Human Monocytes Activated by Immunomodulators in Liposomes Lyse Herpesvirus-Infected but Not Normal Cells

Abstract. *Highly purified peripheral blood monocytes from normal human donors were activated in vitro by incubation with liposomes containing immunomodulators such as recombinant human gamma interferon, human lymphokines, or muramyl dipeptide. The ability of liposomes containing immunomodulators to activate monocytes to a cytotoxic state capable of discriminating between virus-infected and uninfected cells was shown by activated monocytes recognizing and destroying herpes simplex virus type 2–infected cells while leaving uninfected cells unharmed.*

Efforts to treat herpes simplex virus type 2 (HSV-2) infections by antiviral chemotherapeutic agents have been hampered by the inability of the majority of these agents to select between virus-infected and uninfected cells (*1*). The appropriate activation of macrophages is thought to enhance the host defense against various infections (*2*) and cancer (*3*). The encapsulation of immunopotentiating agents within liposomes has been used successfully to achieve an efficient activation of bactericidal and tumoricidal properties in rodent (*4*) and human macrophages (*5*). Moreover, such activated macrophages have been shown to lyse selectively tumorigenic cells without harming normal cells, even under conditions of cocultivation (*6*). Since normal cells acquire sensitivity to macrophage-mediated cytolysis after infection with enveloped viruses (*7*), and since macrophages accumulate early at sites of herpesvirus infections (*8*), we wished to determine whether liposome-activated human monocytes can also distinguish between herpesvirus-infected and normal cells. We were especially interested in those monocytes activated by liposomes containing immunomodulators, since liposomes are a suitable vehicle for the delivery of those compounds to circulating blood monocytes or to fixed macrophages in situ (*4, 9*). We now present evidence that human monocytes can be activated by liposomes containing human recombinant gamma interferon (γ-IFN), lymphokines, or a lipophilic derivative of muramyl dipeptide to lyse HSV-2–infected cells while leaving uninfected control cells unharmed.

Highly purified preparations of human peripheral blood monocytes isolated from normal donors were incubated with immunomodulators for 24 hours in vitro and tested for their ability to lyse HSV-2–infected target cells in an 18-hour ^{51}Cr-release cytotoxicity assay. As shown in Table 1, treatment of monocytes with human recombinant γ-INF and subsequent cocultivation with allogeneic whole human embryo (WHE) cells showed that monocytes were cytotoxic for HSV-2–infected WHE cells but not for uninfected WHE cells. In contrast, monocytes incubated in culture medium alone had no effect on either uninfected or virus-infected cells. In addition, monocytes incubated with a lymphokine preparation containing macrophage-activating factor (MAF) produced from concanavalin A–stimulated lymphocytes (*5*) lysed infected xenogeneic BALB/c 10E2 cells but were not cytotoxic to uninfected 10E2 cells. Significant cytotoxic responses by monocytes activated with MAF against either HSV-2–infected human or murine target cells were observed at effector-to-target cell ratios as low as 12 to 1. Kinetic analyses showed that lysis of HSV-2–infected cells by activated monocytes measured by ^{51}Cr-release occurred as early as 8 hours after cocultivation of effector and target cells (data not shown).

Although monocytes can be activated in vitro by free lymphokines to lyse HSV-2–infected cells without harming uninfected cells, the potential efficacy of this approach for the treatment of viral infections in vivo may be hampered by an inability of free lymphokines to remain in the circulation long enough and at high enough concentrations to render monocytes cytotoxic for virus-infected cells. The intravenous administration of lymphokines containing MAF activity has been shown to be incapable of activating macrophages to a tumoricidal state (*3*). Similar studies with muramyl

Table 1. Lysis of HSV-2–infected cells, but not normal cells, by human monocytes activated with free immunopotentiating compounds. Human peripheral blood monocytes were isolated by Percoll gradient centrifugation followed by adherence (*16*). Purity of monocytes was more than 99 percent on the basis of morphological, biochemical, and immunological criteria (*5, 17*). A total of 5×10^5 monocytes was added to each well of a 96-well flat-bottom Microtest II plate (Falcon Plastics) that had been treated with fetal bovine serum for 1 hour at 37°C. The fetal bovine serum was removed prior to the addition of monocytes. After adherence for 1 hour at 37°C, the cells were washed three times with RPMI 1640 medium. Monocytes were incubated at 37°C for 24 hours with 0.2 ml of culture medium, MAF, or 200 units of human recombinant γ-IFN. After this incubation period, cultures were washed two times before addition of target cells. The WHE cells (originally obtained from HEM Research) and the 10E2 cells (*18*) were used as target cells (*19*). Cell monolayers were inoculated with HSV-2 strain 333 at a multiplicity of infection of 1.0 and adsorbed for 2 hours at 37°C. After the virus adsorption period, target cells were trypsinized and resuspended in minimum essential medium (MEM) plus 5 percent fetal bovine serum. Virus infection of target cells was verified by methylcellulose plaque assays and immunofluorescence staining. Uninfected and HSV-2–infected cells (3×10^6) were labeled with 200 μCi of ^{51}Cr ($NaCrO_4$, New England Nuclear) in a total volume of 0.5 ml for 1 hour at 37°C with intermittent shaking. Cells were washed three times and diluted to 5×10^4 per milliliter in MEM plus 5 percent fetal calf serum. Target cells were then added to designated wells (0.2 ml per well) at an effector-to-target cell ratio of 50 to 1. Culture wells containing target cells alone were used for determination of spontaneous ^{51}Cr release. The total counts available for release were determined by treating the cells with 1 percent Triton X-100. Plates were incubated for 18 hours at 37°C in a 5 percent CO_2 atmosphere. At the termination of assays, plates were centrifuged at 400*g* for 10 minutes. A 100-μl sample was recovered from each well, and the radioactivity was measured in a gamma counter. All samples were assayed in triplicate. The percentage of specific ^{51}Cr release was determined by $[(R_m - R_s)/(R_t - R_s)] \times 100$, where R_m is the mean counts in the presence of monocytes, R_t is the total release with 1 percent Triton X-100, and R_s is the spontaneous release. More than five replicate experiments were performed. Spontaneous ^{51}Cr release for WHE cells (uninfected or HSV-2–infected) ranged from 18 to 31 percent; spontaneous release for 10E2 cells ranged from 15 to 24 percent. Values are mean ± standard deviation. Values in parentheses indicate the percent specific ^{51}Cr release. Student's *t*-test was used to evaluate the significance of observed differences between targets cultured with monocytes pretreated with immunomodulators and those cultured with monocytes treated with culture medium.

Monocyte treatment	^{51}Cr release (count/min)			
	Uninfected cells		HSV-2-infected cells	
	WHE	10E2	WHE	10E2
No monocytes	1690 ± 78	437 ± 20	1322 ± 19	527 ± 41
Culture medium	1708 ± 86 (0.3)	404 ± 7 (−2.5)	1788 ± 23 (7.2)	522 ± 21 (−0.4)
Human γ-INF	1948 ± 186 (4.6)		3425 ± 427 (32.5)*	
MAF		428 ± 14 (−0.6)		761 ± 20 (21.1)†

*$P < 0.005$. †$P < 0.001$.

dipeptide (MDP), a potent monocyte-macrophage activator in vitro (*10*), have shown that MDP is cleared from the circulation within 60 minutes after parenteral administration and that this brief period is insufficient to render macrophages cytotoxic (*10*).

A number of biological materials have been successfully encapsulated in liposomes and delivered to cells in vitro and in vivo (*11*). By entrapping immunomodulators in liposomes, these substances can be protected from degradation and passively targeted to cells of the reticuloendothelial system, including blood monocytes (*12*). Monocytes incubated with liposomes containing MAF lysed HSV-2–infected 10E2 or WHE cells and left uninfected cells unharmed (Table 2). Similarly, monocytes activated by liposomes containing human recombinant γ-INF discriminated between HSV-2–infected cells and uninfected cells, killing only the virus-infected cells (Table 2). In contrast, treatment of monocytes with liposomes containing culture medium did not activate these cells to a cytotoxic state. Relatively high levels of the free immunomodulators (undiluted MAF and 200 units of γ-INF per culture well, respectively) were used to render monocytes cytotoxic for HSV-2–infected cells. When MAF was diluted eightfold, it was no longer effective in activating monocyte-mediated cytotoxicity. Since the amount of liposome-encapsulated MAF and γ-INF presented to the monocytes was nearly a 1000-fold dilution of the free substances (see legend to Table 2), liposome entrapment of immunomodulators and subsequent presentation to monocytes is an efficient method for activating these cells to lyse herpesvirus-infected cells.

Lipophilic compounds form a more stable association with liposomes than do hydrophilic substances (*13*). Lipophilic derivatization of low molecular weight compounds such as MDP makes it possible to insert them directly into the phospholipid bilayer and increase significantly the encapsulation efficiency (*12*). One such substance, *N*-acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanylphosphatidylethanolamine (MTP-PE), has been shown, when incorporated into liposomes, to be efficient in rendering human blood monocytes tumoricidal (*14*). As shown in Table 2, liposomes containing MTP-PE inserted in the phospholipid bilayer can activate monocytes to lyse HSV-2–infected cells but not uninfected cells. Thus, our discoveries show that synthetic and genetically engineered immunomodulators (MTP-PE and human recombinant γ-INF, respectively) encapsulated in liposomes can stimulate human monocyte–mediated destruction of herpesvirus-infected cells. It may also be possible to combine two or more immunopotentiating and chemotherapeutic agents in liposomes as a method for selective destruction of HSV-2–infected cells. As illustrated in Table 2, liposomes containing MAF entrapped in the aqueous space and MTP-PE inserted in the lipid bilayer provided an efficient means for activating monocytes to lyse HSV-2–infected cells. Studies in rodent systems have shown that cytotoxic macrophages capable of nonspecifically lysing a wide variety of virus-infected or neoplastic target cells but not uninfected cells may be generated by treating the animals with a wide range of immunomodulators (*15*). Together with the observation that treatment of human monocytes with liposome-encapsulated MAF or MTP-PE enables these cells to destroy a wide variety of tumorigenic cells without affecting nontumorigenic cells (*5*, *14*), this suggests the possibility that cells infected by viruses distinct from the herpesvirus group may also be susceptible to lysis by human monocytes activated with liposome-encapsulated immunomodulators.

Table 2. Selective lysis of HSV-2–infected cells by human monocytes activated with liposomes containing immunomodulators. Multilamellar vesicle liposomes (MLV) were prepared from a mixture of chromatographically pure egg phosphatidylcholine and beef brain phosphatidylserine (Avanti Biochemicals) at a 7 to 3 molar ratio (*12*). Preparations of MLV containing MAF or human recombinant γ-IFN were centrifuged three times at 15,000*g* to remove unencapsulated material. Liposomes were adjusted to 500 nmole of lipid per milliliter in culture medium, and 100 nmole of phospholipid were added to monocyte cultures for a 24-hour incubation period before adding target cells. The MLV preparations contained approximately 2.5 μl of aqueous phase per micromole of phospholipid, contrasted with the 200 μl of free lymphokines delivered to monocytes during the 24-hour incubation period. The concentration of MTP-PE in the MLV was 4 μg of MTP-PE per micromole of phospholipid. Cytotoxicity assay methodology and statistical evaluations (Student's *t*-test) were as described (see Table 1). All samples were assayed in triplicate.

Monocyte treatment	Specific ^{51}Cr release	
	Uninfected cells	HSV-2–infected cells
WHE cells		
Culture medium	3.5	5.5
Free MAF	8.4	28.0*
Free MAF (1:8)	N.T.†	4.2
MLV-encapsulated MAF	2.7	20.2*
MLV-encapsulated medium	−1.9	0.9
10E2 cells		
Culture medium	−2.0	2.2
Free MAF	1.2	13.9‡
MLV-encapsulated MAF	−0.5	22.5§
MLV-encapsulated medium	−2.7	7.0
Free human recombinant γ-INF	3.0	31.3‖
MLV-encapsulated γ-INF	−1.4	22.5*
MLV containing MTP-PE	−0.9	15.0*
Culture medium	−5.2	3.3
MLV-encapsulated culture medium	−8.0	6.2
MLV-encapsulated MAF	−7.3	13.0§
MLV containing MTP-PE	−8.1	18.0‖
MLV-encapsulated MAF and MTP-PE	−8.9	22.2‖

*$P < 0.01$. †N.T., not tested. ‡$P < 0.05$. §$P < 0.02$. ‖$P < 0.001$.

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17. Monocyte cultures were examined for cell morphology, ability to ingest carbon particles, and uptake of nonspecific esterase stain. More than 99 percent of the cultured monocytes stained positive with monoclonal antihuman monocyte antibody 61D3 (Bethesda Research Laboratories). Culture medium consisted of RPMI 1640 supplemented with 5 percent heat-inactivated human type AB serum. Human lymphokine preparations containing MAF were produced by incubating human mononuclear leukocytes with Sepharose-bound concanavalin A (Pharmacia) for 48 hours and separating the supernatants by centrifugation.
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19. Target cell lines were cultured in Eagle's minimum essential medium supplemented with 10 percent heat-inactivated fetal bovine serum, L-glutamine, penicillin, and streptomycin. None of the reagents contained endotoxins as determined by the *Limulus* amoebocyte lysate assay.
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Androgen Modulation of Adrenal Angiotensin Receptors

Abstract. *Several polar androgens increased the binding of angiotensin and its stimulation of aldosteronogenesis in bovine adrenal glomerulosa cells. The effect was seen only if the steroids were applied to the cells and then washed away. This phenomenon and the technique for demonstrating it may have implications for studies of receptor modulation and for clinical states in which responsiveness to angiotensin is increased.*

In several forms of hypertension, including the majority of patients with low renin essential hypertension (LREH), adrenal secretion of aldosterone in response to angiotensin II (AII) is exaggerated (*1–3*). One possible mechanism for this hyperresponsiveness is an increase in the number or affinity of angiotensin receptors (*4*). We have observed receptor modulation in bovine adrenal glomerulosa cells exposed to cholesteryl hemisuccinate (*5*). Because androgens have been implicated in the pathogenesis of some forms of hypertension (*6–8*), we examined the effects of several androgens on angiotensin receptors and responses in bovine cells.

Collagenase-dispersed adrenal glomerulosa cells from mature cows (*9*) were suspended in Krebs solution containing 20 m*M* Hepes (Sigma). To cell suspensions were added testosterone hemisuccinate (Steraloids), dehydroepiandrosterone (DHEA) sulfate (Sigma), or their ethanol vehicle (1 percent by volume). Androgen concentrations ranged from 8.6×10^{-8} to 8.6×10^{-4}*M*. Cells were incubated with these agents for 2 to 60 minutes at room temperature in a shaking water bath and then washed twice with buffer (*5*). Equal numbers of cells were used in all groups, and there was no between-group difference in cell viability as determined by trypan blue exclusion.

Angiotensin II and angiotensin III (AIII) (Bachem) were labeled with ^{125}I (*10*). Binding to cell suspensions was measured by using the method of Carroll *et al.* (*5*), except that EDTA (15 m*M*) and dithiothreitol (1 m*M*) were added to retard peptide degradation. Binding of AII and AIII was also measured in the presence of testosterone hemisuccinate added in ethanol (1 percent by volume) to a final concentration of 8.6×10^{-4}*M*. Binding experiments were performed at 37°C for 15 (AIII) or 30 (AII) minutes. Saturable binding was defined as the difference between total binding and binding in the presence of 10 μg of unlabeled angiotensin. Binding results were expressed as percentages of control values in each experiment and were subjected to Scatchard analysis.

Effects of testosterone hemisuccinate on aldosterone secretion by bovine cells were determined in quintuplicate (*5*) by direct radioimmunoassay for aldosterone with [^{125}I]aldosterone and antibody to aldosterone (Diagnostic Products). Extraction and chromatography of selected samples with Sephadex LH-20 (Pharmacia Fine Chemicals) did not affect the results. We used angiotensin concentrations of 10^{-9} and 10^{-7}*M* in steroidogenesis experiments because at those levels AII produced submaximal and maximal aldosterone responses, respectively (*5, 9, 11, 12*). Statistical analysis was performed with Student's *t*-test for unpaired values.

When testosterone hemisuccinate was present in the medium during binding

Table 1. Equilibrium binding parameters for angiotensins and bovine adrenal glomerulosa cells. Values are derived from analysis of the data in Figs. 1 and 2 with Scatfit, the nonlinear least-squares program developed by Rodbard (*28*), and are presented as means ± standard errors.

Parameter*	Control		Testosterone hemisuccinate	
	Affinity (nM^{-1})	Capacity (femtomoles per 10^6 cells)	Affinity (nM^{-1})	Capacity (femtomoles per 10^6 cells)
		Angiotensin II		
K_1	1.87 ± 0.61		3.04 ± 0.47	
Q_1		81.2 ± 38.1		104 ± 25
K_2	0.101 ± 0.058		0.278 ± 0.096	
Q_2		653 ± 173		351 ± 38
		Angiotensin III		
K_1	10.7 ± 3.6		4.42 ± 1.20	
Q_1		15.4 ± 3.8		82.2 ± 24.6
K_2	0.0907 ± 0.0235		0.162 ± 0.088	
Q_2		603 ± 115		522 ± 150

*K_1 and K_2 are the affinity constants and Q_1 and Q_2 are the corresponding capacities for each of two independent classes of binding sites.