Synthesis and Secretion of Cystic Fibrosis Ciliary Dyskinesia Substances by Purified Subpopulations of Leukocytes

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ABSTRACT Cultured peripheral blood leukocytes (PBL) from individuals homozygous or heterozygous for the defective gene causing the inherited disease cystic fibrosis (CF) secrete three different ciliary dyskinesia substances (CDS), which can be detected by their activity in vitro in a rabbit mucociliary bioassay. Their PBL also release substances that promote mucus expulsion and destruction of the ciliated epithelium. In the present study the relative numbers of lymphocytes (T, B, and null), monocytes-macrophages (Mϕ), and polymorphonuclear neutrophils were found to be normal in subjects with the CF gene, as were the responses of their PBL to phytohemagglutinin and pokeweed mitogen. Using purified subpopulations of leukocytes, we obtained evidence that both monocytes and T lymphocytes can secrete CDS in vitro with no requirement for cooperation with other lymphocyte subsets, whereas B and "null" lymphocytes probably require either differentiation or cellular cooperation for optimal secretion of CDS. Mucus expulsion and tissue destruction were produced by substances released primarily from polymorphonuclear neutrophils and secondarily from Mϕ. Using cycloheximide and actinomycin D, we obtained evidence that CDS accumulation requires active protein synthesis and is not dependent on newly synthesized RNA, at least in short-term cultures. Gel filtration chromatography of active culture supernates showed that T lymphocytes synthesized only a CF-specific CDS, whereas Mϕ synthesized all three CDS found in PBL cultures. Evidence is presented that one CDS is related structurally to C3a, since it can be removed with rabbit antisera specific for human C3a.

INTRODUCTION

It has been known for some time that the serum and possibly other body fluids of patients with cystic fibrosis (homozygous for the defective gene) and their parents (obligate heterozygotes) contain components designated "ciliary dyskinesia factors" or "ciliary dyskinesia substances" (CDS)[1] (1, 2) that can disrupt the normal synchronous pattern of ciliary beating of rabbit tracheal explants in vitro and promote markedly increased ciliary beating (fast beat), excessive mucus production from goblet cells, and destruction of the ciliary epithelium as evidenced by the extrusion of single cells (3–6). Recent reports by Conover et al. (7, 8) and Wilson and Fudenberg (9) have also shown that components producing similar effects are secreted by short-term mitogen-stimulated peripheral blood leukocytes (PBL) and long-term lymphoblastoid cell lines in vitro; investigators using bioassays with rabbit trachea or other ciliated tissues have demonstrated that CDS or "ciliotoxic factors" are also secreted by fibroblasts from both cystic fibrosis (CF) genotypes (1, 2, 10–12). The possible role of these CDS in the disease process is not understood, nor have the underlying metabolic defects responsible for CDS accumulation in cell cultures in vitro or in serum in vivo been elucidated. To date no systematic evaluation of cellular

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CDS, ciliary dyskinesia substances; CF, cystic fibrosis; C3a, split product from the third component of complement; E, sheep erythrocytes; MNL, mononuclear leukocytes; Mϕ, monocyte-macrophages; MRBC, mouse erythrocytes; NA, nonadherent, PBL, peripheral blood leukocytes; PHA, phytohemagglutinin; PMN, polymorphonuclear neutrophils; PWM, pokeweed mitogen.

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immune function in CF has been reported, and it is not known which subpopulations of PBL secrete the CDS or are responsible for their catabolism.

As evidenced by their effects in vitro, the CDS could possibly be involved in processes promoting chronic obstructive pulmonary disease, which is a clinical characteristic common to almost all CF patients and the primary cause of morbidity and mortality in individuals with this disorder (1, 2). On the other hand, the CDS could be "protective" or could have no role at all in the primary clinical manifestations of CF, since obligate heterozygotes are clinically asymptomatic even through in vitro their body fluids may contain as much CDS activity as those of CF patients (2, 11, 13).

In the present study we have (*a*) determined which subpopulations of leukocytes can secrete CDS without a requirement for cellular cooperation (a first step in obtaining an isolated leukocyte population for the study of CDS metabolism), (*b*) documented the effects of cycloheximide and actinomycin D on CDS accumulation and determined the rate of CDS accumulation in vitro in mitogen-stimulated and unstimulated cultures, and (*c*) determined the types of CDS secreted by each cell type in PBL cultures. In addition, we have determined the cell types whose products are predominantly responsible for mucus production and cellular extrusion in in vitro systems, and we present data documenting the relative numbers of lymphocytes (T, B, and null), monocyte-macrophages (Mϕ), and polymorphonuclear neutrophils (PMN) in CF patients and obligate carriers.

METHODS

Blood samples. Heparinized venous blood (50 U/ml Panheparin, preservative-free, Abbott Laboratories, North Chicago, Ill.) was obtained from CF patients, obligate heterozygotes for CF, and age-matched normal healthy controls. Informed consent was obtained from each subject (or in the case of minors, from their parents). The research was conducted in accordance with the Declaration of Helsinki, and all aspects of this study were approved by the Human Experimentation Committee of the Medical University of South Carolina. Diagnosis of patients with CF was made according to the criteria used in earlier studies (3, 14).

Rabbit tracheal bioassay. Substances in cell-free culture medium that promoted ciliary dyskinesia or other changes in the ciliary beat of tracheal epithelium were identified with a rabbit tracheal bioassay as described previously (9). Supernates from all cell types evaluated in this study were monitored over a 30-min time period after application of any sample to the tissue. Suitable controls were included to evaluate the effects of culture medium or buffer constituents (9).

Preparation of PBL and fractionation to obtain PMN and mononuclear leukocytes (MNL). PBL were obtained from heparinized blood following dextran sedimentation at 1 *g* as described previously (9). PBL were separated into enriched populations of MNL and PMN (>98% pure) by density gradient centrifugation using lymphocyte separation medium solution (Bionetics) (9, 15). Erythrocytes were removed during the washing procedure by hypoosmotic lysis. The same conditions previously specified (9) were utilized throughout the course of the study for all cell populations unless otherwise stated. Briefly, cells prewashed three times in Dulbecco's phosphate-buffered saline (PBS; Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) were cultured for up to 6 d after resuspension in RPMI-1640 medium supplemented with 1% bovine serum albumin (BSA) (Cohn fraction V; Sigma Chemical Co., St. Louis, Mo.), L-glutamine, penicillin, and streptomycin as 1-ml aliquots containing 10^6 MNL per ml. Stimulants or other additives such as fetal calf serum were included as noted. After culture, replicate aliquots were pooled, centrifuged at 150 *g* for 20 min, filtered through a 0.45-μm filter (Millipore Corp., Bedford, Mass.), and either stored at −70°C or lyophilized and stored at −20°C.

Separation of monocytes and lymphocytes. Mϕ-enriched cell preparations were obtained by adherence to plastic, as described previously (15). The purity of the adherent Mϕ was assessed both cytochemically by staining for acid phosphatase (16) and functionally by determining the percentage of cells able to ingest latex particles (17). The purity of the Mϕ preparations obtained in this study did not differ significantly between test groups. Essentially pure Mϕ populations were obtained (>97%), and in all instances results from cytochemical and functional tests were in close agreement. The nonadherent lymphocytes obtained from the procedure never contained >1–3% monocytes as judged by latex particle ingestion, and many preparations were completely devoid of monocytes.

Purification of T, B, and null lymphocytes populations by rosette sedimentation. T lymphocytes, B lymphocytes, and null cells were obtained from nonadherent MNL after separation of rosette-forming cells and nonrosette-forming cells, employing density gradient centrifugation that followed the general methodology outlined by Wahl et al. (18). In each experiment, unless otherwise stated, those cells which formed rosettes were used for studying the synthesis of CDS by isolated lymphocyte subpopulations. The nonrosette-forming cells at the interface, however, were used as starting material for the formation of another type of rosette (e.g., once E-rosette-forming cells were removed, EAC-rosette-forming cells could then be obtained; see below). An aliquot was always removed to enumerate the percentage of rosette-forming cells before removal of nonrosette-forming cells by density gradient centrifugation. In each instance, erythrocytes were removed by hypoosmotic lysis after isolation of the rosetted cells and after dissolution of the rosettes by incubation at 37°C for 15 min followed by vigorous pipetting. Erythrocyte debris was then removed by carefully washing the isolated cells at 4°C in PBS. Purity of the enriched populations was ensured by re-rosetting the isolated cells as necessary.

T lymphocytes. T lymphocytes were isolated after incubation of nonadherent (NA) MNL with unsensitized sheep erythrocytes (E) to form E rosettes following the method of Jondal et al. (19). Purity of the T lymphocyte (E^+) populations obtained was determined by enumerating the percentages of lymphocytes that phagocytosed latex particles or formed EAC rosettes (see below), or were incapable of binding E.

B lymphocytes. B lymphocytes were purified from E^+-depleted NA-MNL by density gradient centrifugation after incubating the cells with either unsensitized DBA/2 mouse erythrocytes (MRBC) (20) or E sensitized with a subagglutinating amount of rabbit IgM anti-E antibody and C5-deficient AKR mouse serum to form EAC (20, 21). To assess the purity of B lymphocytes, we determined the percentage of cells in each preparation capable of phagocytosing latex particles and of binding to an anti-immunoglobulin column

(Ig^+). The preparation and use of the anti-immunoglobulin column was essentially as described by Chess et al. (22), except that anti-immunoglobulin was used instead of anti-Fab. (Monocytes had previously been removed by adherence, and thus an anti-immunoglobulin column seemed adequate for our purposes.) The percentages of B lymphocytes in NA-MNL as detected by EAC or MRBC rosette formation was documented in several instances by determining the percentage of cells with surface immunoglobulin, using fluorescence microscopy as described by Glassman and Bennett (23).

Null cells. Null cells, or "third population cells" (E^-, EAC^-, $MRBC^-$) were enumerated and purified by density gradient centrifugation after incubating NA-MNL depleted of E^+ and EAC^+ cells with human O, Rh^+ erythrocytes sensitized with serum containing high titers of 7S IgG anti-D human erythrocyte antibody to form a "Ripley rosette" (24). Less than 1% of the cells forming Ripley rosettes were either EAC^+ or E^+ (when unfractionated NA-MNL were used, ~2–4% of the cells were EAC^+).

Cell viability. Cell viability of mixed PBL, MNL, NA-MNL, Mϕ, or isolated subpopulations of NA-MNL was determined by trypan blue dye exclusion. The abilities of certain populations of cells to respond to the mitogens phytohemagglutin (PHA) or pokeweed mitogen (PWM) or to antibody-coated O, Rh^+ human E (for null cells only) and to synthesize CDS (where noted) were used as additional criteria for assessing the viability of the cell preparations.

Mitogens and culture conditions. PHA (purified PHA, Burroughs Corp., Detroit, Mich.) was used as a standard stimulant for all studies with PBL, MNL, NA-MNL, or T lymphocytes; PHA was also added to cultures of B lymphocytes, null cells, and monocytes as a control. PHA was used at 2 μg/ml, as pilot experiments using normal control, CF homozygote, and obligate heterozygote cells indicated that 2 μg PHA per ml was optimal and that all test groups responded to the same extent (Fig. 1). PWM (Gibco Laboratories) was used at an optimal dose of 1:100 for stimulation of B lymphocytes. Null cells were stimulated using a 1:20 ratio of lymphocytes to O, Rh^+ human E sensitized with anti-D antibody. To measure the extent of stimulation, 0.2 μCi of [*methyl*-^{3}H]thymidine (6.0 Ci/mmol sp act) was added to aliquots of each culture 4 h before harvesting. Cultures were harvested at 96 h if PHA or PWM was employed. Null cell cultures were harvested after 6 d unless noted otherwise. Cultures were harvested, washed, and counted essentially as described by Janossy et al. (25).

Effects of actinomycin D and cycloheximide on synthesis of CDS. To study the effects of inhibitors of protein and RNA synthesis on the secretion of CDS, MNL were cultured at 4×10^6 cells/ml in special RPMI medium lacking L-lysine, L-leucine, L-isoleucine, and L-valine and supplemented with 2 μCi/ml ^{3}H-labeled amino acid hydrolysate (yeast, Schwarz-Mann, Div. Becton, Dickinson & Co., Orangeburg, N. Y.), 1% bovine serum albumin, glutamine, antibiotics, and PHA (2 μg/ml) with or without cycloheximide or actinomycin D at several different concentrations for up to 48 h. Total protein synthesis was estimated by determining the amount of 10% trichloroacetic acid precipitable material in the filtered culture supernate as described previously (15). The inhibitors were routinely removed before bioassay by dialysis in Spectrapor 3 dialysis tubing (Spectrum Medical Industries, Los Angeles, Calif.) (9). Medium controls supplemented with each inhibitor were included to evaluate the efficiency of removal of the inhibitors by dialysis.

Gel filtration chromatography. Gel permeation chromatography on Bio-gel P-10 (Bio-Rad Laboratories, Richmond, Calif.) was performed as described previously (9). The column effluent was monitored for protein by recording the absorbance at 280 nm, and the column was calibrated with standards of known molecular weight (Results). Column fractions were processed for testing in the rabbit tracheal bioassay as described previously (9, 14).

Evidence that one CDS may be C3a. We have previously presented evidence that one CDS present in CF serum and culture supernates is structurally similar to C3a, since it is of molecular weight 9,000 (9, 14) and since column fractions from serum containing the 9 000-mol wt CDS activity react with monospecific rabbit antisera to human C3a (14). In the present study, to determine whether any of the CDS in culture supernates were structurally similar to C3a, active culture supernates were incubated with monospecific antibody to C3a prior to gel permeation chromatography on Bio-gel P-10 and then tested again for activity. As controls, aliquots of each sample were also incubated with nonimmune rabbit serum. Antisera were routinely heat-inactivated at 56°C for 30 min before use to destroy complement activity and other serum proteases that are thermolabile.

RESULTS

Quantitation of leukocyte subpopulations in peripheral blood samples from CF genotypes. No significant differences were found between the relative numbers of PMN, MNL, Mϕ, and lymphocytes in peripheral blood from normal controls and from the obligate heterozygote or CF homozygote populations tested; however, CF homozygotes had mean PMN and MNL levels slightly lower and higher, respectively, than normal control levels.

Quantitation of lymphocyte populations (T, B, and null cells) by analysis of surface markers using E, MRBC, EAC, or Ripley rosettes provided evidence that both CF homozygotes and obligate heterozygotes had normal compositions of the major lymphocyte populations (Table I). In no instance was a value obtained for the CF homozygote or obligate heterozygote groups outside the normal range of values. Analysis of the T, B, and null lymphocyte subpopulations for contamination by other cell types (e.g., T cells by B cells, null cells, and monocytes) indicated <1–2% contamination for each population. Analysis of NA-MNL populations for B lymphocyte composition by immunofluorescence agreed closely in each instance with the percentage of B lymphocytes (EAC^+, E^- cells) obtained by EAC rosetting.

Reactivity of cells to stimulation under the culture conditions employed. Figs. 1A and 1B show the values obtained for incorporation of [^{3}H]thymidine into DNA (DNA synthesis; Fig. 1A) or ^{14}C-labeled amino acids into protein (protein synthesis; Fig. 1B) for PHA-stimulated and unstimulated cultures from each of the three test groups. No significant difference was found between CF homozygotes, obligate heterozygotes, and normal healthy controls, regardless of whether fetal calf serum or bovine serum albumin (BSA) was employed as a medium supplement. The highest values were obtained with 1.0% BSA. BSA

TABLE I

Relative Percentages of T, B, and Null Cells, as Determined by Rosette Formation, in CF Homozygotes and Obligate Heterozygotes Compared with Normal Healthy Controls

Lymphocyte subpopulation	Normal healthy controls	CF homo-zygotes	CF hetero-zygotes
T cells (SRBC rosetting)			
Subjects	25	15	20
Mean±SD*	73.3±8.4	65.9±10.0	76.0±5.7
Range*	59.5–88.0	54.0–82.0	66.0–85.0
B cells (MRBC rosetting)			
Subjects	16	8	11
Mean±SD*	6.6±2.0	7.2±1.9	7.1±2.5
Range*	3.5–12.5	5.0–14.0	5.0–12.5
B cells (EAC rosetting)			
Subjects	12	10	14
Mean±SD*	9.7±3.5	11.0±4.0	10.1±2.6
Range*	6.0–16.0	8.0–18.0	7.0–15.5
Null cells‡ (Ripley rosetting)			
Subjects	10	10	9
Mean±SD*	14.7±3.5	13.2±5.0	14.8±4.3
Range*	9.5–18.0	7.0–17.0	10.0–18.0

* Percentage of total peripheral blood lymphocytes.
‡ "Third population" lymphocytes.

at a concentration of 1% was also optimal when PWM was used as a stimulate (data not shown). It seems evident, therefore, that the culture conditions employed did not preferentially affect the results for any one test group.

Synthesis of CDS in MNL cultures. Cell-free supernates from cultures of PBL from CF homozygotes and obligate heterozygotes have been shown previously to promote several changes in the movement of rabbit tracheal cilia. These effects occur in a definite order: (*a*) increased beat frequency, (*b*) ciliary dyskinesis, and then (*c*) ciliostasis. Although reaction (*c*) may not be promoted by all samples, (*a*) and (*b*) are produced by almost all CF and carrier samples. After (*b*) or (*c*), or concomitant with (*c*), tissue destruction often occurs with expulsion of mucus from goblet cells and extrusion of cells from the epithelium (3, 9).

Cell-free culture supernates from 6-d PHA-stimulated MNL from 20 CF patients and obligate carriers consistently promoted reactions (*a*) and (*b*), whereas only five of the same samples promoted ciliostasis (Table II). PWM also promoted activation of MNL, resulting in the accumulation of components in the cell-free supernate of CF homozygote or obligate heterozygote cultures that caused all of the changes noted above for PHA-stimulated cultures (*a* to *c*). Supernates from normal MNL failed to promote changes in ciliary function whether PHA or PWM was used as a stimulant.

Cell-free supernates from MNL cultures consistently failed to promote obvious expulsion of mucus or destruction of the tissue during the 30-min observation period. Conversely, cell-free supernates from PMN cultured for 6 d with or without PHA or PWM failed to cause any of the early alterations in ciliary function (*a* and *b* above). PMN supernates from 9 of 20 CF or

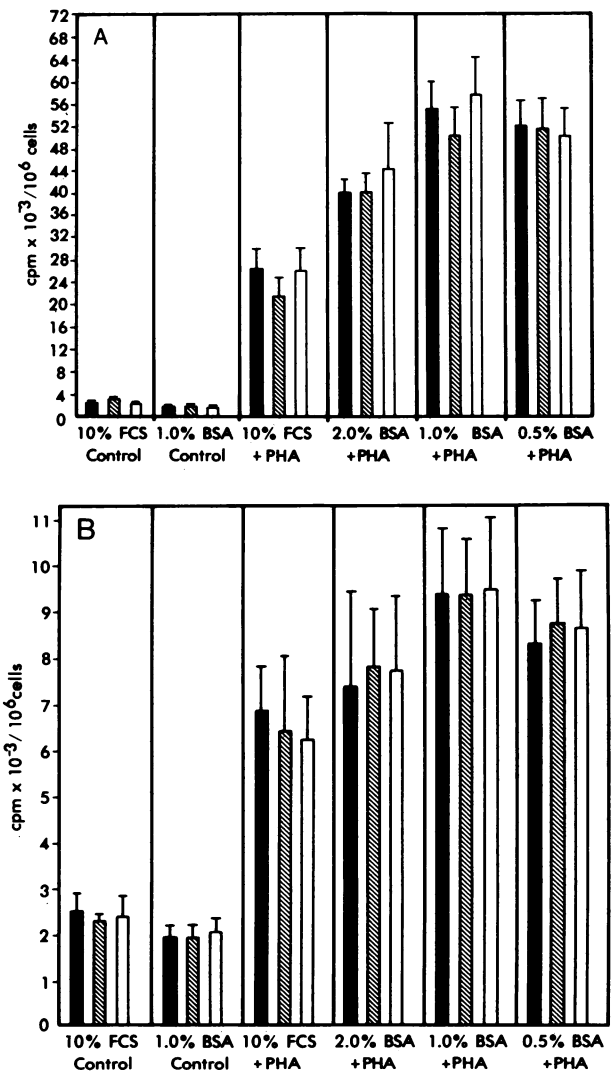

FIGURE 1 Effects of fetal calf serum (FCS) and BSA on the in vitro synthesis of DNA or protein by PHA (2.0 μg/ml) stimulated MNL obtained from CF homozygotes (□) obligate heterozygotes for CF (▨) or normal healthy controls (■). (A) [³H]Thymidine incorporation (counts per minute) into DNA. (B) ¹⁴C-Amino acid incorporation (counts per minute) into protein.

TABLE II

Effects of Cell-free Medium from PBL, MNL, and PMN Cultures on Rabbit Tracheal Epithelium

Source of cultured cells	Number of cultures studied	Cell type*	Number producing effect‡				
			Increased beat frequency	Ciliary dyskinesia	Ciliary dyskinesia and ciliostasis	Mucus expulsion and cell extrusion	No effect
CF homozygote	15	PBL	15	12	3	10	0
	10	MNL	10	7	3	0	0
	10	PMN	0	0	0	5	5
CF heterozygote	15	PBL	15	11	4	8	0
	10	MNL	10	8	2	0	0
	10	PMN	0	0	0	4	6
Normal control	20	PBL	0	0	0	4	16
	11	MNL	0	0	0	0	11
	10	PMN	0	0	0	3	7

* Each cell type was cultured at 10^6 cells/ml for 6 d and stimulated with 2.0 μg/ml phytohemagglutinin.
‡ Changes in ciliary beat of the epithelium within 35 min after addition of the sample. Increased beat frequency was counted as a reaction only if lasting for at least 4 min or directly preceding ciliary dyskinesia.

carrier cultures and 3 of 10 normal cultures did, however, promote obvious expulsion of mucus and release of single cells from epithelial sections (Table II).

To determine whether MNL were actually synthesizing CDS or just releasing preformed material, the effects of actinomycin D and cycloheximide on CDS and protein synthesis were studied. As the results in Table III indicate, cycloheximide at 1.0 μg/ml produced a decrease in the amount of protein synthesized to 11% of that made without inhibitor added, but only a small decrease in viability as compared with untreated cultures (83 vs. 90%). Increased amounts of cycloheximide caused a progressive decrease in viability and a slightly lower level of protein synthesis. Cycloheximide at 1 μg/ml caused a threefold reduction in CDS activity (1:12 dilution vs. 1:4 dilution), and at 10 μg/ml almost completely abrogated CDS accumulation. These results indicate that the majority if not all of the CDS activity in culture supernates was newly synthesized material. In contrast to cycloheximide, actinomycin D seemed to have little effect on CDS accumulation and no effect on protein synthesis which could not be directly accounted for by a proportionate decrease in cell viability (Table III).

TABLE III

Effects of Cycloheximide and Actinomycin D on CDS Production by Mononuclear Leukocytes

Treatment*	Amount	Cell viability‡	Activity§	Total protein‖
	μg/ml	%		
None	—	90	12	9,820
Cycloheximide	1.0	83	3	1,100
Cycloheximide	10.0	75	1	800
Cycloheximide	100.0	45	1	590
Actinomycin D	1.0	60	8	7,920
Actinomycin D	10.0	25	4	3,080

* 4×10^6 MNL/ml from CF patients or heterozygote carriers were cultured for 48 h and stimulated with 2.0 μg/ml PHA.
‡ As determined by trypan blue dye exclusion and by the actual number of cells present in the culture tube at 48 h. Results shown are the mean of five separate cultures.
§ Reciprocal of highest dilution with activity by 35 min. Activity is denoted as the production of an increase in the ciliary beat frequency and the production of ciliary dyskinesia.
‖ Counts per minute per 10^6 MNL. Mean of five determinations is shown.

To determine the rate of CDS synthesis, MNL (10^6 per ml) from five obligate carriers or CF homozygotes were set up as multiple identical aliquots and cultured for 0–6 d. Cultures were stimulated with either PHA (2.0 μg/ml) or PWM (1:100 dilution), or contained no stimulant. Each cell-free supernate was then lyophilized, resuspended at 10 times the original concentration (10×10^6 cell equivalents per ml), and serially diluted down to 0.5 times for testing in the rabbit tracheal assay.

As shown in Fig. 2, by 3 d PHA-stimulated culture supernates promoted ciliary dyskinesia at a mean concentration of IX. The activity increased linearly from 0–4 d for both PHA- and PWM-stimulated cultures. Unstimulated cultures showed markedly less activity, requiring 5 d to accumulate CDS activity equal to that observed for stimulated cultures after 3 d. However, unstimulated reconstituted cultures did show activity

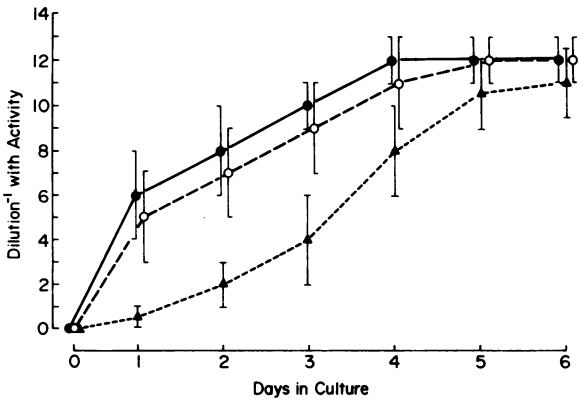

FIGURE 2 Accumulation of ciliary dyskinesia activity in cultures of CF homozygote or obligate carrier MNL (10^6 cells/ml) as a function of time in culture. Cell-free supernates were lyophilized and reconstituted at a 10-fold concentration and then serially diluted for testing. PHA was used at 2.0 μg/ml and PWM at a dilution of 1/100. ●, PHA; ○, PWM; ▲, none.

equal to normal cultures at 1× concentration (10^6 cells per ml) by day 5. Thus, stimulation was not necessary for measurable CDS production.

Synthesis of CDS by subpopulations of MNL. All of 40 T lymphocyte cultures from CF homozygotes or obligate carriers produced fast beat and dyskinesia (Table IV). 16 of 16 obligate heterozygote and 14 of 15 CF homozygote monocyte cultures also produced effects (Table IV); however, monocyte cultures tended to promote ciliostasis in several instances and mucus expulsion and cell extrusion in 17 of 31 experiments. In contrast, purified B lymphocytes and null cells separately failed to produce any changes in the ciliary beat (except in one instance, when an obligate heterozygote null cell culture promoted dyskinesia). The results obtained for B cells and null cells when stimulated with either PWM or O, Rh$^+$ human E (coated with anti-D IgG antibody), respectively, were identical to those obtained with PHA stimulation. Normal control T, B, and null cell cultures in general showed no effect on ciliary motility. Only 1 of 25 T cell and 2 of 20 Mϕ cultures from normal controls caused dyskinesia, which was always accompanied by ciliostasis. 6 of 20 normal control Mϕ cultures did promote mucus expulsion and cell extrusion, and 2 of the 6 were identical to those cultures that promoted ciliary dyskinesia and ciliostasis.

To evaluate the possibility that the failure of B lymphocyte and null cell cultures to synthesize CDS might be due simply to a lack of optimal stimulation or to unusual cell death, representative cultures were evaluated for viability by measuring the incorporation of [^{3}H]thymidine into DNA and by trypan blue dye

TABLE IV

Effects of Cell-free Medium from Monocyte and Lymphocyte Subpopulation Cultures on Rabbit Tracheal Epithelium

Source of cultured cells	Number of cultures studied	Cell type*	Number producing effect‡				
			Increased beat frequency	Ciliary dyskinesia	Ciliary dyskinesia and ciliostasis	Mucus expulsion and cell extrusion	No effect
CF homozygote	18	T	18	16	2	0	0
	6	B	0	0	0	0	6
	5	Null	0	0	0	0	5
	15	MC	14	8	6	8	1
CF heterozygote	22	T	22	22	0	0	0
	4	B	0	0	0	0	4
	5	Null	0	1	0	0	4
	16	MC	16	10	6	9	0
Normal control	25	T	0	0	1	0	24
	7	B	0	0	0	0	7
	5	Null	0	0	0	0	5
	20	MC	0	0	2	6	14

* T, T lymphocytes; B, B lymphocytes; Null, null ("third population") cells; MC, monocytes. All cultures were stimulated with PHA (2.0 μg/ml) for 6 d. Identical results were obtained if MC were not stimulated or if lymphocytes were stimulated with PWM (1/100 dilution) or if null cells were stimulated with O, Rh$^+$ human erythrocytes coated with anti-D IgG antibody.

‡ Changes in ciliary beat of the epithelium within 35 min after addition of the sample. Increased beat frequency was counted as a reaction only if lasting for at least 4 min or directly preceded ciliary dyskinesia.

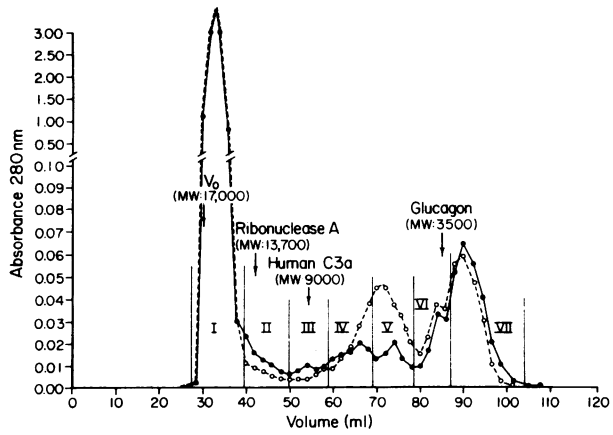

FIGURE 3 Representative chromatogram obtained for Bio-gel P-10 fractionation of 2.0 ml of a 10-fold concentrated solution of cell-free culture medium obtained from either T lymphocytes (○ - - - ○) or monocytes (● —— ●). The column was calibrated with the molecular weight (MW) standards indicated. V_0, void volume. Fractions I–VII indicate pools made for testing in the rabbit tracheal bioassay.

exclusion. The B lymphocytes and null cells were viable (>90%) and showed [^{3}H]thymidine incorporation values indicative of stimulation (but less than that obtained for T lymphocytes). We also tested threefold concentrated culture supernates from CF B cells, null cells, or NA-MNL depleted of E^+ lymphocytes (<1% T lymphocytes); no CDS activity was detected in the rabbit tracheal bioassay. We conclude, therefore, that purified B lymphocytes or null cells do not synthesize significant amounts of CDS under the culture conditions used.

Identification of CDS produced by T lymphocytes and monocytes. After fractionation by Bio-gel P-10 gel permeation chromatography (Fig. 3), T lymphocyte culture supernates from CF homozygotes and obligate heterozygotes showed only one activity, eluting in a fraction of ~4,000–6,000 mol wt (fraction IV-V; Table V). Mϕ cultures containing both ciliary dyskinetic and ciliotoxic activities displayed three peaks of activity (fractions II, III, and IV-V; Table V). Of interest was the fact that both Mϕ and T lymphocytes secreted a CF-specific CDS found in fraction IV-V (9, 14). In contrast, culture supernates from purified B or null cells failed to show activity in any fraction. Also, normal control cultures failed to show any activity regardless of whether T, B, null, or Mϕ cultures were examined (Table V).

The activity in fraction III eluted at the position

TABLE V

Effects on Rabbit Tracheal Ciliated Epithelium Produced by Subfractions Obtained by Chromatography on Bio-gel P-10 of Cell-free Medium from Cultures of Lymphocytes and Monocytes

		Effects produced by*						
		Bio-gel P-10 fraction‡						
Supernate	Crude material	I	II	III	IV	V	VI	VII
CF homozygote								
T lymphocytes	Fast beat, CDA	—	—	—	CDA±	Fast beat, CDA	—	—
Monocytes	Fast beat, CDA, CTA	—	CDA, CTA	CDA	CDA	Fast beat, CDA	—	—
B or null lymphocytes	None	—	—	—	—	—	—	—
CF heterozygote								
T lymphocytes	Fast beat, CDA	—	—	—	CDA	Fast beat, CDA	—	—
Monocytes	Fast beat, CDA, CTA	—	CDA, CTA	CDA	CDA±	Fast beat, CDA	—	—
B or null lymphocytes	None	—	—	—	—	—	—	—
Normal control								
T lymphocytes	None	—	—	—	—	—	—	—
Monocytes	None	—	—	—	—	—	—	—
B or null lymphocytes	None	—	—	—	—	—	—	—

* CDA, CTA; ciliary dyskinetic and ciliotoxic activities observed within 35 min. Fast beat was recorded only if the reaction occurred for at least 4 min or immediately preceding ciliary dyskinesia. All cell-free supernates were tested at double the concentration. Dash indicates no reaction.

‡ Fractions as indicated in Fig. 3.

of the C3a marker. Incubation of active Mϕ culture supernates with rabbit antisera specific for human C3a consistently removed the CDS activity in fraction III, whereas the activity in fraction IV-V was not affected. Prior incubation of active culture supernates with normal nonimmune rabbit serum had no effect on either activity. Elimination of the fraction III CDS activity could not be ascribed to inactivation of this substance by thermostable proteases that may be contained in rabbit serum, since nonimmune rabbit serum failed to remove or destroy the fraction III CDS. These results provide further evidence that the CDS of 9,000 mol wt is structurally similar to C3a, and serve at the very least to distinguish the 9,000 mol wt CDS activity from the CF-specific CDS of 4,000–5,000 mol wt.

DISCUSSION

CF homozygotes and obligate heterozygotes have normal relative percentages of PMN, MNL, Mϕ, and lymphocytes and also normal relative compositions of the major subpopulations of lymphocytes studied, namely, T, B, and null (third population) cells (Table I). Our findings are compatible with the observations that CF is not consistently associated with any particular immunodeficiency syndrome (13) and that CF patients or obligate carriers are not unusually predisposed to systemic infections. We also found that MNL from both CF genotypes respond normally to PHA and PWM when cultured in fetal calf serum or BSA, in general agreement with other investigators (26–30) (Figs. 1A and 1B). We then sought to answer some basic questions concerning the CDS: (*a*) Which cell types are capable of CDS secretion? (*b*) Are they synthesized or just secreted or released from the cell surface? (*c*) Do significant amounts of CDS accumulate in unstimulated cell cultures?

Our studies of PBL subpopulations indicated that only supernates from MNL cultures contained CDS (Table II). PMN cultures did not contain CDS but did contain components that produced mucus expulsion and cell extrusion. When isolated subpopulations of MNL were studied, we found that both Mϕ and T lymphocytes synthesized CDS (Table IV). Mϕ, like PMN, also secreted substances that promoted mucus expulsion and cellular extrusion; we assume that these are lysosomal enzymes or possibly other secretory products (31). Our results using cycloheximide indicated that the bulk of the CDS found in all cultures was newly synthesized (Table III). However, CDS synthesis, at least in short-term MNL cultures, did not require newly formed RNA, since actinomycin D, a suppressor of DNA-dependent RNA synthesis (32), had no effect on CDS synthesis that could not be accounted for by a direct cytotoxic effect of the inhibitor (Table III). In evaluating the kinetics of CDS synthesis (Fig. 2), we observed a rapid increase during the first 96 h in MNL cultures stimulated with either PWM or PHA as compared with unstimulated cultures, thus providing additional evidence that the CDS were newly synthesized. Mitogen stimulation of the cultures was not necessary for CDS accumulation, however, since we also found in agreement with the observations of Conover et al. (7) that unstimulated MNL culture supernatants did contain measurable amounts of CDS.

By using Bio-gel P-10 chromatography, we found that T lymphocytes synthesized only the CF-specific CDS found in fraction IV-V (9, 14; Table V), whereas Mϕ cultures contained all types of CDS initially found in PBL culture supernates (9); i.e., fraction II (~14,000 mol wt), fraction III (9,000 mol wt), and fraction IV-V (5,000 mol wt), thus accounting for the origin of all of these substances (Table V). The possibility that the 9,000-mol wt CDS could be structurally similar to C3a was strengthened by our results showing that this CDS was removed from active supernates by incubation with antisera specific for human C3a. In addition, we have recently completed experiments which demonstrated that this 9,000 mol wt CDS is chemotactic for PMN.[2] Based on its molecular weight, reactivity with anti-C3a antibody and chemotactic activity, we conclude at this point that this CDS is probably identical to a fragment of C3 structurally related to C3a described previously by Ward (33). Alternatively, it may be C3a Des-Arg and could be identical to a ciliotoxic factor affecting oyster gill cilia (34). Additional studies are in progress to resolve its identity. The fact that only Mϕ cultures from CF genotypes contained this "C3a-CDS" activity is not surprising, since only Mϕ are known to synthesize complement components (31). It was surprising, however, that normal Mϕ culture supernates did not contain a similar CDS. Determinations of metabolic abnormalities in CF Mϕ responsible for C3a-CDS accumulation may yield information bearing on the metabolic defects responsible for CDS accumulation in general. Such studies are currently in progress (35).

The fact that both T lymphocytes and Mϕ from CF homozygote and obligate heterozygote carriers synthesized CF-specific CDS, whereas purified B cells and null cells did not (Tables IV and V), warrants an explanation. One possibility is that CF-CDS synthesis may actually be restricted to a subpopulation of T lymphocytes and Mϕ with a common function (such as helper T cells and helper Mϕ, or suppressor T cells and suppressor Mϕ) and that B lymphocytes and null cells do not contain a similar population. Another

[2] Wilson, G. B., H. H. Fudenberg, and E. Floyd. Submitted for publication.

possibility is that B lymphocytes and null cells may actually synthesize the CF-CDS but that optimal synthesis by B lymphocytes may require collaboration with T lymphocytes or Mϕ, whereas synthesis by null cells may first require their differentiation to T or B lymphocytes. Our finding that one obligate heterozygote null cell culture did synthesize CDS (Table IV) could indicate that a large proportion of the null cells from this subject differentiated into T lymphocytes (E^+) after PHA stimulation (35) and began to secrete CDS. Thus, other null cell culture supernates from CF genotypes may also have contained small amounts of CDS, well below the level that could be detected with unconcentrated samples. Further work will be required to fully resolve whether the null cells or only pre-T null cells can secrete CDS. For all MNL subpopulations to have the capacity to secrete CF-CDS would be consistent with the dogma that CF is an autosomal recessive disorder apparently resulting from a somatic mutation affecting all cell types (1, 2). In keeping with this dogma, we would predict that B cells can secrete CF-CDS when fully activated, since long-term lymphoblastoid cell lines synthesize CF-CDS (7, 9) and these are usually rich in immunoglobulin-producing cells (37). In any event, our present results clearly show that Mϕ or T lymphocytes can be used as isolated model cell populations for in vitro studies of the cellular metabolism of the CF-specific CDS and other CDS activities. We are currently investigating the underlying defects responsible for the accumulation of CDS in cultures of these cell types (35, 38).

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