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Running Title: Immunoliposome Binding In Vivo

Target-Specific Binding of Immunoliposomes in vivo*

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I wish to thank the organization committee for inviting me to give this talk.

My group at the University of Tennessee has been concentrating on using monoclonal antibody for targeting of a liposomal drug carrier system (1). Today I will tell you about our initial effort to target these liposomes using an organ-specific monoclonal antibody (for a preliminary account, see 2).

Fig. 1 illustrates a very interesting and challenging problem for liposome targeting. What is shown here are sections of a mouse lung which contain tumor nodules of metastatic line-1 tumor cells. The lung contains a lot of air space as well as the capillary system. The tumor, however, contains no air space nor well developed capillary system. In other words, when an intravenously injected liposomal carrier system is used, immunoliposomes are unlikely to arrive at the tumor masses, even if one coats the liposomal system with a tumor-specific monoclonal antibody. Therefore, the next best thing to do would be to deliver liposomes to normal lung tissue and expect the liposomal encapsulated drug to diffuse freely from this nearby site to the tumor. This is a combination of targeted delivery and sustained release.

In order to achieve this task one would need a monoclonal antibody which is highly specific for the lung capillary endothelium. Dr. Kennel at Oak Ridge National Laboratory has isolated two of these monoclonal antibodies. Shown here (Fig. 1) is the distribution of [^{125}I]MoAb in tumor bearing lung. The

position of [^{125}I]MoAb 201B is detected by autoradiography (black grains) of paraffin sections of lung 3 h. after i.v. injection of the antibody. The normal capillaries are filled with this monoclonal antibody but the tumor mass is not. Thus, this particular antibody can target to the endothelium which is very close to the tumor mass.

At EM level it is possible to resolve the cell type reacting with the MoAb. Gold labelled secondary antibodies detect the presence of the rat MoAb bound specifically to the lumenal side of the endothelial cells (Fig. 2). In fact, when these monoclonal antibodies are injected i.v., more than 80 percent of the injected dose accumulates in the lung (2,4). They are highly specific for the lung endothelium, both in the capillary system and in the arteries.

These monoclonal antibodies bind to a particular antigen gp112 which is highly enriched in the lung (3,4,5). We know that gp112 is also expressed in cultured artery endothelial cells provided for us by Dr. Una Ryan. They display approximately two to three million molecules per cell surface (6).

One of the monoclonal antibodies is 273-34A (abbreviated as 34A), which is a rat IgG_{2a} specific for gp112 (5). Another one which is abbreviated as 201B is also an IgG_{2a} and binds to the same antigen, but to a different epitope. As a control we used another monoclonal antibody, 14, of the same IgG subclass but with unknown specificity.

To prepare our liposomes, we used a lipid composition of egg PC:cholesterol:N-glutaryl PE (5:5:1 molar ratio). N-glutaryl

PE functions as a conjugating lipid with the monoclonal antibody (7). Note that this particular lipid composition does not give the liposomes a "stealth" property (see article by Papahadjopoulos in this issue).

As shown in Fig. 3, liposomes were prepared using the reverse phase evaporation method at low pH (5.5), and then extruded through a 0.4 micron filter four times until they were approximately 250 nm in diameter. The N-glutaryl functional groups on the liposomes were activated by using a water soluble carbodiimide and hydroxysulfosuccinimide. The pH was adjusted to 7.5 before the monoclonal antibody was added and incubated overnight at 4°C. After passage through a gel filtration column to remove the uncoupled MoAb, we obtained immunoliposomes which still maintained the same size of approximately 250 nm. We have used ^{125}I-labeled N-(3-iodo-4-hydroxybenzyl propionyl) PE (Bolton-Hunter PE) as a liposome marker.

First we used our <u>in vitro</u> tissue culture system to test whether these immunoliposomes were specific for the target cells. As shown in Fig. 4, the immunoliposomes conjugated with either 34A or 201B readily bound with the cultured mouse artery endothelial cells. Liposomes which did not contain any antibody (bare liposomes), or immunoliposomes conjugated with the control antibody 14 did not bind. A high level of specificity is shown in this system.

We also observed that the bound immunoliposomes were endocytosed by the endothelial cells in culture. This experiment was done by first incubating cells with immunoliposomes at 4°C

for binding and then, after washing, warming the cells to 37°C. At different times, the cells were treated with a protease to remove any endocytosed liposomes. When liposomes become protease insensitive, they are considered to be internalized by the cells. The lipid and the protein portions of immunoliposomes followed the same kinetics of endocytosis (Fig. 5). Endocytosis did not take place when the cells were incubated at 4°C.

Now let us look at the in vivo behavior of these immunoliposomes. We injected immunoliposomes into the tail vein of the mouse. In the case with bare liposomes (Fig. 6) a large fraction of injected liposomes was found in the blood which was then cleared mainly to the liver. The lung uptake was negligible, particularly when steady state was reached at approximately 15 minutes.

When the liposomes were coated with 34A at the final antibody-to-lipid ratio of 1 to 8 (w/w), a very large fraction of immunoliposomes accumulated in the lung as early as one min after injection which gradually decreased with time (Fig. 7). Thus, the lung binding of immunoliposomes took place during the first few passages of liposomes through the lung capillary system. Accumulation in the blood was much lower at the beginning and was also cleared away rapidly (5 min). The uptake of liver reached a maximum at about 5 minutes. In this particular preparation of immunoliposomes, about 30 percent of the injected dose was present in the lung at 15 min after injection.

If immunoliposomes with a lower antibody content were used,

[antibody-to-lipid ratio of 1 to 25 (w/w)], the amount of lung accumulation was much lower and the steady state of accumulation was only about 10 percent (Fig. 8). However, the accumulation in the liver was drastically increased. As you will recall, with bare liposomes the accumulation in the liver was only about 30 to 40 percent (Fig. 6). Now this was increased to about 50 percent. This means that a large amount of antibody needs to be used to target liposomes to a target epitope. If one uses only a small amount of antibody, there will be an enhanced uptake by the liver. This is probably due to Fc receptor mediated endocytosis by liver macrophages.

Specific accumulation of immunoliposomes in the lung is shown by an inhibition study. We have injected three different ascites fluids containing different monoclonal antibody i.p. one hour before the injection of 34A-immunoliposomes (Table 1). The accumulation in the lung in this particular experiment (22% PBS control) was completely blocked if the same monoclonal antibody 34A was preinjected. The nonspecific antibody 14 did not affect the liposome accumulation in the lung. Preinjection of antibody 201B caused a partial block, not because of competition with 34A-immunoliposomes, but probably because of a modulation of gp112 expression on the cell surface.

The importance of the amount of antibody per liposome was also shown in the next experiment (Table 2). We systematically changed the input monoclonal antibody to lipid ratio. There was approximately 50% antibody conjugation to liposomes in all cases. The antibody content of immunoliposomes had increased from 0

to approximately 900 antibody molecules per liposome. The accumulation in the lung increased with the antibody content of immunoliposomes. Approximately 50% of the injected dose was found in the lung for immunoliposomes containing approximately 900 antibody molecules per liposome. As the lung accumulation increased, the liver accumulation decreased. Therefore, this liposome targeting system is highly sensitive to the valency of MoAb on the liposome.

The next parameter we checked was the size of liposomes. We prepared immunoliposomes of three different sizes: the regular type of 250 nm, 3 microns, or 10 microns or greater. The amount of lung accumulation also increased with size. Very large liposomes showed significantly greater accumulation than the smaller ones (Table 3). Again, the liver uptake decreased as the lung uptake increased. In a control experiment where the bare liposomes were used, the lung accumulation increased only slightly with increased size of liposomes (Table 3). Although immunoliposome accumulation in the lung is mediated by specific antigen-antibody binding, favorable conditions for lung capillary filtration were also favorable for the specific binding of immunoliposomes to the lung target. This interpretation is made because immunoliposomes bind to the lung target in the first few passages. If immunoliposomes pass through the lung capillary too quickly, they may be unable to bind to the antigen. If the rate of passage can be reduced, the chance of binding would be increased. This may be achieved by the use of larger sized liposomes.

Another method used to achieve the same result is called the "crowding" effect which occurs when a high liposome dose is injected. If the dose of injection was increased to 1 mg lipid per mouse, there was also a systematic increase in the percentage of injected dose accumulated in the lung (Table 4). Again, liver accumulation went down. When a large amount of liposomes is injected, a "crowding" effect in the lung may occur, such that the passage rate of the liposome is reduced to provide a greater opportunity for antigen binding.

We continue to optimize the liposome preparation methods, markers, etc. The radioactive marker used in the previous experiments was ^{125}I-labeled Bolton-Hunter PE. This particular marker is metabolized in the liver after an interval as short as 15 minutes. The results presented here may be somewhat underestimated because some of the marker had already been metabolized and excreted.

Recently we have used another marker ^{111}In-DTPA conjugated to stearylamine. This marker is not metabolized (8). When this marker was included in immunoliposomes (200-300 nm) which had been prepared by a dialysis method, there was a very high level of accumulation in the lung. Our record for lung accumulation stands at 76 percent of injected dose (Fig. 9). Liver accumulation is less than 20 percent in this case.

One can also use a water soluble, entrapped marker such as ^{125}I-labelled tyraminyl inulin. A significant level of lung accumulation of 34A-immunoliposomes was again observed (Table 5). Moreover, the half life of 34A-immunoliposome retention in the

lung has been estimated using this more stable marker to be approximately 5 hours.

In conclusion, we have shown that monoclonal antibody coated liposomes specifically accumulate in the lung after tail vein injection. The accumulation takes place in the first or second passage through the lung capillary system. The accumulation level in the lung requires a high antibody-to-lipid ratio, presumably because of the very rapid passage of immunoliposomes through the lung. An important point is that in vivo liposome targeting is different from in vitro liposome targeting. Data from this and other laboratories show that in vitro targeting does not require many antibody molecules per liposome. In general, very good targeting efficiency is obtained if one gets about five to ten antibodies per liposome (1). But in vivo targeting requires much more.

Finally, although the accumulation in the lung is not due to embolism, larger size, higher dose and conditions in favor of a capillary filtration enhance the immunoliposome accumulation. This is, again, because the capillary filtration effect can slow down the passage rate of liposomes through the lung. Therefore, this "flow" mechanism should be considered for in vivo immunoliposome targeting, which is very different from in vitro immunoliposome targeting. Thank you very much.

Dr. Ostro: Questions for Dr. Huang?

Dr. Papahadjopoulos: Leaf, those are very impressive results, but you're at the expense of using very large amounts of antibody and also liposomes of large size and large amounts of

lipids? Do you think you'd achieve the same results if you were to use "stealth" liposomes with much lower doses and much lower amounts of antibody as you have the longer circulation times?

Dr. Huang: That's a very good point. As you see, we have not used the "stealth" liposomes. One of the reasons is that our original antibody coupling method will allow nonspecific coupling of the antibody to any lipids containing carboxylic acid. GM_1, for example, contains carboxylic acid and, therefore, is sensitive to the coupling reagents. Now that we have developed a dialysis method, we can avoid the coupling to the sialic acid and other types of acidic lipids. This work is now underway. You are absolutely right. I think that if one increases the liposome circulation time, one probably will not just rely on the first and second passage of the liposome. The opportunities for immunoliposomes to bind to the lung will be enhanced.

Dr. Weissmann: Weissmann, New York. During the enhanced localization to the lung of liposomes containing large numbers of immunoglobulin molecules which are cationically charged and which then stuck, presumably, to the endothelial cells, did you happen to do white cell counts, namely mononuclear cell counts and granulocyte counts on animals that have been so treated?

Dr. Huang: No. We haven't done that.

Dr. Weissmann: Well, when we did similar experiments, which have been published extensively, it is almost impossible to get immunoglobulin liposomes through a lung capillary without engaging both neutrophils and mononuclear cells to the endothelial cells. And we now know those are caused by specific

adhesive molecules, leukines, on the surface of endothelial cells. I think were you to do neutrophil counts and monocyte counts on such animals you would find that you produce significant granulocytopenia. And this probably has to do with secretion of the cells by virtue of the expression of adhesive molecules both on the neutrophil and on an endothelial cell.

Dr. Huang: We do not know whether gp112 is an adhesive molecule. We are investigating that. So far all the efforts to identify whether gp112 is an adhesive molecule have come out with negative results. We do not know whether the leukocyte counts is normal, and that needs to be examined.

Dr. Papahadjopoulos: Leaf, there have been results from both my laboratory and also from Lee Leserman's indicating that endocytosis of liposomes is size dependent and the size limitation for non-phagocytic cells seems to be about 1000 angstroms. That means liposomes larger than that will not be taken in. I suspect that the endothelial cells that you are talking about probably have the same size effect.

Dr. Huang: I don't know.

Dr. Papahadjopoulos: You don't know. Right. And probably you need certain passage to allow the liposomes to go in further into the tissue for more efficient delivery of the drug that they carry. So the question is whether the results that you presented with the proteolysis really are definitive in showing the endocytosis and whether you have used some other means of establishing whether the liposomes that you used can, in fact, enter the cells, e.g. by some sort of fluorescence measurements.

Dr. Huang: Yes. That's what we have done. We loaded the immunoliposomes with a fluorescent dye and saw that these cells were labeled with fluorescence with a punctate pattern. So, I don't think there's any doubt that these liposomes are endocytosed. I don't know whether the size limitation of 1000 angstroms is true for endothelial cells. We already know that mouse L cells can take in particles much greater than 1000 angstroms in diameter.

A Speaker: University of Toronto. In the immunoliposome treated animals with high lung uptake, do you see evidence of respiratory distress?

Dr. Huang: No, not at all. That's the virtue of this system. I forgot to tell you that there are some negative aspects of this system. First of all, the amount of antibody that has to be used for this type of liposome targeting is really enormous. In the optimal situation, we are injecting about one milligram of liposomal antibody per mouse. For an academic laboratory, it's very expensive. Difficulty number two is that these are rat antibodies and we are injecting them into mice. There are some immunological reactions to the immunoliposomes. We have noticed that if we do a weekly injection, excellent lung accumulations are observed in the first two injections. In the third and fourth injections, there is no accumulation in the lung. The same immunoliposome preparation injected into a fresh mouse accumulates very well in the lung. Somehow, by the third week the mouse has established a immunological reaction and the immunoliposomes fail to accumulate

in the lung. These are the problems that we have to overcome.

Dr. Crommelin: Daan Crommelin, University of Utrecht. Just to elaborate on this story about the large amount of antibody that is used, did you try other techniques where you might have a better position of the antigen binding sites of the antibody to the endothelial cells, because what I understand from your method is that you don't try to really position your antibodies properly to the outside of the liposome structure. The second question is when we tried to load up immunoliposomes with large amounts of antibodies, we ended up, usually, with aggregation of these liposomes. Did you notice that?

Dr. Huang: No. One of the virtues of this coupling scheme is that the aggregation is very low. We extrude the liposomes through the filter before we conjugate them with the antibody. We don't see much aggregation, even when we use a very high level of antibody.

Back to your first question. We have developed, as you probably know, our first method of fatty acid coupling with the antibody (9). That method has the advantage of largely coupling the fatty acids to the Fc portion of the antibody molecule. We have not examined this particular coupling method, whether the lipid is attached to the Fc or not. I suspect that there is no specificity there. However, the last slide (Fig. 9) demonstrated a dialysis method which is really a modification of the original fatty acid coupling method. We first coupled antibody with N-glutaryl PE in detergent. Lipids were then added and the mixture was dialyzed to form liposomes. So, in this

situation, which is very similar to our original fatty acid coupling method, we might be able to enhance the Fc specific coupling.

Dr. Ostro: One more question.

Dr. Ginsberg: Ginsberg, the Liposome Company. Going back to your ultimate goal, which is delivery of drug to a tumor site within the lung, how do you propose that these liposomes that are taken up by endothelial cells in the lung will end up getting drug to the tumor site?

Dr. Huang: That, of course, would depend on the drug. Our idea is to use a lipid soluble drug instead of a water soluble drug because there are still many membrane barriers for the drug to diffuse through. We suspect if one uses a lipid soluble drug, it would have a better chance to reach the tumor. Our preliminary experiment using methotrexate conjugated to a PE anchor has shown that, indeed, it can get to the tumor site and produce a significant therapeutic effect.

Dr. Ostro: All right. Somebody is really anxious. Last question.

Dr. Lopez: Lopez, Houston. He's assuming that the liposomes are going to be taken by the endothelial cells in the lung. Leaf, you mention that depends on the drug, but it depends on many other components also. One, liposome is going to be entrapped in the microcapillary tube, phagocytes are going to take them up. Number one is phagocyte uptake in peripheral blood and two, in the trapped capillary. Then, there will be transport and then there will be lipid exchange with possible

subsequent change of liposomes. I don't think that there is only one component. So I think that in terms of the lung tumor there have to be many components, plus the nature of the tumor itself.

Dr. Huang: That's correct.

FOOTNOTES

Abbreviations used: PE, phosphatidylethanolamine; PC, phosphatidylcholine; DTPA, diethylenetriaminepentaacetic acid; LP, liposomes; BLP, bare liposomes; BHPE, Bolton-Hunter PE.

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Figure Legends:

Fig. 1. Autoradiography of 5 μm tissue sections after injection of [^{125}I]MoAb 201B. Balb/c mice were injected i.v. with 50,000 Line 1 tumor cells 7 d prior to i.v. injection with 10 μg of [^{125}I]MoAb 201B (~10,000 cpm/ng). Animals were sacrificed 3 h after injection and lungs inflated with fixative before tissue processing through paraffin blocks. Sections (5 μm) were cut and deparaffinized before staining with hematoxylin and eosin and processing for autoradiography. Slides dipped in emulsion were developed 3 d later and dehydrated before cover slipd were applied in Permount. Panels a and b were autoradiographs of these sections at different magnifications. Magnification is ~3.5X for panel a and 90X for panel b.

Fig. 2. Immunogold electron microscopy of normal Balb/c lung lightly fixed frozen sections with MoAb 34A (a and b) or MoAb 201B (c); small arrows point to clusters of gold particles bound to endothelial membrane. endo, endothelium; RBC=red blood cell. Taken from (4) with permission.

Fig. 3. Flow chart of immunoliposome preparation.

Fig. 4. Immunoliposome binding to mouse pulmonary artery endothelial cells at 4°C. Binding of 34A-immunoliposomes (open squares), 201B-immunoliposomes (solid squares), 14-immunoliposomes (solid triangles) and bare liposomes (open triangles) are plotted as a function of amount of total lipid added.

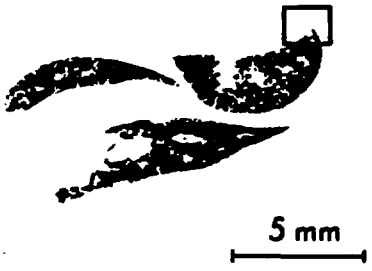
Fig. 5. Endocytosis of 34A- and 201B-immunoliposomes into the mouse pulmonary artery endothelial cells. Data are reported as % insensitivity of bound liposomes to treatment with proteinase K. Both lipid (top) and protein (bottom) portions of immunoliposomes were examined. Prebound 201B (triangles) and 34A (squares) immunoliposomes were incubated at 37°C (open symbols) or 4°C (closed symbols) for different periods of time before enzyme treatment.

Fig. 6. Time course of biodistribution of protein-free, bare liposomes. Liposomes were labeled with ^{125}I-Bolton-Hunter-PE and injected to Balb/c mice via tail vein. Percent of injected radioactivity in liver (triangles), blood (diamonds) and lung (squares) were measured at different times after injection. n=3.

Fig. 7. Time course of biodistribution of 34A-immunoliposomes. The antibody-to-lipid ratio of the immunoliposomes was 1:8 (w/w). Experimental conditions and symbols same as in Fig. 6.

Fig. 8. Time course of biodistribution of 34A-immunoliposomes. The antibody-to-lipid ratio of the immunoliposomes was 1:25 (w/w). Experimental conditions and symbols same as in Fig. 6.

Fig. 9. Biodistribution of 34A-immunolipsoomes in Balb/c mice. Immunoliposomes were labeled with ^{111}In-DTPA-stearylamine and prepared with a dialysis method. Biodistribution was measured 15 min after tail vein injection of immunoliposomes.



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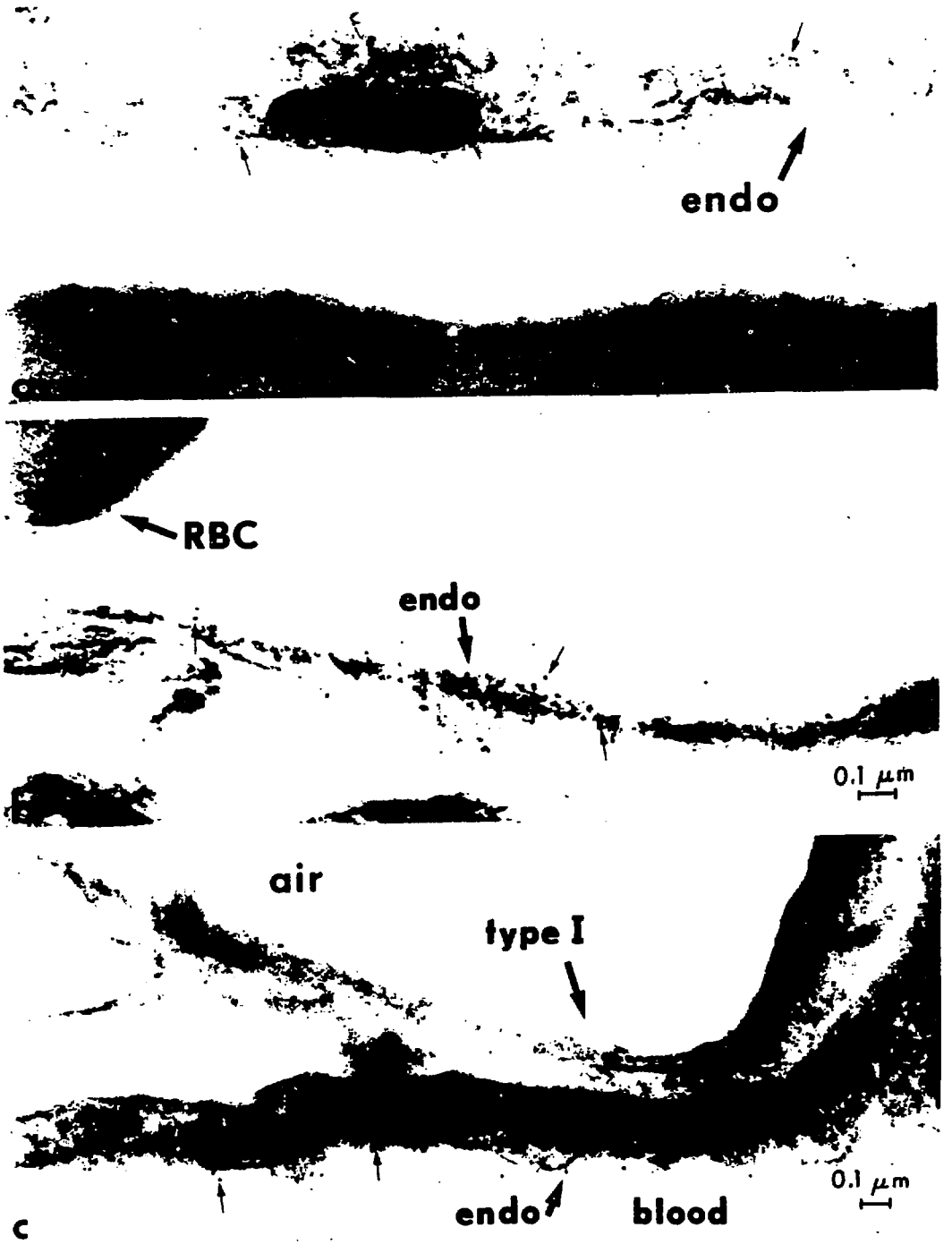
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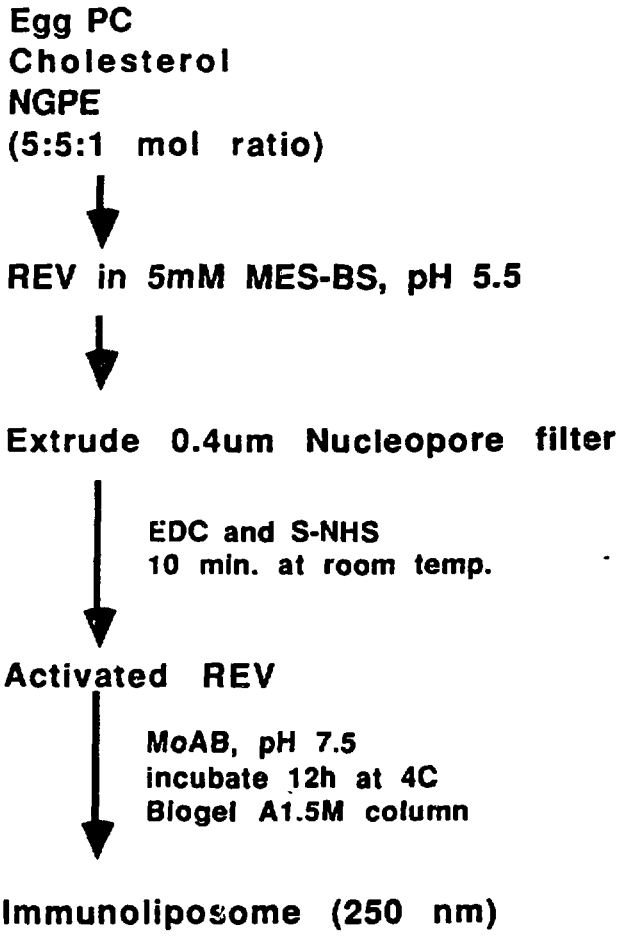

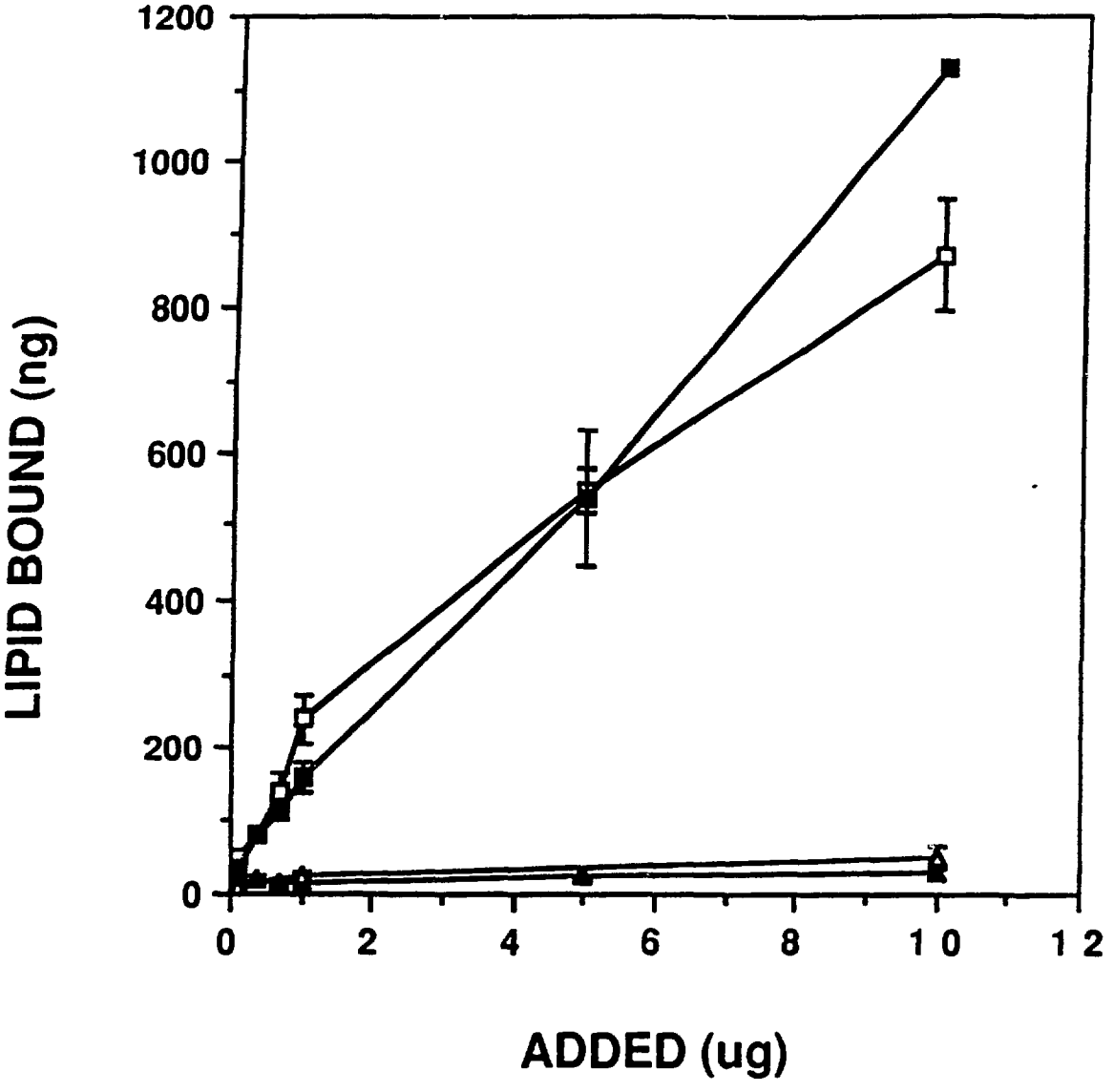
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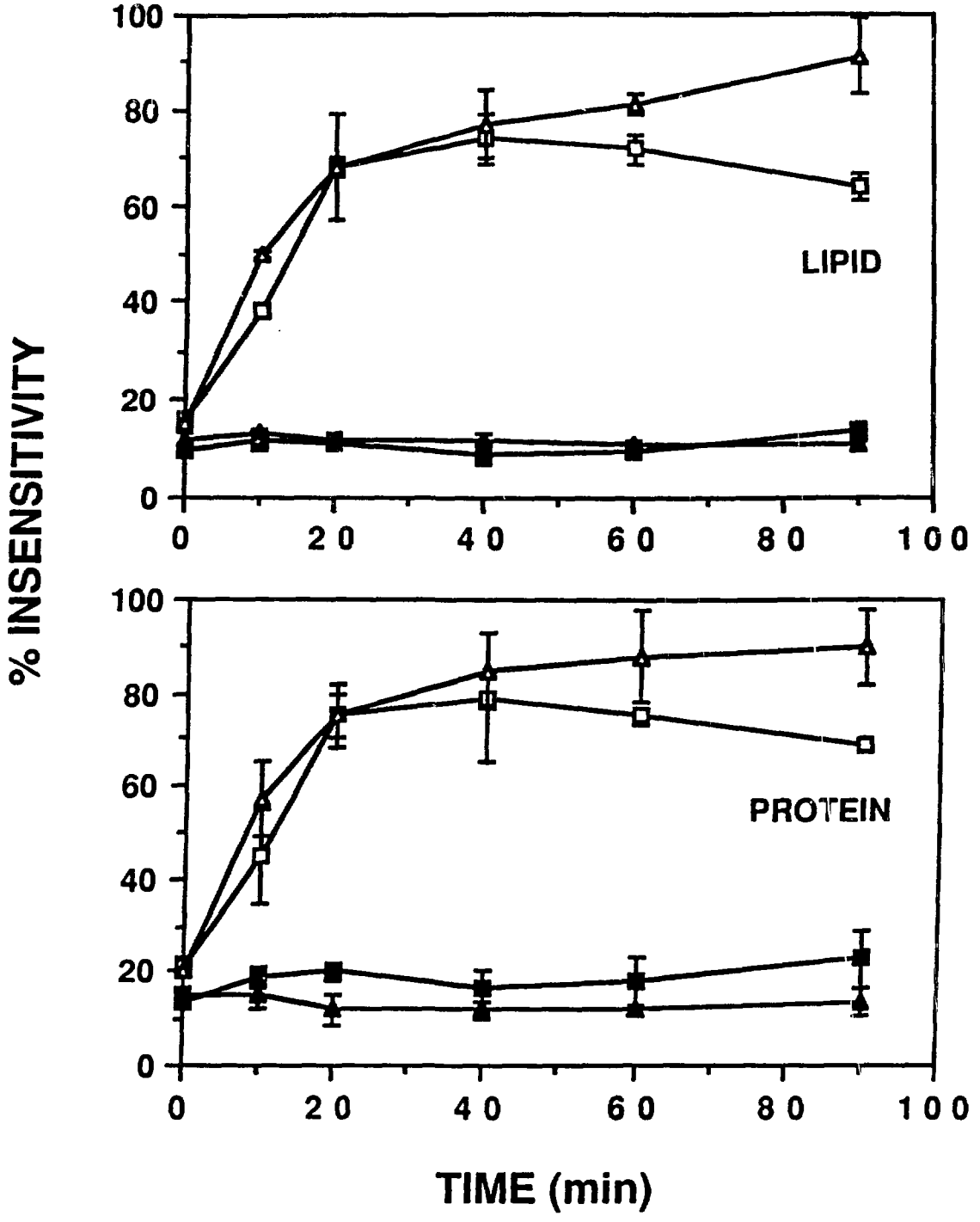
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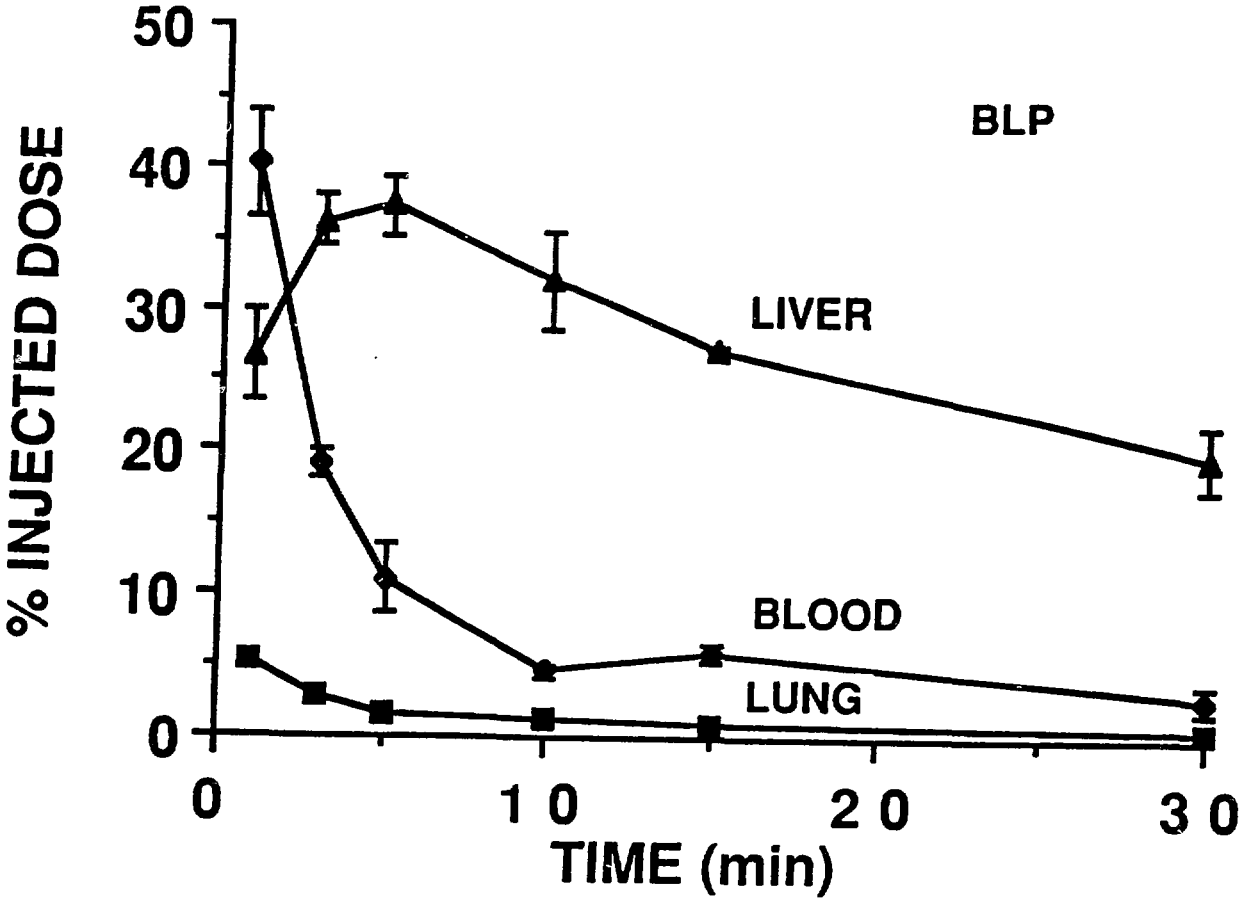
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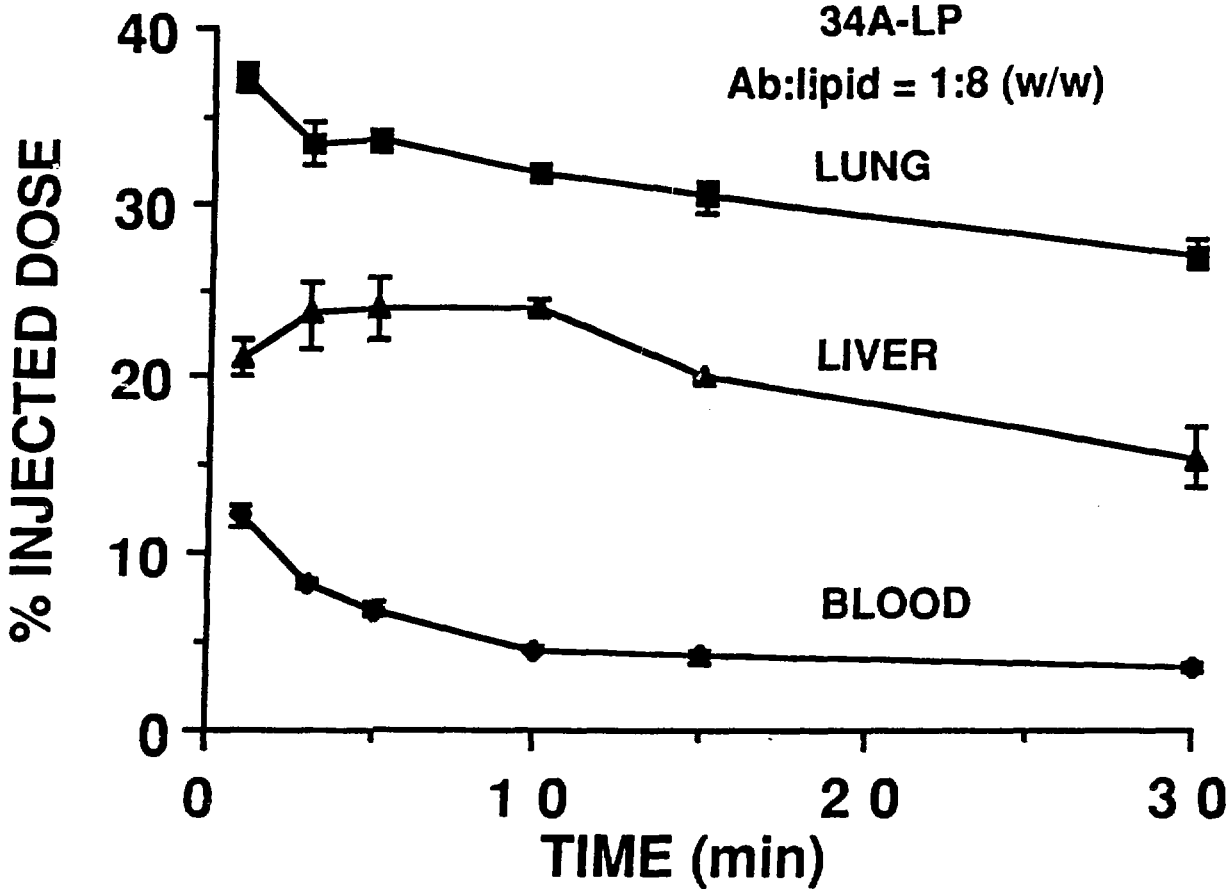
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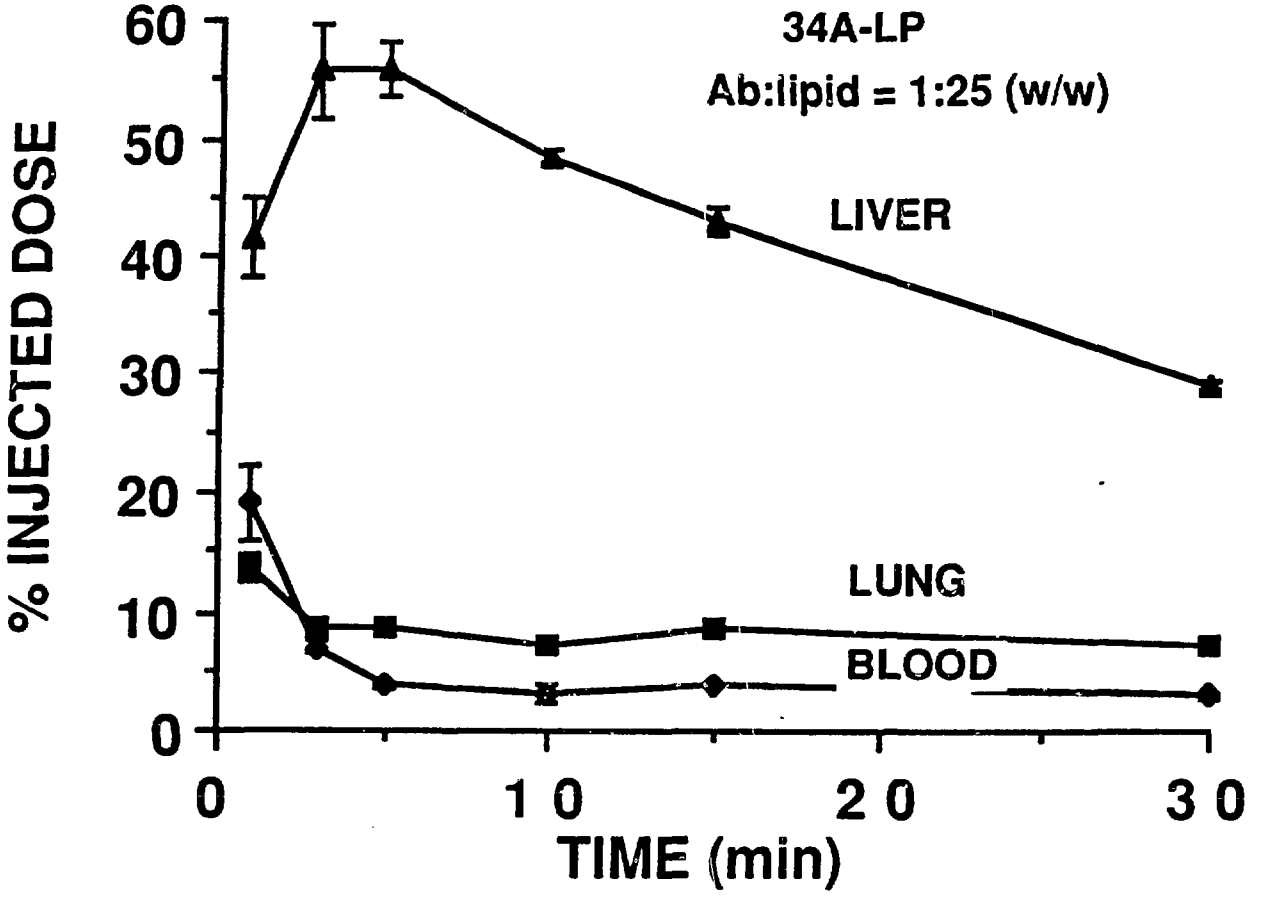
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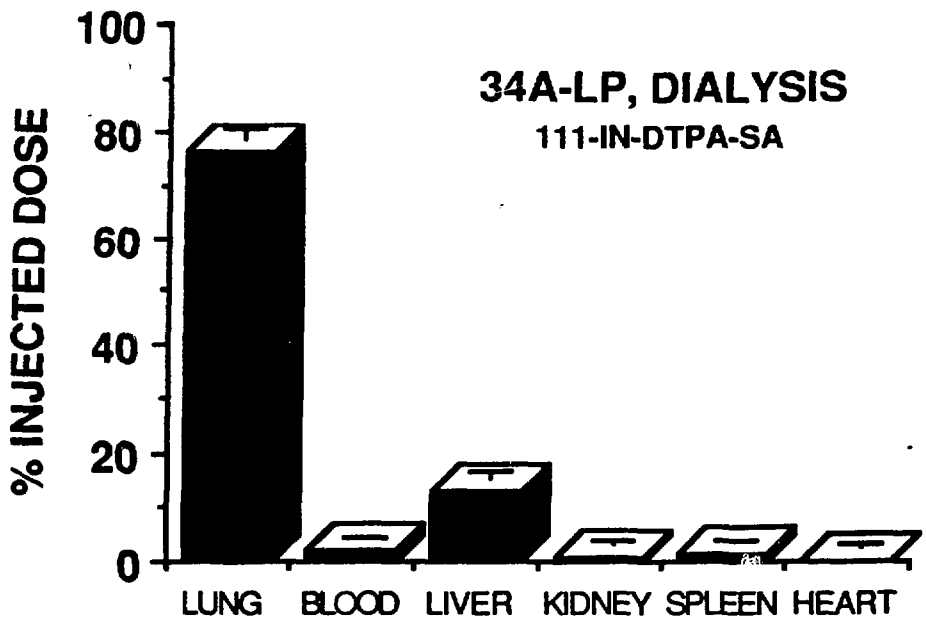
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