are reported to be on the order of 300 m thick (*12*). The data presented here suggest that, even where basement outcrops are absent, advective transport between the oceans and underlying basalt crust may occur.

The flow of water between basement and the oceans off ridge axes has considerable geochemical significance. Off ridge, the upper kilometer of basaltic crust is generally cool [< 50°C (*14*)]. The advective transport of heat under low-temperature conditions is unlikely to have the same heat-mass relations as those observed in the axial hot springs (*5*). Calculation of mass exchange by integration of total advective heat exchange for the oceans solely on the basis of the high-temperature (350° to 400°C) end-member must be incorrect. Should little reaction occur at low temperature, such estimates simply would be too high. If reaction between basalt and seawater occurs at low temperatures, as is likely, then the low-temperature reactions may run counter to the high-temperature ones, at least in some instances [for example, for K^+, Li^+, and possibly Mg^{2+} (*15*)], altering the overall flux estimates significantly.

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The Anticancer Agent Adriamycin Can Be Actively Cytotoxic Without Entering Cells

Abstract. *The antineoplastic agent adriamycin was coupled to an insoluble agarose support. This material was actively cytotoxic to L1210 cells in culture under conditions in which no free adriamycin could enter the cell. It is concluded that an agent whose principal target was previously thought to be DNA can exert its cytotoxic action solely by interaction at the cell surface.*

Adriamycin is one of the most important agents used in the treatment of human cancer. Historically, DNA has been considered to be the primary target for the cytotoxic action of this drug on susceptible cells (*1*). The DNA receptor hypothesis is attractive on structural grounds because adriamycin does bind, with reasonably high affinity, double-stranded nucleic acids by intercalation (*2*, *3*) and because drug fluorescence accumulates in the nuclei of treated cells (*4*).

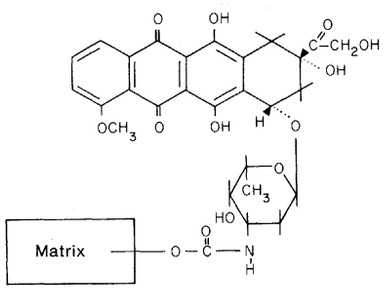

Fig. 1. Structure of adriamycin linked to a polymeric matrix. The activated polymer (Reacti-gel 6x, Pierce Chemical) consists of 6 percent cross-linked agarose beads (40 to 210 μm in diameter) derivatized with 1,1'-carbonyldiimidazole, which can react with free amino groups. In a typical synthesis 1×10^{-8} to 2×10^{-8} mole of adriamycin is allowed to react per milligram of activated agarose (molar ratio of imidazolyl carbamate to adriamycin is about 12:1). The synthesis is carried out for 24 to 72 hours in 0.1*M* borate buffer at *p*H 8.0 and 4°C. Under these conditions 20 to 40 percent of the adriamycin becomes covalently attached to the beads; the remaining unreacted imidazole carbamate groups are eliminated with an excess of hydroxylamine. Unattached adriamycin is removed by exhaustive washing (2 weeks of continuous exposure) with borate buffer, acetonitrile, and methanol. Large volumes (0.5 to 1 liter) from the washings are collected, evaporated under reduced pressure, and assayed for adriamycin by high-pressure liquid chromatography (*14*). These washings do not damage the structural integrity of the beads, as judged by light microscopy. The washings are continued until no free drug can be detected. The resulting immobilized adriamycin is stored in the dark at 4°C. It does not release free drug for at least several months.

There are reasons, however, to question whether DNA interaction is a sufficient explanation for the ability of adriamycin to kill tumor (or any) cells. For example, if one reviews the literature on the hundreds of anthracycline derivatives synthesized, no obvious relation emerges between the ability to inhibit nucleic acid synthesis and cytotoxicity. Moreover, anthracycline synthesis programs in various laboratories have produced active adriamycin-like drugs that have no demonstrable ability to bind DNA, effectively ruling out DNA intercalation as an absolute requirement in cytotoxic action. For example, *N*-trifluoroacetyl adriamycin-14-valerate is an active anthracycline with little or no ability to bind DNA (*5*). Even the effects of the single agent adriamycin on cell viability are not tightly coupled to inhibition of DNA or RNA synthesis (*6*).

Many membrane activities are modulated by adriamycin, including lectin interaction (*7*), glycoprotein synthesis (*8*), phospholipid structure and organization (*9*), fluidity (*10*), fusion properties (*11*), transport of small molecules and ions (*12*), and expression of hormone receptors (*13*). The anthracyclines can act on biological membranes, but this does not necessarily mean that their cytotoxic effects on cancer cells are mediated by membrane interactions.

In this report we present evidence that the cell surface is a target for the action of adriamycin. Our approach was to expose murine cancer cells (line L1210) to large, insoluble, polymeric beads to

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which adriamycin had been covalently attached. Since the support was larger than the cells, the drug could not penetrate to the cytoplasm or nucleus. Any cytotoxic effect under these conditions would be direct evidence that DNA intercalation or any other intracellular binding is not essential for the expression of adriamycin's pharmacological activity.

The structure of the immobilized adriamycin used in these experiments is shown in Fig. 1. The matrix was 6 percent cross-linked agarose beads; adriamycin was attached through an *N*-alkyl carbamate linkage. Cells were exposed to free adriamycin, immobilized adriamycin, or underivatized agarose beads for a specified time and then cloned in soft agar to determine the surviving fraction. As expected, free adriamycin reduced the survival of these cells in a dose-dependent manner (Fig. 2a). The immobilized adriamycin likewise reduced the survival of L1210 cells, while underivatized agarose beads were without effect.

Another measure of cytotoxicity is obtained by growing cells in the continuous presence of drug and determining the growth rate by cell counting. Experiments done in this way also revealed that both free and immobilized adriamycin are cytotoxic to L1210 cells.

It was essential to demonstrate that free adriamycin was not released from the polymer, since such free drug would accumulate in the cellular interiors. We therefore extracted the intracellular contents and analyzed for free drug and its metabolites by high-pressure liquid chromatography (Fig. 2b) (*14*). Cells exposed to free adriamycin showed a dose-dependent intracellular accumulation of free drug. Adriamycin was also present in cells exposed to immobilized adriamycin, but only if the beads were being used for the first time (bar F in Fig. 2a). No detectable drug was released from these extensively washed beads in the absence of cells, suggesting that catalytic action at the cell surface was responsible for the appearance of free drug. The amount of drug released from the polymer was only a tiny fraction (< 0.005 percent) of the total available. If cells were again exposed to the same beads, this second exposure was still cytotoxic (bars G and H in Fig. 2b) but no intracellular drug could be detected, showing that additional leakage of drug from the polymer did not occur. When recycled a third time, the beads remained actively cytotoxic, with no release of free adriamycin.

We repeated this procedure numerous times and always found that freshly prepared polymers needed to be exposed to cells once before all the removable drug disappeared; thereafter the material was still biologically active. Since recycled polymer lacking adriamycin is not cytotoxic, the active principle in the recycled drug-containing polymer must be the remaining adriamycin. Consequently, we conclude that adriamycin can be cytotoxic solely by interaction at the cell surface.

An important consideration is the effective concentration of adriamycin to which cells are exposed. In the case of free drug, all the drug administered is potentially available for interaction with the cell. With immobilized adriamycin, however, we do not know a priori how much is on the polymer surface and how much is in the interior and thus unavailable to cells. The agarose beads themselves range in diameter from 40 to 210 μm, compared to the ~ 15-μm diameter of L1210 cells. Thus, endocytosis of immobilized drug is not possible. Moreover, the beads are freely permeable to low molecular weight substances like adriamycin but not to relatively large objects like cells. In preparing the immobilized adriamycin, we expected that the drug would react uniformly throughout the beads. To confirm this, we embedded agarose-immobilized adriamycin in Epon resin and sectioned the preparation with a microtome. All cross sections of new or recycled beads examined by fluorescence microscopy showed a uniform distribution of drug fluorescence. Assuming that a bead has a diameter of 100 μm, a surface-to-volume calculation shows that less than 0.01 percent of the total adriamycin is available at the bead surface for interaction with the cells. If the total concentration of adriamycin bound to beads is $8.5 \times 10^{-6} M$ (bar G in Fig. 2a), then the effective concentration available to cells is less than $8.5 \times 10^{-10} M$. Thus immobilized adriamycin is 100 to 1000 times more lethal than free adriamycin (bar D in Fig. 2a).

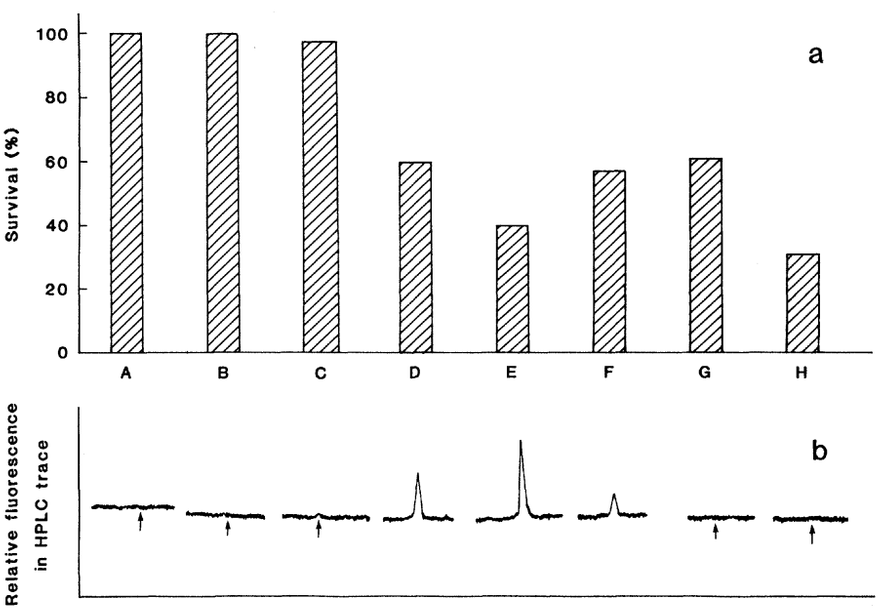

Fig. 2. (a) Survival of L1210 clones in soft agar following exposure to various adriamycin treatments. In each case, 50-ml cultures of cells (1×10^5 per milliliter) grown in Fischer's medium with 10 percent horse serum were exposed to the indicated condition for 2 hours at 37°C. A portion of cells then underwent cloning in soft agar, and the resultant colonies were counted 10 days later (*16*). The results are expressed as the percentage of cells surviving, with survival in the control condition, no adriamycin (A), representing 100 percent. Experimental conditions: (B) 100 mg of agarose without attached adriamycin but with all free imidazolyl carbamate groups removed by reaction with hydroxylamine; (C) $1 \times 10^{-9} M$ free adriamycin; (D) $5 \times 10^{-8} M$ free adriamycin; (E) $1 \times 10^{-7} M$ free adriamycin; (F) 50 mg of freshly prepared, extensively washed immobilized adriamycin; (G) 53 mg of recycled immobilized adriamycin; and (H) 100 μg of recycled, immobilized adriamycin (a preparation of immobilized drug to which cells had previously been exposed). Exposure to media alone is not sufficient to eliminate the dissociable adriamycin. (b) Cellular extracts associated with each experimental condition. Free adriamycin was quantitatively extracted from 5×10^6 L1210 cells and analyzed on a phenyl reversed-phase high-pressure liquid chromatography system (Waters uBondapak) (*14*). The retention time in this system is about 10 minutes; the expected elution position of adriamycin is indicated by an arrow in cases in which no peak is observed. All known metabolites are also separated by this procedure; none were observed. Even at $1 \times 10^{-9} M$ free adriamycin—a concentration causing virtually no reduction in survival of the cells—a small but definite free drug peak can be discerned. No free drug was detectable with the recycled immobilized adriamycin preparation.

One can rationalize this result by considering that the normal fate of free adriamycin is to be taken into the cell and thus removed from interaction with the cell surface. In contrast, immobilized drug cannot enter the cell and remains available for interaction at the surface, which appears to represent the most sensitive drug target.

These experiments do not rule out the possibility that administration of native adriamycin affects cell viability through DNA intercalation. Also, it is possible that free or immobilized adriamycin could lead to the formation of toxic activated oxygen species, as suggested by Bachur *et al.* (*15*). We have shown, however, that a drug heretofore thought to have intracellular DNA as its major site of action can exert its biological activity solely by interaction with the cell surface. If adriamycin does not have to enter cells to be effective, then some form of nonpenetrating adriamycin might be used to avoid the metabolic repercussions of free drug administration. Consequently, it may be important to consider the cell surface as a promising target in the design of a new generation of anticancer agents.

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Antidepressants Alter Cerebrovascular Permeability and Metabolic Rate in Primates

Abstract. *External detection of the annihilation radiation produced by water labeled with oxygen-15 was used to measure cerebrovascular permeability and cerebral blood flow in six rhesus monkeys. Use of oxygen-15 also permitted assessment of cerebral metabolic rate in two of the monkeys. Amitriptyline produced a dose-dependent, reversible increase in permeability at plasma drug concentrations which are therapeutic for depressed patients. At the same concentrations the drug also produced a 20 to 30 percent reduction in cerebral metabolic rate. At higher doses normal autoregulation of cerebral blood flow was suspended, but responsivity to arterial carbon dioxide was normal.*

In studies on rodents, tricyclic antidepressants have enhanced the ability of simple substances such as water and ethanol to cross the blood-brain barrier (*1*). Several observations suggested that this drug action might be clinically relevant (*1*): (i) it occurs at concentrations which are therapeutic for depressed patients, and (ii) chronic administration—patterned after the clinical use of the drugs—produces a sustained and heightened effect. We report that amitriptyline hydrochloride (AMI) produces this alteration in primates as well as in rodents and that the increase in the brain extraction of diffusion-limited substances is primarily the result of a drug-induced increase in cerebrovascular permeability.

The brain extraction of water (*E*)—that is, the percentage of a bolus of radiolabeled water which passes from the vascular compartment into the brain in a single transit through the cerebral microcirculation—is dependent on the permeability coefficient of water across the cerebral capillary (*P*), the mean capillary surface area (*S*), and cerebral blood flow (CBF). The relation of *E* to these variables is expressed by the equation

$$\ln(1 - E) = -PS/\mathrm{CBF} \qquad (1)$$

developed by Renkin and Crone (*2*). The *PS* product is the effective permeability of the blood-brain barrier to the tracer. At physiological flows in the monkey, it appears to depend primarily on changes in *P* (*3*).

Six adult rhesus monkeys (*Macaca mulatta*) weighing 6 to 8 kg were anesthetized with phencyclidine (2 mg/kg, intraperitoneally), paralyzed with gallamine, and passively ventilated with 100 percent oxygen. End-tidal CO_2 pressure, arterial blood pressure, and rectal temperature were continuously monitored.

A femoral artery was cannulated and a catheter advanced to the level of the right internal carotid artery; this position was confirmed fluoroscopically. Through this catheter, 0.2 ml of whole blood labeled with [^{15}O]water was injected into the cerebral microcirculation. The extraction of the tracer bolus into the brain (*E*) and the rate of its washout (CBF) were measured by external detection of the annihilation radiation produced by ^{15}O. Because of its short half-life, sequential injections of ^{15}O-labeled water permitted assessment of changes in *E* and CBF due to the treatment, and these were used in Eq. 1 to calculate changes in *PS* (*3*).

In the first monkey, AMI (10 mg/ml) was administered (7 mg/kg) at 1 minute (Fig. 1). Two additional injections of AMI (3.5 mg/kg) were made at 15 and 25 minutes. The initial injection produced a prompt 22 percent increase in *PS* without altering CBF or mean arterial blood pressure (MABP). Permeability returned to normal within 10 minutes. The second injection of AMI caused a sustained 60 percent increase in *PS* with MABP remaining above 90 mmHg. After the third injection, *PS* dropped but remained above pretreatment values, MABP dropped to 80 mmHg and remained stable, and CBF decreased to 55 percent of baseline.

This decrease in CBF suggests drug-induced suspension of normal CBF autoregulation because in the rhesus monkey CBF is normally independent of MABP until MABP drops below 60 mmHg (*4*). The reactivity of the cerebral vasculature was tested in two ways: (i) 5 percent CO_2 was added to the inspired gas mixture 83 minutes after the first AMI injection, and this produced an increase in CBF without altering MABP; (ii) angiotensin II was administered intravenously at 108 minutes, and this agent produced a transient but marked increase in MABP and CBF (Fig. 1). These results suggest that even though CBF remained responsive to cerebral metabolic demands, as shown by increasing arterial pressure of CO_2, normal autoregulation (CBF is independent of perfusion pressure over a wide range of pressures) was suspended in this subject. Despite the injection of angiotensin II and administration of 5

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