PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **188**, 461–470 (1988)

Modulation of Rat Granulocyte Traffic by a Surface Active Agent *in Vitro* and Bleomycin Injury[1] (42761)

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Abstract. Pluronic F68 (F68) is a nonionic surfactant which has been reported to inhibit the *in vitro* adherence and migration of polymorphonuclear leukocytes (PMN) obtained from some species. We demonstrated similar effects on PMN obtained from rats, with diminished adherence to nylon wool and diminished chemotaxis toward zymosan-activated serum. We then examined the *in vivo* effects of 12-hr F68 infusion on the injury induced by intratracheal bleomycin instillation (ITB) in rats. When sacrificed 24 hr following injury, rats demonstrated neutrophilia, neutrophil-prominent lung lavage cellularity, and increased lung weights. F68 decreased lavage leukocyte counts and lung weight gain in ITB-injured animals. Lung weights of ITB-injured animals correlated ($r = 0.81$, $P < 0.001$) with logarithmic values of lavage PMN. F68 also enhanced neutrophilia and decreased spleen weight gain in injured animals. The acute effects of F68 on circulating leukocyte counts, osmolality, and total complement were also examined. The data demonstrate that F68 can affect PMN traffic both *in vitro* and *in vivo*. The data also confirm the prominence of PMN in lavage fluid early in ITB injury, and suggest that an influx of relatively few PMN is associated with lung weight gain in this model.

Pluronic F68 (F68) is a nonionic surfactant used as an emulsifier in a variety of commercial preparations, including a perfluorocarbon artificial blood substitute (1). Although thought to be relatively inert biologically, recent data have demonstrated that *in vitro* adherence and migration of polymorphonuclear lymphocytes (PMN) obtained from rabbits and humans are inhibited by F68 (2). If PMN are similarly inhibited *in vivo,* then F68 might provide a probe of PMN involvement in lung injury. Demonstration of inhibitory effects *in vivo* might likewise suggest a therapeutic role for F68 in PMN-mediated injury. However, infected patients might be adversely affected if infused with materials containing sufficient F68 to inhibit PMN.

PMN have been noted to be prominent in some animal models of lung injury leading to fibrosis, such as that associated with intratracheal instillation of bleomycin (ITB) (3). Although ITB injury to rodents is frequently employed as a model of idiopathic pulmonary fibrosis (3, 4), a number of features parallel the events of protracted adult respiratory distress syndrome (ARDS). An acute alveolitis as characterized by lung lavage may be found within 24 hr following ITB injury (3). This phase is associated with an increase in lung weights, although not necessarily associated with an increase in wet–dry weight ratios (5). A cellular influx of predominantly PMN has been identified by lung lavage in hamsters within hours following injury (6), and in rats at 24 hr following injury (3). The acute inflammatory process is followed by accumulation of chronic inflammatory cells (3), with increased collagen synthesis noted biochemically as early as Day 4 (7), and fibrosis apparent histologically within 2–4 weeks (8). Similarly, PMN are prominent in ARDS (9–11), and a protracted course of ARDS in humans has been associated with simulation of collagen synthesis (12). A pathogenic role for PMN in ARDS has been suggested by a number of studies in humans and animals (9–12), while the role of PMN in ITB injury is less clear. Several studies (13–15) have suggested that PMN may suppress collagen accumulation and synthesis in

[1] This research was supported in part by an award of the ALA/ATS, (Grant AL 8740), and in part by the Stauffer Foundation.

0037-9727/88 $1.50

the lungs of ITB-injured animals, while the role of the PMN in the initial events following ITB instillation are not well described.

The purposes of the current study were (i) to demonstrate that F68 can inhibit *in vitro* adherence and migration of PMN obtained from rats, and (ii) to examine its ability *in vivo* to modify the PMN-prominent, acute alveolitis induced by ITB instillation, as assessed by lung lavage cellularity and lung weights. We also examined the effects of F68 on circulating blood counts, and recorded the weights of spleens, which represent important reservoirs for marginated PMN (16).

Materials and Methods. Sprague–Dawley rats (Charles River, Worcester, MA, and Simenson, Tustin, CA) weighing 200–250 g were used in this study. All procedures were performed under ketamine (Parke-Davis, Morris Plains, NY) anesthesia. For *in vitro* studies, PMN were harvested from rats by hetastarch exchange transfusion (18). PMN were then isolated by double-density, Ficoll–Hypaque, gradient centrifugation, using methods modified (18) from those previously reported (19). We have previously shown that leukocytes obtained in this manner are approximately 97% PMN, with >98% viability by trypan blue dye exclusion, and demonstrate relatively normal adherence, migration, superoxide generation, and bacterial killing when compared to cells pooled from animals following simple phlebotomy (18). The cells were suspended in basal Eagle's medium with Earle's salts (BME, Flow Labs, St. Louis, MO) at a concentration of 3×10^6 cells/cc. Pluronic F68 (BASF, Wyandotte, MI), was suspended in nonpyrogenic, sterile water in a stock solution of 126 mg/cc and added to the cell suspensions to create concentrations ranging from 0.2 to 2.0 mg/cc in buffered salt solution. All assays were performed in triplicate with each sample.

PMN adherence to nylon wool columns was performed as previously described (2, 18). Briefly, 160 mg nylon wool (leukopak; Fenwall, Deerfield, IL) was packed in a 3-cc syringe at constant volume, and 4 cc of cell suspension was layered into the column with a sterile pipet. Cells eluting from the column were counted with a Coulter counter, and the ratio of eluting to original cell counts representing fractional adherence (Fadh). The decrement in adhesion secondary to F68, termed the percentage adherence (% adh) was calculated as follows:

$$\%\text{adh} = \frac{[\text{Fadh (without F68)} - \text{Fadh (with F68)}]}{\text{Fadh (without F68)}} \times 100.$$

PMN migration toward zymosan-activated serum (ZAS) was assessed with a Boyden microchamber technique (2). Briefly, cell suspensions containing varying concentrations of F68 were placed in the upper chambers and 0–40% ZAS was placed in the lower chambers. The wells were separated by an 8-μm pore size Millipore filter (Millipore Corp.) and incubated at 37°C in incubators with 5% CO_2 for 90 min. The filters were then fixed and stained. Migration was determined by counting the number of cells in four high-powered fields at filter bottom or the nearest depth at which PMN counts reached approximately 150–200. Percentage migration was then determined by the relative number of cells migrating to the same depth, in the same cell preparation, in the presence of varying concentrations of F68. This approach normalized for unavoidable differences in the migration of various cell preparations toward a stimulus derived from a complex biologic system (ZAS).

In the *in vivo* injury study, four groups of animals were examined, matched for weights. Two groups were injured with an intratracheal instillation of ITB, using approximately 1.5 units of outdated bleomycin sulfate (Blenoxane; Brystol, Syracuse, NY) in 0.3 cc NS, as previously described (3). These animals additionally received either Pluronic F68 (F68) or normal saline (NS) via the penile vein, at the time of injury and 12 hr later. Treatments consisted of 450 mg F68 suspended in 1.5 cc sterile NS, or an equal amount of NS without F68, infused over 60 sec. This dose was based on the short circulating half-life of F68 (17), and preliminary data examining other doses. For comparison, two additional groups were not injured, but were given NS or F68 infusions at similar intervals.

Animals were then sacrificed at 24 hr by exsanguination, based on preliminary data

which did not reveal increased lung weights or lavage PMN during the initial 12 hr following injury in these animals. All animals were weighed before injury; most were weighed again at sacrifice and percentage change in body weight was determined for these animals. At sacrifice, blood was collected from the femoral vein in citrate for determination of hematocrit, total circulating white blood cell count (WBC), and differential. Circulating WBC counts were determined with an electronic counter (ZBI; Coulter Electronics, St. Hialeah, FL), and differentials determined by counting 200 cells on a smear using a modified Wright's–Giemsa stain (Leukostat; Fisher, Fairlawn, NJ).

Exsanguination was performed aggressively by phlebotomizing animals of approximately 8 cc of blood, followed by sequential transection of the femoral vessels, abdominal vessels, and apex of the heart. The spleen was dissected free and weighed. The lungs were dissected free, carefully trimmed of nonpulmonary tissue, and weighed. We assumed that residual intravascular volume had been minimized by our phlebotomy, and did not attempt to correct for residual with the hemoglobin content of the lung because of the occasionally hemorrhagic character of this injury. Organ weights were normalized to preinjury body weights. It has been our observation that the weights of these organs increase with animal weight in rats of this age. However, because the relationship is not that of identity, the groups were also matched for preinjury body weight.

The lungs of most animals were then lavaged, using methods previously employed in this lab. Briefly, a 23-gauge, truncated butterfly was ligated in the right mainstem bronchus, and the lung lavaged with 25 cc of NS at ambient temperature using approximately 4-cc aliquots. Lavage fluid was collected into citrated tubes. The cell count was determined by hemocytometry, using toluidine blue; a differential was determined from a Wright's–Giemsa stained, cytospun (Cytospin; Shandon, Astmoor, UK) sample, counting 200 cells. The left lung of some animals was weighed wet, and then reweighed following drying at 85°C for 3–4 days (until the weights were stable). Wet/dry weight ratios were then determined for these animals.

In separate studies, we examined the immediate effects of the F68 infusion on serum osmolality, circulating leukocyte counts, and total complement (CH_{50}) levels on uninjured rats. For these experiments, two groups of 10 rats received either 450 mg F68 in normal saline or saline alone via the tail vein. Blood was drawn from the femoral vein at 0, 5, 15, and 60 min following infusion, collecting 1, 1, 4, and 1, cc, respectively. A 0.2-cc aliquot was placed in EDTA, and leukocyte counts were determined as above. A 0.8-cc aliquot was permitted to clot for determination of serum osmolality from four animals in each group, using an osmometer (Wescor 5100B vapor pressure osmometer). A 3-cc aliquot of the sample at 15 min was then clotted and centrifuged, to obtain serum which was stored at −70°C for subsequent determination of CH_{50} levels. This 3-cc volume of blood was required to obtain 1 cc of serum needed for the CH_{50} assay, and precluded measurement of sequential CH_{50} levels in these small animals. CH_{50} levels were performed by a commercial lab using standard methods, determining 50% lysis of sheep erythrocytes sensitized with rabbit antibody (38).

Analysis was performed with a hand-held calculator and with a personal computer using a statistical software package (BMDP; UCLA, CA). *In vitro* values represent means of triplicate assays in each experiment, and were analyzed by ANOVA. *In vivo* data were first analyzed for variance: When significant, differences between group means were then compared by unpaired *t* tests. The relationship between normalized lung weights and the accumulation of leukocytes in lavage fluid was examined by linear regression. Sequential leukocyte counts were examined for variance and covariance of repeated measures, and *t* tests were applied where variance was significant. Statistical significance was accepted where $P < 0.05$.

Results. The effects of F68 on adherence of rat PMN to nylon wool columns and chemotaxis toward ZAS are shown in Fig. 1. A statistically significant, dose-dependent, marked inhibition of adherence was noted. The dose at which 50% maximal inhibition

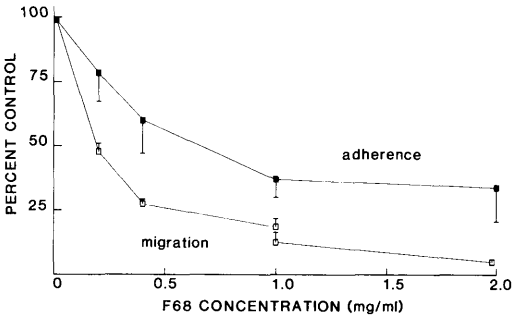

FIG. 1. *In vitro* PMN adherence and migration. Values represent means and standard errors of three or four samples, each run in triplicate. Lines connect data obtained with cells from the same group of rats. Adherence and migration of each cell preparation in the absence of F68 defines 100% (control) adherence and migration. Percentage adherence and migration compared to control values for each cell preparation, in the presence of varying concentrations of F68, are displayed. A significant ($P < 0.05$) reduction in both adherence to nylon wool columns and migration toward zymosan-activated serum were observed in the presence of F68.

(ID_{50}) occurred was approximately 0.6 mg/cc. Similar results were obtained when examining the effects of F68 on PMN chemotaxis, with an ID_{50} at concentrations of approximately 0.2 mg/cc.

The *in vivo* experiments likewise demonstrate an effect on circulating PMN by F68. Examination of peripheral blood is shown in Table I. Differences in hematocrits were not statistically significant. Likewise, although total leukocyte counts varied, the differences were not significant ($P > 0.20$). However, circulating PMN were significantly ($P < 0.05$) increased in ITB-injured animals, and F68 significantly enhanced circulating PMN numbers in injured ($P < 0.01$), but not uninjured animals. Conversely, circulating lymphocytes were decreased in ITB-injured animals ($P < 0.01$), and further decreased by F68 ($P < 0.01$); however, F68 did not significantly alter counts in uninjured animals. Monocytes morphologically represented less than 2% of circulating leukocytes in both groups, and were not specifically enumerated by special stains.

Examination of lung lavage fluid is shown in Table II. The returned volume was virtually the same in each group. However, the nucleated cell (NC) count was significantly increased in the ITB-injured animals receiving NS, and this increase was inhibited by F68 ($P < 0.001$). The decrease in nucleated cells of F68-treated, uninjured animals was not significant ($P > 0.05$). These cells were grouped as either PMN or mononuclear (MN) by stained cytosmear. ITB injury was associated with a marked increased in lavage PMN, consisted largely of neutrophils at 24 hr. This increase was significantly ($P < 0.01$) inhibited by F68 infusions. No difference in lavage PMN was noted when F68 was infused into uninjured animals, which had few lavage PMN. Lavage mononuclear cells, consisting almost exclusively of macrophages and monocytes, were also significantly ($P < 0.01$) decreased by F68 when compared to saline-treated, ITB-injured animals, while the decrease in uninjured animals associated with F68 did not reach statistical significance.

Body weights preinjury and differences in weights of various organs harvested at sacrifice are shown in Table III. The groups were well matched for weights, and organ weights were normalized to preinjury body weights, as explained above. ITB injury was asso-

TABLE I. HEMATOLOGIC DATA

Group	(*n*)	Hct%	WBC (10^3/mm^3)	PMN (10^3/mm^3)	Lymph (10^3/mm^3)
NNS	(15)	34.0 (±1.9)	9.05 (±2.24)	1.94 (±0.72)	7.00 (±1.97)
N68	(10)	36.6 (±1.8)	8.73 (±1.10)	1.97 (±0.81)	6.45 (±1.41)
BNS	(16)	34.3 (±1.9)	8.53 (±2.15)	2.97 (±0.84)**	5.44 (±1.54)*
B68	(16)	34.1 (±5.0)	8.74 (±2.04)	4.83 (±2.27)**.***	3.79 (±1.15)**.***

Note. The groups listed include saline-treated normals (NNS), F68-treated normals (N68), saline-treated injury (BNS), and F68-treated injury (B68). Values represent means (± standard deviation) of hematocrits (Hct), total leukocyte (WBC), neutrophil (PMN), and lymphocyte (lymph) counts.

* $P < 0.05$ and ** $P < 0.01$ compared to NNS; *** $P < 0.01$ compared to BNS.

TABLE II. LUNG LAVAGE DATA

	(*n*)	Volume (cc)	Nucleated cells (No./mm^3)	PMN (No./mm^3)	MN (No./mm^3)
NNS	(9)	22.9 (±1.1)	67.2 (±16.6)	0.4 (±0.5)	66.9 (±16.8)
N68	(10)	22.1 (±1.3)	51.3 (±19.2)	0.7 (±0.7)	50.5 (±18.7)
BNS	(10)	21.4 (±1.7)	145 (±59)**	55.4 (±20.8)**	89.2 (±40.1)**
B68	(11)	21.0 (±1.5)	65.5 (±20.3)***	13.9 (±13.4)**,***	51.5 (±15.2)**,***

Note. The groups are as in Table I. The values obtained are means (± standard deviations) of lavage fluid volume, and counts of nucleated cells, neutrophils (PMN), and mononuclear cells (MN). The groups are those shown in Table I.

* $P < 0.05$ and ** $P < 0.01$, compared to NNS: *** $P < 0.01$, compared to BNS.

ciated with a significant lung weight gain ($P < 0.05$). However, lung weight gain was less ($P < 0.01$) in the ITB-injured animals receiving F68. F68 did not significantly affect lung weights of uninjured animals. Variance in wet/dry lung weight ratios between the four groups did not reach statistical significance, as follows: (a) ITB + NS, 4.80 ± 0.20 ($n = 9$); (b) ITB + F68, 4.73 ± 0.33 ($n = 8$); (c) NS alone, 4.62 ± 0.23 ($n = 11$); (d) F68 alone, 4.54 ± 0.48 ($n = 10$). However, a small increase in wet/dry ratios was noted among ITB-injured animals when compared to animals receiving NS alone ($P < 0.05$ by isolated t test). Spleen weights were significantly increased in ITB-injured animals, and F68 inhibited this increase ($P < 0.01$); F68 did not significantly affect spleen weights of uninjured animals.

We then examined the relationship between lavage PMN and lung weight gain in ITB-injured animals: lung weights were increased with relatively small increases in lavage PMN, with little additional weight gain at higher PMN counts. When log values of lavage PMN counts were compared to normalized lung weights (Fig. 2), a strong linear relationship ($r = 0.81$, $P < 0.001$) was noted. The relationship (not shown) between lavage mononuclear cell counts and normalized lung weights was less strong ($r = 0.44$), with that of total numbers of lavage nucleated cells and normalized lung weights intermediate ($r = 0.64$).

The effects of F68 on circulating leukocyte counts and serum osmolality immediately following injection are shown in Table IV. A decrease in total leukocyte (WBC) counts was noted following injection of both F68 and saline alone, which paralleled a decrease in hematocrits. However, a greater decline in WBC was noted at 5 and 15 min in the F68-treated group, which was accompanied by a significantly greater decrease in PMN counts at 5 min. Hematocrits also were significantly less in the F68 group at 5 and 15 min following injections. Serum osmolality was not significantly affected by F68. Although CH_{50} levels at 15 min tended to be lower in the F68-treated group (40 ± 37) than in the sa-

TABLE III. ORGAN WEIGHTS

	(*n*)	Preinjury body wt (gm)	$\frac{\text{Lung wt}}{\text{Body wt}}$ (10^{-3})	$\frac{\text{Spleen wt}}{\text{Body wt}}$ (10^{-3})
NNS	(15)	238 (±8)	4.99 (±0.30)	2.75 (±0.47)
N68	(10)	240 (±6)	4.88 (±0.28)	2.71 (±0.29)
BNS	(16)	236 (±8)	6.94 (±0.54)**	4.08 (±0.29)**
B68	(16)	237 (±11)	6.13 (±0.78)**,***	3.04 (±0.94)***

Note. The groups are as in Table I. Organ weights of animals are normalized to preinjury body weights. The groups are those shown in Table 1. Values reflect means (± standard deviation).

* $P < 0.05$ and ** $P < 0.01$ compared to NNS; *** $P < 0.01$ compared to BNS.

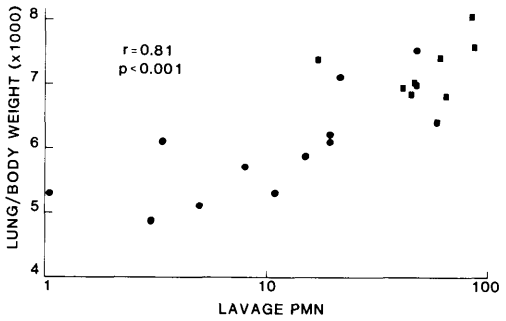

FIG. 2. Lung weight versus lavage PMN in ITB-injured rats. The values include animals receiving saline (squares) and F68 (circles). Lung lavage PMN counts (cell number/mm^3) are compared on a semilogarithmic scale to lung weights normalized to preinjury body weights ($\times 10^3$). A significant ($P < 0.001$) linear correlation ($r = 0.81$) was noted.

line group (92 ± 71), these differences were not statistically significant, and no clinical correlates of these differences (tachypnea, distress) were noted.

Weight loss was generally noted in all groups. Weight loss among uninjured animals was similar in saline-infused rats (2.4% ± 2.0, $n = 15$) and F68 treated rats (2.4% ± 1.6, $n = 10$), while among ITB-injured animals, F68-treated rats tended to lose more weight (9.2% ± 3.0, $n = 10$) than did saline-treated rats (5.9% ± 2.0, $n = 15$). However, we examined the linear relationship between normalized lung weights and change in body weights of ITB-injured animals and found a poor correlation ($r = -0.02$).

Discussion. The data demonstrate that Pluronic F68 (F68) can inhibit *in vitro* adhesion and migration of PMN obtained from rats, as reported in other species (2). It may have similar effects *in vivo* as demonstrated by decreased lavage PMN in ITB-injured rats, although mononuclear cells appeared affected as well. These effects of F68 did not appear related to increased serum osmolality. Although a transient neutropenia was induced by F68 in normal rats, it was associated with a statistically insignificant decrease in circulating complement, and was not of apparent clinical significance. Additionally, the data confirmed a PMN-prominent lung injury at 24 hr following intratracheal injury (ITB) instillation in rats (3), and suggest a strong relationship of modest PMN influx with lung weight gain in this early phase. A small increase in wet/dry lung weight ratios was noted among ITB-injured animals, minimally decreased by F68, but both trends were statistically insignificant.

These effects of F68 on leukocyte trafficking *in vitro* and *in vivo* have not been previously reported in rats. The current data are consistent with that reported by Lane, *et al.* (2), who demonstrated inhibition of rabbit and human PMN adherence and migration toward FMLP *in vitro* (following F68 influsion *in vivo*), and inhibition of PMN influx

TABLE IV. IMMEDIATE RESPONSE TO F68

Time (min):	0	5	15	60
Osmolality (osm/liter)				
Saline	282 (+6)	285 (+7)	285 (+6)	292 (+4)
F68	285 (+6)	286 (+5)	285 (+6)	291 (+6)
Leukocytes ($\times 10^3$/mm^3)				
Saline	9.16 (+1.60)	8.26 (+1.85)	6.70 (+1.57)	5.59 (+1.37)
F68	8.54 (+1.70)	5.34 (+0.74)**	4.73 (+0.82)**	4.53 (+1.33)
PMN ($\times 10^3$/mm^3)				
Saline	1.02 (+0.30)	1.15 (+0.31)	0.98 (+0.35)	1.17 (+0.35)
F68	1.11 (+0.37)	0.76 (+0.29)**	0.75 (+0.37)	1.04 (+0.49)
Hematocrit (%)				
Saline	40.6 (+1.9)	37.2 (+2.2)	36.4 (+1.5)	33.9 (+1.9)
F68	39.9 (+1.9)	29.6 (+5.6)**	31.6 (+4.1)**	34.0 (+2.3)

Note. Shown are sequential determinations of serum osmolality, blood leukocyte and neutrophil (PMN) counts, and hematocrits measured in uninjured rats infused with either Pluronic F68 in normal saline (F68) or saline alone. The values represent means (+ standard deviations). The methods are described in the text.

** $P < 0.01$.

into the peritoneum of mice induced by endotoxin (39). Although the interrelationship between PMN adherence and migration is complex (20), some augmentation of adherence is presumably required for the initial step in migration of PMN through the endothelium and subsequently into the alveoli of ITB-injured animals. Therefore, the reduction in lavage granulocyte counts in our study suggests that F68 in these doses may inhibit adherence and migration of PMN into the lung *in vivo* as well.

These data appear to contrast with those reported by Vercellotti *et al.* (21), who reported pulmonary intravascular sequestration of PMN producing respiratory distress in normal rabbits with infusion of F68 in smaller doses, thought related to complement activation. We also noted a transient leukopenia at 5 and 15 min following infusion. However, we found only a statistically insignificant trend toward decreased CH_{50} levels at 15 min, and the animals were not noted to have respiratory distress. On the other hand, hematocrits were also significantly reduced at 5 and 15 min following infusion, a transient change not likely induced by complement activation. Therefore, the transient leukopenia induced may reflect other factors, including transient fluid shifts (see below), which remain incompletely defined.

Additionally, lung lavage did not identify lung infiltration by PMN in normal or injured rats following F68 infusion in our study, which we would have anticipated if significant pulmonary sequestration of PMN had been induced by complement activation. While we did not examine the ultrastructure of these rat lungs histologically to compare the intravascular PMN numbers, preliminary data in normal rats (Williams, unpublished) have not demonstrated delayed clearance of indium-labeled PMN when infused with small doses of F68. Factors which may explain these contrasts with Vercellotti's findings include differences in the species studied, the duration of infusion, the doses administered, or the methods of sampling. Additionally, potentially toxic by-products were previously reported to contaminate some batches of F68 (22), and might explain the adverse reactions noted by Vercellotti.

Prior stimulation of PMN through complement activation might desensitize PMN for subsequent recruitment into the alveolar space by ITB. However, this would not explain the *in vitro* inhibition of adherence and migration of washed PMN suspended in a nonserum medium. Additionally, it is unlikely that F68 simply accelerated the course of ITB-induced PMN influx in alveoli, inasmuch as ITB-induced PMN influx is known to increase through Day 3 (3), and PMN were not increased in uninjured animals receiving F68. Therefore, we believe that the effects of F68 on alveolar influx of leukocytes following ITB are not simply attributable to stimulation by complement.

The effects of F68 on circulating leukocytes appeared to differ somewhat between injured and uninjured rats at 24 hr following infusion: F68 enhanced the late neutrophilia of ITB-injured rats, while neutrophilia was not found in uninjured rats. Although the acute experiments identified a transient neutropenia following F68 infusion in uninjured animals, this had resolved by 60 min. We cannot exclude the possibility that a rebound neutrophilia may have subsequently occurred, which had resolved by the time of sacrifice. On the other hand, the enhanced neutrophil counts at sacrifice of ITB-injured animals would also be consistent with decreased margination of PMN which had been recruited into the circulation by injury.

Although the lungs are thought to be a significant reservoir of marginated PMN in normal animals (23), the relative magnitude of this role has been questioned (16). During mobilization of PMN from the bone marrow, PMN may transiently sequester in the lung (24), increasing the marginated pool in the lungs. Perhaps the effects of F68 on PMN trafficking are more readily detected during such times. The spleen also appears to be a prominent reservoir for infused, labeled PMN (16, 18), and splenic sequestration of PMN mobilized from the bone marrow during ITB-injury might be inhibited by F68. This might explain inhibition of splenic weight gain in ITB-injured animals by F68, while splenic weights in normals were less affected. However, we did not explore the differences in splenic weight in this study.

The effects of F68 on leukocytes did not

appear limited to PMN. Lavage monocytes and macrophages were also reduced by F68 in ITB-injured animals, and suggest recruitment of additional monocytes (potential macrophages) into inflamed alveoli may be inhibited by F68 as well. These data are consistent with those reported by Lane (2) which demonstrated inhibition of peritoneal macrophage migration *in vitro* by F68. Recent preliminary data suggest that delayed hypersensitivity responses in mice may be inhibited by infusion of F68 containing compounds (25). The decrease in circulating lymphocytes by F68 in the current study is of uncertain significance, but also demonstrates that F68 may alter the traffic pattern of a variety of leukocytes *in vivo*.

The mechanisms by which F68 may inhibit PMN adherence and migration were not the focus of this study, but a number of potential mechanisms might be proposed. Agents which stimulate adenyl cyclase activity may inhibit a variety of PMN functions, including adherence and migration (26–28); however, a direct effect of F68 on adenyl cyclase seems unlikely. Interference with surface glycoproteins or surface charge also may affect cell adherence (29), or might facilitate flow through the microcirculation *in vivo* by increasing PMN deformability: F68, by virtue of its mixed hydrophilic and lipophilic characteristics, may be able to interact with cell membranes in either manner. Whether this interaction could involve specific receptors is not known, but we would suspect the interaction is less specific. It is therefore of interest that bovine surfactant may likewise have antiinflammatory properties in the lung (30). We must also consider a less direct mechanism. For example, adrenal steroids may similarly induce neutrophilia, lymphopenia, and inhibit mononuclear cell influx into sites of inflammation (31). Whether F68 may act directly, or induce an adrenocortical response which secondarily exerts these effects, remains to be established.

Lung weight gain 24 hr following ITB injury may reflect an accumulation of fluid, proteinaceous material, or both. The small and statistically insignificant increase in wet–dry lung weight ratios suggests that both accumulate, as has been reported at 5 days following ITB instillation (5). Of interest, although an alveolitis has been described in hamsters within the first few hours following ITB injury (6), we could not demonstrate alveolitis or lung weight gain during the first 12 hr in these rats (preliminary data not shown), suggesting a species difference in this injury model.

The significant correlation of lung weight gain with lavage granulocytes suggests that these cells may play a role in early ITB injury to rats. The reduction in lung weights and lavage PMN which occurred in F68 treated animals supports such a role. The role of PMN in the acute lung injury following ITB has not been well established, although a pathogenic role in a variety of other models of lung injury has been suggested (9–11). The relatively strong ($r = 0.81$; $r^2 = 0.66$) semilogarithmic relationship suggests that relatively few PMN may be necessary for early lung weight gain, with additional influx having progressively less effect. If so, then a less marked reduction in PMN influx might be expected to have little effect on the acute lung injury. This may be of particular importance when assessing the effects of neutrophil depletion in the rat, in which normally only approximately 20% of circulating leukocytes are PMN. It should be noted that lavage monocytes–macrophages also correlated with lung weight gain, but less strongly ($r = 0.44$; $r^2 = 0.19$).

We considered the possibility that inhibition of lung weight gain and granulocyte influx might not be causally related, but instead reflect unlinked effects of F68 acting in parallel. For example, F68 has been reported to affect platelet adhesion (37); however, the role of platelets in ITB injury remains to be identified. Additionally, although the mean molecular weight of F68 is only 8400 D, some variation exists (product information, BASF Wyandotte, Wyandotte, MI), which might present an osmotic load to the rat. Therefore, fluid shifts related to oncotic gradients might reduce lung weight. Additionally, an osmolar load of 2 mosm/liter could be calculated, based on mean molecular weight and an estimated intravascular volume of 25 cc (18): however, the measured osmolality of 285 was not significantly affected by F68 in our study. Nevertheless, a significantly greater weight loss was noted in

ITB-injured rats receiving F68, consistent with a diuresis. However, the hematocrit of the F68-treated group was not significantly increased at the time of sacrifice, and the poor correlation ($r = -0.02$) between change in body weight and lungs normalized to preinjury body weights did not support this hypothesis. Likewise, differences in lung wet/dry weights did not reach statistical significance, which would be anticipated if only lung water were decreased by F68. Instead, both aqueous and nonaqueous components were reduced, as demonstrated by lung lavage cellularity.

In conclusion, the data demonstrate that F68 can affect rat granulocyte trafficking both *in vitro* and *in vivo*. The inhibitory effects on leukocyte accumulation in the lung *in vivo* would suggest that F68 may provide a probe of leukocyte mediated events; however, the effects did not appear specific for PMN, and other more specific agents have been recently reported effective in other species (38). Although the inhibitory effects of F68 on leukocyte localization in the lung may prove beneficial in lung injury, further data would be required to support this hypothesis. In particular, infusion of a perfluorocarbon emulsion which contains F68 has been reported to adversely affect the outcome of experimental pneumonia (34), a complication which is not uncommon during the course of acute lung injury in clinical medicine.

We thank Thomas Lane, Geraldine Lamkin, Hung Tran and Chris Cha for their assistance in these projects, Hedda Schnur for her assistance in preparing the manuscript, and Brystol Laboratories for providing the Blenoxane used in this study.

1. Schmolka IR. Artificial blood emulsifiers. Fed Proc **34**:1449–1453, 1975.
2. Lane TA, Lampkin GE. Paralysis of phagocytic migration due to an artificial blood substitute. Blood **64**:400–405, 1984.
3. Thrall RS, Barton RW, O'Amato DA, *et al.* Differential cellular analysis of bronchoalveolar lavage fluid obtained at various stages during the development of bleomycin-induced pulmonary fibrosis in the rat. Amer Rev Respir Dis **126**:488–492, 1982.
4. Snider GL. Editorial. Interstitial pulmonary fibrosis —Which cell is the culprit? Amer Rev Respir Dis **127**:535–539, 1983.
5. Wangensteen D, Yankovich R, Hoidal J, Niewoehner D. Bleomycin-induced changes in pulmonary microvascular albumin permeability and extravascular albumin space. Amer Rev Respir Dis **127**:204–208, 1983.
6. Grant MM, Bernardo J. Clearance of intratracheally administered bleomycin from hamster lungs. Amer Rev Respir Dis **129**:A14, 1984. (Abstract)
7. Phan SH, Thrall RS, Ward PA. Bleomycin-induced pulmonary fibrosis: Biochemical demonstration of increased rate of collagen synthesis. Amer Rev Respir Dis **121**:501–506, 1980.
8. Snider Gl, Hayes JA, Korthy AL. Chronic interstitial fibrosis produced in hamsters by endotracheal bleomycin. Amer Rev Respir Dis **117**:1099–1108, 1978.
9. Rinaldo JE. Mediation of ARDS by leukocytes: Clinical evidence and implications for therapy. Chest **89**:590–593, 1986.
10. Tate RM, Repine JE. State of the art. Neutrophils and the adult respiratory distress syndrome. Amer Rev Respir Dis **128**:552–559, 1983.
11. Weiland JE, Davis WB, Holter JF, Mohammed JR, Dorinsky PM, Gadek JE. Lung neutrophils in the adult respiratory distress syndrome: Clinical and pathological significance. Amer Rev Respir Dis **133**:218–225, 1986.
12. Zapol WM, Trelstad RL, Coffey JW, Tsai I, Salvador RA. Pulmonary fibrosis in severe acute respiratory failure. Amer Rev Respir Dis **119**:547–554, 1979.
13. Thrall RS, Phan SH, McCormick JR, Ward PA. The development of bleomycin-induced pulmonary fibrosis in neutrophil-depleted and complement-depleted rats. Amer J Pathol **105**:76–81, 1981.
14. Clark JG, Kuhn C. Bleomycin-induced pulmonary fibrosis in hamsters: Effect of neutrophil depletion on lung collagen synthesis. Amer Rev Respir Dis **126**:737–739, 1982.
15. Phan SH, Schrier D, McGarry B, Duque RE. Effect of the beige mutation on bleomycin-induced pulmonary fibrosis in mice. Amer Rev Respir Dis **127**:456–459, 1983.
16. Peters AM, Saverymuttu SH, Bell RN, Lavender JP. Quantification of the distribution of the marginated granulocyte pool in man. Scand J Haematol **34**:11–20, 1985.
17. Wilcox ML, Newman MM, Paton BC. A study of labeled Pluronic F-68 after intravenous injection into the dog. J Surg Res **25**:349–356, 1978.
18. Williams JH, Moser KM, Ulich T, Cairo MS. Harvesting the non-circulating pool of polymorphonuclear leukocytes in rats by hetastarch exchange transfusion: Yield and functional assessment. J Leukocyte Biol **42**:455–462, 1987.
19. Lane T, Bergun PW, Lichter JP, Spragg RS. The labeling of rabbit neutrophils with [^{111}In]oxine. J Immunol Methods **51**:293–305, 1982.
20. Lackie JM, Smith RPC. Interactions of leukocytes

and endothelium. In: Curtis ASG, Pitts JD, Eds. Cell Adhesion and Motility. Cambridge, Cambridge Univ. Press, pp235–272, 1980.
21. Vercellotti GM, Hammerschmidt DE, Craddock PR, Jacob HS. Activation of plasma complement by perfluorocarbon artificial blood: Probable mechanism of adverse pulmonary reactions in treated patients and rationale for corticosteroid prophylaxis. Blood **59**:1299–1304, 1982.
22. Geyer RD. Perfluorochemical blood replacement preparations. In: Proceedings of the IVth International Symposium on Perfluorochemical Blood Substitutes, Kyoto, pp3–32, 1978.
23. Bierman H, Kelly K, Cordes RL, Byron JA, Polhemus JA, Rappaport S. The release of leukocytes and platelets from the pulmonary circulation by epinephrine. Blood **7**:683–692, 1952.
24. Rothstein G, Christensen RD. Pulmonary sequestration is a part of the normal inflammatory response. Clin Res **32**:79A, 1984.
25. Triolo J, Osterholm JL, Kratky MT. Suppression of delayed type hypersensitivity (DTH) by an emulsion of perfluorobutyltetrahydrofuram (Rm 101) prepared with the detergent F68 in female A/J mice. Fed Proc **44**:1631, 1985. (Abstract)
26. Ulich TR, Dakay EB, Williams JH, Ni RX. In vivo induction of neutrophilia, lymphopenia, and dimination of neutrophil adhesion by stable analogues of prostaglandin E_1, E_2, and F_2. Amer J Pathol **124**:53–58, 1986.
27. Lomitzer R, Rabson AR, Konenhof HJ. The effects of cyclic AMP on leukocyte inhibiting factor (LIF) production and on the inhibition of leukocyte migration. Clin Exp Immunol **24**:42–48, 1976.
28. Fantone J, Maraslo W, Elgas L, Ward P. Stimulus specificity of prostaglandin inhibition of rabbit polymorphonuclear leukocyte lysosomal enzyme release and superoxide production. Amer J Pathol **115**:9–16, 1984.
29. Rutter PR. The physical chemistry of the adhesion of bacteria and other cells. In: Curtis ASG, Pitts JD, Eds. Cell Adhesion and Motility. Cambridge, Cambridge Univ. Press, pp103–135, 1980.
30. Richman PS, Batcher S, Catanzaro A. Pulmonary surfactant (PS) suppresses the immune lung injury response to inhaled antigen in guinea pigs. Amer Rev Respir Dis **133**:A98, 1986. (Abstract)
31. Streeten DHP. Corticosteroid therapy. J Amer Med Assoc **232**:944–947 (part 1), 1046–1049 (part 2), 1975.
32. Gewurz H, Seyehira LA. Complement and immune complexes. In: Rose NR, Friedman H, Eds. Manual of Clinical Immunology. Washington, American Society for Microbiology, pp163–174, 1980.
33. Virmani R, Fink LM, Gunter K, English D. Effect of perfluorochemical blood substitutes on human neutrophil function. Blood **24**:343–347, 1984.
34. Hodges GR, Worley SE, Kemner JM, Reed JS. Effect of exchange transfusion with an oxygen-carrying resuscitation fluid on the efficacy of penicillin therapy of pneumococcal infection in rats. Antimicrob Agents Chemother **26**:903–908, 1984.
35. Bell RC, Coalson JJ, Smith JD, Johanson WG. Multiple organ system failure and infection in Adult Respiratory Distress Syndrome. Ann Intern Med **99**:293–298, 1983.
36. Costello JL, Gadek JE, Dorinsky PM. The effects of acute lung injury on regional blood flow: A possible explanation for organ failure in ARDS Amer Rev Respir Dis **133**:A38, 1986. (Abstract)
37. Grover FL, Heron MW, Newman MM, Paton BC. Effect of a nonionic surface-active agent on blood viscosity and platelet adhesiveness. Circulation Suppl **39–40**:I-249, 1969.
38. Arfors KE, Lundberg C, Lennart L, Lundberg K, Beatty PG, Harlan JM. A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo. Blood **69**:338–340.
39. Lane TA, Lamkin GE. Increased infection mortality and decreased neutrophil migration due to a component of an artificial blood substitute. Blood **68**:351–354, 1986.

Received September 14, 1987. P.S.E.B.M. 1988, Vol. 188.
Accepted April 19, 1988.