Inhibition of *In Vitro* Synthesis of the Second (C2) and Fourth (C4) Components of Complement in Guinea Pig Peritoneal Macrophages by a Soybean Oil Emulsion

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Summary

Recently a soybean oil emulsion (Intralipid) (IL) has been released in the United States for use as a parenteral nutrient. The study reported here was undertaken to determine the effect of ingestion of IL on the synthesis and secretion of the second (C2) and fourth (C4) components of complement by guinea pig peritoneal macrophages *in vitro*. Cells exposed to IL had extensive Oil Red O-positive granular-appearing accumulations of neutral lipid within the cytoplasm. Control cells did not stain with Oil Red O. Incubation of the cells with concentrations of IL from 2.3-37.5 mg/100 ml resulted in a significant decrease in the production of both C2 and C4, which could not be explained by variability between plates. The decrease in total C2 or C4 production by cells incubated with IL for 4 hr was similar to the decrease in production by cells incubated with IL for 48 hr. Several lines of evidence indicated that the decrease of C2 or C4 was the result of decreased synthesis of these proteins and not interference of IL with the detection of the proteins or their secretion from the cells. Exposure of the cells to IL at all concentrations caused reduction of the number of cells having pseudopodia and a rounding-up of the cells. IL did not affect the rate of detachment of the cells from the plates through the 48-hr incubation period or the ability of the cells to exclude trypan blue. Total protein synthesis and total lysozyme production by control and IL-treated cells was similar.

Speculation

Biopsy and autopsy studies of patients who received IL have demonstrated the presence of a fat pigment for up to 5 years in macrophages throughout the reticuloendothelial system, suggesting that macrophages do not process IL efficiently. The effect of IL infusion on C synthesis or serum C levels has not been studied. Inasmuch as macrophages are the major site of synthesis of both C2 and C4, C synthesis may be affected in patients receiving IL. This may be of particular importance in small infants.

Both genetic and nongenetic factors are known to regulate the biosynthesis of serum complement (C) components (2); one of the nongenetic factors is the material ingested by a cell. In early studies, Wyatt *et al.* (16) noted that material used to induce peritoneal exudate affected the proportion of cells producing the second component of C (C2). Fewer C2-producing cells were obtained from mineral oil- or casein-induced exudates than from peritoneal washes or starch-induced exudates, and the average amount of C2 produced by mineral oil- or casein-induced cells was decreased when compared to the amount produced by starch-induced cells. In another study, normal guinea pig macrophages which ingested heat-killed pneumococci *in vitro* demonstrated a

10-fold increase in the rate of C2 synthesis (3). Why the rates of synthesis changed is not clear.

Recently a soybean oil emulsion (Intralipid) has been released in the United States for use as a parenteral nutrient. Biopsy and autopsy studies of patients who received this material have demonstrated the presence of a fat pigment for up to 5 years in macrophages throughout the reticuloendothelial system (9). Those macrophages are the major site of synthesis of both the second (C2) and fourth (C4) components of C. The effect of Intralipid infusion on C synthesis or serum C levels has not previously been studied. The study reported here was undertaken to determine the effect of ingestion of Intralipid on the synthesis and secretion of C2 and C4 by guinea pig peritoneal macrophages *in vitro*.

MATERIALS AND METHODS

MATERIALS

Tissue Culture. Medium 199, penicillin, streptomycin, and fetal calf serum (FCS) were purchased from Grand Island Biological Company, Santa Clara, CA. Tissue culture dishes were purchased from Falcon Plastics, Oxnard, CA. IL was obtained commercially. Noncommercial emulsions of soybean or castor oil were prepared by sonicating commercial soybean or castor oil with egg yolk phospholipid, which was extracted according to the method of Wells and Hanahan (14). Actinomycin D was purchased from Calbiochem, San Diego, CA. Hydrolyzed starch, cholesterol USP, and L- α -lysophosphatidyl choline were purchased from Sigma Chemical Co., St. Louis, MO.

Complement. Veronal-buffered saline sucrose (VBS-S) (pH 7.35; $\mu = 0.065$) was prepared as described by Rapp and Borsos (11). Sheep erythrocytes were purchased from Bolin Labs, Phoenix, AZ and freshly frozen guinea pig and rat sera from Suburban Serum Labs, Silver Springs, MD.

Miscellaneous. A mixture of uniformly labeled [¹⁴C]isoleucine, leucine, lysine, and valine was purchased from New England Nuclear, Boston, MA, aquacide III B grade from Calbiochem, San Diego, CA, and standard egg white lysozyme and lysozyme substrate (*Micrococcus lysodeikticus*) from Difco Laboratories, Detroit, MI.

METHODS

Preparation of Cell Cultures. Normal, randomly bred Hartley male and female guinea pigs, 500-700 g, were injected ip with 7 ml of a sterile 3% aqueous solution of hydrolyzed starch. After 96 hr the guinea pigs were stunned and killed by exsanguination. The peritoneal cavity was opened aseptically and washed with 125 ml of Medium 199 supplemented with 1 mg glucose/ml, 100 U penicillin/ml, and 100 μ g streptomycin/ml (M199). The peritoneal

exudates (PE) were washed once in M199 containing 10% FCS (inactivated at 56° for 2 hr (M199-FCS) by centrifuging at $350 \times g$ for 10 min at 10°. The average cell yield was 30×10^6 /per animal (range, $15\text{--}70 \times 10^6$) with approximately 80% large mononuclear cells. Total white cells, 4×10^6 , were plated on each tissue culture dish (60-x 15-mm size) and incubated for 2 hr at 37° in humidified 5% CO₂:air atmosphere. Nonadherent cells were removed by washing each plate four times with M199-FCS. Finally, 3 ml M199-FCS alone or M199-FCS containing a dose of IL varying from 2.3–37.5 mg/100 ml were added to each plate. Every 24 hr the tissue culture media were sampled, the plate washed once, and fresh M199-FCS or M199-FCS containing IL was added.

Noninduced peritoneal cells were collected by washing the peritoneal cavities of Hartley guinea pigs that had had no prior injection of starch. The cells were processed as described for those from starch-induced PE.

Measurement of C2 and C4 Synthesis. At timed intervals, 0.1 ml of media was removed from each plate, diluted 1:10 in VBS-S and stored at 0° for a maximum of 48 hr. Titers of extracellular functional complement were estimated by hemolytic titrations for C2 and C4 (10). Because the production of C2 and C4 in control plates varied from experiment to experiment, the effect of IL on C2 and C4 production was determined by comparing total C2 or C4 production in a plate with IL to a control plate in the same experiment.

For measurement of extracellular immunochemical C4, macrophages were grown in medium alone or medium with 37.5 mg IL/100 ml, both of which were supplemented with $10 \mu\text{Ci/ml}$ ¹⁴C-amino acids. After 24 and 48 hr aliquots of the labeled media were assayed for immunochemical C4 according to the method of Hall and Colten (7).

Samples for detection of intracellular functional C2 and C4 and intracellular immunochemical C4 were obtained by scraping cells off a plate with a rubber policeman, pelleting the cells at 0° and washing twice with cold VBS-S. The cells were resuspended in 1 ml VBS-S, sonicated for 10 sec (40% duty cycle) with the microtip of a Branson Sonifier model W-350 (Danbury, CT) and stored at 0° for no more than 48 hr. Sonication was found to have no effect on the hemolytic activity of C2 or C4 or the amount of immunochemical C4.

Light Microscopy. To detect morphologic differences between macrophages exposed to IL and normal cells, PE cells were plated on untreated 19-mm circular cover slips and incubated in various media. After 48 hr the cover slips were removed from the media and the cells fixed in phosphate-buffered 3.0% glutaraldehyde for 1 hr at 4° and washed in 0.1 M PO₄ buffer containing 10% sucrose. After being stained for light microscopy with standard Papanicolaou's stain, the cells were categorized by shape (round or non-round) and the number of pseudopodia.

Cell Survival and Viability. The number of adherent large mononuclear cells per plate was determined by scraping the cells from the plate into phosphate-buffered saline and counting with a haemocytometer. The intraexperiment variation in the number of adherent macrophages per plate was 9%. The interexperiment variation in the numbers of cells attached to the plate after the initial 2-hr incubation was $0.77\text{--}2.09 \times 10^6$. Viability was tested by incubating the adherent cells for 10 min in a 0.04% trypan blue solution in saline. Cells considered to be nonviable were those in which the nucleus clearly had taken up the blue dye, a method described by Hard (8).

Total Protein Synthesis. Incorporation of ¹⁴C-amino acids into trichloroacetic acid precipitable proteins was measured as described by Colten and Borsos (4) to calculate total protein synthesis.

Measurement of Lysozyme (EC 3.2.1.17). Media or cells from several plates of macrophages fed IL-containing media or M199-FCS alone were collected and pooled at 24-hr intervals. Cells were washed twice with phosphate-buffered saline, suspended in 1 ml, and sonicated. Media were concentrated using standard dialysis

tubing and Aquacide III B grade. Lysozyme was measured with a lysoplate method (15). Additional assays showed no lysozyme activity in either fresh M199-FCS or M199-FCS with IL.

RESULTS

EFFECT OF IL ON C2 AND C4 PRODUCTION

The effect of IL on the production of hemolytically active C2 and C4 by adherent PE cells is shown in Figure 1. Incubation of the cells with concentrations of IL from 2.3–37.5 mg/100 ml resulted in a significant decrease in the production of both C2 and C4, which could not be explained by variability between plates. This effect was seen at 24 as well as 48 hr for all levels of IL, except for 2.3 mg/100 ml at 24 hr when the decrease of C4 was not significant and the decrease of C2 reached only borderline

Fig. 1. Effect of IL on C2 (A) and C4 (B) production through 24 (●) and 48 (○) hr. Data obtained by comparing total C2 or C4 synthesis for a plate with IL to a control plate in the same experiment. Data are represented as mean $\pm$ SE. All decreases were significant, at least at the 0.01 level except 2.3 mg/100-ml C4 (24 hr), not significant and C2 (24 hr) $P < 0.05$. Data for 0 mg/100 ml represent the intrinsic variation in C2 or C4 production independent of IL and were obtained from comparison of duplicate control plates within single experiments. *Number of experiments at each IL concentration is in parentheses.