in vivo *18*: 181-188 (2004)

Biodistribution of Injected Tritiated Hyaluronic Acid in Mice: A Comparison Between Macromolecules and Hyaluronic Acid-derived Oligosaccharides

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Abstract. *Background: Hyaluronan (HA) has been reported to bind specifically and with high affinity to various cell types and to directly modify cell behaviour. In a previous report we demonstrated that both high molecular weight molecules (HA_H) and HA-derived oligosaccharides were efficient at triggering terminal differentiation of acute myeloid leukemia (AML) blasts, in vitro, through CD44 ligation. Materials and Methods: To explore the possibility of using HA for a differentiation therapy in AML, we investigated whether intravenous injection of tritiated HA_H and/or HA-derived oligosaccharides (HA_{10-20}) into mice accumulated in bone marrow, the main site of AML cell proliferation. Results: The present work showed that the level of HA in bone marrow: 1) was maximum 5 hours after injection of either HA_H or HA_{10-20}; 2) was about 40 times higher after HA_H than after HA_{10-20} injection. The amount of HA in bone marrow (5.8% ID/g) was two-fold higher than in serum, indicating that it was not due to circulating blood. Finally, using chromatographic analysis, we showed that about 34% of tritiated HA present in bone marrow 5 hours after HA_H injection displayed a size higher or equal to HA_{10}. Conclusion: After a single injection of macromolecular hyaluronan in mouse bone marrow we obtained a concentration of oligosaccharides close to the one shown to trigger AML cell differentiation in vitro. A part of the oligosaccharides had a size higher than or equal to the minimal one required to interact with HA receptors.*

Abbreviations: HA, hyaluronic acid; HAH, high molecular weight hyaluronic acid; GAG, glycosaminoglycan; Hyase, hyaluronidase; AML, acute myeloid leukemia.

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Key Words: Bone marrow, hyaluronan, tritiated oligosaccharide size, tissue extract, quantitation.

Hyaluronan (hyaluronic acid, HA) is a large, ubiquitous glycosaminoglycan (GAG) found in extra-cellular matrix and especially abundant in loose connective tissues. It is synthesized in most tissues and partly degraded close to its origin; a high rate of catabolism also occurs in the lymph nodes. The remaining HA enters the blood stream from where it is removed by the endothelial cells of the liver sinuses and further degraded (reviewed in (1,2)).

Numerous works have reported that HA or HA-derived oligosaccharides can directly trigger different properties in cells, through their binding to specific cell surface receptors such as RHAMM and CD44. For example, HA has been shown to stimulate vascular cell proliferation (3-6) and, more recently, it has been evidenced that HA-derived oligosaccharides could inhibit tumor growth in mice (7,8) and tumor cell line proliferation when used as a carrier for drug delivery (9). Interestingly, Charrad *et al.* (10) recently demonstrated that both high molecular weight molecules (HA_H) and HA-derived oligosaccharides were efficient at triggering terminal differentiation, through CD44 ligation, of acute myeloid leukemia (AML) blast *in vitro*, raising the possibility of using HA for a differentiation therapy in AML. Accordingly, it seemed worthwhile to investigate whether, following *in vivo* injection, HA_H and/or HA-derived oligosaccharides (HA_{10-20}) can accumulate in bone marrow, the main site of AML cell proliferation.

So far, it has been well documented that intravenously injected macromolecular HA is rapidly cleared from the blood stream, degraded primarily in the liver and, to a lesser extent, in the spleen, lymph nodes and bone marrow. The reticulo-endothelial cells present in all these organs are thought to be responsible for this uptake and degradation (11-15). In contrast, neither the biodistribution nor the clearance pathways of HA-derived oligosaccharides have been so far analyzed *in vivo*. Considering that the size of an injected molecule might influence its biodistribution, we found it of interest to compare the biodistribution of HA_H and HA-

derived oligomers in mice following intravenous injections. Using tritiated molecules, we here show evidence that injection of macromolecular HA generates, in the bone marrow, a concentration of HA-derived oligosaccharides that is 40-fold higher than that following injection of HA-derived oligomers. Moreover, the size of these oligosaccharides is higher or equal to $HA_{10,}$ indicating that they have the potential to bind HA receptors.

Materials and Methods

Production of tritiated hyaluronan. Production of tritiated hyaluronan was performed according to a previously described procedure (16). Tritiated hyaluronan was produced by the CB193 cell line fed with tritiated acetate (4 MBq / ml of culture medium) in RPMI supplemented with 2mM glutamine and 10% foetal calf serum for 60 h. The cell culture medium was processed as follows: 1) digestion by *Streptomyces griseus* protease (type XIV , 4.6 units / mg, 1 mg / ml) from Sigma (St Quentin Fallavier, France) for 18 h at 37˚C; 2) heating at 100˚C for 20 min; 3) centrifugation at 32,000g for 10 min. The supernatant was concentrated under pressure and chromatographed through Ultrogel A4 (Sepracor, Villeneuve la Garenne, France) in 0.1 M Tris supplemented with 0.1 M NaCl and 0.25 g / l sodium azide at pH 8, packed in a 100 x 1 cm column (Econocolumn, BioRad, Marnes la Coquette, France). The radioactive molecules of high molecular mass (over 10^6 Da) present in the exclusion volume of the chromatography column were checked for their hyaluronan composition by digestion with 200 TRU / ml *Streptomyces* hyaluronidase (a hyaluronan-specific hyaluronidase). Hyaluronidase digestion led to a complete degradation of the radioactive macromolecules in small fragments. Protease digestion had no effect on the radioactive molecules. The preparation was concentrated under pressure to 1 ml, desalted on Biogel P6-DG (BioRad) in RPMI with glutamine, sterilized by ultrafiltration through a 0.22 μm Millipore filter and stored at 4˚C. The hyaluronan concentration of the preparation was determined with the ELSA technique (17). The radioactivity of a 0.1ml sample mixed with 2ml Liquid Scintillation cocktail (Packard Ultima Gold, Rungis, France) was measured with a Packard Scintillation counter. The specific radioactivity of the tritiated hyaluronan was estimated at 28 GBq / g hyaluronan. In the text, this hyaluronan preparation was referred to as High Molecular Weight Hyaluronic Acid (HA_H). The integrity of the tritiated HA_H was maintained for up to one year of storage at 4˚C as demonstrated by the presence of the tritiated molecules in the exclusion volume of the chromatographic elution of an aliquot through Ultrogel A4. The stability of the preparation was checked once a month by gel permeation chromatography.

Production of tritiated hyaluronan oligosaccharides. Tritiated hyaluronan oligosaccharides were prepared according to a previously described method (18). Briefly, a mixture of 150 mg hyaluronan (*Streptococcus equi sp.*, Fluka) and 200 μg tritiated hyaluronan was digested with bovine hyaluronidase type VI-S, Sigma (30 U Hyase / mg HA) at 37˚C for 6 h. Oligosaccharides were separated with regard to their size by chromatography through AcA 202 gel (Sepracor) in 0.25 M glacial acetic acid / 0.28 M pyridine at pH 5 packed in a 200 x 2.5 cm column. The flow rate was 20 ml / h, fractions were 4 ml. Eluted peaks were detected either by the determination of glucuronic acid (GlcA) using the carbazole method of Bitter and Muir (19) or by measurement of the radioactivity in a 50 μl sample mixed with 2 ml of Liquid Scintillation cocktail. Fractions of each peak were pooled and reducing N-acetyl glucosamine (GlcNAc) was determined by the Reissig *et al.* method (20). The size of oligosaccharides in each peak was obtained by calculation of the total GlcA / reducing GlcNAc ratios. Absence of proteins was controlled with the Coomassie Blue dye technique of Bradford (BioRad kit). Peaks containing HA oligosaccharides with a number of disaccharidic units ranging from 5 (HA_{10}) to 10 (HA_{20}) were pooled and concentrated under pressure. Sterility was obtained by ultrafiltration through a 0.22 μm Millipore filter and storage was at 4˚C. This preparation was referred to in the text as $HA_{10\text{-}20}$.

Biodistribution studies. Experiments were carried out with female athymic mice (SWISS-nu/nu), 6-8 weeks old, purchased from Iffa Credo (L'Arbresle, France). All the animals were treated in accordance with the French Ministry of Agriculture's guidelines for the care of laboratory animals. The mice were housed under specific pathogen-free conditions until they received radioactive injections. Then the animals were transferred to the conventional controlled area of the laboratory. They were fed with sterilized food and water and sterile bedding and cages covered with filters were used throughout the experiment.

Groups of 3 mice per time-point received a 200μl intravenous injection of either tritiated HA_H (28 KBq / μg) or tritiated $HA_{10\text{-}20}$ (22 KBq / μg). Each mouse received a total dose of either 420 KBq (HA_H) or 520 KBq ($HA_{10\text{-}20}$). At specific time-points 5h and 24h following injection, mice were sacrificed and tissues (blood, liver, spleen, kidney, lung, heart, brain, lymph nodes and bone marrow) were removed. Three samples per tissue were weighed and completely solubilized in solvable (Packard), W/V=1/5, overnight at 50˚C and the radioactivity was counted in a gamma scintillation counter after being mixed with 2 ml Liquid Scintillation cocktail. Nine results per point (except for the bone marrow the quantity of which allowed only 3 results per point) were expressed as a percentage of the injected dose per gram of tissue (%ID/g) and the *s.e.* was calculated.

Intending to inject the highest quantities of hyaluronan in mice, in one experiment increasing amounts of unlabelled HA (50, 100, 150 or 200 μg) were mixed with 15 μg of radio-labelled HA in 200 μl PBS. Each dose was administered intravenously to mice and, 5 h after the injection, the blood and the bone marrow were removed and processed as described for the other tissues. Radio-labelled HA used as a tracer represented a percentage of the whole injected HA. From the radioactivity found in bone marrow extracts, we calculated the corresponding whole HA amount accumulated in the tissue.

Oligosaccharide size in organ extracts. Tissue samples were solubilized (W/V=1/5) overnight at 50˚C and centrifuged for 20 min at 20,000 g. Two hundred microliters of the supernatant were chromatographed through AcA 202 gel (Sepracor) in PBS packed in a 50 cm / 0.5 cm column. The flow rate was 4 ml / h and 0.5 ml fractions were collected. Eluted peaks were detected by the measure of the radioactivity in 200 μl of each fraction. The column was calibrated with purified HA_4, HA_6, HA_{10} and HA 14, giving elution volumes of 12 ml, 10 ml, 8 ml and 7 ml, respectively. The radioactivity counted in the fractions up to 8 ml was added up and expressed as % of the whole radioactivity present in the fractions of the chromatography. This result was further expressed as % of ID per gram of tissue for each sample and referred

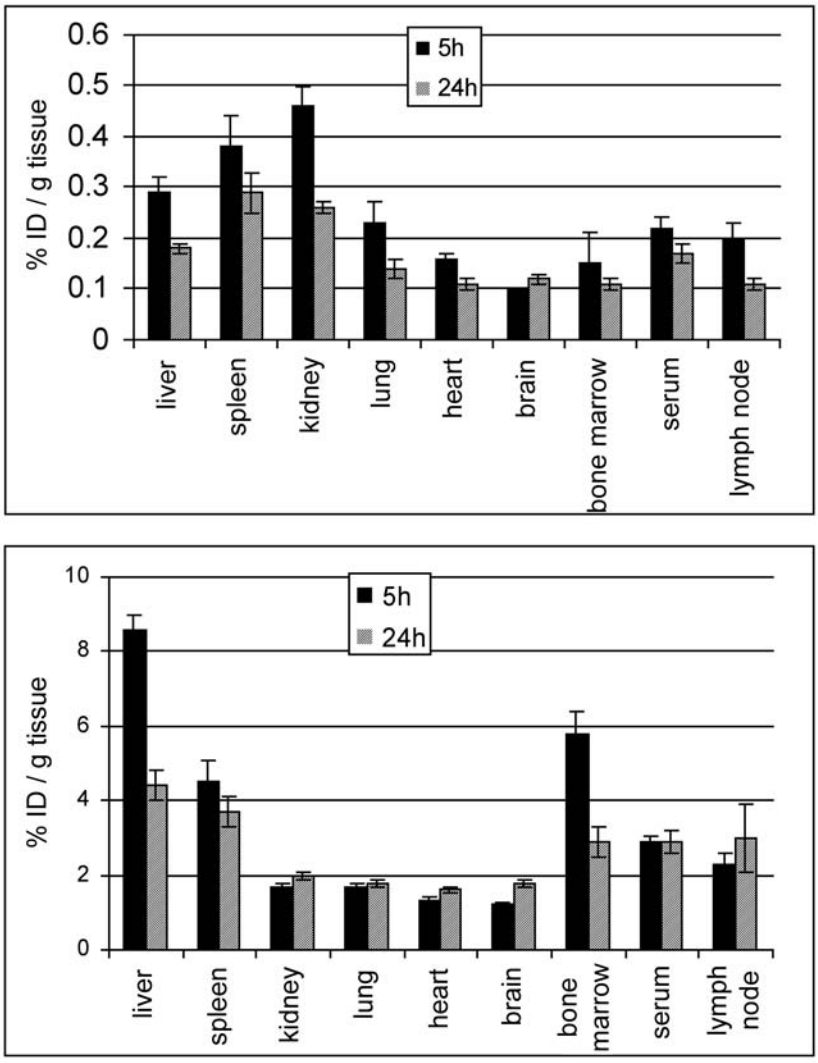

Figure 1. *Organ distribution of tritiated HA_{10-20} (A) or tritiated HA_H (B) 5h (black) and 24h (hatched) following intravenous injection. Organ associated radioactivity is given as % of injected dose per gram of tissue (% ID/g tissue). Values are mean±s.e. (n=9, except for bone marrow n=3).*

to as the "active fraction" ; *i.e.* the fraction containing oligosaccharides that were long enough to interact with the HA receptors of the cells. To ensure that the radioactivity found in organ extracts was associated with HA, a sample of organ extract in deionized water was mixed with *Streptomyces* hyaluronidase (20 TRU / µg HA) overnight at 37˚C and chromatographed through AcA 202 gel. The radioactivity detected in the peaks was associated with small molecules showing a complete digestion of the radioactive molecules by hyaluronidase, demonstrating that they were composed of HA.

Results

The tritiated oligosaccharides were separated with regard to their size by chromatography through AcA 202 gel. Eluted oligosaccharides were detected by the measurement of radioactivity and glucuronic acid concentration. The chromatographic profiles obtained by the two different methods were superimposable, showing that the radioactivity found in the peaks was associated with the oligosaccharides. Peaks from HA_{10} to HA_{20} were pooled and represented the HA_{10-20} fraction injected into the mice.

Mice received *i.v.* injections of either tritiated HA_H or tritiated HA_{10-20}, they were sacrificed 5h or 24h after the injection and organ radioactivity was measured. Following an administration of tritiated HA_{10-20} (Figure 1A), the highest radioactivity levels were found in kidney, spleen and liver 5h after the injection (0.46±0.04 %ID/g, 0.38±0.06 %ID/g and 0.29±0.03 %ID/g, respectively) as well as 24h after the injection (0.26±0.01 %ID/g, 0.29±0.04 %ID/g and 0.18±0.01

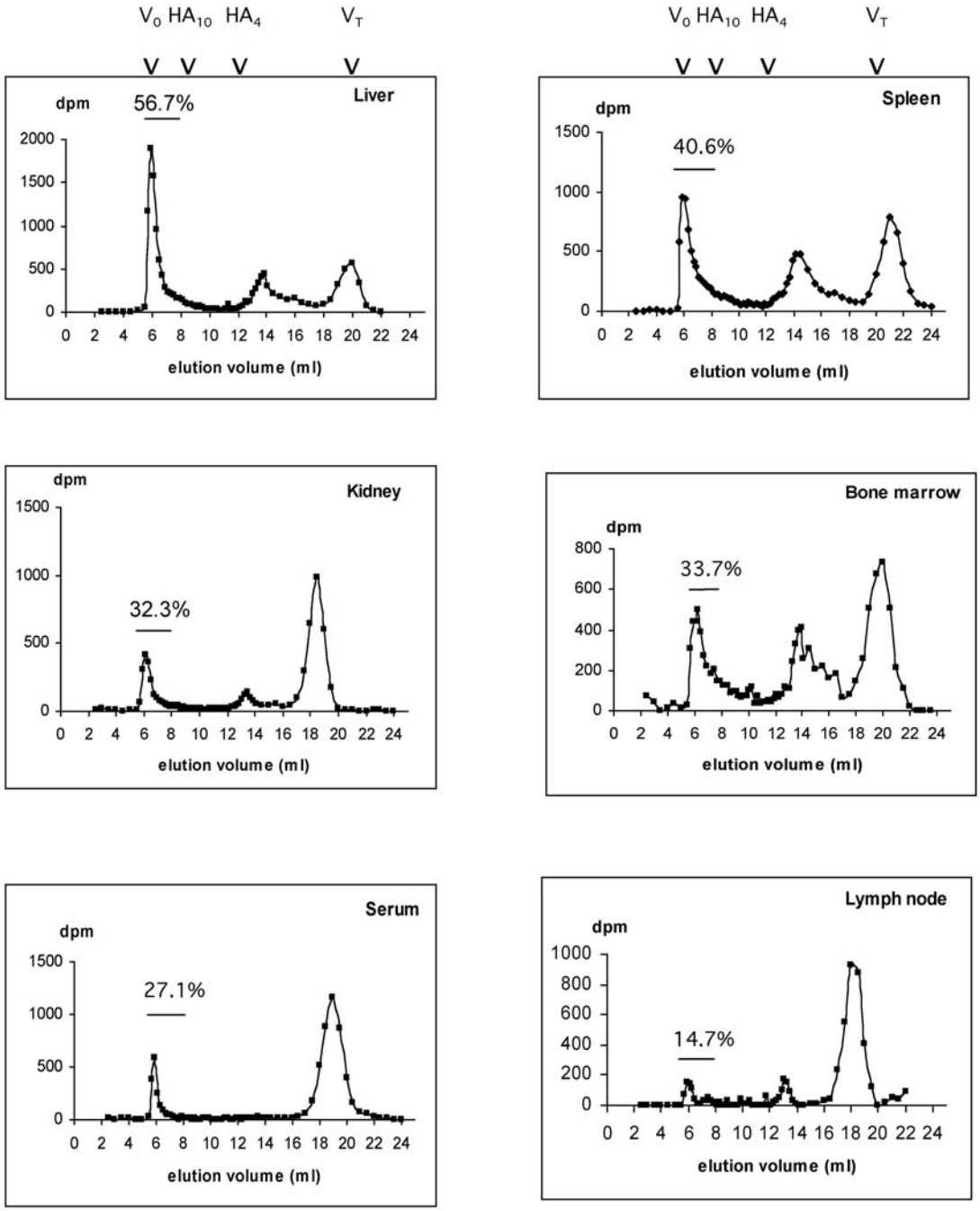

Figure 2. *Organ extract chromatograms through a 50 cm AcA 202 gel column; organs are removed 5h after tritiated HA_H injection. Results are expressed in dpm. Bars represent the " active fractions " (HA molecules with a size higher than or equal to HA_{10}) and are expressed as the percentage of the whole chromatography. Calibration volumes: V_0=6 ml, HA_{10}=8 ml, HA_4=12 ml, V_T=20 ml.*

% ID/g, respectively). Between 5h and 24h, a significant decrease in radioactivity was seen in kidney, liver and spleen: 43 %, 38 % and 24 %, respectively. Only 0.15±0.03 % of the injected dose was found per g of bone marrow 5h after *i.v.* injection and 0.11±0.01 % ID/g at 24h. At both times, bone marrow activities were lower than in serum (0.22±0.02 % ID/g at 5h and 0.17±0.02% ID/g at 24h).

Radioactivity tissue uptake 5h or 24h after an *i.v.* administration of tritiated HA_H is shown in Figure 1B. The highest radioactivity level 8.6±0.4 %ID/g, was found in the

Table I. *HA accumulation in mouse bone marrow extracts following administration of increasing amounts of unlabelled HA_H mixed with 15µg (*) of radio-labelled HA_H.*

Injected HA_H (µg) added to 15 µg HA*	Calculated HA in bone marrow extracts	
	%ID/g	µg/g
0	5.8	0.87
50	4.99	3.20
100	4.8	5.52
150	5.4	8.88
200	5.2	11.18

HA bound to bone marrow was calculated from the radioactivity measured in bone marrow extracts.

Table II. *Comparison of " active fractions " (HA molecules with a size higher than or equal to HA_{10}) in organ extracts 5h after injection of either HA_H or HA_{10-20}. Results are expressed as percentage of injected dose per gram of tissue (%ID/g tissue).*

	HA_H	HA_{10-20}	HA_H/HA_{10-20}
Liver	4.8	0.09	53
Spleen	1.8	0.09	20
Kidney	0.6	0.12	5
Bone marrow	2.2	0.05	44
Serum	0.79	0.05	16
Lymph node	0.3	0.05	6

liver 5h after the injection, decreasing to 4.4±0.4% ID/g at 24h. The amount of radioactivity in the bone marrow was 5.8±0.9 % of the injected dose per g, 5h after the injection. This radioactivity was higher than that of each organ investigated (liver excepted) and represented twice that in serum. At 24h the radioactivity decreased to the same value found in serum *i.e.* 2.9±0.4 % ID/g. To summarize, the highest levels of radioactivity accumulated in bone marrow were observed 5 h following injection of either tritiated HA_H or HA_{10-20}. However, more importantly, the use of HA_H led to a level of radioactivity 40-fold higher than that obtained following HA_{10-20} injection.

The addition of increasing amounts of unlabelled HA to the tritiated HA had no effect upon the percentage of accumulation of radio-labelled-HA within the bone marrow (Table I) measured 5 h after the injection of HA_H, under the conditions studied, but increased the total HA amount found in the bone marrow.

Five hours after tritiated HA_H injection, the size of radioactive molecules in organ extracts was analyzed by chromatography through AcA 202 gel (Figure 2). The chromatogram of each extract (serum excepted) showed three distinct peaks. The first peak corresponded to the exclusion volume of the column (V0=6 ml) and represented molecules with a MM higher than 20,000 Da (*i.e.* higher than HA 100). This peak was predominant in the liver extract (56.7%), whereas in the other organs it corresponded to 1/3 of the total chromatography. The second peak had an average elution volume of 14 ml and represented molecules with MM in the range of 600 Da (the size of HA_3) and the third peak contained small molecules whose size was that of the monosaccharidic constituent or that of tritiated acetyl groups. This last peak was more predominant in kidney and lymph node extracts and in serum. Comparable chromatograms were obtained with organ extracts made 5h after a tritiated HA_{10-20} administration. The macromolecular fraction at the V_0 was indeed composed of HA since it was completely reduced to small molecules by *Streptomyces* hyaluronidase. From each chromatogram the percentage of molecules present in the fraction up to 8 ml was calculated and further expressed in % ID/g of tissue (Table II). This fraction was referred to as the "active fraction" and its HA size was higher or equal to HA 10. In the case of HA_{10-20} injection, the highest percentage of "active fraction" was found in the kidney (0.12% ID/g) and was 0.05 %ID/g in the bone marrow. In the case of HA_H injection, the highest percentage of "active fraction" was obtained in the liver (4.8% ID/g) whereas in the bone marrow it was 2.2 %ID/g, corresponding to 44-fold more than that obtained for this same tissue after an HA_{10-20} injection.

Discussion

The present experiments indicate that, after a single administration of tritiated HA into the blood stream, the biodistribution of the tracer in the mice varies depending on the size of the administered hyaluronan. HA_{10-20} oligomers were more rapidly eliminated from the blood than HA_H, since 5 hours after the injections 10-fold more isotope was present in the blood of mice in the case of HA_H administration. The analysis of the radioactivity extracted from the organs showed that the decrease of HA_{10-20} concentration in the blood from 5 hours to 24 hours after the injection was due to a high uptake of the oligosaccharides by the kidney, where the activity was higher than in all other organs. The rapid rate of HA_{10-20} elimination by the kidney probably prevents the oligomer diffusing into and accumulating in other organs. On the other hand, the HA_H clearance was less rapid and extra-renal, the liver being the main organ responsible for the polysaccharide elimination. These observations are in agreement with those reported by other authors, indicating the preferential uptake of the largest polymers by the liver cells (21,22). Indeed, it is well established that the liver is

the major site of circulating HA uptake and degradation. The hyaluronan fragments are further released into the blood and then eliminated by the kidney. The results of organ extract chromatograms obtained 5 hours after HA_H injection are in agreement with that observation. In the liver, the majority of the radioactivity (56.7%) was associated with HA molecules whose MM was > HA_{10}, whereas in the kidney and the serum the majority of radioactive molecules corresponded to monosaccharidic constituents or to tritiated acetyl groups, assuming that deacetylation had occurred. The chromatogram of each organ extract exhibited three distinct peaks corresponding to macromolecular species and small molecules. The nature of the macromolecular fraction in V_0 was confirmed to be HA by the complete digestion of the molecules by *Streptomyces* hyaluronidase. In organs, the shift of the macromolecular HA towards the low molecular species without intermediate oligosaccharide breakdown indicates that, when the HA degradation process starts, it runs until completion. Hyaluronan degradation to monosaccharidic constituents may occur by the concerted action of three enzymes: a hyaluronidase that generates non-reducing termini and two exoglycosidases that remove sugars sequentially from the non-reducing termini (23).

A significant accumulation of hyaluronan *i.e.* 5.8% of the injected dose per gram of tissue, was found in the bone marrow 5 hours after a 15μg administration of tritiated HA_H and represented twice that obtained in the serum in the same experiment, showing that the tritiated HA detected in the bone marrow was not due to contamination by serum but really represented specific uptake of HA by the bone marrow. It was calculated from the chromatogram that about 34% of these molecules were "active" molecules *i.e.* molecules whose size is higher than or equal to HA_{10}, able to interact with HA receptors on cells. Actually, several authors have shown that the smallest HA fragment that binds to HA receptors on the surface of various cells such as SV-3T3 transformed fibroblasts (24), liver endothelial cells (21) or keratinocytes (25) is the decasaccharide HA_{10}. Administration of increasing amounts of HA_H led to an accumulation of the polysaccharide within the bone marrow, measured 5 hours after the injection, indicating that there was no HA_H saturation of the tissue under the conditions studied. HA_H accumulation reached a mean of 5% of injected dose per gram of bone marrow whatever the injected dose. This percentage became slightly lower if only "active" molecules were considered. In a previous work (10) it was shown that the blockage of human acute myeloid leukemia differentiation was reversed after addition of 10 μg/ml HA_H in cell culture. In that experiment, we obtained a hyaluronan concentration in the bone marrow close to the effective concentration tested in culture, showing that HA can efficiently reach the main site of AML proliferation. Moreover, since the amount found in bone marrow was enhanced by increasing the injected dose of HA, these encouraging results could probably be improved by using longer periods of treatment such as a continuous infusion of hyaluronan instead of a single administration, the concentration of which is limited by the viscosity of the reagent.

In conclusion, in spite of the quick degradation and elimination of most injected tritiated HA from the blood stream, we observed a significant accumulation of hyaluronan in mouse bone marrow. This result was obtained when macromolecular hyaluronan was injected and when, after partial degradation, oligosaccharides with a size able to interact with cell receptors were produced. The oligosaccharide concentration obtained in the bone marrow was close to the one shown to trigger AML cell differentiation *in vitro* and it could probably be enhanced by a continuous infusion of hyaluronan.

Acknowledgements

We thank Dilys Moscato for her help with the english and Patrick Clevers for his skilful assistance with animal handling. This work was supported by a grant from the Conseil Régional de Haute Normandie, France.

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Received August 21, 2003
Revised November 25, 2003
Accepted December 22, 2003