CORNACOFF, JOEL BARRY

A COMPARISON OF THREE MODES OF ADMINISTRATION OF
XENOGENEIC IMMUNE RIBONUCLEIC ACID IN THE IMMUNOTHERAPY
OF A MURINE FIBROSARCOMA

The Ohio State University

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A COMPARISON OF THREE MODES OF ADMINISTRATION OF XENOGENEIC IMMUNE RIBONUCLEIC ACID IN THE IMMUNOTHERAPY OF A MURINE FIBROSARCOMA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * * *

The Ohio State University

1979

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TO MY FAMILY
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LIST OF ABBREVIATIONS AND NOMENCLATURE

syngeneic - genetically identical
allogeneic - genetically non-identical, but within the same species
xenogeneic - genetically non-identical, species different
NL - lymphocytes obtained from the spleens of normal animals
IL - lymphocytes obtained from the spleens of animals which are tumor-immune (resistant to tumor challenge)
TL - lymphocytes obtained from the spleens of animals which are bearing an actively growing tumor
4198 - a polyoma virus-induced fibrosarcoma tumor cell line which is transplantable in the C3H/HeJ mouse
4198V - a clone of the 4198 tumor cell which has been shown to possess approximately 8.8 times more tumor-specific antigen (TSA) than the 4198 cell
S91 - a malignant melanoma tumor cell line which is transplantable in the C3H/HeJ mouse
LM - a non-tumorigenic cell line originally derived from the C3H mouse
RNA - ribonucleic acid
mRNA - messenger RNA
immune RNA (IRNA) - RNA extracted from the lymphoid organs of sensitized animals which has the ability to transfer immunologic activity to naive (nonsensitized to a particular antigen) lymphocytes
poly A⁺ mRNA - messenger RNA which contains a segment (tail) of adenylic acid

poly A⁻ RNA - RNA which lacks an adenylic tail, may contain a small percentage of contaminating mRNA which lacks a tail

DEAE dextran (DEAE) - a polycation which enhances the cellular uptake of RNA
INTRODUCTION

The scientific rationale behind cancer immunotherapy is based on the observations that: 1) neoplastic cells express distinctive cell surface markers which set them apart from their normal counterparts and 2) the immune system is capable of recognizing these tumor-specific antigens (TSA) as "non-self" and is able to mount an immune response. Despite these facts, one frequently sees continued tumor growth in the face of concomitant immunity. This failure to balance the equilibrium between neoplastic growth and immunologic control in favor of the host is currently associated with a wide variety of "factors" (tumor antigen, enhancing antibody, antigen-antibody complexes, suppressor cells and soluble suppressor molecules) which interfere with the immune system's ability to respond. An effective immunotherapeutic agent would attempt to reverse the suppressive nature of the tumor and/or augment the qualitative and quantitative nature of the anti-tumor response.

One form of specific immunotherapy which has been shown to fulfill both of the aforementioned criteria in vitro, is the administration of xenogeneic immune RNA and its associated fractions.
Pennline (1977) has demonstrated that treatment of murine splenocytes (derived from tumor-bearing animals) with xenogeneic immune RNA results in both significant increases in cytotoxic potential above baseline levels and a total abrogation of "factors" which suppress lymphocyte-mediated tumor cell destruction. Extensions of immune RNA immunotherapy to in vivo systems has failed to produce consistent anti-tumor effects. This has been attributed in part to the degradation of active RNA components by endogenous ribonucleases, the failure of immune RNA (or appropriate quantities) to enter the cell, the inability to induce a population of specific "memory" cells, or a lymphoproliferative response and misdirection of RNA toward the appropriate effector and/or effector cells (Magee, et al., 1978).

Recent advances in phospholipid technology may provide a tool which is capable of solving some of the problems associated with immune RNA immunotherapy. Current investigations have shown that liposomes are able to entrap RNA within the aqueous phase while at the same time providing a protective environment from nuclease activity (Ostro, et al., 1977; Dimitriadis, 1978). Lymphocytes and macrophages also are able to internalize the lipid vesicles which contain molecules of biological interest (Magee, et al., 1978; Poste, et al., 1979). The efficiency of
uptake by cells of the immune system may be modified both by altering the lipid composition and by including target-directed immunoglobulin (Gregoriadis, 1976). Techniques are available which enable the production of spherules with a high aqueous space-to-lipid ratio (Szoka and Papahadjopoulos, 1978). All of the above characteristics lend themselves to an analysis and possible solution to the immune RNA system.

The four major objectives of this research project were conducted in the C3H/HeJ mouse-4198 tumor system and were as follows:

1. To determine if there is any potential immunotherapeutic value to the direct intraperitoneal injection of either whole xenogeneic 4198V immune RNA or its polyadenylic fractions.

2. To evaluate the efficacy of using lymphocytes treated with either whole 4198V immune RNA or fractions of RNA as an immunotherapeutic tool.

3. To form positively charged liposomes which are capable of entrapping immune RNA in aqueous solution.

4. To test liposomes immunotherapeutically as an improved method of delivering immune RNA.
REVIEW OF LITERATURE

For the most part, the management of solid tumors has been concentrated in three general areas: surgery, to remove the major tumor load; radiotherapy, to eliminate residual tumor cells at the surgical site and; chemotherapy, to kill neoplastic cells based on their differences in metabolism and cell cycling as compared with their normal counterparts. Although these methods have proved effective in eliminating large numbers of tumor cells, they also may exert an immunosuppressive effect on the host which can be of long duration. This created undesirable circumstance is especially serious in those cases in which clinically undetectable metastatic disease results.

The pioneering work of Foley (1953), Prehn and Main (1957) and others indicated that tumor cells "express" tumor-specific antigens (TSA). These may be recognized by the immune system as foreign or "non-self" and under appropriate circumstances eliminated by various immunological means. These early observations provided the rationale for immunotherapy as a modality for the management of neoplastic diseases. According to Gutterman, et al. (1978), immunotherapy has four major objectives: protection against or reversal of the immunosuppressive effects of radiation, chemotherapy and surgery hyperactivation (immunopotentiation) of the general host defense mechanisms including the reticuloendothelial system (and macrophage
activity), antibody production and cellular immunity; manipulation of the immune system so as to augment specific or positive tumor immunity and abrogate suppressive responses (e.g. blocking factors, soluble mediators, aberrant suppressor cell activity) and; restoration (immunorestoration) of immune responses which are partially or completely suppressed for reasons other than those mentioned above.

Immunotherapy has been classified in a number of ways depending upon the particular author. In this presentation, both experimental non-specific (stimulation of host's immune response by adjuvants) and specific (stimulation of responses directed toward specific TSA) immunotherapy will be considered. Both forms of immunotherapy again may be sub-divided into categories as illustrated in Table 1. This table was compiled for the reader's convenience. A brief discussion of representative examples will be presented with the exception of transfer of specific immunity using immune RNA. This topic will be described in greater detail since it is the major focus of this project.

**Bacillus Calmette-Guérin (BCG)**

A number of genera of bacteria and their sub-cellular fractions have been shown to produce beneficial immunotherapeutic effects in tumorous animals and man. The BCG strain (Bacillus Calmette-Guérin) of *Mycobacterium bovis* has been
TABLE 1

Outline of Immunotherapy

Experimental Non-Specific Immunotherapy

1. Microorganisms and sub-cellular fractions
   - Mycobacterium bovis (Bacillus Calmette-Guérin, BCG)
   - methanol extracted residue (MER)
   - ribosomes
   - Corynebacterium parvum
   - Bordetella pertussis
   endotoxin or lipopolysaccharide

2. Plant polysaccharides
   - zymosan (extract of yeast)
   - lentinan (extract of mushroom)
   - pachymaran (extract of glucan)

3. Polynucleotides
   - polyinosinic-cytidylic acid (poly I·C)
   - polyadenylic-uridylic acid (poly A·U)

4. Small molecules
   - levamisole
   - vitamin A (retinol)

5. Endocrines

6. Interferon

Experimental Specific Immunotherapy

1. Active transfer
   - tumor cells modified with: radiation
   - mitomycin C
   - neuraminidase
   - solubilized membrane antigens
TABLE 1
(Continued)

2. Passive transfer
cytotoxic and opsonic allogeneic or xenogeneic antisera
unblocking antisera
antibody as a carrier for cytotoxic drugs
(immunochemotherapy)

3. Adoptive transfer
presensitized allogeneic or xenogeneic lymphocytes
\textit{in vitro} sensitized syngeneic or autologous lymphocytes

4. Transfer of information
transfer factor
immune RNA
among those evaluated in greatest depth. The three major mechanisms proposed for the antitumor activity of this organism include: 1) "innocent bystander" killing of tumor cells as a consequence of the inflammatory reaction, 2) hyperactivation of the immune system resulting in more efficient processing of tumor specific antigens and increased cytotoxic potential, and 3) cross-reacting immunologic activity due to similar antigenic structure held in common between the microorganism and the tumor cell (Bast and Bast, 1976; Minden, 1976).

One of the earliest tumor models studied was the diethylnitrosamine-induced hepatomas of strain-2 guinea pigs. Zbar, et al. (1972) have reported that the intratumor injection of $2.4 \times 10^7$ live BCG 6 or 7 days post tumor-cell inoculation resulted in tumor nodule regression and a 65% survival rate. The survival decreased to 21% when BCG injection was delayed until Day 12. The factors which seem to contribute to successful immunotherapy in this model include: the tumor burden (size), dose and strain of bacillus, direct contact between the tumor cells and BCG and the host's ability to respond (immunocompetence) to BCG and/or tumor-specific antigens.

Pulmonary metastases which developed spontaneously from subcutaneous grafts of transplanted rat epithelioma Spl were
treated with BCG by Baldwin, et al. (1976). The mean survival time and the number of pulmonary metastases were significantly different from the controls when BCG was administered intravenously.

Gutterman, et al., (1976) have reported the results of a 2 year BCG study involving 52 patients with stage III malignant melanoma (regional lymph node metastases). BCG was administered by scarification weekly for 3 months and then every other week at a dose of either $6 \times 10^7$ or $6 \times 10^8$ viable units after surgical removal of lymph nodes. The data were controlled by comparison with 218 stage III patients who received surgery alone. A significant difference was noted in the high-dose BCG group (but not low-dose BCG group) with respect to the time of relapse ($p < 0.03$) and the survival time ($p < 0.002$) as compared with controls. In addition, the authors noted that patients capable of exhibiting strong skin test reactions to dermatophytin and keyhole limpet hemocyanin presented the best prognosis.

The influence of BCG on inoperable or residual cancer of the liver, lung, breast, colorectum, pancreas, biliary tract, ovary and testis was evaluated by Torisu, et al. (1976). BCG was administered intradermally into the arm or thigh. Mitogen stimulation (PHA), skin test responsiveness (DNCB, PPD, KLH) and percent
T-cells were monitored prior to and after initiation of BCG immunotherapy. The four year study resulted in a prolonged survival (37-58 months) in 11% of the patients. The mean survival in the treatment group was 42.5 months as compared to 8.7 months in the controls. As in the previous study (Gutterm an, et al., 1976), a high correlation was seen between the patient's immunoresponsiveness (as monitored by skin test size, percent T cells and ability to blast in the presence of mitogens) and prognosis.

Sparks (1976) has reported on a number of complications which are frequently associated with BCG immunotherapy. A rash frequently occurs at the site of injection (in greater than 70% of the patient population). This may develop into shallow ulcers which drain for several weeks before healing. "Flu-like" symptoms are frequently noted 2 to 8 hours post-injection and consist of malaise, chills and fever. Systemic BCG infection ("BCGosis") (characterized by persistent fever, weight loss, malaise, nausea and vomiting) has occurred on occasion. This generally responds to therapy with isoniazid, ethambutol or rifampin. Anaphylactic and Arthus-like reactions also have been reported. The frequency and severity of the above reactions are highly dependent upon both the patient's sensitivity to PPD and the route and technique for BCG administration (tine technique, scratch technique, aerosol technique, intradermal
injection or intratumor injection). The author stressed the importance of knowing the clinical setting under which BCG is to be administered in order to offer maximum therapeutic benefit and minimal complication.

**Levamisole**

Levamisole, a synthetic imidazole derivative, has been used for a number of years as an antihelminthic. In 1971 (Renoux and Renoux), it was discovered that the drug had the ability to restore depressed cell-mediated immune responses to tuberculin and to dinitrochlorobenzene (DNCB) in anergic patients with cancer, but did not potentiate this reaction to above normal values. The drug therefore has come to be known as an immunorestorative agent.

A number of reports have been published in reference to the effects of levamisole on tumor growth in animal models. Renoux and Renoux (1972), working with the Lewis lung tumor in C57BL/Rho mice, found that either a single injection (0.5 mg/kg) at 24 hours post-tumor injection or six injections every two days one week after inoculation (tumor) resulted in a 25% and 40% cure rate, respectively. In addition, tumor growth rate and pulmonary metastases were significantly decreased as compared with controls. In contrast, Potter, et al. (1974), reported that the drug was
ineffective (if not enhancing) by a number of regimens in a transplantable adenovirus 12-induced tumor of hamsters, a Maloney leukemia virus-induced lymphoma of Balb/c mice and a chemically-induced metastasising anaplastic neoplasm of inbred rats. The authors suggested that the production of blocking antibody may have contributed to the negative results.

Recently, Amery (1976) reported a double-blind study involving 148 patients with resectable bronchogenic carcinoma. Results seem to indicate that "adequately dosed patients" (2.5 mg/kg/day) presented an almost complete elimination of distant metastases and that the effects were more pronounced in patients with heavier initial tumor load.

Polyadenylic-Polyuridylic Acid (Poly A·U)

Poly A·U is a synthetic double-stranded polynucleotide which has been shown to have a variety of effects on immune functions including: antibody production, delayed hypersensitivity, graft-versus-host reactions and tumor growth and development. The latter function was investigated by Lacour, et al. (1972) using spontaneous mammary tumors in C3H/He mice. Mice bearing 5-10 mm tumors were randomized into groups consisting of: controls, poly A·U treatment, surgery, and poly A·U
treatment as an adjunct to surgery. Poly A·U was given intravenously once a week for 6 weeks. A significant decrease in metastases and mean survival time (as compared to controls) was obtained in the groups treated by surgery or surgery complemented by poly A·U treatment. In addition, the combined treatment was significantly different than surgery alone with respect to mean survival time.

Mechanistically, Webb, et al. (1972) and Braun and Shiozawa (1974) have attributed some of the results of poly A·U treatment to the ability to increase endogenous cyclic adenosine monophosphate (cAMP) via stimulation of adenyl cyclase at either the lymphocyte or tumor cell level. It is interesting to note that synthetic polynucleotides such as poly I·C and poly A·U are inducers of interferon (shown to have an antitumor effect) (discussed in the next section). This may ultimately prove to be the underlying reason for positive effects.

**Interferon**

Vast literature has accumulated on the inhibitory effects on various infectious agents by interferon since its discovery in 1957 by Isaacs and Lindemann. This is especially true in the case of viral infections where it appears to be one of the host's major
defense mechanisms. There are at least three different forms of human interferon: leucocyte, fibroblast and T or immune. All are glycoproteins with a molecular weight of approximately 15,000-20,000 (Marx, 1979).

Gresser, et al., (1969) were one of the first groups to recognize the potential value of interferon in tumor therapy when they observed an increased survival rate and decreased number of tumor takes in Balb/c and C57BL/6 mice injected with RC19 and EL4 tumor cells and then injected daily with mouse interferon.

Crane, et al., (1978) provided some of the impetus for consideration of human clinical trials through work with an osteogenic sarcoma. Although their research involved a murine system (C57BL/6), osteogenic sarcoma is a common primary tumor of the bone in humans. They observed a delay in the initial appearance of tumors and a decrease in the number of animals developing tumors when interferon was injected twice daily for 7 days near the site of tumor cell inoculation.

As with the other forms of tumor immunotherapy, a question exists with regard to the mode of action of interferon. Possibilities include: 1) alteration of the membranes of lymphocyte and/or tumor cells (exposing TSA) resulting in increased cytotoxicity, 2) increased phagocytic activity, 3) inhibition of antibody production.
with a decrease in blocking factors and 4) regulation of cAMP.

One of the major problems associated with interferon research involves the availability of sufficient quantities to perform clinical trials. In September, 1978, the American Cancer Society allocated 2 million dollars for the purchase of 40 billion units of leucocyte interferon (enough to treat 150 patients). Current research involves improving the efficiency of inducing agents and amino acid sequence analysis with hopes of producing the agent synthetically (Marx, 1979).

**Neuraminidase**

As discussed previously, a number of tumors seem to possess TSA which are capable of eliciting an immune response. In some instances, the response seems to be weak or absent and may be a result of "hidden" molecular structures within the tumor cell membrane. This concept led to the use of various enzyme preparations to expose TSA. *Vibrio cholerae* neuraminidase (VCN) has yielded the most significant results to date. Investigators have speculated that the enzyme releases sialic acid residues resulting in reduced negative charges on the tumor cell surface, increased cell deformability, or increased phagocytosis.

Sanford (1967) and Currie and Bagshawe (1968) originally reported that incubation of ascites tumor cells with VCN led to
decreased oncogenicity and increased immunogenicity. Working with methylcholanthrene-induced fibrosarcomas in C3H/HeJ mice, Simmons, et al., (1971) showed temporary cessation of tumor growth in all mice receiving $10^6$ VCN-treated tumors at various time intervals after tumor initiation. In a number of cases, tumors totally regressed and disappeared. The regression was immunospecific and could only be induced with VCN-treated cells identical in type with the growing tumor. Tumor cells treated with heat-inactivated VCN ($65^\circ$C for 30 minutes or $100^\circ$C for 10 minutes) had no effect on growth. In a later study, Simmons and Rios (1972) reported inhibition of tumor growth by direct intratumor injection of VCN and some total regression when the regimen was continued daily over a long period. The same authors have reported similar results with the B16 melanoma in C57BL/6 mice and mammary tumors in C3H/HeJ mice (Rios and Simmons, 1973).

Sedlacek, et al., (1975) have reported that the number of VCN-treated autologous tumor cells injected was a critical factor. Thirteen of fifteen dogs, bearing spontaneous mammary tumors, had a significant regression of their tumors to less than 10% of the original volume when injected with $2 \times 10^7$ cells while 8 out
include: inactivation of specific suppressor thymus-derived (T) cells, and antibody-linked cytotoxic agents.

A paradoxical situation exists in nature in which lymphocytes derived from tumor-bearing animals are specifically cytotoxic in vitro, yet lead to results which are diametrically opposed in vivo. This observation has been attributed to a variety of factors found in the serum which block lymphocyte-mediated cytotoxicity. Although the exact nature of "blocking factors" is as yet unclear, investigators have proposed that either antigen-antibody complexes (Sjogren, et al., 1972), antibody (Hellstrom and Hellstrom, 1969), or free antigen (Baldwin, et al., 1973) are responsible. Recently, specific suppressor T cells have been implicated in abrogating positive anti-tumor immune responses. Fujimota, et al., (1976a) have reported that surgical removal of a methylicholanthrene-induced sarcoma in A/Jax mice, 7 days after implantation, results in tumor-specific immunity as demonstrated by rejection within 15 days of a challenging dose (100-fold higher) of tumor cells. The intravenous administration of $10^7$-$10^8$ thymocytes or splenocytes from tumor-bearing animals at the time of the tumor challenge significantly inhibited the rejection of the tumor while cells from either normal animals or animals bearing an unrelated tumor had no effect. Pre-incubation of thymocytes or splenocytes with
anti-theta serum or with anti-thymocyte serum and complement abolished the effect. These data indicated that tumor-bearing mice possessed populations of T cells which had a negative or suppressive effect on positive tumor-immune responses. The authors later reported that the suppressor T cells were cortisone resistant (Fujimoto, _et al._, 1976b) and were capable of mediating their effect via a soluble factor which shared antigenic determinant(s) of the product(s) of the K end of the major histocompatibility complex (H-2) (Green, _et al._, 1977a). These observations intrigued investigators in that if one were able to eliminate the influence of suppressor T cells, the immune system of the host might be able to alter a neoplastic state. Greene, _et al._, (1977b) have tested this theory by developing anti-I-J antiserum (The mouse H-2 contains four major regions known as K, I, S and D. The I region may be subdivided into five subregions referred to as: A, B, J, E and C. The I-J subregion contains the genetic information for surface-membrane markers found on suppressor T cells and determinants found on soluble inhibitory factors (Broder and Waldmann, 1978)). A/J mice were injected with an MCA-induced sarcoma and treated daily for 15 days with 2 ul doses of antiserum. Statistical analysis indicated that the tumor growth rate of mice receiving anti-I-J serum was markedly decreased when compared
with controls. Histologic examination of animals which had received anti-I-J serum displayed marked mononuclear cell infiltrates at all levels of the tumor with large numbers of dead tumor cells, while controls had but few inflammatory cells and these were limited to the periphery of the tumor. The authors suggested that this approach might be applied to a human system if distinctive suppressor cell alloantigens could be identified.

A second approach to specific immunotherapy with antibody involves chemically binding toxic agents to xenogeneic antibody directed against TSA. This has come to be called immunochemotherapy. Some of the agents tested thus far include: alkylating agents (chlorambucil), antibiotics (adriamycin), antimetabolites (methotrexate), cell surface agents (phospholipases, microbial toxins) and protein synthesis inhibitors (diphtheria toxin). Moolten, et al., (1975) have reported the effects of antibody-diphtheria toxin conjugates on the therapy of simian virus 40 (SV40)-transformed hamster sarcoma and lymphoma cells. Their results indicated that a single dose of the conjugate injected at the same time as the sarcoma cells was capable of reducing tumor incidence, increasing latency and prolonging life-span. No effect was seen in the case of established sarcomas but regressions were noted after repeated treatment with conjugates in 20-56% of hamsters
bearing lymphoma transplants. Ghose, et al., (1977) have evaluated the effectiveness of chlorambucil (alkylating agent) noncovalently linked to goat or rabbit anti-human globulins in patients with inoperable recurrent malignant melanoma. Of the 13 patients treated, 2 had disease confined to lymph nodes and cutaneous sites; 5 showed stabilization of cutaneous, nodal and visceral disease; and 6 showed disease progression. Patients with confined and stabilized disease had a median survival of 20 months as compared with 3.5 months for those showing progressive disease. Eleven patients, treated by standard chemotherapy, exhibited a median survival of 3 months. According to Ghose and Blair (1978), "the inherent problems in chemoimmunotherapy include: 1) the necessity of binding therapeutically effective amounts of antitumor agent, 2) insuring delivery of the drug in active form to target sites, 3) avoidance of host reactions to foreign proteins, and 4) possible emergence of resistant tumor cell populations."

**Lymphocytes**

The transfer of lymphocytes sensitized against TSA has been used effectively in a number of syngeneic animal systems in the immunotherapy of cancer. Unfortunately, the situation becomes complex when considering the human clinical setting since
allogeneic or xenogeneic cells are rapidly rejected due to histocompatibility differences and in immunodepressed patients, one risks severe graft-versus-host reaction.

One possible means of circumventing the above problem is to remove lymphoid cells after surgical removal of a tumor, \textit{in vitro} sensitize the cells and return them to the host at the appropriate time. This concept has been tested by Treves, et al., (1975) using the Lewis lung carcinoma (3LL) in C57BL/6 mice. Splenocytes isolated from normal and tumor-bearing mice were exposed \textit{in vitro} to cobalt-60 irradiated tumor cells. At the end of 5 days, the cells were injected intravenously into mice which had had local tumor removed the previous day. All animals were monitored for survival and death from lung metastases. In four of six independent experiments, normal splenocytes sensitized to 3LL cells were capable of protecting against the development of lethal metastases. On the other hand, lymphocytes derived from syngeneic tumor-bearing mice were only effective in one of three experiments. The authors suggested that suppressor T cells found in the spleen of tumor-bearing mice influenced the ability of lymphocytes to become specifically sensitized \textit{in vitro}. Preliminary data indicated that suppressor cells disappeared from the spleen approximately one week after surgical removal of the local tumor and these cells
might be sensitized after this time.

**Immune RNA**

Ribonucleic acid (RNA) extracted from the lymphoid organs of sensitized (immunized to a specific antigen) animals has the ability to induce normal, non-immune lymphoid cells to produce a wide variety of humoral and cellular immune factors. Examples include: 1) allograft rejection (Mannick and Egdahl, 1964; Rigby, 1968), 2) delayed hypersensitivity (Paque and Dray, 1970; Han, 1973), 3) production of antibody (Fishman, 1961; Friedman, *et al.*, 1965; Bell and Dray, 1973) and 4) tumor immunity (Ramming, *et al.*, 1978; Dodd and Evans, 1978).

The RNA capable of transferring specific immunologic activity (referred to as immunologic or immune RNA) has been located intracellularly in the cytoplasm of splenocytes (deKernion, *et al.*, 1974). Analysis on sucrose density gradients has resulted in sedimentation values of 10-16S (Bilello, *et al.*, 1976; Dodd, *et al.*, 1973; Kern, *et al.*, 1976). Paque (1976) and Greenup (1978) have reported that the RNA capable of transferring delayed-type hypersensitivity and anti-tumor cytotoxicity respectively, contains sequences of polyadenylic acid and is probably messenger RNA. Wang (1978) has suggested that the principal cell type yielding
immune RNA is the macrophage. An unresolved debate exists with regard to the presence or absence of antigen in RNA preparations. It is worth emphasizing that most investigators have reported that their RNA preparations were insensitive to either DNase or pronase, but were unable to transfer activity following exposure to RNase (Evans, 1976; Cornacoff, 1976; Wang, 1978). For the sake of this presentation, we will assume that RNA is the major component responsible for transfer of information in extracted lymphoid preparations.

At this point in time, one can only speculate as to what occurs following the incubation of lymphoid cells with immune RNA. Jachertz (1976) has suggested that if immune RNA is messenger RNA, the molecule could bind with ribosomes and translate protein. The newly formed protein might be in the form of lymphokines, antibody, reverse transcriptase, regulatory protein or cell surface receptor.

In 1971, Deckers and Pilch suggested a model for the immunotherapy of human cancer using xenogeneic immune RNA. They proposed to immunize animals (sheep or guinea pigs) with tumor tissue obtained from the patient at the time of surgery. RNA would be extracted from the lymphoid organs after the appropriate interval, filter sterilized and lyophilized. Whenever possible, each patient would receive immune RNA against the autologous tumor
animals. A significant difference was seen in the incidence of tumor development between the control groups and the group of mice which had received BP-4 immune RNA treated cells. Untreated normal lymphocytes and normal lymphocytes treated with either RNAse-treated BP-4 immune RNA or with RNA extracted from guinea pigs immunized with normal tissue had no effect on tumor development. In addition, BP-4 immune RNA treated splenocytes did not influence the growth of BP-1 tumor (BP-1 does not cross react with BP-4) isografts in C3H/HeN mice.

Deckers and Pilch (1971) treated Fisher 344/N rats, injected with $10^4$ BP-1R tumor cells, by footpad injection of xenogeneic guinea pig BP-1R immune RNA mixed with sodium dextran sulfate (an RNAse inhibitor). RNA-dextran sulfate mixtures were administered every other day for 10 days starting at the time of tumor cell inoculation. The rats which had received immune RNA exhibited significant resistance to the tumor isografts as compared with the groups which had received "non-specific RNA" or dextran sulfate alone.

The influence of immune RNA (rhesus monkey) on a diethyl nitrosamine-induced hepatoma in strain 2 guinea pigs was evaluated
by Schlager and Dray (1976). Guinea pigs were injected intradermally with $10^6$ tumor cells on Day 0 and provided treatment on Day 5. In this system, syngeneic nonsensitive peritoneal exudate cells (PEC) were injected prior to RNA and soluble tumor specific antigen (TSA) injected afterwards. The authors felt that the immune RNA would convert the normal PEC (the lymphoid populations of tumor-bearing animals may be immunodeficient or less responsive to RNA) to immunologic sensitivity and that soluble TSA might amplify the effect by clonal proliferation. A complete regression of the tumor nodule was noted in 100% of the animals which had received the above treatment and all of the guinea pigs were alive in excess of 180 days (controls survived 72 ± 9 days). No regression or increased survival was seen in any of the test groups when the animals were treated with either immune RNA, PEC or TSA alone.

Wang, *et al.*, (1978) evaluated the efficacy of RNA immunotherapy in a model which closely resembles the human clinical setting. The limbs of C57BL/6J mice bearing B16 melanoma isografts were amputated when tumors first became palpable (approximately 2-3 weeks after injection of $2 \times 10^3$ B16 cells). Two days post-surgery, each animal received 5 injections (at 2 day intervals) of $7.5 \times 10^7$ guinea pig immune RNA treated
syngeneic splenocytes. The survival rate was monitored for 100 days following excision of primary isografts. When death occurred it most frequently was from pulmonary metastases. The survival of immune RNA treated animals was significantly improved when compared with that of the control animals (p=0.0001). B16 melanoma immune RNA had no effect on Lewis lung carcinoma isografts and RNAse treatment abolished all beneficial effects, thereby indicating the necessity for an intact RNA molecule.

To date, only one major group of investigators has attempted to use xenogeneic immune RNA in a human clinical circumstance (Y.H. Pilch, D. Fritze, K.P. Ramming, J.B. deKernion and D.H. Kern). The trials have been limited to patients with grossly detectable and/or measurable metastatic disease and patients with "minimum residual disease". The patients in the former category either had no effective therapy available to them or had failed to respond to standard forms of therapy, while the latter category of patients had a greater than 50% chance of developing recurrent and/or metastatic disease within 2 years. The major aims of the trials were to assess toxicity and to establish dose-response relationships. Clinical responses were sought but not expected due to the advanced nature of the disease. The authors admit that their studies were
uncontrolled. The histologic types of tumors treated included malignant melanoma, hypernephroma, sarcoma and carcinoma of the breast, colon, lung, prostate and stomach. All RNA was obtained from the spleens and mesenteric lymph nodes of sheep. The RNA was injected at multiple sites near the lymph nodes of the axillae and/or groin (The investigators would have preferred to admix the RNA—in an RNase inhibitor such as sodium dextran sulfate, but approval has as yet not been obtained from the Food and Drug Administration). Generally, 4-8 mg of immune RNA was injected per week, although doses ranged from 2-60 mg/week in some patients. Patients treated with immune RNA were monitored immunologically by skin tests and lymphocyte-mediated tumor cell cytotoxicity. The investigators concluded that toxicity of immune RNA was minimal or absent. Although complete regression did not occur in any patient treated, stabilization of the growth of metastases was noted in several cases which had been documented as being progressively developing prior to initiation of RNA immunotherapy. Patients with metastatic hypernephroma seemed to exhibit an increased survival based on matched retrospective controls (Pilch, et al., 1976; Ramming, et al., 1978).

In reviewing the data presented for the immunotherapy of cancer in animals and man using specific antitumor immune RNA,
only Schlager and Dray (1976) have reported 100% tumor regression and survival. All of the other reports presented indicate varying degrees of effectiveness substantially below the 100% level. Magee, et al., (1978) attribute some of the problems associated with RNA immunotherapy to degradation of active material by endogenous ribonucleases, ineffective interaction between RNA and lymphoid cells and failure of RNA (or appropriate quantities of RNA) to enter the cell. One might also speculate that immune RNA is not being directed toward the appropriate populations of affector and/or effector cells. Recent advancements in phospholipid technology may be utilized to overcome some or all of the aforementioned problems by incorporating immune RNA into liposomes. The physical nature of the liposomes protects RNA from nuclease activity and at the same time, one may incorporate specific antibody to provide a homing mechanism for the desired cell population.

When various phospholipids are placed in an excess aqueous environment, multilamellar vesicles composed of concentric bilayers form. These may range in size from 5-50 u in diameter (Figure 1). Exposure of the vesicles to ultrasonication tends to convert the sperules to smaller mono- or unilamellar units which average 0.5 u (Figure 2) (Sessa and Weissmann, 1968). During the formation of either type of vesicle, water-soluble substances are entrapped
Figure 1. A depiction of a multiamellar liposome containing two alternating bilayers of polar phospholipids with associated aqueous phase.
Multilamellar Liposome

Neutral, negative or positive charge

- Water soluble molecules
- Lipid soluble molecules
- Water soluble molecules with hydrophobic moiety penetrating lipid phase

Figure 1
between the lipid bilayer(s). The ability of these substances to leak out of the lipid membrane is dependent on both the nature of the solute and the composition of the liposome. In general, large molecules such as protein will be retained within the vesicle until the structure is disrupted (Tyrell, et al., 1976).

Prior to 1971, liposomes were essentially used as models for biological membranes. Small solute molecules were incorporated into the vesicles in order to study the permeability out of or through the membrane. More recently, however, interest has been generated regarding the use of liposomes as carriers of biological materials (enzymes, hormones, drugs, steroids and vitamins). Applications include: enzyme-replacement therapy in inherited metabolic disorders, treatment of metal-storage diseases, cancer therapy, introduction of antibiotics to resistant microorganisms, use as immunologic adjuvants and an improved method by which to treat diabetes mellitus. This is a direct consequence of the observation that liposomes are capable of fusing with cells, being transported across the membrane and releasing their contents into the cell (Theories on the mechanism of fusion have yet to be substantiated). According to Gregoriadis (1976), the uptake of liposomes by a particular cell is "temperature dependent, proportional to lipid concentration and independent of pH (range, 6.8 to 8.7) and lipid
Figure 2. A depiction of unilamellar liposomes containing single layers of polar phospholipids and one aqueous compartment.
Unilamellar Liposomes

Figure 2
composition". An exception does exist in the instance of positively charged liposomes which have an increased chance of being taken into a cell in the absence of serum. One may select for the type of target cell with which the liposome interacts by incorporating specific IgG antibody. It is proposed that the \( F_C \) portion of the antibody molecule penetrates the lipid bilayer while the \( F_{ab} \) or the specific antigen-binding portion extends into the aqueous phase for interaction with the appropriate target cell (Gregoriadis, 1978).

Magee, et al. (1978) have reported that 10-15% of the \( F_{ab} \) sites are available for interaction with the specific target by this method.

There are several published articles on potentiation of anti-tumor effect on drugs in vivo by incorporating the agents into liposomes. These include prolonged survival of tumor-bearing mice treated with liposome-entrapped actinomycin D (Gregoriadis and Neerunjun, 1975), enhanced chemotherapeutic effect of cytosine-arabinoside in liposomes on mouse leukemia L-1201 (Kobayashi, et al., 1975), inhibition of tumor cell growth by 1-beta-D-arabinofuranosylcytosine entrapped within phospholipid (Mayhew, et al., 1976) and reduction of tumor mass in mice by lipid vesicle encapsulated methotrexate (Kosloski, et al., 1978).

Recently, Magee, et al., (1978) reported an in vitro study in which syngeneic guinea pig anti-hepatocarcinoma immune RNA
and antilymphocyte IgG were incorporated into liposomes and incubated with normal strain 2 guinea pig lymphocytes. Treated lymphocytes destroyed chromium-51 labeled tumor target cells 12 times more efficiently than did cells treated with naked (not in liposomes) immune RNA. To date, no published in vivo data exists concerning the use of immune RNA containing liposomes as an improved immunotherapeutic tool.

This latter material is particularly pertinent in as much as traditional modes of administration of immune RNA in the therapy of neoplastic diseases have met with mixed results. It is the purpose of this study to compare the effectiveness of liposomes, as a delivery system for immune RNA, with RNA-treated syngeneic lymphocytes and/or RNA alone in the C3H/HeJ - 4198 tumor system. The results obtained may provide further insight into mechanisms by which one may manipulate the immune system so as to mount an effective anti-tumor response.
MATERIALS AND METHODS

Animals

The mice utilized throughout this study were 4-6 week old male C3H/HeJ (Jackson Laboratories, Bar Harbor, Maine). New Zealand White rabbits and outbred albino guinea pigs were purchased from Kings Wheel, Mt. Vernon, Ohio and Charles River, Wilmington, Massachusetts, respectively. All animals were housed in the Animal Facility of the College of Biological Sciences, The Ohio State University, Columbus, Ohio. Purina Lab Chow and water were provided ad libitum.

Cell Lines

The two major cell lines used in this study were syngeneic fibrosarcomas of C3H mouse origin. The 4198, a polyoma virus-induced cell was originated in vitro as was its cloned variant line-4198V. Both cell lines are similar in morphology, in vitro growth rate and are free from demonstratable virus as determined by hemagglutination-inhibition and plaque assay (Ting and Law, 1965).
Although both cell lines possess tumor-specific cell surface antigen (TSSA), the 4198V has 8.8 times more TSSA than does the 4198 line as determined by isotopic antiglobulin technique (Ting, et al., 1972). In addition, more polyoma T-antigen is found on the 4198V. These quantitative differences in surface antigen led to the use of the 4198V for immunization of test animals as well as for a target cell in evaluating the immunocompetence of lymphocytes in vitro, while the less antigenic 4198 cell was used for tumor induction.

The LM and S91 cell lines were utilized as controls. The nontumorigenic LM cell was originally cloned from Strain L normal C3H/An mouse connective tissue (Kuchler and Merchant, 1956). This was compared with the Cloudman S91 malignant melanoma which originated in a JAX/dba female (Cloudman, 1941) and is transplantable in the C3H system.

**Cell Culture**

All cell lines were cultivated in monolayer in plastic tissue culture flasks (Falcon Plastics) containing RPMI-1640 tissue culture medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco) and 2mM glutamine (Gibco). When necessary, an antibiotic-antimycotic mixture (100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone) (Gibco) was added to prevent
bacterial and/or fungal contamination. The size of the tissue culture flask and the initial cell concentration varied with the experimental need. At regular intervals, the flasks were removed from the 37°C, 5% CO₂ atmosphere and the cultures observed microscopically using an inverted microscope. At confluency, the medium was aspirated, and the monolayer trypsinized with a solution of 0.25% trypsin (Gibco) in Ca²⁺-Mg²⁺ free phosphate-buffered saline. The suspension of cells obtained by triturating with fresh medium was adjusted to the desired concentration after viability testing by the trypan blue dye (Gibco) exclusion method and hemacytometer count. Portions of the resulting cell suspensions were either seeded into new flasks for continued cell culture, frozen for later use, or used in various assays.

Freezing Cells for Storage and Return to Culture

A portion of all cell lines maintained in culture was frozen so as to provide an adequate supply in the case of loss through contamination. The method followed was similar to that of Mullen (1972). Cell suspensions were adjusted to 1 X 10⁷ cells/ml in tissue culture medium containing FCS and 9% dimethylsulfoxide (DMSO) (Eastman Organic Chemicals). Cell suspensions were transferred to 1 ml freezing ampules (Scientific Products) using
a syringe fitted with an 18 gauge needle taking care to prevent any fluid from contacting the neck of the ampule. This minimized the chances of the ampule exploding in the next step in which it was sealed with a propane torch. In order to prevent the formation of large ice crystals which lead to cellular damage, the cells were frozen slowly according to the following schedule:

a) 1 hour in the 4°C refrigerator
b) 1 hour in the -20°C freezer
c) 1 hour to overnight in the -90°C chest freezer
d) storage in liquid nitrogen

When necessary, cells stored in liquid nitrogen were rapidly thawed in 37°C water to retain viability. After wiping the ampule with ethanol, the neck was broken and the contents washed and centrifuged at 400 x g in a tube containing 10 ml of medium with FCS. Upon resuspension, new flasks were seeded and placed into culture.

**Immunization of Mice**

Cells obtained from tissue culture were washed and resuspended in serum-free RPMI 1640 at a final concentration of 3-5 X 10^6 viable cells/ml. C3H mice were injected intraperitoneally (IP) with 1.0 ml aliquots every 5 days for a total of three injections. Previous results (Cornacoff, 1976; Greenup, 1977; Vallera, 1978) have shown that this schedule results in a significant level of lymphocyte-mediated tumor cell cytotoxicity as compared with age-
matched controls.

Tumor Induction

The 4198 tumor was induced in either hind leg of C3H mice by intramuscular injection of $5 \times 10^5$ viable cells per 0.1 ml.

Immunization of Guinea Pigs

Outbred albino guinea pigs were injected 3 times with $1 \times 10^7$ 4198V, LM or S91 cells at 5-7 day intervals. Day 0 preparations were emulsified in Freund's incomplete adjuvant (Gibco) and injected in the footpads and subcutaneously in the back of the neck. The second and third injections were introduced by the IP route. All animals were skin tested intradermally (ID) with $5 \times 10^6$ cells (and medium alone as a control) and observed at 48 hours for erythema and induration. Guinea pigs exhibiting positive skin tests were sacrificed by exsanguination under sodium pentabarbitol anesthesia. The spleens were excised, frozen in dry ice/acetone and stored in the -90°C chest freezer until RNA extraction.

RNA Extraction

A major consideration for RNA research is directed in obtaining a maximum yield of RNA which is as free as possible of DNA and protein. The extraction procedure followed for all experiments most closely resembles that of Thor (1971), with
minor adaptations by Scheetz (1972), Rossio (1973) and Greenup (1977).

All glassware used was thoroughly cleaned in hemosol and 1% HCl, followed by extensive rinses in double distilled demineralized water and triple distilled water. After this, the glassware was sterilized in a hot air oven for 12 hours. In addition, all reagents and buffers were prepared in diethyl pyrocarbonate (DEP, a nuclease inhibitor) (Sigma)-treated triple distilled water. These procedures were necessary to assure nuclease-free glassware and reagents.

High quality phenol was prepared from the liquid chromatographic product (Mallinckrodt) through vacuum distillation. One-tenth percent 8-hydroxyquinoline (8HQ) (Sigma) was added to the constant boiling fraction in order to prevent oxidation. The product was layered with nitrogen gas and stored in flasks at -20°C for use within two weeks.

The 0.01 M acetate extraction, pH 5, consisted of 0.1% 8HQ, 0.5% sodium dodecyl sulfate (SDS, a detergent and ribonuclease inhibitor) (Sigma), and 0.04% polyvinyl sulfate (PVS, a ribonuclease inhibitor) (Sigma) compounded in DEP water.

Purified phenol was adjusted to 90% with respect to triple distilled water and saturated with extraction buffer. One to two
guinea pig spleens (up to 2 grams of tissue) were placed in a chilled Virtis beaker filled with 30 ml of cold buffer-saturated phenol and homogenized on ice for 2.5 minutes. Two milligram (mg) of bentonite (a nuclease inhibitor) (Fisher Scientific) was added along with 30 ml of cold acetate extraction buffer and the contents homogenized for 2.5 additional minutes.

The homogenate was placed in a chilled 250 ml Erlenmeyer flask fitted with a vented stopper housing a thermometer. The contents were slowly swirled in a 60°C water bath so as to reach a temperature of 55°C in exactly 10 minutes. This step was critical in order to maximally dissociate RNA from DNA and protein. Upon completing this step, the flask was agitated in an ice-slush bath to lower the temperature to 10°C or less in 3 minutes. This step provided optimum conditions for the RNA to remain in the aqueous phase.

Equal volumes of homogenate were placed in chilled Corex glass tubes (Corning) and centrifuged at 17,300 x g (12,000 RPM) in a Sorvall model RC2-B at 4°C for 5 minutes. After centrifugation, the clear aqueous layer was removed from the top of the tube exercising care not to disturb the white interfacial material, and placed in a chilled 50 ml graduated cylinder. Two mg of bentonite and one-half volume of cold extraction buffer were added to the non-
aqueous phase in a 250 ml Erlenmeyer flask and the contents were heated, cooled, and centrifuged as before. The aqueous layer which resulted was pooled with that from the first extraction, and the non-aqueous layer was discarded.

The addition of 2 mg of bentonite and one-half volume of chloroform/phenol/isoamyl (1:1:0.2) to the combined aqueous layers was repeated twice following the procedure outlined above. The RNA was precipitated at -20°C by bringing the aqueous phase to 0.3M acetate with 2.5 M pH 5 acetate buffer, and adding 3 volumes of ethanol.

**Ultraviolet (UV) Spectrophotometric Analysis of RNA**

The RNA precipitated from the ethanol-aqueous phase after a minimum of 24 hours was pelleted by centrifugation at 17,300 x g at -20°C for 30 minutes in an RC2-B. The supernatant fluid was discarded and the pellet was washed once with 70% ethanol:30% TNE (0.1 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.4) to remove residual phenol. After centrifugation, the pellet was dissolved in 7 ml of DEP-treated phosphate buffered saline (PBS). A sample of this preparation was placed in a Perkin-Elmer 139 Spectrophotometer and the absorbance of UV light at 230 nanometer (nm), 260 nm and 280 nm measured. A 260/280 nm and 260/230 nm ratio
of 2.0 or greater indicated a relatively protein-free preparation. Any RNA which did not meet this standard was reextracted with phenol and processed again.

The amount of RNA recovered from a particular extraction also was quantitated by spectrophotometric means assuming that a standard 1 mg/ml solution absorbs 24 optical density units at 260 nm. All RNA was adjusted to a concentration of 1 mg/ml. Portions then were treated as follows:

1. Used in in vitro or in vivo assays.
2. Reprecipitated with 3 volumes of ethanol and stored at -20°C until use.
3. Frozen and stored in the -90°C chest freezer
4. Fractionated on oligo (dT)-cellulose
5. Qualitatively analyzed on sucrose density gradients.
6. Incorporated into liposomes.

**Fractionation of RNA on Oligo (dT)-Cellulose**

RNA containing or lacking a segment of polyadenylic acid (poly A) was separated into two distinct populations through affinity chromatography on type 3 oligo (dT)-cellulose (Collaborative Research). Adesnik, et al. (1972) and Lee, et al. (1971) have shown that most eucaryotic messenger RNA's (mRNA) contain a poly A segment which binds to oligo (dT)-cellulose at the proper ionic strength (Mach, et al., 1973). One-tenth gram of dry cellulose was swollen in binding buffer (0.01 M Tris- HCl, 0.5 M NaCl, pH 7.5) and
packed into a 5 3/4 inch ovened Pasteur pipet plugged with glass wool. The cellulose was equilibrated by washing with 5 bed volumes (1 bed volume equals 2 ml) of binding buffer. Two mg of xenogeneic guinea pig splenic RNA, dissolved in binding buffer, was added to the column and the effluent containing poly A-tailed RNA collected. The column was washed with 2 bed volumes of binding buffer to remove unbound material and poly A$^+$-tailed RNA was eluted by adding 1.5 ml of DEP-treated distilled water. The salt concentration in both fractions was adjusted to 0.1 M with 10X TNE (0.1 M Tris, 1.0 M NaCl, 0.01 M ETDA, pH 8.3) and 2 volumes of ethanol were added. The RNA was precipitated at -20°C for a minimum of 24 hours and processed as previously described in the section on UV analysis of RNA. The column was regenerated by sequential washes with 0.1 M NaOH and binding buffer. All fractionations were carried out at room temperature.

The efficiency of the oligo (dT)-cellulose column to bind poly A$^+$-tailed RNA was evaluated prior to the first use and at various intervals thereafter by adding tritiated ($^3$H)-Rauscher leukemia virus. This was provided by Dr. Paul Kimball, Department of Microbiology, The Ohio State University, Columbus, Ohio.
Sucrose Density Ultracentrifugation

In order to determine the degree of degradation of any particular RNA preparation, linear gradients of 5 to 20% w/v sucrose were prepared. An Isco Model 180 gradient former was used to fill 9/16 X 3 1/2 inch cellulose nitrate tubes (Beckman) with crystalline ribonuclease-free sucrose (Sigma) dissolved in 0.01 M pH 7 Tris buffer containing $10^{-3}$ M EDTA and 0.05 M NaCl.

The gradients were carefully layered with 50-100 ug of RNA and centrifuged in a Beckman L2-65B ultracentrifuge at 193,000 x g in a Beckman SW 41 rotor for 18 hours at 4°C. At the end of this period, the gradients were passed through the flow cell of an Isco Model 222 spectrophotometer by forcing 50% w/v sucrose solution through the bottom of the tubes using an Isco 180 density gradient fractionator. The absorbance was recorded at 254 nm. Figures 3 and 4 illustrate typical sucrose density profiles of whole and fractionated 4198V immune RNA respectively.

Isolation of Lymphocytes

The spleens of C3H/HeJ mice were aseptically removed and placed in 8 ml of RPMI-1640 at 4°C. Splenocytes were expressed from the associated capsule and triturated into single cell suspension by successive passage through 18, 21 and 25 gauge needles. At
Figure 3. Sucrose-gradient profile of whole 4198V immune RNA.

One hundred micrograms of RNA was layered on a 5-20% sucrose (w/v) gradient and centrifuged at 193,000 x g for 18 hours at 4°C. The absorbance was monitored at 254 nanometers (nm). The sedimentation values are expressed in Svedberg units (S).
Figure 3

Absorbance at 254 nm

18-43S  8-18S  0-8S

Whole 4198V Immune RNA
Figure 4. Sucrose-density profile of poly A⁻ and poly A⁺ 4198V immune RNA. Seventy-five micrograms of RNA was layered on a 5-20% (w/v) gradient and centrifuged at 193,000 x g for 18 hours at 4°C. The absorbance was monitored at 254 nanometers (nm). The sedimentation values are expressed in Svedberg units (S).
Figure 4
this point, the cell suspension was carefully layered on 3 ml of Ficoll-Paque (Pharmacia) separation fluid. This gradient technique originally described by Boyum (1968), was used to separate splenic lymphocytes from red blood cells.

The layered gradient was slowly brought to 600 x g and centrifuged for 5-10 minutes. After centrifugation, the cloudy interfacial layer was removed with a Pasteur pipet and washed twice in fresh tissue culture medium. Viability was determined by trypan blue dye exclusion and hemacytometer count. Lymphocytes were adjusted to the appropriate concentration in medium with or without FCS based on experimental need.

**Treatment of Lymphocytes with RNA**

Lymphocytes were adjusted to 4 X 10^6 cells/2 ml in serum-free RPMI-1640. Twenty-five ug/ml DEAE-dextran (a polycation which enhances cellular uptake of RNA) (Sigma) was added prior to the addition of 100 ug of xenogeneic immune RNA and the suspension incubated at 37°C in a 5% CO₂ atmosphere while slowly mixing on a magnetic stirrer (The optimum ratio of lymphocytes per volume to DEAE-dextran was previously determined by Scheetz, 1972). After 30 minutes, the cells were centrifuged at 600 x g for 10 minutes, and the supernatant discarded. The cell pellet was resuspended in either RPMI-1640 without FCS for *in vivo* experiments
or RPMI-1640 plus 10% FCS, 2mM glutamine, 10 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) (Gibco) and 200 ug/ml gentamicin (Schering) for in vitro assays.

**Lymphocyte-Mediated Cell Cytotoxicity**

Thirty-five millimeter petri dishes (Corning) were seeded with $1 \times 10^4$ 4198V, LM or S91 cells and incubated at 37° in 5% CO2 for 12-24 hours. After incubation, culture medium and non-adherent cells were aspirated. Lymphocyte suspensions were plated on top of the tumor cells at an effector to target cell ratio of 200:1. The petri dishes were placed in the CO2 incubator for an additional 48 hours. The assay was terminated by washing each plate twice with fresh tissue culture medium and adding 0.5 ml 0.25% trypsin. Viable cells were quantitated by trypan blue dye exclusion and hemacytometer count after triturating the cells in 0.5 ml fresh medium supplemented with FCS. The percent cytotoxicity (%) was calculated from the following formula:

$$%C = \frac{\text{average number of cells in control plates} - \text{average number of cells in test plates}}{\text{average number of cells in control plates}} \times 100$$

**Scanning Electron Microscopy of 4198V Lymphocyte-Mediated Cytotoxicity**

The 4198V tumor cell line was grown on glass coverslips in 35 mm petri dishes and seeded with C3H tumor-immune splenic
lymphocytes as described above. After 48 hours, the coverslips were removed from culture, washed with 0.1 M cacodylate buffer (pH 7.4) and incubated for 1 hour in 0.1 M cacodylate containing 2.5% glutaraldehyde (v/v). The cells were dehydrated in a graded alcohol series after a second wash in cacodylate. Coverslips were incubated overnight in amyl acetate and critical point dried by Mr. John Hanson (College of Biological Sciences Electron Microscopy Facilities) in a Samdri PVT-3 critical point drying apparatus. The coverslips were broken into small pieces and glued (Dag 154) (Acheson) on aluminum pin-type mounts (Ernest Fullam). Control preparations of lymphocytes were prepared similar to above except that glutaraldehyde fixed cells were placed in dialysis tubing along with 3 mm copper discs prior to dehydration.

All preparations were sputter coated with gold, examined and photographed by Mr. David Stutes (under the auspices of Dr. Thomas N. Taylor, Department of Botany, The Ohio State University, Columbus, Ohio) on a Hitachi S-500 Scanning Electron Microscope. Polaroid Type 55 Positive/Negative Film (4 X 5 ins.) was used for all photographs.

Immunotherapy of the 4198 Murine Fibrosarcoma

C3H/HeJ mice were injected IM in the rear hind leg with 2.5 X 10^4 4198 cells/0.1 ml on Day 0 of all in vivo experiments.
Two treatments were given to each group on Days 4 and 6 of the experiment and consisted of an IP injection of one of the following:

1. No treatment (tumor cell control).
2. Lymphocytes from normal mice (NL).
3. Lymphocytes from mice which were tumor-immune (IL).
4. Lymphocytes from mice which were bearing an actively growing tumor (TL).
5. Lymphocytes from normal or tumor-bearing mice treated with RNA.
6. RNA alone.

Each mouse received $2 \times 10^7$ lymphocytes and/or 500 ug RNA (32.5 ug poly A$^+$-tailed or 467.5 ug poly A$^-$-tailed RNA for fractionated preparations) per injection.

Comparisons were made among the groups with regard to tumor size (as measured by a dial caliper gauge), and survival time. Statistical analysis was performed by "Student's" t distribution. All data with a confidence interval equal to or greater than 95% were considered significant.

**Immunization of Rabbits - Preparation of Rabbit Anti-C3H Mouse Lymphocyte Serum**

New Zealand White rabbits were injected at 7 day intervals with $5 \times 10^7$ C3H splenic lymphocytes. The cells were isolated as described earlier. Residual red blood cells were lysed with 0.14 M tris-ammonium chloride. Injections were as follows:

1. Day 0 - IV in the marginal ear vein
2. Day 7 - Subcutaneously in Freund's complete adjuvant
3. Day 14 - IM in the rear hind leg
The rabbits were bled by cardiac puncture 3 weeks after the primary injection. Serum was pooled, heat inactivated at 56°C for 30 minutes and stored at -20°C until used.

**Ammonium Sulfate Precipitation**

Gamma globulin was isolated from whole rabbit serum by the ammonium sulfate precipitation method described by Garvey, *et al.*, 1977. Twenty ml of pH 7.8 saturated ammonium sulfate was added slowly dropwise to 40 ml of serum while constantly stirring. This resulted in 1/3 saturation. The suspension was centrifuged at 1400 x g for 30 minutes at room temperature after an additional 2-3 hours stirring. The precipitate was dissolved in saline to 40 ml and the above procedure repeated twice. The final precipitate was dissolved in 0.1 M potassium phosphate buffer (pH 7.5) to a volume of 20 ml and dialyzed at 4°C against the same buffer for 3 days. The dialyzate was changed twice daily. The final solution was clarified by centrifugation and stored at -20°C.

**Ion-Exchange Chromatography**

Immunoglobulin G (IgG) was isolated from ammonium sulfate precipitated rabbit serum by ion-exchange chromatography on DEAE-Sephadex A-50 (Pharmacia). Ten grams of exchanger was swollen in 300 ml of 0.6 M monobasic potassium phosphate. The supernatant fluid was decanted and the slurry equilibrated with 3 cycles
of pH 7.5, 0.1 M potassium phosphate buffer (Dr. J. Shellhaas, personal communication).

A K 15/30 cm column (Pharmacia) was filled slowly with gel and permitted to settle for 30 minutes. Excess buffer was removed and additional slurry added. This process was repeated until no further settling occurred. The buffer reservoir was connected and the column was allowed to run overnight.

Ten ml of ammonium sulfate precipitated rabbit serum was applied to the top of the column and the flow rate adjusted to 12 ml/hour. The effluent was monitored at 280 nm on an LKB Detector Unit (LKB Instruments) and 2.0 ml fractions collected using an LKB Ultropac Fraction Collector (LKB Instruments). The fractions corresponding to the first major peak were pooled and concentrated to the original volume by pressure dialysis with a Diaflow pressure cell (Amicon) and PM-30 ultrafiltration membrane (Amicon). The concentrate was dialyzed against 0.85% physiological saline for 3 days, filter sterilized, and stored at -20°C.

**Determination of Protein**

The protein content of purified rabbit IgG was determined by the Lowry method (Lowry, et al., 1951). Protein pools of known concentration were used to evaluate the accuracy of the standard curve.
**Immunoelectrophoresis**

Whole rabbit anti-C3H mouse lymphocyte serum, ammonium sulfate precipitated gamma globulin and ion-exchange purified IgG was qualitatively analyzed by immunoelectrophoresis (IEP). Commercially prepared plastic film overlaid with 1.5% agarose in a 0.035 M barbital buffer was used (Kallestad).

Three microliters (ul) of the above serum preparations were placed in the appropriate wells on the agarose sheet. This was set in a Gelman electrophoresis chamber (Gelman Instrument Co.) filled with 0.05 M barbital buffer (pH 8.6). Electrophoresis was conducted for 60 minutes at 110 volts. Bromphenol blue tracking dye (0.4%) complexed to albumin served as a marker to monitor the distance of migration through the agar film. The agar sheet was incubated in a humidified chamber for 18 hours at room temperature after adding 100 ul of goat anti-rabbit whole serum (Miles) to the agar troughs.

**Antilymphocyte Serum Cytotoxicity**

Whole serum and purified IgG rabbit anti-C3H mouse lymphocyte serum was titrated in Falcon 3040 microtest plates (Falcon). Serial two-fold dilutions of serum were performed in 50 ul volumes using RPML-1640 tissue culture medium (w/o FCS). Fifty microliters of splenic lymphocytes adjusted to 5 X 10^7 cells/ml were
added to each well followed by 50 μl of a 1:8 dilution of fresh guinea pig complement. The complement was previously absorbed with $10^8$ C3H splenic lymphocytes for 1 hour at 4°C. The cells were trituated with 0.1 ml trypan blue dye (0.18%) after 45 minutes in the 37°C CO$_2$ incubator. Viability was determined by hemacytometer count and the percent cytotoxicity calculated using cells treated with normal whole rabbit serum or purified IgG as a control.

**Preparation of Liposomes**

Positively charged liposomes were prepared by an adaptation of the ether vaporization method described by Deamer and Bangham (1976). One hundred forty seven micromoles (μmol) of phosphatidyl choline (L-α-lecithin) (Sigma), 18 μmol of cholesterol (Sigma) and 37 μmol of stearylamine (N-octadecylamine) (ICN Pharmaceuticals) were dissolved in 1.17 ml of diethyl ether (Baker). The mixture of lipids were placed in a 1.0 cc glass syringe fitted with a 25 gauge stainless steel needle and injected at 0.1 ml/min. into 4 ml of aqueous phase. This phase contained the materials which were to be incorporated into the liposomes (immune RNA, IgG, etc.). The aqueous phase was gently heated on a hot plate and bubbled with nitrogen gas during the injection process to facilitate vaporization of ether and prevent oxidation of lipids. Nitrogen gas was
continued for 10-20 minutes after injection to remove residual ether and all preparations were then sonicated for 15 seconds. The sonication process converts liposomes from multilamellar to unilamellar and generates vesicles of decreased size (Sessa and Weissmann, 1968). Figure 5 illustrates the mechanical set-up for the preparation of liposomes.

Unincorporated material was separated from formed liposomes by sucrose density gradient ultracentrifugation. A 1.0 ml cushion of 35% w/v sucrose (Sigma) was placed in a 1/2 x 2 inch cellulose nitrate tube (Beckman). This was layered with 4 ml of a 1:1 mixture of 24% w/v sucrose and liposomes. The tubes were centrifuged for 90 minutes at 39,000 RPM in an L2-65B ultracentrifuge (Beckman) using an SW 50.1 rotor (Beckman). At the end of this time, the liposomes which had formed a pellicle at the surface of the gradient were removed, resuspended in sucrose and centrifuged a second time.

**Negative Staining of Liposomes**

Liposomes prepared in phosphate buffer aqueous phase were incubated for approximately 2 minutes in 0.15 M cacodylate buffer (pH 7.2) containing 2% glutaraldehyde (v/v). A single drop of this preparation was placed on a formvar carbon-coated 200 mesh copper grid (Ernest Fullam) and excess moisture removed with
Figure 5. An illustration of the preparation of liposomes.

A. Hot plate  
B. Petri dish containing 40°C water  
C. Scintillation vial containing the aqueous phase (RNA, RNA + IgG or phosphate buffer)  
D. Tuberculin syringe (25 gauge stainless steel needle) containing cholesterol, phosphatidyl choline and stearylamine in ether  
E. Pasteur pipet used to introduce nitrogen gas into the aqueous phase

The ethereal phase was injected at a rate of 0.1 ml/min.
bibulous paper after 5 minutes. Uranyl acetate (2%) was added to the grid, incubated for 1 minute and removed by capillary action.

All preparations were examined by Miss Karin Meng (under the auspices of Dr. Robert M. Pfister, Department of Microbiology, The Ohio State University, Columbus, Ohio) in a Phillips 300 electron microscope (EM) (Phillips Electronics). Kodak 3 1/2 X 4 inch electron microscope film was used for all photographs.

Freeze Etching of Liposomes

Sucrose gradient purified liposomes were suspended in 30% glycerol-phosphate buffered saline (v/v) for 12-24 hours. A drop of this suspension was placed on a gold specimen holder, frozen in freon for 5 seconds and transferred to liquid nitrogen. The specimen was placed in a Balzer's High Vacuum Freeze Etching Device (Balzer High Vac), fractured with a knife (cooled to -196°C), etched for 3.5 minutes, shadowed with platinum and carbon coated. The carbon replicas were floated on distilled water, cleaned in concentrated sulfuric acid, rinsed and recovered on 300 mesh uncoated copper grids (Ernest Fullam). This protocol is similar to that described by Weaver and Dugan (1975). All preparations were examined and photographed by Mr. Jeff Titus as described previously.
Immunotherapy of the 4198 Murine Fibrosarcoma with Positively Charged Liposomes

C3H/HeJ mice were injected IM in the rear hind leg with 2.5 \times 10^4 4198 cells/0.1 ml of Day 0. Immunotherapy was given to each group on Days 4, 6, and 8 of the experiment and consisted of an IP injection of one of the following:

1. No treatment (tumor cell control)
2. Liposomes containing phosphate buffered saline
3. Liposomes containing RNA
4. Liposomes containing IgG
5. Liposomes containing RNA and IgG

Each mouse received 13.5 mg of lipid per injection. Evaluation of tumor size and survival time were performed as described earlier.
RESULTS

One of the major immunologic mechanisms responsible for the destruction of neoplastic cells is the cytotoxic action of T or thymus-derived lymphocytes. Documentation of the in vitro cellular events leading to target cell lysis have been reported by Henney (1974) and by Allison and Ferluga (1976). The former author states that target cell destruction is initiated when a T lymphocyte with a specific antigen receptor interacts with the antigen-bearing cell. This results in the production of a soluble mediator which is delivered from the lymphocyte into or onto the membrane of the target cell. Cytolysis is suppressed by the addition of cytochalasin B (inhibits antigen-lymphocyte interaction), pactamycin (inhibits protein synthesis) and drugs which augment the levels of cyclic 3', 5'-adenosine monophosphate (cAMP) (inhibits cellular secretory events) (Henney, 1974). Apparently, neither the complement system nor antibody plays a role in the lytic process. Allison and Ferluga (1976), on the other hand, do not endorse the concept of the production of a soluble mediator. They summarize the
stages of lymphocyte-mediated killing as follows: a) active movement of viable, metabolizing lymphocytes, b) intimate contact between the plasma membranes of the effector and target cells, c) firm adhesion of the cells in the presence of Mg\(^{++}\), d) intimate contact of the lymphocyte and the tumor cell for a short period in the presence of Ca\(^{++}\), e) lysis of the tumor cells due to osmotic disequilibrium (lymphocytes, Ca\(^{++}\) and Mg\(^{++}\) need not be present in this stage).

A number of systems have been developed to pursue studies as to the mechanism of lymphocyte-mediated tumor cell cytotoxicity in vitro. In the study to be described here, a modification of the method of Takasugi and Klein (1970) was utilized. Splenic lymphocytes derived from normal and tumor-immune C3H/HeJ mice were plated on top of adherent tumor cell targets at a ratio of 200:1. Greenup (1977) has found this ratio to be optimum for maximum activity. After 48 hours, remaining viable targets were quantitated by hemacytometer count using trypan blue dye exclusion. Table 2 presents a series of "criss-cross" experiments in which LM, S91 and 4198V immune lymphocytes (IL) were placed on various distinct targets. The percent cytotoxicity was calculated using normal lymphocytes (NL) effectors as a control. Three replicate 35 mm petri dishes were used for each effector cell type. Immune lymphocytes derived from mice which were given repeated intra-
<table>
<thead>
<tr>
<th>Effector Cell</th>
<th>4198V</th>
<th>LM</th>
<th>S91</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune Lymphocytes (IL) (4198V)</td>
<td>27.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.96</td>
<td>-1.81</td>
</tr>
<tr>
<td>IL(LM)</td>
<td>2.82</td>
<td>20.88&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.49</td>
</tr>
<tr>
<td>IL(S91)</td>
<td>5.48</td>
<td>2.35</td>
<td>55.78&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a. The percent cytotoxicity was calculated using normal lymphocytes as a control.
b. Three replicate 35mm petri dishes were used for each effector cell.
c. p＜0.005
d. p＜0.05
peritoneal injections of 4198V cells were capable of destroying a significant percentage of 4198V targets (27.33%, p < 0.005) but not LM or S91 target cells. Similar results were seen in the case of lymphocytes derived from either LM or S91 immune mice (20.88% and 55.78% respectively), indicating the highly specific nature of the reaction.

The interaction between sensitized lymphocytes and tumor cell targets have been studied by transmission and scanning electron microscopy. Faanes (1978) has reported the appearance of surface microvilli both on the effector and the target cell in the early phase of the cytotoxic reaction. The microvilli appear to interdigitate and thereby provide a firm adhesion between the cells. It has been suggested that this form of contact plays a critical role in lymphocyte activation. Target cells with adhering sensitized lymphocytes undergo drastic changes in surface morphology immediately prior to lysis. Microvilli disappear and blebs (also referred to as zeiosis or "boiling") appear throughout the target cell surface. In the present study, 4198V target cells were grown on glass coverslips for 12 hours prior to the addition of either immune (4198V) or normal C3H/HeJ splenic lymphocytes. These were incubated at 37°C in a 5% CO₂ atmosphere. After 48 hours, the coverslips were thoroughly washed and prepared for scanning electron microscopy.
(SEM). Plate I illustrates a control preparation of 4198V tumor cells while Plate II is a visualization of the interaction of sensitized lymphocytes and tumor cell targets. SEM preparations prepared from 4198V targets incubated with normal splenic lymphocytes showed few adherent lymphocytes.

The ability of RNA to transfer immunologic activity is dependent upon the purity and structural integrity of the preparation. The hot phenol extraction method used in these studies has been shown to meet these requirements. Spectrophotometric analysis routinely resulted in a 260/280 nm ratio of 2.0 or greater indicating a minimal amount of protein contamination. All preparation analyzed for relative integrity by sucrose density ultracentrifugation exhibited the typical 3 peak appearance previously shown (Figure 3). Evans (1976) has shown that treatment of RNA with ribonuclease results in a product which yields a single 4S peak on sucrose density gradients and fails to transfer immunologic activity to normal lymphocytes.

The spleens of outbred albino guinea pigs were chosen as a source of xenogeneic immune RNA. Each animal received a series of three injections of cells and was skin tested prior to sacrifice. Four to five milligrams of RNA was obtained per spleen in the majority of extractions.
Plate I. A scanning electron micrograph of the 4198V murine fibrosarcoma. The magnification is approximately 1875X.
Plate II. A scanning electron micrograph of the 4198V murine fibrosarcoma tumor (TC) exposed to 4198V immune C3H/HeJ mouse splenic lymphocytes (L). The magnification is approximately 1500X.
Scheetz (1972) has previously shown that optimum immunologic activity is transferred to normal lymphocytes when 50 ug of DEAE-dextran (1 mg/ml) is added to 4 X 10^6 lymphocytes/2.0 ml prior to the addition of immune RNA. Figure 6 shows a dose response curve of the cytotoxic activity of normal C3H/HeJ mouse splenic lymphocytes treated with 50 to 500 ug of guinea pig 4198V-immune RNA. Maximum activity was seen at a dose of 100 ug and increasing the dose beyond this level did not increase cell-mediated cytotoxicity.

Table 2 illustrated the point that lymphocyte-mediated cytotoxicity was specific with respect to the cell line used for immunization. This same characteristic is evident with RNA transfer of activity. Normal C3H lymphocytes treated with either 100 ug or 500 ug doses of 4198V immune RNA only resulted in significant cytotoxicity against the 4198V cell line (p< 0.001). The growth of LM and Hela cells was not affected (Table 3). These results are similar to those reported by Greenup (1978) and Pennline (1977) with lymphocytes treated with either LM or with S91 immune RNA.

RNA-treated lymphocytes have been used successfully in immunoprophylactic experiments in the C3H/HeJ-4198 tumor system. Evans (1976) injected mice IP with 2 X 10^7 RNA-treated lymphocytes one day prior to tumor challenge (2.5 X 10^4 4198 cells/0.1
Figure 6. The dose response curve obtained with the transfer of cytotoxicity to normal C3H/HeJ splenic lymphocytes using varying quantities of xenogeneic 4198V immune RNA. Normal lymphocytes were used as a control.
Figure 6

Percent Cytotoxicity

µg RNA

- 0 50 100 200 300 400 500
TABLE 3

The Specificity of Transfer of Lymphocyte-Mediated Cell Cytotoxicity Using Xenogeneic 4198V Immune RNA.

<table>
<thead>
<tr>
<th>Normal Lymphocyte Effector Cells Treated with:</th>
<th>4198V</th>
<th>LM</th>
<th>Hela</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 ug 4198V RNA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.9</td>
<td>5.9</td>
</tr>
<tr>
<td>500 ug 4198V RNA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.8</td>
<td>7.0</td>
</tr>
</tbody>
</table>

---

a. The percent cytotoxicity was calculated using normal lymphocytes as a control.
b. Six replicate 35mm petri dishes were used for each dose of RNA.
c. p<0.001
ml, IM), and two days after tumor challenge. Sixty percent of the treated mice remained free of detectable tumor for greater than 3 months while 100% of the mice which received either no treatment or normal lymphocytes developed tumor within 24 days. Although these results reflect the ability of RNA to prevent the establishment of a percentage of primary tumors in C3H mice, they do not consider the efficacy of immune RNA as a form of immunotherapy.

Is this form of information transfer capable of influencing the immune system in such a manner as to control metastatic disease, slow tumor growth, or cause tumor regression?

**Immunotherapy of the 4198 tumor with IP injected immune RNA**

A series of in vivo experiments was designed to examine the influence of direct IP injection of immune RNA on the growth of the 4198 tumor. In all cases, $2.5 \times 10^4$ 4198 cells were injected IM on Day 0. This dose has previously been determined to be smallest number of cells which would give rise to tumors in 100% of control mice (Evans, 1976). Treatments were administered on Days 4 and 6.

In the first experiment, male C3H/HeJ mice were divided into 4 groups consisting of controls, DEAE-dextran (DEAE) alone, DEAE plus S91 immune RNA and DEAE plus 4198V immune RNA.
A total of 25 mice were used in the experiment--4 controls, 4 DEAE, 8 S91 and 8 4198V. Five hundred micrograms per 0.5 ml of DEAE and 500 µg/0.5 ml of immune RNA was administered at each day of treatment. Control mice received injections of phosphate-buffered saline. Tumor size was measured in one direction with dial calipers and expressed as tumor diameter in centimeters (cm) versus time in days. The results of this experiment are illustrated in Figure 7. There was no significant difference in any group with respect to the type of treatment as compared with the controls. Mice within each group also were monitored for mean survival time, median survival time and 100% mortality. It is worth emphasizing that only the mean survival time is open to statistical analysis. In general, the time points presented for median survival and 100% mortality represent a single mouse within the group. No significant difference was seen between groups with respect to mean survival in Experiment 1. The mice injected with 4198V RNA plus DEAE represented the only group to exhibit some difference in median survival and 100% mortality (3 and 11 days greater than the data from the controls, respectively) (Table 4).

Whole cellular immune RNA may be fractionated by oligo(dT)-cellulose chromatography into two populations, either containing or lacking a segment of polyadenylic acid (poly A tail). The poly
Figure 7. **In Vivo** Experiment 1 - Tumor diameter in centimeters versus time in days. Immune RNA (500 ug/0.5 ml) and/or DEAE-dextran (500 ug/0.5 ml) was injected intraperitoneally (IP) into C3H/HeJ mice on Days 4 and 6 post-tumor cell inoculation. No significant difference was seen between the treatment groups and the control. The controls received IP injections of phosphate-buffered saline.
Figure 7
TABLE 4

The Survival Time (Days) of C3H/HeJ Mice Injected Intraperitoneally with Immune RNA and/or DEAE-Dextran.

<table>
<thead>
<tr>
<th>Treatment Groupa</th>
<th>Mean Survival ± S. D. b, c</th>
<th>Median Survival</th>
<th>100% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td>32.7 ± 8.4</td>
<td>31</td>
<td>44</td>
</tr>
<tr>
<td>DEAE-Dextran (DEAE) (n = 5)</td>
<td>31.8 ± 3.8</td>
<td>31</td>
<td>37</td>
</tr>
<tr>
<td>S91 RNA + DEAE (n = 8)</td>
<td>31.9 ± 5.8</td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>4198V RNA + DEAE (n = 8)</td>
<td>35.1 ± 9.2</td>
<td>34</td>
<td>55</td>
</tr>
</tbody>
</table>

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a. n - The number of mice per group
b. S. D. - standard deviation
c. The mean survival of mice treated with DEAE and/or RNA are not significantly different than the control.
A\(^+\) fraction is known to be messenger RNA (mRNA) which sediments to 9-18S in sucrose and comprises 4-6% of the total cellular immune RNA. A small portion of poly A\(^-\) RNA may be mRNA which lacks the adenylic tail. Greenup (1978) has shown that maximum lymphocyte-mediated 4198V tumor cell cytotoxicity is achieved by treating normal C3H/HeJ mouse splenocytes with 6.5 ug of poly A\(^+\) 4198V immune mRNA (50% cytotoxicity). The highest cytotoxic level obtained with poly A\(^-\) 4198V immune RNA was approximately 20% with a dosage of 200 ug. It was not investigated as to whether or not any part of the activity transferred with poly A\(^-\) was due to mRNA lacking poly A tails, or to contaminating poly A\(^+\) mRNA within the preparation. Additional evidence was presented which suggested that poly A\(^-\) immune RNA might contain information which had a suppressive effect. The high degree of cytotoxic potential exhibited by lymphocytes treated with poly A\(^+\) immune mRNA was diminished significantly with subsequent incubation with poly A\(^-\) RNA. One might suggest that the division of whole immune RNA into various fractions results in information with either a positive or negative regulatory role. Isolation of the appropriate fraction for use in immunotherapy may provide valuable information regarding the immune response on a molecular level.
The second experiment was designed to consider the above observations, and compare the immunotherapeutic effects of whole 4198V-immune RNA with oligo (dT)-cellulose isolated fractions. Five hundred microgram per 0.5 ml of whole 4198V-immune RNA, 32.5 ug/0.5 ml poly A⁺ mRNA and 467.5 ug/0.5 ml poly A⁻ RNA was injected into the appropriate group on each day of treatment. Each group consisted of 5 mice with the exception of the control group which contained 6 mice. The dosage of RNA fractions was derived from the in vitro observations by Greenup (1977), that 6.5% (representing 6.5 ug of poly A⁺ mRNA) of a 100 ug whole immune RNA preparation transfers maximum cytotoxic potential to normal lymphocytes (6.5 ug poly A⁺ + 93.5 ug poly A⁻ RNA = 100 ug whole immune RNA - This is multiplied by a factor of 5 to compare with the 500 ug of whole immune RNA injected). DEAE-dextran was injected with the RNA as described in Experiment 1. Figure 8 illustrates the sizes of the tumor diameters observed in the 4 groups from Days 10-22. The tumor group which had received poly A⁺ RNA was significantly different from the control on Day 10 (p < 0.025). No difference was seen between the groups with respect to either mean or median survival time, although mice within the groups which had received RNA survived (100% mortality) from 6 to 20 days longer than did the controls (Table 5).
Figure 8. **In Vivo** Experiment 2 - Tumor diameter in centimeters versus time in days. Whole 4198V-immune RNA (500 ug/0.5 ml), poly A⁺ mRNA (32.5 ug/0.5 ml) and/or poly A⁻ RNA (467.5 ug/0.5 ml) in combination with DEAE-dextran (500 ug/0.5 ml) was injected intraperitoneally (IP) into C3H/HeJ mice on Days 4 and 6 post-tumor cell inoculation. The controls received IP injections of phosphate-buffered saline. Poly A⁺ mRNA was significantly different than the control on Day 10 (p < 0.025).
Figure 8

Tumor diameter in cm.

Days

Control
RNA (4198V)
RNA (poly A+4198V)
RNA (polyA-4198V)
TABLE 5

The Survival Time (Days) of C3H/HeJ Mice Injected Intraperitoneally with Either Whole or Fractionated 4198V Immune RNA.

<table>
<thead>
<tr>
<th>Treatment Group a</th>
<th>Mean Survival ± S. D. b, c</th>
<th>Median Survival</th>
<th>100% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>29.0 ± 2.8</td>
<td>30</td>
<td>33</td>
</tr>
<tr>
<td>4198V RNA (n = 5)</td>
<td>32.2 ± 5.5</td>
<td>30</td>
<td>39</td>
</tr>
<tr>
<td>Poly A⁺ 4198V RNA + DEAE (n = 5)</td>
<td>37.4 ± 11.8</td>
<td>31</td>
<td>53</td>
</tr>
<tr>
<td>Poly A⁻ 4198V RNA + DEAE (n = 5)</td>
<td>31.4 ± 6.6</td>
<td>31</td>
<td>42</td>
</tr>
</tbody>
</table>

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a. n - The number of mice per group  
b. S. D. - standard deviation  
c. The mean survival of mice treated with RNA or RNA + DEAE are not significantly different than the control.
Immunotherapy of the 4198 tumor with immune RNA-treated splenic lymphocytes

A major problem associated with the direct injection of immune RNA is the presence of endogenous nucleases within the system. One may envision the sequence of events following injection as a race between rapid enzymatic degradation by ribonucleases and uptake by the immune system. It is entirely possible that only a small fraction of injected material is ultimately processed.

There are two available approaches which might permit the by-passing of the problems associated with nuclease activity. The first involves the incorporation of immune RNA into liposomes (The results of which will be covered in a later section), while the second involves incubation of immune RNA with lymphoid cells in vitro. The infusion of RNA-treated lymphocytes from donor to recipient is entirely feasible in a syngeneic animal system but presents major difficulties in the human clinical setting in which histocompatibility differences exist. It is therefore critical to evaluate the efficacy of removing lymphoid cells from a patient just prior to, or shortly after surgery, treating the cells with immune RNA in vitro and returning them to the patient.

Pennline (1977) has studied, in vitro, the influence of immune RNA on lymphoid cells obtained from tumor-bearing mice and has reported that lymphocytes isolated from tumor-bearing mice (tumor
lymphocytes or TL) were capable of both participating in lymphocyte-mediated tumor cell cytotoxicity and releasing "blocking factors" into an antigen-free culture system. Both events were specific in nature. When the tumor lymphocytes were treated with 100 ug of immune RNA per $4 \times 10^6$ cells, the cytotoxic potential was doubled and the production of "blocking factors" was shut-off. One would have an excellent immunotherapeutic tool if these observations could be duplicated in vivo. The continued interference by "blocking factors" would be abrogated thus permitting existing cytotoxic activity to function at an accelerated level.

In Vivo Experiments 3 through 6 were designed to test the above (Pennline, 1977) observations. Treatments were provided on Days 4 and 6 post-tumor cell inoculation ($2.5 \times 10^4$ $4198$ cells/0.1 ml, IM). Twenty million lymphocytes were injected IP into each mouse on each day of treatment and those incubated with RNA were at a ratio of 100 ug of whole immune RNA, 6.5 ug of poly A$^+$ immune mRNA or 93.5 ug of poly A$^-$ immune RNA per $4 \times 10^6$ lymphocytes. All splenic lymphocytes were exposed to DEAE-dextran as discussed previously (Page 75).

C3H/HeJ mice were divided into 6 treatment groups (consisting of either 9 or 10 mice per group) in the third in vivo experiment. The treatment groups included: normal lymphocytes
(NL), 4198V immune RNA-treated NL, tumor immune lymphocytes (IL), lymphocytes from tumor-bearing mice (TL) and 4198V immune RNA-treated TL. Figure 9 illustrates the influence of these cell types on the growth of the 4198 tumor over a 25 day time period. Only 4198V immune RNA-treated NL were ineffective in significantly altering the tumor growth rate when compared with the control. The treatments provided to the four groups which exhibited significant alteration in tumor growth rate also were capable of increasing the 100% mortality from 9 to 35 days beyond the control. No difference was seen in any group with respect to mean or median survival time (Table 6).

The ability of untreated lymphocytes derived from tumor-bearing mice (TL) and 4198V immune RNA-treated TL to influence tumor growth was re-examined in the fourth experiment. The control group was comprised of 6 mice while the TL and TL + 4198V RNA groups contained 9 and 19 mice, respectively. In contrast with the previous experiment, no advantage was provided by either treatment. In fact, incubation of TL with 4198V immune RNA prior to injection resulted in significant tumor enhancement through day 29 of the experiment ($p<0.025$) (Figure 10). The survival times presented in Table 7 would seem to confirm that there was little advantage to be attributed to either treatment.

The fifth in vivo experiment was identical in design to the previous experiment except that lymphocytes derived from C3H mice
Figure 9. **In Vivo** Experiment 3 - Tumor diameter in centimeters versus time in days. Untreated or RNA-treated lymphocytes were injected intraperitoneally (IP) into C3H/HeJ mice on Days 4 and 6 post-tumor cell inoculation. Twenty million lymphocytes were injected per mouse at each treatment. All of the lymphocytes were exposed to DEAE-dextran. The following were significantly different than the control on Day 25: NL (p<0.05), IL (p<0.005), TL (p<0.005) and TL + 4198V RNA (p<0.01).
Figure 9
TABLE 6

The Survival Time (Days) of C3H/HeJ Mice Injected Intraperitoneally with Either Untreated or RNA-Treated Lymphocytes

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mean Survival ± S. D.</th>
<th>Median Survival</th>
<th>100% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
<td>34.6 ± 4.7</td>
<td>35</td>
<td>41</td>
</tr>
<tr>
<td>Normal Lymphocytes (NL) (n = 9)</td>
<td>37.3 ± 9.1</td>
<td>36</td>
<td>54</td>
</tr>
<tr>
<td>NL + 4198V (n = 10)</td>
<td>35.4 ± 6.1</td>
<td>33.5</td>
<td>46</td>
</tr>
<tr>
<td>Immune Lymphocytes (IL) (4198V) (n = 9)</td>
<td>33.2 ± 14.1</td>
<td>38</td>
<td>50</td>
</tr>
<tr>
<td>Tumor Lymphocytes (TL) (4198) (n = 10)</td>
<td>37.0 ± 18.6</td>
<td>39</td>
<td>76</td>
</tr>
<tr>
<td>TL + 4198V RNA (n = 9)</td>
<td>35.9 ± 7.8</td>
<td>33</td>
<td>54</td>
</tr>
</tbody>
</table>

a. n - The number of mice per group  
b. S. D. - standard deviation  
c. The mean survival time of the treatment groups are not significantly different than the control.
Figure 10. In Vivo Experiment 4 - Tumor diameter in centimeters versus time in days. Untreated or 4198V immune RNA-treated tumor lymphocytes (TL) were injected intraperitoneally into C3H/HeJ mice on Days 4 and 6 post-tumor cell inoculation. Twenty million lymphocytes were injected per mouse at each treatment. All of the lymphocytes were exposed to DEAE-dextran. RNA-treated TL were significantly different from the control on Day 29 (p<0.025).
TABLE 7

The Survival Time (Days) of C3H/HeJ Mice Injected Intraperitoneally with Either Untreated or 4198V Immune RNA-Treated Tumor Lymphocytes (TL).

<table>
<thead>
<tr>
<th>Treatment Groupa</th>
<th>Mean Survival ± S. D. b, c</th>
<th>Median Survival</th>
<th>100% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>36.7 ± 6.5</td>
<td>33.5</td>
<td>49</td>
</tr>
<tr>
<td>TL (4198) (n = 9)</td>
<td>40.1 ± 5.2</td>
<td>39</td>
<td>52</td>
</tr>
<tr>
<td>TL (4198) + RNA (4198V) (n = 19)</td>
<td>32.4 ± 3.8</td>
<td>32</td>
<td>40</td>
</tr>
</tbody>
</table>

a. n - The number of mice per group
b. S. D. - standard deviation
c. The mean survival time of the treatment groups are not significantly different than the control.
bearing the S91 malignant melanoma were included as an additional treatment. In this instance, lymphocytes from 4198 tumor-bearing mice (TL) were capable of significantly altering the tumor-growth rate (Day 32, p<0.025) when they were incubated with 4198 immune RNA prior to injection. It is interesting to note that S91 TL, which were "heterologous" for the 4198 tumor, were significantly different than the control (Day 28, p<0.025), while homologous 4198 TL were not (Figure 11). As in the earlier experiments, there was no difference between the control and the treatment groups with respect to mean survival time (Table 8). Although the median survival of mice receiving either S91 or 4198 TL was 10 days greater than was recorded for the control, at least one mouse within the control survived from 7-14 days longer than any in the treatment group.

In Vivo Experiment 6 was the final experiment in the series involving RNA-treated lymphocytes as a form of immunotherapy. Whole 4198V immune RNA was compared with poly A+ and poly A− fractions. Statistical analysis of the data presented in Figure 12 indicated that only whole 4198V immune RNA treated 4198 TL decreased tumor growth rate (Day 23, p<0.05). The incubation of TL with poly A− 4198V RNA resulted in enhanced tumor growth rate (Day 21, p<0.05) as compared with the control. Incubation of TL with the RNA preparations increased median survival time from 6-9 days but had no effect on either mean survival or 100% mortality (Table 9).
Figure 11. *In Vivo Experiment 5 - Tumor diameter in centimeters versus time in days.* Untreated or 4198V immune RNA-treated tumor lymphocytes (TL) were injected intraperitoneally into C3H/HeJ mice on Days 4 and 6 post-tumor cell inoculation. Twenty million lymphocytes were injected per mouse at each treatment. All of the lymphocytes were exposed to DEAE-dextran. RNA-treated TL were significantly different than the control on Day 32 (p<0.025) while TL derived from mice bearing the S91 malignant melanoma were significantly different on Day 28 (p<0.025).
Figure 11

Tumor diameter in cm.

- TL (4199) + RNA (4199v)
- TL (4199)
- TL (591)
- Control

Days

Tumor diameter in cm.

0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
TABLE 8

The survival Time (Days) of C3H/HeJ Mice Treated with Either Untreated or 4198V Immune RNA-Treated Tumor Lymphocytes (TL).

<table>
<thead>
<tr>
<th>Treatment Group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean Survival ± S. D. &lt;sup&gt;b, c&lt;/sup&gt;</th>
<th>Median Survival</th>
<th>100% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
<td>38.1 ± 9.0</td>
<td>34</td>
<td>59</td>
</tr>
<tr>
<td>TL (4198) (n = 9)</td>
<td>42.6 ± 6.4</td>
<td>44</td>
<td>52</td>
</tr>
<tr>
<td>TL (S91) (n = 6)</td>
<td>43.5 ± 1.6</td>
<td>44</td>
<td>45</td>
</tr>
<tr>
<td>TL (4198) + RNA (4198V)</td>
<td>36.4 ± 8.4</td>
<td>34</td>
<td>48</td>
</tr>
<tr>
<td>(n = 11)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

<sup>a</sup> n - The number of mice per group
<sup>b</sup> S. D. - standard deviation
<sup>c</sup> The mean survival time of the treatment groups are not significantly different than the control.
Figure 12. In Vivo Experiment 6 - Tumor diameter in centimeters versus time in days. Whole or fractionated 4198V immune RNA-treated tumor lymphocytes (TL) were injected intraperitoneally into C3H/HeJ mice on Days 4 and 6 post-tumor cell inoculation. Twenty million lymphocytes were injected per mouse at each treatment. All of the lymphocytes were exposed to DEAE-dextran. Poly A+ 4198V immune RNA-treated TL were significantly different than the control on Day 21 (p<0.05) while whole 4198V immune RNA-treated TL were significantly different than the control on Day 23 (p<0.05).
TABLE 9

The Survival Time (Days) of C3H/HeJ Mice Injected Intraperitoneally with Whole or Fractionated 4198V Immune RNA-Treated Tumor Lymphocytes (TL).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mean Survival ± S.D.</th>
<th>Median Survival</th>
<th>100% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
<td>25.2 ± 11.1</td>
<td>24</td>
<td>44</td>
</tr>
<tr>
<td>TL (4198) + RNA (4198V) (n = 5)</td>
<td>30.8 ± 10.3</td>
<td>30</td>
<td>44</td>
</tr>
<tr>
<td>TL (4198) + RNA (Poly A⁺ 4198V) (n = 8)</td>
<td>29.5 ± 4.3</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>TL (4198) + RNA (Poly A⁻ 4198V) (n = 10)</td>
<td>29.6 ± 12.9</td>
<td>32.5</td>
<td>47</td>
</tr>
</tbody>
</table>

a. n - The number of mice per group
b. S.D. - standard deviation
c. The mean survival time of the treatment groups are not significantly different than the control.
Immunotherapy of the 4198 tumor using RNA-containing liposomes

Recent investigations by Ostro, et al., (1977) and Dimitriadis (1978) have shown that RNA entrapped within liposomes is not susceptible to degradation by ribonucleases. Both investigative groups treated RNA-containing liposomes with pancreatic ribonuclease, purified the preparations on Sepharose 4B and re-extracted the RNA. Analysis on either linear sucrose gradients or polyacrylamide gel indicated a structurally intact preparation. In addition, Magee et al., (1978) have reported that Hypaque:Ficoll gradient purified guinea pig lymphocytes were capable of processing syngeneic anti-hepatocarcinoma immune RNA (in vitro) entrapped within positively charged multilamellar liposomes bearing anti-lymphocyte IgG (The IgG provided a specific "homing" mechanism for the liposomes and lymphocyte membrane), as evidenced by a chromium-51 tumor cell cytotoxicity assay. Taken together, the information presented above, suggests that target-directed liposomes containing immune RNA may be a more efficient immunotherapeutic tool than either RNA-treated lymphocytes or RNA alone. Accordingly, it was the intent in this investigation to evaluate this approach in the C3H/HeJ - 4198 tumor system.

In this particular study, a "gamma globulin fraction" or rabbit anti-C3H/HeJ mouse lymphocyte serum was obtained by the ammonium sulfate precipitation method. A pure IgG fraction was isolated from the
preparation by ion-exchange chromatography with DEAE-Sephadex A-50. The whole serum, ammonium sulfate precipitated fraction and final ion-exchange product were qualitatively analyzed by immunoelectrophoresis. Plate III illustrates that a single arc of precipitation was obtained with the ion-exchange purified product. Although not pictured, commercially obtained rabbit IgG migrated to the same location on the agar gel as did the IgG purified in this laboratory.

The activity of the purified IgG was compared with whole rabbit anti-C3H/HeJ mouse lymphocyte serum using a complement-mediated lymphocyte cytotoxicity assay (Shellhaas, 1977). Serial two-fold dilutions of serum were mixed with 2.5 × 10^6 splenic lymphocytes and fresh guinea pig complement. Viability was determined by hemacytometer count using trypan blue dye exclusion after incubation for 45 minutes in a 37°C CO₂ incubator. Normal whole rabbit serum or normal purified IgG served as a control. The titers of whole rabbit anti-C3H/HeJ mouse lymphocyte serum and its purified IgG fraction were 128 and 1024, respectively.

Positively charged liposomes were prepared by an adaptation of the ether vaporization method described by Deamer and Bangham (1976) using a 7:2:1 molar ratio of phosphatidyl choline, stearylamine (imparts the positive charge) and cholesterol. The modification of the original technique has been reported to give rise to numerous
Plate III. The immunoelectrophoretic analysis of whole rabbit serum (wells - 1, 4 and 7), ammonium sulfate precipitated rabbit serum (wells - 2 and 5) and ion-exchange chromatography purified ammonium sulfate precipitated rabbit serum (wells - 3 and 6) against commercial goat anti-rabbit whole serum (troughs A-F). The protein was stained with 0.5% coomassie brilliant blue after electrophoresis for 60 minutes at 110 volts, and precipitation for 18 hours at room temperature.
Plate III

(-) -------- 32 mm -------- (+)

1
2
3
4
5
6
7

A
B
C
D
E
F

No 63051
multilamellar spherules (Deamer and Bangham, 1976). Unincorpo-
rated material was separated from formed liposomes by two
sequential 90 minute centrifugations in sucrose. Plate IV visually
illustrates the purification sequence in which lacmoid blue dye was
incorporated into the liposomes. Entrapment of the blue dye with-
in the liposomes was verified by viewing the preparation under
bright field microscopy (Plate V). Careful evaluation of Plates V
and VI (phase-contrast microscopy) illustrates the presence of
liposomes with several lipid bilayers (multilamellar). While it is
difficult to rule out the presence of unilamellar liposomes within
the preparations based on the two photographs, a freeze-etch prep-
paration of liposomes containing phosphate-buffered saline also
revealed a multilamellar ("membraneous") ultrastructural appear-
ance (Plate VII). It is interesting to note that large liposomes
may entrap smaller vesicles during the vaporization process as
evidenced by the freeze-etch preparation illustrated in Plate VIII.

Two studies were undertaken to determine if any portion of the
IgG molecule extended through the outer lipid bilayer (for interaction
with the specific target) in those preparations in which immunoglobulin
was part of the aqueous phase. Liposomes containing ferritin-labeled
IgG were examined by electron microscopy for typical "electron
dense" areas. None was noted in any of the areas scanned. In a
second experiment, liposomes containing rabbit IgG were incubated
Plate IV. The sucrose-density purification of unsonicated liposomes containing lacmid blue dye. A 1:1 mixture of 24% w/v sucrose and liposomes was layered on a 35% w/v sucrose cushion, and centrifuged at 39,000 RPM for 90 minutes at 4°C.

A. Liposome-sucrose mixture prior to centrifugation
B. The same mixture following 90 minutes centrifugation
C. Resuspension of the liposome pellicle in phosphate buffer and sucrose followed by a 90 minute centrifugation.
Plate IV

A

B

C
Plate V. Unsonicated liposomes containing lacmoid blue dye as viewed by bright field microscopy. The magnification is approximately 1500 diameters.
Plate V
Plate VI. Unsonicated liposomes containing lacmoid blue dye as viewed by phase-contrast microscopy. The magnification is approximately 1400 diameters.
Plate VII. A freeze-etch preparation of unsonicated liposomes as viewed by electron microscopy. The magnification is approximately 73,000X.

S - Direction of platinum shadow
Plate VII
Plate VIII. A freeze-etch preparation of unsonicated liposomes as viewed by electron microscopy. The magnification is approximately 73,250X.

S - Direction of platinum shadow
V - Lipid vesicle
with fluorescein-conjugated goat anti-rabbit IgG. The preparation was examined in a fluorescent microscope and compared with phosphate-buffered saline-containing liposomes which also were incubated with fluorescein-conjugated antibody. Fluorescence of the "peripheral or rim-type" was noted in both preparations although the intensity appeared greater in the liposomes containing rabbit IgG.

A final in vivo experiment was conducted after successfully preparing positively charged liposomes which were capable of entrapping materials in aqueous solution (as evidenced by the presence of lacmoid blue dye within the liposome). Sixty-seven milligrams of lipid (phosphatidyl choline, stearylamine and cholesterol) dissolved in approximately 0.67 ml of ether was injected into 2 ml of aqueous phase at a rate of 0.1 ml/min. The aqueous phase consisted of either phosphate-buffered saline (PBS), 4198V immune RNA, anti-C3H mouse lymphocyte IgG or RNA in combination with IgG. The immune RNA and/or IgG was at a concentration of 1 mg/ml. The mice within each group (each treatment group was comprised of 5 mice with the exception of the IgG group which had 4 mice) received 13.4 mg of sucrose gradient purified liposomes on Day 4, 6 and 8 post-tumor cell inoculation. The liposome preparations were sonically disrupted for 15 seconds just prior to injection into the mice (The size of these vesicles ranged from 0.06 - 0.5 um as determined by photographing electron
microscopic preparations of glutaraldehyde fixed, negatively stained spherules, Plate IX.). Each mouse received $2.5 \times 10^4$ tumor cells/0.1 ml as described in the previous in vivo experiments. Figure 13 illustrates the tumor growth rate over a 25 day time course. A significant difference ($p<0.025$) was noted only between the group of mice which received liposomes containing 4198V immune RNA, and the control group. The difference in tumor growth rate (as monitored by tumor diameter in centimeters) in the RNA-liposome group appeared to be a factor in this experiment since a difference was noted in the mean survival time of this group when compared with the controls ($p<0.05$) (Table 10). The mice receiving liposomes containing immune RNA survived an average of 39.4 days as compared with 29.8 days for the group receiving tumor cells alone. This was the only in vivo experiment of the 7 conducted in which a form of RNA immunotherapy appeared capable of inducing a significant difference in mean survival time. It is worth pointing out that mice which had received liposomes containing PBS would be a more appropriate control than mice which had received tumor cells alone. In this regard, there was no significant difference in any treatment group with respect to mean survival time. One might also note that a difference was seen in the groups of mice which received liposomes containing immune RNA and/or IgG with respect to median survival and 100% mortality.
Plate IX. A negative stain preparation of sonically disrupted (15 seconds) liposomes as viewed by electron microscopy. The magnification is approximately 31,400X.
Plate IX
Figure 13. In Vivo Experiment 7 - Tumor diameter in centimeters versus time in days. Positively charged liposomes containing either phosphate-buffered saline (PBS), rabbit anti-C3H/HeJ mouse lymphocyte IgG, 4198V immune RNA or IgG in combination with immune RNA were injected intraperitoneally into C3H/HeJ mice on Days 4, 6 and 8 post-tumor cell inoculation. Each mouse received 13.4 mg of lipids per injection. The liposomes were formed by injecting 67 mg of lipids (dissolved in ether) into 2 ml of aqueous phase. Immune RNA and/or IgG were adjusted to a concentration of 1 mg/ml in the aqueous phase. The group receiving liposomes containing only 4198V immune RNA were significantly different than the control on Day 25 (p<0.025).
Figure 13

- Control
- PBS Liposomes (Lip.)
- IgG Lip.
- RNA (4198V) Lip.
- [IgG + RNA (4198V)] Lip.
The Survival Time (Days) of C3H/HeJ Mice Injected Intraperitoneally with Liposomes Containing 4198V Immune RNA and/or Anti-Lymphocyte IgG.

<table>
<thead>
<tr>
<th>Treatment Group^a</th>
<th>Mean Survival ± S. D.^b</th>
<th>Median Survival</th>
<th>100% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 5)</td>
<td>29.8 ± 4.1</td>
<td>31</td>
<td>35</td>
</tr>
<tr>
<td>PBS Liposomes (Lip.) (n = 5)</td>
<td>31.8 ± 3.6</td>
<td>30</td>
<td>37</td>
</tr>
<tr>
<td>IgG Lip. (n = 4)</td>
<td>35.5 ± 8.7</td>
<td>37</td>
<td>44</td>
</tr>
<tr>
<td>RNA (4198V) Lip. (n = 5)</td>
<td>39.4 ± 10.8^c</td>
<td>35</td>
<td>55</td>
</tr>
<tr>
<td>(IgG + RNA (4198V)) Lip. (n = 5)</td>
<td>37.4 ± 6.8</td>
<td>37</td>
<td>44</td>
</tr>
</tbody>
</table>

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a. _n_ - The number of mice per group  
b. _S. D._ - Standard deviation  
c. The mean survival time of the mice which received liposomes containing immune RNA alone was significantly different than the control (p<0.05)
DISCUSSION

The first two in vivo experiments conducted in this project were designed to evaluate the influence of direct intraperitoneal injection of whole and/or fractionated 4198V immune RNA on the 4198 fibrosarcoma tumor in male C3H/HeJ mice. From the data obtained, it would appear that this approach was of little or no immunotherapeutic value. Although poly A+ mRNA was capable of causing a significant alteration in tumor growth rate, the effect was transient and was reversed by Day 11 of the experiment (Figure 7). None of the RNA preparations were able to increase the mean survival time as compared with untreated controls (Table 4 and 5). While one might suggest that immune RNA was capable of influencing the immune system in some undefined manner as evidenced by the differences in 100% mortality between treatment groups and the controls, it should be recalled that the 100% figures presented in Tables 4 and 5 represent a single mouse within a group and it is therefore open to interpretation as to whether this is a significant value or not.

Greenup (1977) has suggested that whole preparations of immune RNA might contain a fraction which has a negative or suppressive
regulatory effect on immune functions. Fractionation of whole RNA on oligo d(t)-cellulose results in two populations which either contain or lack a polyadenylic acid tail (poly A). In vitro cytotoxicity experiments have shown that incubation of lymphocytes with poly A\textsuperscript{-} immune RNA following treatment of the cells with poly A\textsuperscript{+} immune RNA results in a decreased cytotoxic response. Reversing the order of treatment (poly A\textsuperscript{-} RNA followed by poly A\textsuperscript{+} mRNA) does not result in a diminished response. This evidence may suggest that the fraction of RNA which lacks a poly A\textsuperscript{+} tail contains a "species" of RNA which is suppressive in nature. The removal of poly A\textsuperscript{-} RNA from the whole 4198V immune RNA used here may offer explanation for the inhibitory effect (through brief) of poly A\textsuperscript{+} mRNA on tumor growth rate in in vivo Experiment 2. In other words, a fraction of RNA containing a "suppressive species" was removed from the preparation, thereby permitting mRNA to exert its influence.

The failure of intraperitoneally administered immune RNA to influence the progression of the 4198 tumor in C3H mice is most probably attributable to the presence of endogenous ribonucleases. It is not unreasonable to assume that the majority of RNA was degraded before it had an opportunity to be processed by the immune system. Molecular virologists have appreciated for quite some time that special precautions are required when handling viral RNA. Nuclease
inhibitors such as polyvinyl sulfate (PVS), bentonite, diethylpyrocarbonate (DEP), proteinase K, DEAE-dextran and sodium dodecyl sulfate (SDS) are often included in buffer preparations. Pipetting is performed with mechanical pipetting devices rather than by mouth (expired air contains nucleases) and gloves are worn when handling all preparations (the skin contains high levels of nuclease activity).

As discussed previously (literature review), the success of immunotherapy involving immune RNA which is directly injected appears to be correlated with the inclusion of an RNAse inhibitor. Deckers and Pilch (1971) reported a significant degree of success in the treatment of BP-1R tumors in rats when RNA was admixed with the RNAse inhibitor sodium dextran sulfate, while results have been less than favorable in human clinical trials where RNA was injected alone (Pilch, et al., 1976; Ramming, et al., 1978).

It is apparent that the injection of DEAE-dextran prior to 4198V immune RNA in this study was ineffective in the C3H/HeJ - 4198 tumor system.

*In vivo* Experiments 3-6 evaluated the efficacy of using whole and/or fractionated 4198V immune RNA-treated syngeneic lymphocytes in the immunotherapy of the 4198 tumor. Previous investigators in these laboratories have shown that RNA may be effectively introduced into lymphoid cells by incubation *in vitro* as
evidenced by the production of either MIF (migration inhibition factor) or lymphocyte-mediated tumor cell cytotoxicity (Scheetz, 1972; Nawrocki, 1977; Greenup, 1977).

The administration of 4198V immune RNA-treated TL (lymphocytes derived from tumor-bearing mice) into C3H/HeJ mice was capable of significantly decreasing tumor growth rate in 3 of 4 in vivo immunotherapy experiments conducted (Figures 9, 10, and 11). This situation did not exert a long term effect though, since none of the groups treated in this manner exhibited a significant difference in mean survival time. Tumor growth was enhanced in the remaining experiment (Figure 10) following the administration of RNA-treated TL while mean survival time was unaffected, as before.

No benefit was provided in terms of tumor growth rate or mean survival time by incubating TL with either the poly A+ or poly A− fractions of whole 4198V immune RNA. In fact, tumor growth rate appeared to be more rapid in the mice which had received the poly A− RNA-treated TL (Figure 12). This latter observation re-emphasizes the points previously made about the possible suppressive nature of RNA which lacks a poly A+ tail.

It is noteworthy that all of the immunotherapy trials reported in the literature utilize "normal" syngeneic lymphocytes as the cells to be treated with immune RNA. The approach taken in this study
was to treat TL with 4198V immune RNA. It was felt that this protocol was more applicable to the human clinical setting in that if one were to treat a patient's lymphoid cells with immune RNA in vitro, the cells would be obtained by leucaphoresis immediately prior to, or shortly after surgical removal of a tumor. In this case, the cells would not be considered "normal". There is in vitro evidence to support this technique as an immunotherapeutic approach. Pennline (1977) has studied the influence of 4198V immune RNA on lymphoid cells obtained from 4198 tumor-bearing C3H/HeJ mice and has reported that lymphocytes isolated from the spleens of tumor-bearing mice were capable of both participating in lymphocyte-mediated tumor cell cytotoxicity and releasing "blocking factors" into an antigen-free culture system. When the tumor lymphocytes were treated with immune RNA, cytotoxic potential was doubled and the production of "blocking factors" was shut-off. These data indicated that immune RNA was capable of manipulating existing immune functions.

It is interesting to note that untreated (no RNA) lymphocytes derived from 4198 tumor-bearing mice provided a beneficial effect which was equivalent to, if not greater than RNA-treated TL in several of the experiments. This was true from the standpoint of either tumor growth rate (Figure 9), median survival (Table 8),
or 100% mortality (Tables 6 and 7), but not mean survival time. **In vitro** studies have indicated that sequential washing of lymphocytes derived from tumor-bearing mice increases cytotoxic potential (Evans, 1974). It is possible that adherent "blocking factors" are removed during the washing procedure and that some benefit might accrue by returning the cells to the host.

As in the case of the direct intraperitoneal injection of RNA, administration of 4198V immune RNA-treated tumor lymphocytes failed to significantly alter the mean survival time of the treatment groups as compared with the controls. Despite this, the latter method appears to be a more efficient mechanism of delivering RNA to the immune system. This statement is based on the **in vitro** data of Scheetz (1972) and Greenup (1977) (previously discussed), and on the observation that RNA-treated tumor lymphocytes were capable of slowing tumor growth for a period of time in 3 of 4 immunotherapy trials. However, this form of therapy was of no greater value than was administration of normal lymphocytes (Figure 9 and Table 6), tumor-immune lymphocytes (Figure 9 and Table 6), or lymphocytes derived from tumor-bearing mice. One must therefore conclude that 4198V immune RNA supplies no additional benefit in the 4198 tumor system.

A number of theories may be proposed to explain the lack
of success of RNA-treated lymphocytes as an immunotherapeutic approach. First and foremost is the question of whether in vitro observations actually reflect the in vivo situation. Treating lymphocytes derived from tumor-bearing hosts with immune RNA as a means of increasing cytotoxic potential and abrogating suppressive effects may be strictly an in vitro phenomenon and, on the other hand, if we assume that it is not, the fact that 100 ug was determined in vitro to be the optimum dose of RNA to treat 4 X 10^6 lymphocytes may be far from correct. Possibly, increasing the numbers of lymphocytes treated with larger doses of RNA and infusing them by differing routes and at more frequent intervals would change the picture. Magee, et al., (1978) attribute some of the problems associated with RNA immunotherapy to ineffective interaction between RNA and lymphoid cells, and to failure of RNA (or appropriate quantities) to enter the cell. At the same time, Vallera (1978) suggests that RNA-treated lymphocytes fail to go through a proliferative or blastogenic phase and therefore do not result in a population of specific "memory cells". It might also be speculated that immune RNA is not being directed toward the appropriate population of affector and/or effector cells.

In the final in vivo immunotherapy experiment immune RNA was administered intraperitoneally to C3H/HeJ mice via positively
charged liposomes. The results indicated that liposomes containing only 4198V immune RNA were capable of both decreasing tumor growth rate (Figure 13) and increasing mean survival time (Table 10) as compared with the controls. It is significant that this was the only in vivo experiment of the 7 conducted in which immune RNA was able to influence the mean survival time (This was not the case if the treatment group was compared with mice which had received liposomes containing phosphate-buffered saline. See page 121). The addition of rabbit anti-C3H/HeJ mouse lymphocyte IgG to RNA-containing liposomes appeared to negate the positive effects discussed above. No effort was made to determine which cell population (polymorphonuclear neutrophiles, macrophages, B cells or T cells) processed the RNA-containing vesicles in vivo.

Recent advancements in phospholipid technology have provided the impetus for evaluating liposomes as a delivery mechanism for immune RNA to the immune system and consequently as an improved immunotherapeutic tool. Ostro, et al., (1977) and Dimitriadis (1978) have shown that RNA entrapped within liposomes is not susceptible to degradation by ribonucleases, while Magee, et al. (1978) and Poste, et al., (1979) have reported that cells of the immune system (lymphocytes, macrophages) are capable of interacting with lipid vesicles in such a manner as to result in the
release of entrapped molecules. Taken together, these data indicate that liposomes should be an appropriate means of introducing immune RNA into the cell in an intact form.

Gregoriadis (1978) has offered evidence that liposomes can be directed toward a particular target by inclusion of specific anti-target immunoglobulin (IgG). The presence of antibody appears to increase the efficiency at which liposomes and their contents are internalized by the target cell. Addition of rabbit anti-C3H/HeJ mouse lymphocyte IgG to RNA-containing vesicles was ineffective in improving the therapeutic value of immune RNA in the 4198 system. The IgG was shown to be chromatographically pure (Plate III) and active in mediating the lysis of lymphocytes in the presence of complement. It is possible that antibody was not available for interaction with lymphocytes since the fluorescent antibody technique used did not reveal a quantitative difference in the fluorescence between the tests and the controls. In addition, no attempt was made to titrate the IgG in order to determine the optimum dose for maximum target cell interaction.

Although only one in vivo experiment was conducted in this instance, it is apparent that liposomes were the most effective mechanism of delivering immune RNA. A number of manipulations can be envisioned to improve the system.
The liposomes utilized in this study were prepared by an adaptation of the ether vaporization method (Deamer and Bangham, 1976). This technique results in the production of numerous multi-lamellar vesicles (Plates V-VIII). Szoka and Papahadjopoulos (1978) have reported that this method yields a low volume of entrapped aqueous space per mole of lipid. This is due to the fact that the majority of lipid is participating in the internal lamellae which are in close apposition to each other thereby restricting the internal aqueous space. Although no attempt was made to quantitate the amount of RNA entrapped within the vesicles, one could certainly improve the efficiency of capture by employing methods which both entrap a large percentage of aqueous material and have a high aqueous space-to-lipid ratio.

It is well established that differences in lipid composition (and ratio) can alter the efficiency of interaction between liposomes and target cells. Magee and Miller (1972) and Magee, et al., (1978) have shown that vesicles which are rendered positively charged by inclusion of stearlyamine in their structure are more avidly bound by various targets than are spherules which have a net negative or neutral charge. In addition, Weissmann, et al., (1975), Cohen, et al., (1976), and Gregoriadis and Neerunjun (1975) have reported that either aggregated immunoglobulin or specific anti-target IgG were
capable of increasing the uptake of various molecules entrapped within liposomes. There is no reason to believe that one will not be able to manipulate the immune response by targeting liposomes containing specific molecules to subsets of B cells, T cells and macrophages by altering antibody and lipid composition.

In conclusion, this study has compared three modes of administration of xenogeneic immune RNA in the immunotherapy of the 4198 fibrosarcoma tumor in male C3H/HeJ mice. Direct intraperitoneal injection of whole and/or fractionated immune RNA was ineffective in altering tumor growth rate and mean survival time in treated mice as compared with controls. This result was attributed to degradation of RNA by endogenous ribonucleases. The administration of RNA-treated lymphocytes was shown to be a more efficient mechanism of delivering RNA to the immune system as compared with direct intraperitoneal injection. This was evidenced by the fact that several preparations were capable of decreasing tumor growth rate. The effect was short-lived and did not cause any significant change in mean survival time. A number of theories were offered as explanation for the observed effect. Finally, immune RNA was incorporated into positively charged multilamellar liposomes and evaluated as an improved immunotherapeutic tool. Significant differences were noted in
both tumor growth rate and mean survival time of mice which received RNA entrapped within vesicles. Inclusion of rabbit anti-C3H/HeJ mouse lymphocyte IgG into RNA-containing spherules nullified the positive effect of RNA alone. It is clear that liposomes should be considered as an improved mechanism of presenting RNA to the immune system and may be of benefit in the therapy of cancer when used in conjunction with other forms of treatment.
SUMMARY

Whole and fractionated xenogeneic 4198V immune RNA, extracted from the spleens of outbred albino guinea pigs, was administered to male C3H/HeJ mice by three different mechanisms and evaluated as a potential immunotherapeutic agent. The criteria used to evaluate the success of a given immunotherapy experiment was the ability of immune RNA to both decrease tumor growth rate and increase mean survival time.

Immune RNA was administered intraperitoneally (IP) along with DEAE-dextran (an RNAse inhibitor and polycation which enhances the cellular uptake of RNA) on Days 4 and 6 post-tumor cell inoculation in the first series of in vivo immunotherapy trials. Twenty-five thousand 4198 tumor cells were administered intramuscularly in all cases. Each mouse within a group received a total of either 1 mg of whole 4198V immune RNA, 65 ug of poly A\(^+\) mRNA, or 935 ug of poly A\(^-\) RNA during the period of treatment. Although poly A\(^+\) mRNA was capable of significantly decreasing the tumor growth rate in the early phase of one experiment, it
was generally concluded that there was little or no advantage to any of the treatments provided.

RNA-treated tumor lymphocytes (lymphocytes derived from tumor-bearing mice) were evaluated as a potential form of immunotherapy in four in vivo experiments. Each mouse within a treatment group received $2 \times 10^7$ splenic lymphocytes (previously incubated with either 500 ug of whole RNA, 32.5 ug of poly $A^+$ mRNA, or 467.5 ug of poly $A^-$ RNA) on Days 4 and 6 post-tumor cell inoculation. The tumor dose was identical to that employed in the previous experiments. RNA-treated tumor lymphocytes were able to significantly alter the tumor growth rate in 3 or 4 cases. However, the effect was temporary and no difference was seen in the mean survival time of treated mice as compared with the controls. Tumor enhancement was noted in the mice which had received poly $A^-$ RNA-treated tumor lymphocytes. This result was attributed to the existence of an RNA "species" which had a suppressive effect on the immune response. Although RNA-treated tumor lymphocytes were capable of slowing tumor growth for a brief period, it was determined that this form of therapy was of no greater value than was administration of normal lymphocytes, tumor-immune lymphocytes or lymphocytes derived from tumor-bearing mice.
Positively charged liposomes were formed by an adaptation of the ether vaporization method using a 7:2:1 molar ratio of phosphatidyl choline, stearylamine and cholesterol. This technique gave rise to numerous multilamellar vesicles which ranged in size from 0.06-0.5 um. The spherules were capable of entrapping materials dissolved in aqueous solution. C3H/HeJ mice were administered 13.4 mg of immune RNA-containing liposomes on Days 4, 6 and 8 post-tumor cell inoculation (2.5 X 10^4 4198 cells/0.1 ml). Rabbit anti-C3H/HeJ mouse lymphocyte IgG was included in some RNA/liposomes preparations as consideration as a specific homing device. The results indicated that liposomes containing only RNA (and not IgG) were capable of both increasing mean survival time and decreasing tumor growth rate as compared with untreated (no liposomes) controls. It was suggested that phospholipid vesicles presented some evidence of being an effective mechanism of delivering RNA to the immune system and might be considered for additional investigations becoming of value in the therapy of cancer.
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