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In vivo expression of rat insulin after intravenous administration of the liposome-entrapped gene for rat insulin I

(recombinant DNA/macrophages/glycemia/external γ-imaging)

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ABSTRACT **A recombinant plasmid encoding rat preproinsulin I was encapsulated in large liposomes and intravenously injected in rats. Glycemia and blood, splenic, and hepatic insulin were assayed at various times beginning 6 hr after inoculation. The results were compared with controls that had received (*i*) empty liposomes, (*ii*) liposomes carrying the *Escherichia coli* pBR322 plasmid, (*iii*) the free rat insulin I gene, and (*iv*) no injection at all. Whereas all controls showed unchanged glucose and insulin levels, the treated animals had, 6 hr after inoculation, a blood glucose level of 72 ± 5 mg of glucose/100 ml of blood as compared with 107 ± 2 mg/ml for controls. Radioimmunoassay of blood insulin gave 61 ± 8 microunits/ml as compared with 43 ± 5 microunits/ml for controls and the spleen and liver values were 242 ± 22 and 204 ± 20 microunits/g of tissue, respectively, as compared with 112 ± 20 and 87 ± 15 microunits/g in controls. External γ-camera imaging of the organ uptake of ^{111}In-labeled liposomes permitted study of the kinetics and extent of uptake of the liposomes by spleen and liver, results that support the findings concerning insulin synthesis in the two organs.**

Recent studies have shown that, when injected intravenously, liposomes are taken up mainly in the liver and spleen by the macrophages of the reticulo-endothelial system (1–4). Intravenous injection of liposome entrapped molecules having long latency periods allows the transport of substantial amounts of such molecules to the cellular sites of liposome uptake—i.e., splenic macrophages and liver Kupffer cells (4, 5).

The use of liposomes to transfer genetic material into culture cells permitted the introduction of significant amounts of exogenous DNA into the cells and led in some cases to expression of the gene thus transported (6–8). We describe in this paper the transport by liposomes of the gene encoding rat preproinsulin I to the spleen and liver of rats. We show its expression in these organs, the release of the expressed hormone in the blood, and its influence on glycemia.

MATERIALS AND METHODS

Plasmid Preparations. The recombinant plasmid used in this study, p(gR19.4) contains a 9.4-kilobase *Eco*RI fragment encoding rat preproinsulin I at the *Eco*RI site of pBR322 (9). Plasmid DNA was prepared from amplified *Escherichia coli* strain HB101 cultures harboring p(gR19.4) or pBR322 according to ref. 10.

Liposome Preparation and Entrapment of DNA. Liposomes consisting of egg yolk phosphatidylcholine (PtdCho; Sigma) purified according to ref. 11/ox brain phosphatidylserine (PtdSer; Sigma)/cholesterol (Chol; Sigma), 8:2:10 (mol/mol) were prepared by reversed-phase evaporation (12). Briefly, a chloroform solution of the lipids (13 μM) was evaporated under nitrogen in a rotary evaporator. The lipids were dissolved in 3 ml of ether under N_2 and mixed with 5 ml of a solution of plasmid p(gR19.4) at a DNA concentration of 10 μg/ml in (50 mM Tris·HCl, pH 7.4/150 mM NaCl). The two-phase system was sonicated briefly and then the ether was removed at reduced pressure in the rotary evaporator. The liposomes thus formed were treated with DNAse I (Boehringer Mannheim) in the presence of 10 mM of $MgCl_2$ at 37°C (6); the mixture was incubated, the suspension was chromatographed on a Sepharose 4B column, and the ^{32}P radioactivity of the DNA was determined. The presence of liposomes was monitored by following the optical density of the samples at 620 nm. The fractions containing the liposomes and the encapsulated DNA were pooled and used for the intravenous inoculations.

Entrapment of ^{111}In-Labeled Bleomycin. To determine the location of the large PtdCho/PtdSer/Chol liposomes in the rat organism, liposomes encapsulating ^{111}In-labeled bleomycin were prepared. The entrapped ^{111}In-labeled bleomycin (Amersham, Radiochemical Centre; 2.5 mCi/ml; 1 Ci = 37 GBq) was separated from the free label by filtration on a Sepharose 4B column. The radioactivity of the liposome-entrapped tracer was ≈80 μCi/ml. The fractions containing the liposomes were pooled and 1-ml aliquots were used for intravenous injection. Controls were injected with free ^{111}In-labeled bleomycin and free $^{111}InCl_3$.

Visualization of the Organ Distribution of Liposome Encapsulated ^{111}In-Labeled Bleomycin by External γ-Camera Imaging. Anesthetized rats (sodium pentobarbital at 40 mg/kg, intraperitoneal) intubated via the jugular vein were placed on a Nuclear Chicago γ camera equipped with a high-resolution 140-keV parallel colimator and a Simis 3 data system (Informatek, Birmingham, AL). Animals were injected with 20 μCi of ^{111}In-labeled bleomycin, $^{111}InCl_3$ (Commissariat à l'Energie Atomique, France; 4 mCi/ml), or liposome-entrapped ^{111}In-labeled bleomycin, in a volume adjusted to 1.5 ml with isotonic saline. Ten-second dynamic scans were carried out continuously for 20 min using the 173-keV photopeak of ^{111}In. From the resultant images, the localization of radioactivity was determined for the areas of interest—the head (circulating blood), liver, spleen, kidneys, and bladder.

For the liposome-entrapped ^{111}In-labeled bleomycin-injected rats, 10-min static scans were carried out 6 hr after injection to estimate the remaining spleen and liver activity.

Injection of the Liposome-Encapsulated Rat Insulin I Gene: Assays of Blood, Spleen, and Liver Insulin and of Blood Glucose. Nonfasting male Wistar rats (300–350 g; from the Institut

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Abbreviations: PtdCho, phosphatidyl choline; PtdSer, phosphatidyl serine; Chol, cholesterol.

National de la Recherche Agronomique, Tours, France) were injected intravenously with 1 ml of the liposome-encapsulated gene for rat insulin I. During the study, the animals were not fed.

Four types of controls were used—animals receiving intravenous injections of 1 ml of "empty liposomes," free plasmid p(gR19.4) at 10 μg/ml, or liposomes having entrapped *E. coli* pBR322 and animals that had not received any inoculation.

The treated animals (five in each of two independent experiments) and all the controls (two by two for each type) were examined for blood glucose and blood, splenic, and hepatic insulin levels. Blood specimens were collected from the tail vein into heparin-containing microtubes 0, 6, 10, 12, and 18 hr after injection. Glycemia was measured immediately by the glucose-oxydase microtest (Merckotest-Glucose; Merck, Darmstadt, Federal Republic of Germany). Immunoreactive blood insulin was assayed by the method of Okajima and Ui (13) on 0.1-ml plasma samples. Rat insulin (Novo Industries, Denmark) was used as a standard.

To assay spleen and liver insulin, the organs were removed and immediately cooled to 2°C in 0.15 M saline. All subsequent procedures were carried out at this temperature. The organs were sliced with scissors and homogenized in a Turrax homogenizer in 0.05 M hypotonic saline (1 ml/g of tissue). The homogenate was sonicated with four 10-sec bursts of a MSE 225 sonicator and then centrifuged (10 min at 10,000 × *g*), and the insulin content was assayed in 0.1-ml aliquots of the supernatant.

RESULTS

Entrapment of DNA and ^{111}In-Labeled Bleomycin in Liposomes. PtdCho/PtdSer/Chol, 8:2:10 (mol/mol) liposomes entrapped typically 300 ng of plasmid DNA per ml of liposome suspension. This result was obtained both by chromatography on Sepharose 4B (Fig. 1) and by flotation in Ficoll gradients (data not shown). The encapsulated DNA, being protected, is insensitive to the action of DNAse I. The level of encapsulation of this 13.9-kilobase plasmid did not exceed 3.5% of the amount dissolved in Tris-buffered saline. ^{111}In-Labeled bleomycin was encapsulated with the same efficiency so that the radioactivity associated with the liposomes corresponded to approximately 80 μCi/ml. This label, encapsulated in liposomes, was used for the γ-imaging of the liposome distribution in the rat, after intravenous injection.

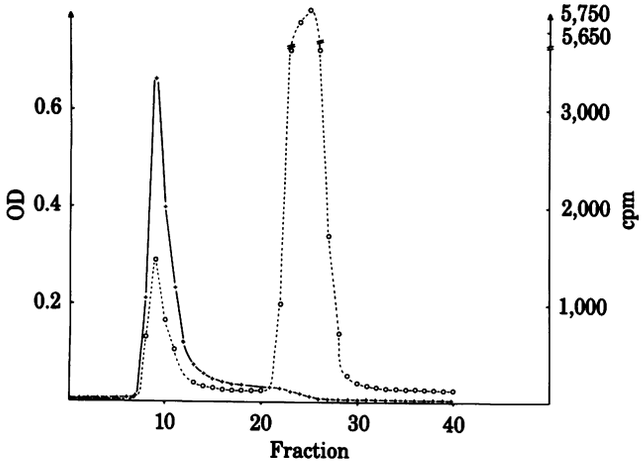


FIG. 1. Assay of macromolecules encapsulated in liposomes: Encapsulation of ^{32}P-labeled p(gR19.4) in reversed-phase evaporation PtdCho/PtdSer/Chol (8:2:10) liposomes. After encapsulation of the DNA, the system was incubated with DNase I for 30 min at 37°C in 10 mM Mg^{2+}/phosphate-buffered saline, pH 7.3. Filtration was through a Sepharose 4B column. ——, OD_{620}; ----, ^{32}P (cpm).

Liposome Distribution in the Rat After Intravenous Injection. The images obtained (Fig. 2 *A–D*) show that, between the injection time and 20 min later, a rapid and intensive uptake of ^{111}In-labeled bleomycin-liposomes by the liver occurred. A weak urinary elimination was observed as well. By 6 hr after injection (Fig. 2*E*), the liposome organ distribution had changed only slightly. A different positioning of the external γ-camera allowed a better view of the spleen, where considerable uptake of the liposome-encapsulated bleomycin also occurs.

The radioactivity in the bladder is probably due to destruction of the liposomes in the liver and concomitant release of the label. Similar observations were made with ^{99}Tc-labeled liposomes (14).

Six hours after injection, the rats were killed and the organs were individually visualized with the γ-camera (Fig. 2*F*). To check whether the liposomes were entirely responsible for the observed organ distribution of bleomycin, a similar experiment was carried out with free ^{111}In-labeled bleomycin and with $^{111}InCl_3$. (The latter was used to determine the pattern of distribution of the free ion, which could have resulted from dissociation of ^{111}In-labeled bleomycin.)

With these controls, the distribution pattern differed strongly from that observed with the liposome-encapsulated label. For ^{111}In-labeled bleomycin, 10 sec after injection, the radioactivity in the heart was high, corresponding to the tracer distribution phase, and two min after injection, bleomycin was already present in liver and in urine. The urinary excretion was particularly strong 5 min after injection and, 20 min after injection, practically all of the radioactivity was found in the urinary bladder. The absence of kidney radioactivity corresponds to the asymptotic phase of plasmatic clearance. The $^{111}InCl_3$ distribution (Fig. 3 *A–D*) differed from that of ^{111}In-labeled bleomycin in that urinary excretion was faster and more significant. Not even a transient liver uptake was observed.

The organ activities were measured individually 20 min after injection and the results are shown in Table 1.

The kinetics of organ uptake and of urinary excretion of the radioactivity are shown in Fig. 4. The kinetics of uptake and excretion were completely different for liposome-encapsulated ^{111}In-labeled bleomycin, free ^{111}In-labeled bleomycin, and $^{111}InCl_3$. Three animals were used for each compound studied.

***In Vivo* Expression of the Liposome-Entrapped Rat Insulin Gene.** The results described above indicated that, should the liposome-encapsulated insulin gene penetrate any cells, the target cells would be mainly in the liver and spleen. Knowing now that the percentage of the total liposome-encapsulated radioactivity injected decreases very little in the target organs (liver and spleen) during the first 6 hr, we examined the animals that had been inoculated with the liposome-encapsulated insulin gene, liposome-encapsulated pBR322, empty liposomes, and free p(gR19.4) 0, 6, 10, 12, and 18 hr after inoculation. Glycemia and blood insulin were measured in the live animals and, at the time points mentioned, in each experiment, three animals were killed, and liver and spleen insulin was assayed (Table 2).

Table 2 shows that injection of the liposome encapsulated insulin gene had a significant effect on the glycemia and insulin levels of the animals so treated. Glycemia in the treated animals showed a significant decrease 6 hr after injection that correlated quite well with the increase in the insulin blood level in these animals. At the same time, 6 hr after injection, the insulin found in the liver and spleen was twice the amount in control animals. An interesting feature is the relatively short time of increased

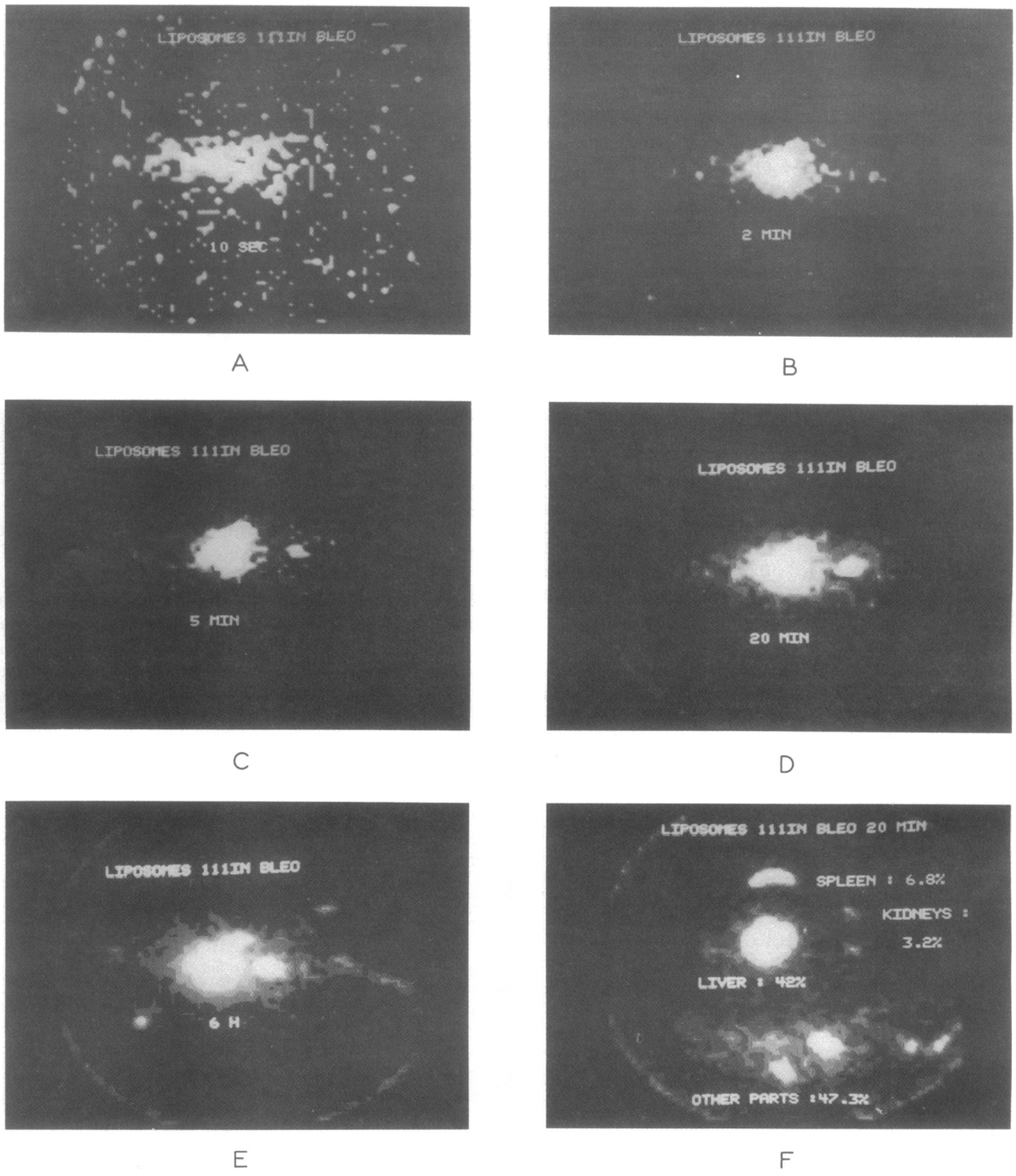


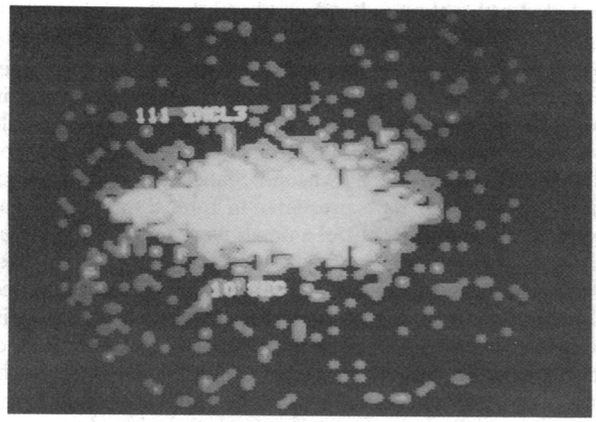
FIG. 2. γ-Imaging with an external camera of the distribution of radioactivity in the rat after intravenous injection of 1 ml of a suspension of liposome-encapsulated ^{111}In-labeled bleomycin (80 μCi) in phosphate-buffered saline (pH 7.3). Time after injection: *A*, 10 sec; *B*, 2 min; *C*, 5 min; *D*, 20 min; *E*, 6 hr. (*F*) Radioactivity of individual organs—liver, spleen, and kidneys—6 hr after injection.

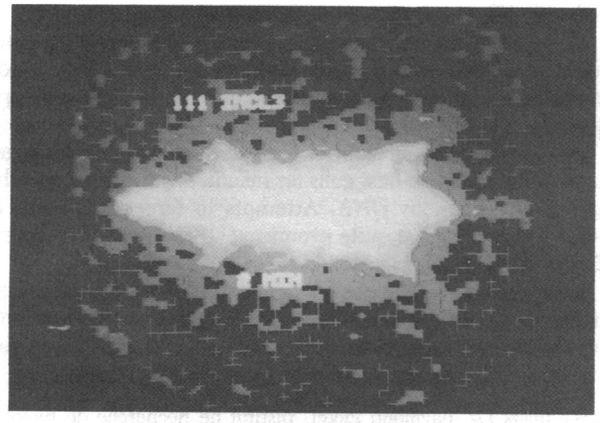
insulin synthesis observed with the treated animals. Glycemia began to increase 10 hr after injection and, 12 hr after injection, it was higher than the control values whereas the blood insulin decreased to less than the control values. The liver and spleen insulin values returned almost to normal by 18 hr after injection.

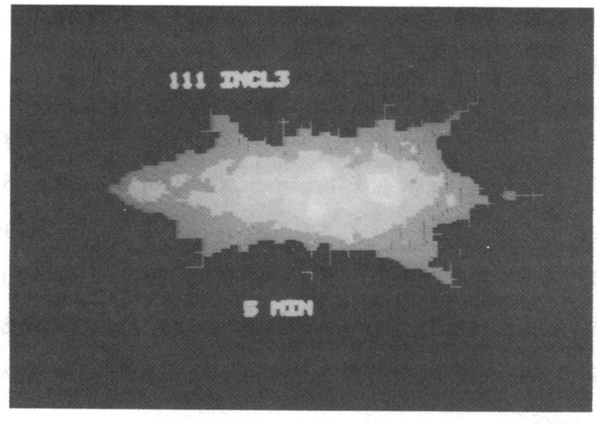
Whatever control materials were injected—free insulin gene, empty liposomes, or liposome-entrapped pBR322—the glycemia and insulin values remained relatively stable over 18 hr, as did those for untreated animals.

DISCUSSION

The data presented here indicate that intravenously injected large liposomes are taken up almost entirely by the livers and spleens of the injected animals. When these liposomes contain high amounts of cholesterol (e.g., phospholipid/cholesterol (mol/mol) ≈1), they are very stable in the blood for at least 18 hr (5). γ-Visualization of liposome-entrapped ^{111}In-labeled bleomycin permitted a kinetic study of the uptake of these liposomes. The kinetics of liposome uptake by the liver is fast







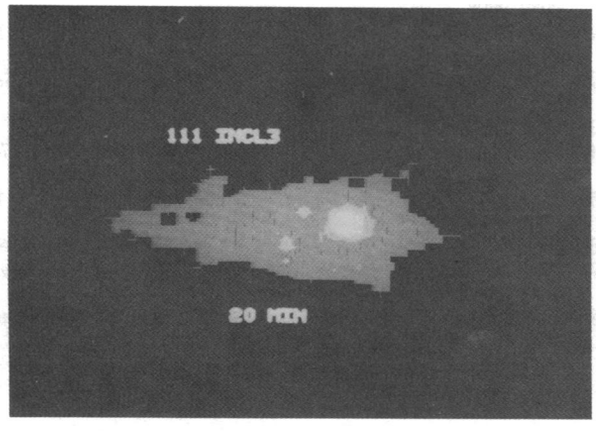


Table 1. Percentage of ^{111}In radioactivity in various organs of the rat 20 min after intravenous injection

Sample	Liver	Kidneys	Bladder
Liposome-encapsulated ^{111}In-labeled bleomycin	42 ± 4	<5	15 ± 4
Free ^{111}In-labeled bleomycin*	10 ± 5	7 ± 5	82 ± 4
$^{111}InCl_3$	0	<5	95 ± 4

Results represent mean ± SD of three measurements on four animals. For values lower than 5%, the statistical analysis does not allow the calculation of a standard deviation with any real significance.

* Liposome-free ^{111}In-labeled bleomycin tends to accumulate to some extent (10–15% of the administered dose) in the rat (as in the human liver; ref. 15). The level of accumulation is well below that of the liposome-encapsulated drug.

($t_{1/2} \approx 2$ min) and, once in the liver, the radioactivity transported by the liposomes decreases very slowly. The DNA segment encoding rat preproinsulin I that we used in this study contains, in addition to the insulin coding and intervening sequences, several kilobases of flanking sequences on each side of the gene (9). This suggests that regulatory signals are also

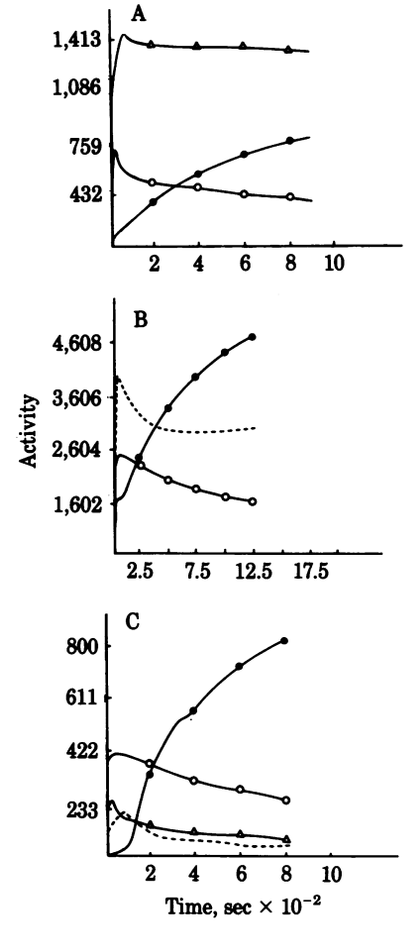


FIG. 4. Kinetics of organ uptake of ^{111}In after intravenous injection to rats in 1 ml of phosphate-buffered saline (pH 7.3). (*A*) Liposome-encapsulated ^{111}In-labeled bleomycin. (*B*) Free ^{111}In-labeled bleomycin. (*C*) $^{111}InCl_3$. △, Liver; ●, urine; ○, blood; ----, kidney. Results represent whole-body determination corrected for background radioactivity.

FIG. 3. γ-Imaging with an external camera of the distribution of radioactivity in the rat after intravenous injection of 1 ml of $^{111}InCl_3$ (80 μCi) in phosphate-buffered saline (pH 7.3). Time after injection: *A*, 10 sec; *B*, 2 min; *C*, 5 min; *D*, 20 min.

Table 2. Glycemia and blood, liver, and spleen insulin in control animals and in rats injected intravenously with the liposome-encapsulated insulin gene

Group	*n*	Glycemia, mg of glucose/100 ml of blood					Insulin								
							Blood, μU/ml				Liver, μU/g of tissue			Spleen, μU/g of tissue		
		0 hr	6 hr	10 hr	12 hr	18 hr	0 hr	6 hr	10 hr	12 hr	18 hr	0 hr	6 hr	18 hr	0 hr	6 hr	18 hr
Empty liposomes	4	110 ± 2	105 ± 2	118 ± 2	112 ± 2	110 ± 2	38 ± 6	40 ± 5	32 ± 8	42 ± 7	43 ± 8	85 ± 13	87 ± 15	84 ± 12	118 ± 15	112 ± 20	127 ± 20
Free insulin gene	4	106 ± 2	109 ± 2	ND	104 ± 2	ND	41 ± 5	45 ± 5	ND	51 ± 7	ND	ND*	ND*	ND*	ND*	ND*	ND*
Liposome-encapsulated pBR322	4	115 ± 3	113 ± 3	ND	107 ± 7	ND	45 ± 5	36 ± 5	ND	46 ± 7	ND	89 ± 12	82 ± 13	82 ± 14	125 ± 20	121 ± 18	123 ± 20
Not injected	4	103 ± 2	107 ± 2	116 ± 2	110 ± 2	103 ± 2	40 ± 6	43 ± 5	45 ± 6	50 ± 5	45 ± 7	ND*	ND*	ND*	ND*	ND*	ND*
Liposome-encapsulated insulin gene	10	108 ± 3	72 ± 6	86 ± 5	129 ± 6	138 ± 9	41 ± 6	61 ± 8	42 ± 6	24 ± 6	30 ± 5	82 ± 10	205 ± 22	105 ± 15	115 ± 18	242 ± 20	143 ± 20

For each animal, two determinations of each value were carried out and the SD was calculated for the mean value per animal. μU, microunit(s).
* Because little variation of the glycemia and insulin levels was observed during 18 hr with these controls, the liver and spleen insulin levels were not assayed.

included within the insulin plasmid and that the gene might be functional when transferred *in vivo* after its entrapment in liposomes. The observed organ distribution of the liposomes permitted us to assay the potentially expressed insulin in the incorporating cells. Indeed, quite enhanced amounts of insulin were found in the livers and spleens of the treated rats whereas those of the controls did not change. Statistical evaluation of the significance of the results presented in Table 2 was not possible because, although 16 control animals were used, the numbers of animals per control group were too low for such evaluation (for instance, by Student's *t* test). Nonetheless, the differences observed in the blood glucose and insulin levels 6 hr after inoculation between controls on one hand and treated animals on the other seem sufficiently clear that statistical tests, in this case, were not absolutely necessary. The insulin normally found in the liver and spleen of control animals is the blood pancreatic insulin that is bound on the insulin receptors present on these cells (16, 17).

These observations suggest that insulin is expressed in liver and spleen for a relatively short time (up to ≈10 hr). The reason might be either the ejection of the gene by the host cells or a quenching of this gene.

We are aware that Kupffer cells and splenic macrophages may not be the ideal host cells for insulin synthesis induced by uptake of exogenous DNA. Attempts to target liposomes to other liver cells seem quite promising (18), but insulin expression in a variety of cells should be investigated.

It appears that intravenously injected liposomes can be used as gene carriers to express the rat insulin I gene *in vivo*. This expression correlates both with the insulin level in blood and with glycemia. The expression is significant but transitory.

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