspecific esterase. The presence of PCA was clearly indicated by the formation of dark blue strands of fibrin about the single cells. This contrasted with the red cytoplasmic reaction for nonspecific esterase.

**Cytologic Staining.** Nonspecific esterase, a highly reliable marker for monocytes and macrophages, was identified as described by Li et al. (22). Cells were fixed for 30 sec in 25% formalin in 45% acetone, buffered with 10 mM phosphate to 6.6 pH, and then were washed with water and dried. To each slide was added a mixture of 0.1 ml of 4 gm% hexazotized pararosanilin, and 0.2 ml napthyl butyrate and incubated for 45 min. The slides were washed, dried, counterstained with 1% methyl green for 3 min and washed. They were immediately examined for the cytoplasmic red reaction product of the esterase reaction. Cell counts were based on examination of 100 cells. Hexazotized pararosanilin was prepared by mixing equal volumes of 4% pararosanilin hydrochloride (J. T. Baker Chemical Co., Phillipsburg, N. J.) in 2 N hydrochloric acid with 4% sodium nitrate for 1 min immediately before use. α-Naptha butyrate (100 mg) was dissolved in 2 ml of ethylene glycol monomethyl ether and stored at 4°C. Methyl green stain was prepared by dissolving 1 gm methyl green in 100 ml of 0.1 N sodium-acetate buffer, pH 5.0. This was titrated to pH 4.2-4.5 with hydrochloric acid, and extracted with chloroform until clear.

**Results**

**Basal PCA.** Viable, as well as disrupted, cells were assayed to establish basal PCA level. The total PCA content of disrupted mononuclear cell preparations was quite consistent at 88 ± 18 milliunits in 10 experiments, whereas the PCA of the viable cells was 23 ± 6 milliunits or 25% of the content (Table I). Total PCA content of the lymphocyte subpopulation, separated on the basis of adherence and defined on the basis of lack of esterase, positive neutral-red uptake, and morphology, was significantly less, but nonetheless positive at 36 ± 12 milliunits. Expression of PCA by viable lymphocytes was 9.5 ± 1.1 milliunits. Recovery of cells was 86 ± 3% and 78 ± 2% for lymphocytes and macrophages, respectively. The macrophages, representing 12% of the average whole splenic mononuclear population based on neutral-red uptake and esterase staining, accounted for 67% of total PCA content and 68% of viable expression. Recovery of total and viable PCA, i.e., the sum of the lymphocyte and macrophage subpopulation, was 109% and 115%, respectively.

**Plaque Analysis.** Cellular distribution of PCA was analyzed directly using plaque assays of both viable and fixed cells. In this assay, cells were overlaid by agarose that contained dilute plasma and calcium for a 10-min assay period to permit formation of local fibrin. After termination and washing, the cells were fixed (if viable) and
TABLE I
Basal Levels of PCA of Splenic Mononuclear Cells

| Cell population          | Viable expression | Total content |
|--------------------------|-------------------|---------------|
|                          | Time (sec)        | Milliunits    | Time (sec)     | Milliunits    |
|                          |                   |               |               |
| Whole mononuclear cells  | 130 ± 6.5         | 23 ± 6        | 90 ± 5        | 88 ± 18       |
| Lymphocytes              | 165 ± 25          | 9.5 ± 1.1     | 115 ± 10      | 36 ± 12       |
| Macrophages              | 76 ± 6            | 150 ± 35      | 55 ± 7        | 535 ± 75      |
| HMEM                     | 225 ± 30          | 0.4 ± 0.1     | 225 ± 30      | 0.4 ± 0.1     |

Splenic mononuclear cells from A/St and BALB/c mice were cultured in HMEM. Lymphocytes were 1% nonspecific-esterase positive, and macrophages after nonadherent cell removal were 98% esterase positive.

Fig. 1. Plaque assay for demonstration of PCA in individual cells from whole, splenic mononuclear-cell preparations either stimulated with LPS or unstimulated. (A) Fixed, esterase-positive cell (macrophage) from cells not exposed to LPS and negative for PCA (no fibrin strands). (B) Fixed, esterase-positive cell (macrophage) from cells stimulated with LPS and positive for PCA (presence of fibrin strands). (C) Fixed esterase-positive cell that is positive for PCA (fibrin strands) and esterase-negative cell (lymphocyte) (arrow) that is negative for PCA from cells exposed to LPS. Note: The esterase reaction cannot be differentiated in the black-and-white photographs.

Stained for esterase. Morphologic identity of the cells by reference to the cytoplasmic red esterase reaction, and the identification of PCA-positive cells by reference to the presence of fine blue strands of fibrin that surrounded the cells was readily apparent (Fig. 1). Only ~1% of cells were PCA positive in the viable state, and of these cells, all were macrophages; i.e., esterase positive. Total PCA content was assessed in fixed-cell preparations and indicated that only ~3% of cells were positive, of which, 80% were macrophages (Table II). The rare esterase-negative but PCA-positive cells appeared cytologically consistent with lymphocytes, though this will require further verification. This is in agreement with assays of cell suspensions which showed that the lymphocyte population possessed low levels of PCA though sufficient to account for 32% of total or 33% of viable expression of the basal PCA of the whole mononuclear cell mixture.

Stimulation of PCA by Bacterial LPS. Whole splenic mononuclear cells were incubated in complete medium with *E. coli* 0111:B4 LPS for 6 h. When assayed for direct viable PCA as well as total PCA content of disrupted cells, there was a significant
FIG. 2. Induction by LPS of PCA in murine spleen cells. (A) 5 × 10⁶ murine splenic leukocytes were incubated with 10 μg LPS (from E. coli 0111:B4) for time intervals of 30 min–20 h, and total PCA content was determined. (B) 5 × 10⁶ murine splenic leukocytes were incubated for 6 h with increasing concentrations of butanol-extracted LPS (from E. coli 0111:B4) from 0.1 to 100 μg, and the total content of PCA was determined.

increase as compared to basal levels. The time-course for optimal amplification of the PCA content of whole mononuclear cells was examined (Fig. 2A). When 10 μg/ml LPS and 5 × 10⁶ cells/ml was used, an increase from basal activity (80 milliunits) was not observed for 2 h, and then increased to a maximum of 800 milliunits by 6 h. Activity declined only slightly during the following 18 h. A representative dose-response curve is shown in Fig. 2B. Incubation of splenic mononuclear cells with serial concentrations of LPS for 6 h at 37°C demonstrated maximum induction of PCA at a concentration of 10 μg LPS/ml, declining sharply at higher concentrations. Subsequent studies uniformly utilized 10 μg LPS/ml and 6 h of incubation. Subsequent pulses of LPS at 6, 12, or 24 h after the initial stimulation did not further increase PCA.

Quantitative analysis of PCA stimulation by LPS in a series of seven experiments is shown in Table III. At 6 h, there was a fourfold increase of expression by whole viable mononuclear cells in contrast to a 10.4-fold increase in total cellular content of PCA. Whereas the viable expression of PCA was ~25% of the total cellular content, in the basal state, after LPS stimulation, the viable PCA was increased fourfold, but represented only 10% of the total content. No significant change was observed at 24 h or by subsequent pulses of LPS at 6, 12, or 24 h.

Plaque assay of PCA of LPS-stimulated viable mononuclear cells (Table II),
Table III

Effects of LPS Stimulation on PCA of Splenic Mononuclear Cells

| LPS-stimulated cell population* | Viable expression | Total content |
|---------------------------------|------------------|--------------|
|                                 | Time             | Milliunits   | Stimulated/ basal | Time             | Milliunits   | Stimulated/ basal |
| Whole mononuclear cells         | 90 ± 10          | 92 ± 18      | 4.0              | 50 ± 5           | 910 ± 110    | 10.4           |
| Lymphocytes†                    | 165 ± 25         | 9.5 ± 1.1    | 1.0              | 115 ± 10         | 36 ± 12      | 1.0            |
| Macrophages§                    | 75 ± 5           | 155 ± 40     | 1.0              | 55 ± 3           | 540 ± 75     | 1.0            |

* 6-h incubation with E. coli 0111:B4 LPS at 10 μg/ml.
† <1% nonspecific-esterase positive.
§ >98% nonspecific-esterase positive.

indicated that 80% of the esterase-positive cells (12–15% of cells) were positive and 5% of the esterase-negative cells were positive for PCA (Fig. 1). The latter appeared cytologically to be lymphocytes. PCA plaque assay of fixed LPS-stimulated mononuclear cells demonstrated that 100% of the esterase-positive cells were PCA positive and 8% of the esterase-negative mononuclear cells were also positive for PCA, suggesting PCA production by a lymphocyte subpopulation.

LPS Stimulation of Cell Populations. In contrast to the LPS stimulation of whole mononuclear cells, when isolated macrophages (>98% esterase positive) were incubated with LPS and assayed 6 h later for PCA, there was no increase in PCA (Table III). Similarly, when lymphocytes were isolated (<1% esterase positive) and incubated with LPS, there was no increase in PCA.

In contrast to the lack of response of isolated lymphocytes or macrophages to LPS, when whole splenic mononuclear cells were incubated for 6 h with LPS and the macrophages and lymphocytes isolated by adherence and assayed for total PCA, a significant response of macrophages was observed. The PCA of the splenic mononuclear cells increased 11.1-fold from 88 milliunits (basal) to 910 milliunits (Table IV). When the two subpopulations were separated by adherence and assayed separately, we could account for 95.8% of PCA. The PCA of macrophages recovered from the stimulated cells increased 12.1-fold from 570 milliunits to 6,920 milliunits and accounted for 95.2% of the PCA of the whole, splenic mononuclear cells. The lymphocytes isolated from the LPS-stimulated mononuclear cells showed only a slight increase of PCA above basal, even though these cells had been stimulated by LPS in the presence of macrophages, and the lymphocyte subpopulation contained 4.8% of total PCA. The validity of separating LPS-stimulated lymphocytes and macrophages by adherence was supported by cytology and esterase reactions.

Lymphocyte Requirement for Generation of Macrophage PCA. To further define the requirements for amplification of PCA content of macrophages, splenic mononuclear cells were separated into macrophages and lymphocytes. The lymphocytes were incubated for 6 h with LPS, then macrophages were incubated for 6 h with either the LPS-stimulated lymphocytes in fresh medium or the medium from the same lymphocytes. When 1 × 10⁶ macrophages that had been incubated with the LPS-stimulated...
Table IV

| Cell subpopulation          | Total PCA per 10^7 cells |            |            |            |
|-----------------------------|--------------------------|------------|------------|------------|
|                             | Basal                    | LPS-stimulated |
|                             | Time Milliunits          | Time Milliunits |
|                             | sec                      | sec        |
| Whole mononuclear cells     | 90 ± 10                  | 50 ± 5     | 910 ± 110  |
| Lymphocytes*                | 115 ± 10                 | 36 ± 12    | 48 ± 14    |
| Macrophages‡                | 50 ± 3                   | 570 ± 154  | 6920 ± 750 |

* Lymphocytes <1% esterase positive. Stimulated with LPS in presence of macrophages and subsequently isolated and assayed for PCA.
‡ Macrophages >98% esterase positive. Stimulated with LPS in presence of lymphocytes and subsequently isolated and assayed for PCA.

Fig. 3. Macrophage PCA recovered from LPS-stimulated murine splenic mononuclear cells. [LPS-SMC]→ Mø (●); splenic mononuclear cells were incubated with 10 μg LPS for 6 h, and then the macrophages were isolated and assayed for PCA content at serial cell concentrations. [LPS-L] + Mø (●); lymphocytes were incubated with LPS for 6 h before their addition to macrophages followed by incubation for 6 h, after which, macrophages were isolated and the total content of PCA was measured. Mø (●); macrophages that were assayed for total PCA content. [LPS-L-S/N] + Mø (●); lymphocytes were incubated with LPS for 6 h after which, the supernate was added to macrophages for a 6-h incubation and the macrophages were assayed for total PCA content. Normal plasma substrate was used in these assays and demonstrated the typically nonproportionality and nonlinearity at <10^5 cells. This demonstrates the collaborative requirement in generating PCA activity and the typical characteristics both at the high and low range.
TABLE V

Cooperative Induction of PCA in Macrophages by LPS-stimulated Lymphocytes

| Added to 1 × 10⁷ macrophages | Total macrophage PCA per 10⁷ cells |
|-----------------------------|----------------------------------|
|                             | Basal & Stimulated               |
|                             | milliunits                       |
| LPS-stimulated lymphocytes  | 770 ± 155                        |
| (9 × 10⁷ cells)             | 6,920 ± 770                      |
| LPS-stimulated lymphocyte   | 770 ± 155                        |
| medium (from 9 × 10⁷ cells, above*) | 755 ± 155                      |

* Supernatant medium clarified at 100,000 g for 1 h.

TABLE VI

Cell Specificity for Induction of LPS-stimulated Macrophage PCA

| Cells added to 1 × 10⁶ macrophages | LPS-stimulated* PCA per 10⁷ macrophages |
|------------------------------------|----------------------------------------|
|                                    | Time (sec) | Milliunits (µg) |
| None                               | 69 ± 3     | 2,300 ± 400     |
| Lymphocyte (9 × 10⁶)                | 49 ± 3     | 7,700 ± 750     |
| Neutrophil (1.3 × 10⁶)              | 69 ± 3     | 2,280 ± 380     |
| RBC (1 × 10⁶)                       | 71 ± 2     | 2,150 ± 300     |

* LPS from E. coli 0111:B4: 10 µg/ml.

Fig. 4. Lymphocyte requirement for amplification of macrophage procoagulant activity. (Δ): LPS-stimulated macrophages (1 × 10⁷) were incubated with increasing numbers of lymphocytes to give the ratio of lymphocytes: macrophages given on the abscissa. The cells were incubated with 10 µg LPS for 6 h, and macrophages were isolated and assayed for total PCA content. (□): macrophages (1 × 10⁷) were incubated with lymphocytes in increasing numbers to give ratios of lymphocytes to macrophage as above. The cells were incubated for 6 h without LPS and the macrophages were assayed for total PCA.
lymphocytes were assayed, there was a ninefold increase in PCA within the linear range of the assay when conducted with normal plasma (Fig. 3). The nonproportionality of the PCA at low cell numbers is similar to that originally described for low concentrations of thromboplastin (20) and paralleled that for LPS-stimulated, whole mononuclear cells from which the macrophages were recovered and assayed. When assayed in Factor VIII-deficient plasma, a typical linear yield of PCA was observed in accord with other studies (21). No increase was observed for macrophages incubated with the medium from the LPS-stimulated lymphocytes (Table V). The molecular aspects will be reported subsequently.

From these experiments, we suggest that the macrophage is the cell responsible for the increased content of PCA; however, amplification of PCA content requires the presence of intact lymphocytes that had been exposed to LPS. Evidence for unilateral collaboration follows from similar experiments in which LPS-stimulated macrophages were added to lymphocytes. No amplification of PCA was observed, indicating that the LPS-lymphocyte can induce macrophage PCA, but that the LPS macrophage does not stimulate lymphocytes, macrophages, or a reciprocal circuit of macrophage-lymphocyte to induce macrophages to increase PCA content.

To assess cellular specificity of the requirement for lymphocyte collaboration, the capacity of neutrophils and erythrocytes (RBC) to collaborate in the induction of macrophage PCA was examined (Table VI). The macrophages stimulated with LPS in the presence of lymphocytes showed a 3.3-fold increase in PCA, whereas no increase was observed for macrophages incubated with RBC or neutrophils in the presence of LPS.

The optimal lymphocyte:macrophage ratio for PCA induction was examined by adding either no lymphocytes, or serial concentrations of lymphocytes from $1 \times 10^7$ through $1 \times 10^9$, to $1 \times 10^7$ macrophages to give lymphocyte ratios of 0:1 to 10:1, and then stimulating with LPS (Fig. 4). Total cellular PCA remained at basal levels for lymphocyte:macrophage ratios of 0:2:1 but increased sharply to 8,864 milliunits at a 4:1 ratio and could not be further augmented.

Discussion

Our study provides direct evidence for cellular collaboration in the induction of the coagulation pathways by the lymphoid system. In our experiments, the model stimulant LPS was used. A variety of in vitro stimuli including allogeneic reactions (10), cell-bound antibody, immune complexes (9) and lectins (3, 12, 23, 24) have been reported to generate PCA of thromboplastin-like character in lymphoid cells (10, 13, 16, 23); and, indeed, certain phenotypic features of immunologically-mediated tissue lesions have suggested that local activation of the coagulation system occurs in vivo (1, 2, 5–7). There is, to date, limited characterization of the phenomenon, and neither the responsible cells nor the cellular requirements for induction of this type of response have been clearly delineated.

Our studies of LPS-induced PCA in murine lymphoid cells provide direct evidence that splenic mononuclear cells possess low basal levels of PCA, which appears to be

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2 Rothberger, H., J. Dunne, and T. S. Zimmerman. Increased production of tissue thromboplastin-like procoagulant activity stimulated by red cells sensitized with human anti-blood alloantibodies. Manuscript submitted for publication.
tissue factor in respect to its dependence on Factor VII. It is observed in both lymphocytes and macrophages by reference to separated cell populations where ~67% was macrophage associated. The development of a procoagulant plaque assay coupled with vital neutral-red uptake and a reaction for nonspecific esterase of macrophages has permitted simultaneous use of cytologic, histochemical, and functional markers on single cells. It has thus been possible to demonstrate that PCA is expressed by only ~1% of viable splenic mononuclear cells, and all were macrophages. About 3% of fixed cells contained PCA and 80% of these were macrophages, and very few (~0.7%) of cells that were esterase negative and appeared cytologically similar to lymphocytes contained PCA. Whether these latter cells are lymphocytes remains to be clarified.

The stimulation of splenic mononuclear cells with LPS led to a significant amplification of total PCA in agreement with Garg and Niemetz (13) and Lerner et al. (23). It has been suggested that as little as 1 ng of LPS might elicit this activity (25), and that it may be observed within 1 h (26) and reach a maximum concentration within 6 h (16, 23). We have observed some variability between preparations of LPS in dose and temporal sequence, but in general, maximal induction is in the range of concentration of LPS necessary for induction of mitogenic activity, and 6 h has been required for maximum effect. We, like Rapaport et al. (27), observed no significant increase of PCA with exposures to LPS of <2 h; however, it should be noted that the relatively brief period of LPS exposure required for PCA generation precludes a proliferative requirement and suggests a direct inductive event.

At 6 h, most of the newly-generated PCA remains intracellular (Table III), and we have observed this to also be true at 24 h. There is a 10-fold increase over basal levels, whereas the activity expressed by viable cells increased only 4-fold over basal. The observation that the increased PCA is for the most part not made available in the viable state is in keeping with the observations of Niemetz et al. (26), who localized tissue factor (thromboplastin) to a light-microsomal fraction consistent with endoplasmic reticulum. Some investigators have suggested that a second pulse of endotoxin enhances generation of PCA; however, we have observed that at an optimal dose of LPS, there is no further increase with a second pulse of LPS at 6, 12, or 24 h after the initial stimulation.

Plaque assays of LPS-stimulated cells demonstrated a significant increase of PCA-positive viable cells. In contrast to the 1% PCA-positive cells in basal cultures, ~14–15% of cells were positive and ~80% of the macrophages expressed PCA within 6 h after LPS stimulation. This study supports the analyses of the fractionated cells after LPS stimulation where 95% of the PCA was recovered with macrophages of 99.5% purity. In addition, it was observed that 8% of esterase-negative cells, cytologically compatible with lymphocytes, also were positive after LPS stimulation, though there was little increase in quantity of PCA in the isolated lymphocytes. Presumably, the PCA content per cell must be much lower than for macrophages. Whether these cells are lymphocytes remains to be established by a more detailed study.

We observed that neither isolated lymphocytes nor isolated macrophages could be independently stimulated by LPS to generate this activity. When macrophages were supplemented with lymphocytes to a minimum ratio of 3:1 (lymphocytes:macrophages) (Fig. 4) and then stimulated with LPS, there was PCA generation. Similarly, if the lymphocytes had previously been stimulated by LPS and were added to macrophages, additional PCA was generated by the macrophages. This is in contrast
to Edwards and Rickles (28), who, using human cells, have suggested in a preliminary report that lymphocytes serve to augment the amplification of PCA but that isolated macrophages could be stimulated to increase PCA. Whether their macrophage preparations had sufficient lymphocyte contamination to permit partial induction is not known.

In our study, it appears that macrophages can be stimulated to increase PCA only when stimulated in the presence of LPS-triggered lymphocytes. All cells, including T and B lymphocytes, RBC, and neutrophils have cell-surface receptors for LPS (29, 31). However, neither neutrophils nor RBC could induce PCA in macrophages; only lymphocytes participated in this collaboration. Thus simple presentation of cell-bound LPS is insufficient. Furthermore, no increase in PCA was observed when lymphocytes were incubated with isolated macrophages that had been previously stimulated by LPS and then washed to remove free LPS. This is strong evidence that lymphocytes must be exposed to the LPS as the primary event and then either present the LPS to the macrophage (or some LPS-stimulated mediator) to induce production of PCA. We were unable to demonstrate that a soluble mediator was produced by lymphocytes and released into the medium from LPS-stimulated lymphocytes, suggesting that lymphocyte collaboration may be mediated via contact. Recent experiments (G. A. Levy and T. S. Edgington. Unpublished results.) have shown that there is genetic restriction in the collaborative interaction between lymphocytes and macrophages in this response. A minimum 3:1 ratio of lymphocytes:macrophages with a sharp rise to maximum at 4:1 is consistent with a stoichiometric interaction, even physical association, with a minor subset of lymphocytes.

It is well recognized (32, 33) that macrophages are required for the proliferative response of murine spleen cells. However, more recently, Tzehoval et al. (34) have shown that T lymphocytes are capable of regulating, or are required for independent induction of the phagocytic properties of macrophages. Thus, although there has been considerable attention to the regulation of a variety of lymphocyte functions by macrophages, the capacity of lymphocytes to regulate macrophage function, including the generation or expression of monokines, is exemplified by the lymphocyte-controlled generation of procoagulant molecules. The significance of this lymphoid pathway has only been suggested by observations of fibrin deposition in a number of types of immunologic lesions; however, the possibility of a direct participant function or requisite role can not be ignored. Fibrinolysis releases a peptide that suppresses lymphocyte function from the α-chain of fibrin (35). Whether this peptide is a toxic cofactor in target cytosis or, on the other hand, suppresses the cellular immune attack, it will be evoked only secondary to local induction of coagulation, a mechanism which can now more clearly be envisioned as a systematic byproduct of immunologic reactions.

Summary

Murine splenic lymphoid cells have been shown to possess basal procoagulant activity. This activity was localized to most macrophages by assay of cell populations, as well as by a direct plaque assay that permitted identification of expressed procoagulant activity of individual viable cells as well as content. Both content and viable expression of procoagulant activity was markedly increased by exposure to bacterial lipopolysaccharide, reaching a maximum after 6 h. The quantitative increase in procoagulant activity content and viable expression was limited to the macrophage
population. Separated populations of lymphocytes or macrophages could not be stimulated by lipopolysaccharide to increase procoagulant activity, whereas the addition of lymphocytes to macrophages at a 3–4:1 ratio maximally reconstituted the amplification of procoagulant activity. Further evidence of cellular collaboration followed from observations that only lymphocytes that had been exposed to lipopolysaccharide were capable of triggering the increase in macrophage procoagulant activity. This appears to represent a new form of lymphocyte-macrophage cooperation in an effector pathway that may participate in some forms of immunologic responses and contribute to the phenotypic features of certain immunologic tissue lesions.

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