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Potentiation of Antitumor Activity of Macrophages by Recombinant Interferon Alpha A/D Contained in Gelatin Microspheres

Yasuhiko TABATA,[*1] Kazuko UNO,[*2,*3] Yoshito IKADA[*1] and Shigeru MURAMATSU[*2]
[*1]*Research Center for Medical Polymers and Biomaterials, Kyoto University, 53, Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606,* [*2]*Department of Zoology, Faculty of Science, Kyoto University, Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606, and* [*3]*Kyoto Pasteur Institute, 425, Yamamoto-cho, Gokomachi-dori, Oshikoji-agaru, Nakagyo-ku, Kyoto 604*

Gelatin microspheres containing recombinant human interferon αA/D (A/D-IFN) (IFN-microspheres) potentiated the antitumor activity of mouse peritoneal macrophages (Mφ) much more efficiently than free A/D-IFN. Mφ acquired the inhibitory activity on tumor cell growth by the ingestion of IFN-microspheres without the aid of lipopolysaccharide (LPS), though LPS was required as a second signal for activating Mφ primed with free IFN. The IFN-microspheres were much more efficient than free IFN plus LPS in respect of the IFN amount and the time required for Mφ activation. Furthermore, Mφ pretreated with the IFN-microspheres maintained their activated state for a much longer period than those pretreated with free A/D-IFN plus LPS. A monoclonal anti-IFN-αA antibody, which was capable of neutralizing A/D-IFN, did not interfere with the Mφ activation by the IFN-microspheres. Even human IFN-αA was effective in activating murine Mφ similarly to A/D-IFN, when given in the form of IFN-microspheres, though human IFN-αA in the free form was ineffective. These results argue that the mechanism of Mφ activation by the IFN-microspheres is different from that by free IFN.

Key words: Macrophage activation — Antitumor activity — Phagocytosis — Gelatin microspheres — Interferon-αA/D

A large amount of evidence has been accumulated that Mφ activation is of potentially great importance in host defense against primary and metastatic tumors.[1–4] Mφ can be activated to manifest antitumor activity by a variety of immunomodulatory agents, such as lymphokines designated as macrophage-activating factors (MAFs),[5,6] interferons (IFNs),[7,8] bacterial substances such as lipopolysaccharide (LPS),[9,10] and muramyl dipeptide.[11–13] Among these agents, IFNs not only act directly on tumor cells to inhibit their multiplication, but also modulate host defense machineries through activation of natural killer cells[14,15] and macrophages/monocytes,[8,16] and induction of antitumor T cell immunity.[17] Thus, IFN may be a promising potentiator of host resistance against tumors and infectious diseases.[18,19] However, several major obstacles, such as its rapid catabolism and pyrogenicity, remain to be overcome for successful use of IFN for immunotherapy. In an attempt to tackle the problems, Fidler and his collaborators have attempted to use liposomes as a vehicle for IFN, leading to efficient tumoricidal activation of Mφ ingesting the liposomes under *in vitro* and also *in vivo* conditions.[20,21]

Gelatin is a biodegradable polymer with extensive industrial, pharmaceutical, and medical uses, and interestingly, has an opsonic ability for macrophage phagocytosis.[22] Therefore, we prepared microspheres of gelatin crosslinked with glutaraldehyde and have explored the applicability of the microspheres as a carrier for delivering immunopotentiators to macrophages in place of liposomes. Gelatin microspheres are capable of holding various drugs including immunopotentiators, and are stable physically as well as chemically under physiological conditions. The rate of drug delivery from microspheres can be readily controlled by changing the extent of gelatin crosslinking. Moreover, the procedure for preparation is simpler and the material cost is lower for gelatin microspheres than for liposomes. Recently, we have demonstrated that muramyl dipeptide (MDP) included in gelatin micro-

spheres was far more efficient in enhancing the inhibitory activity of macrophages for tumor growth than free MDP under *in vitro* and also *in vivo* conditions.[23]

The present study was focused on the potentiation of the inhibitory activity of macrophages on *in vitro* tumor growth by gelatin microspheres containing A/D-IFN. A/D-IFN is known to act not only on human cells but also on murine cells.[24] Incorporation of A/D-IFN in the microspheres allowed macrophages to be effectively activated and to maintain the activity for a long period.

Materials and Methods

Animals Specific pathogen-free inbred male and female BALB/cCrSlc and C3H/HeSlc mice, 2–3 months old, were obtained from Shizuoka Laboratory Animal Center, Shizuoka.

IFN and Anti-IFN Recombinant human IFN-αA/D (A/D-IFN) and recombinant human IFN-αA (A-IFN) were provided by Nippon Roche Research Center, Kamakura. A/D-IFN, the product of a hybrid molecule of A and D clone DNAs, is known to be effective on mouse cells as well as human cells. Monoclonal anti-A-IFN antibody (Lot RYM-001, Nippon Roche Research Center), is able to neutralize both A/D-IFN and A-IFN. The amount of antibody needed to neutralize 1.5×10^5 IU of A/D-IFN was 6.1 μg. For neutralizing A/D-IFN before Mϕ activation, 10 to 20 times more antibody was used.

Media and Reagents All cell cultures were maintained in RPMI-1640 medium (Nissui Seiyaku Co., Ltd., Tokyo) supplemented with 10% fetal calf serum (FCS, M.A. Bioproducts, Walkersville, MD), 5m*M* L-glutamine, and penicillin (100 units/ml), and buffered with 5m*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., Tokyo. Lipopolysaccharide (LPS, *E. coli* 0111:B4) was purchased from Difco Laboratories, Detroit, MI. Gelatin employed here was of alkaline type (pI 4.9, Nitta Gelatine Co., Ltd., Osaka), though any kind of gelatin could be employed for this procedure. Other chemical reagents were of guaranteed grade (purchased from Nakarai Chemicals, Ltd., Kyoto) and used without further purification.

Preparation of Gelatin Microspheres Containing IFN Span 80 (sorbitan monooleate), 0.25 g, was dissolved in a mixture of 2.5 ml of toluene and 2.5 ml of chloroform in a 10 ml sampling tube. To this organic solution, 0.2 ml of aqueous solution consisting of 20 mg of gelatin and 1.5×10^4 IU of A/D-IFN was added, and the mixture was homogenized by sonication (10 W, 10 sec). The result-

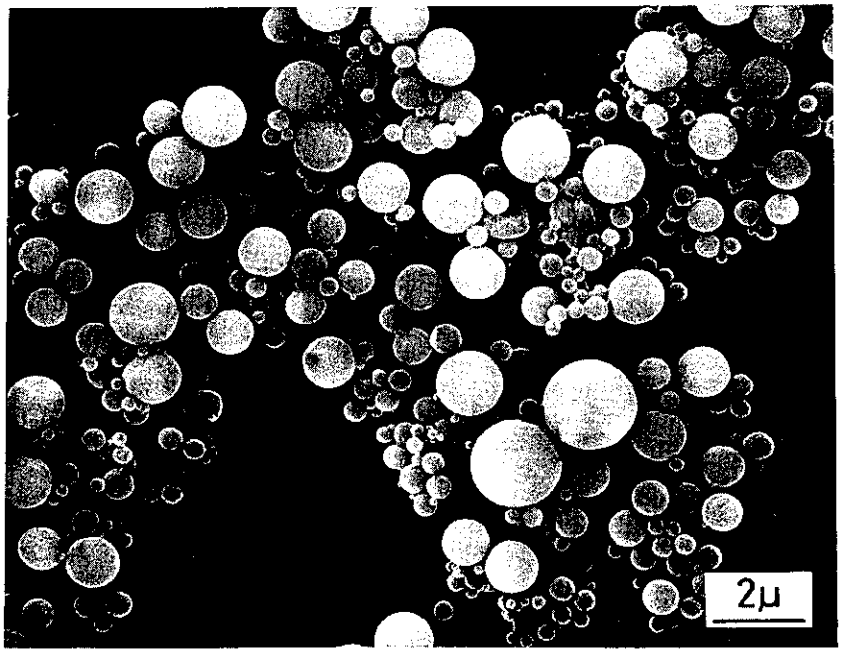

Fig. 1. A scanning electron micrograph of gelatin microspheres containing A/D-IFN.

ing emulsion was poured rapidly into 40 ml of precooled 25% chloroform in toluene containing 2 g of Span 80. Subsequently, gelatin in the emulsion was crosslinked with glutaraldehyde saturated in toluene, which was prepared by vigorous shaking of 10 ml of 25% aqueous glutaraldehyde solution with 10 ml of toluene. The glutaraldehyde concentration in toluene, determined with 3-methyl-2-benzothiazolinone hydrazone,[25] was 5.3 mg/ml. A 5 ml aliquot of the glutaraldehyde-saturated toluene was added to the gelatin emulsion and the mixture was continuously stirred at 0° for 6 hr for crosslinking. Then, the emulsion was centrifuged at 4,000 rpm for 5 min and the supernatant discarded. The resulting microspheres were successively washed with 25% chloroform in toluene, isopropanol, and PBS by centrifugation (4,000 rpm for 5 min) and this was repeated four times. Finally, the washed microspheres were suspended in 3 ml of PBS. Microspheres without IFN and microspheres containing A-IFN were also prepared similarly. A scanning electron micrograph (SEM) (taken with a Hitachi S-450; Hitachi, Tokyo) of gelatin microspheres containing A/D-IFN is shown in Fig. 1. The microspheres are heterogeneous in size with a diameter of 2 μm or less. The amount of A/D-IFN contained in the microspheres was 740 IU per mg of gelatin microspheres as determined by the use of ^{125}I-labeled A/D-IFN.[26]

Preparation and Purification of Mouse Peritoneal Macrophages Mouse peritoneal Mϕ were prepared as described.[27] Four days prior to cell harvesting, 2- to 3-month-old mice were injected intraperitoneally (ip) with 2 ml of thioglycollate medium (Brewer's medium, Difco Laboratories). Peritoneal exudate cells (PEC) were collected by lavage of the peritoneal cavity with cold HBSS. The PEC were washed twice with PRMI- 1640 culture medium by centrifugation (1,000 rpm, 5 min, 0°) and resuspended in RPMI-FCS at 5×10^5 Mϕ/ml. Aliquots of 1 ml of cell suspension were seeded on 16 mm dishes of 24-well multidish culture plates (A/S Nunc, Kamstrup, Roskilde, Denmark). To prepare the material for SEM, Mϕ were seeded on round cover-glass slips (14 mm diameter) placed on the 16 mm dishes. The percentage of macrophages in the PEC collected by this procedure was more than 95%. After incubation at 37° in a 5% CO_2-95% air atmosphere for 2 hr, nonadherent cells were removed by washing three times with HBSS. More than 98% of the adherent cells had the morphological and phagocytic properties of macrophages. These cells were then used for *in vitro* assays.

***In vitro* Mϕ Activation by Gelatin Microspheres Containing A/D-IFN** Mϕ, 5×10^5 in 1 ml of medium per dish, were pretreated for various periods of time up to 30 hr at 37° with free A/D-IFN or IFN-microspheres with or without 10 ng/ml of LPS. Additional control experiments were done in which Mϕ were pretreated with IFN-noncontaining microspheres or free A/D-IFN. In all cases, the dose of microspheres was 71 μg/5×10^5 Mϕ. The dose of free A/D-IFN and that of microspheres applied here were below the cytotoxic level to the cells. Mϕ cultures were rinsed thoroughly with RPMI-1640 medium to remove free A/D-IFN and non-phagocytosed microspheres before the addition of tumor cells.

Inhibitory Activity of Mϕ on Tumor Growth Meth A R1 (R1) cell line, originally established by Uno *et al.*[8] from Meth A cell line (a methylcholanthrene-induced fibrosarcoma of BALB/c mice), was used for assessing the effect of Mϕ on tumor cell growth. The *in vitro* growth of R1 cells is resistant to natural IFN-α/β and also to A/D-IFN, so that we could estimate selectively the effect of activated Mϕ on tumor cell growth without considering the direct effect of free IFN on tumor cells. Assays were always performed on cells in the exponential growth phase. R1 cells, 2.5×10^4 in 1 ml of the culture medium, were added to the Mϕ monolayers prepared as described above. Under these conditions, IFN-untreated Mϕ exerted no inhibitory effect on tumor cell growth and the number of tumor cells grown was similar to that in the case of IFN-untreated Mϕ. The number of viable R1 cells was counted after culture for 48 hr at 37° in a 5% CO_2-95% air atmosphere. The growth-inhibitory activity of Mϕ towards R1 cells was evaluated according to the following formula.[8]

$$\text{Percent growth inhibition} = \frac{\left(\begin{array}{l}\text{No. of tumor cells}\\ \text{cultured with}\\ \text{untreated M}\phi\end{array}\right) - \left(\begin{array}{l}\text{No. of tumor cells}\\ \text{cultured with}\\ \text{activated M}\phi\end{array}\right)}{(\text{No. of tumor cells cultured with untreated M}\phi)} \times 100.$$

Tumoricidal Activity of Mϕ Tumoricidal activity of macrophages was assessed by the method of Meltzer *et al.*[28] with minor modifications. P815 mastocytoma cells (DBA/2 mice) and B16 melanoma cells (C57BL/6 mice) were used as target cells. They were radiolabeled with ^{3}H-thymidine (0.4 μCi/ml or 1 μCi/ml) in RPMI-FCS for 14 hr at 37° and 2.5×10^4 cells were incubated in 0.5 ml of RPMI-FCS on macrophage monolayers, which had been pretreated with free IFN or IFN-microspheres for 8 hr. After 48 hr and 72 hr of incubation for P815 and B16, respectively, the 24-well multidish culture plates were centrifuged, 400 μl of culture supernatant was harvested, and the radioactivity in the supernatant was determined with a liquid scintillation spectrometer. Results

were expressed as the percentage of cytolysis according to the following formula:

$$\text{Percent cytolysis} = \frac{(\text{experimental release}) - (\text{spontaneous release})}{(\text{maximum release}) - (\text{spontaneous release})} \times 100,$$

in which the maximum release, the spontaneous release, and the experimental release indicate, respectively, the radioactivity of the supernatant of SDS-lysed labeled cells, that of the target cell culture (without untreated Mϕ), and that of the culture containing target cells and treated Mϕ. Spontaneous release ranged between 10 and 18% of the maximum.

Statistical Analysis Data were treated statistically with Student's *t*-test ($P < 0.01$).

Results

Macrophage Phagocytosis of Gelatin Microspheres Figure 2 shows an SEM photograph of a macrophage phagocytosing gelatin microspheres after 8 hr of incubation. The microspheres were readily phagocytosed, irrespective of the incorporation of A/D-IFN (unpublished results) and the ingested microspheres were gradually degraded in the cells.

Mϕ Activation by IFN-microspheres or Free A/D-IFN with or without LPS Mϕ were pretreated *in vitro* for 8 hr with various doses of free A/D-IFN or IFN-microspheres with or without 10 ng/ml of LPS, and the inhibitory activity of Mϕ on R1 cell growth was evaluated. The dose responses for Mϕ activation are illustrated in Fig. 3. The Mϕ pretreated with free A/D-IFN alone scarcely inhibited the growth of tumor cells, whereas the inhibitory activity on tumor growth was drastically augmented by addition of 10 ng/ml of LPS, though LPS at this dose alone (data not shown) or with low doses of A/D-IFN (symbols on the abscissa in Fig. 3) could not induce any significant Mϕ activation. On the other hand, a significant augmentation of the activity of Mϕ pretreated with IFN-microspheres was observed even without LPS addition. Moreover, the amount of A/D-IFN in the microspheres required for inducing the activity was about several hundred times less than that of free A/D-IFN given to Mϕ with 10 ng/ml of LPS. The addition of LPS shifted the dose-response curve for the IFN-microspheres toward the lower dose direction by about one order of magnitude. Pretreatment of Mϕ with IFN-free microspheres brought about a feeble activation of Mϕ and addition of IFN-free

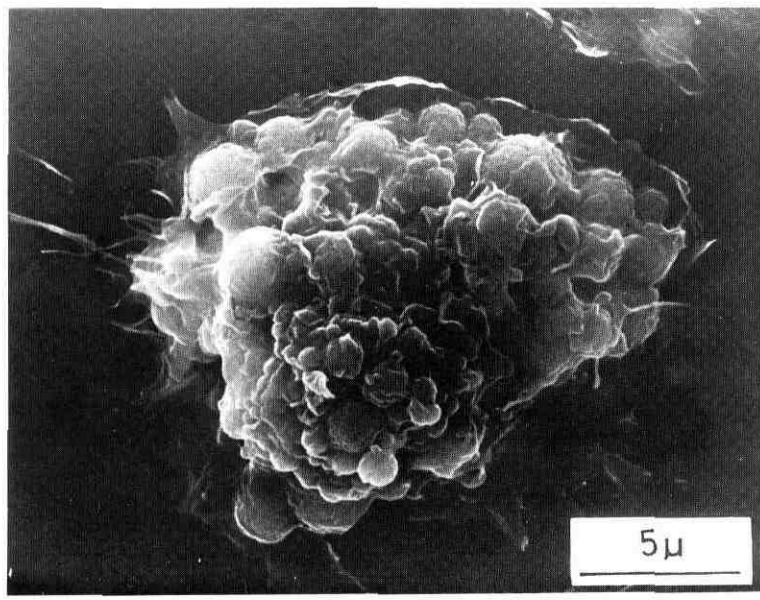

Fig. 2. A scanning electron micrograph of a macrophage phagocytosing gelatin microspheres.

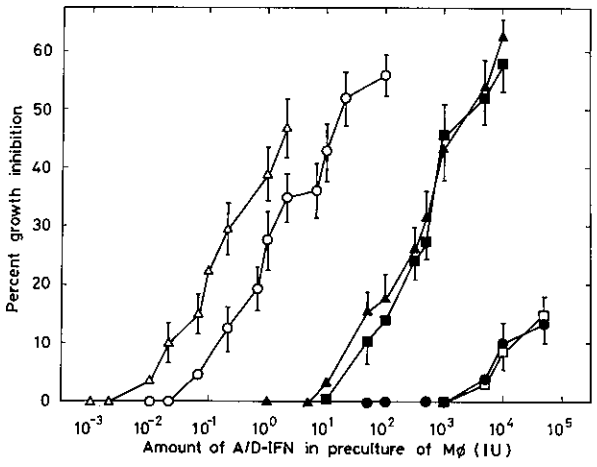

Fig. 3. The inhibitory activity of mouse peritoneal Mφ on *in vitro* tumor growth. Mφ in 1 ml of medium were pretreated for 8 hr at 37° with free A/D-IFN (●), free A/D-IFN plus 10 ng/ml LPS (▲), free A/D-IFN plus IFN-free microspheres (□), free A/D-IFN plus 10 ng/ml LPS plus IFN-free microspheres (■), IFN-microspheres (○), IFN-microspheres plus 10 ng/ml LPS (△). The inhibitory activity on tumor cell growth was evaluated as described in "Materials and Methods." Percent growth inhibition of Mφ pretreated with 10 μg/ml LPS was 51% in this experiment.

microspheres had no influence on the activation induced by free A/D-IFN plus LPS or on the weak activation due to free A/D-IFN. This indicated that the phagocytosis of the microsphere matrix by itself neither contributed to nor interfered with the Mφ activation. Similar results were obtained by the use of microspheres containing heat-inactivated (100°, 30 min), or antibody-neutralized A/D-IFN (data not shown).

Time Course of Mφ Activation To study the time course of Mφ activation, Mφ were pretreated for different time periods of 3 to 30 hr with 1×10^4 IU of free A/D-IFN or with IFN-microspheres containing 5.2 IU of A/D-IFN, in the presence or absence of 10 ng/ml of LPS. The results are shown in Fig. 4. In the case of pretreatment with free A/D-IFN alone, Mφ were activated very slightly in the first 8 hr. When pretreated with free A/D-IFN plus LPS, Mφ were markedly activated to attain the maximal level by an 8 hr pretreatment, and the activity tended to decrease gradually to the basal level in the following 22 hr. On the other hand, Mφ pretreated with IFN-microspheres were activated very rapidly (within only 3 hr) and the activity gradually increased for the following 5 hr to the maximum level which then remained constant. The level of activation was augmented by the addition of LPS.

Durability of Activated State To examine the durability of Mφ activation, Mφ were pretreated for 8 hr with 1×10^4 IU of free A/D-IFN or gelatin microspheres containing 5.2 IU of A/C-IFN, with or without 10 ng/ml of LPS, then thoroughly washed, and cultured in fresh medium. R1 cells were added 1 to 5 days

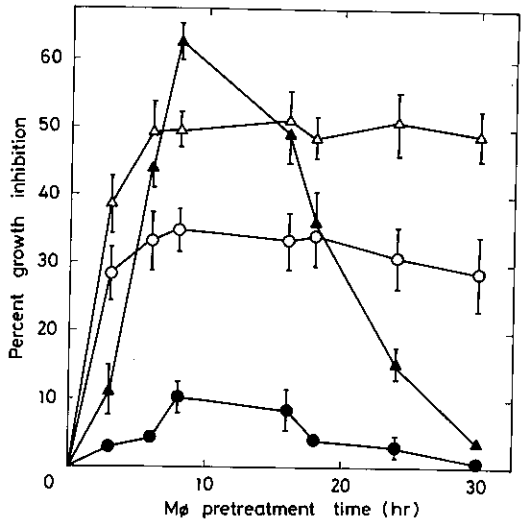

Fig. 4. Time course of Mφ activation by free A/D-IFN or IFN-microspheres with or without LPS. Mφ were pretreated with 1×10^4 IU/ml free A/D-IFN (●), 1×10^4 IU/ml free A/D-IFN plus 10 ng/ml LPS (▲), microspheres containing 5.2 IU A/D-IFN (○), or microspheres containing 5.2 IU A/D-IFN plus 10 ng/ml LPS (△) at 37° for various times as indicated on the abscissa. Pretreated Mφ were washed and submitted to the assay for antitumor activity.

later. The results are illustrated in Fig. 5. The activated state was maintained for much longer in Mφ pretreated with IFN-microspheres with or without LPS than in those pretreated with free A/D-IFN plus LPS.

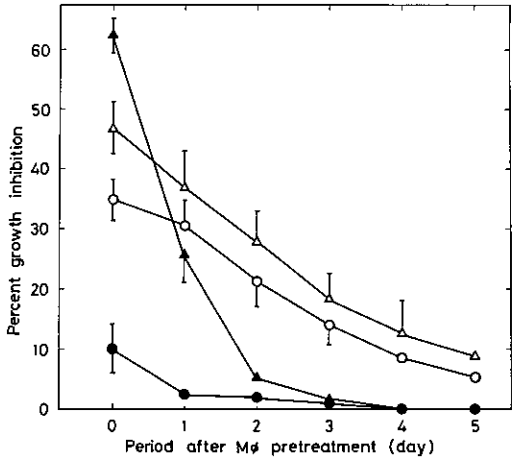

Fig. 5. Decrement patterns of the activated state of Mφ after being pretreated with free A/D-IFN or IFN-microspheres with or without LPS. Mφ in 1 ml of medium were pretreated for 8 hr at 37° with 1×10^4 IU/ml free A/D-IFN (●), 1×10^4 IU/ml free A/D-IFN plus 10 ng/ml LPS (▲), microspheres containing 5.2 IU A/D-IFN (○), or microspheres containing 5.2 IU A/D-IFN plus 10 ng/ml LPS (△).

Activation of LPS-unresponsive Mφ by IFN-microspheres The results in Figs. 3, 4, and 5 seemed to argue that the Mφ activation by IFN-microspheres was independent of LPS. We could not rule out, however, the possibility that the IFN-microspheres collaborated very efficiently with LPS which might contaminate the culture medium. To check this, we conducted an experiment in which the Mφ activation of C3H/He mice was compared with that of C3H/HeJ mice, whose Mφ are known to be directly unresponsive to LPS.[29] As shown in Fig. 6, C3H/HeJ Mφ pretreated with free A/D-IFN plus 10 ng/ml of LPS with or without IFN-free microspheres exhibited only very low tumor growth-inhibitory activity, in comparison with the Mφ of C3H/He mice. However, not only C3H/He but also C3H/HeJ Mφ were activated with IFN-microspheres to inhibit R1 cell growth. Enhancement of Mφ activation by LPS at very small doses of IFN-microspheres, as seen in C3H/He Mφ, was not observed in C3H/HeJ Mφ.

Effect of Anti-IFN Antibody on Mφ Activation In this experiment, we investigated the effect of anti-A-IFN, effective against both A-IFN and A/D-IFN, on the activation of Mφ. Mφ were treated for 8 hr with either free A/D-IFN or IFN-microspheres, each of which had been pretreated with an excess

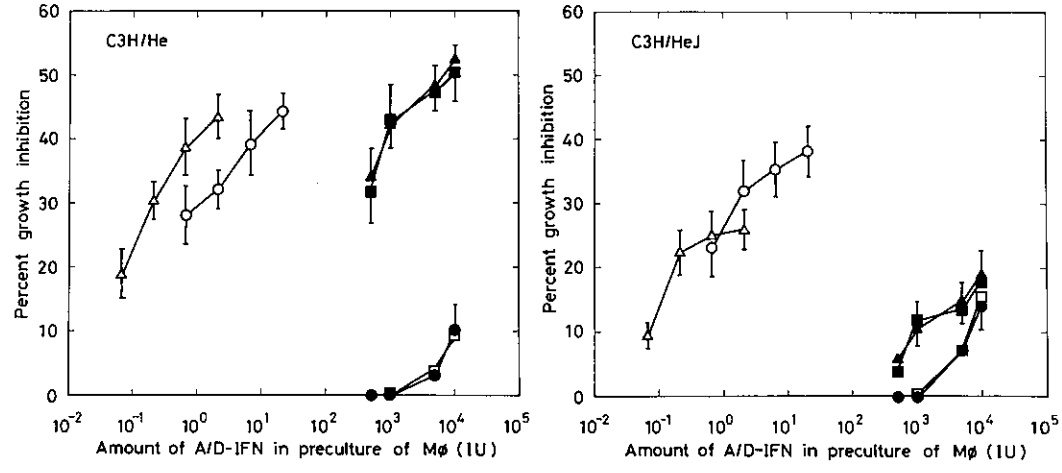

Fig. 6. The inhibitory activity of C3H/He or C3H/HeJ Mφ on *in vitro* tumor growth. Mφ in 1 ml of medium were pretreated for 8 hr at 37° with free A/D-IFN (●), free A/D-IFN plus 10 ng/ml LPS (▲), free A/D-IFN plus IFN-free microspheres (□), free A/D-IFN plus IFN-free microspheres plus 10 ng/ml LPS (■), IFN-microspheres (○), or IFN-microspheres plus 10 ng/ml LPS (△).

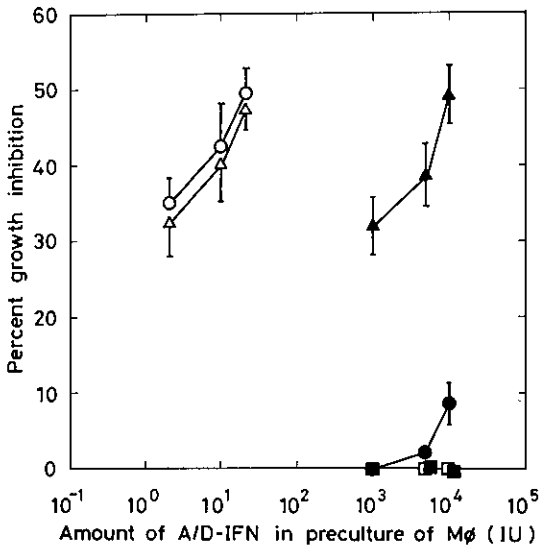

Fig. 7. Effect of pretreatment of free A/D-IFN or IFN-microspheres with monoclonal anti-A-IFN antibody on their activating capacity for Mφ. Mφ in 1 ml of medium were pretreated for 8 hr 37° with free A/D-IFN (●), free A/D-IFN plus 10 ng/ml LPS (▲), free A/D-IFN pretreated with the antibody (□), mixture of free A/D-IFN and 10 ng/ml LPS pretreated with the antibody (■), IFN-microspheres (○), IFN-microspheres pretreated with the antibody (△).

amount of anti-A-IFN antibody as described in "Materials and Methods." As shown in Fig. 7, the antibody prevented the Mφ activation by free A/D-IFN plus LPS, and a slight activation by free A/D-IFN alone was also abrogated. The antibody, however, did not interfere with the Mφ activation by IFN-microspheres.

Abrogation of Species Specificity for Mφ Activation by IFN-microspheres This experiment was conducted to examine whether A-IFN, the soluble form of which is specifically effective for human cells but not for murine cells, could activate mouse Mφ, if A-IFN was included in microspheres. The results given in Fig. 8 show that the A-IFN-microspheres were capable of activating mouse Mφ, though free A-IFN was incapable of doing so even in the presence of LPS.

Tumoricidal Activity of Mouse Mφ Conferred by IFN-microspheres or Free A/D-IFN The results so far presented in this paper are concerned only with the inhibitory activity of Mφ on tumor growth, so that it remained necessary to investigate whether Mφ acquire the tumoricidal activity in response to the stimulation with IFN-microspheres. Thus, Mφ were pretreated for 8 hr

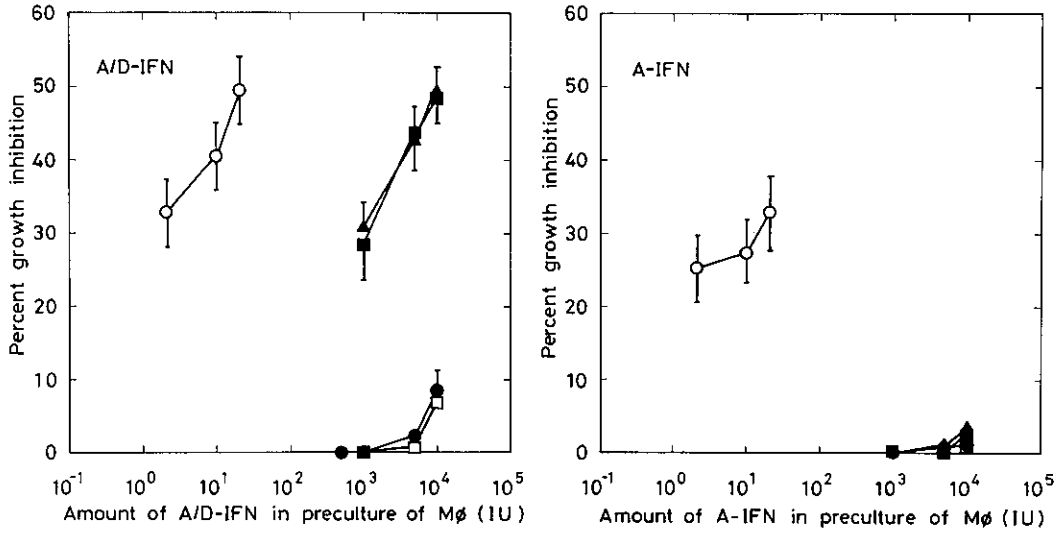

Fig. 8. Effectiveness of human-specific A-IFN for murine Mφ when enclosed in gelatin microspheres. Mφ in 1 ml of medium were pretreated for 8 hr at 37° with free A-IFN or A/D-IFN (●), free A-IFN or A/D-IFN plus 10 ng/ml LPS (▲), free A-IFN or A/D-IFN plus IFN-free microspheres (□), free A-IFN or A/D-IFN plus 10 ng/ml LPS plus IFN-free microspheres (■), microspheres containing A-IFN or A/D-IFN (○).

Table I. *In vitro* Growth-inhibitory and Cytolytic Activities of Mouse Mϕ Pretreated with IFN-microspheres or Free A/D-IFN towards P815 Mastocytoma and B16 Melanoma Cells

Mϕ pretreatment with[a]	LPS/ml	P815 mastocytoma		B16 melanoma
		Percent growth inhibition[b]	Percent cytolysis[c]	Percent cytolysis[c]
—	—	0	−2.6	−3.8
—	10 ng	0	−4.2	−1.2
Free A/D-IFN (1×10^4 IU/ml)	—	6.4	3.8	14
Free A/D-IFN (1×10^4 IU/ml)	10 ng	62	59	43
IFN (52 IU)-microspheres (71 μg/ml)	—	40	1.6	3.3
IFN (52 IU)-microspheres (71 μg/ml)	10 ng	52	−2.4	57
IFN-free microspheres (71 μg/ml)	—	2.2	−1.8	5.0
IFN-free microspheres (71 μg/ml)	10 ng	4.6	0.2	2.6
—	10 μg	54	59	48

a) Mϕ, 5×10^5 in 1 ml of culture medium per dish, were pretreated for 8 hr at 37° before the addition of target cells.
b) The inhibitory activity of Mϕ on P815 cell growth was measured as described in "Materials and Methods."
c) The cytolytic activity of Mϕ towards P815 and B16 cells was measured as described in "Materials and Methods."

with free A/D-IFN, IFN-microspheres, or IFN-noncontaining microspheres with or without LPS, and their cytolytic activity towards P815 mastocytoma and B16 melanoma cells was assessed. Mϕ pretreated with 10 μg of LPS served as positive controls for growth-inhibitory and cytolytic activities towards the tumor cells. The results are shown in Table I. Antitumor activity of Mϕ pretreated with free A/D-IFN plus LPS was manifested both in tumor growth-inhibitory and in cytolytic assays with P815 cells. Mϕ pretreated with IFN-microspheres exhibited growth-inhibitory activity, but no cytolytic activity. Furthermore, it should be noted that the addition of LPS to the Mϕ culture with IFN-microspheres was ineffective in activating Mϕ to be cytolytic. Prolongation of the time of Mϕ pretreatment, increase of the amount of A/D-IFN in the microspheres, and incorporation of A/D-IFN together with LPS in the microspheres were all ineffective (data not shown). These results may appear to indicate that Mϕ cannot acquire the tumoricidal cytolytic activity by phagocytosing IFN-microspheres. This was not the case, however, with B16 target cells for which Mϕ activated with IFN-microspheres exerted a cytolytic effect as potent as that of free IFN-LPS-activated Mϕ.

Discussion

Gelatin has been commonly used as a material for coating and microencapsulating various drugs.[30,31] The drugs thus employed so far have been nonproteins of low molecular weight, and the gelatin particles were too big to be ingested by Mϕ. The ultrasonication of the mixture of gelatin and IFN enabled us to prepare sufficiently small microspheres containing IFN without denaturation. Moreover, we have demonstrated that gelatin microspheres are fairly stable and A/D-IFN contained by the microspheres does not leak out readily unless they are degraded intracellularly. The rate of *in vitro* degradation of microspheres and that of the release of IFN from the microsphere are controllable by changing the extent of crosslinking (unpublished results). One of the advantages of gelatin for targeting a drug to macrophages is that it works as a strong opsonin.[22,32] However, the lack of toxicity of gelatin microspheres crosslinked with glutaraldehyde has

not been conclusively confirmed. Therefore, we shall have to check this carefully before clinical application, though gelatin has been used as a safe plasma expander[33] and the glutaraldehyde content seems to be low enough not to cause any problem.

Results in the present experiment clearly demonstrate that IFN-microspheres enable Mϕ to exhibit inhibitory activity on tumor growth far more efficiently than free A/D-IFN. The total amount of A/D-IFN in the culture required for Mϕ activation by IFN-microspheres is several hundred times lower than that by free A/D-IFN plus LPS (Fig. 3). In addition, the high efficiency of IFN-microspheres, in comparison with free A/D-IFN plus LPS, was also revealed in the short incubation time for Mϕ activation (Fig. 4) and the long retention of the activated state of Mϕ (Fig. 5).

Though the LPS content of A/D-IFN used in the present study was very low (1.6 ng/1.5 $\times 10^7$ IU A/D-IFN) and LPS-free water was employed to prepare the culture medium, the possibility that LPS, which might be derived, for example, from serum and contaminate the culture, contributed to Mϕ activation was checked and ruled out by using LPS-unresponsive C3H/HeJ Mϕ (Fig. 6). In addition, the gelatin microspheres are readily ingested by macrophages (Fig. 2), and it was observed by phase-contrast microscopy that the outline of phagocytosed microspheres disappeared after incubation for 5 days (data not shown). These results strongly suggest that Mϕ which ingest IFN-microspheres are activated by A/D-IFN released from microspheres degraded in the cells.

The results of experiments in which IFN-microspheres were pretreated with anti-IFN antibody seem to indicate that the Mϕ activation by microspheres is independent of the binding of A/D-IFN on the microsphere surface to the IFN-receptor of Mϕ, since, in contrast to the case of antibody-neutralized free A/D-IFN, anti-IFN-treated IFN-microspheres were still effective in activating Mϕ. IFN-free microspheres with or without free A/D-IFN failed to activate Mϕ. Thus, the Mϕ activation by IFN-microspheres can be concluded to be caused by A/D-IFN contained in the microspheres and igested with them by Mϕ.

Mϕ activation by IFN-microspheres does not require the aid of LPS, in contrast with that by free A/D-IFN (Fig. 6). In addition, even A-IFN included in the microspheres is effective in activating murine Mϕ, though the effectiveness of A-IFN is otherwise restricted to human cells. This implies that the species specificity of IFN is attributable to the specificity in the binding between IFN and the IFN-receptor, and that it can be abrogated when the IFN is internalized into macrophages through phagocytosis. A similar result was obtained by Fidler and his collaborators when they applied liposome-encapsulated human IFN-γ to murine Mϕ.[34]

The cytolytic activity of Mϕ pretreated with IFN-microspheres was studied using P815 mastocytoma and B16 melanoma cells as target cells. They exhibited their cytolytic activity towards B16 cells but not P815 cells, while Mϕ pretreated with free A/D-IFN plus LPS were cytolytic to both target cells (Table I). It is uncertain whether Mϕ phagocytosing liposome-encapsulated IFN also behave similarly, since Fidler demonstrated the cytotoxicity for B16 cells but did not examine P815 cells.[35]

At any rate, our present results argue that the activated state of Mϕ is different between Mϕ treated with IFN-microspheres and those treated with free A/D-IFN plus LPS. Two possibilities are envisaged: (1) the difference is qualitative, that is, the effector machanism is different between them, and (2) the difference is quantitative, that is, free A/D-IFN plus LPS is a stronger activator than IFN-microspheres plus LPS, so that the apparent target preference in Mϕ activated with IFN-microspheres plus LPS is ascribable only to the high susceptibility of B16 cells to the activated Mϕ in comparison with P815 cells. Comparative studies are under way on the Mϕ stimulation mechanism in our experimental system between IFN-microspheres and free IFN. In addition, we have obtained results showing an *in vivo* therapeutic effect of IFN-microsphere administration in tumor-bearing mice (data not shown). This will be reported separately in the near future.

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