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ORIGINAL ARTICLE

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Biodistribution of ^{111}indium-labeled macrophages infused intravenously in patients with renal carcinoma

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Abstract **Purpose:** We have previously reported a clinical trial on the intravenous injection of autologous activated macrophages (AAM) in 15 patients with renal carcinoma [11]. The present paper concerns scintigraphic investigations performed in 11 of these patients after injection of ^{111}indium oxinate-radiolabeled AAM. **Methods:** AAM were prepared from mononuclear cells (MNC) collected by apheresis from patients treated simultaneously with granulocyte–macrophage colony-stimulating factor (GM-CSF). MNC were cultured for 6 days in the presence of GM-CSF and exposed for 18 h to gamma-interferon, the AAM were then separated by elutriation and injected. **Results:** After intravenous infusion, radiolabeled AAM were transiently retained in the lungs, where they predominated in the first hour. Later on, radioactivity accumulated in liver and spleen and then decreased from the first and second day, respectively. In one patient, two foci of radioactivity were detected in the lungs 1 h after injection, and persisted thereafter. Their association with tumor lesions was uncertain. This observation possibly resulted from the presence of granulocytes in the radiolabeled AAM populations of this patient. It seems that MNC collected from GM-CSF-treated patients and cultured in the presence of GM-CSF enables the differentiation of granulocytes. **Conclusions:** A series of 11 investigations confirms the previously reported distribution pattern of intravenously injected AAM. It is possible that in patients treated with hematopoietic cell-mobilizing agents, granulocytes develop in cultures designed to produce monocyte-derived antigen-presenting cells.

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Introduction

Autologous activated macrophages (AAM) prepared from leukapheresis-collected mononuclear cells (MNC) were first proposed for cancer treatment by Stevenson et al. [18] and by Andreesen et al. [1]. These cells have been used subsequently by several groups in a variety of cancer localizations [12]. It can be estimated that more than 100 patients have been treated with AAM [2]. We have recently carried out a clinical trial in patients with advanced-stage metastatic renal cell carcinoma [11], in which AAM treatment was combined with injections of granulocyte–macrophage colony-stimulating factor (GM-CSF). Numerous MNC were collected at leukapheresis, since AAM averaged 3×10^9 AAM per injection. Out of 15 patients treated, 8 stabilizations and 1 partial response were observed.

As AAM have been reported to be cytotoxic for tumor cells in vitro [3], the effectiveness of the treatment could depend upon the recruitment of circulating AAM at tumor sites. Such a correlation between tumor localization of effector cells injected and clinical response has already been observed in patients treated with tumor-infiltrating lymphocytes [17]. For these reasons, we investigated the body distribution of ^{111}indium-labeled AAM in our patients.

Patients and methods

Clinical design

Patient selection and the treatment protocol have been published elsewhere [11]. Briefly, 15 patients with metastatic renal cell carcinoma RCC, all at least with one criterion of poor prognosis as defined by Elson et al. [7], received GM-CSF subcutaneously (5 μg/kg/day from day 1 to day 14) and leukapheresis was performed on days 7, 11, and 14. MNC were cultured for 7 days in the presence of

GM-CSF (250 IU/ml) with addition of interferon-gamma (IFN-γ, 500 IU/ml) on day 6. On day 7, AAM were purified by counter-current centrifugation (elutriation) and infused into the patients. Each patient received two to nine AAM infusions. AAM populations contained 95% large cells, as determined by Coulter counter analysis.

AAM radiolabeling

Preliminary work was designed to optimize AAM radiolabeling with ^{111}indium oxinate. Labeling reached a plateau after 5 min of incubation. The labeling efficiency (percentage of radioactivity bound to AAM) was greatly reduced by the presence of human serum albumin in the labeling solution. The following protocol is currently adopted: 3–10×10^8 AAM washed and resuspended in 2 ml RPMI-1640 medium were exposed to 15 MBq ^{111}indium oxinate (Byk-Mallinkrodt, Peten, The Netherlands) for 15 min at room temperature, with intermittent stirring. After centrifugation at 400 *g*, the cells were suspended in 4% human serum albumin. A sample of cell suspension was subjected to in vitro investigations. Labeled cells were mixed with unlabeled ones before intravenous infusion.

Radioactivity measurements

The biodistribution of AAM was investigated 1, 18, 48 and 72 h after injection, using a gamma camera peaked for two photo peaks of ^{111}indium. The same aliquot of injected cells was used to calibrate the decrease in radioactivity. The radioactivity taken up by the organs was expressed as a percentage of the radioactivity injected. Blood radioactivity counts were done on whole blood samples, blood cell pellet and supernatant after high-speed centrifugation, and on mononuclear cells separated by Ficoll centrifugation. The results were related to the blood volume estimated from body weight and size, using correspondence tables.

Characterization of the cells injected

Cell separation by elutriation was monitored by repeated measurements of cell diameter with a Coulter counter ZM. This test and a viability test were performed on AAM before injection, but, due to practical reasons, other tests, cytospin preparations and phenotyping, were performed later on, using frozen cell samples. Phenotyping included determination of CD14, CD11b, CD16, CD34, CD56, CD64, CD66b HLA class I and HLA-DR expression (Immunotech), with reference to specific control isotypes.

Results

In vitro cell labeling

Twelve preparations of ^{111}In-labeled AAM were injected into 11 patients. On average, the patients received 3×10^9 AAM, of which 0.8×10^9 were radiolabeled. Cell labeling efficiency was 88 ± 8%. Viability of labeled AAM averaged 95% soon after labeling. In vitro release of ^{111}In rose from 8% at 1 h to 26% at 24 h.

In vivo biodistribution of cells injected

As shown in Fig. 1, blood recovery of ^{111}In dropped rapidly. The percentage of circulating radioactivity was 18% 1 h after injection and remained stable, around 4%, for at least 3 days. Most radioactivity was present in plasma, suggesting that labeled AAM did not recirculate durably.

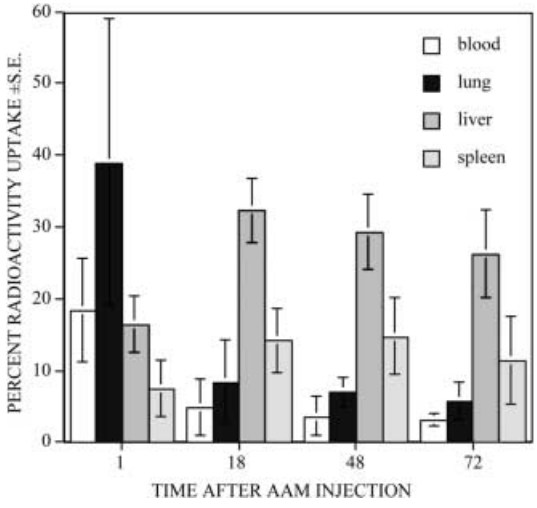

Fig. 1 Biodistribution of ^{111}In-labeled macrophages in 11 patients, at various time intervals after intravenous injection

Organ radioactivity was elevated in the lungs in 1-h images, while predominating subsequently in liver and spleen (Fig. 2A). Measurements at four time points (1, 18, 48 and 72 h) showed that liver radioactivity was maximal at 18 h and decreased slowly, thereafter, while spleen radioactivity peaked on day 2 (Fig. 1). The radioactivity recovered in blood, lung, liver and spleen represented 81.5%, 59.9%, 54.8% and 46.6% of the radioactivity injected at 1, 18, 48 and 72 h after injection, respectively. A slight bone marrow uptake was noted from 24 to 72 h. No digestive or renal elimination of radioactivity was observed.

In one patient (patient RAN), two focal radioactivity retentions were visualized in the lungs, at two different examinations, performed at a three-month interval (Fig. 2B, C). Comparison with CT scan showed that one scintigraphic localization corresponded clearly to a tumor lesion. Another large lung metastasis and bone metastases, however, remained unlabeled.

Investigations on the cell populations injected

Cell size distribution was used to determine the percentage of AAM injected, AAM being defined by a diameter above that of lymphocytes. Cell phenotype and cytospin investigations were performed later on, using frozen samples. The diameter distribution histograms of the cells injected generally showed a two-peak distribution: a minor peak of small cells, considered as a lymphocyte contaminant and a major peak of large cells, considered as macrophages (Fig. 3). In 3 out of the 15 patients treated, three-peak histograms were found in

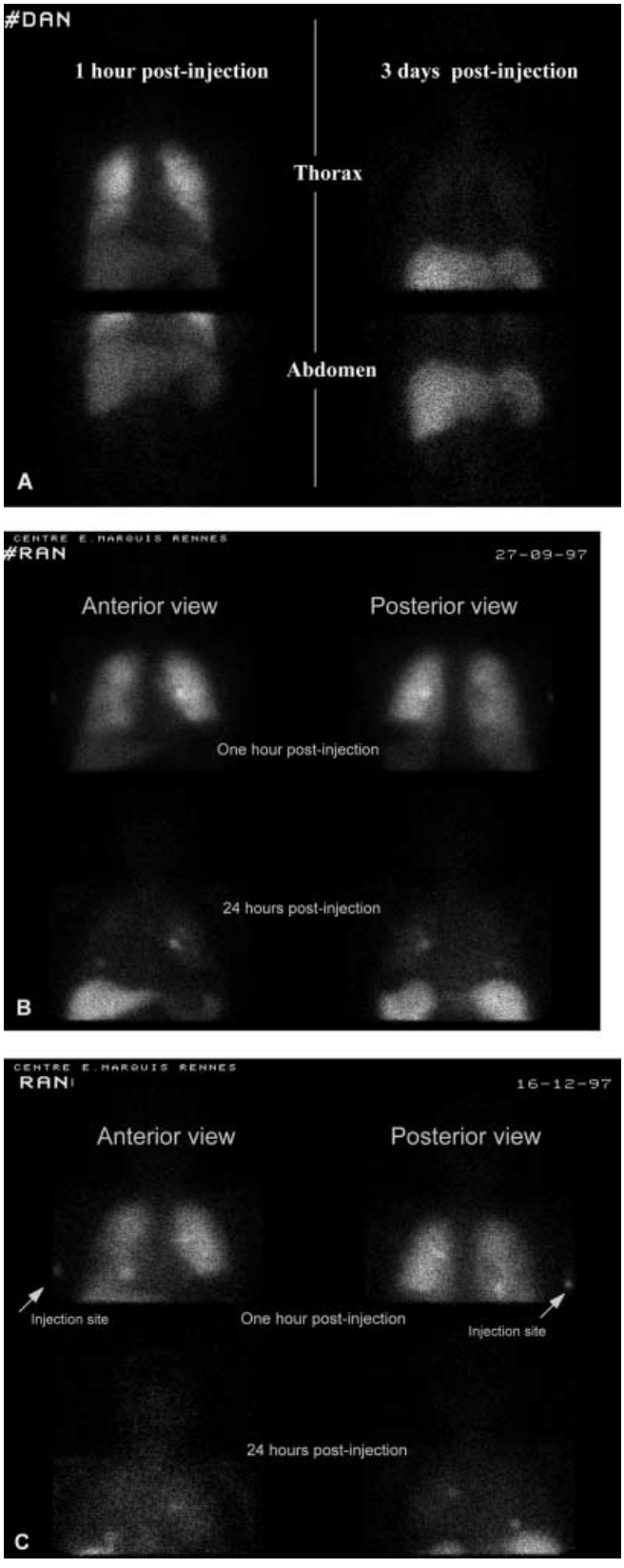

Fig. 2A–C Scintigraphic imaging. **A** Usual appearance, with lung filtration of AAM evidenced in 1-h images, radioactivity accumulation in liver and spleen at later times. **B** In one patient, two spots of radioactivity were clearly detected at 24 h in the lungs. They were already visible 1 h after injection. **C** Same patient as in **B** after an AAM injection performed 3 months later

most of the 6 or the 9 AAM preparations performed for each of them (Fig. 3C, D). Patient RAN was one of these patients.

Frozen samples revealed that the AAM injected contained mostly $CD11b^+$, $CD14^+$, $CD68^+$, $HLA\text{-}DR^+$ cells, and, as reported [11], 0.1–0.8% CD34-positive cells. Cytospin staining showed essentially macrophages, some lymphocytes and burst cells. The morphology of the latter sometimes suggested that they could be the remnants of granulocytes, an observation consistent with the poor resistance of granulocytes to cell freezing.

The median survival of AAM-treated patients was 9 months, but patient RAN survived more than 3 years and relapsed. He again received a treatment with AAM, and again, cell preparations exhibited the three-peaks pattern. This time, fresh cells were analyzed. Cytospin staining showed that the AAM injected contained 18% PMN. The lobularity of these PMN differed from that of PMN purified from a healthy donor's blood. The cells with 2, 3 and 4 or more lobes for patient RAN represented 60%, 36% and 4% of total PMN, respectively, as against 10%, 34% and 66% for the control. Flow cytometer analysis showed that two cell populations could be distinguished on forward against side scattered light plots. The larger population by cell size and number, presumably macrophages, was $CD14^+/CD66b^-$, while the minor population, presumably PMN, was $CD66b^+$, but coexpressed CD14 (Fig. 4).

In contrast with patient RAN, two other patients, who also exhibited a three-peak distribution pattern of AAM, had only 4- and 9-month survivals.

Discussion

Our results on the radiolabeling and the biodistribution of intravenously injected AAM agreed on several points with literature reports. Marienhagen et al. [16] investigated the in vitro radiolabeling of macrophages with indium oxinate, and found 84% labeling efficiency, as against 88% here. They also showed that ^{111}In-radiolabeling did not impair the functional properties of the cells. Our results on the biodistribution of AAM in all but one of 11 patients, did not differ substantially from published data. Faradji et al. [8] conducted investigations in 7 patients, all injected with 2×10^8 ^{111}In-labeled cells. Four patients received AAM, while three received macrophages not submitted to gamma-interferon activation. Radioactivity measurements were done over a 5-day period. The results were similar in the two groups of patients: lungs showed the greatest radioactivity uptake by 1-h post-infusion, liver and spleen uptake rose steadily over the first 24 h and then declined from 72 h

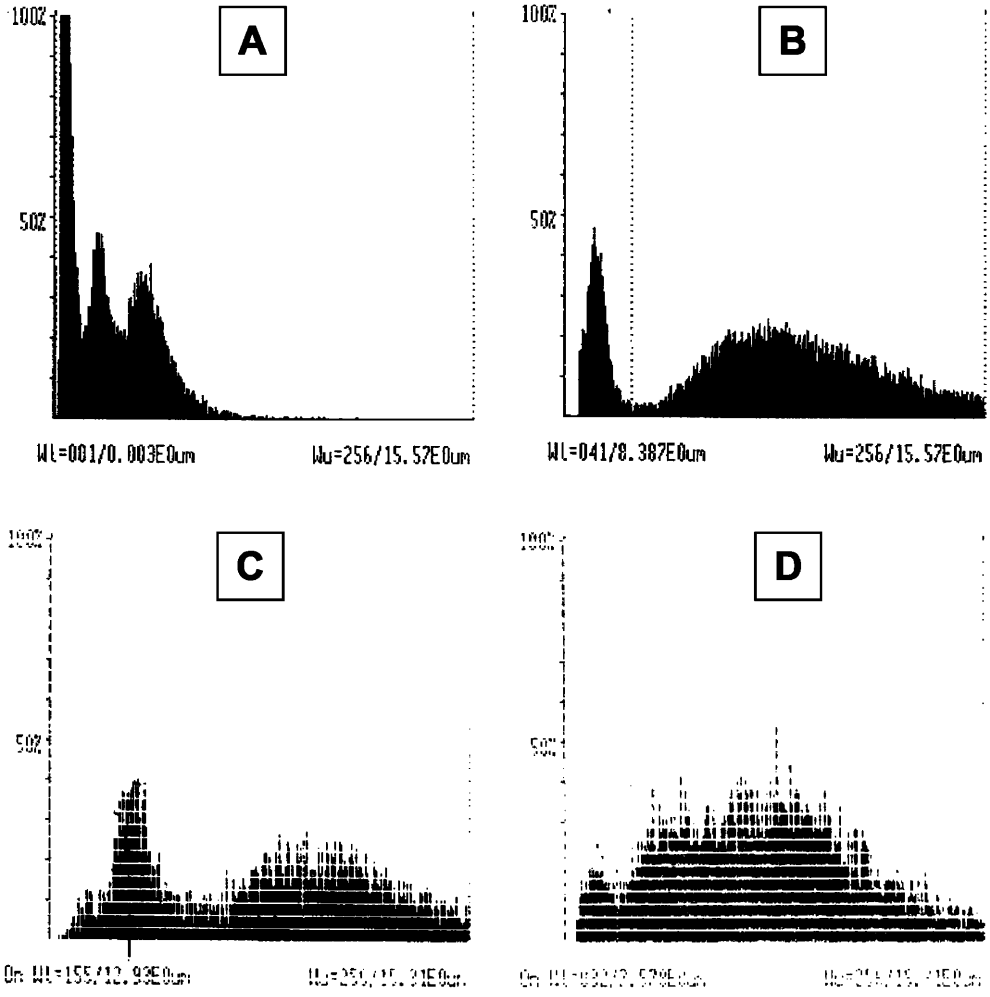

Fig. 3A–D Cell diameter distribution histograms. **A** Leukapheresis product before culture. The three peaks correspond from left to right to: (1) platelets and red cells, (2) lymphocytes, (3) monocytes and PMN. PMN were identified morphologically in leukapheresis product from this patient. **B** AAM before injection. Usual profile with lymphocyte and macrophage peaks (same patient as in **A**). **C, D** AAM of patient RAN. The intermediary cell population between lymphocytes and macrophages was interpreted as containing mainly granulocytes

post-infusion. Andreesen et al. [2] reported similar observations.

The uptake by tumors of injected AAM has only been evidenced when injecting the cells in the peritoneal cavity of patients with peritoneal metastases [2]. Even when injecting radiolabeled AAM in the hepatic artery of patients with hepatic metastases, no tumor radioactivity uptake could be found [10]. These observations correlate with experimental demonstrations showing that macrophages can infiltrate tumor spheroids in vitro [4], but do not migrate substantially in vivo towards inflammatory foci [5].

In the case of patient RAN, in whom focal and persistent accumulation of radioactivity outside the liver and spleen was found, one possible explanation would be that granulocytes contaminating AAM preparations were recruited in acute inflammatory foci. This hypothesis is sustained by: (1) the presence, at least in some AAM preparations, of granulocytes identified by their morphology and CD66b expression; these cells were $CD14^+$, a feature reported for activated granulocytes [6]; (2) the kinetics of radioactivity uptake, which was detectable from the first hour, suggesting granulocyte accumulation in an acute inflammation; (3) the lack of clear correlation between metastatic sites and sites of radioactivity uptake. The presence of granulocytes among AAM is difficult to explain by the survival of PMN collected during leukapheresis and cultured, although GM-CSF has been shown to enhance the median survival of neutrophils in culture from 22 to 28 h [13]. More probably, the weak lobularity of PMN, and the presence of $CD34^+$ cells in the AAM preparations, as reported earlier [11], suggests that the granulocytes differentiated from the hematopoietic precursors mobilized by the in vivo administration of GM-CSF. Moreover, the ex-vivo culture of CD34-positive cells under stroma-free conditions, in the presence of a variety of cytokines, including GM-CSF [15], resulted in the production of granulocytes that could be used for patient treatment.

In conclusion, the present data confirm the biodistribution assigned to AAM injected intravenously with nonrecruitment in tumor foci. The observation of focal uptakes of radioactivity seems artifactual and related to the contamination of the preparations by granulocytes. The latter might have developed from precursor cells contaminating the MNC from patients treated with GM-CSF. Cell therapy protocols that combined

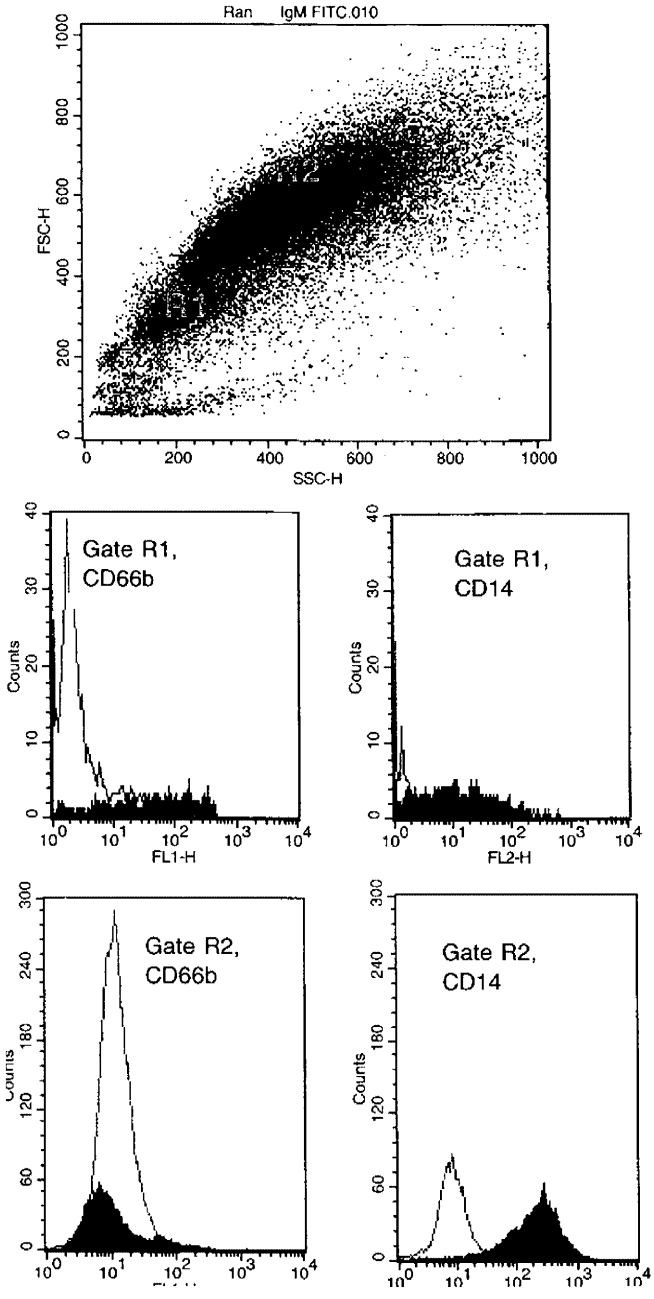

Fig. 4 Flow cytometer analysis of AAM of patient RAN. Population *R1* was interpreted as corresponding to $CD66b^+/CD14^+$ granulocytes, while *R2* corresponded to $CD66b^-/CD14^+$ macrophages

GM-CSF in vivo and in vitro have been reported in the case of macrophage injections [9, 11, 14]. The production of dendritic cells, which are used more and more widely for cancer vaccination, also includes the culture of mononuclear cells with GM-CSF, in addition to IL-4 or IL-13. It seems important to draw attention to the possible occurrence of granulocytes in cultures designed to produce antigen-presenting cells, if the patients are submitted at the same time to a hematopoietic cell-mobilizing treatment.

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