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# Control of the Antitumoral Activity of Human Macrophages Produced in Large Amounts in View of Adoptive Transfer

S. DUMONT,* D. HARTMANN,* P. POINDRON,* F. OBERLING,† A. FARADJI† and J. BARTHOLEYNS*

**Département d'Immunologie et d'Immunopharmacologie, UER de Pharmacie, Université Louis Pasteur, BP 24, 67401 Illkirch-Graffenstaden, France and †CHU Hautepierre, service d'Oncohématologie, 67098 Strasbourg, France*

**Abstract**—*Purified human blood monocytes were grown in hydrophobic bags in RPMI medium containing additional amino acids, indomethacine and growth factors. Autologous serum was added after a few days of culture at* 37°C, 5% $CO_2$. *The antitumoral activity generated by activated monocytes against human tumor cells grown* in vitro *was mediated by soluble effectors in contrast to macrophages which acted by cell contact. Monocyte differentiation into macrophages was achieved after 7 days of culture and characterized by phagocytosis and expression of MAX 1 antigen and non-specific esterases. The macrophages remaining in suspension in the bags were activated by exposure to immunostimulating compounds used alone or in combination (recombinant human gamma-interferon and muramyldipeptide). Activated macrophages were cytotoxic* in vitro *against U* 937 *or ovary carcinoma tumor lines* (95% *cytotoxicity) at a* 1/1 *effector/tumor cell ratio. The antitumoral potency of activated macrophages was confirmed* in vivo *where adoptive transfer of one million human macrophages twice a week to nude mice bearing human ovary carcinoma caused a marked regression of the primary tumor.*

## INTRODUCTION

MONOCYTES and macrophages are involved in the defense of the organism against infections and neoplasia [1]. Monocytes escape from the blood and migrate by chimiotactism [2, 3] to the tumor site where they differentiate into macrophages which are often suppressed by mediators such as PGE2 released by solid tumors. Macrophages can be activated directly by bacteria or their products such as endotoxins [4], muramyldipeptide (MDP) [5, 6] or by their interaction with lymphokines such as recombinant human gamma-interferon (rHuIFN-γ) and tumor necrotizing factor (TNF [7–11]) released by lymphocytes. Immunotherapeutic approaches of cancer include *ex vivo* treatment of plasma to remove suppressive factors [12], *in vivo* injection of immunopotentiators such as interferons or interleukins (IL [13–15]) and *ex vivo* activation of the patient's leukocytes with biological response modifiers followed by reinfusion. Several groups are working with antigen-specific cytotoxic T lymphocytes [16, 17], LAK cells [lymphokine (IL2) activated killer cells] [18], or NK cells activated by IFN-γ or IL2 treatment [19, 20]. Immunotherapy with activated monocytes harvested by cytapheresis and purified by elutriation has recently been attempted [21–23]. In experimental tumors, adoptive transfer of C57BL/6J mice bearing Lewis lung carcinoma with autologous alveolar lung macrophages (cultured for 3–4 weeks) caused clear antitumoral and antimetastatic effects [24]. Peritoneal macrophages expanded and activated *in vitro* caused regression of solid EMT6 sarcoma after adoptive transfer to BALB/c mice [25].

We have now verified the potential antitumoral cytotoxicity *in vitro* and *in vivo* of human blood monocytes and of differentiated macrophages obtained after 7 days of culture. These human macrophages were characterized for their differentiation state: e.a., NS esterases, phagocytosis, cytotoxicity and specific MAX differentiation antigens developed by the group of Andreesen (Freiburg, F.R.G. [26, 27]). Differentiated macrophages were activated to cytotoxicity. This cytotoxicity has been tested *in vitro* against human histiocytic lymphoma U 937 [28] and human ovarian carcinoma cells

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Correspondence and reprint requests should be addressed to S. Dumont, UER Pharmacie 74, Rte du Rhin, 67401 Illkirch-Graffenstaden, France.
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and, *in vivo*, in C57BL/6J nu/nu mice bearing subcutaneous human ovary carcinoma. The aim of the present study is to develop a new immunotherapy for cancer with autologous activated macrophages. Such cells would present less toxicity and more specificity than lymphokines or IFN and would be more effective than adoptive therapy with LAK cells or with activated monocytes.

## MATERIALS AND METHODS

*Human mononuclear leukocytes*

Mononuclear leukocytes were isolated from peripheral blood of healthy volunteers and patients with Crohn disease or with ovarian carcinoma, by cytapheresis of 7–8 l. blood of a COBE 2997 cell separator (COBE Lab., U.S.A.). The number of leukocytes isolated by this technique reached up to $10^9$ cells.

*Purification of human monocytes*

Human blood peripheral monocytes were isolated by 15 min centrifugation at 350 ***g*** on a COBE 2991 blood cell processor, using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) [29] of density 1.070. Human monocytes were obtained with a purity of 65–70% (as determined by May–Gründwald–Giemsa staining) and a yield of 80%.

A second method was used for the purification of a smaller number of monocytes. Centrifugation on Ficoll-Hypaque (density 1.077 for 15 min at 350 ***g***) was followed by a continuous Percoll density gradient [30, 31]. The purity of monocytes reached 90%.

*Long-term culture of monocytes*

The monocytes were cultured in RPMI 1640 medium (Gibco laboratories, Grand Island, NY, U.S.A.) supplemented with penicillin 100 U/ml and streptomycin 100 μg/ml, oxaloacetic acid 132 μg/ml, insulin 8 μg/ml, 2 mM glutamine, 2 mM pyruvic acid, 1 ml non-essential amino acids (Gibco, ref 043-01140H), $5 \times 10^{-6}$M indomethacine.

Monocytes were grown in flexible hydrophobic bags (Lifecell, Fenwall, Travenol, Maurepas, France) suitable for the scale-up and routine cultivation of mammalian cells in suspension [32]. The bags containing 100–300 ml of medium and up to $2 \times 10^6$ cells/ml were incubated at 37°C in a humidified 5% $CO_2$, 95% air incubator. Ten per cent autologous serum was added on day 5 of culture. Macrophage activators were added into the bags on day 7 for up to 24 h before use.

*Tumor cells*

Human ovarian cancer xenografts (OVXF 899) grafted in nude mice were kindly given by Dr H. Fiebig (Univ. Klinik, Freiburg, F.R.G.). These ovarian carcinoma cells were passaged in female C57BL/6J nu/nu mice (Bomholgard, Denmark) and could be grown *in vitro* in RPMI 1640 medium. U 937 monocyte tumor cell line [28] was used as a target for the cytotoxicity assays. These cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum.

*Experimental tumors in animals*

Female C57BL/6J nu/nu mice (5–10 weeks old) were kept on sawdust in plastic cages and inoculated subcutaneously in the abdominal region with $10^6$ human ovarian carcinoma cells dissociated from a solid tumor. Treatment was initiated when the size of the tumor reached 20 $mm^2$. The tumor cross-section was estimated by measurement of two perpendicular diameters ($a$ and $b$) of the tumor according to the formula: $a \times b \times \pi/4$. Mice were injected subcutaneously twice a week with $10^6$ human activated monocytes or macrophages in the peritumoral area. Control mice were injected with phosphate buffered saline (PBS).

*Cytotoxicity assay*

$10^4$ monocytes or macrophages in 0.2 ml RPMI medium supplemented with 10% autologous human serum were seeded in microtiter plates (ref 167008 Nunclon, Denmark) and incubated for 24 h at 37°C. The supernatant was then removed. Tumor target cells were added to the wells in 0.2 ml of medium. After another 24 h, the cells were pulsed for 24 h with 0.2 μCu [$^3$H] thymidine (Dupont NEN Research Products, Boston). Cells were harvested on Titertek glass-fiber filters with a collector (Skatron, Lierbyen, Norway). Thymidine incorporation ($10^4$ to $8 \times 10^4$ dpm/$10^4$ cells in 24 h) was measured by liquid scintillation in a Beckmann scintillation counter. Tumor cells did not adhere to the microtiter plates and could easily be segregated from the adherent macrophages. For estimation of thymidine incorporation, the background radioactivity in the wells containing macrophages only (less than 300 dpm/$10^4$ macrophages) was taken into consideration. Cytostasis was expressed as percentage of [$^3$H]thymidine incorporation in treated tumor cells compared to control tumors.

Cytotoxicity was also assessed by the trypan blue dye-exclusion assay [33]. This internal control minimized the possible artefacts, especially the production by the macrophages of thymidine competing for incorporation [34]. Each value was the mean of six determinations.

*Monocyte differentiation into macrophages*

*Expression of MAX 1 antigens.* Differentiation of macrophages was investigated with the monoclonal antibody MAX 1 (a generous gift from R. Andreesen, Freiburg), specific for antigens present on the membrane of differentiated macrophages and not of monocytes [26, 27]. Immunofluorescent staining

procedure was performed using antibodies diluted in isotonic saline solution containing 0.2% bovine serum albumin and 4% hepes buffer. The monocytes adherent to glass slides were fixed with 2.5% glutaraldehyde (Merck, U.S.A.). For MAX 1 antigen staining, the slides were incubated for 1 h at 37°C in the presence of mouse monoclonal anti-MAX 1 antibodies at 1/10 dilution. The cells were washed twice and incubated for 15 min in buffer containing a 1/100 dilution of peroxidase-conjugated rabbit anti-mouse IgG (Biosys, Compiègne, France). Cells were then washed with PBS and incubated for 15 min at 37°C in diaminobenzidine (DAB)–$H_2O_2$ (30%) solution. Finally, the cells were fixed with 2% osmic acid (Merck) at room temperature for 15–20 min. As negative control, cells were incubated with the second antibody only. After extensive washing in PBS for 1 h, the slides were examined by fluorescence microscopy.

*Phagocytosis.* Macrophages collected in suspension in the bags were allowed to adhere to plastic in microtiter plates. They were cultured for 4 h at 37°C in the presence of a baker's yeast suspension (heat-inactivated for 20 min at 120°C and gently sonicated for 2 min before use, 30 yeast cells per macrophage). Cells were then thoroughly washed with PBS, stained by the May–Grünwald–Giemsa technique before counting the number of intracellular yeast cells.

*Detection of non specific esterases (NSE).* A staining procedure using a commercial kit (Sigma, St. Louis, U.S.A. α-naphthyl acetate esterase N° 90-A1) was applied to cells seeded for 24 h in plastic flasks. The NSE positive cells contained dark brown granulations.

*Activation of monocytes or macrophages to cytotoxicity*

Monocytes and monocyte derived macrophages were activated with rHuIFN-γ (500 U/ml, Hoffmann la Roche, Basel) and MDP (0.5 μg/ml, Ciba Geigy, Basel). The monocytes were maintained for 24 h in contact with the activator before cytotoxicity assay.

## RESULTS

*Evolution of monocyte and macrophage cultures in hydrophobic bags*

Table 1 shows that differentiation of monocytes into macrophages is accompanied by a marked increase in the number of NSE-positive cells, the presence of MAX 1 antigens, and increased phagocytosis. No difference was observed concerning adherence. Usually 35–50% of the initial population seeded was lost during monocyte differentiation. At the end of the incubation period, the cell population consisted essentially of differentiated macrophages with 95% viability. The initial lymphocyte contamination was then negligible, and no cell debris was observed (data not shown).

*Monocyte and macrophage cytotoxicity*

Serum was not required during activation of monocytes to cytotoxicity. Monocytes cultured for 1 day in serum-free medium and activated with MDP plus rHuIFn-γ caused 60% inhibition of [$^3$H]thymidine incorporation by U 937 tumor cells (Fig. 1). This inhibition did not correlate with loss of cell viability as measured by the trypan blue exclusion test (Table 2). In contrast, macrophages cultured for 7 days in serum-free medium were not cytotoxic to tumor cells after activation with rHuIFN-γ and MDP (Fig. 2). When serum was added to the culture on day 5, macrophages were

*Table 1. Functional characterization of human monocyte and macrophages*

| Property | Percentage of positive cells | |
|---|---|---|
| | Monocyte | Macrophage |
| Adherence | + + | + + |
| Non-specific esterases | 40–50% | >70% |
| Phagocytosis | 40–45% | 65% |
| MAX 1 antigen | − | + |

Human blood monocytes (purity between 70 and 80%) were seeded at 2 × $10^6$ cells/ml in bags. Autologous serum was added on day 5 to allow monocyte differentiation into macrophages. Differentiation was assessed on day 7 by morphological and functional analysis (see Materials and Methods). Monocyte analysis was performed after 24 h culture. (−) no reaction. (+) positive reaction. Results are means of six determinations.

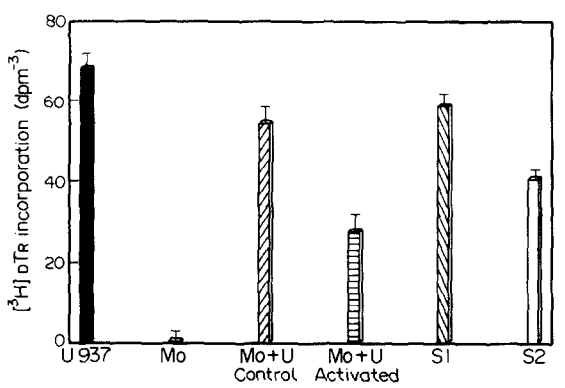


*Fig. 1. Cytotoxicity of human monocytes* in vitro. $10^4$ *monocytes (Mo) were seeded in microtiter plates together with* $10^4$ *U 937 cells (U). S1:* 100 μl *supernatant of non-activated monocytes + U 937 cells. S2:* 100 μl *supernatant of activated* (500 U/ml *rHuIFN-γ*, 0.5 μg/ml *MDP) monocytes + U 937 cells. Mo + U, control: non-activated monocytes in coculture with tumor cells. Mo + U, activated: activated monocytes in coculture with tumor cells. Incorporation of thymidine by tumor cells was measured as described in Materials and Methods. Means of* 10 *data points and standard errors are presented. Statistical analysis by Student's t test gave* $P < 0.001$ *for M + U control or activated and S2, and was not significant for S1.*

*Table 2. Cytostatic or cytotoxic effects of monocyte and macrophages on U 937 and on ovary carcinoma cells*

| | [$^3$H]Thymidine incorporation | Cell viability (trypan blue exclusion) |
|---|---|---|
| Control U 937 cells | 100.0 | 100.0 |
| + non-activated monocytes | 80.4* | 100.0 |
| + activated monocytes | 41.0* | 76.3 |
| + non-activated macrophages | 82.0* | 75.0 |
| + activated macrophages | 4.6* | 12.2 |
| Control ovary carcinoma cells | 100.0 | 100.0 |
| + non-activated monocytes | 89.0** | 100.0 |
| + activated monocytes | 65.2* | 61.0 |
| + non-activated macrophages | 92.5** | 96.6 |
| + activated macrophages | 32.0* | 26.7 |

The cytotoxicity of macrophages and monocytes was evaluated by [$^3$H]thymidine incorporation and cell viability expressed as percentage of control values for a 1/1 effector target cell ratio. Activation was achieved after 16 h pretreatment with 500 U/ml rHuIFN-γ and 0.5 μg/ml MDP. Results are means of six determinations. Statistical analysis by Student's *t* test: $*P < 0.001$, $**P < 0.05$.

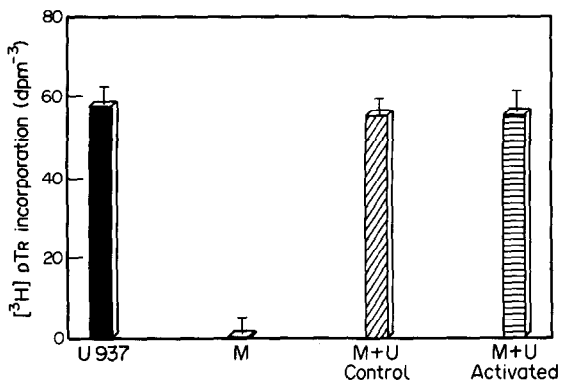


*Fig. 2. Cytotoxicity of human macrophages grown and activated in absence of serum. Macrophages (M) were cultured for 7 days and activated by* 500 U/ml *rHuIFN-γ* + 0.5 μg/ml *MDP in serum-free medium. Macrophages* ($10^4$) *were added to* $10^4$ *U 937 cells and cytotoxicity was measured as described in Materials and Methods. Means of* 10 *samples and standard errors are presented. Student's* t *test indicated no significant difference between the groups except the macrophages alone* ($P < 0.001$).

cytotoxic. After activation, 95% inhibition of radioactive thymidine incorporation by tumor cells was observed (Fig. 3). This antitumoral effect was confirmed by the trypan blue exclusion test, which gave a very good correlation between inhibition of thymidine incorporation by macrophage and loss of viability (Table 2). The cytotoxicity was dependent upon the macrophage/tumor target cell ratio (Fig. 4). A 1/1 effector/target ratio was used for further experiments.

Activated monocytes and activated differentiated macrophages were cytotoxic both to U 937 cells and to human ovary carcinoma cells (Table 2). Macrophages were much more potent than monocytes in killing tumor cells; for monocytes, the decrease in cell viability was smaller than the inhibition of thymidine incorporation (Table 2).

*Are the antitumoral effects mediated by cells or by soluble factors?*

In search of soluble mediators, the cytotoxicity present in the supernatant from activated monocytes or macrophages was measured (Figs. 1 and 3). The supernatant from non-activated cells did not induce cytotoxic effects, the supernatant from activated monocytes produced cytotoxicity, while the supernatant from activated macrophages was not cytotoxic (Figs. 1 and 3). The activators (rHuIFN-γ, MDP) did not induce any cytotoxic effects on tumor cells at the concentration used (data not shown).

Addition of 0.1 ng/ml of recombinant human

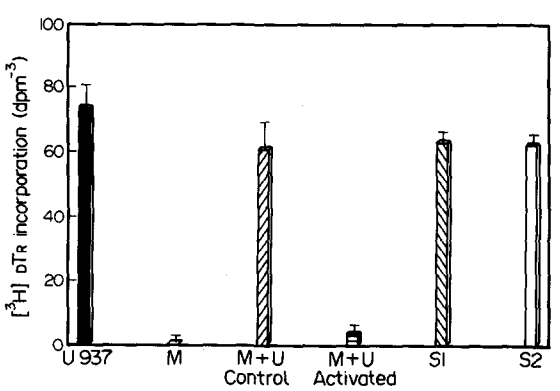


*Fig. 3. Cytotoxicity of macrophages grown and activated in presence of autologous serum.* $10^4$ *macrophages (M) were seeded in microtiter plates together with* $10^4$ *U 937 cells (U). S1:* 100 μl *supernatant of non-activated macrophages (rHuIFN-γ + MDP) + U 937 cells. S2:* 100 μl *supernatant of activated macrophages + U 937 cells. M + U control: non-activated macrophages in coculture with tumor cells. M + U, activated: activated macrophages in coculture with tumor cells. Cytotoxicity was measured as described in Materials and Methods. Means of* 10 *data points and standard errors are presented. Student's* t *test: M + U control* $P < 0.001$, *M + U activated* $P < 0.001$, *S1 and S2 non-significant.*

tumor necrosis factor caused 47% inhibition of [$^3$H]thymidine incorporation. 100 µl supernatant from activated monocytes caused 63% inhibition of [$^3$H]thymidine incorporation. Addition of 2 µg/ml anti-TNF antibodies (a generous gift from Knoll-BASF AC, Ludwigshafen, F.R.G.) to this supernatant neutralized the inhibition, which was reduced to 7%.

*Adoptive transfer of activated human monocytes or of macrophages to nude mice bearing human carcinoma*

We have tested the putative antitumoral effects induced by adoptive transfer of human monocytes or macrophages in nude mice bearing subcutaneous human ovary carcinoma. The results of peritumoral injection of $10^6$ activated macrophages twice a week are shown in Fig. 5. A significant regression of tumor size and mass was observed after five injections of macrophages while control tumors increased three-fold in size during the same time. The subcutaneous injection of $10^6$ macrophages (washed and concentrated in 0.1 ml PBS) in the peritumoral area did not cause any obvious toxicity to the animals. A marked necrosis was observed in the tumor center, not necessarily reflected in the evolution of the tumor cross-section measured before and the day after injection. The antitumoral effects induced by adoptive transfer of activated monocytes and activated or non-activated macrophages are summarized in Table 3. Non-activated macrophages were not cytotoxic *in vitro* and presented no antitumoral effects *in vivo* after adoptive transfer (Table 3), no significant difference was seen between the evolution of the cross-section in control and treated animals. Activated monocytes, which were essentially cytostatic *in vitro*, reduced the growth of the

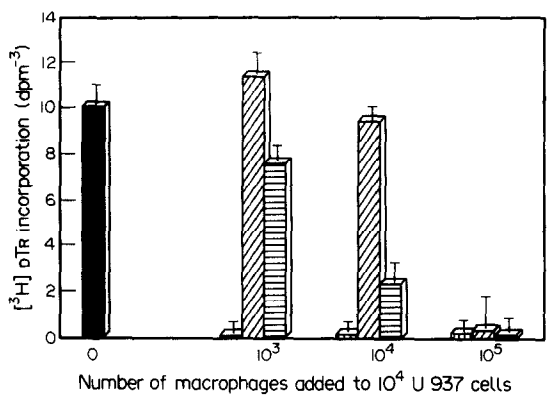


*Fig. 4.* In vitro *cytotoxicity of macrophages on U 937 cells. Cytotoxicity of macrophages for U 937 cells was measured at different effector to target ratios. Increasing number of macrophages were added to U 937 tumor cells ($10^4$ cells). Filled bar: tritiated thymidine incorporation by U 937 tumor cells in the absence of macrophages.* ▥ *thymidine incorporation by macrophages alone.* ▨ *M + U: coculture of non-activated macrophages with U 937 tumor cells (NS at $10^3$ and $10^4$, $P < 0.001$ at $10^5$).* ▤ *M + U + activators: coculture of activated macrophages with U 937 tumor cells (NS at $10^3$, $P < 0.001$ at $10^4$ and $10^5$). Means of 10 data points and standard errors are presented.*

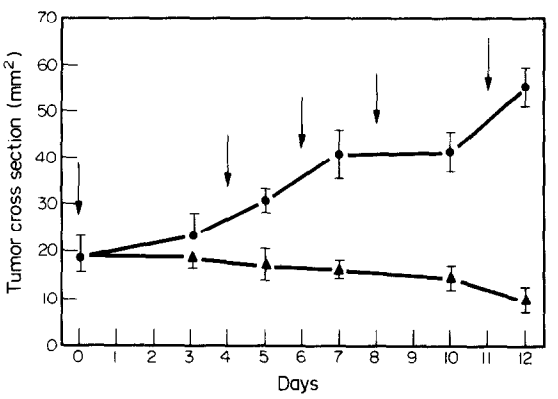


*Fig. 5. Adoptive transfer of activated human macrophages in C57BL/6J nu/nu mice bearing subcutaneous ovary carcinoma. C57BL/6J nu/nu mice having developed a solid carcinoma of 20 mm$^2$ section were injected subcutaneously at the indicated time (arrow) in the peritumoral area either with $10^6$ activated macrophages in 0.1 ml PBS (▲) (n = 6) or with 0.1 ml PBS alone (●) (n = 6). Results are means and standard errors of the means. Statistical analysis of variance indicated $P < 0.04$ for treatment curve compared to control. Student's t test at day 12 gave $P < 0.01$.*

*Table 3. Comparison of* in vitro *and* in vivo *effects of human monocytes and macrophages*

| Cells | Percentage cytotoxicity *in vitro* U 937 | Ovary carcinoma | Effect on ovary carcinoma in nude mice |
|---|---|---|---|
| Activated macrophages | 95% | 68% | Decrease in tumor size |
| Non-activated macrophages | 5% | 8% | No effect |
| Activated monocytes | 59% | 35% | Stabilization |

Macrophages or monocytes were pretreated for 16 h with rHuIFN-γ and MDP, before adding tumor cells for 24 h. *In vivo*, $10^6$ monocytes or macrophages were transferred twice a week to nude mice bearing solid human ovary carcinoma. Results are the means of six determinations (*in vitro* studies and transfer of activated macrophages) or of four determinations (transfer of monocytes and of non-activated macrophages).

tumor *in vivo*, but the difference hardly reached significance. Activated macrophages, with a marked cytotoxicity *in vitro*, induced tumor regression *in vivo* (five mice presented tumor regression and one a total disappearance of the tumor after three injections).

## DISCUSSION

The effects induced by non-activated monocytes on tumor targets are very non-reproducible, depending on the monocyte source and especially the presence of neoplastic disease or inflamation in the donor. In some instances, monocytes were spontaneously cytotoxic (10–20% inhibition of tritiated thymidine incorporation) while in others they did not influence tumor cell growth (Fig. 1). Indomethacine $5 \times 10^{-6}$ M was routinely added to the culture medium to avoid suppression of the macrophages by PGE2 synthesized by endogenous cyclo-oxygenase and to sustain activation [35]. Activated monocytes induced a more important decrease in [$^3$H]thymidine incorporation by tumor cells (Fig. 1) than tumor cell death estimated by the trypan blue dye uptake (Table 2). This suggests that activated monocytes block tumor cell growth essentially by cytostasis rather than cytotoxicity, explaining the dissociation between inhibition of thymidine incorporation and the loss in cell viability. The antitumoral effects of activated monocytes were mediated at least partly by soluble factors (Fig. 1). Preliminary experiments performed with purified anti-TNF antibodies allowed complete reversion of these monocyte-mediated cytotstatic effects, suggesting that TNF was released by monocytes.

Monocytes grown in culture for 1 week in serum-free medium did not produce fully differentiated macrophages (Fig. 2). These cells did not affect tumor cell growth whether activated or not.

Monocytes cultured for 1 week in the presence of 10% autologous serum differentiated into macrophages which became cytotoxic after MDP and rHuIFN-γ activation (Fig. 3). Serum factors were clearly required for macrophage activation and cytotoxicity.

In this case a good correlation was found between inhibition of radioactive thymidine incorporation and loss of tumor cell viability. Macrophages killed tumor cells by intimate contact (Fig. 3) much more effectively than monocytes and at a lower effector/tumoral cell ratio than killer lymphocytes [17] (Table 2). Human U 937 cells were more sensitive than ovary carcinoma cells to activated monocytes or macrophages. The sensitivity of the tumor cells to soluble products released by monocytes or to membrane mediators such as TNF expressed by differentiated macrophages might be very different from one tumor type to another.

### In vivo *antitumoral effects*

*In vivo*, adoptive transfer of differentiated macrophages activated with rHuIFN-γ and MDP, induced a very significant regression of solid macroscopic carcinoma, without obvious side-effects (Fig. 5 and Table 3). Prior activation of the macrophage to cytotoxicity was an absolute requirement for antitumoral effect *in vivo*. Activated monocytes, which *in vitro* released cytotoxic compounds, stabilized tumor growth in nude mice. These data are in agreement with the results of Stevenson and Fauci [36] obtained after adoptive transfer of activated monocytes to patients with ovary carcinoma. According to our data, activated autologous macrophages would represent a better effector cell than monocytes to achieve tumor regression. Antitumoral effects were obtained using a much lower number of macropages than the number of autologous cells required for therapy with LAK cells [18], specific T lymphocytes [17] or NK cells [19, 20]. Furthermore, macrophages differentiated and activated *in vitro* did not require further *in vivo* stimuli such as IL2 injections [13] to induce clear antitumoral effects. The route of administration of macrophages will have to be established for each type of tumor. Local injection will be chosen as much as possible to avoid non-specific biodistribution.

The culture, differentiation and activation of human macrophages can now be accurately controlled. The cytotoxic macrophages obtained induced regression of human tumors in nude mice. Considering the amount of macrophages required for such regression ($10^6$ cells/injection), we can extrapolate that such a treatment is feasible in man ($500 \times 10^6$ activated macrophages can indeed be obtained after differentiation of monocytes from one cytapheresis). We can therefore propose a new kind of cancer immunotherapy using the patient's own macrophages differentiated and activated in culture before adoptive transfusion in the tumor periphery. Preliminary clinical trials are planned in the near future.

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