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Suppressive Effect of Recombinant TNF-gelatin Conjugate on Murine Tumour Growth In-vivo

YASUHIKO TABATA, KAZUKO UNO*, YOSHITO IKADA AND SHIGERU MURAMATSU†

*Research Center for Biomedical Engineering, Kyoto University, 53 Kawahara-cho Shogoin, Sakyo-ku, Kyoto, *Institut Pasteur de Kyoto, 103-5, Monzen-cho, Tanaka, Sakyo-ku, Kyoto, and †Department of Zoology, Faculty of Science, Kyoto University, Kitashirakawa-Oiwakecho, Sakyo-ku, Kyoto 606, Japan*

Abstract—A recombinant human tumour necrosis factor α (TNF) was conjugated to gelatin by means of carbodiimide to improve the in-vivo stability of TNF. About 55% of TNF activity was retained after gelatin conjugation as judged by the cytotoxicity assay using L-M cells in-vitro. Intraperitoneal injection of the TNF-gelatin conjugate significantly suppressed the in-vivo growth of murine Meth A fibrosarcoma cells (SS2 cells) in mouse peritoneum, in comparison with that of free TNF at the same dosage ($P < 0.05$). After intraperitoneal injection, TNF activity of the conjugate was detected in both the serum and the peritoneal cavity of mice for a longer period than was free TNF, irrespective of the presence of SS2 cells. Chromatographic studies of the conjugate demonstrated that the increase in the apparent molecular weight of TNF was consistent with gelatin conjugation. It is likely that this leads to a prolonged retention of TNF activity in-vivo. In addition, the TNF-gelatin conjugate suppressed the in-vitro growth of SS2 cells to the same extent as free TNF. Thus, it is possible that the longer retention period of the conjugate brought about an increase in the chance of contact between TNF and SS2 cells, resulting in the enhanced suppressive effect of TNF on in-vivo tumour cell growth. Gelatin conjugation is an effective method for increasing the in-vivo antitumour activity of TNF.

Tumour necrosis factor α (TNF) was discovered as a cytotoxic factor in the serum of bacillus Calmette-Guerin (BCG)-infected mice after injection of a bacterial lipopolysaccharide (Carswell et al 1975). TNF causes necrosis of certain transplanted tumours without any cytotoxic effect on normal tissues in-vivo. It also manifests a strong in-vitro cytotoxicity to various kinds of tumour cells but not to normal cells (Carswell et al 1975; Helson et al 1975; Matthews & Watkins 1978; Matthews 1981; Ruff & Giffoed 1981; Sohmura et al 1986). The in-vivo antitumour effect of murine TNF has been reported on murine tumours transplanted in syngeneic mice (Carswell et al 1975; Haranaka et al 1984) and human tumours transplanted in nude mice (Helson et al 1979; Haranaka et al 1984; Sohmura et al 1986). Moreover, human TNF was shown to be effective in suppressing the in-vivo growth of murine and human tumours (Sohmura et al 1986).

Recently, the gene for human TNF has been cloned and its product was expressed in bacteria (Wang et al 1985). Because sufficient amounts of recombinant TNF are now available, it is possible to conduct an extensive examination of the in-vivo efficacy of TNF. Clinical application of TNF has posed several problems, such as the rapid catabolism and pyrogenicity of TNF. In addition, the high-dosage regimens required to achieve therapeutic efficacy reversibly heighten the toxic side-effects of TNF itself. Thus, the key to an effective but relatively harmless procedure for in-vivo administration is to increase the in-vivo efficacy of TNF. Stabilization of proteinaceous drugs in-vivo, has been achieved through their conjugation with various water-soluble polymers; it has been demonstrated that the conjugation with dextran or polyethyleneglycol is effective in prolonging their circulation lifetime (Pyataki et al 1980; Kamisaki et al 1981; Wileman et al 1986; Sakuragawa et al 1986). We have already demonstrated that gelatin is also useful as polymer carriers of drugs. The conjugation with gelatin permitted muramyldipeptide (MDP) to augment macrophage activation to acquire the antitumour activity in-vivo as well as in-vitro (Tabata & Ikada 1990, 1991).

In the present study, chemical conjugation of TNF with gelatin was achieved as a method to improve the in-vivo stability of TNF. We describe the antitumour effect of the TNF-gelatin conjugate in tumour-bearing mice.

Correspondence: Y. Ikada, Research Center for Biomedical Engineering, Kyoto University, 53 Kawahara-cho Shogoin, Sakyo-ku, Kyoto 606, Japan.

Materials and Methods

Mice

Specific pathogen-free male and female (BALB/c × DBA/2) F_1 mice (CDF_1/SLC), aged 4–6 months, were obtained from SLC Inc., Shizuoka, Japan. No difference in the in-vivo tumour cell growth was observed between males and females.

Tumour cells

The SS2 cell line employed here was isolated and cloned from methylcholanthrene-induced Meth A fibrosarcoma of BALB/c mice. Their in-vivo as well as in-vitro growth was suppressed by interferon (α, β, or γ) (Uno et al 1985, 1988). The cells were maintained by in-vitro cultures and regularly tested to confirm they were free of mycoplasma.

TNF

Recombinant human tumour necrosis factor (TNF, 3.15×10^6 Japan Relative Units (JRU) (mg protein)$^{-1}$) was kindly provided by the Dainippon Pharmaceutical Co. Ltd, Osaka, Japan. The JRU is equivalent to the conventional unit of TNF. The endotoxin level in the original preparations

was determined by a Limulus test to be 0·04 ng mg^{-1} TNF ($3·15 \times 10^6$ JRU) or less. TNF activity was determined by the conventional cytotoxicity assay using a subline of L929 cells, L-M cells in-vitro according to the method of Nakano et al (1986).

Culture medium and reagents

Cell culture was carried out in complete medium (RPMI-FCS) containing RPMI-1640 (Nissui Seiyaki, Co. Ltd, Tokyo, Japan), 5 mM L-glutamine, 100 int. units mL^{-1} penicillin, 5 mM 4-(2-hydroxyethyl)-1-ethanesulphonic acid, 0·14 wt% $NaHCO_3$, and 10% foetal calf serum (lipopolysaccharide < 0·03 ng mL^{-1} M.A. Bioproducts, Walkersville, MD, USA). Hanks balanced salt solution (HBSS) and phosphate-buffered saline (PBS) were purchased from Nissui Seiyaku Co. Ltd, Tokyo, Japan. The gelatin used was of alkaline type, isolated from pig skin (pI 5·1, mol. wt = 99 000 Da, Nitta Gelatine Co. Ltd, Osaka, Japan), although any kind of gelatin could be used for this procedure. Other chemical reagents (Wako Pure Chemical Industries Ltd, Osaka, Japan) were used as obtained.

Conjugation of TNF to gelatin

The TNF-gelatin conjugate was prepared using carbodiimide according to the method of Sheehan & Hess (1955). Amounts ranging from $2·12 \times 10^{-9}$ mol (1×10^5 JRU) to $4·23 \times 10^{-11}$ mol (2×10^3 JRU) of TNF were dissolved in 2·5 mL of 0·05 M phosphate buffer solution (pH 4·7) containing 1·25 mg gelatin ($1·26 \times 10^{-8}$ mol). After addition of varying amounts of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl salt (EDCI), the conjugation was allowed to proceed under gentle stirring for various time periods up to 30 h at 4°C. The resulting TNF-gelatin conjugate was purified by dialysis against PBS at 4°C for 24 h to remove the chemical reagents used in the conjugation, and sterilized with a Millex-GS Millipore filter (0·22 μm porosity, type: SLGS025OS, Japan Millipore Ltd, Tokyo, Japan) to store at 4°C until use.

Change in the molecular size of TNF-gelatin conjugates with conjugation time was evaluated by measuring differences in the elution time in gel filtration chromatography (GFC, TSK-gel $G6000PW_{XL}$ and $G4000PW_{XL}$ column, Toyo Soda Co. Ltd, Tokyo, Japan). In addition, gel exclusion chromatography using a Sepharose 4B column (1·7 × 47 cm) was performed to measure the molecular size and size distribution of the TNF-gelatin conjugates prepared. The eluted solution was assayed for total amount of protein and TNF activity. The total amount of protein in each fraction was determined by measuring the fluorescence intensity at 308 nm after exposure to ultraviolet light (280 nm) followed by using a calibration curve of known TNF/gelatin mixtures. TNF activity of the conjugate was evaluated by the cytotoxicity assay using L-M cells described previously. In addition, ^{125}I-labelled TNF prepared by the chloramine T method (Greenwood & Hunter 1963) was conjugated to gelatin. The radioactivity was measured to evaluate the amount of TNF incorporated in the conjugate.

In-vitro inhibitory activity of TNF-gelatin conjugate on the growth of SS2 cells

SS2 cells at the density of 5×10^3/200 μL RPMI-FCS were plated into each dish of 96-well multiwell culture plates (Nunc No 1 67008, Kampstrup, Denmark) and were incubated with 0·4 μg of TNF-gelatin conjugate containing different amounts of TNF at 37°C in a 5% CO_2–95% air atmosphere. Varying amounts of free TNF with or without 0·4 μg TNF-free, original gelatin, as well as 0·4 μg TNF-free, original gelatin were used as controls. The number of viable SS2 cells was counted 3 days later to assess the antitumour activity of TNF. The inhibitory effect of TNF was evaluated according to the following formula:

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

Measurement of TNF activity in peritoneal cavity and blood circulation after intraperitoneal injection of TNF-gelatin conjugate

Intraperitoneal injections were made into normal mice and SS2-bearing mice inoculated intraperitoneally with 4×10^6 SS2 cells 8 days previously. Mice received injections of 0·25 mL PBS containing 100 μg of TNF-gelatin conjugate containing 1×10^4 JRU, or 1×10^4 JRU free TNF with or without 100 μg TNF-free gelatin. After different time intervals, blood samples were collected from the retro-orbital plexus of mice and mouse ascites were collected by lavage of the peritoneal cavity with 3 mL HBSS to assess their TNF activity.

In-vivo antitumour activity of TNF-gelatin conjugate

SS2 cells cultured in-vitro were inoculated intraperitoneally to acclimatize them to in-vivo conditions (Uno et al 1985, 1988). The SS2 cells were harvested 6 days later and used as test SS2 cells.

Mice received intraperitoneal inoculation of 4×10^6 SS2 cells in 0·5 mL HBSS at day 0. Injection of TNF-gelatin conjugates and other agents was carried out according to the following protocols: two injections at day 5 and 6 (experiment 1); a single injection at day 11 (experiment 2); three injections at days 3, 5 and 7 (experiment 3). The agents injected were 0·25 mL PBS alone or containing 100 μg gelatin, free TNF (2×10^2 or 1×10^3 JRU), or 100 μg gelatin conjugate containing 2×10^2 or 1×10^3 JRU TNF. As an additional control, the mixture of 1×10^3 JRU free TNF and 100 μg TNF-free gelatin was also injected. The in-vivo antitumour effect was elevated by counting the number of SS2 cells grown in the peritoneal cavity of mice by the trypan blue exclusion test. Viable SS2 cells were counted at the next day of the last injection of the agents: at day 7 (experiment 1); at day 12 (experiment 2); at day 8 (experiment 3). SS2 cells did not form solid tumours in the peritoneum and were readily distinguishable from their host peritoneal exudate cells by their large size.

Statistical analysis

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

Results and Discussion

Characterization of TNF-gelatin conjugate

The GFC elution time of TNF-gelatin conjugates was found to decrease with conjugation time, attaining a constant value

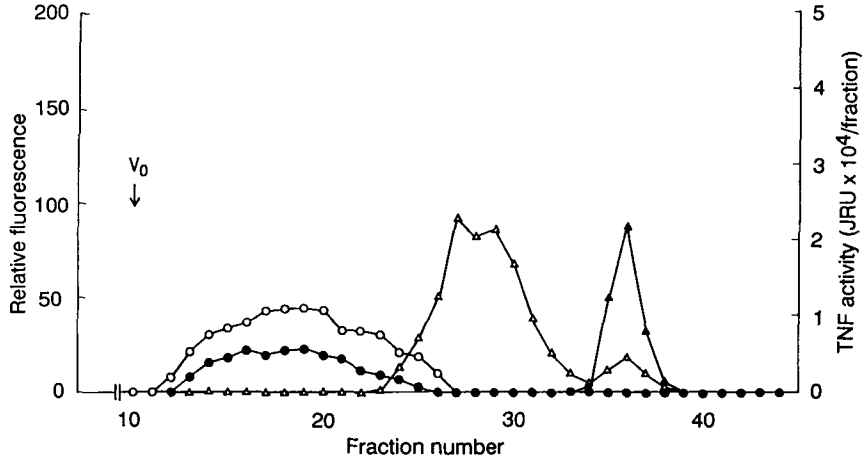

FIG. 1. Gel exclusion chromatography of TNF-gelatin conjugate. $2{\cdot}12 \times 10^{-10}$ mol (1×10^4 JRU) TNF was conjugated to $1{\cdot}26 \times 10^{-8}$ mol gelatin with $6{\cdot}25 \times 10^{-7}$ mol EDCI. 2·6 mL of the conjugate (○, ●) or the mixture of $2{\cdot}12 \times 10^{-10}$ mol free TNF and $1{\cdot}26 \times 10^{-8}$ mol free gelatin (△, ▲) was eluted separately from Sepharose 4B column using 0·5 M potassium phosphate buffer (pH 7·0). The flow rate and volume of each fraction were 20 mL min^{-1} and 2·6 mL, respectively. ○, △, Protein; ●, ▲, TNF activity. V_0 represents the exclusion volume.

at conjugation times greater than 10 h, regardless of the molar ratio of TNF to gelatin or of EDCI to total protein. This implies that conjugation times longer than 10 h are not effective in increasing the molecular size of conjugates. Also, no cloudy appearance was observed in the reaction solution through the conjugation due to intramolecular and mutual cross-linking of their proteins.

Fig. 1 shows the gel filtration profile of a representative TNF-gelatin conjugate. Globular proteins of different molecular weights (e.g. bovine thyroglobulin (mol. wt 670 kDa), bovine serum albumin (mol. wt 67 kDa), bovine pancreatic trypsin (mol. wt 24 kDa), and chicken egg lysozyme (mol. wt 12·5 kDa)) were used to calibrate the column. The elution peaks of the proteins were detected in fractions 23, 31, 34, and 37, respectively. When a mixture of free TNF and TNF-free gelatin was analysed, free gelatin appeared in fractions 24 to 33 without any TNF activity, while free TNF of mol. wt 17 kDa appeared in fractions 35 to 37. On the other hand, the conjugate was eluted in fractions 12 to 26, although showing a broad size distribution. The total amount of proteins collected in the fractions was about 80% of the protein amount initially added for the conjugation. This value was similar to that of TNF incorporated in the conjugate which was evaluated by ^{125}I-labelled TNF. Moreover, about 55% of TNF activity was retained in the conjugate when comparing the activity before and after gelatin conjugation.

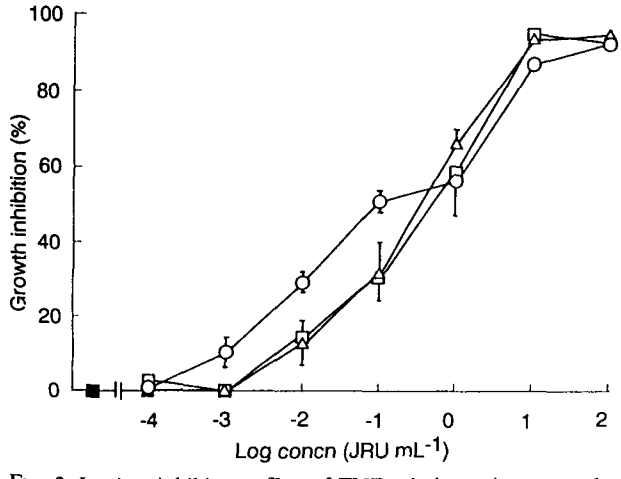

FIG. 2. In-vitro inhibitory effect of TNF-gelatin conjugate on the growth of SS2 cells. 5×10^3 SS2 cells were incubated for 3 days with 0·4 μg TNF-gelatin conjugate containing different amounts of TNF (○) and different amounts of free TNF with (□) or without 0·4 μg TNF-free gelatin (△). The cells were also incubated in the presence of 0·4 μg TNF-free gelatin (■). Points, geometric means of triplicate cultures; bars, s.e.

Effect of TNF-gelatin conjugate on the in-vitro growth of SS2 cells

The suppressive effect of gelatin-conjugated TNF and free TNF on the in-vitro growth of SS2 cells is shown in Fig. 2. The antitumour effect of free TNF increased with an increase in the dosage. Similar dose-dependence was observed for the TNF-gelatin conjugate but over the dose range less than 10^{-1} JRU mL^{-1} the conjugate inhibited the SS2 cell growth more strongly than free TNF. In addition, no effect of TNF-free gelatin addition on the in-vitro growth of SS2 cells induced by free TNF was observed and the presence of gelatin had no influence on the cell growth itself.

TNF activity in the peritoneal cavity and blood circulation after intraperitoneal injection of TNF-gelatin conjugate

The time course of TNF activity in the peritoneal cavity and that in serum after intraperitoneal injection of TNF-gelatin conjugate is shown in Fig. 3. Conjugation with gelatin significantly increased the longevity of activity over free TNF. When conjugated with gelatin, the rate of TNF elimination in the peritoneal cavity decreased (Fig. 3A). Over 20% of the TNF activity of conjugated TNF was retained in the peritoneal cavity 1 h after injection, while the activity for free TNF was around 6% of the activity injected initially. The gelatin conjugation had minimal effect on the time course of TNF activity in serum but the retention of the activity and the time of retention were both slightly increased (Fig. 3B). In addition, simply mixing with gelatin was not effective in prolonging the retention of TNF activity in either mouse peritoneum or serum. Prolonged retention of TNF

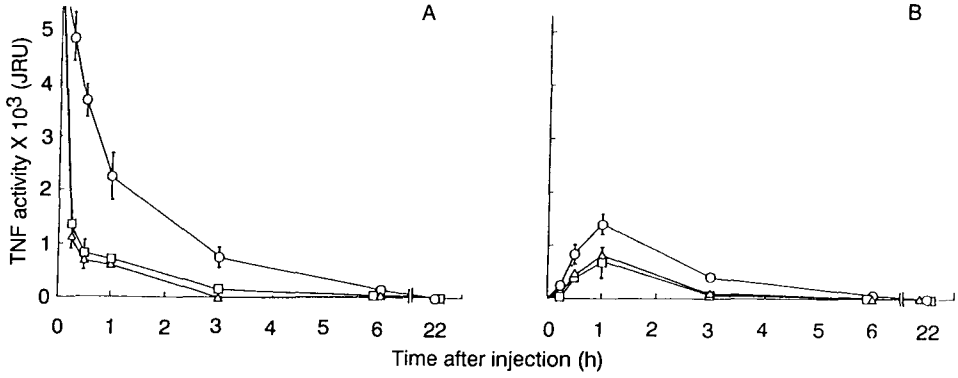

FIG. 3. TNF activity present in the peritoneal cavity and serum of normal mice after intraperitoneal injection of free TNF or TNF-gelatin conjugate. Mice received single injections of 100 μg TNF-gelatin conjugate containing 1×10^4 JRU TNF (○), 1×10^4 JRU free TNF (△), and 1×10^4 JRU free TNF plus 100 μg free gelatin (□) to assess TNF activity remaining in the peritoneal cavity (A) and serum (B) of mice. Points, geometric means of triplicate experiments; bars, s.e.

activity by gelatin conjugation was observed, irrespective of the presence of tumour cells (Table 1). The retention period of TNF in the peritoneal cavity of SS2-bearing mice was longer than that of normal mice; which may be explained by considering clearance by the reticuloendothelial system. The physiological features of SS2-bearing mice are quite different from those of normal mice (Tabata & Ikada 1991). The presence of SS2 tumour cells brings about the impaired functions of the reticuloendothelial system in liver and spleen and the augmented mobilization of host cells to the peritoneum. Thus, it is possible that this causes a reduced transfer of the conjugate to systemic circulation. No significant difference in the TNF activity in serum was observed between normal and SS2-bearing mice.

Effect of TNF-gelatin conjugate on the growth of SS2 cells in the peritoneum

Suppressive effects of gelatin-conjugated TNF and free TNF on the growth of SS2 cells in the peritoneal cavity are shown in Fig. 4. In experiment 1, the growth of SS2 cells was inhibited as a function of TNF dose in the peritoneal cavity of mice treated with either free TNF or TNF-gelatin conjugate. A greater reduction in the number of SS2 cells was observed in mice receiving the conjugate than in those receiving the free TNF when compared at the same dose of TNF ($P < 0.05$). Moreover, the augmentation of the in-vivo suppressive effect was achieved by the gelatin conjugate even if different injection schedules were used (experiment 2, $P < 0.05$ and experiment 3, $P < 0.05$). In addition, the injection of gelatin alone did not suppress the in-vivo growth of SS2 cells and the simple mixing with gelatin produced no change in the suppressive effect induced by injection of free TNF alone (experiment 1).

The present study demonstrates that gelatin conjugation is effective in increasing the in-vivo antitumour activity of TNF. A high suppressive effect on SS2 cell growth in mouse peritoneum was observed by the injection of TNF-gelatin conjugates compared with that of free TNF, regardless of the schedule of conjugate injection. Simply mixing with gelatin neither interfered with nor enhanced the suppressive effect on the in-vivo growth of SS2 cells induced by free TNF. This indicates that the chemical conjugation of TNF with gelatin is required for the enhanced antitumour effect of TNF in-vivo. Gelatin conjugation permitted TNF to retain its activity in mouse peritoneal cavity and serum for a long period in comparison with free TNF. Besides, for the TNF-gelatin conjugate, the in-vitro inhibitory effect on the in-vitro growth of SS2 cells was similar to that induced by free TNF. Thus, it is plausible that the prolonged retention period of the conjugate led to an increase in the chance of contact between TNF and SS2 cells, resulting in the enhancement of direct antitumour effect of TNF.

Table 1. TNF activity present in the peritoneal cavity and serum after intraperitoneal injection of free TNF or TNF-gelatin conjugate to SS2-bearing and normal mice.

	TNF activity ($\times 10^3$ JRU)					
	SS2-bearing mice			Normal mice		
Period after injection (h)	Conjugate	Free TNF	Free TNF +gelatin	Conjugate	Free TNF	Free TNF +gelatin
Peritoneal cavity						
0·5	4·20±0·10	1·12±0·08	1·26±0·20	3·65±0·08	1·10±0·10	1·35±0·12
1	2·71±0·12	0·66±0·06	0·68±0·08	2·25±0·10	0·70±0·05	0·62±0·09
Serum						
0·5	0·82±0·05	0·40±0·03	0·35±0·05	0·80±0·02	0·42±0·06	0·32±0·06
1	1·46±0·12	0·78±0·07	0·80±0·11	1·37±0·08	0·75±0·10	0·75±0·12

Mean ± s.e.

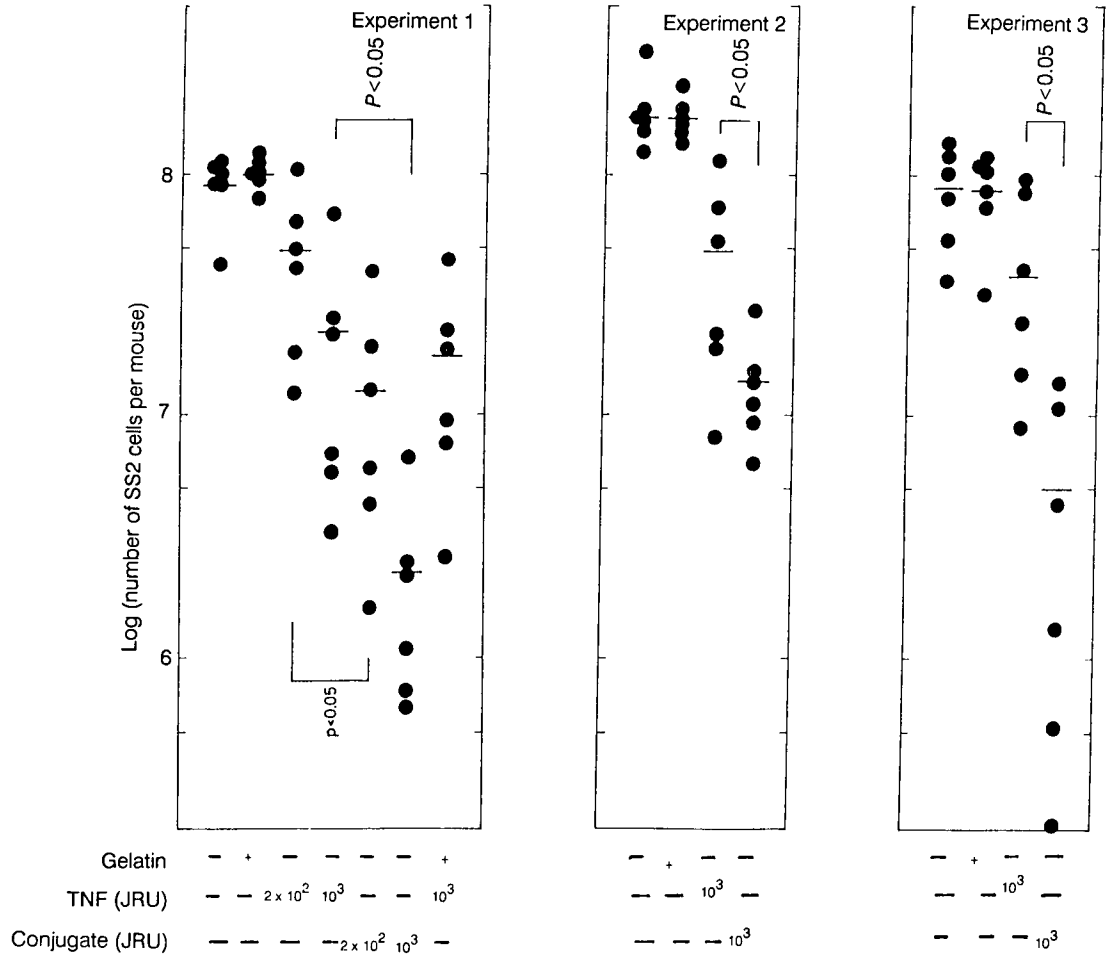

FIG. 4. In-vivo growth of SS2 cells in the peritoneal cavity of mice receiving intraperitoneal injection of free TNF or TNF-gelatin conjugate. SS2 cells (4×10^6) were intraperitoneally inoculated on day 0 and the recipient mice received two injections on days 5 and 6 (experiment 1), a single injection on day 11 (experiment 2), and three injections on days 3, 5, and 7 (experiment 3) of the conjugate or other agents. The number of viable SS2 cells was counted 1 day after the last injection. Points, number of cells in each mouse; bars, mean.

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