Increased Infection Mortality and Decreased Neutrophil Migration Due to a Component of an Artificial Blood Substitute

By Thomas A. Lane and G.E. Lamkin

We previously showed that an artificial blood substitute containing perfluorocarbons, Fluosol-DA, inhibited both neutrophil migration and adherence, due to its detergent component, Pluronic F-68. The purpose of the studies we report here was to determine if Fluosol or Pluronic might also reduce in vivo neutrophil migration and impair host resistance to bacterial infection. We studied in vivo PMN migration by injecting mice intraperitoneally (IP) with glycogen, followed by intravenous (IV) infusion of saline, Fluosol, or Pluronic. Peritoneal lavage after eight hours showed a significant decrease in the accumulation of PMN in lavage fluids of animals given either Fluosol or Pluronic (control - 19 ± 0.03 x 10^6 PMN/mL; glycogen - 1.35 ± 0.14; glycogen/Fluosol - 0.63 ± 0.12; glycogen/Pluronic - 0.69 ± 0.07). We ascertained the effect of Fluosol and Pluronic on infection mortality by injecting mice IV with saline, Fluosol, or Pluronic, followed by a quantity of E coli (0.6 x 10^8) IP shown in preliminary studies to kill 20% to 50% of the mice in 24 hours. The 24-hour mortality was 14/45-saline, 24/32-Fluosol (x^2 = 17.1; P < .001) and 17/23 - Pluronic (x = 11.2; P < .001). Neither Fluosol nor Pluronic caused mortality without E coli. The increase in infection mortality occurred when Fluosol was given either two hours before, or simultaneously with E coli, but only with the simultaneous administration of bacteria and Pluronic. Pluronic did not alter reticuloendothelial system (RES) clearance function. These studies indicate that, in an animal model, Fluosol-DA, due to its detergent component Pluronic F-68, impaired neutrophil delivery to an inflammatory locus, and resulted in an increased rate of infection mortality. Since Pluronic did not result in RES blockade, but did impair the delivery of PMN to an inflammatory locus, our results suggest that the latter effect is responsible for the increase in infection mortality.

METHODS

Neutrophil migration into an inflammatory locus was examined by injecting Swiss white mice (Simmonson Laboratories, Gilroy, Calif) weighing 28 to 32 g intraperitoneally (IP) with 0.5 mL of 10% oyster glycogen (Sigma Chemical Company, St. Louis, Mo) followed by intravenous (IV) injection of 1.6 mL of either (a) 0.85% sterile saline (controls), (b) Fluosol-DA (Alpha Therapeutics, Irvine, Calif) prepared according to the manufacturer’s instructions, or (c) 27 mg/mL of a Pluronic F-68 solution in 0.85% sterile saline (BASF Wyandotte, Wyandotte, Mich). This concentration of Pluronic is equivalent to that found in Fluosol-DA. Just prior to infusion, the pH of all solutions was adjusted to 7.4, followed by millipore (0.2 mm) filtration. Neutrophil accumulation in the peritoneal cavity was quantitated by lavaging the peritoneal cavity with 10 mL of sterile saline followed by white blood cell counting (Coulter Model ZF, Coulter Electronics, Hialeah, Fla). Differential cell counts were made on Wright’s stained smears.

Mortality due to infection was examined using a previously described model of Escherichia coli peritonitis in mice. A local strain of E coli which is resistant to killing by serum alone (VAMC-06, kindly supplied by J. Fierer, MD, Chief Microbiology Lab, VA Medical Center, San Diego, Calif) was grown in trypticase soy broth overnight, then 50 µl were grown for four hours in 5 mL fresh tissue culture medium (Hank’s balanced salt solution). The bacteria were washed three times in saline, and resuspended in saline at 4°C. Bacteria concentrations were ascertained by reading optical density at 610 nm and interpolation using standards of viable (colony-forming) bacteria. In five preliminary experiments, 4 to 6 groups of 4 mice each were injected IP with 0.3 mL of bacteria in concentrations ranging from 1 x 10^8 to 5 x 10^7/mL. In spite of careful attention to...
consistent technique, there was variation in mortality from one experiment to the next. We found that 24-hour mortality was 34 ± 12% after injection of 0.5 ± 0.18 (x 10^7) bacteria. In subsequent experiments the washed bacteria were adjusted to a concentration of 2 ± 10^9/mL with saline. Mice received 0.3 mL of E.coli (total dose = 0.6 x 10^9 bacteria) IP followed by IV injection of 1.6 mL of (a) 0.85% sterile saline, (b) Fluosol-DA, or (c) 27 mg/mL Pluronic F-68 in saline. Mortality was evaluated after 24 hours. In a preliminary peritonitis experiment, injection of 0.3 mL of bacteria IP, followed by infusion of 1.6 mL of the “annex solutions” used to reconstitute the Fluosol-DA resulted in a 24-hour mortality rate similar to that after infusion of 1.6 mL of 0.85% sterile saline (30% mortality, n = 10 each); hence, saline was used in the remaining experiments as the control infusion, and as the solvent for Pluronic F-68. The effect of the time of administration of Fluosol-DA and Pluronic F-68 on E. coli peritonitis mortality was examined in three (Pluronic F-68) or four (Fluosol-DA) groups of animals. Group 1 received 1.6 mL IV of saline or Fluosol-DA, or Pluronic F-68 (27 mg/mL), followed in two hours by E. coli (2 x 10^7/mL) IP. Group 2 received saline, or Fluosol DA or Pluronic, and IP E coli at the same time, and Groups 3 and 4 received IP E coli followed in two or four hours by IV saline, or Fluosol-DA, or Pluronic F-68 (see text). Mortality was evaluated 24 hours after injection of E. coli. Reticuloendothelial system clearance of colloidal carbon (CC) was measured in 2.5 kg New Zealand white rabbits as previously described with minor modification. Briefly, each animal was infused with an approximately 1/2 blood volume dose (55 mL/kg E. coli 2 x 10^7/mL with saline. Mice received 0.3 mL of E. coli (2 x 10^7/mL) IP. Group 2 received saline, or Fluosol DA or Pluronic, and IP E coli at the same time, and Groups 3 and 4 received IP E coli followed in two or four hours by IV saline, or Fluosol-DA, or Pluronic F-68 (see text). Mortality was evaluated 24 hours after injection of E. coli.

Peritonitis experiment, injection of 0.3 mL of bacteria IP, followed by infusion of 1.6 mL of the “annex solutions” used to reconstitute the Fluosol-DA resulted in a 24-hour mortality rate similar to that after infusion of 1.6 mL of 0.85% sterile saline (30% mortality, n = 10 each); hence, saline was used in the remaining experiments as the control infusion, and as the solvent for Pluronic F-68. The effect of the time of administration of Fluosol-DA and Pluronic F-68 on E. coli peritonitis mortality was examined in three (Pluronic F-68) or four (Fluosol-DA) groups of animals. Group 1 received 1.6 mL IV of saline or Fluosol-DA, or Pluronic F-68 (27 mg/mL), followed in two hours by E. coli (2 x 10^7/mL) IP. Group 2 received saline, or Fluosol DA or Pluronic, and IP E coli at the same time, and Groups 3 and 4 received IP E coli followed in two or four hours by IV saline, or Fluosol-DA, or Pluronic F-68 (see text). Mortality was evaluated 24 hours after injection of E. coli.

**RESULTS**

Preliminary experiments revealed that maximum neutrophil (PMN) influx into the peritoneal cavity of control mice given IP glycogen occurred between eight to 12 hours after installation of IP glycogen; consequently, lavage was performed eight hours after glycogen installation in the following experiments. As shown in Table 1, glycogen induced a nearly 7-fold increase in the number of PMN recoverable by peritoneal lavage in mice. The glycogen-induced influx of PMN was significantly blunted by simultaneous infusion of either Fluosol-DA or Pluronic F-68.

**Table 1. Effect of Fluosol DA and Pluronic F-68 on Intraperitoneal Accumulation of Neutrophils**

<table>
<thead>
<tr>
<th>Agents (IP/IV)</th>
<th>PMN Delivery x 10^9/mL</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline/saline</td>
<td>0.19 ± .03</td>
<td>20</td>
</tr>
<tr>
<td>Glycogen/saline</td>
<td>1.35 ± .14</td>
<td>14</td>
</tr>
<tr>
<td>Glycogen/Fluosol-DA</td>
<td>0.63 ± .12†</td>
<td>10</td>
</tr>
<tr>
<td>Glycogen/Pluronic</td>
<td>0.69 ± .07†</td>
<td>10</td>
</tr>
</tbody>
</table>

*P < .001 v saline/saline (two-tailed Student’s t test).
†P < .001 v glycogen/saline (two-tailed Student’s t test).

Having established that Fluosol-DA and its detergent component Pluronic F-68 both decreased the inflammatory response of PMN in the peritoneal cavity, we examined the effect of Fluosol-DA and Pluronic F-68 on mortality, due to E. coli peritonitis, in mice. In two experiments, summarized in Table 2, infusion of a 1/2 blood volume of either Fluosol-DA or Pluronic F-68 (27 mg/mL) at the time of bacterial injection IP resulted in significantly increased mortality after 24 hours, compared to the control animals who were given only saline. Infusion of a 1/2 blood volume of either Fluosol-DA or Pluronic F-68 in animals given no E. coli did not cause mortality (n = 10 each). We also determined that infusion of a 1/2 blood volume of Pluronic (27 mg/mL) did not alter the rate of RES clearance of colloidal carbon in rabbits (mean T/2 = 5.0 min, n = 2) compared to control rabbits (mean T/2 = 5.5 min, n = 3).

The effect of time of administration of Fluosol-DA or Pluronic F-68 on E. coli peritonitis mortality was studied. As shown in Table 3, in one experiment, Fluosol-DA infusion was associated with increased mortality when given prior to or simultaneously with bacteria. Animals injected with saline had insignificant differences in mortality at all time points. In two separate experiments, infusion of Pluronic F-68 was associated with increased mortality only when administered simultaneously with E. coli.

In additional experiments, we compared the effect of Pluronic F-68 and F-108 on neutrophil chemotaxis and adherence in vitro. As shown in Fig 1, Pluronic F-108 was a more potent inhibitor of neutrophil function than Pluronic F-68. In three such studies the mean concentrations of Pluronic F-108 required for 50% inhibition (ID_{50}) of neutrophil chemotaxis and adhesion were 2 ± 1 mmol/L and 21 ± 9 mmol/L respectively. The respective ID_{50} values for Pluronic F-68 were 34 ± 3 mmol/L and 203 ± 24 mmol/L. Finally we found that infusion of Pluronic F-108 (1/2 blood volume of 4.8 mg/mL Pluronic F-108—see Methods) also significantly increased mortality due to E. coli peritonitis in mice (saline

**Table 2. Effect of Fluosol DA and Pluronic F-68 on E. coli Peritonitis Mortality**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Alive (%)</th>
<th>Dead (%)</th>
<th>χ²</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td>14(32)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluosol-DA</td>
<td>8</td>
<td>24(75)</td>
<td>17.1</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Pluronic</td>
<td>6</td>
<td>17(74)</td>
<td>11.2</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

*Numbers refer to number of animals remaining alive or dead, 24 hours after injection of E. coli (see Methods).
†Two-tailed test.
INFECTION AND ARTIFICIAL BLOOD

Mortality refers to number of mice dead at 24 hours/total number of mice. ND, not done.

We showed that these effects occurred after administration of clinically relevant doses in humans.\(^5\) We further showed that, in serum-free media, Fluosol-DA did not disturb other cell functions, that the effect of Fluosol-DA on migration and adherence extended to other cell types (eg, macrophages) and nonhuman phagocytes, that these effects were rapidly reversible both in vitro and in vivo, and that they were due entirely to the detergent component of the artificial blood substitute, Pluronic F-68.\(^3\)

The above findings raised the possibility that any candidate blood substitute containing Pluronic F-68, or other pharmacologic preparations containing Pluronic F-68, or Pluronic F-68 alone, might have a similar deleterious effect on neutrophil migration in vivo. If so, we hypothesized that animals (or potentially humans) given these substances might be particularly susceptible to infection. We report here that infusion of Fluosol-DA, or a corresponding amount of Pluronic F-68 alone, used in doses similar to that which have been used in both animals and humans,\(^2,5\) significantly impaired the accumulation of PMN in the peritoneum after a standard inflammatory challenge. We further demonstrated that animals pretreated with Fluosol-DA or Pluronic F-68 suffered increased mortality due to experimental peritonitis. The association between Pluronic-induced impairment in PMN migration in vivo and increased infection mortality strongly supports a causal relationship between the two. Other investigators have reported that perfluorocarbon emulsions containing pluronic may interfere with other PMN functions in addition to migration.\(^4\) This may also have contributed to the excess mortality in our experiments. Studies in our laboratory did not reveal alterations in other PMN functions.\(^5\) These differences may have been due to variations in methodology, as previously discussed.\(^3\) It has been reported that Fluosol-DA also partially blocks the RES.\(^14,26\) Our finding that Pluronic F-68 infusion did not alter RES clearance indicates that RES blockage cannot explain the effect of Pluronic F-68 on infection mortality observed in these studies.

The increased infection mortality we observed after infusion of Pluronic occurred only when it was infused simultaneously with bacteria injection. In a separate experiment, infusion of Fluosol-DA increased infection mortality even when administered prior to E. coli. This difference may be due to the longer circulating half-life of Pluronic F-68 when given in the form of the Fluosol-DA emulsion (T/2 = 5.7 h) than when given free in solution (T/2 = 1.8 h).\(^1\) Direct comparison of the effect on mortality of infusion of Pluronic v Fluosol two hours prior to bacteria injection is mitigated by the marked difference in mortality of the two control groups. The reason for the failure of Pluronic F-68 or Fluosol-DA to augment infection mortality when given two to four hours after bacteria is unclear but is compatible with earlier observations that the outcome of a bacterial infection is determined within the first few hours of onset.\(^21\)

Pluronic F-68 is but one of many nonionic surfactants that are available for use in the food, drug, and cosmetic industry.\(^4,22\) We found that Pluronic F-108 was a more potent inhibitor of PMN function than F-68. Pluronic F-108 also increased infection mortality in mice. Thus the adverse effects of Pluronic F-68 on PMN function are not unique to

Table 3. Effect of Time of Infusion on Fluosol-DA and Pluronic F-68–Induced Augmentation of E. coli Peritonitis Mortality

<table>
<thead>
<tr>
<th>Time of Infusion</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
</tr>
<tr>
<td>−2 h</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>0</td>
<td>2/7 (29)</td>
</tr>
<tr>
<td>+2 h</td>
<td>2/8 (25)</td>
</tr>
<tr>
<td>+4 h</td>
<td>2/7 (29)</td>
</tr>
</tbody>
</table>

Time of infusion refers to number of hours by which saline, Fluosol-DA, or Pluronic F-68 IV infusion preceded or followed IP injection of E. coli.

\(\*P < .05\) (single tailed significance test), compared to saline group.

Fig 1. Effect of Pluronics F-68 and F-108 on granulocyte adherence (A) and chemotaxis (B). Data points represent mean ± SE of triplicate determinations in a typical experiment (see text). ○, F-68; ●, F-108.
this polymer, but may be a property common to most or all such agents. Further studies in our laboratory on additional Pluronics support this view.14

These studies have potential clinical implications. First, they suggest that the components of future candidate artificial blood substitutes should be carefully studied for inhibition of phagocyte function. Second, although Fluosol-D.A is no longer undergoing trials as a blood substitute, it is currently being investigated in humans as an ultrasound imaging agent15 and for a wide variety of conditions associated with tissue ischemia.3 We are aware of no reports of increased infection mortality in humans to whom Fluosol-D.A has been administered, and we do not advise discontinuation of the investigation of perfluorocarbon emulsions. However, our current studies, and a recent report of increased infection mortality after Fluosol exchange transfusion in an animal model similar to ours,24 support the view that patients who are infused with Pluronic-containing substances should be carefully observed. Finally, these studies suggest that (1) Pluronic F-68 may serve as a useful probe of neutrophil chemotactic function both in vitro and in vivo, and (2) this or a similar agent may potentially be a useful therapeutic agent to specifically and reversibly alter PMN function. Preliminary studies of the effect of Pluronic on a neutrophil-dependent model of acute lung injury support this hypothesis.15

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