Paralysis of Phagocyte Migration Due to an Artificial Blood Substitute

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We investigated the effect of a candidate artificial blood substitute, Fluosol-DA (FDA), on human neutrophil function in a serum-free medium. In a 50% (vol/vol) mixture with polymorphonuclear cells (PMN), FDA had no effect on PMN viability, phagocytosis, superoxide anion generation, degranulation, or bactericidal activity. In striking contrast, the random migration and chemotaxis of PMN to both f-Met-Leu-Phe (fMLP) and activated serum were inhibited by 98% ± 2%, 95% ± 2%, and 88% ± 6%, respectively. Inhibition of chemotaxis by FDA required no preincubation, was dose-dependent (50% inhibition [ID50] with a 14% vol/vol mixture with FDA), and was fully reversible by washing PMN free of FDA after one hour but not after 18 hours of incubation (32% ± 11% inhibition of chemotaxis). FDA itself was not chemotactic and did not impair either the chemotactic activity or binding of fMLP to PMN. FDA also inhibited PMN adhesion (ID50, 9 ± 1 vol/vol %). The inhibitory component of FDA was found to be its detergent additive, Pluronic F-68, which inhibited random migration, chemotaxis, and adhesion with ID50s of 1.4, 2.4, and 2.9 mg/mL, respectively (equivalent to FDA concentrations of 5, 9, and 11 vol/vol %, respectively). All the other components of FDA were noninhibitory. Plasma samples from humans injected with 8 mL/kg FDA and plasma samples from rabbits injected with 16 mL/kg FDA or an equivalent concentration of Pluronic F-68, when mixed with autologous PMN, also severely inhibited PMN chemotaxis. We conclude that exposure of PMN to clinically relevant concentrations of FDA inhibits PMN migration, presumably due to inhibition of adhesion. The inhibitory effect is entirely due to the detergent, Pluronic F-68. Artificial blood substitutes containing Pluronic F-68 may compromise the ability of PMN to prevent or effectively control microbial infections.

MATERIALS AND METHODS

Granulocytes were prepared as previously described.11,12 PMN were suspended in basal Eagle’s medium with Earle’s salts (BME, Flow Labs, McLean, Va) with 0.5% bovine serum albumin (Sigma Chemical Company, St Louis) for all studies, except receptor binding assays, which were performed in phosphate-buffered saline, pH 7.3, with 10 mmol/L glucose, 5 mmol/L KCl, 1.5 mmol/L CaCl2, and 0.5 mmol/L MgCl2, as previously described.13,14 The Fluosol-DA (FDA) perfluorocarbon emulsion (referred to as “stem emulsion”), emulsion without perfluorocarbon (stem emulsion – PF), and “annex” solutions required for reconstitution of Fluosol-DA prior to use were supplied by Alpha Therapeutics, Los Angeles. This preparation was stored, and complete Fluosol-DA was prepared in accordance with the manufacturer’s instructions. Aliquots of the frozen emulsion were thawed just prior to use. For reconstitution, 4 vol of FDA stem emulsion (which contains 17.5 wt/vol % perfluorodecalin, 7.5% wt/vol % perfluorotripropylamine, 3.4% wt/vol % Pluronic F-68, 0.5% wt/vol % yolk phospholipid, and 1.0 wt/vol % glycerol), or stem emulsion without PF, or water (control solution) were mixed with 0.7 vol of annex solution H (which contains 4.28% wt/vol % NaCl, 0.2% wt/vol % CaCl2, 0.144% wt/vol % MgCl2, 1.28% wt/vol % glucose, and 21.44% wt/vol % hydroxyethyl starch) and 0.3 vol of annex solution C (0.567% wt/vol % KCl, 3.5% wt/vol % NaHCO3). This mixture (Fluosol-DA) or its components were adjusted to pH 7.4 and were used in the studies described below. All studies with FDA or control emulsions employed only the reconstituted emulsions as described above. Mixtures of Fluosol-DA (FDA) with PMN are reported as the volume/volume percentage (vol/vol %) of FDA when mixed with the PMN suspension.

Pluronic F-68 (referred to as pluronic) was greater than 99.9% pure and was obtained from Wyandotte Chemical Corporation, Wyandotte, MI; glycerol (Fisher Scientific, Los Angeles), yolk phospholipids, and sodium oleate (Sigma) were of the highest purity available.

In the initial experiments shown in Fig 1 and for experiments in which PMN were incubated with either FDA, stem emulsion without PF, annex solutions, or components of FDA (Fig 4), the incubations were performed at 37 °C in a 5% CO2 incubator for one hour. Otherwise, incubation conditions are as stated in the text.
Prolonged incubation (18 hours) of PMN with FDA was accomplished by adding FDA or reconstituted annex solutions without the stem emulsion (control buffer), in a final concentration of 30% vol/vol to buffy coat leukocyte preparations that were freshly prepared, as previously described, from citrate-phosphate-dextrose (CPD)-anticoagulated blood units. Following storage at room temperature in 150-mL transfer packs (Fenwall, Deerfield, Ill). Assays of PMN function were carried out in the presence of the experimental agent, unless otherwise stated.

PMN viability was studied by trypan blue dye exclusion, determination of cell numbers, and by lactate dehydrogenase (LDH Kit, Sigma) release into the supernatant medium before and after incubation with FDA. Phagocytosis was assessed by enumeration of ingested opsonized zymosan (OZ) and oil red-O, as previously described. Superoxide anion generation was assessed by cytochrome c reduction in response to OZ or after a five-minute, 37°C preincubation with 5 μg/mL cytochalasin B (CB), followed by addition of 10⁻³ mol/L f-Met-Leu-Phe (fMlP, Sigma), as previously described. Degranulation was assessed by release of LDH, β-glucuronidase, and lysozyme, in response to 1 mg/mL OZ, performed as described using commercial kits (Sigma and Worthington). In preliminary experiments, it was determined that both FDA and pluronic interfered with the lysozyme assay, hence, these results are not reported. Neutrophil random migration and chemotaxis toward varying concentrations of fMLP or zymosan-activated serum (ZAS) prepared as previously described, were assessed by the Boyden microchemotaxis technique, as previously described. Peritoneal macrophages were harvested four days after injection of 50 μL of 10% oyster glycogen (Sigma) into 2-kg New Zealand white rabbits, as previously described.

Neutrophil bactericidal capacity toward E. coli (ATTCC 92522) was performed as previously described using a bacteria to PMN ratio of 4:1. PMN were suspended at 1 x 10⁷/mL in a 50% vol/vol mixture with Fluosol-DA or an equal volume of reconstituted annex solutions. Neutrophil adhesion in the presence of a 50% vol/vol mixture with FDA or annex solutions, or Pluronic F-68, was assessed by nylon wool columns, as previously described. Aggregation studies employed a Payton dual-channel aggregation monitor (Buffalo) employing 0.5 μg/mL phorbol myristate acetate (PMA) as the aggregation stimulus. For in vivo studies, New Zealand white rabbits were infused using a syringe pump, via an ear vein, over a 30-minute period with Fluosol-DA (16 mL/kg) or an equal volume of an equivalent amount of pluronic (435 mg/kg). Prior to infusion, each rabbit was heparinized (55 U/kg). Blood samples (10 mL) were obtained at the following times: immediately prior to infusion, immediately following infusion, and at three hours, 20 hours, and 48 hours following infusion. A portion of the blood samples was immediately centrifuged at 1,000 g for 15 minutes, and the plasma samples were immediately frozen at −70°C. For comparison of plasma inhibitory activity of PMN migration before and immediately after infusion, PMN were harvested as described above and resuspended at 3 x 10⁶ PMN/mL in a 100% concentration of the appropriate plasma. Assessment of random migration and chemotaxis (toward 10⁻¹⁰ mol/L fMLP) of PMN suspended in plasma was carried out as described above. For investigation of the time course of inhibition of PMN migration after FDA or pluronic infusion, autologous PMN obtained 48 hours after infusion were suspended at 3 x 10⁶ PMN/mL in the preinfusion and postinfusion plasma samples that had been saved.

Assessment of human PMN migration before and after FDA infusion was performed as above, except that the infusions employed a total dose of 8 mL/kg Fluosol-DA administered IV over a three-hour period. The patients were not heparinized, but the blood samples were drawn into heparinized syringes (final concentration, 1 U/mL heparin), and only preinfusion and 15 to 20 minutes postinfusion samples were drawn. These studies were part of an approved Human Subjects Protocol at UC San Diego Medical Center and the VA Hospital, San Diego.

Unless otherwise stated, all results are reported as the mean ± standard error of the mean (SEM) and statistical comparisons employed a two-tailed Student’s t test.

RESULTS

The effect of incubation of PMN for one hour at 37°C with a 50% volumetric mixture of FDA on PMN function is shown in Fig 1. FDA did not increase cell loss or LDH release during the incubation period. Also, Fluosol did not affect trypan blue dye exclusion, and viability by this criterion was greater than 95% with and without fluosol (n = 3). Likewise, FDA did not influence phagocytosis of OZ or oil red-O, nor did it decrease superoxide anion generation in response to particulate or soluble stimuli. FDA did not reduce the amount of degranulation in response to phagocytosis of OZ. Also, FDA did not inhibit the bactericidal capacity of PMN (103% ± 3% of bacteria killed after 60 minutes, compared to controls; control PMN killed 93% ± 2% of bacteria added; n = 4). FDA itself, in the absence of PMN, had no influence on viability of bacteria. In striking contrast, FDA severely inhibited both the random migration and the chemotaxis of PMN to both synthetic peptides and activated serum (P < .01).

Inhibition of PMN random migration and chemotaxis due to FDA was dose-dependent (Fig 2). In four such studies, the concentration of FDA required to inhibit chemotaxis toward fMLP by 50% (ID₅₀) was
14 ± 2 vol/vol %, while the ID$_{50}$ of FDA for random migration was 12 ± 4 vol/vol %.

The time course of PMN migration inhibition by FDA was essentially immediate, insofar as it could be measured (since the chemotaxis assay itself requires at least a one-hour incubation). In one experiment, in which PMN were preincubated with 50% vol/vol FDA for intervals of up to one hour (samples taken every 15 minutes), the one-hour sample gave no greater inhibition of chemotaxis than the sample without preincubation (98% ± 1% inhibition, respectively). In view of this, subsequent experiments reported here employed no preincubation, unless otherwise stated.

Migration inhibition due to FDA was reversible after a short incubation of PMN with a high concentration (50% vol/vol) of Fluosol (Fig 3). Neutrophils incubated with a more physiologic concentration of FDA (30% vol/vol) for 18 hours at room temperature and washed prior to the assay, however, showed 32% ± 11% irreversible inhibition of chemotaxis (P < .02) compared to PMN incubated for an equal duration without FDA.

In four studies, PMN incubated with 50% vol/vol FDA for one hour at 37 °C bound 109% ± 10% the amount of $^3$H-fMLP as did control PMN (FDA was present during the binding assay). We assessed the effect of FDA on fMLP chemotactic activity itself by incubating a 10$^{-4}$ mol/L concentration of fMLP with 50% vol/vol FDA for one hour at 37 °C, followed by dilution of the fMLP to 10$^{-8}$ mol/L (the usual fMLP concentration for chemotaxis studies). In four studies, fMLP treated in this fashion had 98% ± 2% the chemotactic potency of fMLP treated in a similar fashion without FDA.

In order to determine which of the components of

Fluosol-DA was responsible for neutrophil migration inhibition, we performed the following experiments (summarized in Fig 4): PMN were incubated for 60 minutes at 37 °C with an equal volume of control buffer, following which the cells were washed three times or sham washed (by resuspension in the same medium), and migration was then assessed. In the right panel, Fluosol-DA or control buffer solution was added in a final concentration of 30% vol/vol to buffy coat preparations, which were then stored for 18 hours at 22 to 24 °C. PMN were harvested, washed, and migration assays were performed. Results represent mean ± SEM of four separate studies.

In order to determine which of the components of Fluosol-DA was responsible for neutrophil migration inhibition, we performed the following experiments (summarized in Fig 4): PMN were incubated for 60 minutes at 37 °C with an equal volume of control buffer, following which the cells were washed three times or sham washed (by resuspension in the same medium), and migration was then assessed. In the right panel, Fluosol-DA or control buffer solution was added in a final concentration of 30% vol/vol to buffy coat preparations, which were then stored for 18 hours at 22 to 24 °C. PMN were harvested, washed, and migration assays were performed. Results represent mean ± SEM of four separate studies.
with annex solutions, 13.6 mg/mL pluronic, 4 mg/mL glycerol, 2 mg/mL yolk phospholipids, and 0.16 mg/mL sodium oleate (these concentrations are equivalent to those found in a 50% vol/vol mixture of PMN with FDA). As shown, the stem emulsion without PF inhibited migration equal to the complete FDA; of the remaining components, only pluronic inhibited neutrophil migration. Similar results were obtained for random migration.

Inhibition of random migration and chemotaxis by pluronic was dose-dependent (Fig 5). The ID₉₀ of pluronic for chemotaxis was 2.4 ± 1.2 mg/mL and for random migration, 1.4 ± 0.3 mg/mL (n = 4). These concentrations correspond to 8.8% and 5.1% vol/vol FDA, respectively.

We found that pluronic, when present in a concentration equivalent to that of a 50% vol/vol concentration of FDA (13.6 mg/mL), had no effect on phagocytosis of oil red-O, superoxide anion generation in response to CB/fMLP, or degranulation in response to OZ (n = 4, each). Likewise, the inhibitory effect of pluronic on random migration and chemotaxis toward 10⁻⁸ mol/L fMLP required no preincubation and was fully reversible after a one-hour, 37°C incubation by washing the PMN free of pluronic (n = 4).

The mechanism of migration inhibition induced by FDA and pluronic was studied. Mixture of PMN with either FDA or pluronic resulted in dose-dependent inhibition of PMN adherence to nylon wool, as shown in Fig 5 (n = 3). At the maximum concentrations used (50% vol/vol FDA, and 13.6 mg/mL pluronic), FDA inhibited adherence by 65% ± 8% (ID₉₀ 10 ± 1 vol/vol %), while pluronic inhibited adherence by 78% ± 4% (ID₉₀ 2.8 ± 0.4 mg/mL). Adherence was not inhibited to as great an extent as was chemotaxis. Adherence inhibition by FDA was also reversible by washing (control—67% ± 1% adherent; washed control—67% ± 2% adherent; 50% vol/vol FDA—24% ± 10% adherent; washed FDA—73% ± 5% adherent; n = 3). In four additional studies, pluronic inhibited neutrophil aggregation with an ID₉₀ of 4.5 ± 0.5 mg/mL, but maximum inhibition of aggregation with 13.6 mg/mL Pluronic F-68 (21% ± 1%) was not as great as inhibition of chemotaxis (99% ± 1%) or of adherence (78% ± 4%, see above).

The migration of elicited rabbit peritoneal macrophages was also exquisitely sensitive to FDA. A 50% vol/vol concentration of FDA inhibited random migration by 91% ± 4% and chemotaxis by 93% ± 6% (n = 3).

Washed PMN harvested from rabbits infused with FDA or pluronic migrated as well as control PMN when assessed in the presence of plasma obtained prior to infusion (Table 1); however, PMN obtained prior to or after infusion, when combined with postinfusion plasma, showed severely impaired migratory responses. Inhibition of migration by postinfusion plasma persisted to 20 hours after FDA infusion, but not to 48 hours (n = 3). On the other hand, after pluronic infusion, migration inhibition persisted for three but not 20 hours (n = 3). Plasma also induced migration inhibition after infusion of humans with FDA (Table 2).

**DISCUSSION**

The development of an artificial oxygen-carrying blood substitute (ABS) will have a beneficial effect on

![Fig 5. Effect of Fluosol-DA and Pluronic F-68 on neutrophil adherence and chemotaxis. PMN were mixed with FDA or pluronic in the indicated concentrations, and adherence to nylon wool and chemotaxis toward 10⁻⁸ mol/L fMLP were measured. Results represent mean ± SEM of triplicate determinations.](image)

| Table 1. Effect of Fluosol and Pluronic Infusion on Rabbit Neutrophil Migration |
|-----------------------------------|-----------------|
|                                    | Migration (% Control) | Buffer | fMLP |
| **Fluosol**                       |                  |        |      |
| Pre                               | 100              | 100    |      |
| Pre Post                          | 39 ± 8           | 16 ± 10|      |
| Post Pre                          | 106 ± 35         | 106 ± 19|      |
| Post Post                         | 21 ± 12          | 16 ± 8 |      |
| **Pluronic**                      |                  |        |      |
| Pre                               | 100              | 100    |      |
| Pre Post                          | 30 ± 14          | 16 ± 4 |      |
| Post Pre                          | 78 ± 21          | 92 ± 33|      |
| Post Post                         | 34 ± 1           | 16 ± 1 |      |

Mean ± SEM of three experiments each. Rabbits were infused over a 30-minute period with either Fluosol-DA (16 mL/kg) or Pluronic F-68 (435 mg/kg) as described in Materials and Methods. PMN were harvested, washed, and resuspended in the indicated plasma. Migration assay was carried out without further preincubation with the plasma samples. fMLP was used in a 10⁻¹⁰ mol/L concentration. Pre and Post refer to PMN and plasma obtained either before or after infusion.
The migration of PMN without preincubation. Either buffer or fMLP, 10^-5 mol/L, was placed in the upper wells of the chemotaxis chamber within 20 minutes after completion of infusion. PMN suspended in the indicated plasmas were placed in the upper wells of the chemotaxis chamber.

PMN chemotaxis may be produced by doses of FDA very similar dose-response curves. Pluronic, an agent that has the capacity to paralyze neutrophil migration. Because PMN must migrate to sites of bacterial infection before the invading microbes can be killed, impairment of PMN migration may predispose individuals with such defects to infection or may impair the capacity of PMN from such individuals to control an established infection.

Our studies indicate that inhibition of PMN chemotaxis by FDA is not due to the perfluorocarbons themselves, but to Pluronic F-68; thus, incubation of PMN with pluronic resulted in precisely the same pattern of PMN dysfunction as did Fluosol, and with very similar dose–response curves. Pluronic, an agent that is used to stabilize the emulsion in which the perfluorocarbons are suspended, has been shown to sometimes activate complement and plasminogen.

As our experiments were performed in a serum-free medium, complement activation was not involved in the inhibition of the PMN chemotaxis which we observed. Our in vitro studies suggest that impairment of PMN chemotaxis may be produced by doses of FDA that amount to less than 10% of the circulating blood volume of the individual. Our in vivo studies in rabbits and humans indicate that the inhibitory effects of FDA and pluronic are not limited to in vitro experiments, but also occur with in vivo infusion, and therefore may be of clinical significance. The in vivo studies also confirm that severe inhibition of PMN migration may be caused by infusion of FDA equivalent to 11% to 30% of circulating blood volume and that the effect is almost certainly due to the pluronic component of FDA. Migration inhibition by plasma after FDA infusion was more prolonged than after pluronic infusion. This is consistent with the shorter in vivo persistence of free pluronic (half-life, 1.8 hours) compared to Fluosol-DA (half-life, 5.7 hours).

After FDA infusion, chemotactic impairment may persist for the lifespan of the PMN. Studies designed to ascertain the effect of Fluosol on bacterial infections in animals are currently in progress.

Inhibition of migration by FDA and pluronic may be mediated by impaired PMN adherence. This conclusion is supported by the similar dose–response curves for inhibition of adherence, chemotaxis, and random migration, and the elimination of other major possibilities [eg, normal stimulus (fMLP) binding, normal stimulus–response coupling (superoxide anion generation with fMLP), and normal membrane displacement (phagocytosis)]. The findings that pluronic decreased nylon wool adherence, but did not interfere with adherence of opsonized yeast, are not explained by these studies. However, previously reported rare cases of abnormalities in PMN surface glycoproteins, which have been associated with decreased chemotaxis and adherence to nylon wool, have been associated with either normal or abnormal phagocytosis of opsonized yeast.

Recently, a perfluorocarbon emulsion similar to Fluosol-DA was found to impair phagocytosis of latex beads in the presence of serum. We found no inhibition by FDA of phagocytosis of OZ or oil red-O by PMN in a serum-free medium.

These studies indicate the need for additional investigation of the effect of ABS containing pluronic on PMN function during ABS administration in vivo. Our investigations also suggest the possibility that ABS containing Pluronic F-68 may be potential therapeutic tools to pharmacologically suppress PMN accumulation in pathologic conditions in which PMN may play a pathogenic role. Finally, Pluronic F-68 may serve as a useful probe of the surface requirements for and mechanisms involved in the mediation of neutrophil adhesion and migration.

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