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In vivo Effects of Recombinant Interferon Alpha A/D Incorporated in Gelatin Microspheres on Murine Tumor Cell Growth

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Intraperitoneal (ip) injections of gelatin microspheres containing a very small amount of recombinant human interferon alpha A/D (A/D-IFN) (IFN-microspheres) plus free A/D-IFN improved the survival of mice bearing ascitic Meth A-R1 cells which we had isolated as IFN-resistant cells under *in vitro* conditions. The dose of free A/D-IFN in one injection was 10,000 IU, which was insufficient by itself for manifesting *in vivo* antitumor activity. In these mice, *in vivo* R1 cell growth was suppressed and macrophage recruitment was enhanced in comparison with mice receiving other control agents. Administration of IFN-microspheres alone was also effective but less than that of IFN-microspheres plus free A/D-IFN. Peritoneal macrophages obtained from normal or R1-bearing mice receiving ip injection of IFN-microspheres with or without free A/D-IFN were activated to inhibit the *in vitro* growth of R1 cells. The intratumoral injection of IFN-microspheres strongly inhibited the growth of solid R1 tumors. Intravenous injection of IFN-microspheres was effective in preventing the pulmonary metastasis of B16 melanoma cells. These results indicate that the IFN-microsphere is much more effective against tumors than free A/D-IFN.

Key words: Macrophage activation — *In vivo* antitumor effect — Gelatin microspheres — Interferon-αA/D

The clinical efficacy of natural and recombinant human interferons towards a variety of neoplasms has been extensively investigated. Interferons (IFNs) may act directly or indirectly via activation of host defense mechanisms on tumor cells. Direct inhibitory activity of IFNs against the *in vitro* growth of some tumor cells has been reported.[1-5] There is increasing evidence that IFNs modulate host effector cells so as to inhibit the *in vivo* tumor growth.[4,6] The plausible mechanism is through activation of NK cells,[7-10] macrophages/monocytes,[11-15] and T cells.[16,17] However, the development of effective administration forms should be addressed for successful tumor immunotherapy with IFNs. As a new attempt at delivering IFN to macrophages (Mϕ), we have prepared gelatin microspheres as a carrier of interferon alpha A/D (A/D-IFN) to be ingested by Mϕ. It was demonstrated that the microspheres containing A/D-IFN were far more effective than free A/D-IFN in enhancing the inhibitory activity of mouse peritoneal Mϕ against *in vitro* tumor cell growth.[18]

The present study was undertaken in order to explore the effectiveness of the microspheres containing A/D-IFN in the suppression of *in vivo* tumor cell growth. We also describe the inhibitory effect of Mϕ harvested from tumor-bearing mice treated with the microspheres on *in vitro* tumor cell growth.

MATERIALS AND METHODS

Animals Specific pathogen-free male and female BALB/cCrSlc and C57BL/6CrSlc, 3–5 months old, were obtained from Shizuoka Laboratory Animal Center, Shizuoka. No sexual difference was observed in the *in vivo* tumor cell growth.

Tumor cells R1 cells were isolated and cloned from a Meth-A cell line (methylcholanthrene-induced fibrosarcoma of BALB/c mice) as a subline resistant to murine natural IFN-α/β in *in vitro* cell growth[14] about five years ago, and have been maintained with recloning since then. B16 melanoma cells were obtained from the National Cancer Center Research Institute, Tokyo.

IFN and culture medium Recombinant human interferon alpha A/D (A/D-IFN, 1.5×10^8 IU/mg protein) was provided by Nippon Roche Research Center, Kamakura. Endotoxin level in the original preparation determined by a *Limulus* test was 1.6 ng LPS/1.5×10^7 IU IFN/ml or less. A/D-IFN, the product of a hybrid molecule of A and D clone DNAs, is known to be applicable not only to human cells but also to murine cells.[19] The cell culture was performed in RPMI-1640 medium (RPMI-FCS) (Nissui Seiyaku Co., Ltd., Tokyo) supplemented with 10% fetal calf serum (FCS, M.A. Bioproducts, Walkersville, MD), 5 m*M* L-glutamine and penicillin (100 units/ml), and buffered with 5 m*M* 4-(2-hydroxyethyl)-1-

piperazine-ethanesulfonic acid and $NaHCO_3$ at pH 7.2. Hanks' balanced salt solution (HBSS) and phosphate-buffered saline solution (PBS) were obtained from Nissui Seiyaku Co., Ltd.

Preparation of gelatin microspheres containing A/D-IFN Gelatin microspheres containing A/D-IFN (IFN-microspheres) were prepared as described previously.[18] The microspheres thus prepared were heterogeneous in size with a diameter of 2 μm or less, and contained 1.47×10^3 IU A/D-IFN/mg.

***In vivo* experiments** To acclimatize *in vitro*-cultured cells to *in vivo* conditions and to remove the components of the culture medium, 1 to 2×10^6 R1 cells cultured *in vitro* were inoculated ip into BALB/c mice. The cells were harvested 10 to 14 days later to use for *in vivo* experiments.

Mice were intraperitoneally (ip) inoculated with 2×10^6 R1 cells in 0.5 ml of HBSS. Three days later and thereafter, the mice received ip injections of 0.5 ml of 0.15 *M* NaCl alone or containing 1×10^4 IU of free A/D-IFN, 177 μg of IFN-mircospheres containing 260 IU of A/D-IFN, or a mixture of the IFN-microspheres and 1×10^4 IU of free A/D-IFN. As an additional control, 177 μg of IFN-free microspheres was also injected with and without 1×10^4 IU of free A/D-IFN.

The *in vivo* growth of R1 cells in the peritoneal cavity was determined by counting the number of R1 cells recovered from mice 1 day after the last administration of the IFN-microspheres or other agents. After exsanguination by severing the carotid arteries, the viable R1 cells collected from the peritoneal cavities of mice by lavage with HBSS were counted by a trypan blue dye exclusion test. The R1 cells inoculated intraperitoneally did not form solid tumors and were readily distinguishable from host peritoneal exudate cells by their large size. Cell specimens for microscopic observation were prepared by cytocentrifugation (Model BEST SC-2, Tomy Works, Ltd., Tokyo) with May-Grünwald-Giemsa staining.

Preparation of peritoneal macrophages from mice receiving IFN-microspheres or from control mice Mice were ip injected with various doses of microspheres and A/D-IFN as described above. Peritoneal exudate cells (PEC) were harvested 8 and 24 h later by lavage of the peritoneal cavity with RPMI-1640 medium, washed twice by centrifugation (1,000 rpm, 5 min, 0°C), and resuspended in RPMI-FCS at 5×10^5 Mϕ/ml. The cell suspension (1 ml) was placed in 16 mm dishes of 24-well multidish culture plates (A/S. Nunc, Kamstrup, Roskilde, Denmark). After incubation at 37°C in a 5% CO_2-95% air atmosphere for 2 h, non-adherent cells were removed by aspiration to prepare the monolayer of adherent cells. More than 63% of the adherent cells had morphological and phagocytic properties of Mϕ.

***In vitro* assay for antitumor activity of Mϕ** R1 cells (2.5×10^4) in 1 ml of RPMI-FCS were added to the Mϕ monolayer described above and cultured for 48 h. The number of viable R1 cells was counted at the end of culture by the trypan blue dye exclusion test.

Mϕ populations from the peritoneal cells of R1-bearing mice were prepared according to a modification of Kumagai *et al.*'s method[20] in order to enrich adherent PEC, since the peritoneal cell population comprised R1 cells in addition to PEC. The peritoneal cells were plated on FCS-precoated 10 mm plastic Petri dishes (Falcon No. 3003, Becton Dickinson Co., Oxnard, CA) and incubated at 37°C for 1 h. After removal of the non-adherent cells including R1 cells, adherent cells were taken off by further incubation with PBS containing 10 m*M* EDTA at room temperature for 5 min. The cells recovered were washed three times by centrifugation (1,000 rpm, 5 min, 0°C), and resuspended in RPMI-FCS. These populations contained polymorphs and a vary low percentage of lymphoid cells in addition to Mϕ as the major component, and the Mϕ content varied depending on the substances given to mice. The cell suspensions (200 μl) containing various numbers of Mϕ were placed on 96-well culture dishes (Nunc 16708) and incubated for 2 h. After removal of the non-adherent cells, adherent cells were cultured with 1×10^4 R1 cells in 200 μl of RPMI-FCS and the number of viable R1 cells was determined 24 h later. The inhibitory activity of Mϕ and Mϕ populations on R1 cell growth was evaluated according to the following formula.[18]

Percent growth inhibition = [(No. of tumor cells cultured without Mϕ) − (No. of tumor cells cultured with Mϕ)] × 100/ (No. of tumor cells without Mϕ)

Intratumoral injection of IFN-microspheres R1 cells were suspended at 1×10^6/20 μl of HBSS and inoculated into hind footpads of BALB/c mice. Three days later and thereafter, the footpads received multiple administrations of different agents suspended in 50 μl of saline.

Assessment of effect of IFN-microspheres on tumor pulmonary metastasis The effect of IFN-microspheres on the pulmonary metastasis of B16 melanoma cells was assessed according to the method of Nishimura *et al.*[21] Three days after the intravenous injection of 1×10^5 B16 cells into C57BL/6 mice, the mice were injected intravenously (iv) with 177 μg of IFN-microspheres containing 260 IU of A/D-IFN or other agents.

Statistical analysis Data were treated statistically with Student's *t* test.

RESULTS

Improvement of the survival of tumor-bearing mice by administration of IFN-microspheres plus free A/D-IFN Mice were divided into 6 groups of 14 mice each, in-

Table I. Survival Rate of R1-bearing Mice after Intraperitoneal Injection of Free A/D-IFN or IFN-microspheres

Treatment	14-Day survivors/ total mice	MST (days)	Confidence interval
Saline	0/14	9.75	9.47–10.1
Free A/D-IFN (1×10^4 IU/mouse)	0/14	11.0	10.8–11.3
IFN (260 IU)-microspheres (177 μg/mouse)	0/14	10.3	9.80–10.8
IFN (260 IU)-microspheres (177 μg/mouse) + free A/D-IFN (1×10^4 IU/mouse)	5/14	15.2	13.8–18.0
IFN-free microspheres (177 μg/mouse) + free A/D-IFN (1×10^4 IU/mouse)	0/14	10.3	9.92–10.6
IFN-free microspheres (177 μg/mouse)	0/14	9.92	9.52–10.4

Mice inoculated ip with 2×10^6 R1 cells on day 0 received three ip injections every third day from day 3 to day 9. These mice were thereafter left untreated.

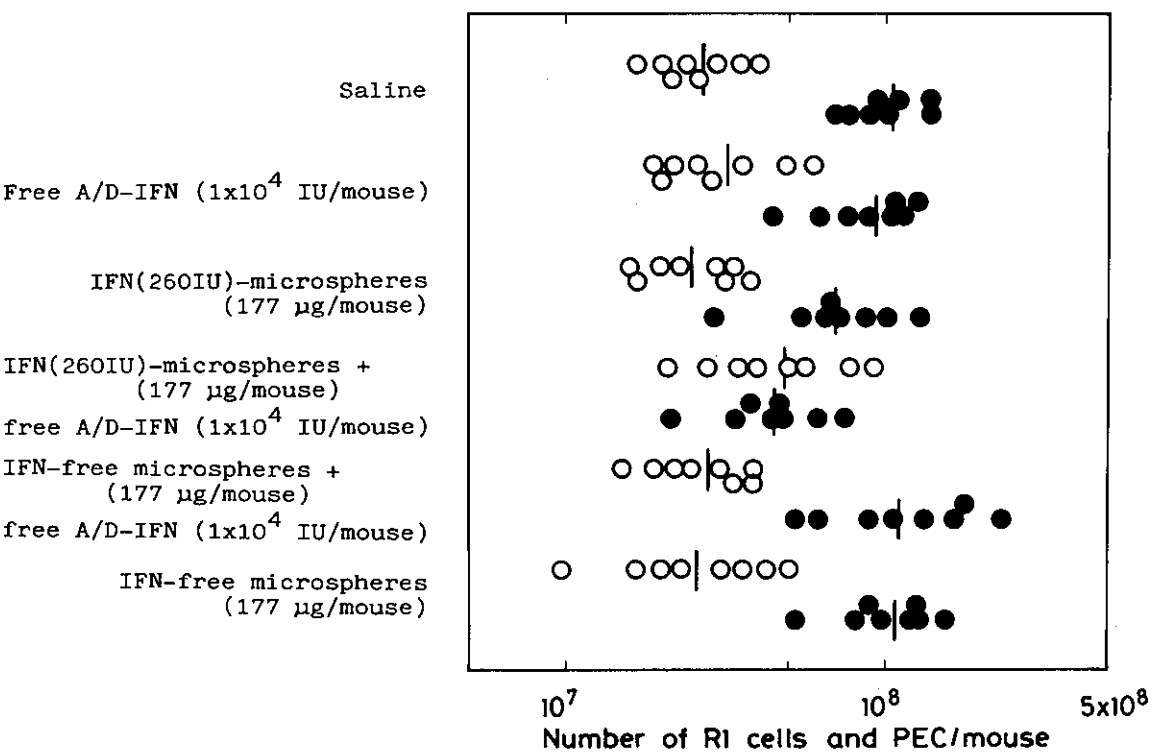

Fig. 1. Number of cells in the peritoneal cavities of R1-bearing mice receiving ip injection of free A/D-IFN or IFN-microspheres: (●) R1 cells and (○) PEC.

oculated ip with 2×10^6 R1 cells at day 0, and given three ip injections of IFN-microspheres or other agents every third day from day 3 to day 9. These mice were left untreated thereafter and the date of death was recorded. Median survival time (MST) was calculated according to Gresser and Bourali.[22] Results are shown in Table I. Under the present experimental conditions, the sole efficacious procedure for improving the survival rate of R1-bearing mice was the administration of IFN-microspheres in combination with free A/D-IFN. The administration of single agents, i.e., free A/D-IFN, IFN-microspheres, IFN-free microspheres, or saline, and that of IFN-free microspheres plus free A/D-IFN were all ineffective. Among 5 mice receiving IFN-microspheres

plus free A/D-IFN and surviving for longer than 14 days after tumor inoculation, 2 mice died within 2 months and the others survived further.

Enhancement of Mϕ recruitment and reduction of tumor cell number in mice receiving IFN-microspheres plus free A/D-IFN The schedule of this experiment was similar to that of the preceding one shown in Table I. Cells in the peritoneal cavities of R1-bearing mice were harvested 1 day after the last injection of various agents, and the R1 cells and PEC were enumerated (Fig. 1). The number of PEC was larger and that of R1 cells was smaller in the group receiving IFN-microspheres plus free A/D-IFN than those in other groups. The injection of IFN-microspheres alone resulted in a slight decrease of the number of R1 cells ($P<0.01$), though this did not improve the survival of mice (Table I).

Mϕ activation in tumor-bearing mice receiving IFN-microspheres Figure 2 is a micrograph of cells from the peritoneal cavity of R1-bearing mice 1 day after the last injection of IFN-microspheres. IFN-microspheres are taken up by Mϕ. Figure 3 shows the effect of Mϕ populations, obtained from R1-bearing mice administered IFN-microspheres and/or free A/D-IFN, on the *in vitro* growth of R1 cells. The results indicate that Mϕ of mice receiving IFN-microspheres were effective in suppressing the growth of R1 cells, regardless of the administration with or without free A/D-IFN, while the administration of IFN-free microspheres, free A/D-IFN, or their combination was ineffective in activating Mϕ.

Mϕ activation in normal mice receiving a single injection of IFN-microspheres In order to check whether IFN-microspheres alone were capable of activating Mϕ within

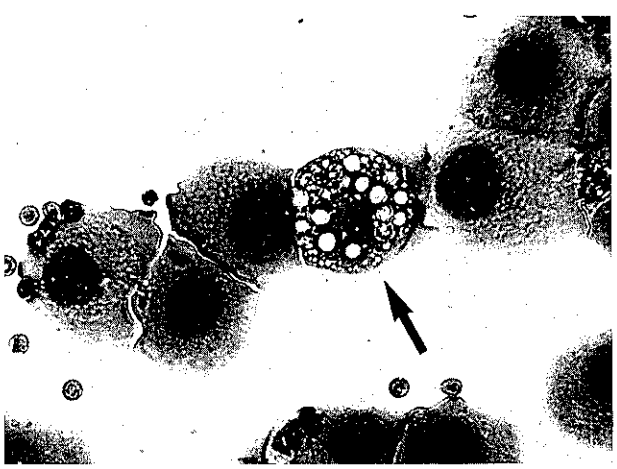

Fig. 2. A micrograph of cells from the peritoneal cavity of R1-bearing mice 1 day after the last injection of IFN-microspheres. An arrow indicates Mϕ phagocytosing IFN-microspheres.

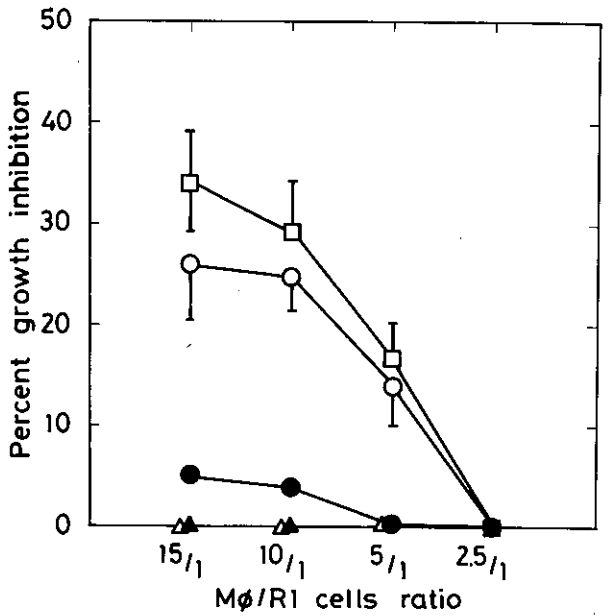

Fig. 3. The inhibitory activity of peritoneal Mϕ obtained from R1-bearing mice receiving ip injection of free A/D-IFN or IFN-microspheres for *in vitro* R1 cell growth: (●) 1×10^4 IU of free A/D-IFN, (○) 177 μg of IFN-microspheres containing 260 IU of A/D-IFN, (△) 177 μg of IFN-free microspheres, (▲) 177 μg of IFN-free microspheres plus 1×10^4 IU of free A/D-IFN, and (□) 177 μg of IFN-microspheres containing 260 IU of A/D-IFN plus 1×10^4 IU of free A/D-IFN.

Table II. Inhibitory Activity of Mouse Peritoneal Mϕ for R1 Cell Growth by Intraperitoneal Injection of Free A/D-IFN or IFN-microspheres

Treatment of Mϕ donor[a]	Percent growth inhibition[b]	
	8 h	24 h
Saline	1.0	2.1
Free A/D-IFN (1×10^4 IU/mouse)	2.3	3.4
IFN(260 IU)-microspheres (177 μg/mouse)	24	32
IFN-free microspheres (177 μg/mouse)	3.0	2.8
IFN-free microspheres (177 μg/mouse) + free A/D-IFN (1×10^4 IU/mouse)	1.0	0.8

a) Mice were injected ip with free A/D-IFN or IFN-microspheres as described in "Materials and Methods." At 8 and 24 h after the injection, the mice were sacrificed to prepare Mϕ monolayers. The initial ratio of Mϕ to R1 cells was 20:1.
b) The inhibitory activity of Mϕ on R1 cell growth was measured as described in "Materials and Methods."

a short time even in the absence of tumor cells, normal mice were administered with a single intraperitoneal injection of IFN-microspheres or other agents as controls, and the antitumor activity of their Mϕ was investigated on the *in vitro* growth of R1 cells. A representative result is shown in Table II. PEC were harvested 8 h or 24 h after injection, and Mϕ were prepared for culture with R1 cells. The result was similar to that in Fig. 3, IFN-microspheres being effective but IFN-free microspheres and/or free A/D-IFN ineffective in activating Mϕ. In addition, the activated state of Mϕ stimulated by IFN-microspheres tended to decline within 4 days.

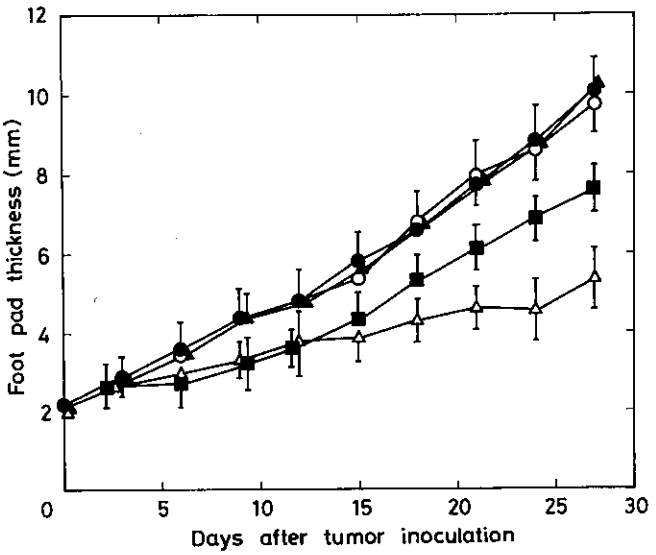

Fig. 4. Suppressive effect of intratumoral injection of free A/D-IFN or IFN-microspheres on the growth of solid R1 tumors: (○) Saline, (■) 5×10^4 IU of free A/D-IFN, (△) 177 μg of IFN-microspheres containing 260 IU of A/D-IFN, (▲) 177 μg of IFN-free microspheres, and (●) 177 μg of IFN-free microspheres plus 260 IU of free A/D-IFN. Each of the groups consisted of 7 mice.

Effect of intratumoral injection of IFN-microspheres Mice were inoculated with 1×10^6 R1 cells into both hind footpads at day 0, and received intratumoral injection of IFN-microspheres or other agents from day 3 at intervals of 2 days for 3 weeks. Thickness of the footpads was measured every 2 days with a dial caliper. The time course of change in the footpad thickness is shown in Fig. 4. The injection of IFN-microspheres containing 260 IU A/D-IFN was effective in suppressing the tumor growth. Free A/D-IFN, 5×10^4 IU per injection, was also effective but less so than the IFN-microspheres, and the injection of IFN-free microspheres with or without 260 IU free A/D-IFN was ineffective, similar to that of saline.

Prevention of pulmonary tumor metastasis by intravenous injection of IFN-microspheres or high dose A/D-IFN From 3 days after the single iv injection of B16 melanoma cells, mice received iv injections of IFN-microspheres or other agents every 2 days for 2 weeks. The number of visible peripheral pulmonary nodules of B16 cells was counted after fixing the lung with formaldehyde 1 week after the last administration of agents. The results are illustrated in Fig. 5. The injection of IFN-microspheres containing 260 IU A/D-IFN or that of 5×10^4 IU free A/D-IFN was similarly effective in suppressing the pulmonary metastasis of B16 cells, al-

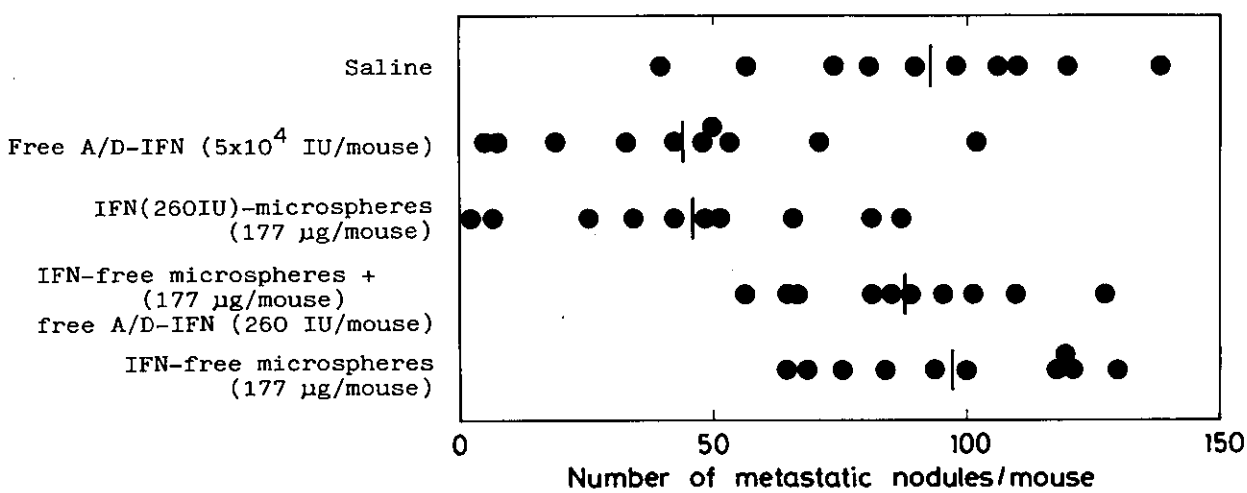

Fig. 5. Preventive effect of intravenous injection of free A/D-IFN or IFN-microspheres on the pulmonary metastasis of B16 melanoma cells.

though the amount of A/D-IFN contained in the IFN-microspheres was approximately 200 times less than that of free A/D-IFN. Injection of IFN-free microspheres with or without 260 IU A/D-IFN was not effective.

DISCUSSION

We have already demonstrated that gelatin microsphers are an efficient carrier for targeting of A/D-IFN to Mϕ, as the microspheres containing A/D-IFN are effective in potentiating the *in vitro* tumor growth-inhibitory activity of Mϕ.[18] The advantages of IFN-microspheres, in comparison with free A/D-IFN, for Mϕ activation are as follows: (1) the amount of A/D-IFN required for Mϕ activation is very small, (2) the treatment period necessary for activation of Mϕ is short, and (3) the activated state of Mϕ induced by IFN-microspheres is maintained for a long time. These results may be explained in terms of the mechanism of Mϕ activation by microspheres. The microspheres are first taken up by Mϕ via phagocytosis, followed by a slow release of A/D-IFN in the cells, leading to efficient Mϕ activation. The present study has demonstrated that IFN-microspheres are also effective in activating Mϕ under *in vivo* conditions.

Mϕ in the peritoneal cavity of R1-bearing mice receiving injection of IFN-microspheres with or without free A/D-IFN were activated to inhibit the *in vitro* growth of R1 cells (Fig. 3). The administration of IFN-microspheres alone resulted in the suppression of *in vivo* R1 cell growth, but less effectively than when IFN-microspheres were given together with free A/D-IFN. In addition, the number of PEC infiltrating the peritoneal cavity of R1-bearing mice was larger in mice receiving the ip injection of IFN-microspheres plus free A/D-IFN than in mice receiving IFN-microspheres alone. The ip injection of 1×10^4 IU of free A/D-IFN to R1-bearing mice could enhance Mϕ recruitment in the peritoneal cavity but to an insufficient degree for manifesting *in vivo* antitumor activity.[15] However, IFN-microspheres became effective in activating Mϕ infiltrating the peritoneal cavity when ip injected with 1×10^4 IU of free A/D-IFN, leading to suppression of the *in vivo* R1 cell growth. This results in a better survival rate of mice given IFN-microspheres plus free A/D-IFN than IFN-microspheres alone.

The suppression of *in vivo* tumor growth was also observed by the intratumoral injection of IFN-microspheres. It was effective in inhibiting the *in vivo* growth of R1 cells (Fig. 4). As is well known, many Mϕ exist in or around neoplastic tissues. They are of prime importance in the host resistance against the tumor cells.[23, 24] Mϕ were effectively activated by injected IFN-microspheres to suppress tumor cell growth and the activated state of Mϕ was maintained for a longer period than that of Mϕ given free A/D-IFN. A distinct preventive effect of the iv injection of the IFN-microspheres on the incidence of pulmonary metastasis was observed (Fig. 5), similar to the finding by Fidler *et al.* that systemic administration of liposomes containing various immunomodulatory agents effectively activated rat alveolar Mϕ to eradicate spontaneous pulmonary metastasis of melanoma cells.[25, 26] A comparative study between gelatin microspheres and liposomes is in progress.

It may be concluded that injected IFN-microspheres are readily phagocytosed by Mϕ at the tumor site, followed by Mϕ activation to acquire antitumor activity, leading to suppression of the *in vivo* tumor cell growth. It should be emphasized that the amount of A/D-IFN in IFN-microspheres required for exerting a significant antitumor effect is much lower than that of free A/D-IFN. These findings clearly indicate the effectiveness of gelatin microspheres as a sustained-release vesicle for targeting of A/D-IFN to Mϕ and subsequent potentiation of Mϕ for *in vivo* antitumor activity.

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