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Phagocytic Activation of Human Neutrophils by the Detergent Component of Fluosol

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Fluosol (Alpha Therapeutic Corporation, Los Angeles, CA) an emulsion of perfluorocarbons with a high oxygen-carrying capacity, was approved as an adjunct to alleviate myocardial ischemia during coronary angioplasty. This drug also significantly enhances myocardial salvage presumably related to an action on the neutrophil. The mechanism by which fluosol and its individual components, including the detergent Pluronic F-68, affected neutrophil function was examined. During the incubation of neutrophils with fluosol, a rapid stimulation of superoxide anion production and degranulation which progressively increased over a 30-minute period was detected. Neutrophils incubated with only Pluronic F-68 produced similar amounts of superoxide anion. Cytochalasin B, an inhibitor of phagocytosis, significantly inhibited this superoxide anion generation. As shown previously, neutrophils incubated with fluosol for 30 minutes and then subsequently stimulated manifested a reduction in lysozyme release as compared with untreated cells. Results of an electron microscopic examination confirmed the cellular uptake of the fluosol within phagocytic vacuoles. Neutrophil viability determined by trypan blue was unaffected after fluosol treatment. These observations show that the fluosol emulsion, primarily through micelles formed by the detergent Pluronic F-68, activates human neutrophils by serving as a phagocytic stimulus, which produces a cell refractory to subsequent stimulation. (Am J Pathol 1992, 140:1081–1087)

Perfluorochemicals are substances with a small particle size, low viscosity, and high oxygen-carrying capacity.[1–3] Because of the insolubility of the perfluorocarbons, an emulsion including detergents and phospholipids is commonly formulated to affect miscibility of these compounds. Fluosol, a formulation of two perfluorochemicals containing egg yolk phospholipids and the Pluronic detergent F-68, was originally investigated in clinical settings as an alternative to blood transfusions.[2,4] Recently, this drug has been released for use as an oxygen carrier to reduce myocardial ischemia during percutaneous transluminal coronary angioplasty.[5,6] Experimental studies have demonstrated that both intracoronary and intravenous administration of fluosol significantly reduces infarct size and improves ventricular function in the canine preparation of regional ischemia.[7–9] Preliminary observations suggest that infusion of fluosol to achieve recanalization of the target artery in patients with acute anterior myocardial infarction significantly enhances regional ventricular function compared with angioplasty without fluosol.[10]

Numerous studies support a role for the neutrophil in mediating further cellular injury during the perireperfusion period after regional myocardial ischemia.[11–13] We have previously demonstrated that fluosol treatment in the canine model reduces the *in vivo* accumulation of neutrophils in the reperfused bed.[9] Neutrophil responses including chemotaxis and degranulation studied *ex vivo* from these animals were markedly suppressed.[7,9] The neutrophil plays an important role in the acute inflammatory response and the healing process after ischemia. Therefore, the mechanisms of the beneficial effects of fluosol must be understood before supporting expanded use of this drug as an adjunctive therapy with thrombolytics in patients with acute myocardial infarction. We examined the effects on human neutrophil superoxide anion responses and lysozyme degranulation of the complete fluosol emulsion, of its components, and of a

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new perfluorochemical preparation FMIQ (perfluoro N-methyldecahydroisoquinoline) which does not contain any detergent.

Materials and Methods

Superoxide dismutase (SOD), cytochrome C, formyl-Met-Leu-Phe (FMLP), cytochalasin B, and 0.4% trypan blue were obtained from Sigma Chemical Company, St. Louis, Missouri. Hanks' balanced salt solution (HBSS) was obtained from Gibco, Grand Island, New York. Fluosol and its components including perfluorodecalin, perfluorotripropylamine, Pluronic F-68, glycerol, and yolk phospholipids were provided by Alpha Therapeutic Corporation, Los Angeles, California. Since the Pluronic detergent also contained butylated hydroxytoluene (BHT) added as an antioxidant during the sterilization, purified F-68 was also obtained from Wyandotte Chemicals, Wyandotte, Michigan. No difference in neutrophil response was observed between the two preparations of the Pluronic. The fluosol percentage indicated in the experiments is the final dilution in the cell incubations of the stock solution made by the manufacturer's recommendation. The amount of components present in the fluosol stock solution includes 2.7% (W:V) Pluronic F-68, 0.8% glycerol, 0.4% phospholipids, 14% perfluorodecalin, and 6% perfluorotripropylamine. The fluosol emulsion and components were prepared on the day of the experiment and dilutions of this stock were made by adding HBSS. FMIQ, an investigational oxygen carrier containing a single perfluorochemical in a phospholipid emulsion with no detergent, was also provided by Alpha Therapeutic Corporation.

Neutrophil Suspensions

Human neutrophils were isolated from heparinized venous blood from healthy adult volunteers by Ficoll-Hypaque density gradient centrifugation, as previously described,[14] and suspended in HBSS at pH 7.4 and kept on ice until incubated.

Superoxide Radical Production

Superoxide radical anion production was assessed by the reduction of ferricytochrome C using 5×10^6 neutrophils/ml. Initially, 500 μl of neutrophils were warmed to 37°C over a 5-minute preincubation. Each treated sample had a corresponding control in which 100 μl of superoxide dismutase was added after the 5-minute preincubation period to prevent reduction of ferricytochrome C. In samples without superoxide dismutase, 100 μl HBSS was added to give equivalent dilutions. After the addition of 25 μl of cytochrome C, 400 μl of fluosol was added to the cells that were treated or untreated with superoxide dismutase to give a final concentration of 1%, 5%, 10%, or 50%. Each treated sample had a corresponding control that received an identical volume of HBSS. For the time courses, only a single concentration was examined compared with its superoxide dismutase control in a single experiment. At the end of each incubation period with fluosol, further cytochrome C reduction was halted by immediately placing the samples on ice. Reduction of cytochrome C by superoxide anion was measured by spectrophotometry at 550 nm wavelength.[9] Additional studies were completed with a 10% concentration of FMIQ and 10% concentrations of the various components of the fluosol emulsion. To address the mechanism of action of fluosol on superoxide anion release, further experiments were done in which cytochalasin B (10^{-5} mol/l), an inhibitor of phagocytosis, was added at the beginning of the initial 5-minute warming period. Each experimental time point was done in triplicate, and the mean difference was calculated by subtracting the average of the corresponding triplicates pretreated with superoxide dismutase. All cell preparations were determined not to be spontaneously activated since no difference in the absorbance occurred over a 60-minute period in cells that were treated or untreated with superoxide dismutase.

Degranulation

Neutrophil degranulation was assessed by assaying the activity of released lysozyme by lysis of *Micrococcus lysodeikticus*.[9] Neutrophils (500 μl) were warmed to 37°C over a 5-minute preincubation. The cells were then exposed over a 60-minute period to an equal volume of 10% fluosol, 10% concentration of detergent component, Pluronic F-68, or 10% FMIQ. Each treated sample had a corresponding control that received an identical volume of HBSS. In other studies, neutrophils (500 μl) were preincubated with an equal volume of a 50% concentration of fluosol or HBSS for 30 minutes. After the incubation period, the neutrophils were washed in HBSS and subsequently stimulated with FMLP for 60 minutes. All samples were centrifuged at 4°C, and lysozyme activity in the supernatant was measured by its ability to lyse *Micrococcus lysodeikticus* as measured spectrophotometrically at 450 nm.

Neutrophil Viability

Neutrophils were incubated with fluosol at the indicated dilutions for 30 minutes. A drop of cells was mixed with a

drop of trypan blue and examined under a coverslip at a magnification of 400×. Each dilution of fluosol was examined in at least three separate preparations of cells. Viability was assessed by examination of 300 cells in each preparation for nuclear staining.

Electron Microscopic Examination

Cells were allowed to incubate for 60 minutes at 37°C with and without an equal volume of fluosol. They were centrifuged, the supernatant was removed, and the cell pellet was fixed with 3% buffered glutaraldehyde and embedded in Epon. Ultrathin sections were cut and stained with uranyl acetate lead citrate, and examined with a Zeiss 109 IGF electron microscope. The number of cells that contained fluosol micelles was quantitated by counting 50 cells randomly.

Statistical Analysis

Each experiment was performed on three to five occasions on separate days using different donor cells. Only one treatment condition was examined compared with its control on a given day. Within each experiment, triplicate treated and corresponding control determinations were completed for each time point, and the values averaged before statistical analysis. Data are expressed as the mean ± standard error of the mean (SEM) of the average value of the triplicates greater than the corresponding control values in each experiment. Significance ($P < 0.05$) was determined by a paired *t*-test comparing the treated with untreated cells within each experiment.

Results

No superoxide radical generation or lysozyme release was detected in neutrophils incubated with HBSS over 60 minutes. As noted previously, neutrophils exposed to fluosol for 30 minutes, washed, and subsequently stimulated with FMLP showed a 67 ± 5% reduction in lysozyme as compared with those cells that were incubated with HBSS alone.[15] In contrast, a rapid and significant generation of superoxide anion was observed from neutrophils incubated with 1% to 50% fluosol (Figure 1). The amount of superoxide anion released plateaued at 30 minutes for all dilutions of fluosol. A significant enhancement of lysozyme release after 15 minutes incubation was also seen with fluosol (Figure 2).

The individual constituents of fluosol were investigated to determine if one could account for the superoxide anion generation from neutrophils. The phospholipids and Pluronic F-68 were found to produce significant superoxide anion release from neutrophils (Figure 3a). The phospholipid component, however, contributed only a small part to the total of the superoxide anion production compared with fluosol, whereas stimulation by the Pluronic detergent F-68 alone equalled that of the entire emulsion. The perfluorochemical preparation FMIQ, which contained only phospholipids and no detergent, produced a minimal stimulation of superoxide anion production equivalent to the amount of the phospholipid component alone. Determination of lysozyme release af-

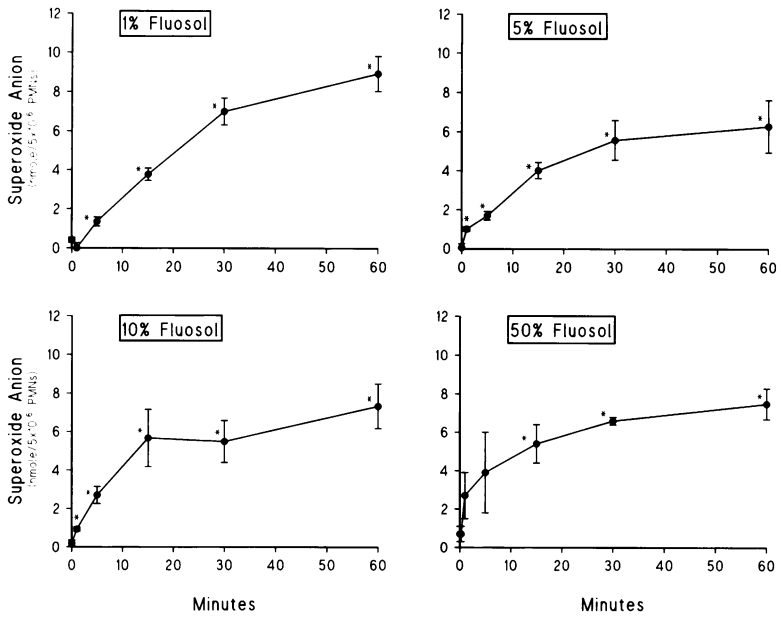

Figure 1. *Effect of fluosol on superoxide anion generation by unstimulated human neutrophils. The amount of superoxide anion is shown at various time points throughout a 60-minute incubation period with 1%, 5%, 10%, or 50% fluosol,* *$P < 0.05$. *Note that the fluosol enhanced release of superoxide anion within 10 minutes of incubation.*

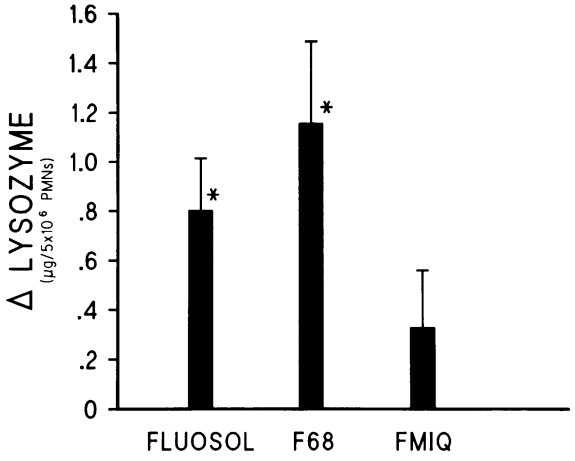

Figure 2. *Degranulation of neutrophils by a 10% dilution of fluosol, Pluronic F-68, or FMIQ. The bars represent the increased release of lysozyme more than untreated neutrophil incubations at 15 minutes,* *$P < 0.05$.

ter 15 minutes incubation also showed that fluosol and Pluronic F-68 significantly enhanced degranulation, whereas no effect was noted with FMIQ (Figure 2).

Both the detergent Pluronic F-68 and phospholipids are present several hundred fold greater than their critical micelle concentrations for dissolution of the dense perfluorocarbons of fluosol. The micelles formed in the emulsion measure up to 0.6 microns in diameter. To investigate whether superoxide anion generation resulted from phagocytosis of the fluosol micelles, we preincubated the neutrophils with cytochalasin B, which disrupts microfilaments, to inhibit phagocytosis before exposure to fluosol. Neutrophils incubated with cytochalasin B showed a significant reduction in the amount of superoxide anion produced for a 30-minute period with all concentrations of fluosol (mean reduction, 64 ± 4%) (Figure 3b). Cytochalasin B significantly reduced the superoxide production to the Pluronic F-68 by 98 ± 1%. To show that the inhibition by cytochalasin B was not due to generalized inhibition in the same preparation of neutrophils, cytochalasin B pretreatment significantly enhanced the amount of superoxide anion generation by 53 ± 3% in response to FMLP (10 μmol/l) which is reported to occur for receptor coupled stimulation of the neutrophil.

The viability of the neutrophils was examined after a 30-minute exposure to fluosol and was not different from the control cells. Neutrophil viability assessed by trypan blue exclusion was observed in 97% of untreated neutrophils and in the preparations treated with 1, 5, 10, and 50% fluosol, 96, 97, 96, and 96%, respectively.

Neutrophils were incubated with and without an equal volume of fluosol for 60 minutes and examined by electron microscopy for evidence of phagocytosis. Neutrophils containing fluosol particles in phagocytic vacuoles are shown in a representative electron micrograph in Figure 4, and this was present in 30% of the cells that were counted.

Discussion

Fluosol has been released as an adjunct therapy to improve oxygen delivery during percutaneous transluminal coronary balloon angioplasty.[5,6] This perfluorochemical preparation has also been shown to significantly reduce infarct size in a canine model of regional ischemia.[7–9] Data from these studies show that intravenous infusion of a low dose of fluosol during the early reperfusion period substantially reduces infarct size and improves regional ventricular function.[7] Adjunctive therapy with fluosol after emergent coronary angioplasty has also been shown to improve regional ventricular function in patients with anterior myocardial infarction.[10] As the oxygen-carrying ability alone of fluosol did not seem to provide an adequate explanation for the striking beneficial effects observed in reperfusion injury, alternative mechanisms of action were investigated.

Neutrophils are known to play an important role in the acute inflammatory response.[16–18] However, numerous studies suggest that these cells may produce further cellular injury after reperfusion of previously ischemic myocardium.[19–21] Activated neutrophils produce a variety of cytotoxic substances, including superoxide anion and proteolytic enzymes, which could damage potentially viable myocardial and endothelial cells during the early reperfusion period.[22,23] This exaggerated inflammatory response has been postulated to play an important role in the pathogenesis of myocardial reperfusion injury.

In our previous studies, we observed that fluosol substantially reduced neutrophil influx into canine myocardial reperfused tissue.[9] Furthermore, in *ex vivo* studies, neutrophils obtained after fluosol infusion showed suppression of various neutrophil functions, such as chemotaxis and lysozyme release.[7,9] In these studies, neutrophils were preincubated with fluosol either *in vitro* or *in vivo*.[7,9,14,15] These neutrophils were subsequently washed and stimulated before measurement of functional responses. Superoxide anion release, degranulation, and chemotaxis were markedly decreased in the neutrophils that were pretreated with fluosol.[7,9,14,15] These observations, along with previous ischemic dog model studies, led to the initial hypothesis that fluosol may suppress neutrophil function.

Since the neutrophil plays a pivotal role in reperfusion injury as well as subsequent healing, we investigated the mechanism of action of fluosol on neutrophil function. In this current study, we initially examined the effects of fluosol on unstimulated neutrophils during various periods of incubation with the drug. Surprisingly, fluosol was found to stimulate neutrophils *per se*. After 5 minutes of

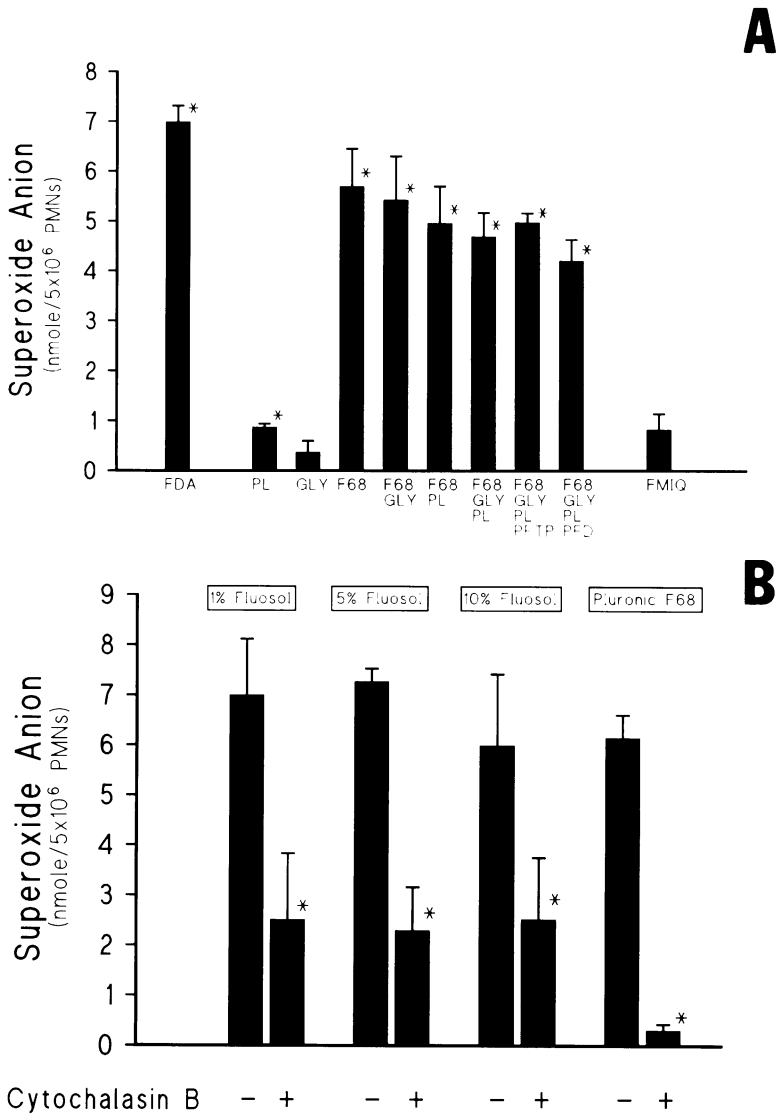

Figure 3. A: *Superoxide anion generation in response to the components of fluosol. The bars show the amount of superoxide anion released from neutrophils at the end of a 30-minute incubation period with 10% fluosol or its various components. FDA = fluosol; F-68 = Pluronic F-68; GLY = glycerol; PL = egg yolk phospholipids; PFD = perfluorodecalin; PFTP = perfluorotripropylamine. The amount of each component added was equivalent to the amount present in a 10% fluosol solution (see Material and Methods for concentrations of each component contained in fluosol). The perfluorocarbon preparations could not be tested directly because they did not dissolve in the cell buffer due to their high density. The superoxide anion production to a new perfluorocarbon preparation FMIQ at a 10% dilution is also shown.* B: *Effect of cytochalasin B on superoxide anion generation by fluosol administration. 1%, 5%, or 10% fluosol or Pluronic F-68 were incubated with neutrophils that either had been preincubated with or without cytochalasin B. The bars illustrate the amount of superoxide anion release from neutrophils incubated with the various concentrations of fluosol or F-68 (the equivalent amount in a 10% fluosol dilution) for a 30-minute time period in the presence or absence of cytochalasin B,* $^*P < 0.05$.

incubation with a 1% concentration of fluosol (which is lower than levels obtained during *in vivo* studies demonstrating myocardial salvage), neutrophils released a significant amount of superoxide anion. This release plateaued at 30 minutes of incubation. Similarly, fluosol was found to stimulate degranulation. These observations demonstrate that neutrophil functions may not be suppressed by fluosol, but rather directly activated by it predominantly through phagocytosis. This is supported by the observation that cytochalasin B, an inhibitor of phagocytosis, significantly attenuates superoxide anion release with various concentrations of fluosol. Furthermore, it appears in our study that the detergent F-68 is the component of fluosol by its formation of micelles which acts as the phagocytic stimulus to activate superoxide anion production. This action is distinct from the known activity of some detergents to activate NADPH (nicotinamide adenine dinucleotide phosphate, reduced) oxidase.[24]

As in the *in vivo* studies, we obtained histologic evidence for the phagocytosis of fluosol *in vitro*.[7,9] Active uptake of the fluosol occurred without evidence of toxicity as trypan blue exclusion remained high. Thus, in previous studies from our laboratory, preincubation with fluosol may have caused the neutrophils to be activated, generating superoxide anion and undergoing degranulation, before addition of the stimulant.[9,14,15] To confirm this hypothesis, we performed additional experiments. Neutrophils that had been preincubated with 50% concentration of fluosol for 30 minutes showed a 67 ± 5% reduction in lysozyme degranulation when stimulated with the chemoattractant FMLP as compared with control cells. This compares favorably with prior *in vitro* studies by Virmani et al who showed a 66% reduction in superoxide release when neutrophils were incubated over the same period.[15] These pretreated neutrophils would be subsequently refractory to additional activation and may

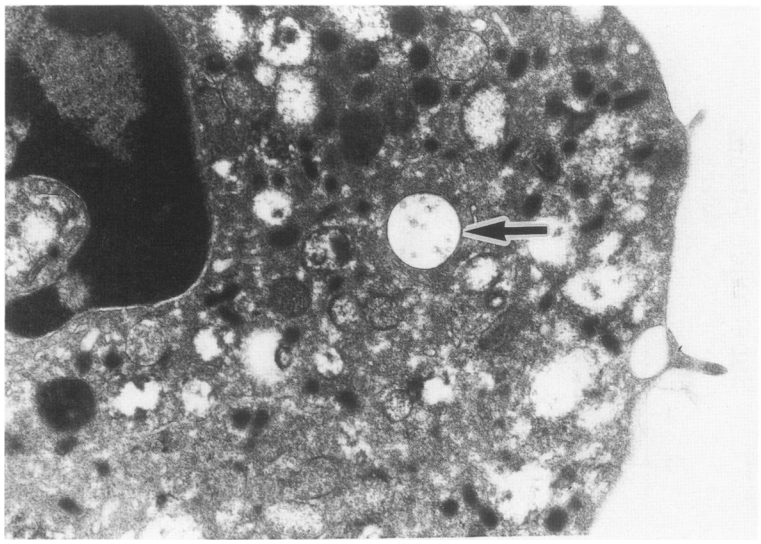

Figure 4. *Electron photomicrograph of neutrophil exposed to fluosol for 30 minutes showing phagocytosed fluosol particles within membrane bound vesicles. Note large vacuole (arrow) with small fluosol particles in it, (magnification ×18,000).*

account for the previously reported "suppression" of neutrophil function by fluosol.

This study demonstrates that fluosol, even in low concentrations, significantly enhances neutrophil superoxide anion production *in vitro*. Uncontrolled neutrophil activation in injured tissue has been implicated in the pathogenesis of numerous clinical processes, including myocardial reperfusion injury. Peripheral activation of neutrophils would result in the release of cytotoxic mediators throughout the circulation with subsequent inactivation via protective circulating enzymes such as superoxide dismutase. Activation by a phagocytic stimulus has the additional advantage of distributing cytotoxic lysozomal contents to a nonreleasable pool located in the phagosome within the cell. Furthermore, activation of these cells would result in the removal from the systemic circulation before reaching the coronary vascular bed during the critical period of reperfusion. These effects may contribute to the enhanced myocardial salvage previously observed, although further *in vivo* studies are required to confirm other potential beneficial effects of fluosol such as oxygen delivery and antiplatelet effects.

However, prolonged suppression of immune responses could have deleterious effects on infarct healing and ventricular function such as observed with corticosteroids.[25,26] In previous studies, we have shown that fluosol does not alter infarct healing or impair ventricular function since its effects on neutrophils are probably transient due to the short plasma half-life (8 hours).[8,27,28] One potential adverse effect of fluosol is the prolonged organ half-life due to retention of the emulsion in the reticuloendothelial system producing potential hepatotoxicity.[29] The demonstration that the detergent component Pluronic F-68 is the component of the emulsion that activates superoxide anion generation suggests that it may be a clinically more appealing drug since it undergoes rapid renal clearance.

This study suggests a possible approach for the treatment of various disease processes in which indiscriminate neutrophil-mediated cellular injury at the site of tissue damage may play an important role in their pathogenesis. Other approaches such as anti-inflammatory agents, neutrophil antibodies, and cobra venom factor have been used to inhibit or deplete the function of neutrophils in an attempt to limit reperfusion injury.[11–13,30] However, these strategies either may not totally abolish all of the potential cytotoxic effects of neutrophils or still leave a critical mass of circulating neutrophils. The development of agents that cause persistent activation of neutrophils throughout the peripheral circulation may prove to be an effective mechanism for reducing neutrophil-mediated injury after reperfusion of the ischemic myocardium.

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References

1. Geyer RP: Fluorocarbon-polyol artificial blood substitutes. N Engl J Med 1973, 289:1077–1082
2. Mitsuno T, Ohyanagi H, Naito R: Clinical studies of a perfluorochemical whole blood substitute (Fluosol-DA). Summary of 186 cases. Ann Surg 1982, 195:60–69
3. Virmani R, Kolodgie F, Osmialowski A, Zimmerman P, Mergner W, Forman M: Myocardial protection by perfluoro-

chemical infusion during transient ischemia produced by balloon coronary occlusion. Am Heart J 1988, 116:421–431
4. Tremper KK, Friedman AE, Levine EM, Lapin R, Camarillo D: The preoperative treatment of severely anemic patients with a perfluorochemical oxygen-transport fluid, Fluosol-DA. N Engl J Med 1982, 307:277–283
5. Cleman M, Jaffe CC, Wohlgelernter D: Prevention of ischemia during percutaneous transluminal coronary angioplasty by transcatheter infusion of oxygenated Fluosol-DA 20%. Circulation 1986, 74:555–562
6. Kent KM, Cleman MW, Cowley MJ, Forman MB, Jaffe C, Kaplan M, King SB, Krucoff MW, Lassar T, McAuley B, Smith R, Wisdom C, Wohlgelemter D: Reduction of myocardial ischemia during percutaneous coronary angioplasty with oxygenated Fluosol R. Am J Cardiol 1990, 66:279–284
7. Forman MB, Pitarys CJ, Vildibill HD, Lambert T, Ingram DA, Virmani R, Murray JJ: Pharmacologic perturbation of neutrophils by Fluosol-DA results in a sustained reduction in infarct size in the canine model of reperfusion. J Am Coll Cardiol 1992, 19:205–216
8. Forman MB, Puett DW, Wilson BH, Friesinger GC, Virmani R: Beneficial long-term effects of intracoronary perfluorochemical on infarct size and ventricular function in a canine reperfusion model. J Am Coll Cardiol 1987, 95:1082–1090
9. Bajaj AK, Cobb MA, Virmani R, Gay JC, Light RT, Forman MB: Limitation of myocardial reperfusion injury by intravenous perfluorochemicals: role of neutrophil activation. Circulation 1989, 79:645–656
10. Forman MB, Perry JM, Wilson BH, Verani MS, Kaplan P, Shawl FA, Friesinger GC: Demonstration of myocardial reperfusion injury in man: results of a pilot study utilizing acute angioplasty with perfluorochemical in anterior myocardial infarction. J Am Coll Cardiol 1991, 18:911–918
11. Romson JL, Hook BG, Kunkel SL, Abrams GD, Schort MA, Lucchesi BR: Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. Circulation 1983, 67:1016–1023
12. Mullane KM, Salmon JA, Kraemer R: Leukocyte-derived metabolites of arachidonic acid in ischemia-induced myocardial injury. Fed Proc 1987, 46:2422–2433
13. Simpson PJ, Todd RF, III, Mickelson JK, Fantone JC, Gallagher KP, Lee KA, Tamura Y, Cronin M, Lucchesi BR: Sustained limitation of myocardial reperfusion injury by a monoclonal antibody that alters leukocyte function. Circulation 1990, 81:226–237
14. Babbitt DG, Forman MB, Jones R, Bajaj AK, Hoover RL: Prevention of neutrophil-mediated injury to endothelial cells by perfluorochemical. Am J Pathol 1990, 136:451–459
15. Virmani R, Fink LM, Gunter K, English D: Effect of perfluorochemical blood substitutes on human neutrophil function. Transfusion 1984, 24:343–347
16. Sommers HM, Jennings RB: Experimental acute myocardial infarction. Histologic and histochemical studies of early myocardial infarcts induced by temporary or permanent occlusion of a coronary artery. Lab Invest 1964, 13:1491–1503
17. Chatelain P, Latour J, Tran D, De Lorgeil M, Dupras G, Bourassa M: Neutrophil accumulation in experimental myocardial infarcts: relation with extent of injury and effect of reperfusion. Circulation 1987, 75:1083–1090
18. Go LO, Murry CE, Richard VJ, Weischedel GR, Jennings RB, Reimer KA: Myocardial neutrophil accumulation during reperfusion after reversible or irreversible ischemic injury. Am J Physiol 1988, 255:H1188–H1198
19. Braunwald E, Kloner RA: Myocardial reperfusion: A double-edged sword? J Clin Invest 1985, 76:1713–1719
20. Forman MB, Puett DW, Virmani R: Endothelial and myocardial injury during ischemia and reperfusion. Pathogenesis and therapeutic implications. J Am Coll Cardiol 1989, 13:450–459
21. Engler RL, Schmid-Schoenbein GW, Pavelec RS: Leukocyte capillary plugging in myocardial ischemia and reperfusion in the dog. Am J Pathol 1983, 111:98–111
22. Fantone JC, Ward PA: Polymorphonuclear leukocyte-mediated cell and tissue injury: oxygen metabolites and their relation to human disease. Hum Pathol 1985, 16:973–978
23. Wright DG, Gallin JI: Secretory responses of human neutrophils: exocytosis of specific (secondary) granules by human neutrophils during adherence *in vitro* and during exudation *in vivo*. J Immunol 1979, 123:285–294
24. Cohen HJ, Chovaniec ME: Superoxide production by digitonin-stimulated guinea pig granulocytes. The effects of n-ethyl maleimide cations, and glycolytic and mitochondrial inhibtors on the activation of the superoxide generating system. J Clin Invest 1978, 61:1088–1096
25. Kloner RA, Fishbein MC, Lew H, Maroko PR, Braunwald E: Mummification of the infarcted myocardium by high dose corticosteroids. Circulation 1978, 57:56–63
26. Maclean D, Maroko PR, Fishbein MC, Braunwald E: Effects of corticosteriods on myocardial infarct size and healing following experimental coronary occlusion. Am J Cardiol 1977, 39:280
27. Virmani R, Kolodgie FD, Osmilowski A, Forman MB: Effect of perfluorochemical Fluosol-DA on myocardial infarct healing in the rabbit. Am J Cardiovasc Pathol 1990, 3:69–80
28. Naito R, Yokoyama K: Perfluorochemical blood substitutes. Fluosol FC-43 emulsion, Fluosol-DA 20% and 35%. The Green Cross Corporation Technical Information Series No. 5. 1978, 20–23
29. Latz J, Metzenauer P: Effects of potential blood substitutes (perfluorochemicals) on rat liver and spleen. Pflugers Arch 1980, 387:175–181
30. Maroko PR, Carpenter CB, Chiariello M, Fishbein MC, Radvany P, Knostman JD, Hale SL: Reduction by cobra venom factor of myocardial necrosis after coronary artery occlusion. J Clin Invest 1978, 61:661–670