JOURNAL OF IMMUNOLOGICAL METHODS

Vol. 152, no. 2, 1992

ELSEVIER SCIENCE PUBLISHERS B.V.
AMSTERDAM – LONDON – NEW YORK – TOKYO

Journal of Immunological Methods, 152 (1992) 209–216

JIM 06374

Evaluation of biological response modifiers in the enhancement of tumor uptake of technetium-99m labeled macromolecules

A preliminary report

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(Received 21 November 1991, revised received 28 February 1992, accepted 9 March 1992)

Imaging tumors with radioactive monoclonal antibodies remains attractive but continues to be challenging. With the hypothesis that the use of biological response modifiers (BRMs) may augment the tumor uptake, technetium-99m(^{99m}Tc)-labeled tumor necrosis factor (TNF) and nuclear histone specific TNT-1-F(ab')$_2$ were evaluated in tumor bearing mice given a single dose of interferon (IFN), Ukrain or pokeweed mitogen as BRMs. As early as 1.5 h post injection (p.i.) of the radioactive macromolecules, the absolute tumor uptake (% administered dose/g) of each agent was enhanced (e.g., TNF, control = 1.8 ± 0.4, Ukrain = 3.2 ± 0.5, $P = 0.006$) and tumor to muscle ratios were elevated (e.g., TNF, control a 4.1 ± 2.2, interferon 8.3 ± 2.7, $P = 0.01$). The absolute tumor uptake remained practically unchanged at 4 h p.i. Generally with BRMs, the blood clearance was rapid and tumor/blood ratios and tumor/muscle ratios were higher than in the control group, increasing to greater than 200% for IFN as a BRM.

The early enhancement in tumor uptake of macromolecules, leading to excellent delineation of tumors by scintigraphy is highly encouraging and warrants further studies to explore the full potential of BRMs.

Key words: Technetium-99m-labeled macromolecule; Biological response modifiers; Tumor uptake enhancement

Introduction

In recent years a battery of monoclonal antibodies (MAbs) have been generated against a variety of human tumors and innovative methods for labeling MAbs with efficient radionuclides such as ^{99m}Tc have been developed (Khaw et al., 1982, Rhodes et al., 1986; Schwarz et al., 1987; Fritzberg et al., 1988; Eckelman et al., 1989; Mather et al., 1990, Thakur et al., 1991). The combination of these events has prompted several investigators to evaluate radiolabeled MAbs for scintigraphic detection of tumors (Eary et al., 1989; Pyrhonen et al., 1990; Baum et al., 1991; Lind et al., 1991). Although some excellent results have been obtained, general concerns remains for a variety of reasons that the full promise of radiolabeled MAbs has yet to be met. The

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challenge therefore is to continue to develop techniques that will enable investigators to identify lesions unequivocally. One approach among several, toward this goal is to seek ways that will enhance the tumor uptake of the radiolabeled MAb itself.

This investigation deals with a preliminary evaluation of certain compounds, generally known as biological response modifiers (BRMs), with a high potential to augment tumor uptake of certain ^{99m}Tc-labeled tumor-specific and non-specific macromolecules.

Materials and methods

The following two agents labeled with ^{99m}Tc were chosen as a specific and non-specific macromolecules.

TNT-1-F(ab')$_2$

This fragmented tumor necrosis treatment antibody (TNT-1), specific for nuclear histones, was prepared by and obtained from Dr. A. Epstein of USC-LA (Chen et al., 1989). The hypothesis was that this MAb will specifically interact with the nuclear histones available from the degenerated cells in the necrotic center of the tumor.

Tumor necrosis factor (α)

Tumor necrosis factor (α, TNF-α) was chosen as a non-specific macromolecule and was obtained from Finn-Tech Industries, NJ. TNF-α, also known as cachectin (Palladino et al., 1987), is a 17 kDa protein, produced primarily by activated macrophages, and to a lesser extent by natural killer cells and bone marrow mast cells. It exists in a trimeric form, consists of 157 amino acid residues and a disulfide bridge (Wanebo et al., 1989). The question whether the trimeric form of the TNF-α is essential for its full biological activity has not yet been fully resolved (Rosenblum et al., 1989).

The following three biological response modifiers (BRMs) were evaluated.

Interferon

Interferon-γ (IFN-γ) is a product of activated T lymphocytes and natural killer cells that was originally described as an antiviral agent (Wleelock et al., 1991). Human recombinant IFN (Sigma Chemicals) is a dimer, and has an α helical structure with six helices in each subunit (Elick et al., 1991). IFN predominantly activates cytotoxic effector cells and has a broad spectrum of biological activities that have drawn considerable attention to its potential therapeutic use against malignant diseases (Koeller et al., 1989). These effects are considered to be exerted by augmentation of macrophage cytotoxic activity, natural killer cell activity and T lymphocyte activity. These activities promote tumor blood flow and enhance capillary permeability (Staren et al., 1989).

Ukrain

Ukrain is a thiophosphoric acid triaziridine derivative of an alkaloid separated from the plant *Chelidonium majus* L. It was prepared by Nowicky et al. (1989), and was obtained from RhoMed N.M. Ukrain has been used in patients in Europe and shown to have anti-cancer activity (Norwicky, 1989). Patients receiving Ukrain have felt warmth and pain at the tumor site, considered to be consistent with Ukrain stimulated blood flow and accumulation of extracellular fluid brought about by infiltrating lymphocytes and macrophages.

Pokeweed

Pokeweed mitogen (PKWD) is a salt extract of ground roots of *Phytolacca America*. PKWD was purified by and obtained from Dr. P. Mann of the University of New Mexico, Albuquerque. PKWD has a long history of cell proliferative response in rodents and is known to induce regression of implanted tumors (Mann et al., 1991). Following inoculation of PKWD, tumor swelling, presumably due to increased extracellular fluid content, has been frequently observed. PKWD consists of five separate proteins each of which is different in biological activity, molecular weight and composition of amino acid residues. The number of cysteine residues, for example, varies from 8 to 50 per molecule (Waxdal, 1974).

Murine embryonal carcinoma

The murine embryonal carcinoma served as a tumor model. Cells (NF-1) were grown in tissue culture in the Department of Cell Biology and

Pathology at our institute (Fox et al., 1983). The cells were pooled, concentrated to approximately 10^8/ml and a 0.1 ml suspension was injected i.m. into the right thigh of BALB/c mice weighing between 20–25 g. Tumors were allowed to grow for 8–10 days reaching 0.5–1 cm in diameter.

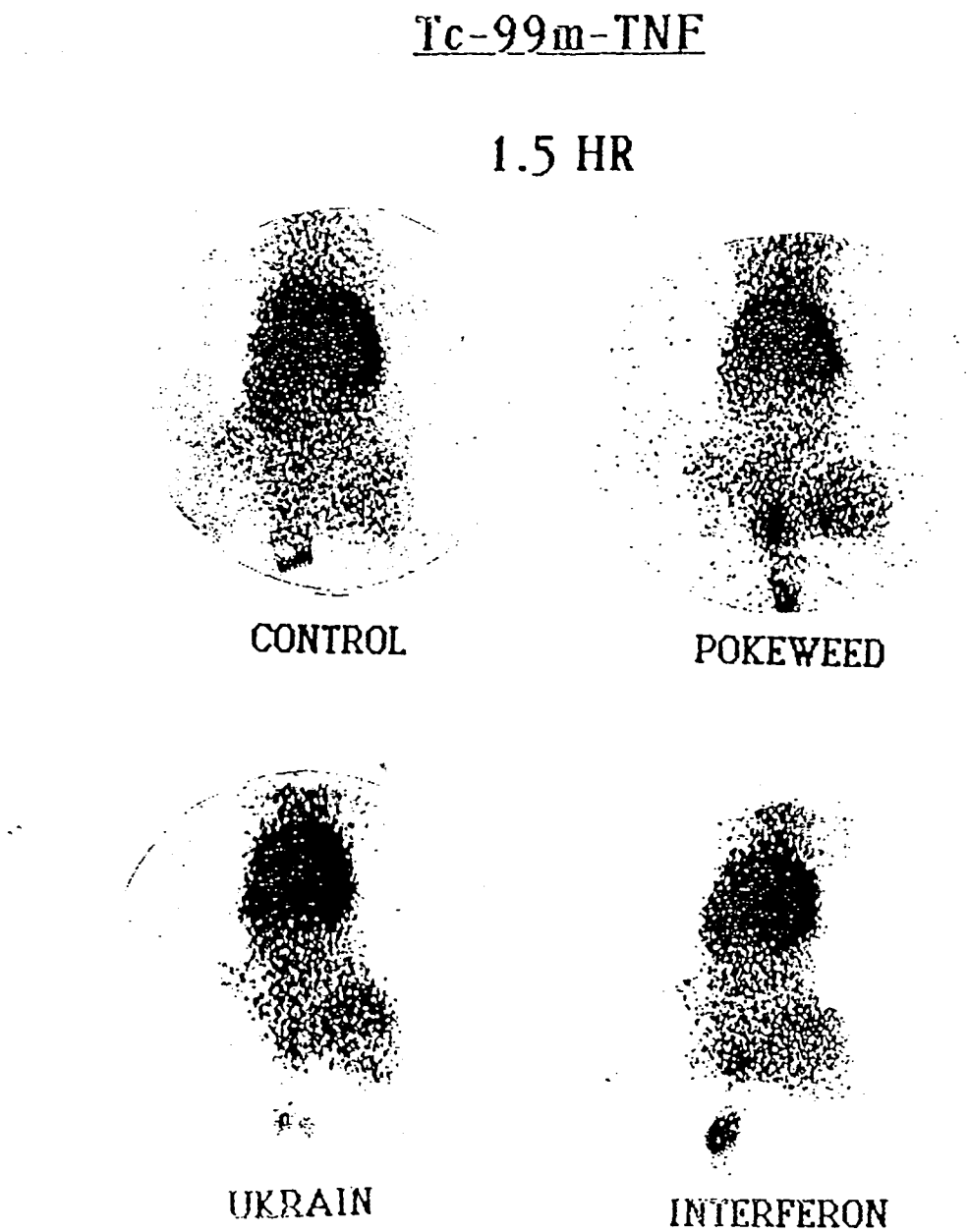

Fig. 1. A composite of four posterior images of four separate tumor bearing animals representing a control group and the three BRM receiving groups. Images were obtained at 1.5 h post injection of ^{99m}Tc tumor necrosis factor. Tumors in the right thigh are detectable and the enhancement in BRM receiving groups is clearly visible.

Labeling macromolecules with ^{99m}Tc

Both agents were labeled with ^{99m}Tc by a method developed recently in our laboratory (Thakur et al., 1991). Briefly, 20–200 μg of the agents were incubated with 3500 times molar excess of ascorbic acid (pH 6.5) for 1 h at 22°C. To a required quantity of freshly eluted ^{99m}Tc solution was then added $Na_2S_2O_4$ (Fluka Chemicals) dissolved freshly in nitrogen purged 0.1 M bicarbonate buffer pH 11. The final concentration of $Na_2S_2O_4$ was 5 μg/μl reaction mixture which was allowed to react for 1 min and then added to the ascorbic acid reduced macromolecule. This was vortexed and further incubated at 22°C for 30 min. In order to eliminate any unbound ^{99m}Tc, the TNT-F(ab')$_2$ was subjected to a molecular filtration through Centricon-30 (Amicon, Boston, MA) and TNF through Centricon-3. The labeled products were collected and subjected to HPLC and two types of instant thin layer chromatographic analysis as described previously (Thakur et al., 1991). The preparations were then dispensed in 300 μl volumes for injections.

Protocol

Animals, each bearing the implanted tumor, were separated into several groups of five mice each. They were subdivided into those receiving ^{99m}Tc-TNF or ^{99m}Tc-TNT-1-F(ab')$_2$ (40 μCi/10 μg/300 μl). 1 h prior to the i.v. administration of ^{99m}Tc-TNF each animal received i.p. either 10 μg of Ukrain, 10 μg PKWD or 1000 IU of IFN. Animals in the control group received 100 μl of sterile isotonic saline. At 90 or 240 min post administration of ^{99m}Tc agents, animals were killed by inhalation of halothane, imaged in the posterior position (pinhole collimator) and dissected for quantitative tissue distribution studies.

Animals receiving ^{99m}Tc-TNT-1-F(ab')$_2$ were given (i.p.) only 1000 IU of IFN in the study group and saline in the control group. These were also imaged and killed at 90 min or 4 h p.i.

During dissection, the entire tail was removed and the associated radioactivity was counted. This was subtracted from the total dose received by the corresponding animal, accounting for any dose infiltrated into the tail during i.v. injection. The radioactivity was then calculated as percent administered dose/g of tissue and also as tumor to tissue ratios. These were evaluated against the control group using the Student's t test.

Results

The ^{99m}Tc-labeling efficiency for both agents averaged approximately 70%. The colloid formation in each preparation as determined by ITLC was less than 3%.

The in vivo results at 1.5 h p.i. given in Table I clearly indicated that with each BRM we were able to observe a definite increase in tumor/muscle (T/M) ratios. The ratios were highest (> 200%, $P = 0.01$) with IFN, modest (> 150%, $P = 0.09$) with Ukrain and marginal (> 125%) with PKWD. Consistent with the increase in the T/M ratios at 1.5 h, the absolute tumor uptake for all BRM animals was also enhanced from $1.8 \pm 0.4\%$ in the control group for example, to as much as $3.2 \pm 0.5\%$ ($P = 0.006$) in the Ukrain group. The net visual effect in imaging tumors is given in Fig. 1. At 4 h p.i. the T/M ratios in the control group had increased from the value at 1.5 h post injection, but the enhancement was statistically insignificant.

The tissue distribution in major organs from animals studied at 1.5 h is given in Table II and that at 4 h in Table III. In Table II the increase in the tumor uptake in all BRM receiving animals

TABLE I

TUMOR/MUSCLE AND ABSOLUTE (% ADMINISTERED DOSE/g) TUMOR UPTAKE OF ^{99m}Tc-TNF-α IN CONTROL AND BRM RECEIVING ANIMALS AT 1.5 AND 4 h p.i.

	Tumor/muscle		Absolute tumor uptake	
	1.5 h	4 h	1.5 h	4 h
Control	4.1 ± 2.2 [a,b]	7.4 ± 2.2 [a]	1.8 ± 0.4 [c]	2.6 ± 0.9
IFN	8.3 ± 2.7 [b]	9.4 ± 0.8	2.5 ± 0.7	2.7 ± 0.15
Ukrain	6.2 ± 2.1	6.1 ± 3.3	3.2 ± 0.5 [c]	2.8 ± 1.3
PKWD	5.1 ± 1.1	5.5 ± 0.9	2.5 ± 0.2	2.3 ± 0.4

[a] T/M ratios at 1.5 h and 4 h p.i. in control animals, $P = 0.15$.
[b] T/M ratios at 1.5 h p.i. in control and INF receiving animals, $P = 0.01$.
[c] Absolute tumor uptake at 1.5 h p.i. in control and Ukrain receiving animals, $P = 0.006$.

TABLE II

TISSUE DISTRIBUTION (% ADMINISTERED DOSE/g) OF ^{99m}Tc TNF-α IN CONTROL AND BRM GIVEN ANIMALS AT 1.5 h p.i.

	Control	IFN	Ukrain	PKWD
Urine [a]	183.9 ± 25.5	47 ± 2.3	147 ± 40.4	186.1 ± 0
Blood	9.6 ± 0.7	5.7 ± 0.5	9.5 ± 3.0	10 ± 1.3
Kidneys	19.2 ± 1	10.9 ± 1.0	16.6 ± 2.5	15.3 ± 2.3
Intestine	2.2 ± 0.1	1.3 ± 0.5	1.8 ± 0.2	1.7 ± 0.7
Muscle	0.45 ± 0.0	0.3 ± 0.0	0.5 ± 0.2	0.5 ± 0.2
Tumor	1.8 ± 0.3	2.5 ± 0.7	3.2 ± 0.5	2.5 ± 0.2
Spleen	16.6 ± 1.1	18.6 ± 2.0	16.9 ± 1.3	15.6 ± 4.8
Lungs	4.9 ± 0.4	4.1 ± 2.2	10 ± 5.4	6.1 ± 0.5
Liver	35 ± 1.5	51.3 ± 2.2	48.9 ± 8.1	33.4 ± 5.4
Heart	2.2 ± 0.2	1.3 ± 0.1	2.6 ± 0.4	2.1 ± 0.4

[a] In some mice urine samples available for counting, weighed just a few milligrams. The resultant high multiplication factor while converting the radioactivity as percentage administered dose per gram, made the urine values very high, frequently greater than 100% of the administered dose.

was clearly evident. In Table III at 4 h p.i., the radioactivity in most tissues had decreased, but the tumor uptake had remained practically unchanged. The liver uptake was high, but it was consistent with the findings of Beatty et al. (1990) who had observed that the tumor bearing BALB/c mice had significantly higher liver uptake than the normal ones.

Among the BRMs studied, IFN produced the highest T/M ratios at 1.5 h post administration of a non-specific macromolecule. Therefore the

TABLE III

TISSUE DISTRIBUTION (% ADMINISTERED DOSE/g) OF ^{99m}Tc-TNF-α IN CONTROL AND BRM GIVEN ANIMALS AT 4 h p.i.

	Control	IFN	Ukrain	PKWD
Urine	NA [a]	NA	42.8 ± 34.0	67.7 ± 38.6
Blood	4.2 ± 0.6	5.1 ± 0.8	4.4 ± 0.9	4 ± 0.3
Kidneys	22.1 ± 3.60	24.4 ± 2.6	33.7 ± 2.8	29.2 ± 3.8
Intestines	1.1 ± 0.10	1.3 ± 0.4	1.9 ± 1.2	1.4 ± 0.3
Muscle	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.4 ± 0.0
Tumors	2.6 ± 0.9	2.7 ± 0.4	2.8 ± 1.3	2.3 ± 0.4
Spleen	14 ± 1.9	15.4 ± 2.0	2.25 ± 3.2	23.3 ± 2.4
Lungs	3.5 ± 0.6	3.4 ± 0.7	7.8 ± 3.8	6.8 ± 1.5
Liver	34.1 ± 2.9	30.5 ± 3.7	43.7 ± 7.3	37.5 ± 2.0
Heart	1.4 ± 0.1	2 ± 0.8	2 ± 1	1.5 ± 0.1

[a] NA = not available.

TABLE IV

TISSUE DISTRIBUTION (% ADMINISTERED DOSE/g) AND TUMOR/MUSCLE RATIOS OF ^{99m}Tc-TNT-1-F(ab')$_2$ AT 1.5 h POST ADMINISTRATION.

	Control	With IFN
Muscle	0.5 ± 0.1	0.4 ± 0.0
Intestine	1.5 ± 0.1	1.4 ± 0.2
Urine [a]	59.3 ± 11.9	122.4 ± 14
Heart	2.1 ± 0.2	1.5 ± 0.3
Lungs	6.5 ± 2.0	5.3 ± 0.7
Blood	6.7 ± 0.7	3.9 ± 0.6
Spleen	23.7 ± 2.4	23.8 ± 2.9
Kidneys	38.5 ± 2.5	49.4 ± 3.8
Liver	41.1 ± 3.3	29.6 ± 3.1
Tumor	4.2 ± 1.0	5.1 ± 1.3
Tumor/muscle	10.5 ± 2.1	12.7 ± 2.6

[a] In some mice urine samples available for counting weighed just a few milligrams. The resultant high multiplication factor while converting the radioactivity as percentage administered dose per gram, made the urine values very high, frequently greater than 100% of the administered dose.

distribution of histone-specific ^{99m}Tc-TNT-1-F(ab')$_2$, was also studied at 1.5 h p.i. with IFN. With this specific agent, (Table IV), the T/M ratios at 1.5 h p.i. were much higher (10.5 ± 2.1) in the control group than the non-specific ^{99m}Tc-TNF group. With IFN, these ratios increased to 12.7 ± 2.6. With IFN the absolute tumor uptake (% administered dose/g) also enhanced for both macromolecules (Table II and IV). Although this increase was statistically insignificant, the point remained that BRMs have the potential to augment the tumor uptake of macromolecules.

Discussion

The advent of hybridoma technology and genetic engineering has led investigators to the development of monoclonal antibodies (MAbs) specific for a variety of malignant tumors. When labeled with γ emitting radionuclides, these MAbs, in principle, can be excellent agents specific for imaging primary lesions as well as secondary malignant foci.

The clinical results of ^{111}In or ^{99m}Tc MAbs however have not yet lived up to their theoretically perceived potential. Several key factors lim-

iting the effectiveness of radiolabeled MAbs have been identified. These include but are not limited to the blood flow to the tumor, the concentration gradient between the blood and tumor, permeability of the radiopharmaceuticals to the tumors through the circulation and to the tumor cell receptor binding specificity. Other problems include the excessively high liver uptake of the radiolabeled MAbs, loss of the MAb specificity during radiolabeling and the in vivo instability of the tracer (Goodwin, 1989; Jain et al., 1989).

Some excellent research has been carried out that addresses some of these drawbacks. For example, the development of innovative bifunctional chelates has stabilized the tracers in vivo (Hnatowich et al., 1983; Meares, 1988), metabolizable linkers placed between the antibody and radiometal complexes have reduced the liver uptake (Paik et al., 1989), and techniques have been adapted that will preserve the biological activity of antibodies (Zalutsky et al., 1987). However, relatively less attention has been given in the diagnostic applications of antibodies to investigate methods that will enhance the tumor uptake of radiolabeled antibodies. Augmented tumor uptake will increase the tumor to background radioactivity ratios and thereby help to delineate the malignant foci small in size. If this enhancement can be apparent sooner than currently possible, the earlier can the patient be imaged. The sooner the diagnosis, the better it is for patient care and cost effectiveness.

The preliminary data described here have strongly suggested that with the use of certain biological response modifiers (BRMs), the tumor uptake can be enhanced. In a prototype system we used ^{99m}Tc-TNF as a non-specific agent for mouse embryonal carcinoma and ^{99m}Tc-TNT-1-F(ab')$_2$ as a specific agent to nuclear histones that may be released in the necrotic center of the tumor. Supported by their excellent properties interferon, Ukrain and PKWD mitogen were chosen as biological response modifiers. Our objectives were to assess if the use of these BRMs would facilitate tumor imaging by augmenting tumor uptake of ^{99m}Tc-labeled specific and non-specific macromolecules. When BRMs were administered to the animals 1 h prior to the administration of ^{99m}Tc macromolecules, a consistent enhancement in tumor to muscle ratios was obtained within 1.5 h of administration of the latter. This lead to the unequivocal delineation of tumors in all animals studied. In these tumor bearing BALB/c mice the liver uptake was high but it was consistent with previous findings by other investigators (Beatty et al., 1990).

The use of BRMs is a rapidly evolving field. These agents are considered to activate tumor infiltrating lymphocytes and/or macrophages that release cell produced cytokines locally, within the tumor. These cytokines temporarily increase tumor blood flow, and capillary permeability, as well as the accumulation of extracellular fluid. Our hypothesis was that these parameters will augment the tumor uptake of radiolabeled antibodies as well as other agents, specific and non-specific alike. The net result of this sequence will be increased uptake of radioactivity in the tumor, increased tumor to background or target-to-non-target ratio and improved delineation of lesions.

The net gain we have achieved currently is modest but statistically significant and is consistent with observations by other investigators giving multiple BRM doses aimed at therapeutic applications (Hnatowich et al., 1983; Zalutsky et al., 1987; Paik et al., 1989; Quesada, 1989; Wimer, 1989; Scholm et al., 1990; DeNardo et al., 1991; LeBerthon et al., 1991; Nakamura et al., 1991).

The exact mechanism by which this enhancement occurs has not been validated. We believe that with radiolabeled BRMs, it should be possible to understand the mechanism by which the BRMs interact with tumors. With this aim in mind we already have separated the five proteins in PKWD and labeled them with ^{99m}Tc (McDevitt, 1991).

We believe that the further evaluation of BRMs will stimulate growth and generate new directions in the field of radionuclide imaging and perhaps in the therapeutic treatment of cancer.

Acknowledgements

Mr. James Finn, Finn Tech. Industries, NJ, provided TNF-α, Dr. A. Epstein, UCLA, TNT-1-F(ab')$_2$, Dr. B. Rhodes, RhoMed Inc. Ukrain,

Dr. P. Mann, University of New Mexico, Albuquerque, Pokeweed and Mrs. A. Dawjanov, Thomas Jefferson University Hospital, NF-1 cells. The authors thank them and are also grateful to Dr. W. Schelman for his helpful suggestions and to Mrs. Renee King for typing the manuscript.

The work was supported by NIH grants, 1R01CA51960-01 and CA 05137-10.

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