New Application of Human Tumor Clonogenic Assay to *in Vitro* Evaluation of Tumor-Targeting Efficiency of Immunoconjugates

Shunji NAGATA,* Kazuyoshi MASUDA, Hideo NOGUSA, Koichiro HIRANO, and Yasushi TAKAGISHI

Shionogi Research Laboratories, Shionogi & Co., Ltd., 5–12–4 Sagisu, Fukushima-ku, Osaka 553, Japan. *Received December 6, 1991*

This report proposes an efficient *in vitro* method for the evaluation of drug targeting with monoclonal antibody as a carrier to tumor cells. Monoclonal antibody (35G; IgG_{2a}) selectively binding to α-fetoprotein (AFP) from human hepatoma cells (HuH-7) was conjugated with an anticancer drug, vindesine (VDS). Human tumor clonogenic assay (HTCA) with some modifications was applied to estimate the targeting efficiency of a conjugate (VDS–35G) for the first time. In this assay, VDS–35G was cytotoxically active against HuH-7 cells at a lower concentration (0.5 ng/ml) and for a shorter contact time than VDS (50 ng/ml), while 35G and VDS–normal mouse immunogloblin conjugate (VDS–n-IgG) were not active against the cells. Both VDS–35G and VDS–n-IgG were inactive against HuH-13 cells established from a human hepatocellular carcinoma producing no AFP. In the conventional monolayer culture assay (MCA), VDS–35G showed little effect on HuH-7 cells at the concentration effective in HTCA. The cytotoxic activity of VDS in MCA was similar to that in HTCA but the cytotoxic activity of VDS–35G in MCA was considerably different from that in HTCA. This discrepancy could be explained by the hypothesis that VDS–35G was directed at stem cells of the HuH-7 cell population sensitively and selectively. HTCA was shown to be a useful *in vitro* evaluation method for drug targeting.

Keywords α-fetoprotein; monoclonal antibody; human hepatoma cell; vindesine; human tumor clonogenic assay; monolayer culture assay; immunoconjugate; tumor-targeting

The ideal drug delivery system in cancer chemotherapy is one in which the anticancer drug is selectively delivered to the neoplastic cells and has no influence on healthy tissue. Many selective delivery systems of anticancer drugs have been reported: liposomes,[1] emulsions,[2] and monoclonal antibody (MoAb) carrier systems.[3–6] The extent of delivery of anticancer drugs included in liposomes or emulsions to tumor tissues depends on physical factors such as particle size and surface charge, and their targeting efficiency is not always satisfactory.[1b,c] MoAb is expected to selectively deliver coupled anticancer drugs to tumor tissues if the mutual recognition between MoAb and tumor cells is not disturbed. MoAb against the antigens on the surface of tumor cells or the tumor-associated antigens is considered to be one of the most selective carriers to the neoplastic cells among the many delivery systems proposed. For example, MoAbs against human squamous carcinoma,[4] carcinoembryonic antigen (CEA),[5] and human adenocarcinoma[6] have been used as carriers for tumor-targeting.

MoAbs conjugated with anticancer drugs have usually been evaluated by *in vivo* methods. Only sparse information is available on *in vitro* methods for primary evaluation of MoAb-related carriers. Not only anti-tumor activity but also selectivity to tumor cells should be quantitatively examined in the case of tumor-targeting. There is a need for an appropriate *in vitro* method for evaluating the efficiency of drug targeting systems from the viewpoint of pharmaceutics.

In the present study, human hepatoma cells were used as a target because, although hepatocellular carcinoma is one of the common cancers, few drugs are known to combat it; development of more effective and less toxic drugs for cancer chemotherapy is truly desired.

A new conjugate between murine MoAb against human α-fetoprotein (AFP) and a vinca alkaloid was synthesized. AFP is a major serum protein synthesized in liver, gastrointestinal tract, and yolk sac during fetal life. AFP is similar to albumin in overall amino acid sequence, but certain parts of their predicted secondary structures are significantly different.[7] AFP is a carcinoembryonic protein, which is known to be produced by approximately 80% of clinically diagnosed hepatoma cells.

A vinca alkaloid was chosen because of its unique mechanism of action. Most of the biological activities of vinca alkaloids seem to be explained by their ability to bind specifically to tubulin and to block the ability of the protein to polymerize into microtubules.[8] Anti-AFP antibodies have been studied as carriers of daunorubicin[9] and doxorubicin,[10] but there has been no report on vinca alkaloids.

The *in vitro* activity of our new conjugate was evaluated using a method of modified human tumor clonogenic assay (HTCA). HTCA was originally developed to study the kinetic and biological properties of human primary tumor stem cells, and was applied to the *in vitro* sensitivity testing of anticancer drugs.[11] The assay had not previously been used for evaluation of drug targeting systems. The cells are placed in contact with a drug for a limited period in HTCA, while in a conventional monolayer culture assay (MCA) they must remain in contact during the entire cultivation period. This paper presents a brief description of the modified HTCA and then evaluates the characteristics of a new conjugate synthesized for tumor-targeting. The results are used to discuss the usefulness of the modified HTCA.

Experimental

Materials Human serum albumin was purchased from Sigma (Missouri, U.S.A.) and normal human serum from Miles Laboratories (Indiana, U.S.A.). Normal mouse immunogloblin G (n-IgG) was obtained from Jackson Immunoresearch Laboratories (Pennsylvania, U.S.A.); agar from Oxoid (Hampshire, England). Vinblastine sulfate was purchased from Wako Pure Chemical Industries (Osaka, Japan). Vindesine sulfate (VDS) was obtained from Shionogi and Co., Ltd. (Osaka, Japan). All other chemicals were of reagent grade.

Hepatoma Cells An AFP-producing human hepatoma cell line (HuH-7) and an AFP-non-producing human hepatoma cell line (HuH-13) were

provided by Dr. H. Hirai (Foundation for Basic Oncological Research, Tokyo, Japan). HuH-7 cells were cultured in serum-free medium (IS-RPMI[12]) that was improved for cultivation, and HuH-13 cells were grown in RPMI 1640 medium (GIBCO, New York, U.S.A.) supplemented with 15% fetal calf serum (FCS; Flow Laboratories, North Ryde, Australia).

Production of MoAb AFP was purified from supernatants of *in vitro* cultured HuH-7 cells by affinity chromatography on Sepharose 4B coupled with anti-AFP rabbit polyclonal antibodies. Mice were immunized with AFP in Freund's complete adjuvant (Difco Laboratories, Detroit, U.S.A.) and their spleen cells were fused with mouse myeloma cells (NS-1), using 50% solution of polyethylene glycol 4000 (Merck, Darmstadt, Germany). The hybridoma clone (clone line no. H-35G) selected in the usual way was inoculated i.p. into pristane-primed BALB/c mice, and ascites fluid of the mice was harvested a few weeks later. MoAb (35G) was purified from the ascites fluid by sodium sulfate fractionation followed by Protein A column chromatography.

Synthesis of Conjugates 35G or n-IgG was bound to 4-desacetylvinblastine acid azide that was derived from vinblastine *via* two steps according to the procedure described for VDS–bovine serum albumin conjugate by Conrad *et al.*[13] The synthesized conjugates (VDS–35G, VDS–n-IgG) were purified using Sephadex G-100 (Pharmacia LKB, Uppsala, Sweden) and concentrated using Collodion bags (Sartorius Gmbh, Göttingen, Germany).

Characteristics of MoAb and Conjugates The subclass of 35G was determined by the Ouchterlony test. The purity and affinity of 35G and its conjugates were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and enzyme-linked immunoadsorbent assay (ELISA), respectively. Chemically bound VDS analogue per mole of the protein was determined from its characteristic absorption spectra (270, 280 nm) of the conjugates. The protein of the conjugates was measured by the Lowry method.[14]

HTCA Procedure A two-layer soft agar culture system described by Hamburger and Salmon[11] was adopted with several modifications. The medium in both layers was changed to IS-RPMI without FCS from McCoy's 5A medium with 15% FCS in the lower layer and CMRL 1066 medium with 20% FCS in the upper layer.[11b] HuH-7 cells cultured in IS-RPMI were harvested by trypsin-EDTA treatment. The cells (1×10^6) dispersed in 2 ml of IS-RPMI were exposed to a test drug (VDS, VDS–35G, or VDS–n-IgG) for 60 min unless otherwise stated. After exposure, they were centrifuged at room temperature and 1000 rpm for 5 min to remove the drug remaining in the bulk medium. They were then rinsed with 5 ml of cell-free conditioned medium (CFCM) and centrifuged under the same conditions as above. Two ml of CFCM suspending the cells was mixed with 3 ml of 5% agar diluted with IS-RPMI at 45 °C and the mixture was superposed onto the 5% basic agar prelayered in a 35-mm tissue culture dish (Nunc, Inc., Illinois, U.S.A.), giving 2×10^4 cells/dish. The cultures were incubated at 37 °C in a 5% CO_2 atmosphere for about 2 weeks, then colonies larger than 20 μm in diameter were counted by microscopic observation. IS-RPMI without a drug was used as a control. When HuH-13 cells were tested, RPMI 1640 supplemented with 15% FCS was used instead of IS-RPMI and CFCM. The sensitivity index of a test drug against tumor cells was calculated by the equation described below. A drug with an index greater than 50% was considered to be effective based on the criterion by Kawamura *et al.*[15]

$$\text{sensitivity index} = \left(1 - \frac{\text{number of colonies for a test sample}}{\text{number of colonies for control}}\right) \times 100$$

MCA Procedure HuH-7 cells (1×10^5) were pre-cultured in 0.5 ml of IS-RPMI consisting of 50% CFCM at 37 °C in a 5% CO_2 atmosphere. Following 2 d of pre-culture, the cells were incubated with various concentrations of a test drug dissolved in IS-RPMI under the above conditions and the medium was replaced at 2 or 3-d intervals. Eight days after initial exposure, the cells were counted under a microscope using the trypan blue exclusion method.

Results

Characteristics of Conjugates 35G (IgG_{2a}) produced by H-35G was bound to 4-desacetylvinblastine acid azide as well as n-IgG. The conjugates were fractionated from an unbound drug by Sephadex G-100 chromatography. The first peak was assigned to the conjugate and the second to small molecular compounds including a modified VDS using the molecular weight calibration kits of Pharmacia (Uppsala, Sweden). The fractions coinciding with the first peak were collected, concentrated to an appropriate level, and were used after sterilization with a 0.22-μm filter. SDS–PAGE analysis of VDS–35G showed only a single band stained with Coomassie Blue, which migrated near the same molecular weight as 35G. The molar ratio of VDS to 35G or n-IgG in the conjugates was 3.1 or 2.4, respectively. The affinity of 35G and VDS–35G to AFP and that to normal human serum are shown in Fig. 1.

Neither 35G nor VDS–35G reacted with human serum albumin or normal human serum. The binding activity of VDS–35G to AFP was almost equal to that of 35G. Therefore, the covalent binding between vindesine and 35G did not affect the antibody activity of 35G.

Evaluation of Cytotoxic Activity against HuH-7 Cells of VDS, VDS–35G, and VDS–n-IgG in HTCA The plating efficiency in HTCA is defined as the percentage of the number of colonies to the number of plated cells.[16] Under the present experimental conditions, the plating efficiency of HuH-7 cells ranged from 0.53 to 0.68% for 2×10^4 cells/dish, and the number of colonies of HuH-7 cells depended on the number of plated cells as shown in Table I.

The sensitivity indices of VDS and VDS–35G are plotted against the concentration of VDS equivalent in Fig. 2. Plots of both VDS and VDS–35G showed sigmoid-shaped curves. The effective concentration of VDS–35G was more than 0.5 ng/ml, one-hundredth that of VDS.

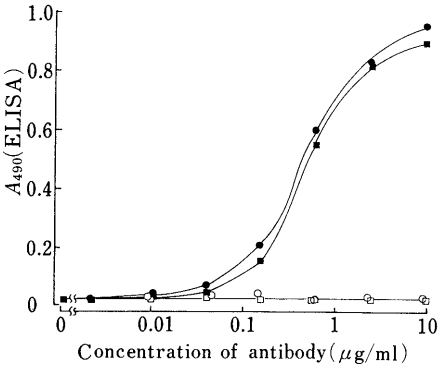

Fig. 1. Affinity of 35G and VDS–35G to AFP and to Normal Human Serum Measured by ELISA

ELISA plates were coated with 50 μl of the solution of AFP (10 μg/ml) or normal human serum, ●, 35G to AFP; ■, VDS–35G to AFP; ○, 35G to normal human serum; □, VDS–35G to normal human serum.

TABLE I. Relationship between Number of Cells Plated and Number of Colonies Formed in HTCA

Plated cells (/dish)	Colonies[a]	Plating efficiency (%)
1×10^4	74±13	0.74
2×10^4	136±14	0.68
	114± 5	0.57
	106±14	0.53
	121±20	0.61
4×10^4	130±11	0.33
	139±24	0.35
11×10^4	467±36	0.42

a) Mean±S.D. of three dishes.

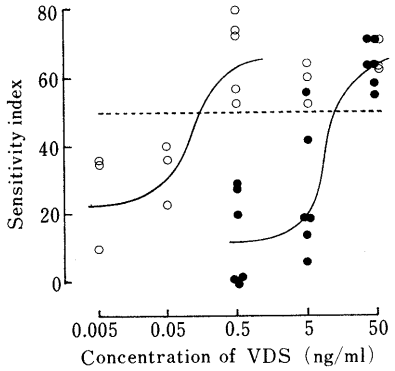

Fig. 2. Sensitivity Index of VDS and VDS–35G against HuH-7 Cells in HTCA

HuH-7 cells (1×10^6) remained in contact with 2 ml of VDS or VDS–35G solution for 1 h at 37 °C. ●, VDS; ○, VDS–35G. A drug is effective above the dotted line (50%).

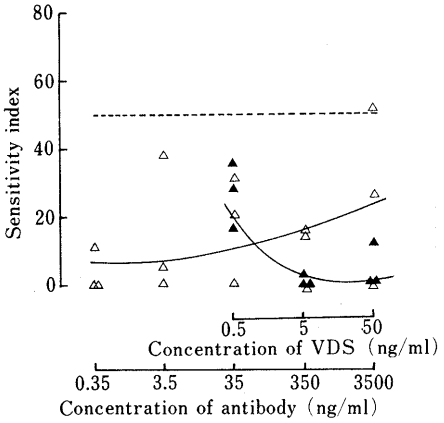

Fig. 3. Sensitivity Index of 35G and VDS–n-IgG against HuH-7 Cells in HTCA

HuH-7 cells (1×10^6) remained in contact with 2 ml of 35G or VDS–n-IgG solution for 1 h at 37°C. △, 35G; ▲, VDS–n-IgG.

The sensitivity indices of 35G and VDS–n-IgG against HuH-7 cells are shown in Fig. 3. The range of protein concentration of 35G was similar to that of VDS–35G shown in Fig. 2. The sensitivity indices of VDS–n-IgG were less than 50% in the concentration range tested. At the protein concentration of 3500 ng/ml of 35G, one of the three trials showed an index of 52%, while the others were less than 50%. The indices for the other protein concentrations were also less than 50%. Although the index of 52% of 35G may show that it became effective at higher concentrations, neither 35G nor VDS–n-IgG was effective against HuH-7 cells in the concentration range tested in HTCA. The sensitivity indices of the mixture of VDS and 35G (its molar ratio was the same as VDS–35G) were less than 50% at the concentration of 0.5 ng/ml of VDS (data not shown). The simple mixture of VDS and 35G was not effective at this concentration.

Evaluation of Cytotoxic Activity against HuH-13 of VDS, VDS–35G, and VDS–n-IgG in HTCA The sensitivity indices of VDS bound to either 35G or n-IgG against HuH-13 cells were lower than 50%, as shown in Fig. 4. Thus, neither VDS–35G nor VDS–n-IgG showed effective cytotoxic activity to HuH-13 producing no AFP.

Effect of Contact Period on Sensitivity of VDS and VDS–35G in HTCA In Figs. 2—4, HuH-7 cells were exposed to a test drug for 60 min. In Fig. 5, VDS–35G and VDS were kept in contact with HuH-7 cells for 5 to 120 min at the concentrations of 5 (of VDS equivalent) and 50 ng/ml, respectively, at which they were estimated to be effective (Fig. 2). VDS needed to remain in contact with the cells for 60 min to become positive in HTCA, while VDS–35G required only 10 min.

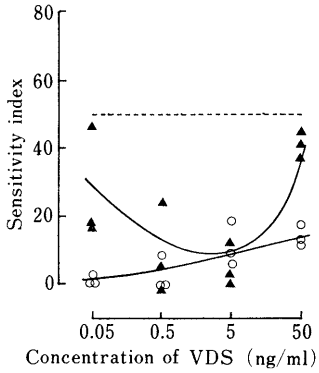

Fig. 4. Sensitivity Index of VDS–35G and VDS–n-IgG against HuH-13 Cells Producing No AFP in HTCA

HuH-13 cells (1×10^6) remained in contact with 2 ml of VDS–35G or VDS–n-IgG solution for 1 h at 37 °C. ○, VDS–35G; ▲, VDS–n-IgG.

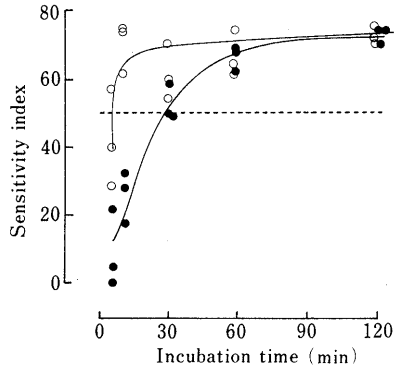

Fig. 5. Effect of Contact Time on Sensitivity

HuH-7 cells (1×10^6) remained in contact with 2 ml of VDS or VDS–35G solution (each containing 50 or 5 ng/ml of VDS equivalent, respectively) for 5 to 120 min at 37 °C. ●, VDS; ○, VDS–35G.

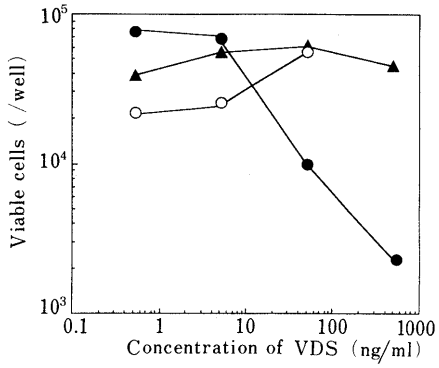

Fig. 6. Sensitivity of VDS and VDS–35G against HuH-7 Cells in MCA

HuH-7 cells (1×10^5) in IS-RPMI (50% CFCM) were plated on 24-well multiplates. Two days later, the medium was replaced by IS-RPMI containing VDS or VDS–35G. The drug-containing medium was replaced at 2 or 3-d intervals and viable cells were counted using trypan blue exclusion, 8 d after initial exposure. $n=2$. ●, VDS; ○, VDS–35G; ▲, control.

Evaluation of Cytotoxic Activity against HuH-7 of VDS

and VDS–35G in MCA Drug activity is considered positive in MCA where the number of viable cells is less than 50% of the control. As shown in Fig. 6, the active concentration of VDS was more than 50 ng/ml, which was similar to the result of HTCA. However, VDS–35G was not active at the same concentration as VDS. We could not use a higher concentration of VDS–35G as this resulted in aggregation.

Discussion

HuH-7 and HuH-13 cell lines were used in this study. Many primary human tumor cells were reported to yield a plating efficiency of 0.001—0.1%.[11b,16] Although HuH-7 cells were tested in serum-free medium, their plating efficiency was 0.3—0.7%. HuH-7 is considered to be one of the cell lines which easily forms colonies. The number of colonies in HTCA was correlated to the number of cells plated (correlation coefficient = 0.972).

VDS–35G was 100 times more active than VDS in HTCA (Fig. 2). It was also examined in MCA, and the results (shown in Fig. 6) differed from those in HTCA. The difference in results can be discussed by making some assumptions. There are three cell categories within the total tumor cell population: a) nonproliferating, differentiated (end) cells, b) proliferating, nonrenewing (transitional) cells, and c) proliferating, self-renewing (stem) cells.[17] Stem cells are considered to represent a small subpopulation of the total cells in solid tumors. If self-renewing stem cells express more AFP on their surfaces, more anti-AFP MoAb may accumulate on these cells. In HTCA, VDS–35G might be concentrated in stem cells forming colonies, although there is no direct evidence that tumor colony-forming units are the same as tumor stem cells.[16] On the other hand, the cells in MCA remained in contact with the drug for 4 d. During this period, VDS was non-selectively taken up into the cells. In MCA, non-stem cells which are little affected by VDS–35G may be cultivated and counted. Although the assumptions described above cannot be backed with any evidence at present, they may offer a good explanation of the discrepancy between HTCA and MCA.

In HTCA, VDS–35G was effective against HuH-7 cells at the concentration of 0.5 ng/ml of VDS equivalent, but the simple mixture of VDS and 35G was not effective at the same concentration. VDS–n-IgG was not effective in the concentration range tested. Neither conjugate was effective against HuH-13 cells. These results suggest that VDS–35G is attracted to HuH-7 cells by the affinity of 35G to AFP present on the surface of the cells and is then easily taken up into the cells.

The cytotoxic activities of VDS and VDS–35G became effective when they were kept in contact with HuH-7 cells over 60 and 10 min, respectively. The difference shows that 35G is more sensitively directed to HuH-7 cells. It is an advantage of HTCA that the contact time between a drug and tumor cells can be varied. It is possible to estimate one of the functions of tumor-targeting by comparing the contact time.

The HTCA procedure is complex and tedious. However, the contact time is adjustable and the remaining drug can be removed after exposure. The most important difference is that in HTCA it is mainly colony-forming cells that are evaluated. The results of this study strongly suggest that the modified HTCA proposed here is a reasonable and useful method for *in vitro* evaluation of targeting drugs like MoAb-anticancer agent conjugates. The feasibility of this method should be confirmed by clarifying the correlation between *in vitro* and *in vivo* results. The gathering such data is now in progress.

Acknowledgments The authors are indebted to Dr. H. Hirai for supplying HuH-7 and HuH-13. They would like to acknowledge the continuing guidance and encouragement of Dr. S. Harada, Dr. T. Sakamoto (Shionogi), and Dr. H. Maekawa.

References

1) *a*) D. Layton, J. De Meyere, M. Collard, and A. Trouet, *Eur. J. Cancer*, **15**, 1475 (1979); *b*) R. L. Juliano and D. Layton, "Drug Delivery Systems," ed. by R. L. Juliano, Oxford University Press, New York, 1980, Chapter 6; *c*) J. N. Weinstein, *Cancer Treat. Rep.*, **68**, 127 (1984).
2) *a*) G. W. Halbert, J. F. B. Stuart, and A. T. Florence, *Int. J. Pharm.*, **21**, 219 (1984); *b*) R. J. Prankerd and V. J. Stella, *J. Parenter. Sci. Technol.*, **44**, 139 (1990).
3) B. P. Ram and P. Tyle, *Pharm. Res.*, **4**, 181 (1987); V. V. Ranade, *J. Clin. Pharmacol.*, **29**, 873 (1989); C. Kosmas, H. Kalofonos, and A. A. Epenetos, *Drugs*, **38**, 645 (1989).
4) D. A. Johnson and B. C. Laguzza, *Cancer Res.*, **47**, 3118 (1987).
5) J. R. Johnson, C. H. J. Ford, C. E. Newman, C. S. Woodhouse, G. F. Rowland, and R. G. Simmonds, *Br. J. Cancer*, **44**, 372 (1981); C. H. J. Ford, C. E. Newman, J. R. Johnson, C. S. Woodhouse, T. A. Reeder, G. F. Rowland, and R. G. Simmonds, *ibid.*, **47**, 35 (1983); G. F. Rowland, J. R. F. Corvalan, C. A. Axton, V. A. Gore, C. H. Marsden, W. Smith, and R. G. Simmonds, *Prot. Biol. Fluids*, **31**, 783 (1984).
6) K. Fukuda, *Akita J. Med.*, **12**, 451 (1985); B. C. Laguzza, C. L. Nichols, S. L. Briggs, G. J. Cullinan, D. A. Johnson, J. J. Starling, A. L. Baker, T. F. Bumol, and J. R. F. Corvalan, *J. Med. Chem.*, **32**, 548 (1989).
7) T. Morinaga, M. Sakai, T. G. Wegmann, and T. Tamaoki, *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 4604 (1983).
8) P. Calabresi and B. A. Chabner, "The Pharmacological Basis of Therapeutics," 8th ed., ed. by A. G. Gilman, T. W. Rall, A. S. Nies, and P. Taylor, Pergamon Press Inc., New York, 1990, Chapter 52.
9) Y. Kato, N. Umemoto, Y. Kayama, H. Fukushima, Y. Takeda, T. Hara, and Y. Tsukada, *J. Med. Chem.*, **27**, 1602 (1984); Y. Tsukada, K. Ohkawa, and N. Hibi, *Cancer Res.*, **47**, 4293 (1987).
10) H. Konno, H. Suzuki, T. Tadakuma, K. Kumai, T. Yasuda, T. Kubota, S. Ohta, K. Nagaike, S. Hosokawa, K. Ishibiki, O. Abe, and K. Saito, *Cancer Res.*, **47**, 4471 (1987).
11) *a*) A. W. Hamburger and S. E. Salmon, *Science*, **197**, 461 (1977); *b*) A. W. Hamburger and S. E. Salmon, *J. Clin. Invest.*, **60**, 846 (1977); *c*) A. W. Hamburger, *Int. J. Cell Cloning*, **3**, 89 (1987).
12) H. Nakabayashi, K. Taketa, T. Yamane, M. Miyazaki, K. Miyano, and J. Sato, *Gann*, **75**, 151 (1984).
13) R. A. Conrad, G. J. Cullinan, K. Gerzon, and G. A. Poore, *J. Med. Chem.*, **22**, 391 (1979).
14) O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
15) M. Kawamura, K. Yamashita, T. Sagawa, Y. Yorozu, and T. Shimizu, *Obstetrical and Gynecological Practice*, **35**, 1907 (1986).
16) K. Inoue, M. Arakawa, and M. Ogawa, *Jpn. J. Cancer Chemother.*, **9**, 599 (1982).
17) W. J. Mackillop, A. Ciampi, J. E. Till, and R. N. Buick, *J. Natl. Cancer Inst.*, **70**, 9 (1983).