

**A**bstract. Experiments were carried out to examine relationships between alveolar macrophage maturity and amounts of tissue factor (Clotting Factor III) in these cells under physiologic conditions and during immunologically induced pneumonitis. Using discontinuous density gradient centrifugation, alveolar macrophages from healthy rabbits were rapidly isolated into five subpopulations at different stages of maturation, as demonstrated by morphologic and morphometric evaluation. Very large amounts of tissue factor activity were found in fully mature cells that were purified in the lowest density subpopulation and assayed without preliminary *in vitro* stimulation or culture. In the remaining four subpopulations of increasing density, amounts of tissue factor were found to progressively diminish in direct correlation with declines of cell maturity. These differences at mean levels were as great as 35-fold. In addition, blood monocytes had  $<1/219$  and  $<1/6$  of the activity of the fully mature and the least mature subpopulations, respectively. After 16 h culture of the five isolated subpopulations in the absence of lymphokines or of significant numbers of lymphocytes, tissue factor activity increased in inverse correlation with the preincubation stage of cell maturity (2,387 and 109% in the least mature and most mature subpopulations, respectively). These increases required protein synthesis and were accompanied by morphologic and morphometric changes which indicated cellular maturation during the period of tissue factor activity generation *in vitro*, thus further demonstrating relationships between macrophage maturity and tissue factor content. In additional experiments, direct correlations between cell maturity and tissue factor activity content

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## Tissue Factor Activity

### A Marker of Alveolar Macrophage Maturation in Rabbits. Effects of Granulomatous Pneumonitis

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were also found in activated alveolar macrophage populations from rabbits with *Bacillus Calmette Guering* (BCG)-induced granulomatous pneumonitis. However, as compared with controls, the BCG populations had increased total amounts of tissue factor activity due to the presence of large numbers of mature alveolar macrophage forms that had high levels of the procoagulant. Thus, tissue factor activity in alveolar macrophages is a marker of cellular maturation *in vivo* and *in vitro*. Increased amounts of this initiator of the extrinsic clotting pathway, as found in alveolar macrophage populations from animals with granulomatous pneumonitis induced by BCG hypersensitivity, suggest that alveolar macrophage tissue factor may contribute to the pathology of immune lung diseases.

#### Introduction

Alveolar macrophages are phagocytic and secretory cells important in pulmonary defense and inflammation (1-3). As found in human and animal lungs, these cells have been shown to be heterogeneous with regard to phenotype. The cells are highly variable in enzyme content, oxidative metabolism, bactericidal activity, lysosomal inclusions, surface receptors, cytoplasmic morphology, size, and other properties (4-7). This heterogeneity reflects the rapid turnover of the alveolar macrophage, which continuously and randomly enters the lung as a monocyte that matures into macrophages in a few hours. The turnover of alveolar macrophages has been recently calculated to be 5.5 d in the mouse (8). Phenotypic heterogeneity in these macrophages is augmented after cell exposure to inhaled stimuli, which include bacteria, toxins, and particulate matter, or by systemic stimuli as in *Bacillus Calmette Guering* (BCG)<sup>1</sup> pneumonitis (3, 7).

In earlier experiments we reported that the alveolar macrophage contains tissue factor. This initiator of the extrinsic pathway of coagulation (9) was present in alveolar macrophages at increased levels relative to amounts in several populations of lymphoid cells, especially populations from marrow and blood

1. Abbreviations used in this paper: BCG, *Bacillus Calmette Guering*; TFU, tissue factor units.

that contained immature monocyte/macrophage forms (10). These findings suggested the possibility that macrophages may be heterogeneous with regard to tissue factor content and that levels of this activity may vary according to monocyte/macrophage differentiation or maturation. Therefore, in experiments reported here using animals with BCG pneumonitis and controls, we directly examined relationships between alveolar macrophage maturity and tissue factor as present in vivo and in vitro.

## Methods

**Alveolar macrophage and blood monocyte isolation.** Alveolar macrophages were collected by bronchoalveolar lavage from New Zealand White female rabbits weighing 2–3 kg, as previously described (11, 12). Briefly, the animals were anesthetized by intravenous injection with 3 ml of pentobarbital, and the lungs were exised and lavaged with 80 ml of sterile saline. The lavaged macrophages were centrifuged, and to lyse any red cells, 10 ml of 0.015 M  $\text{NH}_4\text{Cl}$  in 0.017 M Tris buffer prewarmed to 37°C was added to the cell pellet. The suspended pellet was incubated at 37°C for 5 min, washed, and resuspended at  $20\text{--}40 \times 10^6$  leukocytes/ml in RPMI media containing 25 mM Hepes buffer, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. To obtain in vivo activated alveolar macrophages, chronic granulomatous pulmonary reactions were induced by intravenous immunization using 150  $\mu\text{g}$  of heat-killed BCG suspended in light mineral oil. Animals in the BCG treatment group were sacrificed 21 d after BCG injection. The lungs of these animals, all showing the typical gross pathology of BCG pneumonitis (13), were processed as described above. In experiments using blood monocytes, mononuclear cell populations were isolated from rabbit blood on Ficoll-Hypaque gradients. These populations contained monocytes, lymphocytes, and neutrophils at 20, 80, and <1%, respectively, as previously described (10).

Washed alveolar macrophage populations obtained by lavage were further purified using centrifugation on discontinuous Percoll gradients (of density 1.055, 1.060, 1.064, 1.068, and 1.070 g/ml). The gradients were made by diluting a stock Percoll solution (nine parts Percoll, one part  $10\times$  medium), to 35, 40, 45, 48, and 50% in RPMI medium. In order of decreasing density, 2.25 ml of each Percoll dilution was serially layered in a 15-ml conical test tube using a peristaltic pump. An additional 2.25 ml of 5% Percoll was placed above the 35% Percoll layer to separate any low density subcellular material from the intact cells centrifuged on these gradients. Approximately  $25 \times 10^6$  lavage cells were layered over the top of the gradient and the cells were centrifuged at 400 g for 15 min at 4°C. Cell subpopulations banding at each density interface were collected by aspiration using the peristaltic pump. The subpopulations were washed, counted, and resuspended in fresh medium at a concentration of  $1 \times 10^6$  cells/ml. Samples were cytocentrifuged and stained using nonspecific esterase (14) and Wright's staining reagents. Unseparated populations from healthy animals and cells banding in the 35–50% Percoll fractions were >98% macrophages, as reported in Results. The gradient pellet contained a few large and small macrophages and rare lymphocytes or neutrophils. Cells in the pellet, always <10% of those recovered from gradient centrifugation, were excluded from morphometric and functional studies since these cells were of mixed type and density. By trypan blue exclusion, viabilities in the unseparated and 1.055-g/ml gradient fractions were, respectively,  $98.8 \pm 29$  and  $86.3 \pm 3.1\%$ . Viability was >99% in the remaining four subpopulations. The unfractio-nated population from BCG-inoculated animals contained 78% mac-

rophages, and the subpopulations, in order of increasing density, contained 85, 93, 89, 79, and 69% macrophages, respectively. Remaining cells were neutrophils and lymphocytes in  $\sim 2:1$  ratios.

**Measurements of cell diameters.** Morphometric determinations of nuclear and cytoplasmic diameters were made by projecting microscope slides on the surface of a Graf Pen-Gp/3 digitizer (Scientific Accessories Corp., South Port, CT) that was interfaced with a microcomputer (Hewlett-Packard-HP9825S; Hewlett-Packard Co., Calculator Products Div., Loveland, CO). Programs were written for data storage and analysis, and linear measurements were standardized using a marker of known length. Nuclear and cytoplasmic diameters of cytocentrifuged and stained macrophages from each fraction were measured in duplicate, and results for 200 cells were averaged. Care was taken to randomly select the macrophages counted.

**Cell culture.** Macrophages from each fraction were incubated in tissue culture grade polypropylene tubes at  $1 \times 10^6$  cells/ml in 0.5 ml of the medium used for cell isolation, which was supplemented with 10% fetal calf serum. This medium contained <0.005 ng/ml of endotoxin as determined by Limulus testing. Incubation was for 16 h at 37°C in humidified 5%  $\text{CO}_2$  and 95% air. After incubation, cells were assayed for procoagulant activity. Although we chose to culture macrophages with serum under nonadherent conditions, preliminary studies showed similar effects on in vitro generation of tissue factor under alternative conditions of adherent culture and culture without serum.

**Procoagulant activity measurement and characterization.** Macrophage procoagulant activity was measured in lysates after a cycle of cell freezing and thawing. (Amounts of expressed procoagulant activity as measured with viable cells compared with total amounts measured after cell lysis were 61–89% and 69–80% for subpopulations from healthy and BCG animals, respectively. The two highest density fractions from healthy animals were exceptional in that these were found to express as little as 27% of the total activity.) The assays (10) were initiated by adding  $0.6 \times 10^5$  macrophages in 60  $\mu\text{l}$  of medium to a clear polystyrene 12  $\times$  75-mm test tube, followed by 60  $\mu\text{l}$  of 0.025 M calcium chloride and 60  $\mu\text{l}$  of platelet poor rabbit plasma. Clotting times were determined in duplicate by the manual tilt method and the average of the two measurements was used to calculate procoagulant activity units. Under these conditions, which resembled the nonactivated partial thromboplastin test, contact activation of substrate plasmas during preparation and during the assay is insignificant, as shown by long buffer blank times that were >500's in all of the normal and deficient plasmas used, with or without addition of exogenous lipid in the assay mixture (15, 16). When added to buffer blanks, the lipids were at 0.02–0.2 nmol P per assay (rabbit brain cephalin, Sigma Chemical Co., St. Louis, MO), which corresponded to dilutions recommended by the manufacturer for an activated partial thromboplastin test. Our methods of standardizing the assay with commercial thromboplastin, of preparing calibration curves, defining TFU, and confirming low intraassay variability (<6% between replicate samples throughout the range of the calibration curve) are the same as previously reported (10). Tested cells yielded clotting times within the linear portions of the calibration curves. The assay consistently detects as little as 1 TFU. Characterization of tissue factor in macrophage subpopulations before and after incubation relied on one-stage assays using human plasmas deficient in single clotting factors and inhibition by Concanavalin A and phospholipase C, as previously reported (10). For example, one-stage clotting times in assays of the cultured 1.064-g/ml alveolar macrophage subpopulation in substrate plasmas mono-deficient in Factors VII, X, II, and V were >370 s. By contrast, clotting times in normal human plasma and in the plasmas deficient in Factor

XII, XI, IX, and VIII were much shorter (106–130 s). Since short clotting times were not induced in plasmas deficient in Factors VII, X, II, and V, specific requirements for these factors are shown, and the macrophage procoagulant activity thereby fulfills classic definitions of tissue factor (9, 10). In the panel of deficient plasmas, brain tissue factor (diluted to shortened clotting times in normal human plasma to the same extent as tested cells) gave differential results identical to those seen with the cells.

*Miscellaneous.* Protein determinations were made using the Lowry method in diluted samples obtained from cell suspensions immediately after fractionation. Under the conditions of culture, total cellular protein did not increase detectably by the Lowry assay. Lipid determinations were made by the method of Rouser et al. (17). Puromycin (Sigma Chemical Co.) added to culture at doses of 1–10  $\mu\text{g}/\text{ml}$  had no effect on cell viability or DNA content. Addition of puromycin at 10  $\mu\text{g}/\text{ml}$  led to a reduction of total cellular protein by 15–25% compared with totals found after culture without puromycin. Cellular DNA was measured by the diphenylamine method (18). Data are shown as means  $\pm$  SE of at least three experiments unless otherwise indicated.

## Results

*Alveolar macrophage isolation as subpopulations of different maturity.* To study the relation between alveolar macrophage maturation in vivo and procoagulant activity content, cells freshly obtained by bronchoalveolar lavage were isolated into five subpopulations of different density by centrifugation on discontinuous Percoll gradients. By using morphologic examination, macrophages in the lowest density band (density = 1.055 g/ml) were large cells of mature appearance that had highly vacuolated and granulated cytoplasm. Consistent with the very mature appearance of these cells, morphometric studies of this subpopulation demonstrated large overall cytoplasmic diameters and low ratios of nuclear/cytoplasmic diameters (Fig. 1). Morphologically, macrophages in the remaining four macrophage bands of progressively increasing density showed less mature appearance, i.e., they showed decreasing cell size, cytoplasmic granulation, and vacuolation. Concomitantly, morphometric studies showed decreasing cytoplasmic diameters and increasing nuclear/cytoplasmic ratios, which confirmed the stepwise declines of macrophage maturity as cell density increased from the 1.055 to the 1.070 g/ml band (Fig. 1). Macrophages in these preparations were readily distinguishable from any rare neutrophils and lymphocytes that were present by noting characteristic differences in cell size, cytoplasmic vacuolation, and nonspecific esterase staining. Macrophages comprised >98% of the cells isolated.

*Correlation of alveolar macrophage maturity and procoagulant activity as measured in unincubated cells.* Procoagulant activity was measured in each of the five alveolar macrophage subpopulations isolated on Percoll gradients. The subpopulation of greatest maturity (density = 1.055 g/ml) was found to have the largest amount of procoagulant activity (Table I). This activity was characterized as tissue factor by showing specific dependence on Factors VII, X, V, and II (for characterization see Methods). Procoagulant activity identified as tissue factor was found in the remaining four subpopulations as well. Amounts of this

Cytoplasmic and Nuclear Diameters  
and Ratios of Nuclear/Cytoplasmic Diameters  
of Alveolar Macrophages from Healthy Control Rabbits

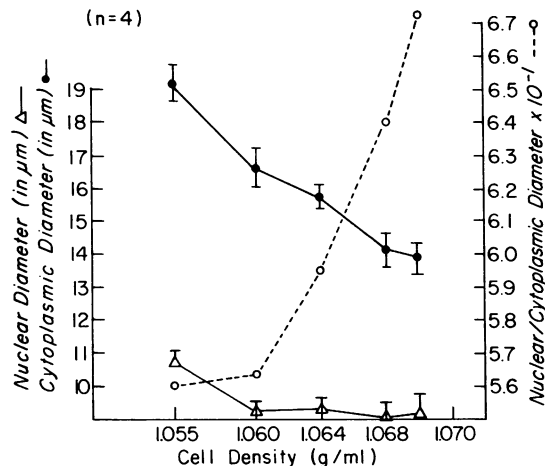


Figure 1. Alveolar macrophages were isolated using discontinuous Percoll gradients of densities indicated on the abscissa. Nuclear and cytoplasmic diameters were determined by morphometric techniques described in Methods. Nuclear/cytoplasmic ratios were calculated from mean diameters measured in experiments with four animals.

activity became progressively smaller with declining cell maturity (Table I). When calculated as TFU/ $10^6$  macrophages, differences between mean amounts of procoagulant activity in subpopulations of different maturity were found to be as great as 35-fold, as seen by comparing results for the most and least dense cell bands (Table I). Furthermore, even the least mature subpopulation had more procoagulant activity than blood mono-

Table I. Procoagulant Activity of Alveolar Macrophage Subpopulations Isolated from Healthy Animals by Density Gradient Centrifugation

Density	TFU
g/ml	
1.055	9,846 $\pm$ 3,439
1.060	1,994 $\pm$ 866
1.064	1,347 $\pm$ 928
1.068	591 $\pm$ 334
1.070	278 $\pm$ 69
Unseparated cells	1,075 $\pm$ 280

Total number of cells recovered,  $91.3 \pm 11 \times 10^6$ ; total TFU recovered,  $99.6 \pm 29.3 \times 10^3$ ; lung weight,  $10.3 \pm 0.36$  g;  $n = 7$ . TFU is normalized for  $1 \times 10^6$  cells. Total TFU recovered equals total number of cells recovered  $\times$  TFU/ $10^6$  unseparated cells. TFU assayed with peripheral blood mononuclear cells and normalized for  $10^6$  monocytes was <45 in four experiments.

cytes (Table I). As compared with monocytes, the most and least mature macrophage subpopulations had >219- and >6.2-fold higher mean procoagulant activities, respectively.

*Total macrophage lipid and protein concentrations and normalization of procoagulant activity relative to these constituents.* Macrophage lipid content in the subpopulations showed progressive decreases from the least dense band (1.055 g/ml) to the most dense band (1.070 g/ml) (Table II). Thus, like TFU, lipid content was directly correlated with subpopulation maturity. Normalization of TFU per nmol P (total cellular lipids) rather than cell number showed that gains of tissue factor activity in the more mature alveolar macrophages outpaced the lipid increases accompanying maturation and enlargement (Table II). Total macrophage protein and DNA content did not vary appreciably from one band to the other. Results obtained after normalization of TFU for cell protein are shown (Table II).

*Changes of alveolar macrophage procoagulant activity in vitro: inverse correlation between percentage increases of tissue factor and stage of maturity before cellular incubation.* The five subpopulations of macrophages were incubated as suspension cultures in vitro for 16 h and procoagulant activity was then measured. All showed increases of tissue factor as compared with preincubation (0 h) levels (Table III). These increases were inversely correlated with the stage of cell maturity. For example, the least mature subpopulation increased in procoagulant activity

Table II. Procoagulant Activity of Alveolar Macrophage Subpopulations: Normalization for Protein and Lipid Content

Density	Tot. protein	Tot. lipid*	TFU/mg protein‡	TFU/nmol P
<i>g/ml</i>	<i>mg</i>	<i>nmol P</i>		
1.055	1.3±0.08	29.1±8.5	3,220±518	312±75
1.060	1.3±0.10	26.8±6.7	668±160	59±11
1.064	1.3±0.09	21.9±3.5	422±101	34±5
1.068	1.2±0.10	15.3±4.1	249±51	29±6
1.070	1.3±0.09	12.2±3.3	162±44	31±6
Unseparated cells	1.5±0.11	25.7±6.8	2,026±677	160±44
	<i>n = 4</i>	<i>n = 6</i>	<i>n = 4</i>	<i>n = 6</i>

\* In three additional experiments, exogenous lipid (rabbit brain cephalin) was added to higher density subpopulations just before assay in amounts sufficient to bring total lipids up to the same levels found in the freshly isolated 1.055 g/ml subpopulations. The presence of added lipid had no effect on levels of procoagulant activity measured in these subpopulations, which indicated that differences of TFU as shown above were not due to variations in cellular lipid content.

‡ Results obtained after normalizing TFU for cellular DNA in three additional experiments were similar to those shown above for normalization with respect to total protein content. DNA was present in equivalent amounts in all of the subpopulations (11.0–12.0 µg DNA/10<sup>6</sup> macrophages).

Table III. Procoagulant Activity of Alveolar Macrophage Subpopulations from Healthy Animals: Increases After In Vitro Incubation\*

Density	TFU 0 h	TFU 16 h	TFU increase
<i>g/ml</i>			<i>%</i>
1.055	6,103±191	12,773±3,193	109
1.060	1,569±414	11,534±1,020	635
1.064	740±212	8,867±1,753	1,198
1.068	525±253	12,909±2,929	2,358
1.070	479±15	11,915±1,284	2,387
Unseparated cells	2,224±518	14,306±2,665	543

*n = 6.*

\* The TFU increases during culture were inhibited up to 96.2% in the presence of 10 µg/ml of puromycin, which indicates that protein synthesis is required for tissue factor generation by alveolar macrophages at each of the maturation stages represented by the different subpopulations.

by 2,387% after incubation, as contrasted with only a 109% increase in the most mature subpopulation (see the 1.055 and 1.070 g/ml fractions, respectively, Table III). During incubation, macrophage diameters increased and nuclear/cytoplasmic ratios decreased (Table IV), which demonstrated that gains in procoagulant activity in vitro were concomitant with these changes characteristic of cellular maturation. The procoagulant activity generated in vitro by each subpopulation was identified as tissue factor using methods similar to those used for freshly isolated cells. Since lymphocytes were absent from the subpopulations (<2% of the isolated cells), these in vitro procoagulant activity increases in the alveolar macrophage subpopulations were independent of direct lymphocyte-macrophage interaction. Addition of 10 µg/ml of puromycin to the cultures inhibited the increases in tissue factor in all subpopulations to 4% of control values, which indicated a requirement for protein synthesis.

*Procoagulant activity of alveolar macrophages from animals*

Table IV. Effects of In Vitro Incubation on Macrophage Diameter (D) and Nucleus/Cytoplasmic Ratio (N/C)

Density	0 h		16 h	
<i>g/ml</i>	<i>D (µm/cell)</i>	<i>N/C</i>	<i>D (µm/cell)</i>	<i>N/C</i>
1.060	13.5±2.5	5.1	17.9±3.2	4.2
1.064	10.5±2.0	6.2	15.3±2.7	4.5
1.068	9.6±2.0	6.8	14.4±2.7	5.0
1.070	10.5±2.2	7.8	12.6±1.9	6.7

Results are shown for one of three similar experiments. 200 cells were sized in each subpopulation.

with BCG pneumonitis. Procoagulant activity in alveolar macrophages lavaged from rabbits with BCG-induced granulomatous pneumonitis was investigated using methods similar to those used for the studies of healthy animals. Again, macrophage tissue factor levels correlated directly with cell maturity. Activity was highest in the least dense subpopulation and progressive declines were seen as subpopulation density increased (Table V). Quantitative comparison of TFU (Tables I and V) showed that the low density subpopulation of macrophages from BCG-inoculated rabbits had less (4.8-fold) tissue factor per cell than the same density fraction from the control animals. Remaining subpopulations from the two groups had nearly equivalent activity per  $1 \times 10^6$  cells (see Tables I and V). For BCG animals the total numbers of macrophages recovered were increased by 6.1-fold (Tables I and V). Contrasting with the predominance of high density/relatively immature macrophages found in normal populations, the majority of cells isolated from BCG animals were in low density/more mature subpopulations (Fig. 2). Total amounts of macrophage tissue factor recovered from the BCG animals were 11.2-fold greater than amounts found under control conditions (see Tables I and V).

## Discussion

The present experiments examine relationships between alveolar macrophage maturation and tissue factor activity. Five subpopulations of macrophages, each at a different stage of mat-

Table V. Procoagulant Activity of Alveolar Macrophages from Animals with BCG-induced Granulomatous Pneumonitis

Density	TFU	n*
g/ml		
1.055	2,069±276	6
1.060	1,557±471	5
1.064	1,037±345	4
1.068	796±374	3
1.070	768±234	3
Unseparated cells	1,933±243	6

Total number of cells recovered,  $554 \times 10^6$ ; total TFU recovered,  $1,112 \times 10^3$ ; lung weight,  $37 \pm 6.6$  g. TFU is normalized for  $10^6$  freshly isolated cells in experiments with a total of six BCG-inoculated animals. If results as shown here were normalized for  $10^6$  macrophages instead of  $10^6$  cells, TFU in the five subpopulations would be ~20% higher. (BCG subpopulations, as contrasted with subpopulations from control animals, contained significant numbers of neutrophils and lymphocytes; see Methods.)

\* n equals number of animals with subpopulations isolating in the density bands shown. While all animals had mature cells isolating in the lowest density band, fewer animals had relatively immature cells recoverable in higher density fractions.

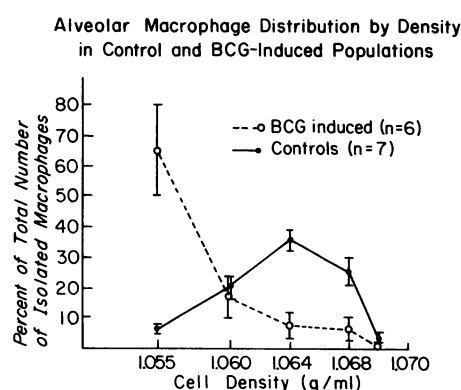


Figure 2. Density distributions of alveolar macrophages from control and BCG-inoculated animals are compared. The percentage of macrophages isolated at each different density band on a discontinuous density gradient is shown relative to the total number of cells recovered from the gradient.

uration, were isolated using bronchoalveolar lavage techniques followed by centrifugation on discontinuous density gradients. In the five cell subpopulations obtained from healthy animals and assayed for procoagulant activity without prior in vitro culture or stimulation, the stage of cell maturity as determined by morphologic and morphometric criteria correlated directly with tissue factor levels. Thus, alveolar macrophages characterized as fully mature were found to have very high levels of tissue factor activity, and progressively less mature cells in the remaining four subpopulations had proportionally smaller amounts. In these populations, cells with advanced maturity and high activity comprised only a minor component of the total macrophages isolated, and less mature cells with relatively small amounts of tissue factor predominated in number. Consistent with observations relating tissue factor levels to maturation, even the least mature of the five subpopulations studied was found to have more tissue factor activity than blood monocytes, which are cells considered to be precursors of alveolar macrophages (8, 19).

The subpopulations isolated from healthy control animals were subsequently incubated in vitro to compare potential for production of additional procoagulant activity. During incubation, tissue factor activity was observed to increase by amounts that were inversely correlated with the degree of maturity of each subpopulation. Thus, subpopulations that were relatively immature at the onset of incubation showed the greatest percentage increases. Maturation, defined by morphologic and morphometric criteria, was shown to occur in the immature cells during the incubation period, which again indicated the relationship of macrophage maturity to tissue factor levels.

Of further interest is the finding that lymphocytes were not required for the in vitro tissue factor activity increases seen, since lymphocytes comprised <2% of the cells isolated in our experiments with control animals used for these studies. More-

over, subpopulations isolated from some animals were completely devoid of lymphocytes. This demonstration of lymphocyte-independent procoagulant activity increases, which at mean levels were as great as 2,387%, differs from reports describing lymphocyte or lymphokine requirements for significant *in vitro* procoagulant activity generation by murine and human cells of macrophage lineage obtained from blood, peritoneum, spleen, and cell line sources (20–26). Although tissue factor increases in incubated alveolar macrophages were independent of added lymphocytes or lymphokines, it is possible that critical interactions with accessory cells or products of such cells occurred in the complex environment of the lung before macrophage isolation.

To examine effects of *in vivo* macrophage activation on tissue factor activity, experiments were carried out using animals with chronic granulomatous pneumonitis that was induced with heat-killed BCG (13). Alveolar macrophages from animals with this type of immune pulmonary reaction consistently differ from normal controls with respect to oxygen consumption, enzyme content, number and affinity of complement and Fc receptors, and other markers considered to indicate a state of cell activation, as shown by previous experience in our laboratory and by other investigators (13, 27–29, for a review see reference 1). Our present findings with these activated macrophages closely resembled results obtained with healthy animals, in that tissue factor levels as assayed using freshly isolated subpopulations directly correlated with stages of cell maturity. However, as compared with controls, total amounts of alveolar macrophage tissue factor were found to be greatly increased. This was in part due to recovery of more cells than from controls, a finding likely explained by the characteristic accumulation of alveolar macrophages in BCG granulomatous pneumonitis (13). Further, the tissue factor increases in this model of chronic immunologic pulmonary inflammation were also a result of an intrapopulation shift to mature macrophage forms that have relatively high levels of the procoagulant.

The possibility that macrophage tissue factor levels might be related to cell maturity was suggested to us by our earlier experiments (10). These studies showed that tissue factor activity is present in different amounts in lymphoid populations isolated from six distinct anatomic sites. Differences of activity were found to be especially great in comparing alveolar macrophages with blood and marrow cells, populations which contain alveolar macrophage precursors. Another finding was that, in marrow and blood populations, tissue factor increased *in vitro* by far greater percentages than in alveolar macrophages, which suggests that relatively immature cells might have the largest potential for producing additional activity (30). However, from these earlier studies it was not possible to conclusively determine whether tissue factor activity was related to cell maturation, since the investigated lymphoid populations contained monocytes/macrophages of varying maturity as well as leukocytes of other classes. In another series of earlier experiments we found increased tissue factor activity in monocytes/macrophages isolated

from rejected canine kidney allografts relative to amounts in precursors of these cells from blood (31). The very limited numbers of monocytes/macrophages obtained from these kidney grafts precluded subfractionation experiments for quantitation of intrapopulation heterogeneity with regard to parameters of cell maturation and tissue factor content.

To overcome difficulties encountered in studies comparing populations containing mixed classes and/or limited numbers of cells, we studied, in the experiments described here, the effects of maturation using alveolar macrophages. As obtained from control animals, this is a highly purified population of  $\sim 90 \times 10^6$  cells that did not require depletion steps to remove lymphocytes or other cell types. The Percoll fractionation technique selected for separation of these macrophages into subpopulations of different maturities has the advantages of being rapid and relatively atraumatic. In addition, cellular incubation under conditions that might induce generation of procoagulant activity *in vitro* are avoided during the procedure. In view of the conditions used for obtaining alveolar macrophages and earlier work showing that several hours of *in vitro* culture are needed for production of leukocyte procoagulants (21–23, 32), it is likely that tissue factor levels, as measured here in these unincubated cells, are representative of amounts present *in vivo*.

Criteria for advanced cell maturity in the present studies included findings of a large cytoplasmic diameter and a low ratio of nuclear-cytoplasmic diameters. These criteria were adopted because of the general agreement that during monocyte/macrophage maturation *in vivo* and *in vitro* there is overall cell enlargement that mainly results from cytoplasmic rather than nuclear growth (7, 33, 34). Measurements of diameters were carried out by tracing cell dimensions, using optically projected stained smears for image enlargement and a light pen digitizer with output to a computer. This methodology provided resolution of cell size differences in the range required for discriminating the macrophage subpopulations. In evaluating maturity we also included comparisons of cytoplasmic granulation and vacuolation, since it is well known that these readily detectable morphologic features become increasingly prominent during monocyte/macrophage maturation (33, 34).

Total cell lipids were studied as an additional marker of maturation, since alveolar macrophage lipids increase as these cells phagocytose surfactant in the alveolar space (3, 35). Indeed, in the present studies we found that lipid content correlated directly with the degree of alveolar macrophage maturation. By contrast, total cell protein content did not change appreciably at the various stages of maturation examined. In comparing indices of maturation in the alveolar macrophage, it was of interest to find that differences in amounts of tissue factor activity in the subpopulations studied were very pronounced in proportion to changes in other parameters including total cell lipids, protein, DNA, density, and cytoplasmic or nuclear/cytoplasmic diameters.

Subpopulations of mature alveolar macrophages found to have the largest amounts of tissue factor are not necessarily

cells that also express maximum levels of classical metabolic or functional activation markers. Indeed, available studies using rabbit alveolar macrophages that are subfractionated by density show that several activation markers are maximal in high or intermediate rather than low density fully mature cells. These markers include hexose monophosphate shunt utilization, phagocytosis, and content of  $\beta$ -glucuronidase, acid protease, neutral protease, and acid phosphatase (4, 36). An exception is nitroblue tetrazolium reduction, which is greatest in fully mature alveolar macrophages (4). Thus, in alveolar macrophages not all activation markers peak at the same stage of maturation, which is a finding consistent with current information about the heterogeneity of macrophages from sources outside the lung (37, 38). Whether induction of tissue factor in the lung environment occurs at specific stages of the cell cycle together with characteristic types of metabolic or functional activation is unknown. It is possible that this induction occurs as a discrete event or repetitively at different times during the macrophage lifespan in response to activation by ambient stimuli such as the aerobic environment, inhaled particulate matter, and microbes in the airway. Either discrete or repetitive activation would be consistent with the present finding that isolated immature and mature forms of alveolar macrophages are capable of generating additional tissue factor.

The precise physiologic role of tissue factor activity in alveolar macrophages remains to be determined. However, from the present experiments, it is clear that tissue factor activity becomes available in progressively increased amounts in these cells during maturation from blood monocytes and immature alveolar macrophage forms. Thus, tissue factor is a marker of alveolar macrophage maturation in vivo. Low levels of tissue factor activity as found in blood monocytes and in precursors of alveolar macrophages would seem to be a property which allows circulation of these cells without thrombosis, since increases of this activity in monocytes may trigger intravascular coagulation, as observed during endotoxemia and meningococemia (10, 39, 40). The higher levels of the activity, as found in maturing alveolar macrophages, cells with limited physiologic potential for interaction with the clotting system in blood due to extravascular localization, suggest an adaptation for protective functions in the event of capillary injury and extravasation of blood. Indeed, from current evidence it appears that in a variety of diseases producing pulmonary capillary injury, plasma coagulation proteins leak into the alveolar space where alveolar macrophages are localized, and that this type of injury can be associated with coagulopathies and fibrin deposition on alveolar membranes (41, 42). The increases of tissue factor activity demonstrated here in cell populations from animals with granulomatous hypersensitivity pneumonitis show that this activator of the extrinsic coagulation pathway can be induced in vivo by immune pulmonary reactions that effect alveolar macrophage accumulation and maturation. These increases suggest that alveolar macrophage tissue factor may contribute to the fibrin deposition, which is found in immune lung diseases such as tuberculosis and allergic alveolitis

and in experimental models of delayed hypersensitivity (43-46).

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## References

1. Mason, A. J. 1977. Metabolism of alveolar macrophages. *In* Respiratory Defense Mechanisms. J. D. Brain, D. F. Proctor, and L. M. Reid, editors. Marcel Dekker, Inc., New York. 893-926.
2. Gee, J. B. L., and A. S. Khandwala. 1977. Motility, transport and endocytosis in lung defense cells. *In* Respiratory Defense Mechanisms. II. J. D. Brain, D. F. Proctor, and L. M. Reid, editors. Marcel Dekker, Inc., New York. 927-982.
3. Bowden, D. H., and I. Y. Adamson. 1980. Pulmonary macrophages. *In* The Reticuloendothelial System. I. Morphology. I. Carr and W. T. Daems, editors. Plenum Press, New York. 607-633.
4. McGee, M. P., Q. N. Myrvik, B. Y. Thomson, L. E. Eaton, and J. Ockers. 1980. Macrophage Heterogeneity in BCG-induced granulomas. *In* Workshop on Basic and Clinical Aspects of Granulomatous Disease. T. Yoshida, editor. Elsevier/North Holland, Inc., New York. 52-63.
5. Dauber, J. H., A. H. Holian, M. A. Rosemiller, and R. P. Daniele. 1983. Separation of bronchoalveolar cells from guinea pig on continuous density gradients of Percoll: morphology and cytochemical properties of fractionated lung macrophages. *J. Reticuloendothel. Soc.* 33:119-126.
6. Zwilling, B. S., L. B. Campolito, and N. A. Reiches. 1982. Alveolar macrophage subpopulations identified by differential centrifugation on a discontinuous albumin density gradient. *Am. Rev. Respir. Dis.* 125:448-452.
7. Adams, D. O. 1974. The structure of mononuclear phagocytes differentiating in vivo. I. Sequential fine and histologic studies of the effect of bacillus calmette-guerin (BCG). *Am. J. Pathol.* 76:17-39.
8. Blusse, A., A. Van Oud, and R. Van Furth. 1982. The origin of pulmonary macrophages. *Immunobiology.* 161:186-192.
9. Nemerson, Y., and R. Bach. 1982. Tissue factor revisited. *Prog. Hemostasis Thromb.* 6:237-261.
10. Rothberger, H., F. Dove, T.-K. Lee, M. P. McGee, and B. Kardon. 1983. Procoagulant activity of lymphocyte-macrophage populations in rabbits: selective increases in marrow, blood and spleen cells during Shwartzman reactions. *Blood.* 61:712-717.
11. McGee, M. P., and Q. N. Myrvik. 1981. Collection of alveolar macrophages from rabbit lungs. *In* Manual of Macrophage Methodology. H. Herscovitz, H. T. Holden, J. A. Bellanti, and A. Gaffar, editors. Marcel Dekker, Inc., New York. 17-22.
12. Soranzo, M. R., H. Koerter, and W. T. H. Daems. 1978. Peroxidatic and morphometric analysis of alveolar macrophages in guinea pigs. *J. Reticuloendothel. Soc.* 23:346-359.
13. McGee, M. P., and Q. N. Myrvik. 1976. Hydrolase levels in necrotizing and non-necrotizing BCG-induced pulmonary granuloma. *J. Reticuloendothel. Soc.* 20:187-195.
14. Li, C. Y., K. W. Lan, and L. T. Yan. 1973. Esterases in human leukocytes. *J. Histochem. Cytochem.* 21:1-12.

15. Kingdon, H. S., R. L. Lundbald, J. J. Ventkamp, and D. L. Aronson. 1975. Potentially thrombogenic materials in factor IX concentrates. *Thromb. Diath. Haemorrh.* 33:617-631.
16. Brinkhouse, K. M., and F. A. Dombrose. 1980. Partial thromboplastin time. In CRC Handbook Series in Clinical Laboratory Science, Section I Hematology, III. D. Seligson and R. M. Schmidt, editors. CRC Press, Inc., Boca Raton, FL. 221-246.
17. Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* 5:494-496.
18. Burton, K. 1968. Determination of DNA concentration with diphenylamine. In Methods in Enzymology, XII. L. Grossman and K. Moldave, editors. Academic Press, Inc., New York. 163-166.
19. Van Furth, R., Z. A. Cohn, J. G. Hirsch, J. H. Humphrey, W. G. Spector, and H. L. Langevoort. 1972. The mononuclear phagocyte system: a new classification of macrophages, monocytes and their precursor cells. *Bull. W. H. O.* 46:845-852.
20. Edwards, R. L., and F. R. Rickles. 1980. The role of human T cells (and T cell products) for monocyte tissue factor generation. *J. Immunol.* 125:606-609.
21. Schwartz, B. S., and T. S. Edgington. 1981. Lymphocyte collaboration is required for induction of murine monocyte procoagulant activity by immune complexes. *J. Immunol.* 127:438-443.
22. Levy, G. A., and T. S. Edgington. 1980. Lymphocyte cooperation is required for amplification of macrophage procoagulant activity. *J. Exp. Med.* 151:1232-1244.
23. Levy, G. A., B. S. Schwartz, and T. S. Edgington. 1981. The kinetics and metabolic requirements for direct lymphocyte induction of human procoagulant monokines by bacterial lipopolysaccharide. *J. Immunol.* 127:357-363.
24. Farram, E., C. L. Geczy, D. K. Moon, and K. Hopper. 1983. The ability of lymphokine and lipopolysaccharide to induce procoagulant activity in mouse macrophage cell lines. *J. Immunol.* 130:2750-2756.
25. Geczy, C. L., and K. E. Hopper. 1981. A mechanism of migration inhibition in delayed-type hypersensitivity reactions. II. Lymphokines promote procoagulant activity of macrophages in vitro. *J. Immunol.* 126:1059-1065.
26. Osterud, B., U. Lindahl, and R. Seljelid. 1980. Macrophages produce blood coagulation factors. *FEBS (Fed. Eur. Biochem. Soc.) Letters.* 120:41-43.
27. Montarroso, A. M., and Q. N. Myrvik. 1979. Oxidative metabolism of BCG-activated alveolar macrophages. *J. Reticuloendothel. Soc.* 25:559-574.
28. Montarroso, A. M., and Q. N. Myrvik. 1978. Effect of BCG vaccination on the IgG and complement receptors on rabbit alveolar macrophages. *J. Reticuloendothel. Soc.* 24:93-99.
29. Suga, M., A. M. Dannenberg, and S. Higuchi. 1980. Macrophage functional heterogeneity in vivo: macrolocal and microlocal macrophage activation. Identification by double-staining tissue sections of BCG granulomas for pairs of enzymes. *Am. J. Pathol.* 99:305-324.
30. McGee, M. P., H. Rothberger, and T.-K. Lee. 1984. Selective increases of procoagulant activity in rabbit lymphoid populations in vitro following stimulation with endotoxin: dependence on anatomic source. *Thromb. Haemostasis.* In press.
31. Rothberger, H., M. Barringer, and J. Meredith. 1984. Increased tissue factor activity of monocytes/macrophages isolated from canine renal allografts. *Blood.* 63:623-628.
32. Rothberger, H., T. S. Zimmerman, H. L. Spiegelberg, and J. H. Vaughan. 1977. Leukocyte procoagulant activity: enhancement of production in vitro by IgG and antigen-antibody complexes. *J. Clin. Invest.* 59:549-557.
33. Ebert, R. H., and H. M. Florey. 1939. The extravascular development of the monocyte observed in vivo. *Br. J. Exp. Pathol.* 20:342-356.
34. Fisher, D., M. B. Van der Weyden, R. Snyderman, and W. N. Kelly. 1976. A role for adenosine deaminase in human monocytes maturation. *J. Clin. Invest.* 58:399-407.
35. Nichols, B. A. 1976. Normal rabbit alveolar macrophages. I. The phagocytosis of tubular myelin. *J. Exp. Med.* 144:906-919.
36. Thompson, B. Y. 1982. Morphologic and functional characteristics of gradient separated pulmonary macrophages obtained from BCG-induced rabbits. Ph.D. thesis. Bowman Gray School of Medicine, Winston-Salem, NC. 50-77.
37. Walker, W. S. 1980. Macrophage heterogeneity: membrane markers and properties of macrophage subpopulations. In *Macrophage Regulation of Immunity*. A. S. Rosenthal, editor. Academic Press, Inc., New York. 307-332.
38. Hopper, K. E., P. R. Wood, and D. S. Nelson. 1979. Macrophage heterogeneity. *Vox Sang.* 36:257-274.
39. Thiagarajan, P., and J. Niemetz. 1980. Procoagulant-tissue factor activity of circulating peripheral blood leukocytes. Results of in vivo studies. *Thromb. Res.* 17:891-895.
40. Osterud, B., and T. Flaegstad. 1983. Increased tissue thromboplastin activity in monocytes of patients with meningococcal infection: related to an unfavorable prognosis. *Thromb. Haemostasis.* 49:1-4.
41. Staub, N. C. 1981. Pulmonary edema due to increased microvascular permeability. *Annu. Rev. Med.* 32:291-312.
42. Meyer, E. C. 1978. Acute and chronic clearance of lung fluids, proteins and cells. In *Lung Water and Solute Exchange*. N. C. Staub, editor. Marcell Dekker, Inc., New York. 277-321.
43. Jenkins, D. E., and E. Wolinsky. 1974. Mycobacterial diseases of the lung and bronchial tree: clinical and laboratory aspects of tuberculosis. In *Textbook of Pulmonary Diseases*. G. L. Baum, editor. Little, Brown & Co., Boston. 257-321.
44. Kleinerman, J. Pulmonary diseases related to organic materials and radiation. In *Textbook of Pulmonary Diseases*. G. L. Baum, editor. Little, Brown & Co., Boston. 525-537.
45. Hopper, K. E., C. Geczy, and W. Davies. 1981. A mechanism of migration inhibition in delayed-type hypersensitivity reactions. *J. Immunol.* 126:1052-1058.
46. Cohen, S., B. Benacerraf, R. McCluskey, and Z. Ouary. 1967. Effect of anticoagulants on delayed hypersensitivity reactions. *J. Immunol.* 92:351-358.