amino acid analysis of the interferon in the gel band was virtually identical to that obtained directly on the purified sample of human leukocyte interferon (*9*) that was applied to the gel.

We conclude that we have purified one species of human leukocyte interferon to homogeneity. The precise relation of this species to the others, to the secreted material, and to the primary gene product requires further study.

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Design of Liposomes for Enhanced Local Release of Drugs by Hyperthermia

Abstract. *Liposomes can be designed to release an entrapped drug preferentially at temperatures attainable by mild local hyperthermia. In a test system in vitro, protein synthesis by* Escherichia coli *is inhibited and killing of the cells is enhanced by heating neomycin-containing liposomes to their phase transition temperature to maximize drug release. In the presence of serum the ratio of release at 44°C to that at 37°C can be made greater than 100:1, suggesting possible applications in the treatment of tumors or local infection.*

Liposomes are microscopic particles consisting of one lipid bilayer enclosing a single aqueous compartment (unilamellar vesicles) or a number of concentric bilayers enclosing an equal number of aqueous spaces (multilamellar vesicles). They are currently being studied as vehicles for delivery of pharmaceutical agents (*1*), but a major barrier to their use is the difficulty of directing them to specific target sites. The distribution of liposomes in vivo can be influenced somewhat by varying such nonspecific factors as the size, charge, fluidity, and route of administration, but not to a degree permitting flexible control of the site of delivery.

More specific "targeting" has been attempted by use of a "recognition macromolecule" to bind liposomes selectively to particular cells. Antibodies (*2–4*), plant lectins (*5*), and desialylated glycoproteins (*3*) have been tried, most often in model systems in vitro. This type of targeting faces special problems: the paucity of appropriate cell surface markers potentially useful for therapy; the possibility of immune sensitization of the host to the targeting moiety; the requirement in most cases that liposomes cross endothelial barriers; and the difficulty that bound liposomes do not always deliver their contents into the cells (*2, 4*). In this report we suggest a different approach, the use of local hyperthermia to promote selective delivery of liposome-encapsulated drugs to target areas.

Local hyperthermia is currently receiving increased attention as a therapeutic tool, for use either alone or in conjunction with radiation (*6*) or drugs (*7, 8*). Since many normal mammalian cells begin to show damage at about 42°C (*9*) the aim has been to achieve therapeutic results just a few degrees above physiological temperature. In addition to older methods of heating (for instance, in a warm bath or with warmed perfusate), microwaves and ultrasonic energy are now being investigated, especially for the local heating of deeper structures (*6*).

We can distinguish five ways in which local hyperthermia might increase the therapeutic effectiveness of drug-containing liposomes, for example in the treatment of neoplasms: (i) by promoting selective drug release at temperatures near that of the lipid phase transition of the liposomes; (ii) by increasing local blood flow; (iii) by increasing endothelial permeability to particles, thereby enhancing accumulation of liposomes in the target tissues; (iv) by increasing the permeability or susceptibility of target cells to the drug released from the liposomes; and (v) by increasing direct transfer of drug from vesicles to cells—for example, by fusion or endocytosis (*10*).

This study focuses primarily on the first of these possibilities. Near their liquid-crystalline transition temperatures (T_c), liposomes become highly leaky to water-soluble contents (*11*), a phenomenon generally attributed to disorder at the boundaries between solid and fluid domains in the lipid. Our basic strategy was to design liposomes with T_c above physiological temperature but in a range attainable by mild local hyperthermia. On passing through the heated area in the circulation, the liposomes would be expected to release their contents at a greater rate than elsewhere and thus to develop higher local concentrations.

Dipalmitoyl phosphatidylcholine (DPPC) appears to be a reasonable choice for the primary liposomal lipid. It has 16-carbon saturated fatty acid chains and a T_c of 41°C (*12*). By adding various proportions of distearoyl phosphatidylcholine (DSPC; 18-carbon chains; T_c = 54°C), it is then possible to obtain any desired T_c between 41° and 54°C. Since the two lipids are miscible in all proportions (in both "solid" and "fluid" states) only a single broad transition is observed (*13*). Small, primarily unilamellar vesicles of these lipids were prepared by a procedure involving sonication, Millipore filtration, and gel chromatography, essentially as described elsewhere (*14*) but with two important changes necessitated by the high T_c of the lipids: vortex mixing and sonication were done at 50° to 55°C,

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and the suspension was cooled as rapidly as possible to about 3°C after sonication to minimize changes at the temperature of transition (*15*). Although such a preparative procedure results in a suspension of small, mostly unilamellar vesicles, aggregation and changes in state can occur with time (*16*). All vesicles for our studies were prepared on the day of the experiment, and no systematic differences in results were seen between early and late experiments on that day. When dispersed in excess water (that is, to form multilamellar vesicles), the DPPC and DSPC used (*17*) showed sharp transition peaks at 42° and 55°C, respectively, by differential scanning calorimetry. The DPPC showed a transition at 42°C by fluorescence depolarization (*18*).

To obtain an index of the release of solute from the vesicles as a function of temperature, we used an approach based on self-quenching of fluorescence (*19*).

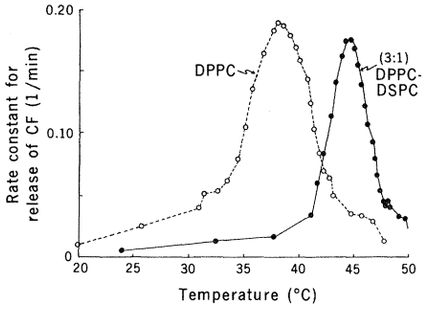

Fig. 1. Temperature dependence of CF release from small unilamellar vesicles, determined from continuous temperature scans. Release rates were greatest near the liquid-crystalline phase transitions of the respective vesicles. Suspensions of vesicles (0.135 ml) were placed in 3 by 3 mm (inner dimension) cuvettes in the optical path of a fluorometer (Aminco-Bowman, Silver Spring, Maryland) and heated from an initial temperature of 20°C to a final equilibrium value of 55°C with time constants of 18.5 seconds (*DPPC*) and 12.1 seconds (*DPPC-DSPC*). The rate of temperature increase near the phase transition temperature was about 14°C per minute. The temperature in the cuvette was monitored continuously with a thermocouple probe, and a progressive increase in fluorescence indicated relief of self-quenching by release of CF into the medium (*14*). At the end of each scan the total amount of dye present was measured after disrupting the vesicles with 5 μl of 20 percent Triton X-100 detergent. The points were calculated by taking the time derivative of fluorescence, normalizing it in accordance with the amount of dye remaining in the vesicles, and plotting the resulting release rate constant (k_r) at each time against the prevailing temperature. Corrections were made for the temperature dependence of CF fluorescence and for the dilution with Triton (*24*). The vesicles were formed in 100 m*M* CF. Each suspension contained approximately 1 μM dye and 31 μM lipid. In some experiments the peak of release was wider for 3:1 DPPC-DSPC than for DPPC.

The highly water-soluble fluorophore carboxyfluorescein (CF) (Eastman, Rochester, New York) was encapsulated in sonicated vesicles at sufficiently high concentration (100 m*M*) that its fluorescence was almost entirely self-quenched by interaction between neighboring fluorophore molecules. Release of CF from the vesicles could then be related directly to the progressive increase in fluorescence with time as the temperature was scanned through T_c. Figure 1 shows the rate of CF release from DPPC and 3:1 DPPC-DSPC vesicles as a function of temperature. For DPPC the rate constant of release (*kr*) was highest at 38°C, a value almost 4° below that obtained by scanning calorimetry for the phase transition of hydrated DPPC. This result was expected since small unilamellar vesicles have lower-temperature, broader transitions than their multilamellar counterparts (*13*), probably as a result of strains in lipid packing associated with the small radius of curvature of the bilayer.

The data in Fig. 1 (and Fig. 4) should not be taken to imply that release is a simple, single-valued function of temperature. In fact, the temperature of maximum release varied by as much as 1° or 2°, and the amount of dye released was sensitive to details of the protocol for making vesicles, to the history of the vesicle suspension (for example, number of cycles through T_c), and, as discussed later, to the rate of scanning.

Small unilamellar DPPC vesicles would clearly not be effective in attempts at selective release in areas of hyperthermia if the baseline temperature were 37°C. The 3:1 DPPC-DSPC vesicles, on the other hand, released solute maximally in the range 42.5° to 44.5°C. For the experiment shown in Fig. 1 the ratio of release at the peak to that at 37°C was 11:1. On the basis of these results, we chose the mixed-lipid vesicles for model studies in vitro in which neomycin-containing vesicles were preincubated at various temperatures and then tested for their capacity (i) to inhibit synthesis of protein by *Escherichia coli* B/r and (ii) to kill the bacteria (that is, render them incapable of forming colonies) (*20*).

Protein synthesis, assessed by uptake of [^{3}H]leucine into the cells, was inhibited most markedly by vesicle suspensions preincubated at 42° to 44°C (Fig. 2). The correlation between this result and leakage of CF strongly suggests that neomycin released near the transition temperature was responsible for the inhibition. If transfer of drug directly from vesicles to cells had been the principal mechanism of increased inhibition, vesicles preincubated at temperatures far removed from the transition would presumably have been more effective. Vesicles containing only *p*H buffer had no effect on uptake of [^{3}H]leucine or on the inhibition seen with free neomycin.

Studies of survival of *E. coli* exposed to neomycin-containing 3:1 DPPC-DSPC vesicles gave analogous results: maximal killing was seen with vesicles preincubated in the range 42° to 46°C, as shown in Fig. 3. This observation leads one to hope that therapeutic advantage could be obtained as a result of local drug release within the temperature range realistically obtainable by hyperthermic techniques. When the vesicles and cells were heated to various temperatures together (results also shown in Fig. 3), killing was considerably greater than when the vesicles were preincubated alone. Hyperthermic treatment of the cells for 10 minutes did not itself cause significant cell death in the absence of liposomes and drug (at temperatures below 46°C); it did, however,

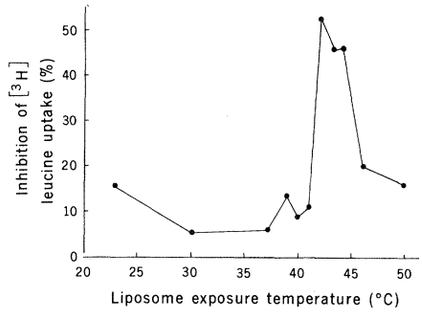

Fig. 2. Inhibition of protein synthesis in *E. coli* by neomycin released from small unilamellar 3:1 DPPC-DSPC vesicles preincubated at different temperatures. Inhibition of [^{3}H]leucine incorporation, expressed as percentage of inhibition with respect to a control not exposed to vesicles, was greatest after preincubations near the phase transition temperature. Vesicle suspensions (0.85 ml) containing 120 m*M* neomycin (approximately 20 mg of lipid and 0.365 mg of neomycin) were heated with a time constant of 15 seconds to various temperatures between 23° and 50°C, and preincubated at the temperature for 10 minutes in phosphate-buffered saline. Each suspension, plus 0.1 ml of a tenfold concentrate of acetate medium, was then added to 0.5 ml of a suspension of *E. coli* B/r (10^8 cells). Each tube was kept for an additional 30 minutes in a shaking water bath at 37°C. Tritiated leucine (0.625 μCi) in 0.05 ml was added to each tube 15 minutes before the end of this incubation. Uptake of [^{3}H]leucine was terminated by chilling in an ice bath. The cells were washed twice by centrifugation with iced phosphate-buffered saline, precipitated with two portions of 10 percent perchloric acid, and resuspended in scintillation fluid for counting. The background inhibitions of 5 to 15 percent seen for low-temperature preincubation probably resulted from release of drug during the later incubation with cells at 37°C.

potentiate the killing effect of free drug (data not shown). Potentiation of killing has also been found with other cells and drugs (*7*). Such enhanced susceptibility of the cells to the leaked neomycin at high temperatures would suffice to explain the greater killing observed when vesicles and cells were heated together. However, a direct interaction between vesicles and cells, or increased leakage of drug at the transition because of the presence of cells, cannot be ruled out. Vesicles containing only buffer had no effect on protein synthesis or viability, and did not alter the effects seen with free drug.

The complex nature of the release process at transition, and of the effort required to exploit it optimally, was indicated by studies in which the rate of temperature scanning was varied. At the scan rate in Fig. 1, 3:1 DPPC-DSPC vesicles released their contents at transition about ten times as fast as they did in the steady state at the same temperature. Downward scanning of temperature caused less pronounced leakage. These findings were consistent with those for release of glucose from multilamellar vesicles of DPPC (*21*). In our experiments with bacteria the relative roles of the initial approach to temperature equilibrium (taking about 15 seconds) and of the 10-minute steady-state period of exposure are not clear at present. The rapid increase in temperature in vivo as vesicles passed into a heated area of the bloodstream should amplify the drug release at transition.

We used small unilamellar vesicles in this study because they circulate in the bloodstream longer than their multilamellar counterparts (*22*). However, the temperature dependence of release could be amplified again if multilamellar forms, perhaps of DPPC alone, were used. Their transitions are sharper and their leakage generally much greater at a particular rate of scanning, as shown in Fig. 4.

Before application of these findings in vivo, the possible effects of serum must be considered. A number of serum components have been reported to interact with liposomes, in some cases causing them to release their contents more rapidly (*23*). We were concerned that serum might complicate the effort to obtain preferential release in areas of hyperthermia, but instead it appeared to potentiate the effect. As indicated in Fig. 4, the presence of 10 percent fetal calf serum dramatically increased release in the region of the transition but had little effect at lower temperatures. The temperature of maximal release could not be determined with confidence because most of the CF (85 percent) was released during a scan, but the ratio of the peak value to that at 37°C was greater than 100:1. Release was even more rapid in the presence of 100 percent fetal calf serum. Note that in Fig. 4 the closed circles represent the same experimental points as those for DPPC-DSPC in Fig. 1.

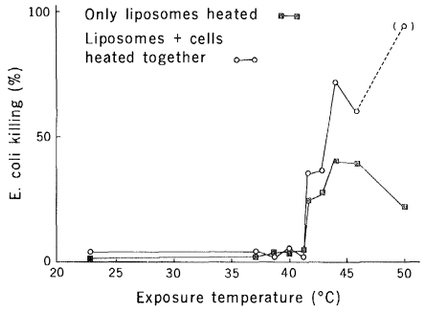

Fig. 3. Killing of *E. coli* by neomycin from small, sonicated 3:1 DPPC-DSPC vesicles heated to various temperatures separately from, or together with, the bacteria. Killing was greatest in both cases near the phase transition temperature. Preincubations and incubations were done as in Fig. 2 but in some cases (○) the bacteria were added at the beginning. Killing was assessed by comparing the number of colonies obtained on nutrient agar after incubation with the number obtained when a control cell suspension not exposed to vesicles was used. Controls (not shown) indicated that the increased effect when cells and vesicles were heated together could be due to an increased susceptibility of the bacteria to neomycin at higher temperatures but not to the effect of hyperthermia alone. Since killing by heat alone was significant at 50°C, that experimental point is shown in parentheses.

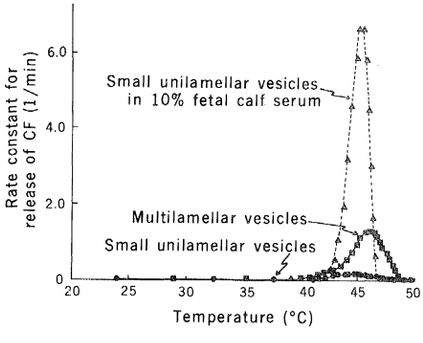

Fig. 4. Temperature dependence of carboxyfluorescein release from small unilamellar 3:1 DPPC-DSPC vesicles. Release at the transition temperature was enhanced by substituting multilamellar for small unilamellar vesicles and, more strikingly, by the presence of heat-inactivated fetal calf serum (Gibco, Grand Island, New York). The closed circles represent the same data as in Fig. 1 but with a different scale on the ordinate. Experimental methods, calculations, and concentrations were as for Fig. 1. The rates of temperature increase were about the same as in Fig. 1. In some experiments significant leakage began at about 37°C in the presence of serum. Multilamellar vesicles were made by omitting sonication and Millipore filtration from the protocol used for small unilamellar vesicles.

It might be important for practical applications in vivo to achieve a large percentage release during a single circulation of liposomes through the heated area; most of the blood volume is in the venous system at any particular time and only release induced in the arterial, arteriolar, and capillary systems of the area of heating would be effective in promoting local accumulation of drug. The effectiveness of preferential release at the transition temperature must now be tested in animal systems, and a combination of studies in vivo and in vitro will be required to explore possibilities (ii) to (v) for synergistic interaction between hyperthermia and liposomal drug carriers.

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Nicotinamide Adenine Dinucleotide Splitting Enzyme: A Characteristic of the Mouse Macrophage

Abstract. *Murine macrophages are endowed with nicotinamide adenine dinucleotide splitting activity that is markedly higher than that of other cells, tissues, or organs of the mouse. This enzyme therefore can be used as a biochemical marker for distinguishing macrophages from other cells of the lymphoreticular system and from polymorphonuclear leukocytes.*

In this report we present evidence to show that murine macrophages are endowed with NAD splitting activity (*1*) (NADase) that is much higher than that of other cells, tissues, and organs of the mouse. Moreover, within the elements of the biological defense system of the mouse, activity of this enzyme is confined almost entirely to macrophages. In contrast, polymorphonuclear leukocytes and T and B lymphocytes have very low or undetectable NAD splitting activity.

Since one of the problems in the preparation of macrophages for study is distinguishing them biochemically from lymphocytes and polymorphonuclear leukocytes, the presence of NADase can serve as a biochemical marker for murine macrophages.

Figure 1 illustrates the results of experiments on NADase activity of homogenates prepared from various mouse organs. All homogenates contained 500 μg of protein per milliliter, and the reaction was followed for 25 minutes at 37°C. Spleen had the highest activity, 110 units, and brain and heart the lowest, 15 and 7 units, respectively. Intermediate activity was found in lung (45 units), liver (36 units), and kidney (31 units) homogenates. These results are in agreement with reports of the high NADase activity of spleen (*2*).

To analyze the distribution of this enzyme among the various cells that make up the spleen, we fractionated splenic cells (*3*). The first step consisted of preparing purified nonadherent cells by filtering splenic cells through a glass wool column. This procedure removes macrophages and adherent subclasses of T and B lymphocytes.

Nonadherent cells (a mixed population of T and B lymphocytes and "null" cells) are practically devoid of NADase activity (Fig. 2). The removal of adherent cells similarly affected acid phosphatase (a lysosomal enzyme of monocytes and macrophages) and NADase activities. In some experiments, adherent cells were displaced from glass wool columns by squeezing the column with several 10-ml portions of phosphate-buffered saline without Ca^{2+} and Mg^{2+} until more than 95 percent of adherent cells were recovered. Splenic cells were also separated into adherent and nonadherent populations by plating them in petri dishes, with subsequent recovery of both types of cells. The results obtained confirmed our original finding that nonadherent cells were almost completely devoid of

Table 1. Evidence that NADase activity does not reside in adherent T or B lymphocytes. Activity was assayed in murine splenic and peritoneal cells depleted of T and B lymphocytes by complenent-dependent cytolysis. Nonfractionated and adherent splenic cells were prepared as described in the legend to Fig. 2. Peritoneal cells from noninduced mice were plated as described in the legend to Fig. 3 and used as monolayers. Cells (1×10^7/ml or per monolayer; ^{51}Cr-labeled, 40 minutes, 100 μCi/ml) in RPMI 1640 medium plus Hepes plus 0.3 percent BSA were incubated with antiserum to T cells (anti-T) or antiserum to mouse immunoglobulin (anti-B) at a final concentration of 1:10 for 60 minutes at 4°C, washed once in the same medium, and incubated with 1 ml of freshly reconstituted lyophilized rabbit complement diluted 1:10 in RPMI 1640-Hepes-BSA. After incubation for 45 minutes at 37°C the supernatants were aspirated and portions (0.1 ml) were counted in a well-type gamma counter. The lytic effect was determined by the formula:

$$\frac{\text{experimental } {}^{51}\text{Cr release} - \text{spontaneous release}}{\text{maximal release (freeze thaw)} - \text{spontaneous release}} \times 100$$

The sedimented splenic cells were brought to the original volume in 0.1*M* phosphate buffer, *p*H 7.2, and assayed. Peritoneal cells were assayed in the form of monolayers. Control cells (incubated without antiserum, or complement or both) were identically treated. The number of experiments is shown in parentheses.

Treatment	Cell lysis (%)	NAD split (μmole/25 minute)
Splenic cells, nonfractionated		
Control	< 2	0.31 ± 0.2 (6)
Anti-T + C	32	0.32 ± 0.3 (6)
Anti-B + C	21	0.33 ± 0.3 (6)
Splenic cells, adherent		
Control		0.42 ± 0.3 (6)
Anti-T + C		0.42 ± 0.4 (6)
Anti-B + C		0.43 ± 0.3 (6)
Peritoneal cells		
Control	0.2	0.36 ± 0.4 (4)
Anti-T + C	4.0	0.38 ± 0.4 (4)
Anti-B + C	3.5	0.37 ± 0.3 (4)

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