to be effective in decreasing those suppressor mechanisms activated in vivo by tumor antigens that interfere with specific adoptive immunotherapy (*10*). We realize, however, that administering low doses of cyclophosphamide or radiation may have a synergistic effect when combined with LAK cells plus RIL-2.

The combination of normal splenocytes and RIL-2 or RIL-2 alone was ineffective in reducing established pulmonary sarcoma metastases. This finding suggests that LAK cells are not generated in vivo in sufficient amounts by intraperitoneal administration of RIL-2 at the doses used in these experiments. The serum half-life of IL-2 in vivo is short (3 to 4 minutes) in the murine host (*11*), and serum inhibition of IL-2 activity, as reported previously (*12*), may limit the usefulness of IL-2 administration in vivo.

Because LAK cells are nonspecifically generated by activation with IL-2 alone, they are more easily obtainable than lymphoid cells specifically sensitized in vivo or in vitro to unique tumor antigens. This is particularly important when considering human tumors that have poor immunogenicity and in which the availability and the preparation of suitable human tumor material for sensitization in vitro is a major problem. By using recombinant IL-2, human LAK cells with properties in vitro identical to those of murine LAK cells can readily be generated in vitro (*2, 3, 5*). Clinical trials of the infusion of human LAK cells generated with RIL-2 as well as Phase I trials of the infusion of RIL-2 systemically into humans have recently begun.

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Inhibition of Collagen Fibril Formation in Vitro and Subsequent Cross-Linking by Glucose

Abstract. *Glucose inhibits collagen fibril formation in vitro. A linear dose response was observed, with half-maximum inhibition of fibril formation occurring at 50 mM glucose. Nonfibrillar collagen cannot be cross-linked by lysyl oxidase, an enzyme that catalyzes the initial cross-linking reaction. The degree of decreased fibril formation correlated with the loss of ability of the collagen to serve as a substrate for lysyl oxidase. Collagen that is not cross-linked is unstable and more susceptible to collagenolytic attack. Interference with collagen cross-linking and more rapid degradation may explain the decreased amounts of interstitial collagen and the poor healing of wounds associated with diabetes mellitus.*

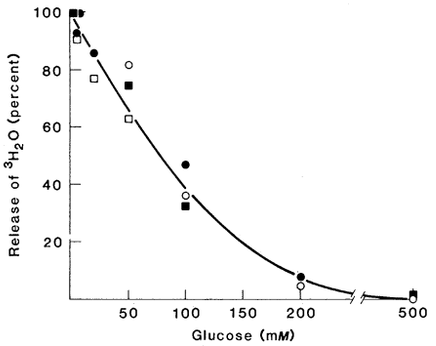
A compromised vascular supply due to thickened capillary basement membranes is the mechanism usually invoked to explain the slow and poor healing of wounds in diabetic patients. However, there is a lower content of interstitial collagen in wounds (*1*) and the skin (*2*) of diabetics than in normal subjects. The tensile strength of wounds and the collagen content of wound chambers is reduced in streptozotocin-induced diabetic rats as compared to control animals (*1*). The mechanism by which this occurs is unknown, but accelerated catabolism of interstitial collagen in streptozotocin-induced diabetic rats probably acts in this process (*3*).

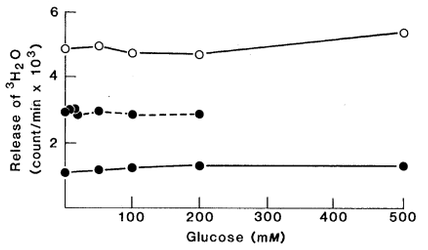
The rate of degradation of interstitial collagen is related to the degree of collagen cross-linking. Cross-linked collagen is more resistant to collagenase degradation than the polymer that is not cross-linked (*4*). Fibril formation is the critical step in the cross-linking of collagen (*5*), and glucose and other sugars have been shown to inhibit collagen fibril formation in vitro (*6*). We now describe our experiments to test the hypothesis that glucose may decrease cross-linking through the inhibition of fibril formation and present evidence that decreased cross-linking and enhanced susceptibility to proteolytic attack may be the mechanism of the connective tissue defect in diabetes.

The effect of glucose on collagen cross-linking was investigated with the use of an in vitro model. Isotopically labeled native collagen was obtained from cultured embryonic chick calvaria incubated in vitro with [6-^{3}H]lysine. The initial reaction in the cross-linking of collagen by the enzyme lysyl oxidase is the formation of ε-aldehydes from the ε-amino groups in certain lysyl and hydroxylysyl residues (*7*). Formation of aldehydes at the 6 position of lysyl or hydroxylysyl residues of the [6-^{3}H]lysine-labeled collagen substrate results in release of ^{3}H from that position and formation of ^3H_2O (*7*). The amount of released ^3H_2O correlates directly with the formation of aldehydes, which are the actual precursors of the lysine-derived cross-linkages in collagen (*7*). Therefore, by measuring the amount of released ^3H_2O, we estimated the rate of aldehyde formation obtained under our experimental conditions.

Lysyl oxidase activity is dependent on the physical state of the collagen substrate. This activity is high with precipitated collagen fibrils and lower with soluble collagen (*5*). The effect of glucose on fibril formation and the subsequent substrate activity was measured by incubating the substrate with various concentrations of glucose at 37°C for 1 hour and then adding lysyl oxidase and incubating the mixture again. The amount of ^3H_2O released after the reaction with lysyl oxidase was suppressed in the presence of glucose (Fig. 1). A linear relation between glucose concentration and dose response was observed up to 100 m*M*. Glucose inhibited release of ^3H_2O at concentrations as low as 5 m*M*, and half-maximum inhibition occurred at 50 m*M*. At 200 m*M*, the amount of ^3H_2O released was less than 10 percent of the control reaction.

To determine whether glucose affected the cross-linking reaction by acting directly on the inhibiting lysyl oxidase, we added glucose to the isotopically labeled collagen substrate after the collagen had been precipitated and reconstituted as a fibril. The rate of ^3H_2O release was not affected by the presence of glucose, even at concentrations as high as 500 m*M* (Fig. 2). To determine whether the inhibitory effect of glucose was reversible, we added glucose to the substrate and





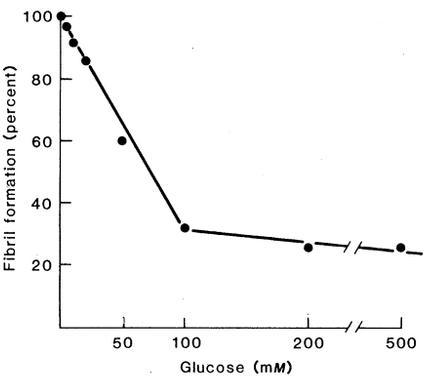


Fig. 1 (left). Inhibitory effect of glucose on the lysyl oxidase reaction as measured by the amount of ^3H_2O released from [6-^{3}H]lysine–labeled collagen. The isotopically labeled collagen was prepared from 17-day-old chick calvaria (*6*). The partially purified lysyl oxidase enzyme preparation was derived from the femoral and tibial epiphyseal cartilage of 17-day-old chick embryos (*7*). Briefly, the enzyme fraction was extracted with 6*M* urea and 50 m*M* tris (*p*H 7.6) from an epiphyseal cartilage homogenate and was partially purified with a DE-52 cellulose column (Whatman) by means of a linear gradient of NaCl from 0 to 0.5*M*. Fractions from the peak of enzyme activity were pooled and dialyzed overnight at 4°C against phosphate-buffered saline (PBS; 0.15*M* NaCl, 0.1*M* Na_2HPO_4; *p*H 7.5). The lysyl oxidase enzyme assays were performed as described (*6*). In each assay tube, 0.5 to 0.6 nmol of collagen (specific activity, 1.0×10^{15} count/min per mole) was used. Various amounts of glucose stock solution (1.25*M* glucose in PBS) were added to the assay tube to attain the final concentrations of 5 to 500 m*M*. In control tubes, PBS alone was added. The final reaction volume was 250 μl. Tubes were incubated at 37°C for 1 hour to permit collagen fibril formation. Enzyme fractions of 0.5 ml were then added, and incubation continued for 2 hours more. After distillation, the amount of ^3H_2O was determined in 10 ml of Aquasol (New England Nuclear) in a Beckman liquid scintillation counter with a counting efficiency for tritium of 30 percent. The ^3H_2O release for control experiments was considered as 100 percent. Each symbol represents an independent experiment. Fig. 2 (top right). The direct effect of glucose on the lysyl oxidase assay evaluated in three control experiments. When collagen fibril had formed after incubation of the substrate at 37°C for 1 hour, glucose was added. The ^3H_2O release was expressed as counts per minute per 5×10^5 count/min of collagen substrate. No inhibitory effect of glucose was observed (○—○). The reversibility of the glucose effect on the release of ^3H_2O catalyzed by lysyl oxidase was evaluated with [6-^{3}H]lysine–labeled collagen. In control groups no glucose was added, and the volume was adjusted by adding PBS. The collagen substrate and glucose mixture were placed in dialysis tubes and dialyzed against the same concentration of glucose for 1 hour at 37°C to permit fibril formation. However, no increase of the turbidity was observed in the presence of glucose. The samples were then thoroughly dialyzed against PBS at 4°C to remove glucose, at which time the fibril that had formed dissolved. The collagen substrates were then harvested. A 50-μl portion was removed for measurement of the amount of label (5×10^5 count/min of each group was used for the assay). Collagen substrates were incubated for 1 hour at 37°C at that time. Fibril formed to the same extent in control and experimental groups. Enzyme was then added as described (see legend to Fig. 1). The ^3H_2O release is expressed as counts per minute per 5×10^5 count/min of collagen substrate (●—●). As noted, the zero glucose point showed a value lower than that for the groups treated with glucose. This can be explained by the presence of trace amounts of endogenous lysyl oxidase, an enzyme known to be tightly bound to its collagenous substrate (*5, 8*). During the preliminary incubation at 37°C, the endogenous lysyl oxidase may have released some ^3H_2O as fibrils formed. The ^3H_2O was then lost during the subsequent dialysis. When glucose was present in the preliminary incubation, loss of ^3H_2O was limited because of the inhibition of fibril formation. Therefore, when the subsequent assay was performed, the amount of ^3H_2O appeared to be slightly higher in those samples that had been treated with glucose. The effect of glucose on the lysyl oxidase reaction was measured with the use of insoluble elastin as a substrate. [4,5-^{3}H]Lysine–labeled elastin was prepared from 17-day-old chick embryo aortas (*6*), and 5×10^5 count/min was incubated with various concentrations of glucose for 1 hour at 37°C. Lysyl oxidase was then added, and incubation continued for 2 hours more. The ^3H_2O release is expressed as counts per minute per 5×10^5 count/min of elastin substrate. No inhibitory effect of glucose on ^3H_2O release was observed (●- -●). Fig. 3 (bottom right). Inhibitory effect of glucose on fibril formation of ^{3}H-labeled collagen. Chick calvarium collagen labeled with [6-^{3}H]lysine (500,000 count/min) was incubated with glucose in a microfuge tube in a final volume of 250 μl for 1 hour with shaking at 37°C. Samples were then centrifuged in a Beckman microfuge for 4 minutes. The amount of label in a 50-μl portion of the supernatant was measured by scintillation counting. The percentage of fibril formation was calculated by $(L_t - L_s/L_t) \times 100$, where L_t is the total label added and L_s is the label found in the supernatant. The volume of the pellet after centrifugation was assumed to be negligible. The extent of fibril formation in control groups was considered as 100 percent.

incubated the mixture at 37°C for 1 hour. The glucose was then removed by dialysis, and the collagen substrate was allowed to form fibrils by incubation at 37°C for 1 hour before performing the lysyl oxidase assay. The release of ^3H_2O in the substrate treated with glucose was not suppressed (Fig. 2).

To test whether glucose inhibits ^3H_2O release by interfering with fibril formation, we chose elastin, an amorphous nonfibrillar substrate, for the enzyme. When insoluble elastin was incubated with glucose at 37°C for 1 hour and then assayed, no effect of glucose was observed (Fig. 2). Even at 500 m*M* glucose, the amount of ^3H_2O released was the same as in controls to which no glucose had been added.

Glucose has been observed by spectrophotometry to inhibit collagen fibril formation (*6*). Both the rate of fibril formation and the final opacity are suppressed by glucose. To compare the effect of glucose on ^3H_2O release to its effect on fibril formation, we incubated collagen substrates at 37°C for 1 hour and then centrifuged them to separate reconstituted fibrils. Without glucose, fibril formation was in the range of 65 to 75 percent, compatible with results reported earlier (*8*). Glucose inhibited fibril formation in a linear fashion at concentrations up to 100 m*M* (Fig. 3), with half-maximum inhibition at 50 m*M*. The inhibitory effect reached a maximum of 75 percent at 200 m*M*, and no further inhibition occurred as the glucose concentration increased up to 500 m*M*.

We conclude from these studies that glucose inhibits collagen cross-linking by interfering with fibril formation. The physiological range of blood glucose in humans is 3.3 to 5 m*M*. Because the first effect we observed on cross-linking in vitro occurred at 5 m*M* glucose, and because the inhibitory effect was linear up to 100 m*M*, both fibril formation and cross-linking of collagen could conceivably be suppressed when the concentration of glucose in blood [which also reflects its concentration in tissues and wound fluid (*1*)] is elevated to three to four times the normal amount, as is observed in some diabetic patients. The nonenzymatic glycosylation of collagen, which is increased in both interstitial collagen (*9*) and basement collagen (*10*) in diabetic tissue, is not likely to play a role in our in vitro model because it is a slow reaction (*11*).

An explanation for the ability of glucose to inhibit collagen fibrillogenesis comes from a recent model. A hydrophobic cluster in the carboxyl terminal extrahelical peptide of type I collagen di-

rects lateral aggregation of chains during fibrillogenesis (*12*). Glucose, a polyol, might interfere with such hydrophobic interactions.

Since fibril formation is an early event in collagen synthesis (*13*), we speculate that the high glucose environment in diabetes may prevent fibril formation and the subsequent cross-linking reaction that occurs soon after synthesis of collagen. It has been shown that 30 percent of newly synthesized collagen is degraded in the skin of streptozotocin-induced diabetic rats, while only 13 percent is degraded in control rats (*3*). Our observations may explain the enhanced catabolism of newly synthesized collagen in diabetics. Collagen from diabetic tissues has been suggested to be more highly cross-linked because of its observed higher resistance to collagenase and to extraction with acetic acid in comparison to normal tissue (*14*). Because collagen becomes progressively more cross-linked with age, and because newly synthesized collagen is not cross-linked and is less stable than the preexisting collagen in diabetic tissue, the selective depletion of newly synthesized collagen may leave tissues composed predominantly of preexisting, more highly cross-linked collagen. The net result then would be an increase in the degree of cross-linking and a decrease in the quantity of interstitial collagen in diabetic tissues.

The specific effect of glucose on fibril formation may also explain the tissue-specific changes of collagen in diabetics. A net loss of interstitial collagen mass occurs in intact skin (*2*), wounds (*1*), and bone (*15*), whereas a net accumulation of basement membrane collagen mass occurs in the intestine (*2*) and glomerulus (*16*). Interstitial collagen forms fibrils, whereas basement membrane collagen does not (*17*). The accumulation of basement membrane collagen in diabetic tissue may be due to enhanced synthesis (*17*), decreased degradation (*18*), and some additional mechanisms (*19*). Thus, glucose specifically stimulates the catabolism of interstitial collagen—which requires cross-linking for persistence—and does not affect the catabolism of basement membrane collagen.

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Flotation of the Bivalve *Corbicula fluminea* as a Means of Dispersal

Abstract. *Small specimens of the Asiatic bivalve* Corbicula *c.f.* fluminea *(Müller) secrete long mucous threads through their exhalent siphons that act as draglines to buoy the animal into a water column. These mucous strands, secreted in response to water current stimuli, are produced by dense accumulations of ctenidial mucocytes and may help in the downstream or interstream dispersal of this rapidly spreading exotic clam.*

The spread of the freshwater Asiatic clam *Corbicula* c.f. *fluminea* (Müller, 1774) across North America since its accidental introduction about 50 years ago (*1*) has led to controversy over the mode of transport that could account for its invasiveness. Most often cited are theories of human transport (*2–4*), incidental transport of small byssate juveniles attached to bird feet or feathers or carried within fish or bird gastrointestinal tracts (*3, 5*), and direct transport of larvae or juveniles swept along stream bottoms with water currents (*2, 3, 6*). Although each of these transportation modes is feasible, there is little evidence to support any one of them as a primary means of dispersal. For instance, the viability of *Corbicula* in bird gastrointestinal tracts during transport is questionable, and high clam mortality has been suggested to occur (*5, 7*). Mackie (*8*), discussing possible methods of dispersion for other sphaeriid bivalves, concluded that their transport within bird intestinal tracts is unlikely (again because of mortality) but that external transport on bird feet or wings or on insect legs are still viable options. While this might be possible, although with probably low frequency, it is still, as with all the other proposed transport modes, a passive activity. In each case the transport is accidental, and the bivalves play no active role in dispersion. We now report an unusual active participation in transport by small adult *Corbicula* that may account for much of their downstream and perhaps interstream invasiveness.

Small adult *Corbicula* (shell length, 7 to 14 mm) collected in March 1984 from Tallahala Creek near Runnelstown, Mississippi, were found to be capable of floating after being exposed to gentle water currents produced by an aquarium filtration system (current speeds, 10 to 20 cm/sec). The behavior of the clams was consistent (Fig. 1). After initial exposure to the water flow, the clams would right themselves from the bare aquarium floor with their active and muscular foot. The clams would come to lie with their valves perpendicular to the substratum and with siphons directed upward. In contrast to its normal pumping activities (as seen during feeding or respiration), the exhalent siphon of each clam was abnormally distended with a wide lumenal space. After a period of 2 to 8 minutes, during which the clams continued to pump actively, they would gently lift off the substratum, often with their foot extended, and drift into the water column of the aquarium with the current flow. The upright posture of the