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EFFECT OF LIPOPOLYSACCHARIDE ON C3 AND C5 PRODUCTION BY HUMAN LUNG CELLS[1]

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Although studies to date have demonstrated the ability of the monocyte/macrophage to produce C components in vitro, very few studies on C production by nonhepatic tissue cells have been reported. Recently, using ^{35}S-methionine incorporation and immunoprecipitation techniques our laboratory has demonstrated the ability of tissue cells, i.e., the human lung type II pneumocyte (A549) and human lung fibroblast (WI-38), to synthesize and secrete a variety of early and terminal complement components, as well as several regulatory proteins in vitro, i.e., Clr, Cls, C4, C3, C5, C6, C7, C8, C9, factor B, factor H, factor I, and Cls inactivator. In our studies, we extended these observations by demonstrating the capability of LPS to modulate C3 production by A549 pneumocytes. Specifically, using a sensitive ELISA we demonstrated that A549 pneumocytes exposed to LPS induced an 80 to 180% increase in C3 levels when compared to untreated A549 cells. Interestingly, LPS had no effect on C5 production or total protein synthesis by A549 pneumocytes. In the case of the WI-38 fibroblast, LPS had no effect on 1) C3 production, 2) C5 production, or 3) total protein synthesis in vitro. These studies demonstrate that agents such as LPS have the potential to selectively regulate C production (i.e., C3) in individual lung cells in vitro, and suggests that in vivo LPS may alter the local tissue reservoir of C components during infection and lung injury, thus impacting on pulmonary inflammation and host defense.

Although previous studies have focused on various aspects of C component production by inflammatory cells (i.e., monocyte/macrophage), very little is known about the capability of normal or injured tissue cells to produce C components in vivo or in vitro (1). Specifically, studies on tissue cell production of C have demonstrated: 1) epithelial cells from the gastrointestinal and genitourinary tracts produce C1 (2); 2) fibroblasts can synthesize C1, C3, factor B and factor H (3–7); and 3) lung macrophages produce C2, C4, C3, and factor B (8–10). Our laboratory has studied complement production (i.e., synthesis, secretion, and metabolism) by prototypes of tissue cells, i.e., the lung type II pneumocyte (A549) and lung fibroblast (WI-38) (11–13). Using ^{35}S-methionine incorporation and immunoprecipitation of specific C components we demonstrated the capability of these lung cells to synthesize and secrete numerous C components and regulatory proteins including Clr, Cls, C4, C3, C5, C6, C7, C8, C9, factor B, factor H, factor I, and Cls inactivator in vitro (11). Additionally, we have further characterized the physical and antigenic nature of the C3 and C5 produced by these cells, as well as the kinetics of C3 and C5 production (11). These studies revealed four important findings: 1) in vitro, the type II pneumocyte and lung fibroblast can synthesize and secrete a wide variety of early and terminal C components; 2) these lung cells synthesize and secrete a number of C components (e.g., C6, C7, C8, or C9) which are not thought to be produced by the monocyte/macrophage; 3) type II pneumocytes produce significantly greater amounts of C3 (20-fold), as quantitated by an ELISA, than that reported for the monocyte (14); and 4) using ^{35}S-methionine incorporation and immunoprecipitation techniques, the C3 and C5 produced by the type II pneumocytes is antigenically and physically (i.e., m.w.) identical to serum C3 and C5 (11). Recent studies by Strunk et al. (15) using primary cultures of type II pneumocytes have confirmed our observations with the A549 pneumocyte, i.e., human type II cells synthesize C2, C4, C3, C5, and factor B. These data not only demonstrate the in vitro ability of tissue cells to produce C but clearly underscore the possible impact of the C components produced by lung cells to the local C reservoir within lung tissue. Additionally, these studies suggest that compartmentalization of local C production by individual lung cell and inflammatory cell populations may occur, i.e., different C components being produced by different cell types. Thus, local tissue C levels likely represent the result of the contribution of not only humoral C components, but also tissue and inflammatory cell-derived C components.

Clearly, alterations in the balance of tissue C levels could result from stimulation and/or suppression of C component synthesis, secretion and/or metabolism within individual cell populations. Thus, agents or factors that enhance or suppress production of C components by recruited or tissue cells would likely have a significant impact on tissue C levels and the resulting tissue inflammation and host defense. Endotoxin (LPS) has been demonstrated to induce pulmonary inflammation, activate the C system, and alter C synthesis in the

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monocyte/macrophage (16). In our studies we examined the effects of LPS on C production by type II pneumocytes and lung fibroblasts. These studies demonstrate that type II pneumocytes exposed to LPS produced 80 to 180% greater C3 levels in comparison to untreated cells. Interestingly, LPS had no effect on C5 production or protein synthesis by type II pneumocytes. Additionally, we demonstrated that LPS could not regulate C3 or C5 production by human lung fibroblasts. Thus, these studies not only demonstrate the capability of LPS to selectively regulate complement production (i.e., C3) by type II pneumocytes, but that this regulation may be unique to the type II cell, because it does not occur in the lung fibroblast. These observations suggest that during pulmonary disease associated with gram-negative bacterial infection, resident lung cells (such as type II pneumocytes) may contribute to pulmonary host defense and inflammation by directly altering tissue levels of important C components such as C3.

MATERIALS AND METHODS

A549 and WI-38 cell cultures. Passage 76 human lung type II pneumocyte cell line (A549: ATCC CCL 185), and passage 13 human lung fibroblast cell line (WI-38: ATCC CCL 75) were purchased from American Type Culture Collection (Rockville, MD). The A549 cell line was maintained in F12 Ham's medium (Kansas City Biological, Kansas City, MO) and the WI-38 cell line was maintained in Eagle's MEM (Kansas City Biological). Both types of media were supplemented with 15% FBS[3] (Sterile Systems, Inc., Logan, UT), 20 μg/ml gentamicin, 0.5 μg/ml amphotericin B, 0.1 mM nonessential amino acids, 4 mM glutamine, and 1 ml/100 ml medium of MEM vitamins (100×) (GIBCO, Grand Island, NY). Routine subcultures for A549 pneumocytes (passage 76 to 94) and WI-38 fibroblasts (passage 13 to 22) were done at 1:3 split ratios by incubation with 0.05% trypsin 0.02% ethylene diaminetetraacetic acid in calcium-magnesium free HBSS (GIBCO) for 5 to 9 min at 37°C. Cell cultures were incubated at 37°C in a humidified incubator with 5% CO_2 in air.

Cell culture conditions for sample collection. The A549 and WI-38 cell lines were grown in 75 cm^2 tissue culture flasks (Falcon, Oxnard, CA) in the appropriate complete media described above. Confluent WI-38 fibroblast cultures (1×10^7 cells/flask) or confluent A549 pneumocyte cultures (1×10^7 cells/flask) were used for all studies. C production experiments were begun by rinsing the confluent cell cultures three times with 10 ml sterile saline, followed by the addition of 10 ml of serum free or 15% FBS supplemented media with or without LPS. These flasks were then incubated at 37°C in a humidified incubator with 5% CO_2 in air for 11 days. On day 1, 3, 5, 7 and 9, a 5-ml sample of medium from each cell culture flask was collected and replaced with 5 ml of the appropriate fresh medium. On the last day of culture (i.e., day 11), the total cell culture supernatant was harvested. All culture supernatant samples were centrifuged at 400 ×*g* for 10 min to remove cellular debri then divided into 1-ml aliquots and stored at −70°C.

Cell culture with LPS. Experiments examining the effects of LPS on C production by lung cells were performed by adding 100 μg/ml TCA extracted LPS from *Escherichia* serotype no. 055:B5 (LPS) (Sigma Chemical Co., St. Louis, MO).

Antiserum. The IgG fraction of goat anti-human C3 was purchased from Cappel Laboratories (Malvern, PA). Rabbit anti-human C5 antibody was prepared by immunizing rabbits subcutaneously with 250 μg purified human C5 (Calbiochem Biochemicals, San Diego, CA) in CFA (Difco Laboratories, Detroit, MI) (17). Serum was isolated from rabbit blood and tested for anti-human C5 antibody titer by double immunodiffusion analysis. Both normal human serum and C5 deficient human serum (18) were used to validate the specificity of the anti-human C5 antiserum. Rabbit anti-human C5 IgG was purified using Protein A Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), and assayed for purity by immunoelectrophoresis against purified C5, normal human serum, or C5-deficient human serum. Alkaline phosphatase conjugated to antiserum or Ag was prepared by the method of Voller et al. (19). The enzyme preparation used was alkaline phosphatase, Sigma type VII-S of sp. act. 1000 U/mg.

[3] Abbreviations used in this paper: FBS, fetal bovine serum; LDH, lactate dehydrogenase; SI, stimulation index.

ELISA for human C3 Ag. The immunoassay to quantitate human C3 Ag was performed in 96-well flat-bottom microtiter plates (Flow Laboratories, McLean, VA) using a competitive ELISA technique (11, 20, 21). The lower limit of detection for this ELISA was 50 ng C3/ml. Because the A549 pneumocyte and WI-38 fibroblast cells were grown in media containing 15% FBS, the cross-reactivity of the FBS was tested in this ELISA and was found to have no detectable cross-reactivity.

ELISA for human C5 Ag. The immunoassay to quantitate C5 Ag was performed in 96-well flat-bottom microtiter plates (Flow Laboratories, McLean, VA) using a competitive ELISA procedure (11, 20). The lower limit of detection for this ELISA was 50 ng C5/ml. Because the A549 pneumocyte and WI-38 fibroblast cells were grown in media containing 15% FBS, the cross-reactivity of the FBS in this ELISA was tested and no significant cross-reactivity was found.

Functional assay for human C5. A hemolytic assay to quantitate functional C5 (C_5H_{50}) was performed using antibody-coated sheep E in veronal-buffered saline with 0.1% gelatin, 0.15 mM Ca^{++}, and 1.0 mM Mg^{++}. Generally, 250 μl of purified C5 (Calbiochem Biochemicals, San Diego, CA) or test sample was added to 250 μl of a 4.0% solution of C5-deficient serum (18). Next, 125 μl of antibody-coated sheep E (3.33×10^7 cells/ml) was added to the mixture, followed by a 60-min incubation at 37°C. After incubation, 1.0 ml isotonic saline was added and the samples were centrifuged at 450 ×*g* for 6 min. Hemolysis was determined by evaluation of the OD at 412 nm of the resulting supernatants. Results were calculated by comparing the dilution at which 50% RBC lysis occurred in relation to the highly purified C5 which was used as the standard in each assay. The 50% lysis point for this assay was approximately 1 to 5 ng C5/ml, and no cross reactivity between human C5 and FBS was detected using this functional assay.

Protein synthesis by A549 pneumocytes. To incorporate radioactive amino acids into proteins synthesized by A549 pneumocytes, the cells were grown to confluence in 48-well plates and maintained in serum-free, methionine-free F12 Ham's medium for 16 h in the presence of 50 μCi/ml L-[^{35}S]methionine (1120 μCi/mmol, New England Nuclear, Boston, MA) and LPS. At selected times in culture, after exposure to LPS, cell supernatants were collected, centrifuged 400 ×*g* for 10 min, aliquoted, and stored at −70°C. These culture supernatants were used for analysis of total protein synthesis using TCA precipitation, i.e., equal volumes of [^{35}S]methionine labeled cell culture supernatant and 10% TCA, and 1% albumin as carrier protein, were incubated at 4°C for 30 min and then centrifuged at 400 ×*g* for 20 min to precipitate [^{35}S]methionine incorporated proteins.

Protein assay. A549 pneumocyte and WI-38 serum free culture supernatants were analyzed for total protein content using a microprotein assay with a sensitivity range of 1 to 10 μg protein/0.1 ml as described by Bradford (22).

Cell viability. Cell viability was examined by uptake of vital dye Erythrocin B (J. T. Baker Chemical Co., Phillipsburg, NJ) or Trypan Blue (GIBCO) and visualized by light microscopy. In addition, LDH levels in cell culture supernatants were also used as an indicator of cell death or cell integrity (23). All LDH data was expressed as the percent of total LDH, where total LDH equaled the LDH released into the supernatant from 1×10^7 A549 pneumocytes after exposure to Triton X-100. The percent LDH was calculated using the following equation:

$$\% \text{ LDH} = \frac{\text{LDH content in experimental culture supernatant}}{\text{Total LDH}} \times 100$$

Data analysis. Quantitation of absolute Complement component levels in individual cell culture supernatants were determined by direct comparison to the standard curves of known C3 and C5 Ag in the appropriate ELISA system and the final data were expressed as ng/ml.

Quantitation of the total accumulation Complement component levels in each culture supernatant were determined by using the equation below and the final data were expressed as ng/ml:

$$Y_n = X_n + \sum_{j=1}^{\frac{n-1}{2}} \frac{X_{(2j-1)}}{2}$$

n, days in culture, i.e. day 1, 3, 5, 7, 9 or 11; X_n, absolute component protein detected in a culture supernatant harvested on day n; Y_n, total accumulated complement protein produced by cells in culture from day 0 to day n.

All data are expressed as the mean ± SEM, and statistical signif-

icance of the data was determined using the unpaired Student's *t*-test (24). SI was calculated using the following general equation:

$$SI = \frac{\text{experimental value}}{\text{control value}}$$

SI_R, SI for radioactive amino acid incorporation; SI_{C3}, SI for antigenic C3; SI_{C5}, SI for antigenic C5; and SI_{C5f}, SI for functional C5.

Percent functional C5 levels were determined using the following equation:

$$\% \text{ functional C5} = \frac{\text{total accumulated functional C5 (ng/ml) on day n}}{\text{total accumulated antigenic C5 (ng/ml) on day n}} \times 100$$

RESULTS

Effect of FBS on C component production by A549 pneumocytes. During the course of our initial investigations on C component production by type II pneumocytes we noted that different lots of FBS influenced the absolute amount of the third (C3) and fifth (C5) C components produced by A549 pneumocytes in vitro. To control for this effect in our studies, we cultured A549 pneumocytes in two different lots of FBS for 11 days and analyzed culture supernatants obtained on days 1, 3, 5, 7, 9, and 11 for antigenic C3 and C5 levels by ELISA. These studies demonstrated that lot no. 2 stimulated a significantly greater rate of antigenic C3 production when compared to the stimulatory effect of lot no. 1, i.e., 335 vs 135 ng C3/ml/24 h. Additionally, baseline C3 production in serum free medium was similar in all the studies, i.e., cultures of A549 pneumocytes maintained in serum-free medium produced antigenic C3 at similar rates 62 ng C3/ml/24 h (lot no. 1 studies) vs 70 ng C3/ml/24 h (lot no. 2 studies). Further investigations with a third lot of FBS revealed similar effects on C3 production by A549 cells as described for lot no. 2 (data not shown). Similar data were obtained when these same A549 pneumocyte culture supernatants were analyzed for C5 production. Specifically, lot no. 2 stimulated antigenic C5 production at a rate of 320 ng C5/ml/24 h in comparison to the rate of 245 ng C5/ml/24 h determined for lot no. 1. Once again control cultures of A549 pneumocytes cultured in serum-free medium produced antigenic C5 at similar rates (148 ng vs 153 ng C5/ml/24 h). Interestingly, although there were detectable variations in the absolute amount of C3 and C5 produced by A549 pneumocytes cultured in different lots of FBS, the absolute amounts of C3 and C5 produced by A549 pneumocytes cultured in an individual lot of FBS remained consistent from experiment to experiment. Additionally, when experimental results were expressed as SI the modulation of C component production during exposure to LPS was constant, with less than 10% variation under any experimental condition, regardless of the lot of FBS used (see below). Additionally, baseline C3 and C5 production in serum-free medium was similar in all the studies.

Toxicity of LPS on A549 pneumocytes. Although LPS modulation of C production by monocyte/macrophage has been demonstrated (16), only recently has a single study on the effects of LPS on C3 or C5 production by tissue cells been reported (15). Therefore, the effects of LPS on C component production by human type II pneumocytes in vitro was studied by culturing A549 pneumocytes in the presence of LPS. To begin these studies, we evaluated the viability of A549 pneumocytes cultured in the presence or absence of LPS. Morphologically, A549 pneumocytes cultured in the presence of FBS with or without LPS (10 to 500 μg/ml) remained a tightly packed epithelial cell monolayer. A549 cells cultured in the absence of FBS, appeared spindle shaped and irregular, and addition of LPS (10 to 500 μg/ml) to serum free cultures did not alter cell morphology (data not shown). In addition, A549 pneumocytes cultured in the presence of LPS displayed excellent cell viability and cell integrity as determined by: 1) exclusion of vital dyes; 2) no significant release of total LDH during LPS exposure, e.g., the total LDH release from A549 pneumocytes cultured in serum-free medium alone was 1.26 vs 3.77% total LDH release by A549 cells cultured in serum free medium and LPS (100 μg/ml) on day 3, with no additional increase above control levels detected on day 7; and 3) electron microscopic analysis which showed A549 pneumocytes exposed to LPS (100 μg/ml) had normal ultrastructural characteristics, similar to untreated cells (data not shown).

LPS and C3 production by A549 pneumocytes. To begin our studies on LPS modulation of C production by type II pneumocytes in vitro, we initially examined the effects of LPS concentration on C3 production by A549 pneumocytes. Figure 1 demonstrates that various concentrations of LPS stimulated increased production of total accumulated antigenic C3 by A549 pneumocytes during 11 days in culture, and that on day 3 of culture maximum stimulation of C3 was detected for all concentrations of LPS examined. Although there appeared to be a dose-dependent increase in the stimulation of C3 by A549 pneumocytes in response to various concentrations of LPS, 100 μg/ml LPS was used in all the following studies because 100 μg/ml LPS was the lowest concentration analyzed that resulted in the most constant stimulation response during 11 days in culture.

We further investigated the effects of LPS (100 μg/ml) on the kinetics of C3 production by A549 pneumocytes by analyzing culture supernatants obtained on day 1, 3, 5, 7, 9 and 11 for antigenic C3 levels. As shown in Figure

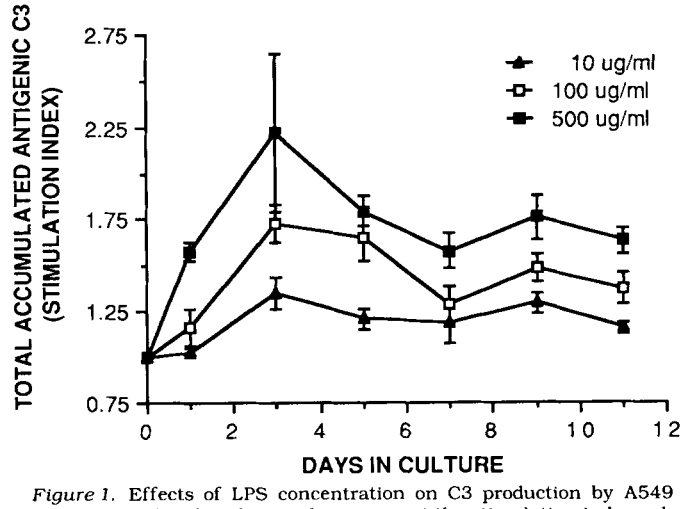

Figure 1. Effects of LPS concentration on C3 production by A549 pneumocytes. The *closed triangles* represent the stimulation index calculated for C3 production (SI_{c3}: mean ± SEM) by A549 pneumocytes cultured with 10 μg LPS/ml ($n = 4$). The *open squares* represent the SI_{c3} for A549 pneumocytes cultured with 100 μg LPS/ml ($n = 4$). The *closed squares* represent the SI_{c3} for A549 pneumocytes cultured with 500 μg LPS/ml ($n = 3$). All data are from culture supernatants obtained from cell cultures maintained in the presence of 15% FBS.

2 LPS stimulated A549 pneumocytes to produce greater absolute and total accumulated antigenic C3 levels, whether the cells were cultured in serum-free or FBS-containing culture conditions. Analysis of data in Figure 2 demonstrated that during 11 days in culture, the rate of antigenic C3 production was 347.2 ± 54.6 ng C3/ml/24 h for A549 cells cultured in FBS containing medium vs 486.3 ± 85.7 ng C3/ml/24 h for cells cultured with LPS. In comparison, A549 pneumocytes cultured in serum-free medium and LPS exhibited a significant enhancement in the rate of C3 production. Specifically, A549 pneumocytes cultured in serum-free medium for 11 days produced antigenic C3 at a rate of 90.3 ± 22.3 ng C3/ml/24 h, which increased to 210.8 ± 33.4 ng C3/ml/24 h when A549 cells were cultured with LPS ($p < 0.02$). Additionally, analysis of nine independent experiments for the SI for antigenic C3 production for each time point demonstrated that A549 pneumocytes cultured in FBS-containing medium and LPS had a maximum $SI_{C3} = 1.83 \pm 0.10$, whereas A549 cells cultured in serum-free medium plus LPS had a maximum $SI_{C3} = 2.78 \pm 0.19$ (Fig. 3).

LPS and C5 production by A549 pneumocytes. A549 pneumocyte culture supernatants were also analyzed for effects of LPS on antigenic C5 and functional C5 production. Generally, LPS had no enhancing or suppressing effects on antigenic C5 levels or functional C5 activity detected in A549 pneumocyte culture supernatants. A549 pneumocytes cultured in serum-free medium for 11 days, with or without LPS, had almost identical rates of antigenic C5 production (116.4 ± 17.6 vs 118.9 ± 19.8 ng C5/ml/24 h), and similar rates of functional C5 production (124.3 ± 7.1 vs 131.1 ± 9.5 ng C5/ml/24 h). Similarly, when A549 pneumocytes were cultured for 11 days in medium supplemented with FBS, with or without LPS, LPS had no effect on the rate of antigenic C5 production (342.3 ± 57.8 vs 314.1 ± 69.2 ng C5/ml/24 h), or on the rate of functional C5 production (284.8 ± 58.0 vs 290.9 ± 47.5 ng C5/ml/24 h). These data are further substantiated by examination of several independent experiments as shown in Figure 3. For example, A549 pneumocytes cultured in serum-free medium plus LPS had an SI for antigenic C5 production of $SI_{C5} = 1.16 \pm 0.04$ ($n = 8$), and an SI for functional C5 production of $SI_{C5f} = 0.98 \pm 0.01$ ($n = 6$) during 11 days in culture. Additionally, analysis of culture supernatants from A549 cells cultured in medium supplemented with FBS and LPS demonstrated that LPS had no effect on either antigenic C5 or functional C5 production, i.e., $SI_{C5} = 0.93 \pm 0.02$ ($n = 9$) and $SI_{C5f} = 0.98 \pm 0.01$ ($n = 6$), respectively. Investigation of the percent functional C5 activity detected in these A549 pneumocyte culture supernatants revealed that LPS had no effect on functional C5 activity during 11 days in culture. For example, on day 5 in culture when maximum functional C5 activity was generally observed, A549 cells maintained in serum-free medium produced 70.0 ± 6.9% functional C5 activity ($n = 6$) and A549 cells cocultured with LPS produced 75.9 ± 4.6% functional C5 activity ($n = 3$). A549 pneumocytes maintained in FBS-containing medium, with or without LPS, also had similar percent functional C5 activities on day 5 (113.1 ± 4.4 ($n = 3$) vs 108.6 ± 14.8 ($n = 4$), respectively).

LPS and protein synthesis by A549 pneumocytes. Our studies demonstrated that LPS significantly stimulated C3 production by type II pneumocytes, but had no effect on C5 production. To further investigate the specificity of this response, and to determine if this response was related to effects on protein synthesis, we examined the effects of LPS on total protein synthesis by A549 pneumocytes. This was accomplished using two methods: 1) analysis of the total accumulated protein levels in serum-free cell culture supernatants using a sensitive protein-dye binding assay, and 2) analysis of the total synthesis of proteins as measured by incorporation of [^{35}S]methionine into TCA-precipitable proteins which is

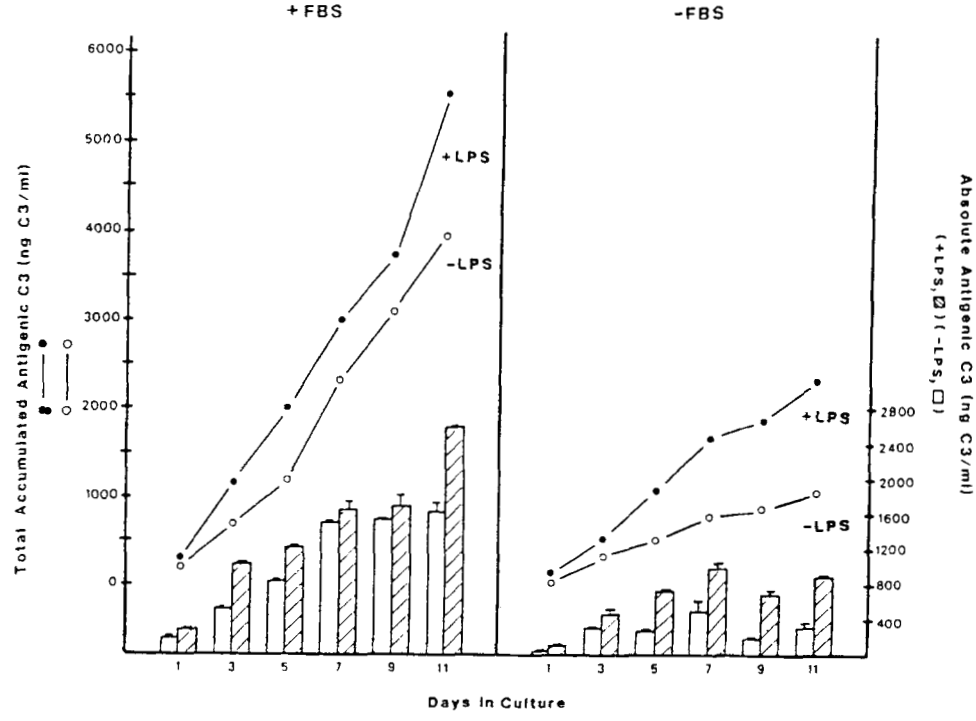

Figure 2. Effect of LPS on C3 production by A549 pneumocytes. The *open bars* represent absolute antigenic C3 levels (ng/ml) detected in culture supernatants obtained from A549 pneumocyte cultures maintained in medium supplemented with or without 15% FBS for 11 days. The *hatched bars* represent absolute antigenic C3 levels (ng/ml) in supernatants obtained from A549 pneumocyte cultures maintained in medium supplemented with or without 15% FBS, and 100 μg/ml LPS for 11 days. The line graphs represent the total accumulated antigenic C3 (ng/ml) produced by A549 pneumocytes under the conditions described above.

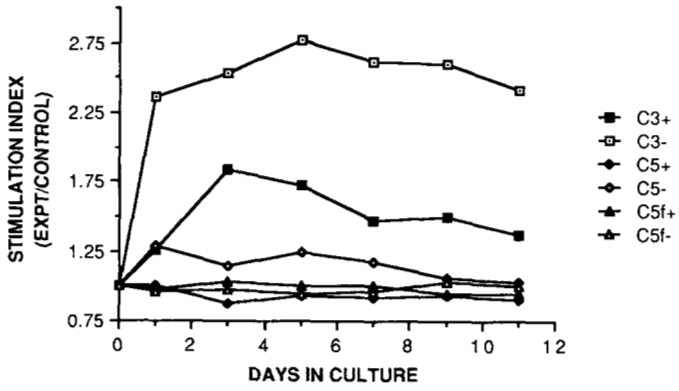

Figure 3. Effect of LPS on C3 and C5 production by A549 pneumocytes. The *open symbols* represent the mean stimulation index (SI) calculated for culture supernatants obtained from A549 pneumocyte cultures maintained in medium without 15% FBS (−) plus 100 μg/ml LPS for 11 days. The line graphs represent SI for antigenic C3 (n = 10), antigenic C5 (n = 8), and functional C5 (n = 6). The *closed symbols* represent the mean SI calculated for culture supernatants obtained from A549 pneumocyte cultures maintained in medium with 15% FBS (+) plus 100 μg/ml LPS for 11 days. The line graphs represent SI for antigenic C3 (n = 9), antigenic C5 (n = 9), and functional C5 (n = 6). The SEM was less than 10% for all data presented.

a direct measurement of protein synthesis. Analysis of total accumulated protein levels in serum-free cell culture supernatants using a sensitive protein-dye binding method demonstrated that A549 cells cultured in serum free medium with or without LPS had identical rates of protein synthesis, 10.3 μg protein/ml/24 h. For the radioactive amino acid incorporation studies, A549 cells were cultured with LPS in serum-free conditions for 5 days and given a 16-h pulse period with [^{35}S]methionine. The results of these studies demonstrated that the SI of radioactive amino acid incorporation for LPS-treated A549 cells was $SI_R = 0.83 \pm 0.02$ (n = 4). A549 cells cultured with LPS in FBS containing conditions had a stimulation index of $SI_R = 1.01 \pm 0.01$ (n = 3). Inasmuch as alterations in the C3 and C5 levels in these cultures could be influenced by C3/C5 catabolism (i.e., proteolytic degradation), studies to address the stability of these C proteins were undertaken. For these studies, ^{125}I-labeled C3 or C5 was added to control and LPS-stimulated cell cultures maintained in the absence and presence of FBS. After 6 days in culture, the culture supernatants were analyzed by SDS-PAGE and the radiolabeled C3 and C5 were found to be intact and not fragmented, documenting the stability of these C proteins. These observations suggest that LPS stimulation of C3 production by A549 pneumocytes may be a reflection of specific effects on: 1) induction of C3 gene expression; 2) stabilization of C3 mRNA levels; or 3) increased C3 secretion (i.e., posttranslational effects), and not a result of generalized effects on the cells' protein synthetic capabilities or proteolytic degradation. Regardless of the exact mechanism(s), it is clear that LPS selectively enhances production of C3, but not C5 by A549 pneumocytes.

Effect of LPS on C component production by lung fibroblasts. To determine if the effects of LPS on C component production by A549 pneumocytes were specific to this cell population or generalized effects of LPS on all human lung cells, we evaluated the effects of LPS on human lung fibroblast production of C3 and C5. Confluent cultures of WI-38 fibroblasts were maintained for 11 days in serum-free medium or FBS-containing medium, with or without LPS. Addition of LPS did not alter the morphology of the WI-38 fibroblasts as determined by phase contrast microscopy. Analysis of C3 and C5 production demonstrated that LPS did not stimulate or suppress C3 or C5 production by WI-38 fibroblasts, e.g., WI-38 cells cultured in FBS had a $SI_{C3} = 1.09 \pm 0.07$ (n = 12), and a $SI_{C5} = 1.02 \pm 0.04$ (n = 12) during 11 days in culture (i.e., days 1, 3, 5, 7, 9, and 11). Thus, LPS at a concentration of 100 μg/ml does not appear to be an agent that regulates C component production by all human lung cells in vitro.

DISCUSSION

Our understanding of the importance of the C system in host defense and inflammation has grown significantly from the original observations over a century ago that serum contained heat labile proteins which were bactericidal. Today, the C system is seen as a complex cascade of biochemical reactions which not only play a central role in host defense and inflammation, but have been implicated as regulatory molecules in immune responses, as well as the coagulation pathway (25). Inasmuch as the activation of the C system is dependent on a variety of biochemical and enzymatic reactions, the functional expression of the C system is clearly controlled by both the absolute amounts, as well as relative concentrations, of the various C components and regulatory molecules present within the tissue. Previously, control of the tissue levels of individual C components was generally believed to be a result of only changes in vasopermeability. More recently, questions have been raised about the existence and significance of local C component synthesis and secretion in the regulation of host defense and inflammation within tissues (26). Generally, these investigations into cellular synthesis and secretion of C components have been limited to the monocyte/macrophage (27). These studies have demonstrated that the monocyte/macrophage has the capability to synthesize many C components and regulatory proteins, including C1, C4, C2, C3, C5, factor B, factor D, factor H, factor I, properdin, and C1 inhibitor (28–31). Interestingly, the monocyte/macrophage has not yet been demonstrated to produce the terminal components of the C system, i.e., the membrane attack complex (C6, C7, C8, C9) (32). More recently, studies have described a variety of factors or agents that can regulate the ability of the monocyte/macrophage to produce C components in vitro, e.g., Ag-sensitized SRBC, immune complexes, LPS, and IFN-γ (16, 29, 33, 34). Thus, these studies clearly demonstrate the ability of at least one population of inflammatory cells to synthesize and secrete numerous C components in vitro, and that this production can be regulated by various factors. Based on these data various investigators have suggested that in vivo, local production of these C components within the tissue may play an important role in controlling host defense, inflammation, and tissue repair.

Although these studies have provided important insights into the potential contribution of recruited cells to local concentrations of C components, very little is known about the contribution of tissue cells to local C levels. To begin to fill this important gap area, our laboratory has recently demonstrated the capability of human type II pneumocytes, i.e., primary cultures and a continuous cell

line, to synthesize and secrete C3 and C5 in vitro (11–13). Additionally, these studies also demonstrated the ability of human type II pneumocytes (A549) and human lung fibroblasts (WI-38) to synthesize and secrete a number of other C components in vitro, e.g., Clr, Cls, C4, C6, C7, C8, C9, factor B, and the regulatory proteins factor H, factor I, and Cls inactivator (11). Recent studies by Strunk et al. (15) using primary cultures of human type II pneumocytes have supported our observations on C production (i.e., C2, C4, C3, C5, and factor B) by pneumocytes. Comparison of our in vitro studies on C production by A549 pneumocytes with studies of the monocyte/macrophage indicates: 1) that A549 pneumocytes and WI-38 fibroblasts produce 20- to 50-fold more C3 (antigenic) than isolated human mononuclear cells, and 2) A549 pneumocytes and WI-38 fibroblasts produce 10–20 fold more C3 (antigenic) than the human monocyte-like cell line U937 (14). These data clearly indicate that on a per cell basis, the type II pneumocyte and fibroblast likely represent a major source of C component production within the lung. These data assume additional importance in light of the fact that: 1) within the normal lung alveoli, tissue epithelial cells are far more numerous than alveolar macrophages, and 2) that the number of type II pneumocytes markedly increases as a result of lung injury (35–39). Therefore, in addition to alveolar macrophages, it is likely that in vivo type II pneumocytes and fibroblasts may represent a significant source of C components within the lung.

Our present studies have focused on the capability of LPS to regulate C component production by lung cells. Initially our studies demonstrated that A549 pneumocytes cultured in medium supplemented with FBS produced greater levels of C3 and C5 when compared to cells cultured in serum-free medium. This difference is likely a result of nutrional effects of the FBS, as well as the effects of hormones, growth factors, or cytokines in the FBS, which may have an influence on C production by the cells. Additionally, our studies demonstrated that although LPS had no effect on C3 and C5 production by WI-38 fibroblasts, LPS stimulated a two- to threefold increase in C3 production by A549 pneumocytes. Specifically, the observation that LPS stimulated a greater increase in C3 production by A549 pneumocytes cultured in serum free conditions compared to cells in FBS conditions may be a result of: 1) lower baseline production of C3 by A549 pneumocytes cultured in serum free medium vs FBS containing medium, and/or 2) LPS inactivation by factor(s) in FBS or factors secreted by A549 pneumocytes cultured in the presence of FBS. The possibility that LPS may be inactivated by factors in FBS is supported by studies of Ulevitch and Johnston (40) which demonstrated that interactions of LPS with serum inhibit a number of endotoxic activities of LPS. Interestingly, although LPS had a stimulatory effect on C3 production by the A549 cell, LPS had no effect on either antigenic or functional C5 production or total protein synthesis. Recently, studies by Strunk et al. (15) demonstrated that LPS stimulated C3 production in primary cultures of type II pneumocytes, but not C5 production. These data demonstrate that the effects of LPS on C3 production were not generalized but appear specific, in that they occurred without a comparable effect on total protein synthesis or on C5 production by the type II pneumocytes. The increase in C3 production induced by LPS in A549 cells may be the result of a specific increase in gene expression and/or secretion of C3. Therefore, although C3 gene expression may be inducible in human type II pneumocytes by LPS, the C5 gene is either not LPS inducible or it is a constitutive gene that is already maximally induced. In several previous studies, in vitro and in vivo regulation of C3 production have been described. For example, Strunk et al. (16) have reported that LPS and specifically the lipid A component of LPS, increased C3 protein synthesis in adult human mononuclear phagocytes, and enhanced factor B synthesis, yet did not increase synthesis of C2, lysozyme, or effect total protein synthesis in these cells. The mechanism responsible for this enhanced C3 synthesis in the monocyte was determined to be largely at the pretranslational level because the concentration of C3-specific mRNA was increased at least fivefold. In other studies, hydrocortisone has been documented as another specific stimulus for C3 production in a rat hepatoma cell line whereas production of C2 and C5 was not effected (41). These observations, as well as our own, support the concept that C3 production can be regulated independently from other component proteins. Recently, Katz et al. (6) have demonstrated the ability of LPS and IFN-γ to increase C2 and factor B synthesis in fibroblasts. Therefore, factors that would enhance the local production of C components, i.e., LPS, would likely enhance local pulmonary host defense. Alternatively, factors that would suppress the local production of C components and regulatory proteins would likely compromise primary lung defense against infection.

Thus, our studies not only demonstrate the ability of LPS to selectively regulate complement production (i.e., C3) by A549 pneumocytes, but that this regulation may be unique to the type II cell, because it does not occur in the lung fibroblast. Additionally, the recent studies by Strunk et al. (15), which suggest that LPS can regulate C3 production, but not C5 production in primary type II pneumocyte cultures support the usefulness of the A549 pneumocyte cell line as a valid model for investigating C production and mechanisms of regulation. Furthermore, the A549 cell line has the advantage of being readily available, as well as lacking other contaminating cells (e.g., macrophages) which can complicate data interpretation when primary cell cultures are studied. Clearly, further studies to delineate the capability of lung cells such as the type II pneumocyte, alveolar macrophage, and fibroblast to synthesize, secrete, and activate factors of the C system, as well as to identify regulatory mechanisms, will likely add to our understanding of the molecular basis of inflammation and host defense in the lungs.

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