PRODUCTION OF THE SECOND COMPONENT OF COMPLEMENT BY HUMAN MONOCYTES: STIMULATION BY ANTIGEN-ACTIVATED LYMPHOCYTES OR LYMPHOKINES*

By BRUCE H. LITTMAN and SHAUN RUDDY

(From the Division of Immunology and Connective Tissue Diseases, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298)

Human monocytes and animal peritoneal macrophages have been demonstrated to produce various complement components including C2, C3, C4, and properdin factor B (1–3). Production of complement components by monocytes or macrophages is enhanced by prolonged culture on glass cover slips (1) and by phagocytosis of bacteria or bacterial products (2), factors that have been shown to activate macrophages by other criteria (4, 5). Activation of monocytes and macrophages, manifested by increased bactericidal or tumoricidal activity, inhibition of migration, and alterations in size and shape can also be induced by soluble mediators from antigen-stimulated lymphocytes (6). To determine whether or not lymphokine-mediated monocyte activation is associated with enhanced production of complement components, we studied the synthesis of C2 by monocytes co-cultured with lymphocytes with and without specific antigens and by monocytes cultured with lymphokine-rich supernates. We report here that monocytes cultured with lymphocytes in the presence of antigen produce hemolytically active C2 earlier and in larger amounts than do control cultures without antigen. Further, increased C2 production by adherent monocytes is mediated by a soluble substance present in lymphokine-rich supernates from antigen-stimulated cultures but not by supernates from unstimulated control cultures.

Materials and Methods

Cell Separations ana Cultures. Ficoll-separated peripheral blood mononuclear cells were obtained as previously described (7). These cells were washed three times with Hanks' balanced salt solution (HBSS,[1] Microbiological Associates, Bethesda, Md.) and resuspended in medium 199 (TC 199, Microbiological Associates) at 1×10^7 cells/ml. 2-ml cultures of mononuclear cells were performed in 15 × 100-mm round bottom screw cap culture tubes with 1×10^7 cells in 15% heat-inactivated (56°C, 60 min) AB+ serum-TC 199. Either streptokinase-streptodornase antigen (dialyzed Varidase, Lederle Labs, Pearle River, N. Y.) at a final concentration of 50 U/ml or purified protein derivative antigen (kindly supplied by the U. S.-Japan Cooperative Science

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[1] *Abbreviations used in this paper:* HBSS, Hanks' balanced salt solution; MIF, migration inhibitory factor.

Program, National Institutes of Health, Bethesda, Md.) at 10 μg/ml final dilution was added to control culture supernates. C2 was measured in the supernates of these cultures at various times, or the culture supernates were harvested at 48 h and dialyzed against fresh media to serve as a source of lymphokine-rich culture medium.

Purified monocyte monolayers were prepared in 35 × 10-mm plastic Petri dishes by adding 1 ml of mononuclear cells at 10^7 per ml in 10% heat-inactivated AB+ serum-TC 199. These were cultured for 90 min, and nonadherent cells were removed with a Pasteur pipette followed by washing each dish three times with warm HBSS. Replicate dishes were prepared in parallel for DNA determination. Either 2 ml of fresh 15% heat-inactivated AB+ serum-TC 199, dialyzed lymphokine-rich or control supernates, or dilutions of these were added to dishes containing adherent monocytes. All cultures were performed in 5% CO_2 humidified air at 37°C.

In experiments using various sugars to inhibit C2 production, [^{3}H]thymidine incorporation was determined as described previously (7) by transferring cultured cells to microtiter plate wells and pulsing for 18 h with [^{3}H]thymidine (sp act 6.7 Ci/mM, New England Nuclear, Boston, Mass.). The sugars and amino-sugars tested included α-L(−) fucose, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine (Sigma Chemical Co., St. Louis, Mo.).

C2 Determination. 100 μl of culture supernate was removed at various time points from all cultures and usually frozen at −70°C for later use. Hemolytically active C2 was measured using an effective molecule titration (8). The efficiency of the assay was such that it detected 2.5×10^{12} effective molecules in 1 ml normal human serum. Results are expressed as average number of effective molecules of C2 produced per culture or per microgram of DNA.

DNA Assay. A fluorometric assay for DNA was utilized as an index of the number of adherent cells remaining on Petri dishes (1). After removal of nonadherent cells, 2 ml of 2% sodium dodecyl sulfate (Bio-Rad Laboratories, Richmond, Calif.) in deionized water was added to the monocyte monolayer and incubated for 1 h at 37°C, solubilized cells were mixed, and 0.45-ml aliquots were frozen at −20°C for later DNA determinations. Known numbers of cells were similarly treated to determine the amount of DNA per cell. Staining for nonspecific esterase (9) and determination of percentages of phagocytic cells (10) were performed as described elsewhere.

Results

C2 Production by Mononuclear Cells: Antigen Stimulation. In nine separate experiments, 1×10^7 mononuclear cells composed of 80–93% lymphocytes and 7–20% esterase-positive monocytes were cultured with and without antigen. As in the experiment illustrated in Fig. 1 (left), measurable C2 production by unstimulated mononuclear cells was noted by 72 h, and C2 accumulated in the culture supernate linearly thereafter. In contrast, in the presence of specific antigen, C2 was produced earlier and in greater amount. In nine experiments the mean ± SEM accumulated C2 after 96 h of culture (Table I) was $11 \pm 4 \times 10^8$ effective molecules per culture for unstimulated cultures and $44 \pm 8 \times 10^8$ effective molecules per culture for antigen-stimulated cultures ($t = 3.52$, $P < 0.005$). Although this represents a fourfold increase in C2 production, the stimulation by antigen in individual experiments ranged from 1.3 to 34.5 times the C2 produced without antigen; the mean stimulation was eightfold. This large range was due both to individual differences in the basal level of C2 production and response to antigen.

The rate of C2 production was also determined. 1×10^7 mononuclear cells were cultured in triplicate with and without antigen for an initial 2 days. Each day thereafter the cells were washed and resuspended in fresh medium with or without antigen corresponding to initial culture conditions. The rates of C2 production as measured over five daily intervals are shown in Fig. 1 (right). The rate of antigen-stimulated C2 production rapidly increased through day 3 but

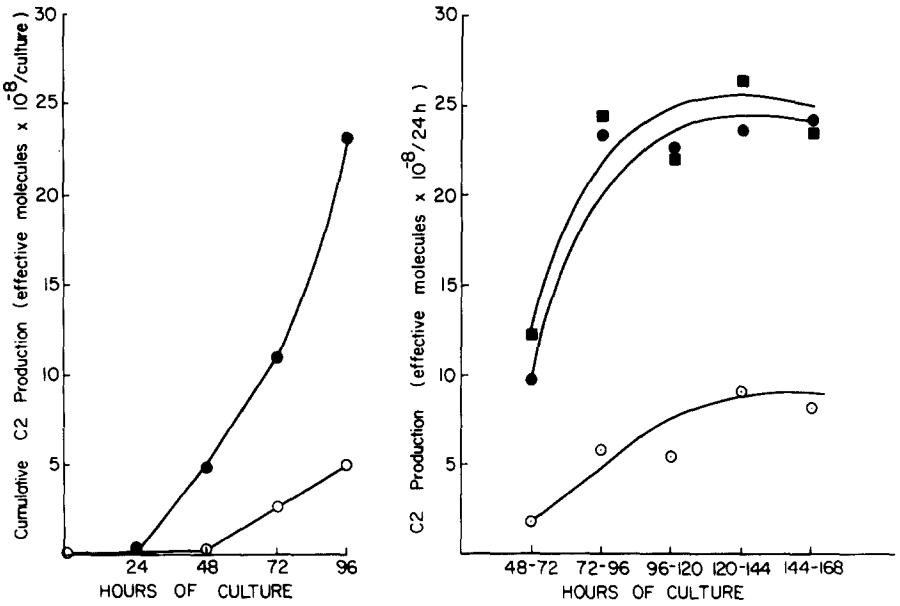

FIG. 1. C2 production by mononuclear cells. Antigen stimulation. Left hand panel: kinetics of C2 production. (●) SK-SD antigen added; (○) No antigen added. Right hand panel: rate of C2 production by mononuclear cells. (○) No antigen added; (●) SK-SD antigen added; (■) purified protein derivative added.

TABLE I
Cumulative C2 Production

	Effective molecules × 10^{-8}/culture after 96 h	
	Antigen-stimulated mononuclear cells*	Lymphokine-stimulated adherent monocytes‡
Control	11 ± 4 (0.2 ± 0.08/μg DNA)	8 ± 2 (1.3 ± 0.3/μg DNA)
Stimulated	44 ± 8 (0.9 ± 0.02/μg DNA)	39 ± 13 (6.5 ± 2.2/μg DNA)

* Nine experiments.
‡ Six experiments.

leveled off thereafter. Unstimulated cultures increased their rate of C2 production more slowly and reached a maximal rate only after 6 days of culture.

The measured C2 in the supernates of these mononuclear cell cultures was actively synthesized and not simply released. Cells were cultured for 72 h with and without SK-SD antigen, washed, and resuspended in fresh medium with and without 10^{-4} M cycloheximide. The results given in Table II indicate that C2 production was virtually completely inhibited by this agent.

C2 production by mononuclear cells could be attributed primarily to an adherent cell population. 10^7 mononuclear cells cultured with or without SK-SD antigen for 24 h were separated into adherent and nonadherent populations, washed, and recultured with or without antigen corresponding to the makeup of the initial culture. After a total incubation time of 96 h, C2 production during the interval between 24 and 96 h was measured. Antigen-stimulated nonadherent

TABLE II
Cumulative C2 Production Effect of Cycloheximide

Treatment day 3	Antigen	C2 production, effective molecules $\times 10^{-8}$/culture		
		Day 4	Day 5	Day 6
TC 199	None	4.0	11.5	22.2
	SK-SD	7.6	24.9	38.5
10^{-4} M cycloheximide	None	0.7	0.9	1.1
	SK-SD	1.7	1.2	2.2

cells, mainly lymphocytes, produced 6.3×10^8 effective molecules of C2 per culture, while the corresponding adherent population, consisting of many fewer cells, chiefly monocytes, produced 16.9×10^8 effective C2 molecules. Thus, 73% of the C2 was produced by the small numbers of adherent cells.

C2 Production by Adherent Monocytes: Lymphokine Stimulation. Medium containing dialyzed 48-h mononuclear cell culture supernates from antigen-stimulated or control cultures were used in cultures of purified adherent monocytes which were 98% phagocytic cells. The response of adherent monocytes to various dilutions of control and active supernates is given in Fig. 2 (left). A maximal fivefold increase in C2 production occurred at a 1:4 dilution. Cumulative C2 production by unstimulated adherent monocytes (Fig. 2, right) was barely detectable after 72 h but increased linearly thereafter. Dialyzed control lymphocyte culture supernate only slightly stimulated C2 production as compared to fresh medium, while active lymphokine-rich supernate greatly stimulated C2 production. Between 72 and 96 h, the maximal rate of C2 production was calculated to be 9×10^8 effective molecules of C2 per μg DNA per 24 h. Since 1 μg DNA was equivalent to 2×10^5 mononuclear cells, this corresponded to approximately 4,500 molecules/cell per 24 h.

Results were similar in five other experiments (Table I). The average cumulative C2 production by monocytes after 96 h of culture with control supernate was $1.3 \pm 0.3 \times 10^8$ effective molecules/μg DNA while active supernates resulted in $6.5 \pm 2.2 \times 10^8$ effective molecules/μg DNA ($t = 2.56$, $P < 0.025$). The range of lymphokine stimulation was from 4.3 to 8.9-fold with an average stimulation for individual experiments of 5 ± 0.4.

Inhibition of C2 Production by α-L(−) Fucose. 0.1 M α-L(−) fucose, a concentration previously shown to inhibit the response of human monocytes to macrophage migration inhibitory factor (MIF) (Rocklin, R. E. Personal communication), was used in an effort to inhibit the antigen-induced stimulation of mononuclear cell C2 synthesis. This resulted in almost complete inhibition of C2 production although the lymphocytes in the same culture were able to incorporate [^{3}H]thymidine and were, therefore, viable (Table III). When other sugars were tested, 0.1 M *N*-acetyl glucosamine was found also to be somewhat inhibitory, but 0.1 M *N*-acetyl galactosamine was not. In an experiment comparing the inhibitory effect of varying concentrations of α-L(−) fucose and *N*-acetyl glucosamine, 0.025 M *N*-acetyl glucosamine was found to have little inhibitory effect while the same concentration of α-L(−) fucose inhibited 52% of the C2 pro-

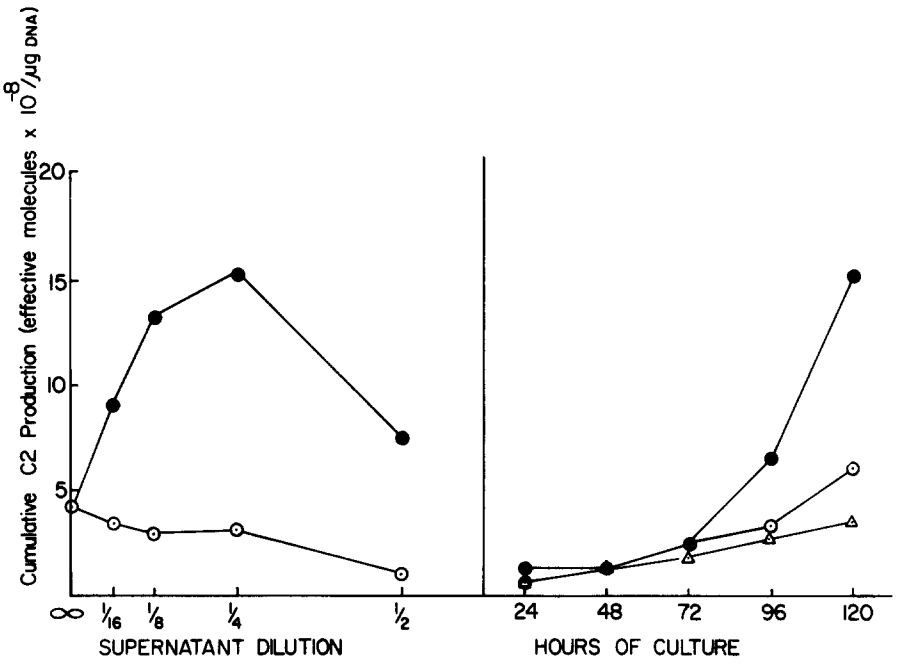

FIG. 2. C2 production by adherent monocytes: lymphokine stimulation. Left hand panel: dose response 5 days of culture. (●) Active supernate; (○) control supernate with antigen added. Right hand panel: kinetics of response to 1:4 diluted supernates. (●) Active supernate; (○) control supernate; (△) fresh media.

TABLE III
Inhibition of C2 Production by Fucose

	Added to 10^7 mononuclear cells	C2 produced (effective molecules × 10^{-8})			[^{3}H]thymidine incorporated
		72 h	96 h	120 h	120–144 h
					cpm
Donor A	SK-SD	7.0	19.7	44.3	24,951
	SK-SD and 0.1 M fucose	2.4	3.6	2.5	8,083
Donor B	SK-SD	74.1	170.2	174.6	12,518
	SK-SD and 0.1 M fucose	12.8	14.2	14.3	11,334

duction. Spontaneous C2 production was also inhibited by α-L(−) fucose. The inhibition of C2 production by α-L(−) fucose was reversed by washing. Table IV gives the mean and standard error for C2 production in this experiment performed in triplicate. At the end of a 3-day culture with these sugars (day 0 after wash), 0.025 M fucose inhibited spontaneous C2 production by 35% and antigen-stimulated C2 production by 63%. After washing, fucose-treated SK-SD-stimulated mononuclear cells produced the same small amount of C2 as did freshly isolated mononuclear cells (Table IV), while cells treated with other amino sugars or medium alone and then washed began producing C2 at a much greater

TABLE IV
Cumulative C2 Produced (Effective Molecules × 10^{-8})

Sugar present first 3 days	Antigen	Days after wash			
		0	+1	+2	+3
None	None	5.4 ± 1.1	10.2 ± 1.4	13.2 ± 2.0	21.3 ± 4.1
	SK-SD	27.6 ± 6.6	18.3 ± 1.5	24.5 ± 2.9	36.0 ± 4.8
0.025 M α-L(−) fucose	None	3.5 ± 0.1	6.8 ± 1.3	9.3 ± 0.2	14.9 ± 0.9
	SK-SD	10.2 ± 0.6	7.5 ± 1.3	14.3 ± 1.1	21.1 ± 3.3
0.025 M N-acetyl galactosamine	None	6.6 ± 0.2	7.0 ± 0.4	14.6 ± 0.6	22.1 ± 1.3
	SK-SD	21.4 ± 2.1	15.3 ± 0.4	31.7 ± 2.2	44.3 ± 1.4
0.025 M N-acetyl glucosamine	None	5.6 ± 0.6	4.3 ± 0.3	7.3 ± 0.8	11.7 ± 0.6
	SK-SD	29.1 ± 2.4	17.0 ± 1.1	26.6 ± 0.7	46.8 ± 1.0

	Days of culture		
	1	2	3
Fresh cells + SK-SD	5.1 ± 0.5	11.6 ± 1.5	27.6 ± 6.6

rate. Thus the 3-day culture before washing served to activate these cells while α-L(−) fucose prevented this state of activation.

Discussion

It is clear from the data presented here and elsewhere that human monocytes can produce C2 (1). Previous studies by many workers have demonstrated that lymphocyte products can alter macrophage (monocyte) functions. These include increased adherence, ruffled membrane movement, glucose oxidation, altered rates of phagocytosis, decreased lysosomal enzymes (acid phosphatase, cathepsin D, and β-glucuronidase), increased cytoplasmic granules, increased lactic dehydrogenase and adenylate cyclase, increased glucosamine incorporation, bacteriostasis, and tumoricidal activity (6). The new and interesting finding reported here is that the production of C2 can also be greatly enhanced by coculture with lymphocytes and antigen or by culture with lymphokine-rich medium. This observation links a cell-mediated immune phenomenon with synthesis of a humoral mediator of inflammation. The same active supernates which stimulated adherent monocytes to produce more C2 and to produce C2 earlier also had activity in an assay for leukocyte inhibitory factor and lymphocyte mitogenic factor (data not shown).

Stimulation of monocytes to produce C2 was greater when they were cocultured with antigen and lymphocytes than when cultured in lymphokine-rich supernate. This is analogous to the observation that guinea pig macrophages responded better to antigen-stimulated lymphocytes than to MIF-rich supernatants in terms of migration inhibition, adherence, and bactericidal activity (11).

Macrophage activation by adherence to glass has been reported previously in

an assay measuring erythrocyte lysis by macrophages (5). A comparison of the rate of C2 synthesis by monocytes stimulated by lymphokine-rich supernate with the rate of C2 synthesis by monocytes after prolonged culture adhered to glass as reported by Einstein et al. (1) is instructive. The rates of C2 production at 2, 4, and 8 wk of culture were 5, 6.3, and 15 $\times$ 10^7 effective molecules C2/μg DNA per day (1). In our study, adherent monocytes stimulated by lymphokine-rich supernate produced 90 $\times$ 10^7 effective molecules C2/μg DNA per day, and this occurred after only 5 days in culture. The efficiency of our C2 assay and our values for unstimulated C2 production by adherent monocytes were very close to those previously reported (1). Thus lymphokine-rich supernate is a more potent stimulus of C2 synthesis than is prolonged culture. Like Einstein et al., we also noted a lag period of 6 days before the rate of spontaneous C2 production became constant. We found, however, that this lag period was reduced to 3–4 days when mononuclear cells were stimulated by antigen (Fig. 1, left). Significant C2 production was never observed before 48–72 h of culture. A preliminary approach to the characterization and identification of the lymphokine responsible for the stimulation of C2 production by human monocytes was presented here. α-L(−) fucose has been shown to inhibit the response of guinea pig macrophages to guinea pig (11) and human MIF and to inhibit the response of monocytes to human MIF (Rocklin, R. E. Personal communication). The data presented here demonstrates a similar inhibition of monocyte C2 synthesis by α-L(−) fucose. At the appropriate concentration, 0.025 M, this effect was specific for fucose. However, it was not only the antigen-stimulated increase in C2 synthesis which was inhibited but also the spontaneous increase in C2 synthesis associated with cultivating human mononuclear cells. Washing these cells after culture with fucose for 3 days enabled them to increase C2 production, but they behaved like freshly obtained monocytes rather than monocytes partially stimulated by 3 days of culture. Therefore, fucose seemed to inhibit activation of monocytes due to any cause and was not specific for lymphokine (lymphocyte)-mediated activation. This hypothesis may be tested using other measures of monocyte activation such as Listeria killing (12) and will be tested further using the system reported here. For these reasons MIF cannot yet be identified as the lymphokine responsible for the stimulation of C2 production by monocytes.

Recently Schorlemmer and Allison reported that the cleavage products of C3, C3b, and C3a can stimulate mouse and guinea pig macrophages to release lysosomal enzymes (13). Further, enzymes released from activated macrophages can cleave C3 into these products. This is, therefore, a self-amplifying system and would be further amplified by C3 cleavage either via the classical or alternative pathway of complement activation. Lymphocyte-mediated monocyte activation resulting in enhanced complement component synthesis must, therefore, be viewed in terms of the local environment where this activation occurs. Perhaps with increased utilization, new synthesis of complement components is even more enhanced.

Finally, monocytes and macrophages have been shown to produce many other complement components besides C2. These include C3, C4, and properdin factor B (2, 3). It is entirely possible that lymphocytes control the production of these as well as C2 via the secretion of lymphokines. The role of lymphocytes in the

regulation of inflammation via control of antibody production and cell-mediated immune phenomena is well established and now complement component synthesis appears also to be under similar control.

Summary

Human peripheral blood mononuclear cells cultured in the presence of antigen produced hemolytically active second complement component earlier and in larger amounts than did control cultures of the same cells without antigen. The increased amount of C2 in culture supernates came primarily from the adherent cell population and was due to increased synthesis as demonstrated by inhibition with 10^{-4} M cycloheximide. Purified adherent monocytes produced more C2 when exposed to lymphokine-rich supernates from antigen-stimulated lymphocytes than when exposed to control supernates from unstimulated lymphocyte cultures. The increased synthesis of C2, which appeared to be mediated by a lymphokine, was partially inhibited specifically by 0.025 M α-L(−) fucose, a sugar which has previously been shown to inhibit the response of macrophages to migration inhibitory factor.

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