Our study shows that long-term growth of the *Babesia* parasites in vitro did not appear to reduce their ability to produce protective antigens. The absence of erythrocyte stroma from the immunogen makes it useful for cattle of all ages. Vaccinated cattle were still immune to babesiosis when circulating antibody titers had stabilized at low levels, suggesting that protection persisted beyond the interval studied in this report. Subsequent exposure to tick-borne *Babesia* infection would probably confer species-specific protection for life (*1*). The finding that *B. bigemina*–immune cattle are susceptible to *B. bovis* suggests that protection of cattle from both species of parasite in the field will require vaccination with species-specific antigens.

The concentration of soluble antigen derived from the *B. bovis* culture system that we used is probably much lower than that attainable with the recently developed microaerophilus stationary phase (MASP) culture system (*19*). Studies are now needed on the duration of immunity after various doses and injection regimens with cell-free immunogens derived from the more efficient MASP culture system.

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14. The parasites were actively growing in vitro approximately 50 percent of the time with a generation time of approximately 24 hours alternating with periods of storage at 4°C.
15. The Quil A, containing 1.5 percent dry matter, was obtained from Superfos Export Co. a/s, Vedbaek, Denmark.
16. *Babesia bigemina* was isolated from pooled blood collected from 70 cattle in Tizimin, Yucatan, Mexico. Adult female ticks were infected by allowing them to feed on an animal during peak parasitemia, and their larval progeny were used to establish carrier infections in the cattle that were subsequently challenged with tick-borne *B. bovis* in this report.
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Alcohol-Dependent Liver Cell Necrosis in vitro: A New Model

Abstract. *In alcoholic liver injury, necrosis is involved in the progression from benign fatty liver to alcoholic hepatitis and cirrhosis. However, there is no practical model of alcohol-dependent liver cell necrosis. The calcium-dependent killing of cultured rat hepatocytes by two different membrane-active hepatotoxins, galactosamine and phalloidin, is potentiated by ethyl alcohol. This indicates that some general physical effect of alcohol on cellular membranes renders cells susceptible to otherwise nonlethal injuries. The in vitro model described in this report may thus be used to search for a general mechanism underlying alcohol-related tissue injury.*

Alcoholic liver disease is primarily restricted to the benign and asymptomatic fatty liver. After years of alcohol (ethanol) abuse, a minority of alcoholics suddenly develop the potentially lethal condition of alcoholic hepatitis, an acute disorder characterized by necrosis and inflammation (*1*). Similarly, the transformation of fatty liver into cirrhosis—whether or not mediated by alcoholic hepatitis—involves the establishment of a necrotizing process (*2*). Thus the key to understanding the development of serious liver disease in chronic alcoholics is probably an understanding of the pathogenesis of liver cell necrosis.

A practical experimental model with which to study alcohol-dependent necrosis of hepatocytes has been lacking. The unpredictable occurrence of alcoholic hepatitis and the small amount of tissue obtainable by needle biopsy preclude functional studies at the cellular level. The baboon model of alcoholic hepatitis and cirrhosis (*3*) is also of limited usefulness because of the small proportion of animals developing these disorders, the unpredictability of the time at which necrosis occurs, and the expense of maintaining these primates.

In attempting to develop a model of alcohol-dependent necrosis, we were guided by several considerations: (i) alcohol does not kill hepatocytes at the concentrations reached in chronic alcoholics (~ 100 m*M*) (*4, 5*); (ii) alcohol can damage organs in which it is not metabolized, such as the heart (*6*) and pancreas (*7*); (iii) the presence of alcohol "fluidizes" or "disorders" various biological membranes in a dose-dependent fashion (*8*) and interferes with certain membrane functions (*5, 9*); (iv) toxic liver necrosis generally involves damage to the plasma membrane, resulting in an influx of lethal amounts of calcium ions (*10, 11*); and (v) alcohol enhances the liver cell necrosis produced by a wide variety of chemicals (*12*). In view of these points, we hypothesized that the physical effects of alcohol on biological membranes promote liver cell necrosis by potentiating the action of other membrane-active hepatotoxins.

We have used primary cultures of adult rat hepatocytes to explore the mechanisms underlying the liver cell death produced by various toxins (*10, 11*). Two of these, galactosamine and phalloidin, act on the plasma membrane to cause reversible cell injury in the absence of extracellular Ca^{2+} (*11, 13*) and lethal injury in its presence (*10, 11*). In both cases there is probably disruption of the permeability barrier function of the plasma membrane. With each toxin, however, the mechanism of such damage is different. Phalloidin, a bicyclic heptapeptide isolated from the mushroom *Amanita phalloides* (*14*), polymerizes actin monomers intimately associat-

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ed with or embedded in the plasma membrane (*15*). Galactosamine is metabolized by cytosolic enzymes through reaction with uridine 5′-triphosphate and accumulation of uridine 5′-diphosphate (UDP) and galactosamine (*13, 16*). This induces a deficiency of uridine nucleotides and UDP hexoses. The accumulation of nonphysiological UDP and galactosamine (hexosamines are normally present as *N*-acetyl-UDP derivatives) or the depletion of normal substrates is probably associated with disordered glycoprotein or glycolipid metabolism—reflected in the altered plasma membrane function. Cytochalasin B reverses the effect of phalloidin on actin polymerization and prevents the lethal consequences of adding extracellular Ca^{2+} to liver cells reversibly injured by phalloidin (*10, 11*). Similarly, uridine reverses the effects of galactosamine on uridine-containing compounds (*15*) and prevents the cell death accompanying the addition of extracellular Ca^{2+} ions to cells previously treated with galactosamine (*13, 16*).

We treated primary cultures of rat hepatocytes with galactosamine or phalloidin in the presence or absence of 100 m*M* ethyl alcohol. Table 1 shows the results for treatment with galactosamine at 100 μg/ml for 18 hours, a dose lower than that used previously (*10, 11*) in order to reduce the extent of killing in the absence of alcohol. Alcohol was added 6 and 16 hours after the addition of galactosamine. The data indicate that alcohol potentiates cell killing by galactosamine and that this effect is dependent on the concentration of Ca^{2+} ions in the culture medium. Neither galactosamine alone nor galactosamine plus alcohol killed cells in medium containing 0.13 m*M*, as opposed to 3.6 m*M* $CaCl_2$. Alcohol alone had no effect, irrespective of the Ca^{2+} concentration of the culture medium.

The addition of alcohol to phalloidin-treated hepatocytes also produced significant cell death over 2 hours (Table 2). This effect was again dependent on the concentration of Ca^{2+} ions. In this case, the cells were treated with phalloidin in medium containing 1.8 or 0.02 m*M* Ca^{2+}. Cell death with phalloidin alone and with phalloidin plus alcohol occurred only when the medium contained the high concentration of Ca^{2+}. As in the experiment with galactosamine, alcohol alone had no effect.

These experiments establish a potentially useful, new, in vitro model of alcohol-dependent cell death. The data are consistent with those presented in many previous reports of alcohol's ability to potentiate the toxicity of various chemicals (*12*). The experimental model used in the present study, however, has several advantages over previous ones. The use of cultured hepatocytes has the obvious advantages associated with tissue culture systems. In addition, the demonstration that the lethal effect of membrane-active hepatotoxins plus alcohol is dependent on the presence of extracellular Ca^{2+} implicates the action of toxins and alcohol on the permeability properties of the plasma membrane as a site of their interaction. This provides a new system for studying the mechanisms of membrane injury and death in hepatocytes and permits speculation regarding the pathogenesis of alcoholic hepatitis and cirrhosis.

The nature of the synergism between alcohol and membrane-active toxins is not clear. The lethal increase in membrane permeability to Ca^{2+} might result from a specific interaction of alcohol with each toxin or its specific site of attack. This would require a different mode of action of alcohol for each toxin. While such a possibility cannot be excluded, it seems more likely that calcium entry (and thus, toxicity) is potentiated by a general physical effect of alcohol on the cell membrane. Such an effect could potentiate the membrane consequences of poisoning with two agents as diverse as galactosamine and phalloidin. It is also possible that alcohol impairs the extrusion of Ca^{2+} by its effect on adenosinetriphosphatase in the membrane (*17*), thus interfering with the means of counteracting the accumulation of cytoplasmic Ca^{2+}. Changes in the lipid composition of membranes after chronic alcohol ingestion (*18*) may further complicate explanation of the phenomenon.

The data presented here may be relevant to the problem of the sudden appearance of necrosis with alcoholic hepatitis after years of alcohol ingestion. Random exposure of the liver to membrane-active toxins may ordinarily be well tolerated. Examples of such toxins may include products of intestinal bacteria, ingested xenobiotics, viruses, and

Table 1. Alcohol potentiation of galactosamine-induced liver cell death. Hepatocytes were isolated from the livers of food-deprived female Wistar rats (150 to 200 g) by collagenase perfusion (*10, 11*). The hepatocytes were plated in plastic 25-cm^2 dishes at a density of 10^5 cells per square centimeter in Williams E medium (Flow) containing 10 percent fetal calf serum (Flow) inactivated by heating to 56°C for 10 minutes, Garamycin (50 μg/ml; Schering) and insulin (0.02 U/ml; Squibb). The cells were incubated at 37°C in a humidified atmosphere of 5 percent CO_2 amd 95 percent air for 90 minutes to allow the cells to attach. Cultures were then rinsed three times with Hanks balanced salt solution without Mg^{2+} or Ca^{2+} and placed in Williams E medium with serum, Garamycin, insulin, and either with 3.6 m*M* $CaCl_2$ or without Ca^{2+}—except for that added with the serum (final Ca^{2+} concentration, 0.13 m*M* by atomic absorption spectroscopy). They were then treated immediately with D-galactosamine (Sigma). Cultured hepatocytes rapidly lose the ability to metabolize galactosamine, and maximum toxicity is obtained by adding galactosamine with the initial change of medium. Ethyl alcohol (Pharmco) was added directly to the medium to a final concentration of 100 m*M* 6 and 16 hours after the addition of galactosamine. Controls received no additions or alcohol alone. Cell survival was determined by Trypan blue exclusion 18 hours after galactosamine was added. Trypan blue (0.4 percent in 0.15 percent NaCl; Gibco) was added to cultures to a final concentration of 0.02 percent. The attached cells that excluded the dye were counted with a 10-mm^2 eyepiece grid in an inverted microscope (×200). Cell survival is expressed as a percentage of the number of unstained cells in untreated cultures. All measurements were made by counting five fields in each of triplicate cultures.

Treatment	Cell survival (% of control)	
	High $[Ca^{2+}]$	Low $[Ca^{2+}]$
None	100 ± 3	100 ± 2
Alcohol	96 ± 2	99 ± 3
Galactosamine	83 ± 6	96 ± 5
Galactosamine plus alcohol	49 ± 5	91 ± 5

Table 2. Alcohol potentiation of phalloidin-induced liver cell death. Hepatocytes were prepared and plated as described in the legend to Table 1. After being incubated for 90 minutes, the cultures were rinsed once with Hanks balanced salt solution and placed in Williams E medium, Garamycin, and insulin for 18 hours. Unlike their response to galactosamine, cultured hepatocytes do not lose their ability to metabolize phalloidin. Furthermore, after the cells have been in culture for some time and have attached and spread on the culture plate, the effect of phalloidin on the cells in the absence of extracellular Ca^{2+} can be evaluated by the formation of plasma membrane evaginations (*10, 11*). The cultures were rinsed three times with Hanks balanced salt solution without Mg^{2+} or Ca^{2+} and placed in Williams E medium without serum and with or without 1.8 m*M* $CaCl_2$. The cultures were then treated with phalloidin (30 μg/ml; Boehringer Mannheim) and 100 m*M* ethyl alcohol as indicated. Cell survival was determined after 2 hours.

Treatment	Cell survival (% of control)	
	High $[Ca^{2+}]$	Low $[Ca^{2+}]$
None	100 ± 2	100 ± 3
Alcohol	100 ± 3	101 ± 3
Phalloidin	86 ± 3	100 ± 2
Phalloidin plus alcohol	54 ± 3	100 ± 3

drugs. However, in the presence of alcohol, such agents may overwhelm the homeostatic mechanisms of the cell by permitting an influx of lethal amounts of calcium. Such a mechanism might also explain alcohol-induced disease in organs that do not metabolize alcohol, including the heart, pancreas, and central nervous system.

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Astatine-211–Tellurium Radiocolloid Cures Experimental Malignant Ascites

Abstract. *An investigation of the efficacy of astatine-211–tellurium colloid for the treatment of experimental malignant ascites in mice reveals that this α-emitting radiocolloid can be curative without causing undue toxicity to normal tissue. By comparison, negatron-emitting phosphorus-32 as colloidal chromic phosphate had no antineoplastic activity. The most compelling explanation for this striking difference is the dense ionization and short range of action associated with α-emission. These results have important implications for the development and use of α-emitters as radiocolloid therapy for the treatment of human tumors.*

Although the potential of directed but unsealed sources of radiation for cancer therapy was recognized early (*1*), the therapeutic potential of such sources remains largely unrealized. The shortcomings have been both physical and biological; not only must the radionuclide deposit its energy within a short range of action, but it must also be localized preferentially within or in close proximity to tumor cells. If appropriate carriers can be devised and labeled with α-emitting radionuclides, these requirements should be satisfied.

The α-particles emitted in the process of radioactive decay (i) are directly ionizing, (ii) have energies (E_α) of 5 to 8 MeV, (iii) have a range of several cell diameters, and (iv) have a high linear energy transfer which results in high specific ionization; their radiobiological effects are largely independent of cellular oxygenation. Among the available α-emitters, ^{211}At appears the most promising (*2*). The average E_α is 6.8 MeV, and the range in water is 60 μm; the average linear energy transfer is 113 keV/μm. The chemical properties of astatine are quite different from those of iodine, its nearest halogen neighbor; nonetheless, astatine is concentrated by thyroid tissue, albeit less avidly than iodine (*3*).

We have prepared ^{211}At-tellurium colloid and investigated its therapeutic efficacy in a malignant ascites tumor model. The therapeutic ratio should be highly favorable in this system because the radionuclide is administered directly into the peritoneal cavity and is brought directly into contact with tumor cells. The decay characteristics of ^{211}At emissions are such that the critical normal tissue, intestinal mucosa, is largely spared the cytotoxic effects of the emitted α-radiations because of the thickness of the serosa and muscularis relative to the α-particle range. This model provides a quantitative experimental system in which to assess risk-benefit considerations that may be directly applicable for evaluating human radiocolloid therapy.

The ^{211}At was produced on the 60-inch cyclotron of the Brookhaven National Laboratory. Targets were prepared by melting bismuth-209 onto circular aluminum disks. These water-cooled targets were irradiated with α-particles (21 to 28 MeV) to produce the reaction ^{209}Bi(α,2n)^{211}At. The beam current was 10 to 15 μA, and the irradiation time was 2 to 6 hours. We isolated the ^{211}At from the target by distillation at 700°C and collected it in a trap containing 0.1*N* sodium hydroxide and 0.01*N* sodium bisulfite. Preparations were determined to be chemically pure by elemental analysis

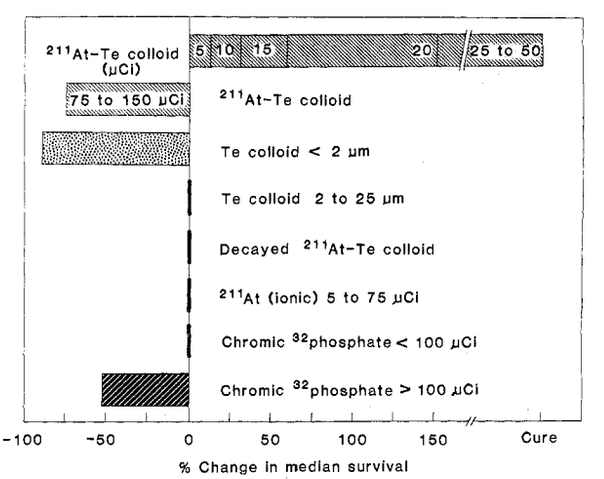

Fig. 1. Results of radiocolloid therapy on experimental malignant ascites in mice, expressed as the percentage of change in median survival. Each experimental group contained 10 to 12 mice; experiments were performed three times. Nonradioactive tellurium colloid < 2 μm in size is uniformly lethal in 3 days, presumably the result of pulmonary insufficiency.

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