Duration of Intralipid's Suppressive Effect on NK Cell's Functional Activity

Roumen G. Roussev[1], Brian Acacio[2], Siu C. Ng[1], Carolyn B. Coulam[1,3]

[1]Millenova Immunology Laboratories, Chicago, IL, USA;
[2]Acacio Fertility Center, Laguna Niguel, CA, USA;
[3]Rinehart Center for Reproductive Medicine, Chicago, IL, USA

Keywords
Intralipid, NK cell activity, suppression

Correspondence
Roumen Roussev, 233 East Erie St, Suite 510, Chicago, IL 60611, USA.
E-mail: rgroussev@yahoo.com

Submitted March 25, 2008;
accepted April 25, 2008.

Citation
Roussev RG, Acacio B, Ng SC, Coulam CB. Duration of intralipid's suppressive effect on NK cell's functional activity. Am J Reprod Immunol 2008; 60: 258–263

doi:10.1111/j.1600-0897.2008.00621.x

Background
In vitro investigations have revealed the ability of intralipids to suppress natural killer (NK) cytotoxicity. Evidence from both animal and human studies suggests that intralipid administered intravenously may enhance implantation and maintenance of pregnancy when the patient has an abnormal NK cell level or function.

Problem
The aim of this study was to establish the duration and efficacy of Intralipids suppressive effect on NK cell functional activity.

Method of study
Fifty patients with abnormal NK activity results (NKa) received intralipid 20% i.v. (9 mg/mL total blood volume -corresponds to 2 mL of intralipid 20% diluted in 250 mL saline; or 18 mg/mL – corresponds to 4 mL of intralipid 20% diluted in 250 mL saline) infusions and their NKa were tested periodically. The determination of NK cell function was performed by flow cytometry using K562 cells as targets.

Results
Fifty women with abnormal NKa-testing received intralipid infusions. 39 (78%) showed NKa suppression within the normal range the first week after infusion, 11 (22%), showed suppression, but still above the normal threshold. They received second infusion 2–3 weeks later. In 10, the Nka activity was normalized the following week. Four patients had three intralipid infusions in 2-week periods in between and after the third infusion, and all showed NKa normal activity. In 47 patients the suppressive effect of the Intralipid after the normalization of NKa lasted between 6 and 9 weeks, in two patients this benefit lasted 5 weeks, and in one patient the effect was 4 weeks.

Conclusion
Intralipid is effective in suppressing *in vivo* abnormal NK-cell functional activity. The results suggest that Intralipid can be used successfully as a therapeutic option to modulate abnormal NK activity in women with reproductive failure.

Introduction

Reproductive failure is a serious problem in any society, particularly among women over 30 years of age.[1] In these women pregnancy loss occurs in both natural and assisted reproductive techniques. Whereas the majority of pregnancies that are lost are on account of chromosomal abnormalities[2,3] immunologic causes have been considered more then 80% of the otherwise unexplained reproductive failures.[4–7] Most studies addressing the problems of immunologic reproductive failures have focused on the role of natural killer (NK) cells.[8–12] Women with reproductive failures have significantly elevated numbers and functional activity of peripheral NK cells compared with normal fertile controls,[7,13,14] and suppression of NK cell's abnormal activity during these pregnancies determine reproductive success.[15–17] Intravenous immunoglobulin down-regulates NK-cell killing activity both *in vitro* and *in vivo*[15,18] and it has been used successfully for treatment of recurrent spontaneous abortions and recurrent implantation failure after *in vitro* fertilization (IVF) and embryo transfer.[19]

We have recently reported the ability of Intralipid 20% (i.v. fat emulsion) to suppress abnormal NK cytotoxic activity in peripheral NK cells from women with recurrent reproductive failure *in vitro*.[20]

The purpose of this study is to evaluate *in vivo* the level and duration of Intralipid 20% suppressive effect on abnormal NK activity (NKa) in patients with reproductive failure. Also, the expression of some activation and inhibition NK-cell markers has been compared between the untreated and those treated with Intralipid lymphocytes.

Materials and methods

Blood Samples

Blood samples from 50 patients experiencing recurrent reproductive failure including recurrent implantation failure after IVF and embryo transfer referred to Millenova Immunology Laboratories for NK functional activity evaluation were included in the IRB approved study. All patients with abnormal NK-cell activity were followed on a weekly basis after being selected for Intralipid infusions.

Intralipid Infusion

A quantity of 2–4 mL of 20% intralipid solution (Frezenius, Clayton, NC, USA) was injected into 250 mL sterile i.v. saline. The solution was administered i.v. at approximately 250 cm^3/hr.

NK Cytotoxicity Assay

The determination of NK-cell function was performed by flow cytometry using a previously described technique.[21] Briefly, K562 cells were grown as stationary cultures at 37°C in 5% CO_2. The cells were subcultured for 3 days before the assay, to be certain they were in the log phase. Before use in the assay, cells were incubated with 10 μL of 30 mmol/L dioctadecyloxacarbocyanine perchlorate (DiO) per mL for 20 min at 37°C, 5% CO_2. Effector cells were isolated from the buffy coat of heparinized blood using Ficol–Hypaque centrifugation. Target cells at the standard concentration and effector cells were added to create effector/target ratios of 50:1 and the mixture was centrifuged for 30 s at 1000 ***g*** to pellet the target and effector cells. The mixture was incubated for 2 and a half hours at 37°C, 5% CO_2 and 15 min before flow-cytometry acquisition a total of 100 μL of propidium iodide (PI) was added to the tubes to label the dead cells. Data were collected for analysis on the Becton-Dickinson fluorescent-activated cell sorter flow cytometer, using the CELLQUEST program and LYSIS software. Spontaneous lysis was subtracted from the actual lysis for each sample. Based upon the control population, increased (abnormal) NK killing activity was defined as >10% killing. The 10% threshold was established as the 99th percentile from NKa results of 55 normal healthy individuals.

Suppression of NK Cytotoxicity Assay

Intralipid 20% (Frezenius), was used to measure the NK cell's response to suppression. A total of four 12 × 75 B-D tubes per patient was used: target cells at standard concentration (10 μL from 1×10^6) were mixed with 100 μL of 5×10^6 peripheral blood mononuclear cells (effector cells) to create T:E ratio 1:50. A separate tube with target cells only was used for background control. Two tubes were used to measure Intralipid 20% suspension. In the first tube 18 mg/mL Intralipid in culture media (CM) and 9 mg/mL in the second tube was used. All tubes were incubated at

37°C in 5% Co_2 for 2 and a half hours and analyzed by flow cytometry. Fifteen min. before analyses100 μL of PI was added to all tubes. The controls for the background contained only DiO-stained target (T) cells and PI.

NK Cell Activation and Suppressive Markers

Lymphocytes from 20 patients have been labeled for some activation and suppressive markers before (background) and after incubation for 2 and a half hours with intralipid. The antibodies used in combination were CD56, CD16, CD69, CD94, CD161, and CD11c.

Fluorescent-activated Cell Sorter Analyses

The forward- and side-scatter parameters were adjusted to accommodate the inclusion of both target and effector cells within the acquisition gate and 10,000 cells minimum were counted. Quadrant markers were drawn to distinguish DiO-labeled cells and cells with incorporated PI (dead cells). Percent lysis was calculated by the equation:

$$\%\ \text{lysis} = (\ \text{quadrant2 events/quadrant2} + \text{quadrant4 events}) \times 100$$

$$\%\ \text{changes in NK cytolysis} = \frac{(\text{Natural NK lysis - NK cytolysis after Intralipid}) \times 100}{\text{Natural NK cytolysis}}$$

or

Statistical analyses were performed by ANOVA and $P < 0.05$ was considered statistically significant. Data were expressed as mean ± S.D.

Results

Fifty patients with abnormal NK functional activity were treated with Intralipid infusions and NKa assay was performed weekly for follow-up levels of NK-cytotoxicity suppression. The patients were divided into two groups based on the level of abnormal NKa percentage: first, with NKa above 15%, and second, <15% (Figs 1 and 2). Almost all (89.3%) of the patients with NK abnormal activity <15% after the first Intralipid infusion normalized their NKa below the normal threshold of 10%. Some of the patients

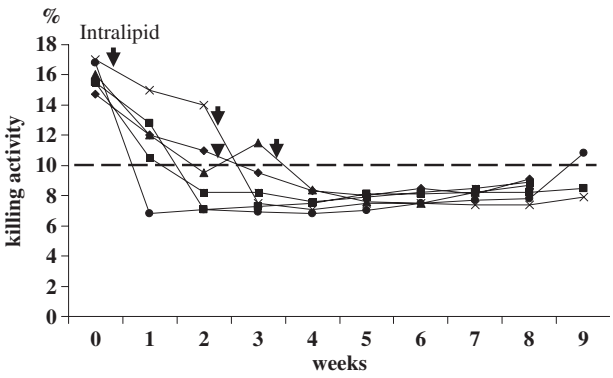

Fig. 1 Duration of intralipid suppression of natural killer (NK) cell activity in patients with abnormal NK cytolytic activity higher then 15%. The arrows show the time of intralipid infusions (— normal level threshold).

(43.8%) with higher then 15% NKa required more than two infusions to have their activity suppressed to normal range.

Of 50 women with abnormal NKa testing that received Intralipid infusions, 39 (78%) showed NKa suppression to within normal levels after the first week, and of these 50, 11 (22%), showed suppression, but still above the normal threshold (Table I). Those with suppression above the normal threshold received a second infusion 2–3 weeks later. In 10, the NKa was normalized the following week. Four patients had three intralipid infusions in 2-week

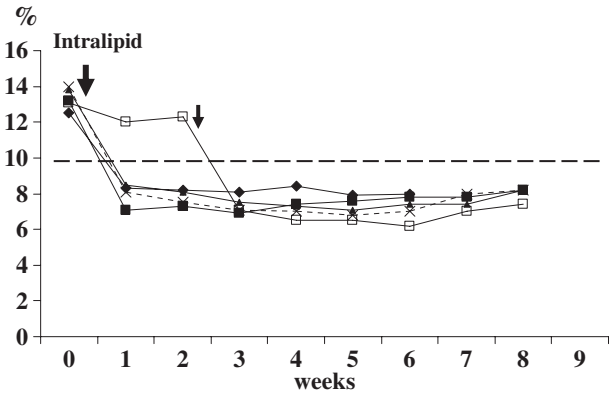

Fig. 2 Duration of intralipid suppression of natural killer (NK) cell activity in patients with abnormal NK cytolytic activity between 11% and 15%. The arrows show the time of intralipid infusions (— normal level threshold).

American Journal of Reproductive Immunology **60** (2008) 258–263

Table I Intralipid Suppression of Abnormal Natural Killer Cell Functional Activity *In Vivo*

	Suppressed' NKa after first infusion		Suppressed NKa after second infusion		Duration of the suppression	
Abnormal NKa	>10%	<10%	>10%	<10%	4–6 week	6–9 week
>15% (*n* = 16)	7 (43.8%)	9 (56.2%)	1	6	1 (6.3%)	15 (92.7%)
<15% (*n* = 34)	4 (11.7%)	30 (89.3%)	–	4	2 (5.9%)	32 (94.1%)
Total (*n* = 50)	11 (22%)	39 (78%)	1	10	3 (6%)	47 (94%)

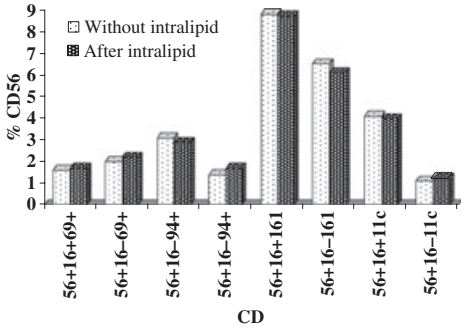

Fig. 3 Expression of some activation and suppression markers on $CD56^+$ NK cells before and after incubation with intralipid. There is no significant statistical difference in the markers' expression (*n* = 20).

periods in between and after the third infusion, and all showed normal NKa. In 47 patients the suppressive effect of the Intralipid after the normalization of NKa lasted between 6 and 9 weeks, in two patients this benefit lasted 5 weeks, and in one patient the effect lasted for 4 weeks.

There are no differences in the expression of $CD56^+/CD16^-$ or $CD16^+$, CD69, CD94, CD161, and CD11c before and after incubation with Intralipid *in vitro* (Fig. 3).

Discussion

The immunologic causes for reproductive failure are the results of abnormal activities of white blood cells and antibody responses. Increased numbers and cytotoxic activity of NK cells have been reported in women with reproductive failures.[9,22,23] A number of studies reported that Intralipid might modulate immune function with suppression of NK cytotoxicity[24,25] and pro-inflammatory cytokine generation.[26,27] Intralipid fat infusions are standard component of total parenteral nutrition in trauma and severely burned patients. It has been reported that intravenous fat emulsion infusions during early post-injury period increased susceptibility to infection, prolonged pulmonary failure, and delayed recovery in critically injured patients suggesting that one possible way of acting is by suppression of NK activity.[28] Sedman et al. have found a significant fall of NK activity and lymphokine-activated killer activity after total parenteral nutrition regimens with long-chain triglycerides.[29] Parenteral fat emulsions are known to accumulate in macrophages and to impair various functions of macrophages and those of the reticuloendothelial system. It was shown that the administration of fat emulsion, intralipid 20%, to recipient mice could suppress genetic resistance to bone marrow grafts and NK cell activity probably through the impairment of the macrophage function.[30]

While intralipids have been shown to stimulate the reticulo-endothelial system and remove 'danger signals' that can lead to pregnancy loss,[31] the exact mechanism by which intralipids suppresses NK function is not known. NK cells possess both cell surface[32] and nuclear receptors.[33] We found no differences in the expression of $CD56^+/CD16^-$ or $CD16^+$, CD69, CD94, CD161, and CD11c before and after incubation with intralipid *in vitro* suggesting that intralipid does not act on these cell surface receptors. These observations are consistent with previous reports.[34,35]

Natural killer cells have also been shown to express nuclear receptors known as peroxisome proliferator-activated receptors (PPARs).[36,37] PPARs are ligand-activated transcription factors belonging to the nuclear receptor superfamily that form dimers with the retinoid X receptors.[36] When these receptors are activated by their fatty acid ligands, they have been shown to regulate inflammation.[38–40] In NK cells, PPARs-ligand interaction results in decreased interferon-γ secretion and cytotoxicity.[33] Increased NK cytotoxicity has been associated with

implantation failure[14,15] and recurrent pregnancy loss[9–13] in humans. In mice, PPAR gene deletions were associated with reduced implantation[41] and decreased litter size.[42]

Taken together, these observations lead to the following hypothesis: intralipids administered intravenously engage their receptors, PPARs, in NK cells and decrease their cytotoxic response enhancing implantation and maintenance of pregnancy. Intralipid has been shown to be effective in suppressing the abnormal NK cell functional activity both *in vitro*[20] and *in vivo* (this study). Thus, the results suggest that Intralipid can be used successfully as a therapeutic option to modulate abnormal NK activity in women with reproductive problems.

References

1 Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M: Maternal age and fetal loss: population based register linkage study. *BMJ* 2000; 320:1708.

2 Coulam CB, Stephenson M, Stern JJ, Clark DA: Immunotherapy for recurrent pregnancy loss: analysis of results from clinical trials. *Am J Reprod Immunol* 1996; 35:352–359.

3 Stern JJ, Dorfmann A, Gutierrez-Najar AJ, Cirrello M, Coulam CB: Frequency of abnormal karyotypes among abortuses from women with and without a history of recurrent spontaneous abortion. *Fertil Steril* 1996; 65:250–253.

4 Cramer DW, Wise LA: The epidemiology of recurrent pregnancy loss. *Semin reprod Med* 2000; 18:331–339.

5 Tempelton A, Morris JK, Parslow W: Factors that affect outcome of in-vitro fertilization treatment. *Lancet* 1996; 348:1402–1406.

6 Clark DA: Immunology of pregnancy. In Medical Complications during Pregnancy, 6th edn, Burrow GN, Duffy T, Copel J (eds). Yale University, WB Saunders, 2004, pp 592.

7 Clark DA, Arck P, Chaoaut G: Why did your mother reject you? Immunolgenetic determinant of the response to environmental selective pressure expressed at the uterine level. *Am J Reprod Immunol* 1999; 41:5–22.

8 King A, Loke YW: On the nature and function of human uterine granular lymphocytes. *Immunol Today* 1991; 12:432–435.

9 Coulam CB, Goodman C, Roussev RG, Thomason EJ, Beaman KG: Systemic CD56+ cells can predict pregnancy outcome. *Am J Reprod Immunol* 1995; 33:40–46.

10 Aoki K, Kajijura S, Matsumoto Y, Okada S, Yaghami Y, Gleicher N: Preconceptual natural killer cells activation as a predictor of miscarriage. *Lancet* 1995; 135:1340–1342.

11 Kaider AS, Kaider BD, Janowicz PB, Roussev RG: Immunologic evaluation in women with reproductive failure. *Am J Reprod Immunol* 1999; 42:335–346.

12 Ntrivalas EI, Kwak-Kim JY, Gilman-Sachs A, Chung-Bang H, Ng SC, Beaman KD, Mantouvalos HP, Beer A: Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology. *Hum Reprod* 2001; 16:855–861.

13 Kwak JYH, Beaman KD, Gilman-Sachs A, Ruiz JE, Schewitz D, Beer AE: Up-regulated expression of CD56+, CD56+/CD16+ and CD19+ cells in peripheral blood Lymphocytes in women with recurrent pregnancy losses. *Am J Reprod Immunol* 1995; 34:93–99.

14 Matsubayashi H, Hosaka T, Sugiama Y, Suzuki T, Arai T, Kondo A, Sugi T, Izumi S, Makino T: Increased natural killer-cell activity is associated with infertile women. *Am J Reprod Immunol* 2001; 46:318–322.

15 Coulam CB, Roussev RG: Correlation of NK cell activation and inhibition markers with NK cytotoxicity among women experiencing immunologic implantation failure after *in vitro* fertilization and embryo transfer. *J Assist Reprod Genet* 2003; 20:58–62.

16 Stricker RB, Steinleitner A, Winger EE: Intravenous immunoglobulin (IVIG) therapy for immunologic abortion. *Clin Applied Immunol Rev* 2002; 2:187–199.

17 Coulam CB, Krysa L, Stern JJ, Bustillo M: Intravenous immunoglobulin for treatment of *in vitro* fertilization failure. *Hum Reprod* 1994; 9:2265–2269.

18 Kwak JYH, Kwak FMY, Ainbinder SW, Ruiz AM, Beer A: Elevated peripheral blood natural killer cells are effectively suppressed by immunoglobulin G infusions in women with recurrent spontaneous abortions. *Am J Reprod Immunol* 1996; 35:363–369.

19 Clark DA, Coulam CB, Stricker RB: Is IVIg efficacious in early pregnancy failure? *J Assis Reprod Genet* 2006; 23:1–13.

20 Roussev RG, Ng Sc, Coulam CB: Natural killer cell functional activity suppression by intravenous immunoglobulin, intralipid and soluble human leukocyte antigen-G. *Am J Reprod Immunol* 2007; 57:262–269.

21 Kane KL, Ashton FA, Schmitz JL, Folds JD: Determinations of natural killer cell function by flow cytometry. *Clin Diag Lab Immunol* 1996; 3:295–300.

22 Lachapelle MH, Miron P, Hemmings R, Roy DC: Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. *J Immunol* 1996; 156:4027–4034.

23 Haddad EK, Duclos AJ, Lapp WS, Baines MG: Early embryo loss is associated with the prior expression of macrophage activation markers in the deciduas. *J Immunol* 1997; 158:4886.
24 Granato D, Blum S, Rossle C, Le Boucher J, Malnos A, Dutot G: Effects of parenteral lipid emulsions with different fatty acid composition on immune cell functions *in vitro*. *J Parenter Enterak Nutr* 2000; 24:113–118.
25 Mayer K, Meyer S, Reinholz-Muhly M, Maus U, Merfels M, Lohmeyer J, Grimminger F, Seeger W: Short-time infusion of fish-oil based lipid emulsions, approved for parenteral nutrition, reduces monocyte proinflammatory cytokine generation and adhesive iomteraction with endothelium in humans. *J Immunol* 2003; 171:4837–4843.
26 Krog J, Hoklland M, Ahlburg P, Parner E, Tonnesen E: Lipid solubility and concentration dependent attenuation of *in vitro* natural killer cell cytotoxicity by local anesthetics. *Acta Anaesthesiol Scand* 2002; 46:875–881.
27 Inoue H, Miyaji M, Kosugi A, Nagafukku M, Okazaki T, Mimori T, Amakawa R, Fukuhara S, Domae N, Bloom ET, Umehara H: Lipid rafts as the signaling scaffold for NK cell activation: tyrosine phosphorylation and association of LAT with phosphotidlyinositol 3-kinase and phospholipase C-gamma following CD2 stimulation. *Eur J Immunol* 2002; 32:2188–2198.
28 Battistella FD, Widergren JT, Anderson JT, Siepler JK, Weber JC, MacColl K: A prospective, randomized trial of intravenous fat emulsion administration in trauma victims requiring total parenteral nutrition. *J Trauma* 1997; 43:52–58.
29 Sedman PC, Somers SS, Ramsden CW, Brennan TG, Guillou PJ: Effects of different lipid emulsions on lymphocyte function during total parenteral nutrition. *Br J Surg* 1991; 78:1396–1399.
30 Tezuka H, Sawada H, Sakoda H, Itoh K, Nishikori M, Amagai T, Uchino H, Mori KJ: Suppression of genetic resistance to bone marrow grafts and natural killer activity by administration of fat emulsion. *Exp Hematol* 1988; 12:609–612.
31 Clark DA: Intralipid as a treatment for recurrent unexplained abortion? *Am J Reprod Immunol* 1994; 32:290–293.
32 Farag SS, Fehniger TA, Ruggeri L, Velardi A, Caligiuri MA: Natural killer cell receptors: new biology and insights into the graft-versus-host effect. *Blood* 2002; 100:1935–1947.
33 Zhang X, Rodriguez-Galan MC, Subleski JJ, Ortaldo JR, Hodge DL, Wang JM, Shimozato O, Reynolds DA, Young HA: Peroxisome proliferator-activated receptor-γ and its ligands attenuate biologic functions of human natural killer cells. *Blood* 2004; 104:3276–3284.
34 Versleijen M, Roelofs H, Preijers F, Roos D, Wanten G: Parenteral lipids modulate leukocytes phenotypes in whole blood, depending on their fatty acid composition. *Clin Nutr* 2005; 24:822–829.
35 Rodriguez JM, Ariaz-Diaz J, Figueredo MA, Torres-Molero J, Garcia-Carreras C, Gomez de la Concha E, Balibrea JL: Lymphocyte subpopulation and surgery. The role of postoperative parental nutrition. *Nutr Hosp* 1994; 9:324–330.
36 Desvergne B, Wahli W: Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocrin Rev* 1999; 20:649–688.
37 Khan SA, Vanden-Heuvel JP: Role of nuclear receptors in the regulation of gene expression by dietary fatty acids (review). *J Nutr Biochem* 2003; 14:554–567.
38 Delerove P, Fruchart JC, Staels B: Peroxisome proliferator-activated receptors in inflammation control. *J Endocrinol* 2001; 169:453–459.
39 Clark RB: The role of PPARs in inflammation and immunity. *J Leukoc Biol* 2002; 71:388–400.
40 Daynes RA, Jones DC: Emerging roles of PPARs in inflammation and immunity. *Nat Rev Immunol* 2002; 2:748–759.
41 Akiyama TE, Sakai G, Lambert G, Nicol CJ, Matsusue K, Pimprale S, Lee YH, Ricote M, Glass CK, Brewer HB Jr, Gonzalez FJ: Conditional disruption of the peroxisome proliferator-activator γ gene in mice results in lowered expression of ABCA1, ABCG1 and apoE in macrophages and reduced cholesterol efflux. *Mol Cell Biol* 2002; 22:2607–2619.
42 Koutnikova H, Cock TA, Watanabe M, Houten SM, Champy MF, Dierich A, Auwerx J. Compensation by the muscle limits the metabolic consequences of lipodystrophy in PPARγ hypomorphic mice. *Proc Nat Acad Sci USA* 2003; 24:14457–14462.