Pharmaceutical Research, Vol. 6, No. 5, 1989

Report

Synthesis of Gelatin Microspheres Containing Interferon

Yasuhiko Tabata[1] and Yoshito Ikada[1,2]

Received July 7, 1987; accepted January 9, 1989

Gelatin microspheres with a diameter less than 2 μm were synthesized by means of cross-linking with glutaraldehyde. When the microspheres were subjected to degradation in phosphate-buffered saline solution containing collagenase, the digestion of microspheres was found to decrease with increasing cross-linking. Interferon was incorporated in the microspheres at a high trapping efficiency, and the rate of interferon release from the microspheres was regulated by the extent of cross-linking with glutaraldehyde. Gelatin microspheres incorporating interferon-α were readily phagocytosed by macrophages, regardless of the extent of cross-linking, and the phagocytosed microspheres were observed to be degraded gradually in the interior of macrophages, resulting in the slow release of the incorporated interferon in the cells.

KEY WORDS: gelatin microspheres; macrophages; phagocytosis; degradation; interferon.

INTRODUCTION

Interferon-α (IFN) is an immunomodulatory agent which activates natural killer cells (1) and macrophages/monocytes (2,3) and induces antitumor T-cell immunity (4). Thus, it may be a promising potentiator of host resistance against tumors and infectious diseases. However, several obstacles, such as its rapid catabolism and pyrogenicity, remain to be overcome for successful use of IFN in immunotherapy. Therefore, it is important to develop sustained-release formulations or IFN preparations targeted to macrophages.

Encapsulation of drugs in particulate carriers has recently attracted attention in drug delivery systems (5–7). For successful application, the carrier itself should be nontoxic, with a suitable shape and size. Among biodegradable carriers, gelatin has commonly been used for microencapsulation (8–13), and it enhances macrophage phagocytosis (14).

Recently we have found that gelatin microspheres incorporating a muramyl dipeptide are readily phagocytosed by macrophages, which are, in turn, activated to inhibit selectively and efficiently the growth of neoplastic cells under *in vitro* and *in vivo* conditions (15).

In the present work, we prepared gelatin microspheres cross-linked with glutaraldehyde as a sustained-release vesicle for targeting of IFN to macrophages. The objective of this work was to examine the degradation of the microspheres, the release of IFN from the microspheres as a function of the extent of gelatin cross-linking, and phagocytic behaviors of the microspheres by macrophages. Degradation of the microspheres and release profiles of IFN in macrophages are also described.

MATERIALS AND METHODS

Reagents. Gelatin was kindly supplied by Nitta Gelatine Co., Ltd., Osaka, Japan. Collagenase was kindly supplied by Amano Seiyaku Co., Ltd., Nagoya, Japan. Recombinant human interferon alpha A/D (IFN) (1.5×10^8 IU/mg protein) was kindly supplied by Nippon Roche Research Center, Kamakura, Japan. The medium for macrophage culture is RPMI-FCS prepared by supplementing RPMI-1640 medium with 10% fetal calf serum (FCS).

Preparation of Gelatin Microspheres (MS). A mixture of 2.5 ml toluene and 2.5 ml chloroform containing 0.25 g Span 80 (sorbitan monooleate) was placed in a 10-ml sampling tube, and then 0.2 ml of gelatin aqueous solution with 1.5×10^4 IU of IFN or 2 mg of rhodamine 6GX (6GX) was added to the organic mixture, followed by emulsification by sonification (10 W, 10 min). The resulting emulsion was quickly poured into 40 ml of a precooled mixture of 25% chloroform and 75% toluene containing 2 g of Span 80. Then gelatin in the emulsion was cross-linked with glutaraldehyde-saturated toluene which had been prepared by vigorous shaking of 10 ml of toluene with 10 ml of glutaraldehyde aqueous solution. The concentration of glutaraldehyde saturated in the toluene phase was determined with 3-methyl-2-benzothiazolone hydrazone (16). The cross-linking reaction was performed at 0°C for predetermined periods of time with continuous stirring. The resulting MS were successively washed with 25% chloroform in toluene solution, isopropanol, and phosphate-buffered saline (PBS), by centrifugation (4000 rpm, 5 min), and were finally suspended in PBS.

MS Degradation and Release Test. One-half milligram of MS with or without IFN and 6GX was suspended in 1 ml of phosphate-buffered saline solution (PBS) or magnesium

[1] Research Center for Medical Polymers and Biomaterials, Kyoto University, 53, Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, Japan, 606.

[2] To whom correspondence should be addressed.

0724-8741/89/0500-0422$06.00/0

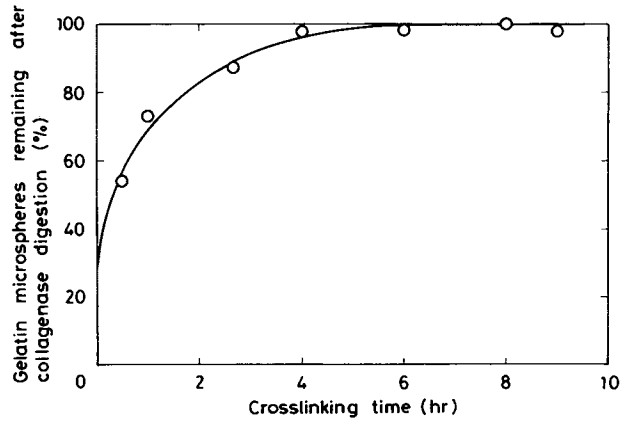

Fig. 1. Effect of the cross-linking reaction time on the amount of G-1-1 gelatin microspheres remaining after 30 min of collagenase digestion.

and calcium-containing PBS [PBS(+)] with 0.1 mg of collagenase and agitated at 37°C. At regular time intervals, the suspension was centrifuged at 8000 rpm for 10 min, and the amount of gelatin in the sediments was determined by the ninhydrin method (17). For release tests, the amount of IFN or 6GX released from the MS was determined by measuring the radioactivity in the sediments or by measuring the fluorescence intensity in the supernatants, respectively. ^{125}I-labeled IFN was prepared by the method of Greenwood and Hunter (18). The total amount of 6GX was determined after complete alkaline hydrolysis of MS.

Macrophage Collection and Phagocytosis Assay. Mac-

Table I. Preparation of Gelatin Microspheres

Code No.	Gelatin Conc. in feed (w/v%)	Glutaraldehyde added (mg/mg gelatin)	Yield of Microspheres (%)[a]	Trapping of IFN (%)[b]
G-1-1	20	1.33	53.2	52.4
G-1-2	20	0.71	52.4	49.4
G-1-3	20	0.28	52.6	48.4
G1 G-1-4	20	0.14	50.9	45.9
G-1-5	20	0.05	44.5	41.4
G-1-6	20	0.03	40.8	37.8
G-2-1	10	1.33	55.2	47.7
G-2-2	10	0.71	60.7	51.2
G-2-3	10	0.28	63.4	50.1
G2 G-2-4	10	0.14	64.5	50.9
G-2-5	10	0.05	64.8	47.9
G-2-6	10	0.03	57.3	35.9
G-3-1	5	1.33	58.0	42.7
G-3-2	5	0.71	54.8	39.4
G-3-3	5	0.28	52.8	40.0
G3 G-3-4	5	0.14	44.3	32.9
G-3-5	5	0.05	46.6	26.4
G-3-6	5	0.03	32.4	12.2
G-4-1	2	1.33	29.4	19.4
G-4-2	2	0.71	30.1	19.9
G-4-3	2	0.28	16.5	9.5
G4 G-4-4	2	0.14	15.6	6.3
G-4-5	2	0.05	5.6	1.9
G-4-6	2	0.03	7.4	0.8

[a] (Wt of microsphere/wt of gelatin in feed) × 100.
[b] (Wt of IFN in microsphere/wt of IFN added) × 100.

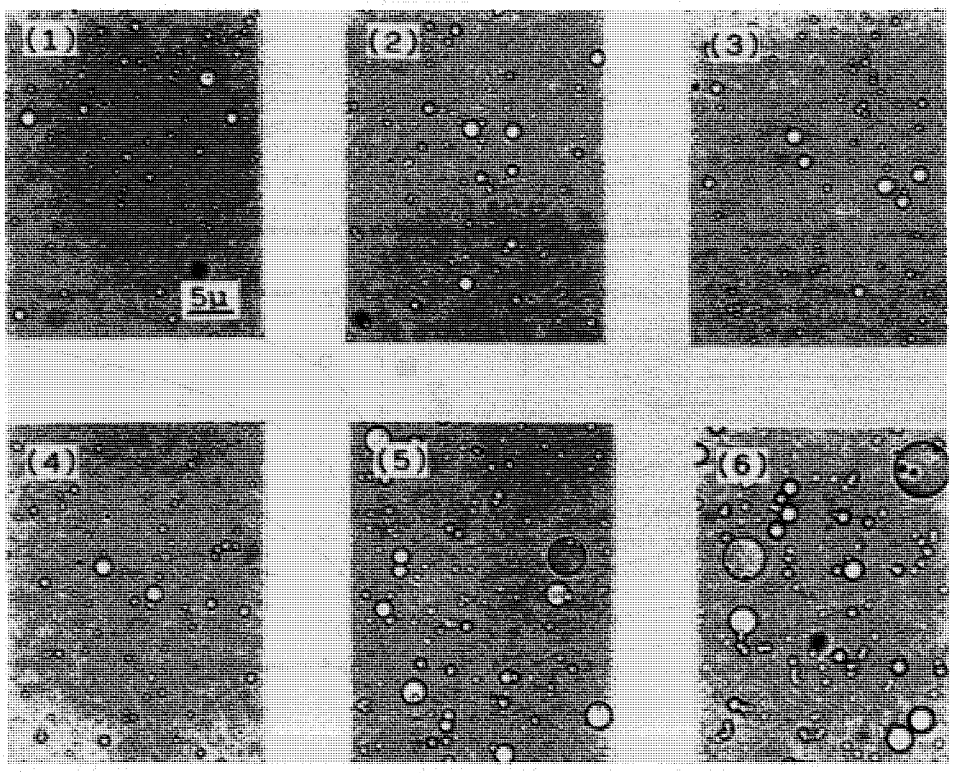

Fig. 2. Scanning electron micrographs of gelatin microspheres: (1) G-1-1, (2) G-1-3, (3) G-2-3, (4) G-2-4, (5) G-3-3, and (6) G-4-3.

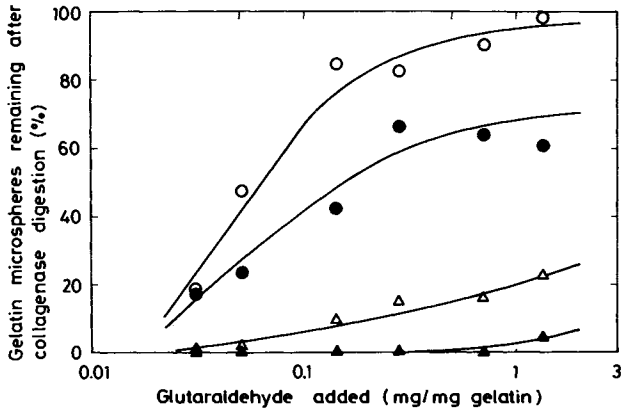

Fig. 3. Effect of gelatin and glutaraldehyde concentrations at cross-linking on collagenase digestion of gelatin microspheres for 30 min: (○) G-1, (●) G-2, (△) G-3, and (▲) G-4.

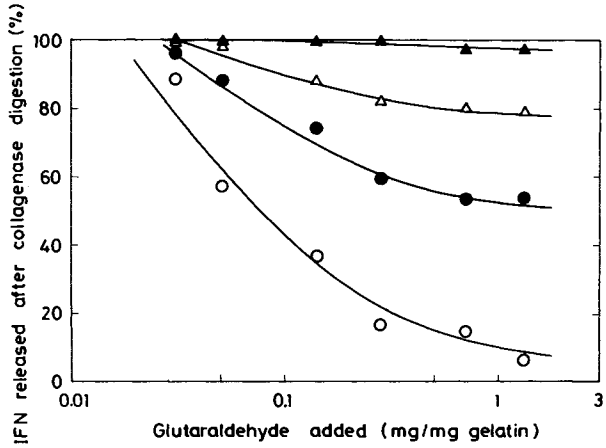

Fig. 5. A/D release from gelatin microspheres after collagenase digestion for 30 min: (○) G-1, (●) G-2, (△) G-3, and (▲) G-4.

rophages (Mϕ) used here were mouse peritoneal macrophages, prepared according to the method described previously (14). One milliliter of cell suspensions of 5×10^5 Mϕ/ml RPMI-FCS was plated into 24-well multidish culture plates. Mϕ monolayers were obtained by the removal of nonadherent cells 2 hr after incubation at 37°C and 5% CO_2. Phagocytosis of MS was evaluated according to the method reported by Carpenter and Barsales (19). Briefly, the MS containing ^{125}I-labeled IFN were cultivated with Mϕ in the dose range from 65 to 300 μg. After incubation for 1, 3, or 6 hr, the monolayers were thoroughly washed to eliminate nonphagocytosed MS and were lysed with 2% SDS in PBS. The radioactivity of the cell lysates was measured, and the percentage phagocytosis was calculated as previously described (20): percentage phagocytosis = (cpm of Mϕ with MS phagocytosed)/(cpm of MS added to Mϕ cultures) × 100.

MS Degradation in Macrophages. MS containing ^{125}I-labeled IFN or microspheres prepared from ^{125}I-labeled gelatin were incubated with Mϕ monolayers for 6 hr. After removal of nonphagocytosed MS, Mϕ were further incubated in fresh RPMI-FCS. Then 6, 18, or 30 hr and 2 or 3 days later, Mϕ were washed and lysed with 2% sodium dodecyl sulfate (SDS) in PBS. After precipitation of the cell lysate solutions with 10% trichloroacetic acid aqueous solution and subsequent centrifugation at 13,500 rpm for 5 min, the radioactivity of both supernatants and sediments was measured. The amounts of gelatin remaining and IFN released were calculated according to the following formula: gelatin remaining = (cpm of sediments)/[(cpm of supernatants) − (cpm of sediments)] × 100 and IFN released = (cpm of supernatants)/[(cpm of supernatants) − (cpm of sediments)] × 100.

RESULTS

Characterization of MS. Figure 1 indicates that cross-linking reaction periods longer than 4 hr did not further increase the resistance of gelatin microspheres to collagenase. Thus, the reaction time was fixed at 6 hr throughout the present work. The yield of MS and the percentage of IFN trapped in the MS (Table I) decreased in parallel as the concentration of glutaraldehyde added became low. Light mi-

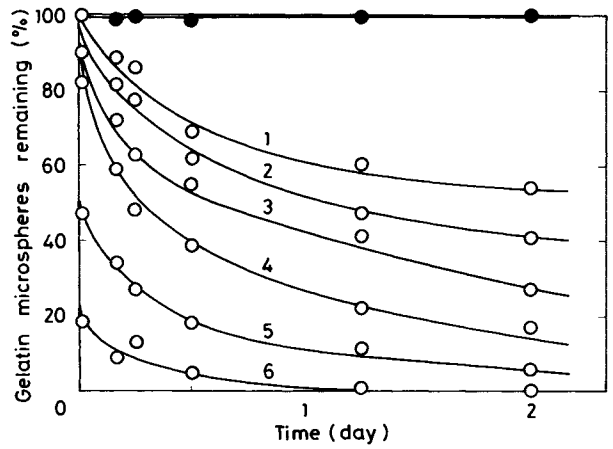

Fig. 4. Degradation profiles of G-1 gelatin microspheres: (1) G-1-1, (2) G-1-2, (3) G-1-3, (4) G-1-4, (5) G-1-5, and (6) G-1-6 microspheres. Open symbols, in PBS(+) containing collagenase; filled symbols, in PBS.

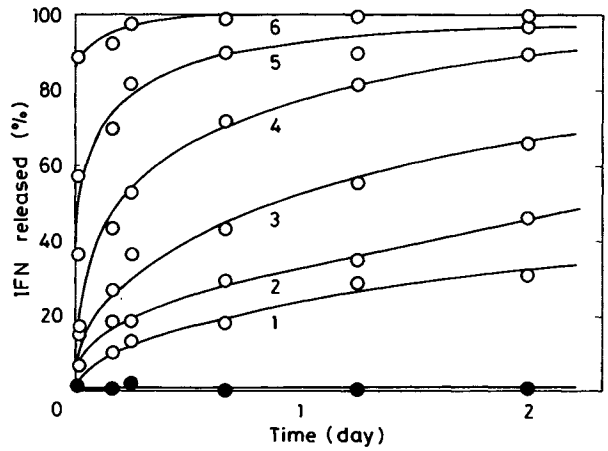

Fig. 6. Release profiles of A/D from G-1 gelatin microspheres in the presence of collagenase: (1) G-1-1, (2) G-1-2, (3) G-1-3, (4) G-1-4, (5) G-1-5, and (6) G-1-6 microspheres. Open symbols, in PBS(+) containing collagenase; filled symbols, in PBS.

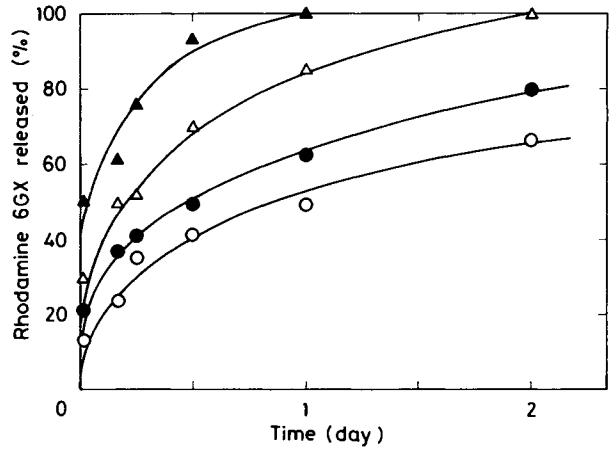

Fig. 7. Release profiles of rhodamine 6GX from G-1 gelatin microspheres in the absence of collagenase: (○) G-1-1, (●) G-1-2, (△) G-1-3, and (▲) G-1-4.

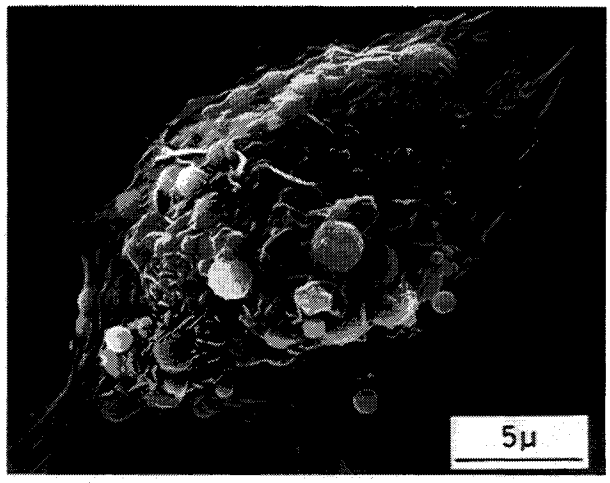

Fig. 8. Scanning electron micrograph of a macrophage phagocytosing G-1-1 gelatin microspheres 6 hr after incubation.

croscope photographs of MS suspended in PBS are shown in Fig. 2. The MS were swollen at low concentrations of gelatin and glutaraldehyde. No morphological change was observed by incorporation of IFN or 6GX and by the use of ^{125}I-labeled gelatin for MS preparation.

MS Degradation and IFN Release from MS. Degradation of MS of the G-1 series is shown in Figs. 3 and 4. No degradation was observed in PBS within 2 days. Further, in PBS(+) containing collagenase, the degradation of MS decreased with increasing concentrations of gelatin and glutaraldehyde. Results given in Figs. 5 and 6 indicate that the released amount of IFN from MS decreased with increasing amounts of gelatin or glutaraldehyde. IFN was released slowly from every MS with time, and the rate was regulated by changing the extent of gelatin cross-linking in PBS(+) containing collagenase, in contrast with that in PBS. However, 6GX was released from the MS, even when placed in PBS without collagenase, as shown in Fig. 7. The release rate was regulated by changing the concentration of glutaraldehyde added, similarly to that of IFN in collagenase/PBS(+) solution. This result may be caused by its low molecular weight and lack of functional groups reactive to glutaradehyde.

Table II. Macrophage Phagocytosis of Gelatin Microspheres at 37 and 4°C

Code No.	Percentage Phagocytosis[a] 1 hr	3 hr	6 hr
G-1-1	25.8 (2.0)	28.8 (2.1)	35.6 (2.4)
G-1-2	22.2 (1.9)	30.1 (2.2)	32.7 (1.2)
G-1-3	24.9 (2.5)	23.3 (1.8)	35.7 (2.0)
G1 G-1-4	22.9 (2.4)	24.7 (1.6)	35.2 (2.3)
G-1-5	18.1 (1.8)	26.6 (1.5)	25.3 (1.8)
G-1-6	14.6 (2.3)	27.7 (1.9)	23.3 (1.4)
G-2-1	28.3 (2.0)	33.7 (2.4)	37.4 (2.5)
G-2-2	26.2 (1.9)	28.9 (2.1)	36.1 (2.0)
G-2-3	23.1 (1.9)	24.0 (2.3)	32.9 (2.0)
G2 G-2-4	17.6 (2.2)	23.2 (1.9)	36.6 (1.9)
G-2-5	16.0 (1.6)	26.8 (1.8)	28.6 (1.5)
G-2-6	14.9 (1.2)	23.4 (1.5)	20.9 (1.8)
G-3-1	22.6 (1.8)	29.1 (2.4)	29.4 (1.9)
G-3-2	21.9 (1.6)	21.5 (2.5)	26.4 (2.0)
G-3-3	20.3 (1.5)	24.4 (1.9)	28.8 (2.1)
G3 G-3-4	24.6 (1.4)	27.6 (2.2)	26.0 (2.0)
G-3-5	25.5 (1.2)	24.0 (1.7)	30.4 (1.7)
G-3-6	7.0 (1.0)	8.1 (1.6)	19.2 (2.5)
G-4-1	22.0 (2.0)	22.5 (1.9)	24.5 (1.8)
G-4-2	17.9 (1.7)	21.9 (2.0)	24.9 (2.0)
G-4-3	14.4 (1.5)	18.3 (1.9)	23.7 (2.4)
G4 G-4-4	4.8 (1.0)	7.8 (1.8)	9.3 (1.0)
G-4-5	2.4 (1.6)	2.4 (1.6)	8.1 (1.2)
G-4-6	1.4 (0.9)	2.8 (1.1)	3.2 (1.0)

[a] The values denote the percentage of the radioactivity of gelatin microspheres in macrophages to that of gelatin microsphere added to cultures at 37°C, while those in parentheses denote the percentage at 4°C.

Phagocytosis of MS by Macrophages. Results of Mϕ phagocytosis of MS are tabulated in Table II. Values of percentage phagocytosis at 4°C were always low in comparison with those at 37°C, and hence, this assay system can distinguish the phagocytosed MS from those not internalized but simply adsorbed on the surface of macrophages, since par-

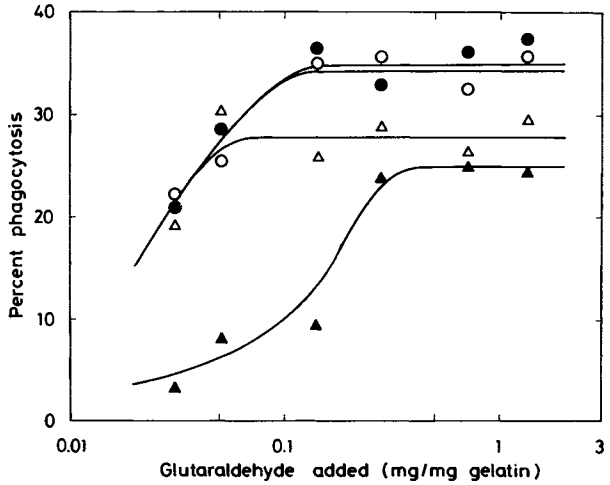

Fig. 9. Macrophage phagocytosis of gelatin microspheres of different extents of cross-linking: (○) G-1, (●) G-2, (△) G-3, and (▲) G-4.

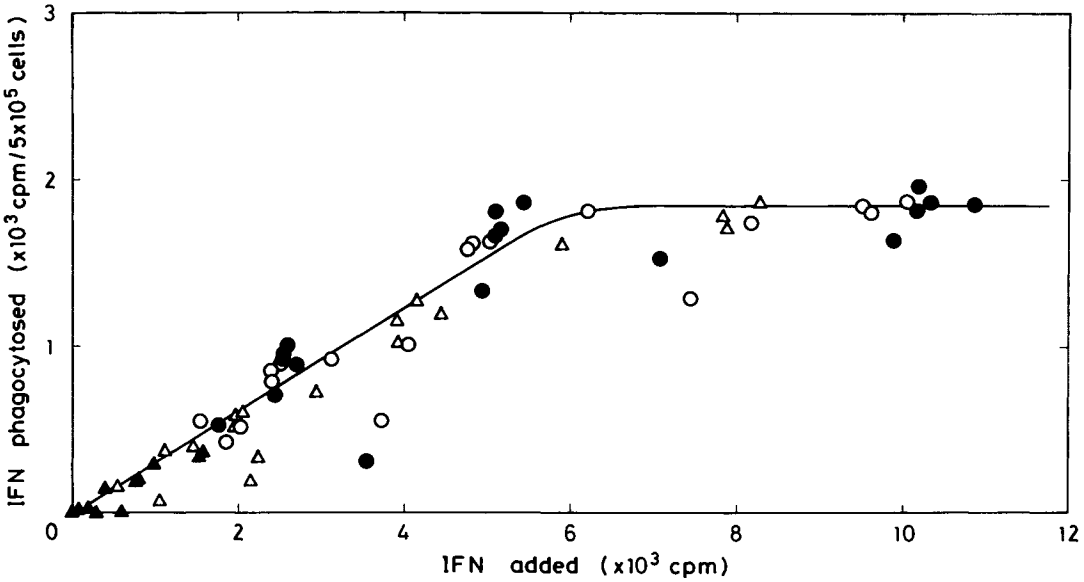

Fig. 10. Relationship between ^{125}I-IFN in gelatin microspheres added to cultures and ^{125}I-IFN in gelatin microspheres phagocytosed by macrophages: (○) G-1, (●) G-2, (△) G-3, and (▲) G-4.

ticles only bind to the surface of macrophages and are not ingested by the cells (21). The SEM micrograph shown in Fig. 8 shows Mϕ phagocytosis of MS. Phagocytosis decreased with decreasing concentrations of gelatin and glutaraldehyde (Fig. 9). Further, the amount of MS phagocytosed was proportional to the MS added, until saturation of phagocytosis was observed at high MS doses (Fig. 10).

MS Degradation in Macrophages. Figures 11 and 12 show the degradation of MS of the G-1 series and the subsequent release of IFN from the MS in Mϕ, respectively. The MS phagocytosed were gradually degraded in Mϕ, leading to the slow release of IFN in the cells. Both the degradation and the IFN release rates were affected by changing the glutaraldehyde concentration.

DISCUSSION

Gelatin serves as a carrier matrix for pharmaceutical uses. However, it has been applied only to nonproteins of low molecular weight, and the gelatin particles were too big to be ingested by Mϕ. Ultrasonication of the gelatin–IFN mixture afforded sufficiently small microspheres containing IFN at a high trapping efficiency without denaturation. Moreover, gelatin microspheres (MS) are fairly stable, and IFN contained by the MS does not leak out readily unless they are degraded in PBS(+) containing collagenase or intracellularly. The amount of IFN released from the MS was inversely proportional to that of IFN remaining in the MS, as shown in Fig. 13. Neither MS degradation nor IFN release was observed without collagenase, suggesting that enzymatic hydrolysis is responsible for the degradation of gelatin. Further, the rate of MS degradation and that of IFN release from the MS are controllable by changing the extent of gelatin cross-linking by changing the concentration of glutaraldehyde.

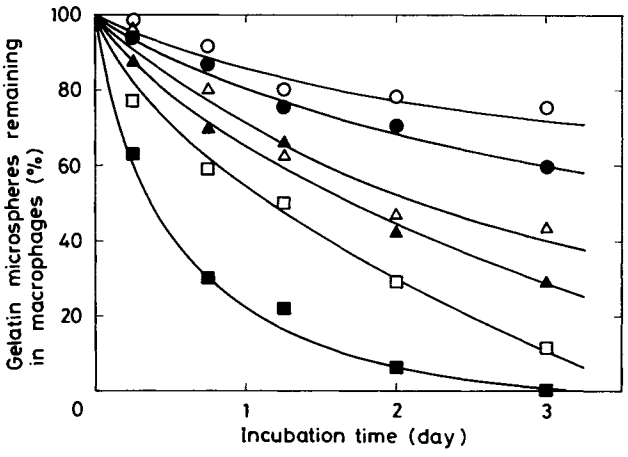

Fig. 11. Degradation profiles of G-1 gelatin microspheres in macrophages: (○) G-1-1, (●) G-1-2, (△) G-1-3, (▲) G-1-4, (□) G-1-5, and (■) G-1-6 microspheres.

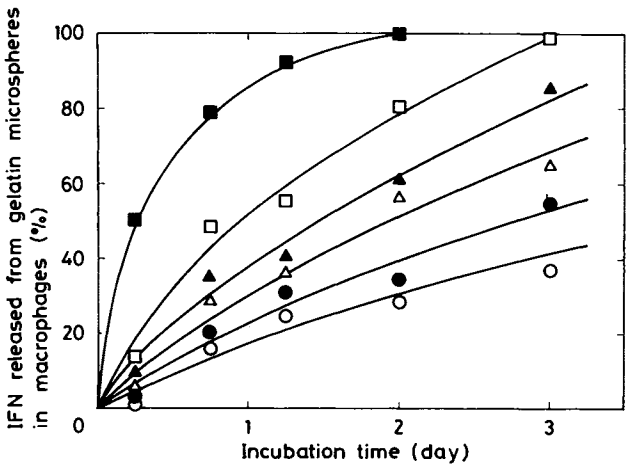

Fig. 12. Release profiles of A/D from G-1 gelatin microspheres in macrophages: (○) G-1-1, (●) G-1-2, (△) G-1-3, (▲) G-1-4, (□) G-1-5, and (■) G-1-6 microspheres.

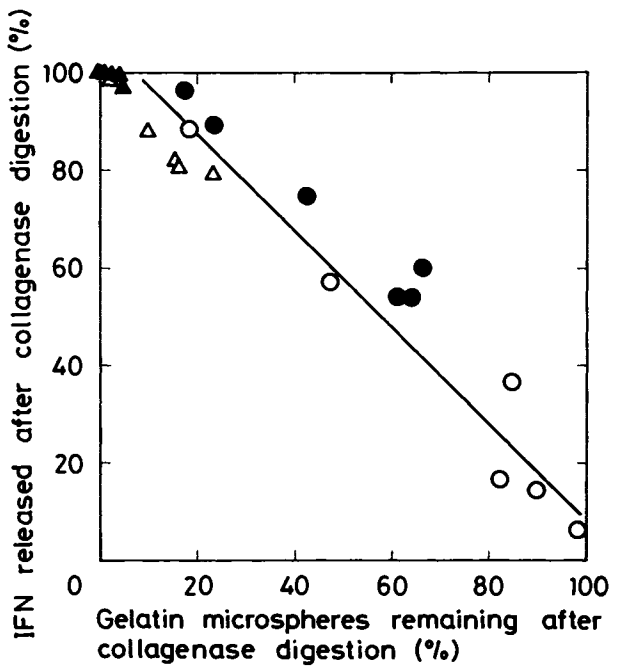

Fig. 13. Dependence of IFN release on collagenase digestion of gelatin microspheres for 30 min: (○) G-1, (●) G-2, (△) G-3, and (▲) G-4.

Gelatin functions as an accelerator of Mϕ phagocytosis, and gelatin precoating of microspheres enhanced their phagocytosis by Mϕ (14). Hence, fibronectin and other cell-adhesive proteins contained in FCS may become bound to the MS surface, since such proteins have a high affinity to gelatin. As a result, the MS undergoes increased opsonization in the presence of FCS. All of the MS prepared were similarly ingested by Mϕ. However, at low concentrations of gelatin and glutaradehyde, the MS were greatly swollen, resulting in a reduction of phagocytosis. The MS were phagocytosed in proportion to the amount of MS added to the Mϕ culture, to the dose of 130 μg MS for 5×10^5 Mϕ. Above this dose, the adhered macrophages detached from culture dishes 6 hr after the addition of the MS but were found to be still viable by the trypan blue exclusion test.

Phagocytosed MS gradually underwent degradation in the Mϕ, followed by the subsequent release of IFN from MS in the cells. The release rate of IFN again correlated well with the degradation rate in the cells and was sensitive to the cross-linking extent of the MS. No release of IFN was observed in RPMI-FCS culture medium (data not shown), demonstrating that MS containing IFN were first phagocytosed without being degraded, followed by gradual degradation to release IFN in Mϕ. In addition, the IFN, delivered intracellularly with this MS, was demonstrated effectively to activate Mϕ for antitumor function (22).

REFERENCES

1. T. Barlozzari, J. Leonhardt, R. H. Wiltrout, R. B. Herberman, and C. W. Reynold, *J. Immunol.* 134:2783–2789 (1985).
2. J. R. Jett, A. Mantovani, and R. B. Herberman. *Cell Immunol.* 54:425–436 (1980).
3. K. Uno, S. Shimizu, M. Ido, K. Naito, K. Inaba, T. Oka, T. Kishida, and S. Muramatsu. *Cancer Res.* 45:1320–1326 (1985).
4. T. Kataoka, N. Matsuura, F. Ohnishi, and Y. Suhara. *Cancer Res.* 45:3548–3553 (1985).
5. R. L. Juliano. *Drug Delivery Systems,* Oxford University Press, New York and Oxford, 1980.
6. S. S. Davis, L. Illum, J. G. McVie, and E. Tomlinson. *Microspheres and Drug Therapy,* Elsevier, Amsterdam, New York, and Oxford, 1984.
7. D. A. Tirrell, L. G. Donaruma, and A. B. Turek. *Ann. N.Y. Acad. Sci.* 446 (1985).
8. P. L. Madan, L. A. Luzzi, and J. C. Price. *J. Pharm. Sci.* 63:280–284 (1974).
9. J. R. Nixon, S. A. Khalil, and J. E. Carless. *J. Pharm. Pharmacol.* 20:528–538 (1968).
10. N. Tanaka, S. Takino, and I. Utsumi. *J. Pharm. Sci.* 52:664–667 (1963).
11. M. Hashida, Y. Takahashi, S. Muranishi, and H. Sezaki. *J. Pharmacokin. Biopharm.* 5:241–255 (1977).
12. M. Hashida, M. Egawa, S. Muranishi, and H. Sezaki. *J. Pharmacokin. Biopharm.* 5:225–239 (1977).
13. M. Hashida, S. Muranishi, H. Sezaki, N. Tanigawa, K. Satomura, and Y. Hikasa. *Int. J. Pharm.* 2:245–256 (1979).
14. Y. Tabata and Y. Ikada. *J. Bioact. Compat. Polymers* 1:32–46 (1986).
15. Y. Tabata and Y. Ikada. *J. Pharm. Pharmacol.* 39:698–704 (1987).
16. E. Sawicki, T. R. Hanser, T. W. Stanley, and W. Elbert. *Anal. Chem.* 33:93–96 (1961).
17. R. McGrath. *Anal. Biochem.* 49:95–102 (1972).
18. F. C. Greenwood and W. M. Hunter. *Biochem. J.* 89:114–123 (1963).
19. R. R. Carpenter and P. B. Barsales. *J. Immunol.* 98:844–853 (1967).
20. R. I. Kavet and J. D. Brain. *J. Reticuloendothel. Soc.* 27:201–221 (1980).
21. M. Rabinovitch. *Exp. Cell Res.* 46:19–28 (1967).
22. Y. Tabata, K. Uno, Y. Ikada, and S. Muramatsu. *Jpn. J. Cancer Res.* 79:636–646 (1988).