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Release of Soluble TNF/LT Receptors from a Human Ovarian Tumor Cell Line (PA-1) by Stimulation with Cytokines *in Vitro*

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ABSTRACT

We have demonstrated the presence of the 55- and 75-kDa receptor for tumor necrosis factor (TNF) and lymphotoxin (LT) (TNF-R) in serum, and ascites from women with ovarian cancer. The present studies were initiated to begin to examine the possible cellular source of these receptors in women with ovarian cancer. Human ovarian tumor cells (PA-1) were cocultured for 24–48 hr with various levels of recombinant human cytokines (IL-1β, IL-4, IFN-γ) and the supernatants were assayed by ELISA for the soluble forms of each receptor. PA-1 cells spontaneously release the 55-kDa TNF-R and low levels of the 75-kDa TNF-R. The release of both 55- and 75-kDa TNF-R was stimulated when PA-1 cells were cultured with IL-1β and IFN-γ but unaffected by IL-4. The level of 55-kDa TNF-R was elevated slightly over spontaneous release but the level of 75-kDa TNF-R increased dramatically. IFN-γ was the most potent stimulator of receptor release particularly of the 75-kDa TNF-R. IFN-γ also induced increased expression of cell membrane TNF-R measured by binding of ^{125} I-labeled TNF. Membrane TNF-Rs which were induced by IFN-γ were the 75-kDa type, because TNF binding was blocked by anti-75-kDa TNF-R antibody. These data suggest that IFN-γ selectively induced release and expression of 75-kDa TNF-Rs.

INTRODUCTION

Recently, soluble fragments of two tumor necrosis factor receptor (TNF-R) have been identified in human urine and in the serum of human cancer patients. These molecules are capable of specifically binding to and inactivating human TNF and LT cytolytic activity *in vitro* (1–5). Once bound to the TNF/LT molecule they appear to physically block the ability of the cytokine to bind to specific receptors on susceptible cells and tissue, thus neutralizing its activities. These soluble TNF/LT receptors (sTNF-R) have molecular weights of 30 to 40 kDa, and are derived from the N-terminal, extracellular region of the 55- and 75-kDa TNF membrane TNF-R (6,7).

In addition, other groups have identified cell surface TNF-Rs in biological fluids that specifically bind to and inactivate other cytokines such as IL-2, IL-4 and growth factors *in vitro* (8–10). Collectively, these studies have led to the concept that release of the extracellular portions of cell membrane TNF-Rs may represent a new mechanism to specifically control cytokine activity. This concept is supported by the recent report of Gatanaga *et al.* (6) that human sTNF-R derived from the 55-kDa molecule can block the antitumor activity of recombinant human TNF-α when injected into Meth-A tumor bearing BALB/c mice and also the injection of recombinant TNF-R derived from 55-kDa TNF-R decreases mortability in animal models of septic shock (11). It is not yet clear what is the cell and tissue source of these sTNF-R *in vivo*. However, a recent report by Porteu and Nathan (12) indicates that activated human neutrophils can release soluble forms derived from both 55- and 68- to 75-kDa TNF-R *in vitro*. Gatanaga *et al.* (13) also reported that the human monocytic cell line can release sTNF-R when stimulated

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with PMA and LPS. Neither of these cell types spontaneously releases these TNF-Rs but had to be stimulated.

Recently, Cappuccini *et al.* (14) identified sTNF-R activity in the cell-free ascites derived from 15 patients with ovarian cancer but the cell source of these sTNF-R was not identified. The present studies were designed to examine the capacity of a human ovarian cancer cell line to release these sTNF-R *in vitro*. We showed ovarian cancer cells may be one of the sources of sTNF-R in the ascites fluid *in vivo* and cytokines can further up-regulate their release.

MATERIALS AND METHODS

Reagents

Recombinant TNF-α and IL-1β were supplied by Genentech Corp, (South San Francisco, CA). IL-4 and IFN-γ were purchased from Genzyme (Boston MA). All other reagent were obtained from SIGMA Chemical Co. (St. Louis, MO).

Human ovarian cancer cell line

The human ovarian cancer cell (PA-1) was obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained as adherent monolayers in tissue culture flasks (Corning, Corning, NY) in RPMI-1640 containing 10% fetal bovine serum (FBS) (GIBCO, Inc. Grand Island, NY). Cultures were maintained at 37°C in an atmosphere of 5% CO_2–95% air. Cells were passed twice weekly.

Treatment of cells with various agents

PA-1 cells were seeded into T25-cm^2 tissue culture flasks (Corning, Corning, NY) at a density of 0.6×10^6 cells per flask in 5 ml of RPMI- 1640 containing 10% FBS. After 24 hr of incubation at 37°C, cell monolayers were washed with PBS and incubated with preselected levels of the different agents in RPMI-1640 containing 10% FBS for 0 to 72 hr at 37°C. Culture supernatants were collected and centrifuged for 10 min at 1500 rpm to remove cells or cell debris. After centrifugation, the supernatants were concentrated 10 times by Centriprep 10 concentrator (Amicon, Beverly, MA).

ELISA for TNF-R

Antibody specific for the extracellular domain of the human 55- and 75-kDa TNF-Rs were generated in New Zealand white female rabbits according to the technique of Yamamoto *et al.* (15). The recombinant forms of the extracellular domain of the human 55- and 75-kDa TNF-R (7) were kindly provided by Synergen Inc. (Boulder, CO). Briefly, 100 μg of these proteins was injected into rabbits with Freund's complete or incomplete adjuvant (DIFCO, Detroit, MI) every 2 weeks. After six injections, animals were bled and the antisera collected, aliquoted, and stored at −20°C until use. IgGs against the 55- and 75-kDa TNF-Rs were purified by protein G sepharose column by the method of Ey *et al.* (16). The IgG antibodies were coupled with horseradish peroxidase by the method of Nakane and Kawaoi (17) and Tijssen and Kurstok (18). The procedures for the TNF-R ELISA was performed as follows. First, 0.5 μg of IgG in 100 μl of 0.05 *M* carbonate buffer (pH 9.6) to either the 55- or 75-kDa TNF-R was bound to a 96-well ELISA microplate (Corning, Corning, NY) by overnight incubation at 4°C. After three washes with 0.2% Tween 20 in PBS, 100 μl of various concentrations of either recombinant TNF-R or 100 μl of sample was added to each well and the plate was incubated overnight at 4°C. The plate was then washed with 0.2% Tween-20 in PBS. One hundred mocroliters of horseradish peroxidase coupled anti-TNF-R IgG to either the 55- or 75-kDa TNF-R was added to each well and incubated for 1 hr at 37°C. The wells were washed three times with 0.2% Tween-20 in PBS. The color was developed for 20 min at room temperature (RT) with 100 μl of substrate which was prepared by dissolving 1 tablet of ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) diammonium salts (Pierce, Rockford, IL) in 10 ml of 0.1 *M* sodium acetate (pH 4.2) with 3 μl 30% H_2O_2 (Fisher Scientific, FairLawn, NJ). The amount of 55- or 75-kDa TNF-R in the unknown sample was determined by comparing it to a standard curve for the recombinant 55- or 75-kDa TNF-R by reading the absorbance at 405 nm in an EAR AT ELISA plate reader (SLT-Lab instruments, Salzburg, Austria). Duplicate wells of each dilution or sample were tested and the average of these results is shown.

TNF binding assay

One million monolayer PA-1 cells into 24-well culture plate were first incubated with or without IFN-γ for 2 and 18 hr. They were then incubated with 2–30 ng of ^{125}I-labeled human recombinant TNF (Amersham, IL, specific activity 400 Ci/mmol) in the presence or absence of a 100-fold excess of unlabeled human TNF (Cetus Corp., CA) at 4°C for 3–4 hr. After three washes with cold PBS, cells were solubilized in 1 ml of 0.3 *N* NaOH and radioactivity was determined in a Pharmacia Clinigamma counter (Uppsala, Sweden).

RESULTS

The identification and quantitation of 55-kDa and 75-kDa TNF-Rs in cell-free supernatants of PA-1 cells stimulated with various cytokines

Various levels of recombinant human IL-1β, IL-4, and IFN-γ were tested for their ability to affect the release of 55- and 75-kDa TNF-Rs. An optimum level of each cytokine that induced receptor release was selected and subsequent studies were conducted with that single dose. PA-1 cells were cocultured with optimal levels of each cytokine and after 72 hr, cell-free supernatants were tested for the presence of 55- and 75-kDa TNF-Rs by ELISA. The results of one experiment are shown in Fig. 1. Human IL-1β had a weak effect on the release of 55-kDa TNF-R, however, induced release of the 75-kDa TNF-R. Human IFN-γ weakly stimulated the release of the 55-kDa receptor but was a powerful stimulator of the release of the 75-kDa TNF-R. These studies were repeated with identical results.

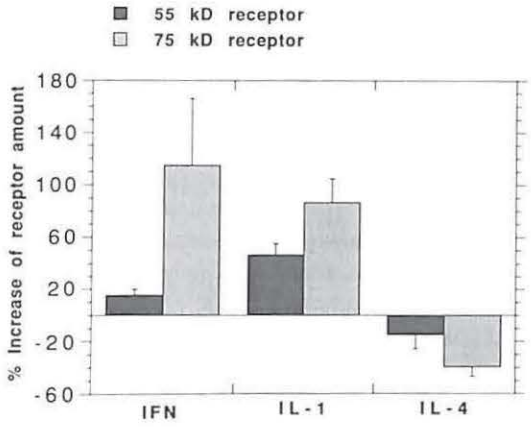

FIG. 1. The identification and quantitation of 55-kDa and 75-kDa TNF-Rs in cell-free supernatants of PA-1 cells stimulated with various cytokines. PA-1 cells were stimulated with IFN-γ, IL-1β, and IL-4 as described under Materials and Methods. The amount of TNF-R was assayed by ELISA. The concentrations of the cytokines were IFN-γ (100 ng/ml), IL-1β (100 ng/ml), and IL-4 (10 ng/ml).

Kinetics of the release of soluble 55-kDa and 75-kDa TNF-R by human IFN-γ stimulated PA-1 cells in vitro

First IFN-γ concentration ranging from 4 to 100 ng/ml was tested on releasing TNF-R of PA-1 cell for 72 hr, as described in Materials and Methods. A representative experiment is shown in Fig. 2. It is clear that these cells spontaneously release soluble forms of both receptors; however the 55-kDa TNF-R was the major form released. Spontaneous release does not appear to be the result of cell death because viability was high in all PA-1 cell cultures established by staining with eosin and trypan blue. Increasing the dose of IFN-γ had only a moderate effect in release of the 55-kDa TNF-R; however, release of the 75-kDa TNF-R is strongly stimulated. Next, cell-free culture supernatants were collected at 3, 6, 24, 48, and 72 hr from PA-1 cells cocultured with 100 ng/ml of IFN-γ. These supernatants were assayed for the presence of 55- and 75-kDa TNF-Rs by ELISA. The results of a single experiment are shown in Fig. 3. The release of the 75-kDa TNF-R began within 3 hr and continued to 72 hr.

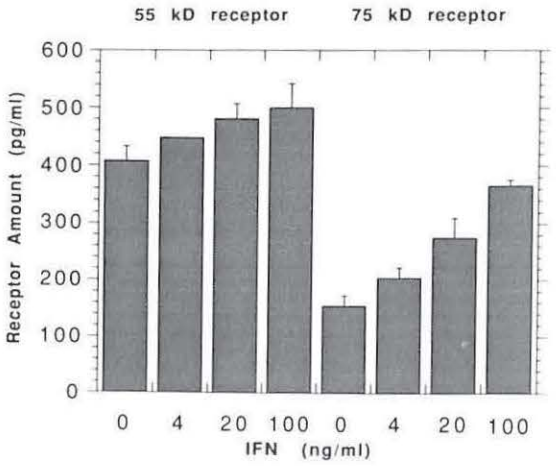

FIG. 2. Effects of IFN-γ on 55-kDa and 75-kDa TNF-R release. PA-1 cells were stimulated with various concentrations of IFN-γ as described under Materials and Methods. The range of IFN-γ concentration from 4, 20, 100 ng/ml was tested. The amount of TNF-Rs was assayed by ELISA after 72 hr of culture.

Effect of exposure to human IFN-γ on membrane expression of 55-kDa and 75-kDa TNF-R by PA-1 cells in vitro

The total number of TNF/LT membrane TNF-Rs was established by binding ^{125}I-TNF-α to PA-1 cells as described in Materials and Methods. Next the effects of INF-γ on ^{125}I-TNF binding by PA-1 cells was investigated. PA-1 monolayer cells were incubated in wells for 2 or 18 hr with 10 ng IFN-γ as outlined in Materials and Methods. The number of plasma membrane TNF-Rs was established. We found receptor number increased 34% after 2 hr incubation with IFN-γ and increased 65% of the 18 hr of incubations (Fig. 4).

We next employed rabbit anti-55-kDa and anti-75-kDa TNF-R IgG as specific competitive inhibitors of ^{125}I-TNF-binding PA-1 cells after IFN-γ stimulation. The data in Fig. 5 indicate that receptor number increased (534/cell to 878/cell: 64%) after 18 hr incubation with IFN-γ. Coincubation with IgG against the 75-kDa TNF-R in binding assay only increased 24%

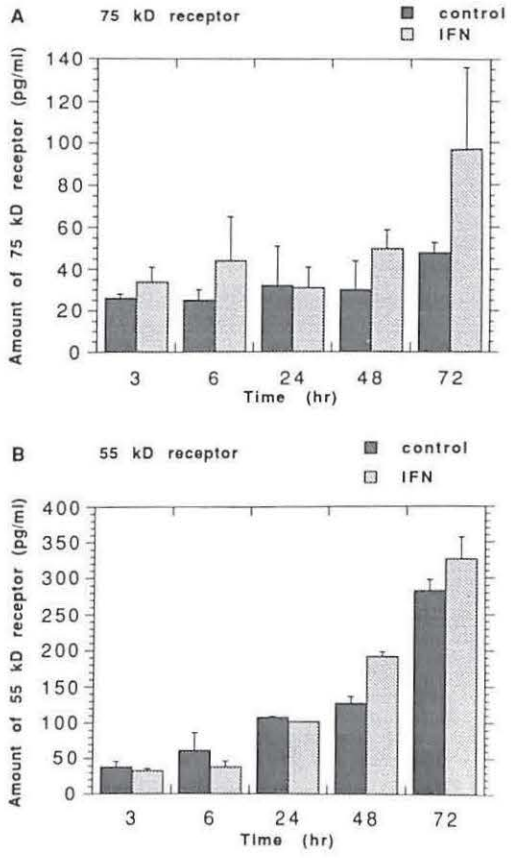

FIG. 3. Kinetics of the release of soluble 55-kDa and 75-kDa TNF-R by IFN-γ-stimulated PA-1 cells *in vitro*. PA-1 cells were stimulated with IFN-γ (100 ng/ml) as described under Materials and Methods. The amount of TNF-Rs was assayed by ELISA after 3, 6, 24, 48, and 72 hr of culture. (A) 75-kDa TNF-R; (B) 55-kDa TNF-R.

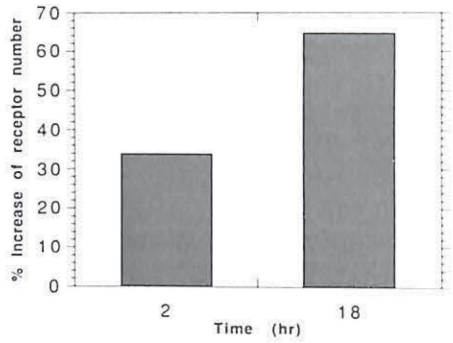

FIG. 4. The effect of IFN-γ on the expression of TNF/LT membrane receptors on PA-1 cells. The indicated amounts of ^{125}I-TNF were added to 10^6 PA-1 cells at 4°C for 3 hr as described under Materials and Methods. The results of the calculated percent increase of the number of receptors after treatment with IFN-γ for 2 and 18 hr are shown.

of receptor numbers (456/cell to 564/cell) after 18 hr incubation with IFN-γ. In contrast, coincubation with IgG against the 55-kDa TNF-R in binding assay increased 152% (283/cell to 714/cell) after 18 hr incubation with IFN-γ. Increased receptor binding by IFN-γ was mainly blocked by antibodies to the 75-kDa TNF-R.

DISCUSSION

Shedding or release of membrane receptors for cytokines may represent a new mechanism for control of these highly active proteins. Release of IL-2 and IL-6 receptors has been reported by Rubin *et al.* (8) and Novick *et al.* (9), respectively. Weisman *et al.* (19) recently identified a soluble type 1 human complement membrane receptor that is biologically functional *in vivo*. sTNF-R were first identified in the urine of febrile patients and in minimal amounts in the urine of normal subjects (1–4). We recently identified and characterized sTNF-R in the serum of cancer patients (5) and identified blocking factor (BF) activity and sTNF-R in the ascites of ovarian cancer patients (14). These BF are able to bind to and inhibit human TNF and LT cytolytic activity *in vitro* and interfering with the ability of TNF/LT to interact with their specific cell membrane receptors. It is not yet clear but appears that "all" materials with BF activity are "secreted" or "shed" extracellular domains of the 55- and/or 75-kDa membrane TNF-Rs (6,7,12). Clearly these sTNF-R may have an important role in controlling the action of TNF-α and LT *in vitro* and perhaps *in vivo*.

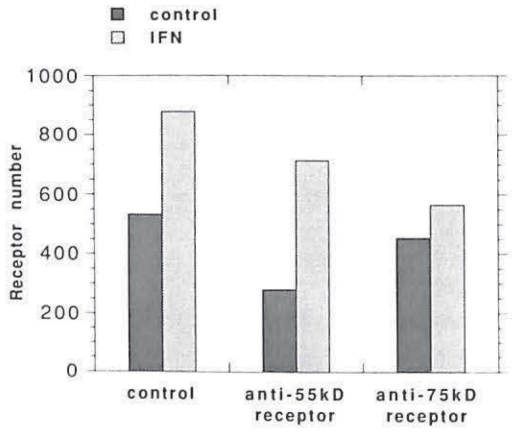

FIG. 5. Anti-55-kDa, 75-kDa TNF-Rs antibody block the binding of TNF to IFN-γ-treated PA-1 cells. Ten micrograms of anti-55 and 75-kDa antibody was added to the assays to compete with ^{125}I-labeled TNF on PA-1 cells after being treated with IFN-γ for 18 hr. The results of the calculated number of receptor after treatment with or without antibody for 55- and 75-kDa TNF-R are shown.

The cell and tissue source of these materials and the cellular processes involved in release *in vivo* are not yet known. A recent report by Porteu and Nathan (12) indicates that human neutrophils can release sTNF-R derived from both 55- and 75-kDa TNF-R *in vitro* when activated by coculturing with FMLP. Gatanaga *et al.* (13) reported that the human monocytic cell THP-1 can release sTNF-R derived from cell membrane TNF-R when activated by coculturing with PMA and LPS. It is interesting that neither of these cell types spontaneously released receptors but had to be activated before they released these sTNF-R.

We are particularly interested in the cell and tissue source and the role of these sTNF-Rs in human cancer patients. We have identified high levels of TNF/LT BF and soluble 55- and 75-kDa membrane TNF-R in the ascites of women with ovarian cancer (14). Our recent data indicate both BF activity and sTNF-Rs are spontaneously released in the first 48 hr by primary cultures of fresh ascites cells and ovarian cancer tissues (20); however, BF levels decrease after 48–72 hr. These data support the concept that release of soluble receptors occurs in a local tumor site(s) *in vivo* and may be a source of both local and systemic levels of BF in these patients.

The present studies were initiated to begin to determine if TNF-R was released into the supernatants by resting and stimulated continuous human ovarian tumor cell lines. We found PA-1 cells spontaneously release both 55- and 75-kDa TNF-Rs; however, the 55-kDa TNF-R is the predominant form that is spontaneously released.

All of the cytokines tested except IL-4 were stimulatory, but IFN-γ was the most active for induction of release of the 75-kDa TNF-R. The results support the concept that receptor release is a selective process and cells may be able to specifically release one or both of these receptors in response to different inducing stimuli. It is important to note that in all supernatants the 55-kDa TNF-R was the most prevalent soluble form even though the 75-kDa TNF-R was the major form that was up-regulated. Exposure of cells to IFN-γ resulted in up-regulation of the production and the release of the soluble form of the 75-kDa TNF-R more than those of the 55-kDa TNF-R. After the PA-1 cell treated by IFN-γ, the receptor numbers were increased on binding assay in short time exposure. INF-γ causes the production and release of TNF-R on cell surface. While the data are not shown, we found similar results with these cytokines when tested on different ovarian tumor cell lines (i.e., 222 cell).

These studies reveal an important new concept; the tumor cell may be able to defend itself when confronted with immunologic attack. Tumor infiltrating macrophages and lymphocytes

become activated *in situ* and release cytokines into the tumor microenvironment where these effector molecules initiate and orchestrate both local and systemic antitumor activity. However, tumor cells may not be passive but can respond defensively by releasing or shedding of specific membrane TNF-R, which can bind to and inactivate the soluble cytokines. How does this occur? The mechanisms of control and levels and types of receptors released may hold the key to the outcome of the tumor–host interaction and the clinical status of the patient.

REFERENCES

1. **Peetre, C., H. Thysell, A. Grabb, and I. Olsson.** 1988. A tumor necrosis factor binding protein in presents in human biological fluids. *Eur. J. Haematol. 41:414.*
2. **Seckinger, P., S. Isaaz, and J. M. Dayer.** 1988. A human inhibitor of tumor necrosis factor-α. *J. Exp. Med. 167:1511.*
3. **Seckinger, P., S. Isaaz, and J. M. Dayer.** 1989. Purification and biologic characterization of a specific tumor necrosis factor alpha inhibitor. *J. Biol. Chem. 264:11966.*
4. **Engelman, H., D. Aderka, M. Rubinstain, D. Rotman, and D. Wallach.** 1989. A tumor necrosis factor-binding protein purified to homogeneity from human urine protects cells from tumor necrosis factor. *J. Biol. Chem. 264:11974.*
5. **Gatanaga, T., R. Lentz, I. Masunaka, J. Tomich, E. W. Jeffes, M. Baird, and G. A. Granger.** 1990. Identification of TNF-LT blocking factor(s) in the serum and ultrafiltrates of cancer patients. *Lymphokine Res. 9:225.*
6. **Gatanaga, T., C. Hwang, W. Kohr, F. Cappuccini, J. A. Lucci, E. W. Jeffes, R. S. Yamamoto, and G. A. Granger.** 1990. Purification and characterization of an inhibitor (soluble tumor necrosis factor receptor) for the tumor necrosis factor and lymphotoxin obtained from the serum ultrafiltrates of human cancer patients. *Proc. Natl. Acad. Sci. U.S.A. 87:8781.*
7. **Kohno, T., M. T. Brewer, S. L. Baker, P. E. Schwartz, M. W. King, K. M. Hale, C. M. Squired, R. C. Thompson, and J. L. Vannice.** 1990. A second tumor necrosis factor receptor gene product can shed a naturally occurring tumor necrosis factor inhibitor. *Proc. Natl. Acad. Sci. U.S.A. 87:8331.*
8. **Rubin, L. A., C. C. Kurman, E. M. Fritz, W. E. Biddison, B. Boutin, R. Yarchoan, and D. L. Nelson.** 1985. Soluble interleukin 2 receptors are released from activated human lymphoid cells *in vitro*. *J. Immunol. 135:3172.*
9. **Novick, D., H. Engelman, D. Wallach, and M. Rubinstein.** 1989. Soluble cytokine receptors are present in normal human urine. *J. Exp. Med. 170:1409.*
10. **Zupan, A. A., P. A. Osborne, C. E. Smith, N. R. Siegal, R. M. Leimgruber, and E. M. Johnson.** 1989. Identification, purification, and characterization of truncated forms of the human nerve growth factor receptor. *J. Biol. Chem. 264:1171.*
11. **Ashkenazi, A., S. A. Marsters, D. L. Capon, S. M. Chamow, I. S. Figari, D. Pennica, D. V. Goeddel, M. A. Palladino, and D. M. Smith.** 1991. Protection against endotoxic shock by tumor necrosis factor receptor immunoadhesin *Proc. Natl. Acad. Sci. U.S.A. 88:10535.*
12. **Proteu, F., and C. Nathan.** 1990. Shedding of tumor necrosis factor receptors by activated human neutrophils. *J. Exp. Med. 172:599.*
13. **Gatanaga, T., C. Hwang, M. Gatanaga, F. Cappuccini, R. Yamamoto, and G. Granger.** 1990. The regulation of TNF-R in mRNA synthesis, membrane expression and release by PMA and LPS-stimulated human monocytic THP-1 cells *in vitro*. *Cell Immunol. 138:1.*
14. **Cappuccini, F., R. Yamamoto, P. J. Disaia, E. Grosen, M. Gatanaga, L. A. Lucci, E. Ininns, T. Gatanaga, and G. Granger.** 1991. Identification of tumor necrosis factor and lymphotoxin blocking factor(s) in ascites of patients with advanced and recurrent ovarian cancer. *Lymphokine Cytokine Res. 10:252.*
15. **Yamamoto, R. S., J. C. Hiserodt, J. E. Lewis, C. E. Carmack, and G. Granger.** 1978. The human LT system. II. Immunological relationships of LT molecules released by mitogen activated human lymphocytes *in vitro*. *Cell. Immunol. 38:403.*
16. **Ey, P. L., S. L. Pruwse, and C. R. Jenkin.** 1976. Isolation of pure IgG1, IgG2a, and IgG2b immunoglobulines from mouse serum using protein A Sepharose. *Biochemistry 15:429.*
17. **Nakane, P. K. and A. Kawaoi.** 1974. Peroxidase-labeled antibody: A new method of conjugation. *J. Histochem. Cytochem. 22:1084.*
18. **Tijssen, P., and E. Kurstok.** 1984. Highly efficient and simple methods for the preparation of peroxidase and active peroxidase-antibody conjugates for enzyme immunoassays. *Anal. Biochem. 136:451.*
19. **Weisman, H. F., T. Bartow, M. K. Leppo, H. C. Marsh Jr., G. R. Carson, M. F. Concino, M. P. Boyle, K. H. Roux, M. L. Weisfeldt, and D. L. Fearon.** 1990. Soluble human complement receptor Type 1: *In vivo* inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science 249:146.*
20. **Grosen, E. A., R. S. Yamamoto, G. Ioli, E. K. Ininns, M. Gatanaga, T. Gatanaga. P. J. Disaia, M. Berman, A. Manetta, and G. A. Granger.** 1992. Blocking factor (soluble membrane receptors) for tumor necrosis factor and lymphotoxin detected in ascites and released in short-term cultures obtained from ascites and solid tumor in women with gynecologic malignancy. *Lymphokine Cytokine Res. 11:347.*

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