Original Article

Perflubron Emulsion Does Not Cause Neutrophil (PMN) Activation In-Vivo.

Donald S. Wilson, III, BS; Jason Y. Hokama, MS; George Lai, BS; Grace F. Gorman, BS; Nancy J. Susa; Jack G. Copeland, MD; Paul F. McDonagh, PhD

Section of Cardiovascular and Thoracic Surgery, Department of Surgery, University of Arizona, Tucson, Arizona

Keywords: perfluorocarbons, oxygen carriers, leukocytes, CD11b adhesion molecules, reactive oxygen species

Presented at the American Society of Extra-Corporeal Technology 35th International Conference, April 3-6, 1997, Phoenix, Arizona, by Donald Wilson, winner of the 1997 Scientific Presentation Award.

ABSTRACT

Earlier, we reported that in-vitro incubation of blood for ten minutes with the perfluorocarbon (PFC) emulsion Fluosol increased leukocyte activation as determined by adhesion to nylon fiber. In this study, we examined if in-vivo treatment with these PFC emulsions affected the expression of the leukocyte adhesion protein CD11b (primarily found on PMNs) and the generation of leukocyte-derived reactive oxygen species (ROS, oxygen free radicals). Rats were anesthetized and catheterized. Three groups were studied: 1) a phosphate buffered saline (PBS) control group (n=6), 2) a group treated with Fluosol emulsion (1.08g PFC/kg, n=6) and 3) a group treated with perflubron emulsion (1.08g PFC/kg, n=6). Blood samples were taken before and 10, 20, 40 and 60 minutes after treatment for hematology and analysis of PMN CD11b expression and ROS production using flow cytometry. We found that Fluosol caused significant increases in both neutrophil surface expression of CD11b and ROS generation ($p<0.05$, ANOVA). In the Fluosol group, the peak responses in PMN CD11b expression and ROS production were observed ten minutes after treatment. In contrast, treatment with perflubron emulsion did not cause a significant increase in CD11b expression nor an increase in ROS production at any time after treatment. These findings suggest that Fluosol causes a transient PMN activation in-vivo. The activation of circulating PMNs, in-vivo, is sufficient to significantly enhance oxygen derived free radical production. The lack of a PMN response to perflubron emulsion in-vivo suggests that this agent is not likely to induce a leukocyte-mediated inflammatory response.

Address correspondence to:
Paul McDonagh, PhD
Section of Cardiovascular and Thoracic Surgery
University of Arizona
P.O. Box 245071
Tucson, AZ 85724

INTRODUCTION

Due to their oxygen solubility properties, perfluorocarbon emulsions were developed for use as oxygen carrying solutions to serve as temporary blood substitutes (1,2). Perfluorocarbons (PFCs) are compounds with cyclic or straight chain carbon backbones and have complete replacement of hydrogen with fluorine. Perfluorocarbons are insoluble in blood and require an emulsification process to increase solubility. Perfluorocarbon emulsions may be useful in the settings of cardiac surgery and extracorporeal circulation for several reasons. One PFC emulsion, Fluosol-DA, has been reported to have cardioprotective properties, limiting myocardial ischemia-reperfusion injury (3,4,5,6). The beneficial effect appears to be independent of Fluosol's oxygen carrying capacity, low viscosity and small particle size (0.1-0.3 um) (7,8). A mechanism proposed to explain the cardioprotective effects of Fluosol is modulation of leukocyte activation, limiting the leukocyte contribution to ischemia-reperfusion injury (3,9,10). Some studies indicate that Fluosol provides cardioprotection by inhibiting neutrophil function (7), including neutrophil chemotaxis, phagocytosis and superoxide radical production (11,12). This agent may also interfere with neutrophil expression of adhesion molecules limiting adherence to endothelial cells and decreasing the neutrophil's involvement in the acute inflammatory response during reperfusion (11).

In follow-up studies, it was later learned that, rather than inhibit neutrophil activation, Fluosol activates neutrophils (10,13). A different hypothesis was then proposed to explain the observed cardioprotective effects of Fluosol during cardiac surgery and extracorporeal circulation. That is, Fluosol may possibly protect the cardiac tissue by causing a release of damaging oxygen free radicals and proteases from the circulating neutrophils, while the heart is crossclamped. The neutrophils would then be in a "refractory" state when the aortic crossclamp is released and the heart is reperfused, limiting the neutrophil contribution to reperfusion injury (9,13). Another hypothesis proposed to explain how activating neutrophils could be cardioprotective is that Fluosol-induced activation may cause neutrophils to marginate in other vascular beds, thereby "distracting" the neutrophils from participating in myocardial reperfusion injury following crossclamp release (13). It is possible that the cardioprotective effects of Fluosol are via modulating neutrophil function, but the exact mechanism(s) by which Fluosol provides cardiac protection from myocardial ischemia-reperfusion injury remains unclear.

The initial step in the neutrophil contribution to myocardial ischemia-reperfusion injury is leukocyte deposition in the microcirculation of the heart (14,15). Following neutrophil activation there is an increase in adhesion molecule expression and neutrophil adherence. The neutrophil's surface membrane expresses a glycoprotein (CD11b) associated with neutrophil adhesion. In the presence of a chemotactic stimulator, such as formyl-methionyl-leucyl-phenylalanine (fMLP), CD11b expression is upregulated (16). Previous reports have confirmed two to ten fold increases of CD11b over resting values following neutrophil stimulation (17,18). Neutrophils also respond to stimulation by generating an oxidative burst, whereby reactive oxygen species (ROS) are produced. Release of significant ROS by the neutrophil can cause oxygen radical-mediated injury to the tissues (19).

The purpose of this study was to compare the effects of two perfluorocarbon emulsions, on leukocyte function and activation in-vivo. We compared the white blood cell response to a first generation agent, Fluosol-DA[a] and a second generation agent, perflubron emulsion[b]. In a previous study, we compared the effects of Fluosol and perflubron emulsion on leukocyte adhesion in-vitro, finding that Fluosol had a direct stimulatory effect on leukocyte function (20). Fluosol increased leukocyte adhesion while perflubron emulsion had no effect on granulocyte adhesion compared to a phosphate buffered saline (PBS) control. The effects of PFC emulsions on neutrophil function and activation in-vivo are less clear. As mentioned, earlier studies found that in-vivo administration of Fluosol either suppressed neutrophil function (3,12) or activated neutrophils (10,21,22). Fewer studies have been performed to determine the effects of second generation PFC emulsions on neutrophil function in-vivo. In this study, we found that intravascular administration of Fluosol caused a transient neutrophil activation, as demonstrated by significant increases in both the expression of the neutrophil adhesion protein CD11b and the production of oxygen free radicals. Administration of perflubron emulsion, on the other hand, did not cause neutrophil activation.

MATERIALS AND METHODS

Experimental Protocol. All animal experiments in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals (23). Adult Sprague-Dawley rats (400-600g), were anesthetized with sodium pentobarbital (50mg/kg, IP). The animals were placed in a supine position on a Deltaphase Isothermal Pad[c]. The femoral artery was cannulated and connected to a blood pressure transducer[d] for measuring arterial blood pressure and heart rate. The rectal temperature was monitored with a telethermometer and body temperature was maintained with the isothermal pad.

Baseline, control measurements of heart rate, blood pressure and body temperature were collected and a one milliliter blood sample was taken for arterial blood gas analysis, hematology and flow cytometry. Then phosphate buffer saline (PBS, 5.4ml/kg, n=6), or Fluosol (1.08g/kg, n=6), or perflubron emulsion (1.08g/kg, n=6) was infused through the arterial cannula. Fluosol and perflubron emulsions have different perfluorocarbon

a Green Cross, Osaka, Japan
b Alliance Pharmaceutical Corp., San Diego, CA
c Deltaphase, Braintree Scientific, Braintree, MA
d DTX Model TNF-R, Ohmeda Medical, Madison, WI

Table 1: Heart rate, MAP, and arterial blood gases for the PBS, Fluosol and perflubron treated groups

	PRE			POST 20min			POST 60min		
	PBS	Fluosol	Perflubron	PBS	Fluosol	Perflubron	PBS	Fluosol	Perflubron
Heart Rate	347±10	317±22	335±13	318±12	355±22*	295±5	325±18	340±24	317±9
MAP	127±4	132±8	140±4	101±11	103±10	94±5	103±11	111±11	84±6
pH	7.34±0.01	7.31±0.01	7.36±0.02	7.37±0.01	7.33±0.02	7.37±0.01	7.41±0.02	7.36±0.01	7.39±0.01
PCO_2	46.6±2.3	48.9±2.1	44.3±2.1	44.6±1.8	48.4±3.6	42.2±1.0	36.9±2.5	42.2±0.9	39.1±1.3
PO_2	71.7±4.1	61.4±7.7	74.5±2.2	71.1±2.7	68.2±4.8	73.0±2.3	77.7±5.8	75.6±2.3	78.1±1.0

*Fluosol significantly different from the perflubron group($P<0.05$)

concentrations (20% vs. 60% w/v, respectively), thus the emulsion doses were adjusted to deliver the same PFC dose of 1.08g PFC/kg. Following treatment, arterial blood pressure, heart rate and body temperature were measured continuously for one hour. At 10, 20, 40 and 60 minutes after agent administration, blood samples were taken for blood gas analysis, hematology, and flow cytometry. Following the 60 min measurements, 7 ml of blood were withdrawn into a heparinized syringe for the in-vitro leukocyte adhesion assay.

Blood Gases and Hematology. Arterial blood gases (pH, pCO_2, PO_2) were measured in a blood gas analyzer[e]. Blood cell counts (total white cells, granulocytes, lymphocytes, platelets, hematocrit) were made using an automated hematology analyzer[f].

Expression of CD11b and ROS Production. DCFH-DA (2'7'-dichlorofluorescin diacetate) is a fluorescent probe used to quantify intracellular ROS production. DCFH-DA diffuses into the neutrophil's cytoplasm where it is enzymatically deacetylated to nonfluorescent 2'7'-dichlorofluorescin (DCFH), trapping it within the cell. In the presence of ROS, DCFH is oxidized to the fluorescent compound dichlorofluorescein (DCF). DCF fluorescence can be quantified using flow cytometry analysis (24,25). At each time point, blood samples were collected for analysis of the neutrophil's CD11b adhesion molecule surface expression (26) and the ROS production. Blood samples were first incubated with LDS-751 (1.0 ug/ml), a fluorescent nuclear stain, at 37°C for 10 minutes. LDS-751 staining allows the separation of fluorescent leukocytes (nucleated) from non-fluorescent erythrocytes (non-nucleated) when using flow cytometry (27). The whole blood and LDS mixture samples were divided into five 250 ul aliquots for flow cytometry analysis: 1) PBS, 2) anti-CD11b/FITC mAb (5.0 ug/ml), 3) anti-CD11b/FITC mAb and fMLP (10-7M), 4) DCFH-DA (80uM) or 5) DCFH-DA and fMLP.

Blood samples treated with anti-CD11b mAb or DCFH-DA were incubated for 15 minutes at 37°C, followed by incubation with either fMLP (10-7M) or PBS for 10 minutes at 37°C. The samples were then diluted with 4 ml PBS (4°C) and placed on ice until analysis. Measurement of anti-CD11b/FITC and DCF fluorescence was performed using flow cytometry[g]. Neutrophils and lymphocytes were identified on the basis of their size (forward scatter) and granularity (side scatter). Back-gating was used to focus on the sub-population of granulocytes (26). The neutrophils CD11b surface expression and ROS production were expressed as the total fluorescence intensity (TFI) which was the product of mean channel fluorescence and percent positive events of the fluorescent probes.

Leukocyte Adhesion Assay. The leukocyte adhesion assay was described by us previously (20,28). Briefly, 40 mg of scrubbed nylon fiber was packed to the 0.2 ml mark of 1 ml tuberculin syringe barrels. A 25 gauge needle was attached to the syringe barrel and the barrels were suspended over 2 ml collecting tubes. In a 37°C warm room, 0.9 ml of blood was pipetted onto each column and the effluents were collected for ten minutes. Blood cell counts (total white cells, red blood cells, platelets) were performed of the blood that was applied to the columns, as well as the blood effluent from the columns. Manual differential blood cell counts were made for all blood samples. The percent WBC adherence is calculated as: $1-(WBC_{post}/WBC_{pre})$, where *pre* is the blood sample applied to the column and *post* is the effluent from the column. For each animal, at least three columns were run for each blood-PFC mixture evaluated.

e Model ABL-330, Radiometer, LaVerne, CA
f Model 9018, Serono Diagnostics, Allentown, PA
g FACScan Flow Cytometer, Becton Dickenson, San Jose, CA

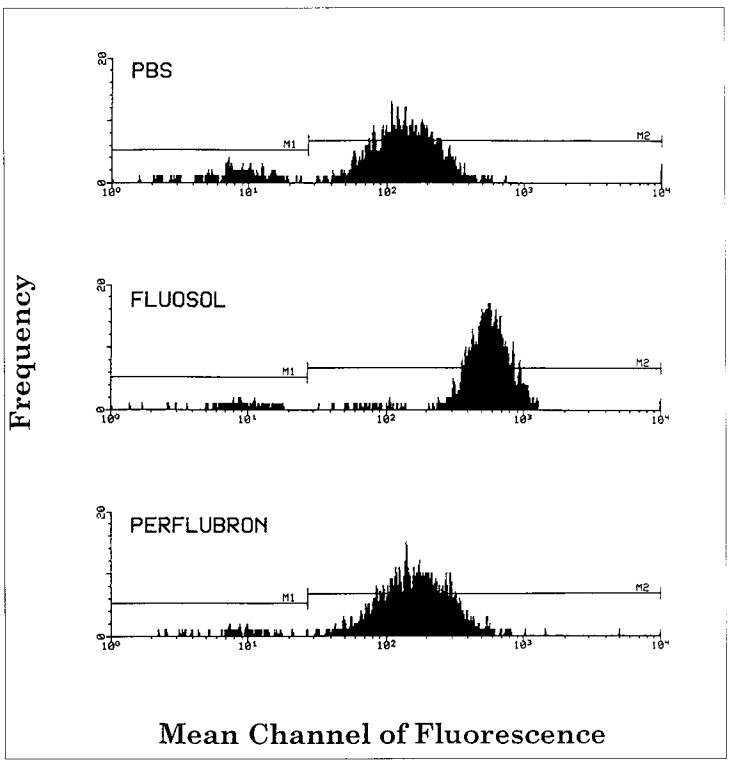
Table 2: Leukocyte, granulocyte, and lymphocyte counts as a percent of the pre-treatment control for the PBS-control, Fluosol, and perflubron treated groups 20, 40, and 60 minutes after treatment.

	Leukocyte Count			Granulocyte Count			Lymphocyte Count		
	20min	40min	60min	20min	40min	60min	20min	40min	60min
PBS	87.4±3.8	84.3±4.4	89.9±6.1	92.7±12.8	87.1±8.2	115.9±9.3	88.2±4.5	85.9±7.9	84.7±10.0
Fluosol	123.6±6.1*	108.2±12.3	100.9±11.1	133.7±10.7	108.7±17.2	132.0±25.5	120.5±6.4*	107.6±11.3	89.4±5.5
Perflubron	98.6±5.4	93.1±4.5	108.4±5.0	110.8±14.9	100.9±5.3	160.6±38.1	96.5±5.6	90.4±5.4	97.7±6.6

*Fluosol significantly different from the perflubron and PBS groups ($P<0.05$)

Data Analysis. Data was collected and tabulated on computer spreadsheets[h]. Statistical analyses were performed using a computer program[i]. The summary group data were expressed as mean ± SE. Differences among groups were assessed with a ANOVA. Post hoc testing was performed when the results of the ANOVA were significant. Differences were considered statistically significant at the $p<0.05$ level.

Figure 1: Representative CD11b fluorescence histograms from a PBS (top), Fluosol (middle) and perflubron (bottom) treated animal at 10 minutes following treatment. The rightward shift in the mean channel of fluorescence for the Fluosol treated animal indicates a higher density of CD11b receptors on the neutrophil's surface. M1 and M2 markers represent background fluorescence and positive events (fluorescence above background) respectively.



RESULTS

Table 1 summarizes the results of the measurements of heart rate, mean arterial blood pressure (MAP), and arterial blood gases for the PBS-control, Fluosol and perflubron treated groups prior to and 20 and 60 minutes after treatment. There were no differences in the heart rate, MAP, or blood gases between the three treatment groups before drug administration. Body temperature was maintained at 37°C (range 36.0 - 37.5°C) for all three groups during all time points in the study. The PBS, Fluosol and perflubron groups had no statistically significant changes in MAP, or blood gases during the 60 minutes following treatment. All groups demonstrated a decrease in MAP with time, perhaps due to the anesthetic, but there were no statistically significant differences in MAP among the three groups. The Fluosol group had a significantly higher heart rate then the perflubron group 20 minutes after treatment ($p<0.05$). There was no difference in the heart rate among the three groups 60 minutes following treatment.

Table 2 gives the circulating leukocyte counts for the PBS, Fluosol and perflubron groups, expressed as the percent of pre-treatment control. Prior to treatment, all blood cell counts, leukocytes, platelets and red cells, were quite similar for all groups. Following treatment, the platelet counts and hematocrits were not different among the groups. However, there was a significant increase in the leukocyte count in the Fluosol group by 20 minutes ($p<0.05$). By 40 and 60 minutes, the leukocyte, granu-

h Excel v5.0, Microsoft, Redmond, WA
i Statistica v3.1, Statsoft, Tulsa, OK

Figure 2: The flow cytometry analysis of the PMN's CD11b surface expression for the PBS, Fluosol, and perflubron groups before and 10, 20, 40, and 60 minutes after treatment

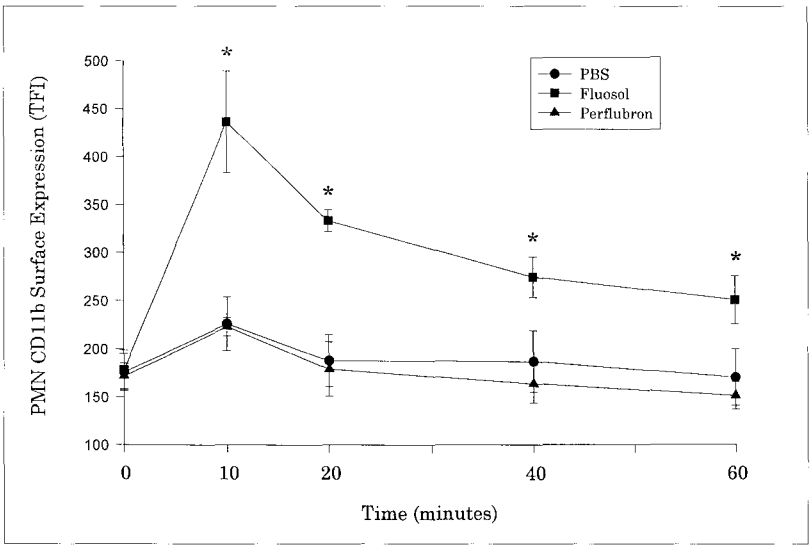
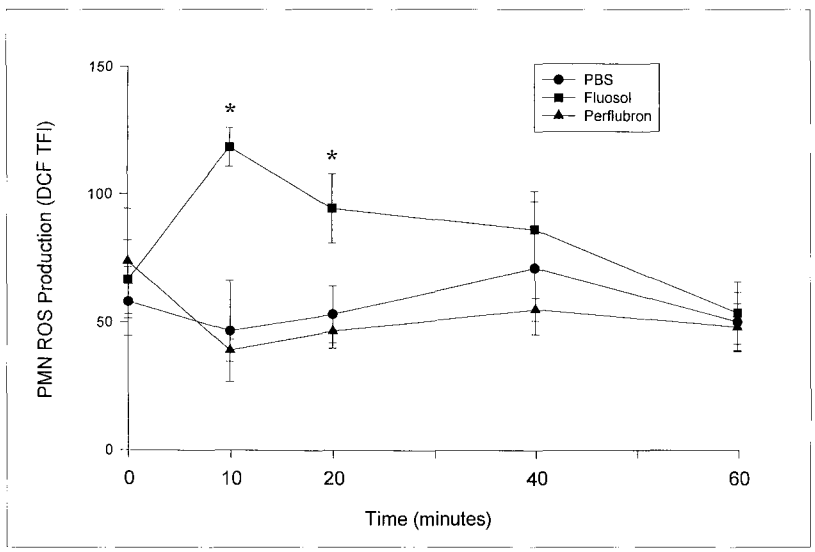


Figure 3: The flow cytometric analysis of the PMN's ROS production for the PBS, Fluosol and perflubron groups before and 10, 20, 40, and 60 minutes after treatment



locyte and lymphocyte counts were no longer significantly elevated in any of the treatment groups.

Figure 1 gives a representative result from the flow cytometry analysis of neutrophil CD11b expression for the PBS, Fluosol and perflubron groups. In this case, the frequency distribution of the CD11b mean channel fluorescence (MCF) was sampled from each treatment group ten minutes after treatment. Fluosol treatment increased the CD11b MCF, as demonstrated by the rightward shift of the frequency distribution along the log scale for MCF.

Figure 2 summarizes the results of the flow cytometry analysis for the PMN expression of CD11b. The pre-treatment values were similar for all three groups. There was no significant increase in the CD11b TFI during the 60 minute observation period in the PBS and perflubron groups. In contrast, we observed a rapid increase in CD11b TFI at 10-20 minutes in the Fluosol group, which remained elevated throughout the 60 minute observation period ($p<0.05$).

Figure 3 summarizes the effect of treatment on PMN oxygen free radical production (ROS). All three groups had similar levels of ROS production prior to treatment. The Fluosol group had a rapid increase in PMN ROS 10 minutes after treatment ($p<0.05$). This increase in ROS remained elevated at 20 and 40 minutes but returned to approximately the pretreatment level after 60 minutes. There were no significant changes from baseline in PMN ROS for the PBS or perflubron groups, throughout the 60 minute observation period following treatment.

Table 3 contains the flow cytometry results for the fMLP-stimulated PMN CD11b expression and ROS production. All three groups had a similar response to stimulation with a 200-400% increase in CD11B expression and ROS production.

From the blood obtained after the final (t=60min) measurements were taken, no differences in leukocyte adhesion in the nylon column assay were observed among the three groups. The percent PMN adhesion for the PBS, Fluosol and perflubron groups were (mean ± SEM): 27.9±2.1, 26.2±2.7, 25.5±1.9, respectively.

DISCUSSION

Fluosol-DA, a first generation PFC emulsion, consists of two perfluorocarbons, perfluorodecalin and perfluorotripropylamine in a 20% weight per volume emulsion consisting of a detergent, pluronic F-68, and a surfactant, egg-yolk phospholipids (29). Perflubron emulsion, a second generation PFC emulsion, consists almost entirely of a single perfluorocarbon, perfluoroocytlbromide, in an emulsion consisting of egg-yolk phospholipids (30). Currently perflubron is being tested in Phase II clinical studies for use in conjunction with hemodilution during elective surgery or cardiopulmonary bypass procedures (31). Early studies with Fluosol

Table 3: PMN CD11b expression and ROS production in unstimulated and fMLP stimulated blood samples for the PBS, Fluosol, and perflubron treated groups

CD11b Expression

	PBS		Fluosol		Perflubron	
Time	Unstimulated	Stimulated	Unstimulated	Stimulated	Unstimulated	Stimulated
PRE	175.9±19.5	696.8±47.9	178.2±20.8	727.6±70.0	171.9±13.1	631.0±59.2
10min	226.2±27.2	724.0±42.4	436.5±53.3	975.1±55.5	223.3±9.6	695.4±32.2
20min	187.9±27.2	767.5±33.4	333.8±11.6	850.2±72.9	179.2±25.9	735.2±64.6
40min	187.1±31.9	783.0±74.9	274.6±21.2	760.1±53.9	163.9±20.1	699.6±63.6
60min	170.6±29.5	641.7±171	250.6±24.9	902.2±60.0	151.3±14.5	723.9±50.3

ROS production

	PBS		Fluosol		Perflubron	
Time	Unstimulated	Stimulated	Unstimulated	Stimulated	Unstimulated	Stimulated
PRE	58.1±13.4	164.9±20.3	66.7±15.2	163.3±24.1	73.8±20.5	147.9±19.2
10min	46.7±20.0	180.6±8.1	118.6±7.6	343.8±27.1	39.1±4.4	173.2±18.7
20min	53.3±11.2	178.1±14.2	94.6±13.4	283.7±49.0	46.7±6.8	163.4±6.4
40min	71.4±26.0	197.8±28.7	86.4±14.9	274.6±58.6	55.2±4.4	217.0±11.6
60min	50.3±11.6	178.0±38.0	53.9±12.3	230.7±48.9	48.3±9.2	218.2±30.8

suggested that this PFC emulsion inhibited leukocyte function (3, 11, 32). However, later studies found that incubation of blood with Fluosol stimulated PMNs (10, 13, 20). In the current in-vivo study, we found that Fluosol caused a PMN activation as demonstrated by both a significant increase in CD11b adhesion molecule expression and ROS production by the phagocytic PMNs.

The PMN response to Fluosol appeared to vary with time following treatment (Figures 2, 3). For PMN CD11b, the expression was significantly increased throughout the 60 minute observation period. However, PMN ROS production was significantly increased only 10-20 minutes after treatment. The transient nature of the PMN response to Fluosol treatment may be explained in two ways. First, the CD11b adhesion molecules expressed on the surface of the PMNs may be shed or returned to internal cellular compartments. However, this possibility is unlikely because other studies suggest that expressed CD11b is not shed rapidly. For example, Miller et al (33) found a rapid increase in the PMN CD11b expression ten minutes following fMLP[10^{-7}M] stimulation. There was a further increase in the PMN CD11b expression by 60 minutes following stimulation with no indication of a decline of expression (33). Thus, in the one hour observation period of our study, it is unlikely that CD11b adhesion molecules were significantly shed or re-internalized, causing CD11b expression to decrease.

A second possibility is that the neutrophils sampled later during the observation period are from a different cell population than those initially sampled. The increased PMN CD11b adhesion protein expression following Fluosol treatment may have caused PMN adherence to the vasculature. Thus, the activated blood cells will effectively leave the circulating pool of PMNs. Then the blood sampled later in the experiment contained those PMNs that did not adhere in response to the earlier stimulation. The notion of changes in the circulating pool of PMNs following stimulation is not novel. Appen et al measured the effects of hemodialysis membrane materials on leukocyte activation (34). Following exposure of blood to the membrane surfaces, neutrophil CD11b expression was increased within the first three minutes, but returned to pre-exposure levels after 60 minutes. Appen et al concluded that the neutrophils sampled three minutes after exposure were not the same cells sampled at 15 and 60 minutes after exposure. The activated neutrophils (increased CD11b expression) could adhere to the vascular endothelium, causing the measured CD11b fluorescence of the residual circulating population of cells to be lower.

In contrast to the sustained increase in CD11b expression and transient increase in ROS production with Fluosol, treatment with perflubron emulsion did not stimulate the PMNs. Other studies also report no increase in PMN activity with perflubron. In fact, at higher concentrations, perflubron may decrease PMN activity. In a recent in-vitro study, Rossman et al (35) reported that human neutrophils treated with perflubron produced significantly less ROS and had a significantly decreased chemotactic response to PMA stimulation. In another study, Smith et al (36) reported a decrease in ROS production by alveolar macrophages after in-vitro exposure to perflubron emulsion. Previously, we

compared the effects of Fluosol and perflubron emulsion on leukocyte adhesion in-vitro, finding that Fluosol had a direct effect on leukocyte function (20). In a separate, in-vivo study, we found that treatment with perflubron emulsion significantly decreased neutrophil adhesion (37). The lack of a perflubron effect on neutrophil adhesion in the current study versus a significant decrease in PMN adhesion in our earlier in-vivo study may be due to the dose of perflubron emulsion administered. In the earlier in-vivo study, the dose of perflubron emulsion was two and a half times more than the dose used in the current study. Thus, the ability of perflubron emulsion to limit leukocyte stimulation may be a function of dose. The dose of perflubron emulsion used in the current study is the same dose currently being evaluated in clinical trials.

All three groups demonstrated a decrease in MAP with time. This decrease may be related to the deep plane of pentobarbitol anesthesia, but all MAPs were within normal limits for anesthetized animals.

There was a significant increase in the number of circulating leukocytes in the Fluosol treated group at 20 minutes following treatment. Fluosol treatment may have caused a net demargination of leukocytes increasing the number of circulating leukocytes. Neither of the perfluorocarbon treatments had any effect on hematocrit or platelet count.

Previous studies suggested that activation of neutrophils by Fluosol may cause the cells to be refractory to further stimulation (9,13). This hypothesis is not supported by our findings. As indicated in Table 3, before and at all times after emulsion treatment, all groups were quite responsive to fMLP stimulation, as indicated by significant increases in both CD11b expression and ROS production (~200-400% of unstimulated samples). At no time during the one hour observation period did either emulsion limit the PMNs from responding to the chemotactic peptide fMLP. Perhaps another mechanism, such as "distracting" the activated leukocytes to non-coronary vasculatures, is the way in which Fluosol exerts cardioprotection.

In contrast to our earlier report, in the current study, at t=60min, we did not observe a difference in granulocyte adhesion for the Fluosol and perflubron emulsion groups. The different findings between our in-vitro and in-vivo studies may be explained by differences in the experimental protocols. That is, both the PFC doses and the incubation periods were different in the two studies. The dose of PFC emulsions incubated with the blood in this in-vivo study was less than the dose used in the previous in-vitro study. Also, in the previous study, blood was incubated in-vitro with the PFCs for 10 minutes before being tested in the nylon fiber adhesion columns. In this study, blood was tested 60 minutes after treatment. Perhaps the lower dose of emulsion was not sufficient to effect granulocyte adhesion, or by 60 minutes after treatment those leukocytes in the circulating blood pool were less active. The results from the nylon column adhesion assay support the flow cytometry results for PMN ROS that, 60 minutes after treatment, those PMNs in the circulating blood pool were not significantly activated.

There have been previous reports that cardiopulmonary bypass (CPB) stimulates a leukocyte-mediated inflammatory response (38,39). PFCs are currently being investigated for use in conjunction with hemodilution during CPB procedures (31,40). Previous studies using animal models of CPB suggest that PFC emulsions are effective oxygen carrying solutions and may serve as excellent prime solutions during cardiac surgery (41,42). The effects of PFC emulsions on leukocyte activation are not yet fully understood. An added benefit of some PFC emulsions may be to help limit the inflammatory response during and after CPB. Future studies are required to determine if addition of PFC emulsions to the CPB circuit will effect the inflammatory response.

In conclusion, these findings indicate that both in-vivo and in-vitro blood treatment with Fluosol causes leukocyte activation, as demonstrated by an increase in CD11b adhesion molecule expression and ROS production. The lack of a PMN response to the clinical dose of perflubron emulsion, in-vivo, suggests that this agent is not likely to induce a leukocyte-mediated inflammatory response. Perflubron is an oxygen carrying agent that might be well suited as a priming solution for the CPB circuit.

REFERENCES

1. Millard RW. Oxygen solubility, rheology and hemodynamics of perfluorocarbon emulsion blood substitutes. Art Cells, Blood Subs, and Immob Biotech. 1994; 22:235-244.
2. Marchbank A. Fluorocarbon emulsions. Perfusion. 1995; 10:67- 88.
3. Bajaj AK, Cobb MA, Virmani R, Gay JC, Light RC, Forman MB. Limitation of myocardial reperfusion injury by intravenous perfluorochemicals: Role of neutrophil activation. Circulation. 1989; 79:645-656.
4. Forman MB, Puett DW, Wilson BH, Vaughn WK, Friesinger GC, Virmani R. Beneficial long-term effect of intracoronary perfluorochemical on infarct size and ventricular function in a canine reperfusion model. J Am Coll Cardiol. 1987; 9:1082- 1090.
5. Martin SM, Laks H, Drinkwater DC, et al. Perfluorochemical reperfusion yields improved myocardial recovery after global ischemia. Ann Thorac Surg. 1993; 55:954-960.
6. Mehvar R, Reynolds JM, Shepard TL. Disposition of fluorescein-labelled dextran (150 KD) in isolated perfused livers from control and diabetic rats. Life Sciences. 1991; 49:1699- 1706.
7. Forman MB, Bingham S, Kopelman HA, et al. Reduction of infarct size with intracoronary perfluorochemical in a canine preparation of reperfusion. Circulation. 1985; 71:1060-1068.
8. Kolodgie FD, Virmani R, Farb A. Limitation of no reflow injury by blood-free reperfusion with oxygenated perfluorochemical (Fluosol-DA 20%). J Am Coll Cardiol. 1991; 18:215-223.

9. Forman MB, Ingram DA, Murray JJ. Role of perfluorochemical emulsions in the treatment of myocardial reperfusion injury. Am Heart J. 1992; 124:1347-1357.
10. Forman MB, Pitarys II CJ, Vildibill HD, et al. Pharmacologic perturbation of neutrophils by fluosol results in a sustained reduction in infarct size in the canine model of reperfusion. JACC. 1992; 19:205-216.
11. Virmani R, Fink LM, Gunter K, English D. Effect of perfluorochemical blood substitutes on human neutrophil function. Transfusion. 1984; 24:343-347.
12. Virmani R, Warren D, Rees R, Fink LM, English D. Effects of perfluorochemical on phagocytic function of leukocytes. Transfusion. 1983; 23:512-515.
13. Ingram DA, Forman MB, Murray JJ. Phagocytic activation of human neutrophils by the detergent component of fluosol. Am J Pathol. 1992; 140:1081-1087.
14. Ritter LS, Wilson DS, Williams SK, Copeland JG, McDonagh PF. Early in reperfusion following myocardial ischemia, leukocyte activation is necessary for venular adhesion but not capillary retention. Microcirculation. 1995; 2: 315-327.
15. Ritter LS, Wilson DS, Copeland JG, Williams SK, McDonagh PF. Pentoxifylline reduces leukocyte retention in the coronary microcirculation early in reperfusion following ischemia. Internation J Microcirc Clin Exp, 1996; 16:170-179.
16. Buyon JP, Filips MR, Abramson SB, Slade SG, Weissman G, Winchester R. Mechanism regulating recruitment of CD11b/CD18 to the cell surface is distinct from that which induces adhesion in homotypic neutrophil aggregation. In: Springer TA, Anderson DC, Rosenthal AS, Rothleim R, eds. Leukocyte Adhesion Molecules Structure, Function, and Regulation. New York: Springer-Verlag, 1990; 72-83.
17. Todd III RF, Amaout MA, Rosin RE, Crowley CA, Peters WA, Bablor BM. Subcellular localization of the large subunit of Mo1 (Mo1-alpha; formerly gp 110), a surface glycoprotein associated with neutrophil adhesion. J Clin Invest. 1984; 74:1280-1290.
18. Amaout MA, Spits H, Terhorst C, Pitt J, Todd III RF. Deficiency of a leukocyte surface glycoprotein (LFA-1) in two patients with Mo1 deficiency. Effects of cell activation on Mo1/LFA-1 surface expression in normal and deficient leukocytes. J Clin Invest. 1984; 74:1291-1300.
19. Bolli R. Oxygen-derived free radicals and postischemic myocardial dysfunction ("stunned myocardium"). J Am Coll Cardiol. 1988; 12:239-249.
20. McDonagh PF, Wilson DS. The initial response of blood leukocytes to incubation with perfluorocarbon blood substitute emulsion. Art Cells, Blood Subs, and Immob Biotech. 1995; 23(3):439-447.
21. Kolodgie FD, Farb A, Carlson GC, Wilson PS, Virmani R. Hyperoxic reperfusion is required to reduce infarct size after intravenous therapy with perfluorochemical (Fluosol-DA 20%) or its detergent component (Poloxamer 188) in a poorly collateralized animal model. Absence of a role of polymorphonuclear leukocytes. J Am Coll Cardiol. 1994; 24:1098-108.
22. Tsai AG, Nolte D, Messmer K, Intagliette M. Effect of Oxygent (perflubron emulsion) on leukocyte-endothelial interaction in postcapillary venules. Biomat, Art Cells, and Immob Biotech. 1992; 20:959-961.
23. US Department of Health and Human Services P.H.S. National Institutes of Health: Guide for the care and use of laboratory animals. NIH Publication. 1985; 80:23.
24. Himmelfarb J, Hakim RM, Holbrook DG, Leeber DA, Ault KA. Detection of granulocyte reactive oxygen species formation in whole blood using flow cytometry. Cytometry. 1992; 13:83- 89.
25. Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol. 1993; 130:1910-1917.
26. McCarthy DA, Macey MG. A simple flow cytometric procedure for the determination of surface antigens on unfixed leucocytes in whole blood. J Immunol Meth. 1993; 163:155-160.
27. Simon SI, Chambers JD, Butcher E, Sklar LA. Neutrophil aggregation is B2-integrin- and L-selectin-dependent in blood and isolated cells. J Immunol. 1992; 149:2765-2771.
28. Tomlinson KC, Gardiner SM, Hebden RA, Bennett T. Functional consequences of streptozotocin-induced diabetes mellitus, with particular reference to the cardiovascular system. Pharmacol Rev. 1992; 44:103-150.
29. The Green Cross Corporation. Fluosol product information. 1992; 1-8.
30. Chapman KW, Keipert PE, Graham HA. Commercial-scale production of perfluorochemical emulsions. Art Cells, Blood Subs, and Immob Biotech. 1996; 24 (abstract):318.
31. Keipert PE, Conlan MG. Advances in perflubron emulsion development: potential use during surgery and cardiopulmonary bypass to avoid donor blood transfusion and prevent tissue hypoxia. Art Cells, Blood Subs, and Immob Biotech. 1996; 24 (abstract):359.
32. 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.
33. Miller LJ, Bainton DF, Borregaard N, Springer TA. Stimulated mobilization of monocyte mac-1 and p150,95 adhesion proteins from an intracellular vesicular compartment to the cell surface. J Clin Invest. 1987; 80:535-544.
34. Appen KV, Goolsby C, Mehl P, Goewert R, Ivanovich P. Leukocyte adhesion molecules as biocompatibility markers for hemodialysis membranes. ASAIO Journal. 1994; 40:M609-M615.
35. Rossman JE, Caty MG, Rich GA, Karamanoukian HL, Azizkhan RG. Neutrophil activation and chemotaxis after in vitro treatment with perfluorocarbon. J Ped Surg. 1996; 31:1147-1151.

36. Smith TM, Steinhorn DM, Thusu K, Furhman DP. A liquid perfluorochemical decreases the in vitro production of reactive oxygen species by alveolar macrophages. Crit Care Med. 1995; 23:1533-1539.
37. Wilson DS, McDonagh PF. Granulocyte adherence is reduced by in-vivo incubation with the perfluorocarbon blood substitute, perflubron emulsion. J Leuk Biol Supplement. 1994; 27.
38. Larson DF, Bowers M, Schechner HW. Neutrophil activation during cardiopulmonary bypass in paediatric and adult patients. Perfusion. 1996; 11:21-27.
39. McQueen PJ, Bowers MC, Larson DF. Neutrophil activation subsequent to cardiopulmonary bypass: a sensitive method for determining neutrophil activation by quantification of lactoferrin concentrations. J Extra-Corpor Technol. 1995; 1:11-14.
40. Spiess BD, Cochran RP. Perfluorocarbon emulsions and cardiopulmonary bypass: a technique for the future. J Cardiothorac Vasc Anesth. 1996; 10:83-90.
41. Spruell RD, Ferguson ER, Clymer JJ, Vicente WV, Murrah CP, Holman WL. Perfluorocarbons are effective oxygen carriers in cardiopulmonary bypass. ASAIO Journal. 1995; 41:M636-M641.
42. Vocelka C, Spiess B, Soltow L, et al. A perfluorocarbon emulsion prime additive improvews the electroencephalogram and cerebral blood flow at the intiation of cardiopulmonary bypass. J Extra-Corpor Technol. 1995; 27:6-10.