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THE OHIO STATE UNIVERSITY, PH.D., 1978

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1978
Role of RNA in Influencing T and B Cell Responses

DISSERTATION

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

by

Joseph Paul Dalmasso, BS, MS

* * * * * * * * * * * * * * * * * * * *

The Ohio State University

1978

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DEDICATION

I wish to dedicate this dissertation to Dr. Matthew C. Dodd whose personality and wisdom strongly influenced my desire to achieve this level of education. I feel his selfless dedication to his students and discipline are an achievement only a very few will ever equal. I wish to personally thank him for his "natural" method of graduate education which encourages independent thought, allows one to mingle curiosity and freedom of direction in experimentation, and fosters strong inferential processes in those fortunate enough to be under his tutelage.
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Candace Greenup, John Nawrocki and Dan Vallera offered extremely helpful suggestions and encouragement after reading early ROUGH drafts of this dissertation. To these employed individuals I am especially appreciative.

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Enormous thanks to Dr. Melvin S. Rheins, who accepted responsibility for guidance and editing during the composition of this dissertation in its latter stages.

I wish to thank Xerox Corporation, IBM Corporation, The Christian Brothers and the automobile industry, without whose existence and wonderful products this dissertation could not have been completed as scheduled.

To two very close friends, Sharon Maraffa and Fred Vogel, I will have rememberances of endless research jags, much talk, and exotic cuisine at Columbus' most famous restaurants. To Sharon, I wish you the best luck in completing your degrees—with your enthusiasm and endurance you shouldn't have a bit of trouble. To Fred, I leave you my vitamins, in hopes they will
help prevent recurrences of "puss out syndrome" and hasten achievement of degree status.

To my parents I wish to express what I have often failed to express in the past; that is, I do appreciate the early guidance in leading me to become independent and somewhat self-sufficient, and I am thankful they allowed and encouraged an early interest and curiosity in science and mechanical devices.

To Vicki I wish to express my gratitude for your capacity for endurance of my crazy lifestyle while work on this degree progressed. I still don't quite understand how you do it.
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INTRODUCTION

The potential of immunogenic ribonucleic acid (RNA) as a therapeutic adjunct to other therapies in the management of malignancies has been in controversy. Much of this has centered about the failure of RNA, although functional \textit{in vitro}, to consistently demonstrate \textit{in vivo} anti-tumor activity. Accordingly, it has been necessary to examine the component facets of the manipulative procedures regarding RNA to determine the role each of the facets examined exert on other compartments of the immune system.

Currently, a variety of \textit{in vitro} responses can be initiated using the appropriate RNA. RNA extracted from the spleens of mice immunized with sheep erythrocytes can be used to treat normal lymphocytes. These cells then may be observed for a specific humoral (B cell) response, the production of antibody. Specific cellular responses exhibited by the T cell population can be transferred with the proper RNA, e.g., splenic RNA from animals immune to a tumor. This has been consistently demonstrated in many animal systems (Pennline 1977, Kern and Chow 1978). Greenup (1977) showed that selective cells can express the informational message when she demonstrated that \textit{in vitro} cytotoxic responses to a murine fibrosarcoma were only effective if the RNA were presented to T cells. The implication that the RNA specificity used may only function in a cell that is geared for that message dictates that a significant number of cells in a population possess the potential for processing
particular messages, viz., T cell messages. Conceivably, most informational RNA may be processed by the T cell, and a stimulus from this specifically activated T cell may in turn direct the B cell.

Based on the above, it seemed entirely appropriate that immune RNA could then be utilized to initiate in vivo anti-tumor responses. This intriguing possibility properly led to the proposition that lymphoid cells from a tumor-bearing animal could be directed against the autochthonous malignancy. From this view, this laboratory had undertaken the adoptive transfer of immune RNA-treated lymphocytes to tumor-bearing animals. Surprisingly, enhancement of tumor growth followed.

A logical consequence was to examine the effects of RNA treatment on lymphoid cell populations. Could the RNA be transferring anti-tumor activity as well as regulatory functions which culminated in suppression? Or, perhaps the activity of RNA treated T cells was dictating processes that were detrimental to the tumor-compromised host.

The decision to probe this and associated problems followed several attempts to transfer humoral immunity with RNA. When anti-sheep erythrocyte RNA treatment of normal lymphocytes provided only variable results, the need became apparent to examine further the degree to which an RNA species might be able to modify the activities of several different cells within a treated population. This was accomplished by treating splenic lymphocytes from mice demonstrating a primary or secondary response to the sheep erythrocyte antigen and monitoring their T and B cell responses in vitro for several days. Also investigated was the effect of RNA treatment on in vitro T and B cell function as...
measured by Jerne plaque and migration inhibitory factor assays. Additionally, the effect of antigen in culture was examined to determine its effect on RNA-treated immunocompetent cells.

An improved understanding of T and B cell function during Jerne plaque activity was accomplished by investigating B cell responses following lipopolysaccharide blastogenic stimulation. Similarly, tritiated thymidine uptake studies during the plaque response provided understanding of T and B cell interactions in vitro and clarified the effect of RNA on lymphoid cells.
LITERATURE REVIEW

The extraction protocol (Scheetz 1972), analyses for purity and integrity (Rossio 1973) and characterization of the Poly(A) tailed RNA moiety in which biological transfer of immunity resides (Greenup 1977) have been extensively reviewed by others from this laboratory. This review, therefore, is intended to orient the reader from the historical to the current perspective regarding the biological and biochemical progress that has evolved in the area of RNA research.

It is generally accepted by all but the staunchest opponents that messenger RNA from the lymphoid tissues of immune animals (immune RNA) can and does transfer this immunity to other normal lymphocytes. This was demonstrated early when Fishman and Adler (1963) induced specific immunity with RNA which had been extracted from macrophages infected with T2 phage. The criticism was that antigen was being transferred along with the RNA, thus acting as a direct stimulus or creating, in combination with the RNA, a superantigen (Friedman 1963, 1965). The question was seemingly resolved when immune macrophage RNA from rabbits of one allotype and recipient lymphoid cells from another allotype were combined. The IgM antibody formed by the recipient cells had the allotype of the donor macrophages (Adler 1966). Bell and Dray (1971) used immune RNA donors of one allotype and the recipient lymphoid cells of another allotype to show the synthesis of both IgM and IgG heavy and light chains of the foreign allotype.
The major concern regarding the superantigen concept centers about the inadequacies of the extraction procedure in completely eradicating protein contamination. Excessive protein has not been a problem since the hot phenol process for extraction of RNA results in a product of exceptional quality (Rossio 1973). The RNA preparations prepared in these laboratories have been used to transfer humoral immunity (Nawrocki 1977), cellular immunity to tuberculin (Dodd 1973) and antibody-dependent cell-mediated cytotoxicity to tumor cells (Pennline 1977). The specific activity of this RNA has been demonstrated (Greenup 1978) as well as its susceptibility to RNAase treatment (Pollock 1970). Others have expressed that antigen contamination, which is consistently associated with protein complexes, may be important in transport or translation of the mRNA (Barrieux 1976). Other laboratories indicate that deproteinization by phenol extraction removes these fragments (Sundquist 1977). Treatment with pronase does not alter the efficiency of RNA preparations in transferring immunity (Cornacoff 1976).

Both xenogeneic RNA and syngeneic RNA have been used in the in vitro transfer of immunity (Kern and Chow 1978). Wang (1974) demonstrated the transfer of tuberculin sensitivity, Pennline (1977) reported the transfer of specific anti-tumor lymphocyte cytotoxicity and Kern and Pilch (1978) illustrated the synthesis of anti-tumor antibody. These successful in vitro experiments preceded in vivo attempts to transfer activity with RNA preparations.

RNA has an adventitious quality when considering passive transfer of specific immunity to an antigen that is individually unique (i.e., a
cancer antigen) in that immunization with autochthonous tumor, a potentially lethal situation, would not be necessary. A specific immune response can be transferred with RNA treated patient lymphocytes which could specifically instruct cells to immunocompetency without the problems of sensitization to histocompatibility antigens or the dangers of involving a graft versus host reaction. Repeated injections of RNA itself should not sensitize the recipient since RNA is a poor antigen. RNA also could be effective in certain types of host anergy, i.e., antigen recognition or processing (Pilch 1976).

Early non-human in vivo experiments (Jureziz 1970) showed the transfer of correlates of cellular immunity (MIF) when animals were directly injected with immune RNA. Schlager (1975) achieved tumor regression in guinea pigs with a mixture of peritoneal exudate cells, antigen and specific immune RNA. Investigators grasping the potential for RNA immunotherapy of human tumors directly administered RNA which had shown specific immunity in vitro (deKernion 1975). These earlier studies suggested a positive change in the patient's clinical course, while later trials indicated an increased survival and lower recurrence rates in cancer patients (deKernion 1977). However, some RNA immunotherapy in the treatment of neoplasms has met with only mixed success. Close analysis of data presented indicated no improvement, or a decrease in the survival of time of some groups studied (Schlager 1976). Throughout all of this work is the obvious need for information concerning the exact mechanism by which RNA exerts its effects in vivo. This, then, might enable potentially marginal therapy to become routinely successful. In this regard our
laboratory has injected tumor-bearing mice with anti-tumor RNA-treated lymphocytes from tumor-bearing syngeneic donors. These treated cells have been shown to transfer specific cytotoxicity in vitro (Evans 1977). This adoptive transfer approach revealed no decrease in tumor growth, but rather tended to show enhancement (Cornacoff 1977). Scrutiny of these data prompted the question as to whether other responses being transferred in vivo were detrimental to the host. These RNA treatments were conducted on whole populations of mouse spleen cells. Greenup (1977) was able to show that activity of immune RNA in anti-tumor cell cytotoxicity tests was transferred to the T cell population. Recently, thymus RNA was used to induce T cell specific antigens on rabbit bone marrow cells (Archer 1978). It is tenable, then, that other T cell capabilities such as suppression are being transferred. The effects these suppressive messages may exert on lymphocytes, as well as the relative influences of these suppressor messages in RNA preparations derived from different lymphoid cell sources, perhaps could explain the disparity observed with the in vivo experimentation.

A review of the lymphoid cells capable of regulating immune responses is pertinent, since it is these cells from which the RNA is ultimately extracted. Their presence in splenic cell populations as well as their efficiency in regulation dictate their importance.

Before discussing the role of T cells, the role of macrophages will be briefly noted. Several properties of the macrophage indicate its importance in the inductive phases of the immune response. Macrophages can bind antigens for presentation to other cells of the immune system; they can ingest and degrade antigens and alter their immunogenicity; they have receptors for B and T cell products as well as for
lymphocytes themselves; and they can release products capable of suppressing or enhancing an immune response (Basten 1976). Quan (1978) demonstrated that the depressed response to phytohemagglutinin of cancer patients' lymphocytes was due to a carrageenan sensitive cell, thus implicating a monocyte as the suppressor cell.

The importance of macrophages in T cell-B cell collaboration leading to antibody synthesis lies in the pre-T cell stage, where the association of antigen with macrophages selectively induces cell-mediated reactions and the priming of T helper cells that are involved in facilitating antibody production (Oppenheim 1976). Macrophages also are needed in conjunction with T cells to deliver a second signal, after induction, for antibody production to occur. Two acceptable theories as to the mechanisms are 1) information transfer, implicating specific immunological information in the form of an RNA molecule, and 2) the release of soluble mediators. Soluble mediators, whether specific or non-specific, have the key feature of being able to enhance antibody production to a given antigen by activating a large number of T cells, which in turn influence B cell responsiveness (Basten 1976). Beyond their importance in the induction of the immune response, the role of the macrophage in non-specific immunoregulation (Nelson 1976) and non-specific phagocytic scavenging functions has been extensively reviewed (Oehler 1978).

Current thinking suggests that the immune response is no longer a simple matter of introducing an antigen and having T or B cells, respectively, respond with cell-mediated or humoral immunity; but rather, it is a response controlled by a complex regulatory system with
T cell function assuming a role of extreme importance.

With the help of macrophages, a newly introduced antigen confronts various antigen-reactive cells (T or B) that pre-exist in the organism. These cells are stimulated to undergo clonal expansion. Among the progeny cells are those which eventually secrete antibody (B cells) having the same combining site for antigen that was present on the progenitor cell of this clone. T cells also are stimulated to undergo clonal expansion in the presence of the correct antigen. Helper T cells and suppressor T cells, which can recognize receptors on the first set of cells, are stimulated to proliferate and function to modulate their responses. Additionally, the activities of this second clone stimulates expansion of a third clone which modulates the second clone. Anti-idiotype antibodies and regulatory cells bearing anti-idiotype receptors are important in this fine control of the immune response (Cosenza 1977).

Studies with helper T cells by Waldmann (1977) indicate two basic types of interaction, the first being mediated by a non-specific soluble factor. T cells educated to an antigen will, in the presence of antigen and macrophages, produce a non-specific mediator that activates B cells to respond to at least two antigens (sheep rbc's and donkey rbc's), but will not replace T cell function for responses to soluble proteins. The second type of interaction, which appears most plausible from experimental data, indicates that specific T helper cooperation occurs by responding to one B cell only. This monogamous phenomenon occurred when one T cell was presented with a possible 15 hapten-specific B cells to which it could have responded. The explanation stated was that physiologically this T cell interacted
directly with B cells of one specificity and was somehow affected by this interaction such that communication could occur with no other B cell specificities (Waldmann 1977).

Both the switch from IgM to IgG production and the rise in affinity of antibody produced seem to be under T cell control (Miller 1975). Antigen as well as receptor-receptor interactions are important in directly stimulating regulatory cells (Siegal 1978). In the B cell response, antibody exhibits two distinct mechanisms of control. High concentrations of antibody exhibit direct negative feedback by interacting with antigen and masking antigenic sites that stimulate B cells. However, the non-specific Fc portion of the antibody molecule also exhibits suppressive activity by affecting the cooperation of T and B cells (Hoffman 1974, 1978). The net effect of a mixture of helper and suppressor T cells is dependent on the helper-suppressor ratio (Cantor 1975). His results indicated that suppressor T cells predominate six days after priming. Elson and Taylor (1974) showed that after activation T cells went first through a phase when the net effect was helper and secondarily through a phase when the net effect was suppression. Basten (1975) has indicated that the thymus contains relatively few suppressor T cells when compared with the spleen, and therefore cells from this organ are inefficient for use as a suppressor population. Ford (1975) showed that despite continuing presence of antigen in an immunogenic form, recruitment of lymphocytes was suppressed by cells involved in the antibody response. Subsequently it was shown that the depressed recruitment was a result of suppressor T cells (Elson 1977). Others have
reported that low zone tolerance is primarily T cell mediated, and results in prevention of IgG production by B memory cells (Stumpf 1977). Athymic mice demonstrated a ten-fold higher PFC response to pneumococcal polysaccharide than did intact controls (Hopkins 1978). Control experiments with anti-thymocyte serum indicated the enhanced responses resulted from a lack of suppressor T cells. Reconstitution of the athymic mice with normal thymocytes restored the T helper activity within 24 hours, and suppressor activity within six days.

From this the degree to which both helper and suppressor T cell functions extend their controls on the the immune response becomes apparent. T suppressor cells also play an important role in neoplastic processes. The animal bearing a tumor has both a compromised cell-mediated (Whittaker 1971) and humoral (data presented later) immune response. Patients with lung carcinoma had depressed lymphocyte responses to phytohemagglutinin mitogen (Rees 1975). Responses to several of the commercial skin test antigens (Candida, mumps virus, tuberculin purified protein derivative and streptokinase-streptodornase) were also less than those observed in normal volunteers. Non-specific suppression of T lymphocyte responses have been shown in mice with progressively growing tumors (Kilburn 1974). However, this work suggested the unresponsiveness of T cells from the spleens of these animals was suppressed by a cell of B lymphocyte origin as it was responsive to lipopolysaccharide. Broder (1978) suggested that suppression required interaction between two T cell populations and demonstrated that a separate T cell population controlled activation of the suppressor T cell population. Recently, Waldmann
(1978) has presented evidence for T-T cell interaction prior to achieving suppression in a human leukemia. These investigations with leukemic patients revealed depressed immunoglobulin production which was present before any immunosuppressive chemotherapy was initiated. Hellstrom (1978) has presented evidence suggesting that tumor antigens enhance tumor growth in vivo by interacting with a suppressor cell population that is Thy-1 positive.

From the preceding, one becomes increasingly cognizant of the varied regulatory controls T cells can exert. A delicate balance exists in vivo between helper functions necessary for inductive phases of the immune response and suppressor functions responsible for holding these positive processes in check. The product of these interactions can be a beneficial response or a disfunctional immune process detrimental to the individual. The role that RNA from lymphocytes may play in transferring "immune" responses must also include then the regulatory functions which have been discussed above. In this respect, macrophages may be less important because of their relative low numbers in the spleen.

Work to be reported later involved the culturing of lymphoid cells treated with various RNA preparations and gave indications that increases in the number of plaque-forming cells might be due to blastogenic responses. At that time in the investigation, the possibility of reverse transcription of the RNA to permanent DNA message was an intriguing concept. This would have enabled a population of cells that would proliferate to form cells which could express a permanent immune state. Work by Vallera (1978) has
indicated RNA treatments were unable to induce blastogenesis, an
effect that would be necessary to establish immunological memory
in an animal. Previous work by Bluestein (1970) also has not
supported the transfer of blastogenic ability to lymphocytes with RNA
in the presence of antigen.

To provide further understanding of the role RNA may play in
stimulating cells to show enhanced responses such as plaque-forming
ability, mitogenic studies were employed using *Escherichia coli*
lipopolysaccharide (LPS). LPS, although thought to be strictly a
B cell mitogen, has been shown to affect T helper cell function
(Armerding 1974, Norcross 1977). Braun (1965) observed the enhanced
sheep cell response to normal cells treated with bacterial endotoxin,
and Jones (1972) showed that the increased Jerne plaque response was
due to an effect of the LPS upon the B cell. Effects due to the
T cell were seemingly ruled out when cell cultures were treated with
anti-theta antiserum without influencing the plaque-forming capability
of the population. Gronowicz (1974) indicated the B cell population
was a heterogeneous group with respect to their different stages
of differentiation, and consisted of two subsets. One subset could
readily be activated to divide, but were not high rate antibody
secreting cells. Activation of the other subset resulted in end
cells that exhibited a high rate of antibody secretion, but were
insensitive to further stimulation by mitogen. Both subsets of cells
were activated by LPS.

In *vitro* work by Armerding (1974) suggested that in the absence
of antigen LPS acted primarily on B lymphocytes. However, in the
presence of antigen the influence of LPS was on helper T cell function which was predominantly responsible for the enhancing effect on antigen-specific antibody responses. This conclusion was the result of work with hapten-carrier primed spleen cells whose response in vitro with LPS and several antigens was enhanced only if the carrier of the challenging stimulus was the same as that of the priming stimulus. They offered no evidence stating that the T cell was DIRECTLY affected by the LPS, but only that its function was altered. Andersson (1972), using anti-theta treated spleen cells or spleen cells from nude mice, concluded that LPS does not cause proliferation of T cells. Norcross (1977) indicated B cell proliferative responses to LPS were regulated by a low Thy-1, non-Concanavalin-A T cell subpopulation which could be separated on bovine serum albumin gradients by centrifugation.

The present body of knowledge concerning T and B cell interrelationships is being constantly expanded. This discussion was completed according to current knowledge that best satisfied hypotheses presented and was compatible with data obtained.
MATERIALS AND METHODS

IMMUNIZATION OF ANIMALS TO OBTAIN PRIMED LYMPHOCYTES

Six to 12 week old male Swiss white or C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Maine) were injected intraperitoneally with 5% sheep erythrocytes which were washed and suspended in 0.01M phosphate buffered saline (PBS). The erythrocytes were obtained from resident sheep in the animal facility of the Department of Microbiology, The Ohio State University. Animals were given food and water ad libitum.

MOUSE SPLENIC LYMPHOCYTE CULTURE

Lymphocytes were teased from the splenic capsule by "nicking" one end of the spleen and gently expressing the splenic contents with smooth curved forceps. The lymphocytes were separated from other blood elements by centrifugation through a Hypaque-Ficoll gradient at 600 x g for 10 minutes (Boyum 1968). The lymphocytes were collected from the interface, washed once in fresh RPMI 1640 (GIBCO) supplemented with 10% virus- and mycoplasma-screened fetal calf serum (GIBCO), and then centrifuged at 800 x g for 20 minutes. Approximately 30% more lymphocytes could be recovered by resuspending the pellet from the Hypaque-Ficoll gradient in 7 ml RPMI 1640 and recentrifuging on another Hypaque-Ficoll gradient. All media used routinely contained additionally
292 ug/ml 1-glutamine (GIBCO), 30 ug/ml gentamycin (Schering), 100 units/ml Penicillin G (Squibb), and 50 ug/ml Streptomycin Sulfate (Lilly). Centrifuged lymphocytes were resuspended in RPMI 1640 with 3 mg/ml HEPES buffer (N-2-Hydroxyethylpiperazine-N'-2-Ethansulfonic Acid, GIBCO), but without fetal calf serum if RNA treatments were to be performed. Alternately, HEPES buffer and 10% fetal calf serum supplements were utilized if lymphocytes were to be used in the Jerne plaque assay. HEPES buffer and 20% fetal calf serum additions were used to culture lymphocytes. Total cell and viability counts were performed on this final suspension by using the trypan blue dye exclusion method (GIBCO, 0.04% trypan blue in 0.01M phosphate buffered saline). Viability routinely surpassed 96%. Lymphocytes to be cultured were placed in 25 ml erlenmeyer flasks in 5 ml aliquots at a concentration of $6 \times 10^6$ cells per ml. Other cellular elements such as macrophages, sheep erythrocytes, or tumor cells were added at this point to the culture suspension. The flasks were loosely covered with "caps" of aluminum foil and incubated in 5% CO$_2$ at 37°C.

Culture supernatants were collected by careful aspiration with a 5 ml pipet to prevent disturbance of the sedimented cells. Best results were achieved by leaving approximately 0.5 ml of supernatant in each flask. This minimized reduction of total cell numbers during continuous culture.

An especial comment would be beneficial regarding "long term" culture of lymphocytes. Initial concern over the capacity of lymphocytes placed in culture for six to seven days to exhibit
substantial immune phenomenon were dispelled when it was shown that high numbers of direct (IgM) and indirect (IgG) plaque-forming cells (PFCs) were present after 4-5 days. Cultured cells were monitored for viability and the total number of cells remaining in culture. In a typical viability curve (Figure 1), cell viabilities decrease rapidly the first 24-48 hours to approximately 2-3 million cells per ml, then decline at a slower rate during the next four days to 1-2 million per ml. A more reliable meter of population change, especially if one were concerned with blastogenic activities, would be to record the total number of cells surviving each day (Figure 2). Typical survivorship results depicted indicate an initial drop followed by a constant slow decline in total cell numbers.

TUMOR CELL CULTURE

Murine C3H cell lines were used for immunization and tumor induction in six to twelve week old male C3H/HeJ mice. Ting (1965) originally cloned the polyoma-induced C3H murine fibrosarcoma (4198) used for tumor induction in the following experiments. An intramuscular dose of 1-5 x 10⁵ cells into the thigh resulted in palpable tumors in 8-12 days. Ten thousand cells were the fewest that proved tumorigenic for the C3H mouse.

A highly antigenic variant (4198V) cloned from the 4198 cell line by Ting (1972) was an effective immunogen when mice were given three or more weekly injections of 2-5 x 10⁶ cells intraperitoneally. Antitumor immunity could be demonstrated by challenging animals intramuscularly with a tumorigenic dose of the 4198 tumor cells (1-5 x 10⁵ cells).
Figure 1. Per cent viable cells during six days of culture as determined by trypan blue dye exclusion.

Primary immune lymphocytes
Primary immune lymphocytes + Srbc
Primary immune lymphocytes + Srbc + Normal RNA
Figure 1.
Figure 2. Total viable cells per ml of media during six days of culture.

- Primary immune lymphocytes
- Primary immune lymphocytes + Srbcs
- Primary immune lymphocytes + Srbcs + Normal RNA
Figure 2
The cell lines were cultured in 75 cm² plastic flasks (Falcon 3024) in 15 ml of RPMI 1640 plus 10% fetal calf serum, and were incubated in 5% CO₂ at 37°C. Flasks inoculated with 1-2 x 10⁶ cells demonstrated confluency in 48-72 hours and were trypsinized with 0.25% trypsin (GIBCO) prepared with Ca⁺⁺-Mg⁺⁺ free 0.01 M phosphate buffered saline. Trypsinized cells were collected in RPMI 1640, centrifuged at 600 x g for 10 minutes, and resuspended in RPMI 1640 for injection.

EXTRACTION AND CHARACTERIZATION OF RNA

All reagents for RNA extraction were prepared with DEP-treated (Diethyl pyrocarbonate, Sigma D5758) double distilled demineralized water (50 ul DEP/liter), and autoclaved for 30 minutes to facilitate destruction of nucleases (Ehrenborg 1976). Ribonucleic acid was extracted according to a modification of the method of Mach and Vassali (1965). Frozen spleens or lymphoid cell pellets were homogenized in a Virtis 45 homogenizer at 30% power for 10 minutes in 30 ml buffer-saturated phenol (7 parts vacuum distilled chromatographic grade, 88% phenol [Mallincrodt], diluted to 90% with DEP water, and 3 parts extraction buffer (EB)) [0.1 M sodium acetate, 0.1% 8-hydroxyquinoline, 0.5% sodium dodecyl sulfate, 0.04% polyvinyl sulfate, pH 5.0]). To this homogenate, 2 mg bentonite (Bentonite USP, Fisher; washed and centrifuged 7 times to purify) and 30 ml EB were added and the mixture homogenized an additional 10 minutes at 30% power. The homogenate was placed into a chilled 250 ml erlenmeyer flask with thermometer inserted.
and heat extracted with wrist action shaking. A 60°C water bath was used to quickly raise the temperature of the extraction mixture to 54.5° - 55.0°C for a ten minute time period. The homogenate was then chilled in a salt brine to 0°C or lower, centrifuged at 12,000 x g for 8 minutes, and the clear aqueous supernatant collected. The remaining interphase material and cell pellet were transferred to a 250 ml flask to which 2 mg bentonite and a half volume of EB were added, and the flask was chilled to 0°C or below. This mixture was extracted in the hot water bath as above. After centrifugation at 12,000 x g for 8 minutes, this supernatant was pooled with the initial supernatant. The pooled aqueous phases were combined with a half volume of 1:1 phenol:chloroform in a 250 ml erlenmeyer flask and 2 mg bentonite were added. The mixture was extracted in the hot water bath and chilled as described above. After centrifugation at 12,000 x g for 5 minutes, this supernatant was collected into a 250 ml erlenmeyer flask. Again, a half volume of 1:1 phenol:chloroform and 2 mg bentonite were added, the mixture chilled, extracted in the hot water bath, and chilled again to 0°C or lower. This final supernatant was brought to 0.3M acetate concentration with 2.5M acetate buffer (2.5M sodium acetate, pH 5.0), and the RNA was precipitated by adding 3 volumes of cold (-20°C) 95% ethanol and storing overnight at -20°C. After centrifugation of the precipitated RNA at 12,000 x g for 10 minutes, the ethanol was decanted, and the pellet dried with high purity nitrogen. The pellet was again dissolved in Ca++-Mg++free 0.1 M phosphate buffered saline prepared with DEP water and quantitated
by preparing a 1:101 dilution of the preparation and measuring the absorbancy at 260 nm. The RNA yield was calculated according to the following formula:

\[
\text{mg RNA/ml} = \frac{\text{Optical Density at 260 nm}}{24} \times \text{dilution factor.}
\]

The criteria for RNA purity in these experiments dictated that the 260 nm peak be twice the OD reading of the 280 nm peak, indicating an RNA preparation free of detectable protein or DNA. Further exacting quality control analyses indicated protein content to be less than 5% using the Lowry method (Williams and Chase 1968, p 273) and the DNA content to be below detectable levels by Burton's diphenylamine analysis (Williams and Chase 1968, p 320).

The physical integrity of the extracted RNA as determined by sucrose density centrifugation was the final indication of a preparation that contained intact messenger RNA (8-18S) and would be suitable for experimentation. One hundred ug RNA was layered on a 6-21% sucrose gradient (Sigma S9878 Grade 1 crystalline sucrose) which had been formed in Beckman 4" x 5/8" cellulose nitrate tubes with the aid of an Isco Model 180 gradient former. The layered gradients were centrifuged 18 hours in a Beckman Model L-2 centrifuge at 116,000 x g at 4°C in an SW27 swinging bucket rotor.

The RNA gradients were scanned at 254 nm by puncturing the bottom of the tube with an 18 ga hypodermic needle and passing the gradient through an Isco Model 222 Ultraviolet Analyzer. The RNA profile was simultaneously recorded with an Isco Model 610 chart recorder. A typical profile is illustrated in Figure 3. The middle peak is the 8-18S messenger RNA peak, the larger peak is
Figure 3. Ultraviolet (260 nm) analysis of RNA banding following ultracentrifugation on a sucrose density gradient.
the 18-43S ribosomal RNA peak, and the smaller 0-8S peak represents transfer and degraded RNA. Nawrocki (1977) and Svedersky (1977) have indicated that an increase in the small peak is indicative of a partially or fully degraded preparation. The RNA extracted in this manner is suitable for lymphocyte treatment, or may be stored for 2-6 months at -70°C, lyophilized for indefinite periods at -20°C, or again placed under ethanol for protracted periods (months or years). RNA was reconstituted in 0.01 M DEP-phosphate buffered saline for use in treating lymphocyte suspensions.

RNA TREATMENTS

Normal or immune lymphocytes were treated with RNA in the following manner. Hypaque-Ficoll purified lymphocytes were resuspended in RPMI 1640 with HEPES buffer at a cell concentration of 2 x 10^6 lymphocytes per ml. Lymphocytes at a concentration of 4 x 10^6 per 2 ml were treated with 50 ug DEAE dextran (Pharmacia) and a measured amount of RNA, e.g., 25 ug, 50 ug, etc. The RNA was added immediately and the cell suspension mixed and incubated at 37°C for 30 minutes. The treated lymphocytes were centrifuged at 600 x g for 10 minutes and resuspended in medium as required for each experiment.

MOUSE PERITONEAL EXUDATE CELL SUSPENSIONS

Normal male Swiss mice were injected intraperitoneally with 1.5 ml of a 5% solution of industrial grade dextran (5-40 x 10^6 mol wt, Sigma D5501) in 0.01 M phosphate buffered saline. Sixty to one hundred twenty hours later, the animals were nearly exsanguinated
by the intracardial route and the peritoneum exposed. The peritoneal
exudate cells were lavaged from the abdominal cavity with 10 ml
aliquots of Earle's minimum essential medium (MEM). A 10 ml glass
syringe and 18 ga needle facilitated the filling and draining of
the abdominal cavity. The collected cells were centrifuged at
1300 x g for 10 minutes, washed once, and the pellet resuspended
in RPMI 1640 with 20% fetal calf serum. If excessive numbers of
erthrocytes were present, they were lysed by resuspending the
pellet in 4-5 ml of 0.14M NH₄Cl and incubating at 37°C for 10
minutes, then diluting with 5 volumes of MEM. This cell suspension
was centrifuged and the cells resuspended in RPMI 1640 with 20%
fetal calf serum at a concentration of 8-10 x 10⁶ cells per ml.
The concentration of macrophages was routinely 50-60%, with a higher
yield and lower purity evident at 60-96 hours, and a higher purity
and lower yield apparent at the 96-120 hour recovery period.

Peritoneal exudate cells also were induced as above in C3H/HeJ
mice bearing palpable tumors. These cells were washed as above, or
pelleted and frozen at -20°C until needed for an RNA extraction.

MIGRATION INHIBITION FACTOR ASSAYS (George 1962)

Peritoneal exudate cell suspensions (8-10 x 10⁶ cells per ml)
were used to fill 0.8-1.1 x 100 mm capillary tubes. The ends were
sealed with Clay-Adams Critoseal, and the tubes were centrifuged
at 100 x g for 3 minutes. The capillary tubes then were etched
with a diamond pencil at the cell-fluid interface. The cell pack
was broken off and inserted in Seitz-Moore chambers modified by
Scheetz and Rossio (1972) and capable of holding up to 8 capillary
tubes. Indirect MIF assays were conducted by filling chambers with supernatants from lymphocyte cultures of normal, immune or RNA-treated cells and incubating the chambers in a level position at 37°C for 48 hours. Culture supernatants were previously heated for 15 minutes at 56°C. The area of migration was projected and traced onto paper, later to be accurately measured with a compensating polar planimeter (K & E). Controls were comprised of supernatants from normal lymphocytes. Inhibition of migration was calculated according to the following formula:

\[
\% \text{ Inhibition} = \frac{\text{Control Value} - \text{Test Value}}{\text{Control Value}} \times 100.
\]

LYMPHOBLASTOGENESIS ASSAY

In order to measure the proliferative response of activated lymphocytes, a radioactive microassay for DNA synthesis was used (Oppenheim 1977, Penhale 1974). Lymphoblastogenesis assays were conducted to determine the extent of DNA synthesis of primary and secondary immune cells after exposure to RNA or antigen. Falcon 3040 Microtest II plates were used throughout the blastogenesis assays and consist of an 8 x 12 grid of 0.3 ml capacity wells. Aliquots of the test lymphocytes were seeded in replicates in six wells at a concentration of 2 x 10^5 cells per 0.2 ml RPMI 1640 with 10% fetal calf serum. Control wells contained normal cells. Sheep erythrocytes were controlled for thymidine uptake when used as an antigen to stimulate primed lymphocytes. The plates were covered with Falcon 3041 lids and incubated in 5% CO₂ at 37°C.
Twenty hours before harvesting, the wells were pulsed with one microcurie [methyl-\textsuperscript{3}H]-thymidine (New England Nuclear) introduced with a 500 ul Hamilton repeating syringe. At time zero, the lymphocytes were removed from the wells with the aid of a Mash II unit and filtered onto fiberglass filter strips. The individual cell filtrates were excised intact with the glass filter material and placed in 3 ml Filmwarc scintillation tubes (Nalgene #500-0030). One tenth ml of 0.5M Protosol Tissue and Gel Solubilizer (New England Nuclear) was added to the still moist filter discs and the tubes were incubated at 55°C for 30 minutes. After a brief cooling period, 0.05 ml glacial acetic acid was added to each tube to facilitate emulsification of the cell debris. Three ml of scintillation cocktail (42 ml PPO-PPOP toluene concentrate [Liquifluor, New England Nuclear] per liter of scintillation grade toluene) was added to each sample and the plastic tubes were heat sealed and placed in scintillation vials. Samples were counted twice on a liquid scintillation spectrophotometer (Packard Tri-Carb) for five minutes, and the results expressed as counts per minute.

JERNE PLAQUE ASSAY

A modified (Yamada 1969) agarose-drop hemolysis-in-gel method (Jerne 1963) was employed to measure the ability of treated and untreated lymphocytes to produce IgM or IgG antibodies. One tenth ml quantities of lymphocytes were mixed in a 40°C water bath with 0.3 ml of 7% sheep erythrocytes in phosphate buffered saline. To this was quickly added 0.272 ml 2x MEM, 0.272 ml of melted 1.4%
agarose (Agarose B grade, Calbiochem) and 0.04 ml fetal calf serum that previously had been twice absorbed for one hour at 4°C with packed sheep erythrocytes. A 5 3/4" Pasteur pipet was used to thoroughly mix the cell-agarose suspension and dispense the mixture dropwise onto petri dishes from a height of 60 cm. The petri dish lids contained 9 cm Whatman filter paper circles moistened with distilled water to prevent desiccation of the droplets. The plates were incubated 3-4 hours in 5% CO₂ at 37°C.

To develop direct (IgM) plaques, guinea pig complement (GIBCO) or freshly frozen guinea pig serum recovered from animals in our laboratory facility was twice absorbed for 1 hour at 4°C with packed sheep erythrocytes. This complement then was diluted 1:10 in MEM and two drops dispensed onto each agarose drop. The plates again were incubated in 5% CO₂ at 37°C for 1 hour, removed and the plaques counted with the aid of a Quebec colony counter.

To develop IgG (indirect) plaques, the procedure for direct plaques was followed except that rabbit anti-mouse immunoglobulin diluted 1:15 in the above complement solution was substituted for complement only. The anti-mouse immunoglobulin previously had been twice absorbed for 1 hour at 4°C with packed sheep erythrocytes.

A digression concerning the selection of this method of assessing antibody production is in order. The modified agarose-drop technique was the most practical and sensitive assay which could be performed in this culture system. Cornacoff (1978) indicated that this method was slightly more sensitive than the slide technique of Cunningham (1968). Cunningham stated his slide technique
was three times more sensitive than the Jerne plate method (Jerne 1963). Also, Lynch (1973) determined the modified drop technique also showed more sensitivity than the original plate method of Jerne. This increased sensitivity is accompanied by an irregular background level of plaque forming cells. Generally, because of methods used in counting viable lymphocytes, sample sizes used for counting plaque-forming cells over long periods of culture and the decrease in numbers of cells remaining in culture, PFC counts of 100 or less may be doubtful indices of transferred immunity and should be interpreted relative to controls in each experiment. That values below approximately 100 PFCs per million lymphocytes may approach the limits of reliability may be more acceptable if it is realized that plaque-forming cell counts of 10,000 and greater are common to many of the experiments.

PREPARATION OF RABBIT ANTI-MOUSE IMMUNOGLOBULIN
(Williams and Chase 1967, p 316)

Intracardial blood from six to twelve week old Swiss mice was drawn into citrated saline and the plasma removed after centrifugation. The plasma was diluted with three volumes of distilled water, and the pH adjusted to 7.7. The solution was cooled to 0°C, and sufficient precooled (-20°C) 50% ethanol was added slowly with vigorous stirring to achieve a final concentration of 20%. As the ethanol was added, the temperature of the plasma solution was lowered with brine and kept near the freezing point of the aqueous-ethanol mixture until -5°C to -6°C was reached. The insoluble proteins were removed by centrifugation at this
temperature. The majority of this material was IgG, with lesser amounts of IgM and IgA (Williams and Chase 1967, p 317). This precipitate was quantitated using the Lowry method and resuspended for injection at 1 mg/ml in 0.01M phosphate buffered saline. Aliquots for future use were stored at -20°C.

A normal New Zealand white rabbit was injected intradermally and intramuscularly with 1 ml of the immunoglobulin concentrate at 10 day intervals. The first two injections were emulsified in Freund's incomplete adjuvant. The animal was bled seven days after each injection of immunoglobulin concentrate. The blood was allowed to clot at 37°C in sterile tubes, the clot was loosened and the tubes placed at 4°C for 4-6 hours. Serum was pooled, centrifuged to remove cells and debris, and the clear serum drawn off and stored at -20°C until needed. Ouchterlony gel diffusion assays (Ouchterlony 1973) conducted on the antiserum against whole mouse serum gave two lines of precipitate (Plate I). As a control, commercial rabbit anti-IgG (Cappel) was tested against the whole mouse serum. The activity of the anti-IgG in developing IgG hemolysis-in-gel plaques was demonstrated readily with 1:15 dilutions of the antiserum.
Plate I. Ouchterlony gel diffusion plate.

A. Prepared rabbit anti-mouse immunoglobulin
B. Commercial goat anti-mouse immunoglobulin
C. Normal mouse serum
RESULTS

The potential for altering the outcome of unfavorable immunological responses with RNA gives tremendous significance to the therapeutic possibilities of diseases involving malignancy, autoimmune reactions and deficient lymphoid cell function. In these situations there is a definite malfunction or depression of one or both arms of the immune response. Variability has been encountered as investigators have employed RNA preparations from immunocompetent cells to alter these phenomena (Schlager 1976, Cornacoff 1977). The reason for this inconsistency is at question. Clearly, "informational" RNA could be capable of directing specific cells, e.g., T suppressor lymphocytes, to regulate immune responses. In the present investigation, the early experiments involved the transfer of specific humoral activity to normal lymphocytes with RNA. The data gathered generated other investigations which were designed to further clarify the regulatory effect of RNA on the T and B cell responses.

A. RNA TREATMENT OF NORMAL LYMPHOCYTES

Normal Swiss splenic lymphocytes were treated with 100 ug of $^{1}$Srbc IRNA per four million cells and were assessed daily for direct plaque production while in culture. No significant increases in plaque production occurred as shown in Figure 4.
Normal lymphocytes again were treated with 100 ug 1°Srbc IRNA or with 100 ug 2°Srbc IRNA and mixed with sheep erythrocyte antigen (Figure 5). It was conceivable that antigen homologous for the RNA species might be necessary for total expression of the immune response. Plaque-forming cells were recorded as great as 75 per million lymphocytes, but did not differ from background control levels.

Primary Srbc IRNA and 2°Srbc IRNA again were used to treat normal cells, but in 50 ug quantities, an amount which Nawrocki (1977) indicated would induce a PFC response in CD-1 mice. From Figure 6 it may be seen that the direct plaque response approximated the control values. When these same treated lymphocytes were cultured in the presence of Srbc's the PFC response did not exceed control values.

An experiment was initiated that paralleled the in vivo therapeutic experiments in these laboratories in which tumor-bearing mice had been injected with lymphocytes exposed to tumor-immune RNA and monitored for immunity as measured by tumor regression. In the present study, 10^8 normal Swiss lymphocytes were subjected to 50 ug treatments of 1°Srbc IRNA or 2°Srbc IRNA and were injected intraperitoneally into normal recipients. One week later these recipient mice were sacrificed and their splenic lymphocytes placed in culture with and without Srbc antigen. The cultures were monitored for direct Jerne plaque production at days zero and three. The direct PFC response of splenocytes from mice which had been injected one week previously with 50 ug of 1°Srbc IRNA-treated, or with 2°Srbc IRNA-treated normal lymphocytes is shown in Table 1. An increase was noted only if these cells were cultured in the presence
Figure 4. Direct plaque responses from cultured normal Swiss splenic lymphocytes treated as follows:

- No treatment
- 100 ug 1°Srbc IRNA
- 100 ug 1°Srbc IRNA + Srbc
Figure 4

$\log_{10} \text{PFCs} / 10^6 \text{CELLS}$

DAYS

0 1 2 3 4
PLEASE NOTE:

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UNIVERSITY MICROFILMS
Figure 5

LOG$_{10}$ PFCs / 10$^6$ CELLS

DAYS

0 1 2 3
Figure 6. Direct plaque response from cultured normal Swiss splenic lymphocytes treated as follows:

Normal cells

Normal cells + 50 ug 1°Srbc IRNA

Normal cells + 50 ug 2°Srbc IRNA
Table 1

Direct Plaque Responses$^a$ from Cultured Lymphocytes Taken from Mice Injected One Week Previously with RNA-treated or Untreated (Control) Normal Lymphocytes

<table>
<thead>
<tr>
<th>DAYS IN CULTURE</th>
<th>Zero</th>
<th>Three with Srbc$^c$</th>
<th>Three without Srbc$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA treatment$^b$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4.5</td>
<td>3.9</td>
<td>3.2</td>
</tr>
<tr>
<td>1$^o$Srbc IRNA</td>
<td>15.0</td>
<td>42.0</td>
<td>0</td>
</tr>
<tr>
<td>2$^o$Srbc IRNA</td>
<td>8.0</td>
<td>33.0</td>
<td>0</td>
</tr>
</tbody>
</table>

a. Plaque-forming cells per $10^6$ lymphocytes.
b. 50 µg RNA/4 × $10^6$ cells/2 ml culture media.
c. 6 × $10^6$ Srbc/10$^6$ lymphocytes.
of Srbc antigen for three days.

The variability observed thus far indicated that the immunological information was not being consistently transferred following direct exposure of normal lymphocytes with immune RNA. This information, coupled with input from the ineffective therapeutic approach in vivo seen in the murine tumor system, led to the general premise that the in vitro cytotoxic responses to tumor cells following RNA treatment with anti-tumor RNA perhaps were not to be demonstrated in an overt manner with $1^\circ$Srbc IRNA or $2^\circ$Srbc IRNA. This is not to say that RNA was not affecting some lymphocyte function, but rather led to the question of which immune function was being affected.

B. RESPONSES OF PRIMARY IMMUNE LYMPHOCYTES IN CULTURE

To further investigate the possibility that RNA was influencing a variety of lymphocyte responses, studies were undertaken to detect changes attributable to RNA in ongoing immune responses. Again normal Swiss mice were given intraperitoneal injections of 5% Srbc's and the primary response was measured to determine the time of maximum direct plaque production. The data demonstrated that peak production of direct plaques occurred on day four (Figure 7). This then would seem to be the most sensitive time in the primary immune response in which to attempt to measure any effects RNA might have on this particular response.

A dose response curve was determined (Figure 8) to measure the effects of sheep erythrocyte antigen in culture with $1^\circ$Srbc immune splenic lymphocytes. These interesting results encouraged the
Figure 7. Direct (IgM) plaque response of splenic lymphocytes from Swiss mice injected intraperitoneally with 1 ml of 5% sheep erythrocytes. Mice were sacrificed on days 0 through 6 post-injection.
Figure 7
Figure 8. Direct plaque responses from primary Srbc immune Swiss splenic lymphocytes cultured with various numbers of sheep erythrocytes. Numbers of erythrocytes used per $10^6$ lymphocytes in culture are as follows:

- $2.047 \times 10^9$
- $5.2 \times 10^7$
- $6.0 \times 10^4$
- $8.0 \times 10^2$
- $6.2 \times 10^0$
- No Srbc
Figure 8

LOG$_{10}$ PFCs / $10^6$ CELLS

DAYS

0 1 2 3 4 5 6
conclusions that there are several doses of antigen, from approximately $6 \times 10^4$ to $2 \times 10^9$ Srbcs per million lymphocytes cultured, which enhance the number of direct plaque-producing cells five-fold higher than recorded at day zero. Subsequent additions of Srbcs to lymphocyte cultures were routinely $5-10 \times 10^6$ per $10^6$ lymphocytes. This response was not seen with normal lymphocytes incubated with antigen. Neither was it felt to be in vitro sensitization, as no 2-mercaptoethanol (Click 1965) or nutrient cocktail used for in vitro sensitization was employed. As previously stated, decreases in total cell numbers in culture did not coincide with the observed dramatic decreases in numbers of PFCs.

C. RNA TREATMENT AND CULTURE OF IMMUNE LYMPHOCYTES

Svedersky (1977) reported that small dosages of splenic RNA from tumor-bearing animals (C3H TRNA) had the capacity to suppress direct plaque formation. Based on this study, protocols were devised to assess the effect of C3H TRNA, as well as normal and immune RNA, on the immune lymphocytes in vitro.

Primary Srbc immune lymphocytes were treated with 25 µg quantities of NRNA, 1°Srbc IRNA, or C3H TRNA and cultured in the presence and in the absence of Srbcs. The direct Jerne plaque response obtained over a six day culture period (Figure 9) indicated that lymphocytes cultured in the absence of antigen gave direct plaque-forming responses similar to those recorded for control cells and therefore demonstrated a continued decline in PFCs. Treated lymphocytes cultured with Srbcs showed a rise in the number of direct PFCs which was followed by a decline after four days in culture. No differences between treated cells and controls were evident.
Figure 9. Direct plaque responses from cultured primary Srbc immune Swiss splenic lymphocytes treated with RNA and cultured with (or without) Srbc.

1° immune cells only

1° immune cells + 25 ug NRNA

1° immune cells + 25 ug 1°Srbc IRNA

1° immune cells + 25 ug C3H TRNA

1° immune cells + Srbc

1° immune cells + 25 ug NRNA + Srbc

1° immune cells + 25 ug 1°Srbc IRNA + Srbc

1° immune cells + 25 ug C3H TRNA + Srbc
Figure 9
Primary Srbc immune lymphocytes were treated with 50 ug NRNA or with 4198V IRNA, and were subsequently cultured with and without Srbcse for six days (Figure 10). 4198V IRNA-treated lymphocytes cultured with Srbcse demonstrated direct plaque values approximating control values. However, cells treated with NRNA and cultured with antigen exhibited a peak response that was ten times lower than was the peak of the control culture, suggesting a suppressive capability of NRNA which occurred only in the presence of antigen. Others (Vallera 1978, Greenup 1977) have noted immune suppression following NRNA treatments. Treated cells cultured without Srbcse manifested a decrease in the number of direct PFCs.

Since other studies in this laboratory have centered around the effects of C3H TRNA (Vallera 1978, Pennlino 1977) or lymphocyte products from human patients and tumor-bearing laboratory animals (Svedersky 1977), it seemed appropriate to investigate the anti-Srbc responses of tumor-bearing animals in an attempt to elucidate further the results obtained after lymphocyte treatment with C3H TRNA.

Initially, C3H mice, either normal or bearing palpable 4198 tumors, were injected intraperitoneally with Srbcse and were sacrificed four days after injection to determine the magnitude of response to antigen. At time zero, tumor-bearing animals exhibit a PFC response that is ten times lower than that of normal animals (Figure 11). Subsequently, normal C3H animals which have received Srbc injections can develop direct Jerne plaque responses ranging from 800 to 1500 PFCs per million lymphocytes plated. Primary Srbc immune lymphocytes from tumor-bearing mice, although obviously
Figure 10. Direct plaque responses from cultured primary Srbc immune Swiss splenic lymphocytes treated with RNA and cultured with and without Srbc.

1° immune cells

1° immune cells + 50 ug NRNA

1° immune cells + 50 ug 4198V IRNA

1° immune cells + Srbc

1° immune cells + 50 ug NRNA + Srbc

1° immune cells + 50 ug 4198V IRNA + Srbc
Figure 11. Direct plaque response from cultured primary immune splenic lymphocytes from C3H tumor-bearing mice.

Arrow ( ) indicates direct PFC response of primary Srbc immune splenic lymphocytes from normal mice.

1° immune cells

1° immune cells + Srbc
suppressed, responded with a slight elevation in the number of PFCs after two days. Lymphocytes cultured with Srbcs showed only a threefold rise in PFCs at day four. This augmentation is less pronounced than were the responses previously seen in the lymphocyte cultures from 1°Srbc immune mice. Therefore, the suppressed state, at least with respect to primary anti-Srbc responses, seemed to be a distinct possibility in the tumor-bearing host.

D. CULTURE OF TUMOR RNA-TREATED IMMUNE LYMPHOCYTES

Primary Srbc immune lymphocytes treated with 50 μg C3H TRNA were cultured with and without Srbcs and were evaluated for PFCs (Figure 12). The number of plaques associated with untreated cells declined, as previously noted, while numbers from treated cells cultured with antigen rose. Additionally, immune lymphocytes treated with C3H TRNA only also showed an increase in the number of PFCs, a value which peaked on day four. Thus, it was concluded that this tumor RNA stimulated immune cells to enhanced IgM plaque production.

E. EFFECTS OF TUMOR MACROPHAGES ON IMMUNE LYMPHOCYTES IN CULTURE

In view of the suppressed anti-Srbc response seen in the tumor-bearing animal experiments were undertaken to determine if any similar responses could be attributed to the macrophage population present in these mice. Peritoneal exudate cell preparations containing predominantly macrophages were recovered from Swiss mice after intraperitoneal injection of dextran and subsequent lavage
Figure 12. Direct plaque responses from cultured primary Srbc immune Swiss splenic lymphocytes treated with C3H TRNA.

1° immune cells

1° immune cells + 50 ug C3H TRNA

1° immune cells + 50 ug C3H TRNA + Srbc
Figure 12
three to six days later. When suspensions of these macrophages were incubated with 1°Srbc immune Swiss lymphocytes at a 1:10 ratio (Figure 13) and cultured with Srbc's the anticipated increase in PFC's indeed occurred. Lymphocytes cultured only with tumor macrophages exhibited a more rapid and more prolonged decline than did control cultures. The viability of the lymphocytes did not seem to be affected by the presence of macrophages from tumor-bearing animals.

In another experiment the effect of co-cultivation of tumor macrophages and 2°Srbc immune Swiss lymphocytes was investigated (Figure 14). Both control cultures without macrophages present demonstrated increases in the number of indirect PFC's produced on day two. Secondary Srbc immune lymphocyte-tumor macrophage cultures incubated with Srbc's demonstrated a suppression of this response. From other data, the direct plaque response was not suppressed by the presence of the tumor macrophage in culture with secondary immune cells.

F. CULTURE OF TUMOR MACROPHAGE RNA-(TMO RNA)-TREATED IMMUNE LYMPHOCYTES

The rationale was established for investigating the role TMO RNA could have in regulating an ongoing immune response. Fifty ug of TMO RNA were used to treat 1°Srbc immune Swiss lymphocytes which were cultured with and without Srbc's (Figure 15). The control cultures showed an increase in PFC's when cultured with Srbc's, and a decrease in PFC's when cultured without Srbc's. The number of PFC's from cells treated with TMO RNA tended to be similar.
Figure 13. Direct plaque responses from primary Srbc immune Swiss splenic lymphocytes cultured with C3H normal and tumor macrophages. Cultures were also incubated with and without Srbc's.

1° immune cells

1° immune cells + normal macrophages

1° immune cells + normal macrophages + Srbc's

1° immune cells + tumor macrophages

1° immune cells + tumor macrophages + Srbc's
Figure 13
Figure 14. Indirect (IgG) plaque responses from secondary Srbc immune Swiss splenic lymphocytes cultured with C3H normal and tumor macrophages. Cultures were also incubated with and without Srbc.

- 2° immune cells
- 2° immune cells + Srbc
- 2° immune cells + normal macrophages
- 2° immune cells + normal macrophages + Srbc
- 2° immune cells + tumor macrophages
- 2° immune cells + tumor macrophages + Srbc
Figure 15. Direct plaque response from primary Srbc immune Swiss splenic lymphocytes treated with tumor macrophage RNA and cultured with and without Srbc.

1° immune cells

1° immune cells + Srbc

1° immune cells + TMØ RNA

1° immune cells + TMØ RNA + Srbc
to the number assigned the controls. However, 1°Srbc immune lymphocytes treated with TMØ RNA and cultured with Srbcs failed to show a significant increase in PFCs during culture and the numbers declined to a level lower than any recorded for the control cultures.

G. CULTURE OF S91 RNA-TREATED IMMUNE LYMPHOCYTES

Since splenic or macrophage RNA extracted from animals bearing 4198 tumors influences the ongoing PFC response of primary immune cells, another tumor RNA preparation was tested to determine the commonality of this phenomenon. RNA was extracted from the spleens of animals with the S91 tumor, a C3H melanoma which does not cross-react with the 4198 murine fibrosarcoma (Vallera 1978). Svedersky (1977) already has demonstrated suppression of direct plaque production after primary Srbc immune lymphocytes were treated with this RNA preparation.

The number of PFCs from primary Srbc immune lymphocytes treated with 50 ug S91 TRNA and cultured with sheep erythrocyte antigen rose and declined rapidly (Figure 16). Lymphocytes treated with S91 TRNA showed a three-fold increase in numbers of PFCs over those of untreated controls. This enhancement in plaque-forming ability is reminiscent of that recorded after C3H TRNA treatment (Figure 12).

H. RNA TREATMENT AND CULTURE OF SECONDARY IMMUNE LYMPHOCYTES

Following the above, the effect of RNA on 2°Srbc immune cells was evaluated. Secondary immune splenic lymphocytes from
Figure 16. Direct plaque response from primary Srbc immuno Swiss splenic lymphocytes treated with S91 TRNA and cultured with and without Srbc.

1° immune cells

1° immune cells + Srbc

1° immune cells + S91 TRNA

1° immune cells + S91 TRNA + Srbc
Figure 16
hyperimmune Swiss mice treated with 1°Srbc IRNA, 2°Srbc IRNA, or
C3H TRNA, were cultured and the production of both direct and indirect
Jerne plaques was monitored. When these treated lymphocytes were
cultured in the absence of Srbc, there was an eight-fold increase
in the number of indirect plaques formed (Figure 17), however, there
were no significant differences in the IgM plaque response of treated
2°Srbc immune lymphocytes when compared with control values (not shown).

Secondary Srbc immune lymphocytes treated with 1° Srbc IRNA,
2°Srbc IRNA, or C3H TRNA and cultured in the presence of Srbc
demonstrated only slightly increased capabilities as measured by
the number of indirect plaque-forming cells (Figure 18). This
implies that the RNA does not control expression of indirect plaques
when antigen is present. Observations of direct plaque production
during culture of 2°Srbc immune cells and antigen showed the
early and complete suppression of direct PFCs in lymphocytes
treated with 1°Srbc IRNA or 2°Srbc IRNA (Figure 19).

I. ANALYSIS OF T CELL ACTIVITY OF RNA-TREATED IMMUNE LYMPHOCYTES

THROUGH MIF PRODUCTION

To this point in the study the results indicate that an
alteration in the responses of lymphocytes in culture occurs under
the influence of RNA. The B cell activities examined (PFCs) would
seem to be suppressed (Figures 10,15,19) or enhanced (Table 1,
Figures 12,16,17) depending upon the specificity of RNA used.
Interestingly, RNA from tumor-bearing animals (C3H TRNA, S91 TRNA)
showed some enhancement or increase in plaque production without the
Figure 17. Indirect (IgG) plaque response from secondary Srbc immune splenic lymphocytes treated with RNA and cultured without Srbc.

- 2° immune cells
- 2° immune cells + 1°Srbc IRNA
- 2° immune cells + 2°Srbc IRNA
- 2° immune cells + C3H TRNA
Figure 18. Indirect (IgG) plaque response from secondary Srbc immune Swiss splenic lymphocytes treated with RNA and cultured with Srbc.

2° immune cells + Srbc

2° immune cells + 1°Srbc IRNA + Srbc

2° immune cells + 2°Srbc IRNA + Srbc

2° immune cells + C3H TRNA + Srbc
Figure 18

LOG\textsubscript{10} PFCs / 10\textsuperscript{6} CELLS

DAYS

0 1 2 3 4 5 6 7 8
Figure 19. Direct (IgM) plaque response from secondary immune Swiss splenic lymphocytes treated with RNA and cultured with Srbc.

2° immune cells + Srbc 
2° immune cells + 1°Srbc IRNA + Srbc 
2° immune cells + 2°Srbc IRNA + Srbc 
2° immune cells + C3H TRNA + Srbc
Figure 19

Log_{10} PFCs / 10^6 CELLS

DAYS
necessary stimulus of antigen (Figures 12, 16). Since it also has been reported that RNA influences T cell responses (Greenup 1978, Vallera 1978), it was thought that selective analysis of culture supernatants (in this instance, MIF) might well yield information regarding the effect of RNA on T cell responses which might have occurred during culture of the treated cells. Numbers in brackets refer to the Jerne plaque graph from which the supernatants were derived. Migration inhibition values above 20% are considered significant.

Primary immune cells usually exhibit low MIF values during the first 24-36 hours in culture, after which the values rise rapidly to significant levels, whether antigen is present or not (Figure 20 [8]). However, normal lymphocytes or normal lymphocytes treated with 100 μg 1°Srbc IRNA showed no significant MIF production over a four day culture period (Figure 21 [4]). From the values in Table 2 [6] cells treated with 50 μg 2°Srbc IRNA demonstrated slight MIF production at 72 hours with and without the antigen being present in the culture.

The MIF production remained significant throughout the culture period with 1°Srbc immune lymphocytes were treated with either 50 μg NRNA or 4198V IRNA and cultured with Srbcs (Figure 22 [10]). However, when the same treated cells were cultured without Srbcs a significant decrease in MIF production occurred at 48 hours (Figure 23 [10]). This possibly was attributed to the direct effects of the RNA, since 1°Srbc immune cells alone (not depicted here) exhibit continued MIF production. This suppressive effect
Figure 20. Production of migration inhibition factor by primary Srbc immune Swiss splenic lymphocytes cultured with and without Srbc.

1° immune cells

1° immune cells + Srbc
Figure 20
Figure 21. Production of migration inhibition factor by cultured RNA-treated normal Swiss splenic lymphocytes.

Normal cells
Normal cells + 1°Srbc IRNA
Figure 21
Table 2

Production of Migration Inhibition Factor by Normal RNA-treated Swiss Splenic Lymphocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cultured with Srbc antigen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cultured without Srbc antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs</td>
<td>72 hrs</td>
</tr>
<tr>
<td>No treatment</td>
<td>26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12</td>
</tr>
<tr>
<td>1&lt;sup&gt;o&lt;/sup&gt;Srbc IRNA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>2&lt;sup&gt;o&lt;/sup&gt;Srbc IRNA</td>
<td>21</td>
<td>28</td>
</tr>
</tbody>
</table>

a. 5-10 x 10<sup>6</sup> Srbc/10<sup>6</sup> lymphocytes

b. % Migration inhibition

c. 50 ug/4 x 10<sup>6</sup> cells/2ml culture medium
Figure 22. Production of migration inhibition factor by RNA-treated primary Srbc immune Swiss splenic lymphocytes cultured with Srbc.

1º immune cells + 50 ug NRNA + Srbc

1º immune cells + 50 ug 4198V RNA + Srbc
Figure 23. Production of migration inhibition factor by RNA-treated primary Srbc immune Swiss splenic lymphocytes cultured without Srbc.

1° immune cells + 50 ug RNA

1° immune cells + 50 ug 4198V IRNA
did not alter ongoing B cell activities as measured by the direct Jerne plaque technique.

When 1°Srbc immune lymphocytes were treated with 50 ug C3H TRNA and cultured with antigen, a normal MIF curve was noted (Figure 24[12]). However, when immune cells were treated with C3H TRNA and cultured without Srbc, the MIF response was delayed until the fourth day in culture. This is suggestive evidence that RNA from tumor-bearing animals can suppress T cell phenomena and enhance the B cell response.

Figure 25[15] illustrates the level of MIF production by 1°Srbc immune cells treated with 50 ug TMØ RNA and cultured with and without Srbc. Neither group of RNA-treated cells produced significant levels of MIF even though the treated lymphocytes cultured with antigen demonstrated a strong, constant increase in MIF production.

MIF production by S91 TRNA-treated 1°Srbc immune lymphocytes was depressed (Figure 26[16]). The treated cells cultured with Srbc antigen exhibited significant MIF production, but only after five days in culture.

MIF production by 2° Srbc immune cells cultured with and without Srbc exhibited similar curves (Figure 27[17,18]). In comparison, a complete suppression of MIF activity was noted in 2° immune cells treated with 50 ug of 2°Srbc IRNA, while other RNA treatments resulted in variable MIF production (Figure 28[17]). No MIF production results when 2° Srbc immune lymphocytes are treated with 50 ug of 1°Srbc IRNA, 2°Srbc IRNA, or C3H TRNA and cultured with Srbc antigen (Figure 29[18,19]).
Figure 24. Production of migration inhibition factor by cultured C3H TRNA-treated primary Srbc immune Swiss splenic lymphocytes.

1° immune cells + 50 ug C3H TRNA

1° immune cells + 50 ug C3H TRNA + Srbc
Figure 24
**Figure 25.** Production of migration inhibition factor by cultured C3H TM0 RNA-treated primary Srbc immune Swiss splenic lymphocytes.

1° immune cells + TM0 RNA

1° immune cells + TM0 RNA + Srbc
Figure 25
Figure 26. Production of migration inhibition factor by cultured S91 TRNA-treated primary Srbc immune Swiss splenic lymphocytes.

1° immune cells + S91 TRNA

1° immune cells + S91 TRNA + Srbscs
Figure 26
Figure 27. Production of migration inhibition factor by primary Srbc immune Swiss splenic lymphocytes cultured with and without Srbc cells.

2° Srbc immune cells

2° Srbc immune cells + Srbc cells
Figure 28. Production of migration inhibition factor by cultured secondary Srbc immune Swiss splenic lymphocytes.

2° immune cells + 1°Srbc IRNA

2° immune cells + 2°Srbc IRNA

2° immune cells + C3H TRNA
**Figure 29.** Production of migration inhibition factor by secondary Srbc immune Swiss splenic lymphocytes cultured with Srbc.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2^o$ immune cells + $1^o$Srbc IRNA</td>
<td>~~~~~~~~~~~~~~~</td>
</tr>
<tr>
<td>$2^o$ immune cells + $2^o$Srbc IRNA</td>
<td>~~~~~~~~~~~~~~~</td>
</tr>
<tr>
<td>$2^o$ immune cells + C3H TRNA</td>
<td>~~~~~~~~~~~~~~~~~~</td>
</tr>
</tbody>
</table>
Figure 29
J. BLASTOGENESIS ASSAY OF IMMUNE LYMPHOCYTES UTILIZING TRITIATED THYMIDINE UPTAKE

In an effort to explain the increased plaque production an investigation was undertaken to determine whether this response could be due to an inapparent increase in cell numbers. This was approached by examining blastogenesis of primary Srbc immune Swiss lymphocytes cultured with and without antigen and measuring DNA synthesis through the incorporation of radiolabeled thymidine (Figure 30). Concurrently it was noted that the peak Jerne plaque response occurred on day four (Table 3). During the entire Jerne response, primary Srbc immune lymphocytes incubated with Srbc exhibited a thymidine uptake similar to that observed for the control lymphocytes. If uptake values for the erythrocytes are subtracted, a curve can be constructed in which the last three points are identical to the curve representing normal control lymphocytes. From this, the increase in the numbers of direct plaque-forming cells noted when 1° Srbc immune cells are cultured with antigen is a reflection of activity of the existing cell population and does not appear to be an immediate result of cell division.

Since a notable increase in the relative number of indirect plaque-forming cells was seen when 2° immune cells were treated with RNA and cultured for eight days (Figure 17), it was felt this provided an opportunity to determine if RNA could nonspecifically initiate blastogenesis. Accordingly, the appropriate systems were assayed. While lymphocytes in this assay are metabolically more
Figure 30. Blastogenesis of primary Srbc immune Swiss splenic lymphocytes cultured with Srbc. Tritiated thymidine uptake was measured in CPM/2 x 10^5 lymphocytes.

Srbc

Normal cells

1° immune cells

1° immune cells + Srbc
Table 3

Direct Plaque Response from Primary Srbc Immune Swiss Splenic Lymphocytes Cultured With and Without Srbc and Monitored for Blastogenesis as Seen in Figure 30.

<table>
<thead>
<tr>
<th>Days in Culture</th>
<th>Without Srbc</th>
<th>With Srbc&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2072&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2072</td>
</tr>
<tr>
<td>1</td>
<td>447</td>
<td>889</td>
</tr>
<tr>
<td>2</td>
<td>79</td>
<td>2377</td>
</tr>
<tr>
<td>3</td>
<td>142</td>
<td>3902</td>
</tr>
<tr>
<td>4</td>
<td>168</td>
<td>2215</td>
</tr>
<tr>
<td>5</td>
<td>122</td>
<td>753</td>
</tr>
</tbody>
</table>

<sup>a</sup> 5-10 x 10<sup>6</sup> Srbc/10<sup>6</sup> lymphocytes

<sup>b</sup> Plaque-forming cells per 10<sup>6</sup> lymphocytes
active than those in the primary response, more thymidine was taken up during the culture period (Figure 31). With the exception of tumor RNA-treated cells assayed on day six, the activity of RNA-treated immune cells followed the control curve. The number of indirect plaque-forming cells per million lymphocytes in culture on day eight was 19 for 2° Srbc immune cells only, 538 for 2° Srbc IRNA-treated immune lymphocytes and 205 for C3H TRNA-treated immune lymphocytes, reflecting the increased PFC trend seen in Figure 17.

K. LIPOPOLYSACCHARIDE INDUCED ENHANCEMENT OF PLAQUE FORMING CELLS

From the foregoing it was necessary to proceed further in examining the nature of the enhanced Jerne plaque response seen throughout this study. Lipopolysaccharide, an essentially B cell mitogen, was employed with 1° Srbc immune cells in culture to test whether increases in PFCs could occur as a result of blastogenesis. It was thought that this experiment might aid in establishing the role B cells play in the differential plaque responses seen when primary immune cells are cultured with and without homologous antigen.

Primary Srbc immune cell controls incubated with and without antigen were consistent with past assays, i.e., the antigen stimulated cells demonstrated a ten-fold increase in PFCs (Figure 32). Primary Srbc immune lymphocytes treated with an optimal dose of 10 ug/ml of LPS (Ling 1975, p 242) resulted in a three-fold increase in plaques, while plaques from cells incubated with both LPS and Srbc were increased 85-fold.
Figure 31. Blastogenesis of RNA-treated secondary Srbc immune Swiss splenic lymphocytes. Tritiated thymidine uptake was measured in CPM/2 x 10^5 lymphocytes.

- Normal cells
- 2° immune cells
- 2° immune cells + 2°Srbc IRNA
- 2° immune cells + C3H TRNA
Figure 31
Figure 32. Direct plaque response from primary Srbc immune Swiss splenic lymphocytes cultured with and without lipopolysaccharide (10 μg/ml) and Srbc.
Figure 32
A MIF assay conducted on the supernatants from 1°Srbc immune lymphocytes cultured with LPS and with LPS plus Srbc is shown in Figure 33[32]. Both cultures immediately exhibited significant MIF production, but these values declined after five days.
Figure 33. Production of migration inhibition factor by primary Srbc immune Swiss splenic lymphocytes cultured with LPS (10 μg/ml) and Srbc.

1° immune cells + LPS

1° immune cells + LPS + Srbc
DISCUSSION

Poly(A) tailed messenger RNA, the predominant RNA molecule taken up in vitro by lymphoid cells, can induce the transfer of specific immunological activities, e.g., immunoglobulin production in B cells and antibody dependent cell-mediated cytotoxic responses in T cells. Very likely, mRNA directing regulatory activity for lymphoid cells, i.e., suppressor or helper activity, is present in all lymphoid RNA preparations and is transferred simultaneously. It is conceivable that in vitro the recipient cell population affected by a regulatory RNA might not always be present. Or perhaps the regulatory messages are translated, but the protein products affect a subset of cells whose altered activities would go undetected as a result of test parameters being measured in the particular assay used.

Two occurrences triggered investigations into the function of T and B cells following RNA treatment. Adoptive transfer of RNA-treated lymphocytes to tumor-bearing animals in the murine fibrosarcoma system had an enhancing effect on tumor growth. The nature of this enhancement in these "therapeutic" studies was not realized since in vitro assays of RNA-treated lymphocytes indicated consistent cytotoxicity. In other studies, the in vitro transfer of B cell responses (immunoglobulin production) to normal lymphocytes with Srbc immune RNA produced irregular results. The variable MIF production coupled with inconsistent plaque production failed to offer conclusive evidence linking T and B cell responses.
DISCUSSION

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Two occurrences triggered investigations into the function of T and B cells following RNA treatment. Adoptive transfer of RNA-treated lymphocytes to tumor-bearing animals in the murine fibrosarcoma system had an enhancing effect on tumor growth. The nature of this enhancement in these "therapeutic" studies was not realized since in vitro assays of RNA-treated lymphocytes indicated consistent cytotoxicity. In other studies, the in vitro transfer of B cell responses (immunoglobulin production) to normal lymphocytes with Srbc immune RNA produced irregular results. The variable MIF production coupled with inconsistent plaque production failed to offer conclusive evidence linking T and B cell responses
following the RNA treatment. The likelihood that this variability could be related to the uncertainties found in the in vivo therapeutic studies (mentioned above) led to further examination of the functions of RNA-treated lymphoid cells.

The establishment of a system to evaluate this RNA treatment led to the use of Srbc primed mouse lymphocytes placed in culture four days following injection of antigen. The elevated plaque response associated with immune lymphocyte culture plus homologous antigen is much higher than can be produced in the animal with one injection of antigen, since the 1°Srbc immune lymphocytes came from pooled splenocytes from mice already expressing a maximal response to Srbc (Figure 8). This suggests that in vivo there are regulatory mechanisms that only allow the primary response to proceed to a certain level. This was noted when mice were given injections of 15% erythrocytes and showed similar numbers of direct PFCs at four days post-injection. Neither do multiple injections of Srbc raise the direct (IgM) plaque count higher at day zero.

In the antigen culture system, the cells are committed to produce anti-Srbc immunoglobulin, but the presence of antigen is sufficient to trigger increased PFC production, which is dependent to some extent upon the concentration of antigen (Figure 8). Several events could explain this enhanced plaque phenomenon seen above. There could be increased blastogenic activity of B lymphocytes in the presence of antigen, either from direct contact of Srbc with primed B cells, or as a result of T cell stimulation. Alternatively, primed B lymphocytes could be stimulated to begin
antibody production through the action of T helper cells plus associated antigen. An efficient response, such as helper cell activity, is probably important here since levels of antigen as low as $6 \times 10^4$ rbcs per $10^6$ lymphocytes are effective. Waldmann (1977) has indicated the number of PFCs evoked by one T helper cell over a 5 day culture period is equivalent to that generated by one responding B cell in vivo (usually 15-25 PFCs). Hoffman (1975) indicated spleen cells from immunized animals continued to produce antibody to Srbc in vitro only as long as T cells were not eliminated from the cell population. Alternatively, there could be present a suppressor T cell population which functions to prevent expression of a population of Srbc-primed B cells. Selective death of these suppressors or the activation of helper T cells with antigen would abrogate or mask this effect. Lastly, primed B lymphocytes repressed in vivo may require the presence of antigen in vitro for derepression to occur. These primary Srbc immune lymphocytes cultured with and without sheep erythrocyte antigen were treated with RNA preparations and observed for alterations in B and T cell functions.

Primary Srbc immune lymphocytes were treated with small amounts of NRNA, $1^\circ$Srbc IRNA and C3H TRNA demonstrated by others to cause suppression (Svedersky 1977). In those studies, direct plaque responses for all treated cells followed those of normal controls (Figure 9). Larger doses of NRNA and 4198V IRNA were used again to treat $1^\circ$Srbc immune lymphocytes. Direct plaque values were similar to control values. Exceptional was the suppressed PFC
response observed when NRNA-treated cells were cultured with antigen (Figure 10).

Vallera (1978) and Greenup (1977) have seen suppression of T cell responses using normal RNA. The above suppression of plaque production appears to be non-specific, as MIF production (Figure 22) suggests no direct positive relationship to T cell function.

In the compromised host with a malignancy, it is acknowledged that an anergic state exists (Rees 1975). The diminished primary response to Srbcs in mice compromised with 4198 tumor (Figure 11) was examined by looking toward the suppressive nature of splenic RNA from these tumor-bearing mice. It will be recalled that immune lymphocytes treated with C3H TRNA showed a significant increase in direct plaque production during culture (Figure 12), and MIF production by these treated cells was depressed for four days (Figure 24). This was the first instance where enhanced plaque production (B cell activity) due to RNA treatment was inversely related to an alteration in the T cell response. If the RNA contained information which coded for the same general suppression seen in the neoplastic compromised host, then this suppressive effect may have been directed toward T cell function. This depressed T cell function could alter B cell activities by one of several mechanisms. First would be the direct derepression of B cells that are committed but cannot express themselves except when antigen is present (with or without functional T helpers). As discussed above, there could be a suppressor T cell population
which, in the absence of antigen, functions to prevent Srbc primed B cells from expressing themselves. Broder (1978) has shown that one population of T cells regulates suppressors. This information suggests regulatory RNA could have the potential for control at two levels, that of the suppressor cell and that of the suppressor inducing cell. Lastly, the RNA could be exerting a non-specific effect causing all B cells to express themselves, however, previous data does not support this hypothesis (Figure 9,10).

Another instance where a tumor RNA has altered the plaque-forming ability of primed lymphocytes in vitro is seen with S91 TNRA treatment (Figure 16). An enhancement of the plaque-forming response inversely related to MIF production (Figure 26) is seen that is identical to results observed with C3H TRNA (Figure 15).

Macrophages from tumor-bearing animals, when cultured with Srbc immune lymphocytes, also exerted a suppressive effect on plaque production. This action was more pronounced in modifying the secondary or IgG plaque response (Figure 14) than it was in altering the primary response (Figure 13). The controlling ability of the macrophage seen here parallels information illustrating this cell's role in immune regulation (Oehler 1978). Tumor macrophage RNA demonstrated an ability to suppress the enhanced B cell response to antigen (Figure 15) while causing a suppressed MIF or T cell response (Figure 19).

Although RNA purportedly would have an affinity for the cell type representative of that from which the RNA was extracted (Greenup 1977), RNA molecules from macrophages have been implicated as the informational link to T cells in the immune response (White 1976).
Explanations for the suppression therefore tend to fall within these limits. Assuming the enhanced plaque response in the presence of antigen is due to T helper activity, one could postulate a transfer of suppressor message to the helper cell. The feasibility of this is augmented by the finding of monocyte infiltration of tumors suggesting a transfer of suppressive information to T cells in vivo at the same time they are, or should be, receiving specific information regarding recognition of tumor antigens (Evans 1976). Or, the TMØ RNA could affect the T cells such that T-B cooperation would not occur. Alternatively, the B cell could directly take up the informational RNA and be rendered non-functional. This possibility is not born out, however, if one observes cultured TMØ RNA treated lymphocyte values (Figure 15).

The effect of several RNA preparations was directed to the secondary Srbc immune response. Secondary immune cells treated with various RNAs showed a significant and equal increase in the number of indirect PFCs present in culture (Figure 17). The non-specific nature of the response must be attributed to the RNA treatment. This effect could be due to generalized stimulation at the cell surface by the nucleotide preparation (Braun 1965) although this phenomenon has not been seen throughout this study. Scheid (1973) showed that nucleotide agents such as poly A:U are probably inducing differentiation of T precursor cells into T helper cells. In light of the long culture period for this experiment, a non-specific stimulation of this nature is possible. Secondary immune lymphocytes treated with these same RNAs and cultured with Srbc showed no
significant changes in indirect plaques (Figure 18). However, direct plaque production was quickly depressed by the RNAs specific for the Srbc antigen (Figure 19). This could imply that RNA regulates the "shutdown" of IgM production, similar to what one could expect when a cell "switches" from IgM to IgG production. Primary or secondary Srbc IRNA could contain this information, as both populations of cells could have control cells expressing regulatory proteins governing the "switch". MIF data for the secondary immune lymphocytes treated with RNA and cultured with (Figure 29) and without (Figure 28) antigen show MIF responses that are depressed. This general suppression of T cell activity occurring during the secondary response appears to be non-specific.

One could postulate blastogenesis as a possible mechanism for plaque enhancement when culturing primary immune cells with antigen. A blastogenic assay employing tritiated thymidine uptake was performed on primary immune lymphocytes (Figure 30). However, immune lymphocytes incubated with antigen do not show an increase in blastogenic activity in the 24 hour period preceding maximal plaque production. This is not to imply that blastogenesis did not occur earlier, but only indicates that cell division does not immediately precede peak plaque production. Dutton (1968) reported that, assuming a minimum cell division period of six hours for mammalian cells, cell proliferation could not occur rapidly enough to give this increase in plaque-forming cells, thus suggesting that some of the PFCs must arise by a process of differentiation of non-antibody forming cells.
A thymidine uptake assay also was conducted on secondary Srbc immune lymphocytes treated with RNA to determine if the RNA stimulated blastogenesis of the immune lymphocytes (Figure 31). The steady decline in thymidine uptake by RNA-treated cells reveals that increased blastogenesis did not parallel the observed increase in the indirect plaque production. One must therefore assume that the increase in plaque-forming B cells did not arise through immediate cell division, but rather may have resulted from increased differentiation of Srbc primed lymphocytes, or differentiation of non-antibody forming cells. The latter hypothesis is marginally acceptable since antigen is not present in culture and is suggestive that the RNA may be stimulating a B cell activity that is not noted with the untreated but primed lymphocytes.

To further delineate the cell(s) important in the enhancement of plaques, primary immune cells were cultured with E. coli lipopolysaccharide, a B cell mitogen (Ortiz 1970). Incubation with LPS enhanced the number of direct plaques seen, but resulted in values significantly below the levels induced when sheep erythrocyte antigen was present. Vogel (1978) showed that primary Srbc immune Swiss lymphocytes treated with LPS demonstrated a peak Jerne plaque response at day 3, and a stimulation index also indicating maximum DNA synthesis on day 3. Immune lymphocytes cultured with both LPS and antigen showed an 85-fold increase in the number of plaque-forming cells as compared to zero time values. In the absence of antigen, LPS appeared to act primarily on B lymphocytes (Armerding 1974). Armerding (1974) also implicated LPS as influencing specific
helper T cell function in the presence of antigen. Gronowicz (1974) implied LPS was a stimulator of T cells, while Scheid (1973) indicated the mitogen could induce maturation of T cells from precursor cells. Norcross' (1977) explanation of events following LPS treatment involved the direct stimulation of B cells, resulting in the activation of a subpopulation of T cells, which in turn exerted a stimulatory effect on the ongoing LPS-induced B cell proliferation. Thus, in light of the potential regulatory cells LPS could affect, it appeared feasible that the mitogen plus antigen could have the double effect of stimulating helper T cells as well as initiating blastogenesis in the residual B cell population and could lead to the synergistic enhancement of plaques.

From the above it appears enhanced plaque formation is a B cell response and is probably initiated by T helper cells. Ortiz (1970) indicated plaque enhancement by endotoxin was associated primarily with the period after antigen interaction but before cell proliferation. This period, which occurred during the first 24 hours of culture, was characterized by active RNA synthesis and was postulated to be a period of differentiation. Active DNA synthesis was evident 24-96 hours after stimulation with Srbcs or with Srbcs and endotoxin. Whether the antigen acts to trigger the T or B cell directly, or causes the B cell to trigger the T cell which would further stimulate and/or regulate the B cell response is not known. From Norcross' model explaining LPS activation of B cells, this latter possibility is plausible.
This work elucidating possible T-B cell interactions in vitro should aid in integrating information from RNA treatments into a framework that can explain possible mechanisms by which RNA might alter the ongoing immune response. Throughout this study RNA-treated cells have shown variable T and B cell responses with only several instances of consistent agreement, e.g., tumor RNA treatments (Figures 12,16). In a rare instance altered B cell activity can be attributed to possible non-specific activity of the RNA (Figure 17), suggesting a general alteration of some regulatory activity (Scheid 1973) unrelated to cell proliferation.

Complete comprehension of the exact mechanism of enhanced PFC production is still conjectural, even in lieu of information gained from LPS and blastogenesis studies. However, it seems apparent that the regulatory mechanisms present in vivo, as well as in vitro, involve a delicate balance between positive and negative signals. The data suggested that RNA extracted from lymphoid cells was capable of regulating the response of other similar cells. These treated cells then exerted suppressive or stimulatory control over other cells residual in the population. Naturally, T cell subpopulations were implicated as the cells being affected by the RNA treatments. This approach would explain the enhanced tumor growth seen with therapeutic adoptive transfer of RNA-treated cells to tumor-bearing recipients. Fine control of the immune response through T-T cell interaction was noted by Turkin (1977) who indicated a very small part of an immunogen molecule was responsible for eliciting cells which suppressed the
immune response to any determinant attached to the entire molecule. Thus, the proper informational molecule may have to exert influence on only a very few cells in a treated lymphoid cell population to exercise a great deal of control over the ultimate expression of immunocompetency exhibited by that population. If the suppressive nature of RNA could be revealed through such studies, circumstances of disease which regard suppressive processes as therapeutic would benefit, as with situations involving transplantation rejection or autoimmune disorders.
SUMMARY

RNA from the spleens of normal, immune or tumor-bearing mice can alter the responses of immune lymphoid cells cultured in vitro. When sheep erythrocyte immune splenic lymphocytes are cultured in vitro with homologous antigen, an enhanced B cell response (antibody production) is seen which is probably the result of a T helper function. The response seemingly is not the result of concurrent blastogenic processes. B cell responses may be enhanced or suppressed if sheep erythrocyte immune splenic lymphocytes are treated with RNA from tumor-bearing animals and cultured with or without sheep erythrocyte antigen.

RNA-treated hyperimmune anti-sheep erythrocyte lymphocytes were stimulated non-specifically to produce indirect IgG plaques when cultured in the absence of sheep erythrocyte antigen. RNA treatments from any source can result in variable T cell responses in an in vitro immune lymphocyte population without altering B cell responses. Paradoxically, tumor RNA can suppress in vitro T cell responses and enhance B cell responses.

It is felt RNA modifies the ongoing immune response exhibited in vitro, and most probably in vivo, by affecting T cell function. The implication is that RNA preparations contain regulatory message that can readily be assimilated by T cells, which then may exert suppressive control over other lymphoid cells.
LIST OF REFERENCES


Cantor, H, FW Shen and EA Boyse. Separation of helper T cells from suppressor T cells expressing different Ly components. II. Activation by antigen after immunization indicates antigen-specific suppressor and helper activities are mediated by distinct T cell subclasses. J. Exp. Med. 143:1391, 1975.


Cornacoff, JB. A comparison of the anti-tumor cytotoxicity of lymphocytes treated with syngeneic RNA derived from immune and tumor-bearing mice. Master's thesis. The Ohio State University, Columbus, Ohio, 1976.

Cornacoff, JB. Personal Communication. 1978


Evans, SB. Immunological properties of anti-tumor "immune" RNA. PhD dissertation. The Ohio State University, Columbus, Ohio, 1976.


Greenup, CJ. The immunological role of Poly(A) tailed messenger RNA isolated from tumor-bearing and tumor-immune lymphoid tissue. PhD dissertation. The Ohio State University, Columbus, Ohio, 1977.


Lynch, JJ. Personal communication. 1978.


Nawrocki, JF. Transfer of an antigen-dependent suppression of immune responses by RNA from sensitized lymphocytes: Implications for immunological control. PhD dissertation. The Ohio State University, Columbus, Ohio, 1977.


Pennline, KJ. Distinctive immunological properties of RNA extracted from the spleens of tumor-immune and tumor-bearing mice. PhD dissertation. The Ohio State University, Columbus, Ohio, 1977.


Pollock, HM. RNA transfer of immunity between inbred strains of mice. PhD dissertation. The Ohio State University, Columbus, Ohio, 1970.


Rossio, JL. Characterization of transducing "immunogenic" RNA. PhD dissertation. The Ohio State University, Columbus, Ohio, 1973.


Scheetz, ME II. The transfer of tuberculin sensitivity and cytotoxicity against tumor cells to human peripheral blood lymphocytes using xenogenic RNA. PhD dissertation. The Ohio State University, Columbus, Ohio, 1972.


Svedersky, LP. Non-specific lymphocyte induced suppression of immune responses by tumor-bearing hosts. Master's thesis. The Ohio State University, Columbus, Ohio, 1977.


