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Transfer of an Antigen-Dependent Suppression Of Immune Responses by RNA From Sensitized Lymphocytes: Implications for Immunological Control

Dissertation

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

by

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1977

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Production of MIF by normal splenic guinea pig lymphocytes treated with Fraction III of guinea pig tuberculin IRNA, with antigen exposure
Introduction

The study of the control of the immune response in vivo is an area of immunology with far reaching importance for the understanding of immune pathological conditions and the disease states which are manifested, as well as their reversal. In this respect, suppression of the immune response can be considered an active control response which must be maintained in balance with that response which is thought of as the positive expression of immunity (i.e. antibody production). With too much suppressive control, arresting and elimination of the intruding antigen is impossible - too little suppression and the likelihood of autoimmune or allergic disease increases. The demonstration of known measureable immune correlates, such as in the case of tuberculosis, has been insufficient in describing either the presence or absence of protective immunity. Likewise, even though lymphocytes from tumor bearing animals have been shown to be cytotoxic for tumor cells in vitro, there is no control of the growth of the tumor in vivo. Attempts to describe
protective immunity in these diseases have led to the modern conception of the cellular immune response and its protective role. Yet still there is no applicable immunotherapy program by which the immune system can be induced to reverse pathology in either tuberculosis or cancer. Thus, there is good reason why so many investigators in immunology now approach these problems by investigating the control and interrelationships between the different arms of the immune system.

As early as 1956, Medawar was conceiving a diverse immune system, with interacting units, establishing a homeostatic balance resulting in the measured responses to antigen. Most of what is known about the control of this system can now be described by the interaction between well defined subpopulations. The founding work on which this system is based demonstrated some populations of lymphocytes came from the thymus (Davis, 1966), while others came from an unknown origin or the so called "bursa like" organ (Copper, 1967). Thymic derived populations, T cells, effect cellular immunity (Mitchell and Miller, 1968). T cells have since been divided into those that help induce the immune response (Gershon, 1968), while others direct a suppressive role
(Gershon, 1970). B cells and their products have also been assigned suppressive controlling function (Uhr, 1968).

Concurrently, there are studies which deal with the afferent control of the immune response. These involve the role of macrophages, particularly concerning the presentation of antigen to lymphocytes, and the importance of the physical structure of the antigen itself in the induction of the immune response. Macrophages have been shown to be essential for the induction of sensitized cells (Unanue, 1975a). Studies show that the antigen, presented by the macrophage, is bound to the surface of the macrophage (Rosenthal, 1975) and that it is in a partial state of degradation (Unanue, 1975b). Exactly what state the antigen is in is not known, however, it is known that antigenic structures which are composed of few repeating determinants will tolerize or suppress sensitized cells, while more complex forms will not (Feldman, 1971). These ideas have lead to the concept of hapten and carrier responses. Bretchner and Cohen (1970) have proposed a molecular model to explain this phenomenon and indicate the controlling factors of the immune response.

These reports are of particular interest since this investigation is concerned with a suppressive control
mechanism effected by antigen, controlling the responses of either IRNA sensitized lymphocytes, or lymphocytes sensitized in vivo by injections of antigen. It is interesting that these phenomena were noted only after an antigen free system had been developed which demonstrated the production of immune factors by sensitized lymphocytes cultured in medium without any antigen stimulation. It was only when antigen was introduced that suppression was seen. Interestingly, not all amounts of antigen were suppressive. Certain proportions of antigen to the amount of IRNA used to sensitize the lymphocytes, or to the sensitized lymphocytes themselves, allowed production of immune factors at least as significant as that produced by lymphocytes without antigen. Perhaps this is another indication of the importance of antigen presentation to sensitized lymphocytes described in the macrophage studies (Rosenthal, 1976). The phenomenon described here as antigen-dependent suppression has been most completely described in the sheep erythrocyte system, but only after the initial phenomenon was observed in the BCG and tumor antigen systems. The in vivo implications of this study suggest that if there were to be an antigen overload of the immune system, such as in the tumor situation, suppression of lymphocyte activity
might ensue limiting or even creating an aberrant response such as that described in the "enhancement" of tumor (Prehn, 1971). Through these perspectives, it is interesting that there is agreement that the regression of tumor, and thus perhaps reinstatement of an operative immune response, is dependent upon the reduction of tumor antigen.

In this regard, one potential that these IRNA studies have is the illumination of complex interrelationships between the immune system and antigen on a molecular level. Ultimately the hope is that IRNA may be used to protect against tumor or to be used for the reversal of the suppressed immune state apparent in the tumor condition. In this sense the immunization with IRNA would be directed towards the manipulation of a measured response, or towards the "adoptive" transfer of an immune state. This is a novel approach considering that present immunotherapy programs aim to stimulate an immune response against an etiological agent. In the case of tumor this agent has proved to be elusive. Moreover, as described, immune responses by cells of individuals who have tumor can be measured, as well as in tuberculosis, but paradoxically in either situation the response is of limited or no value for the elimination of antigen. These problems are better
related to control, not establishment, or protective immunity. In this investigation most of the considerations of the control pertain to responses to sheep erythocytes. However, demonstration of the same antigen-dependent suppression in the BCG and tumor antigen systems indicate that characterization of the sheep erythrocte suppression of sensitized lymphocytes will apply to the study of the immune pathology of tuberculosis and cancer.
Literature Review

For many years it has been known that "molecules" could be extracted from sensitized cells which were able to induce recipient normal cells to "mimic" immunological capabilities of the donor cell. Stertzl and Hrubesova (1956) produced crude and undefined extracts from spleen cells of sensitized animals which were able to transform normal cell populations into antibody producing cells. The specificity was the same as the original sensitized animals. Attempts to characterize that extract has lead investigators to the conclusion that the molecule responsible was either IRNA (Fishman, 1967), a superantigen, RNA-antigen complex (Solomon, 1963), transfer factor (Lawrence, 1969) or small amounts of contaminating antigen in the preparations.

Many investigators have shown that antigen is not responsible for the transfer of immunity. By treating the RNA preparation with RNA nucleases Fishman (M1961) and Evans (1976) were able to show that there was complete abrogation of activity. Treatment with proteases had no effect.
Fishman and Adler (1967) demonstrated small amounts of antigen in their preparation but found in separate studies that the amount of antigen was too small to incite transfer of sensitivity to normal lymphocytes in vitro. Perhaps the most convincing evidence that antigen plays no role in transfer of immunity comes from the work by Saito (1967). IRNA was extracted from mice immunized with Salmonella enteritidis. The IRNA was then used to protectively immunize a second group of mice. From this set of mice, another RNA preparation was extracted and used to immunize a third set of mice. This serial transfer of IRNA was repeated six times. In every set of mice, protection against the Salmonella was found along with antibody producing spleen cells. At no time, except in the instance of the initial immunization, was antigen used to immunize IRNA donor animals. Finally, it has been shown by Mishell and Dutton (1967) that antigen alone is able to sensitize normal populations of lymphocytes in vitro. However, if the protocol for this sensitization is examined it will be found that the conditions required are much more complex than that used for IRNA transfer of immunity, including substitutions for macrophage activity, and the time required for sensitization (3-5 days) is much
longer than an IRNA transfer of sensitivity (30-60 minutes). Even if it is found that antigen is required (superantigen) for the IRNA activity, there is no question that without the IRNA there would be no transfer of immunity.

The remaining choices for the "moiety" which is transferring the sensitivity, all have RNA in their structure, and it is possible that they represent different points in the sequence leading to the sensitized state in vivo. Thus they may all be able to transfer sensitivity to normal lymphocytes in vitro. However, controversy has developed over the reports dealing with transfer factor. There has been difficulty repeating the work, even to the point where extraction of the factor has not been consistently successful. Techniques are available, however, for the reliable extraction of IRNA and successful transfer of immunity with RNA is widespread in the literature. This laboratory has likewise consistently been able to produce IRNA which transfers immunity.

There are many systems in which transfer of humoral immunity with IRNA was studied. These, as well as systems designed to study cellular immunity, have been extensively reviewed by Rossio (1973) and Scheetz (1971). Fishman (1963) and Fishman and Adler (1963) were among the first to demon-
strate transfer of humoral immunity with RNA. RNA extracted from macrophages infected with T2 phage transferred the ability to produce antibody to normal lymphocytes in vitro. Abramoff and Brun (1968), likewise, have shown that RNA, extracted from spleens of animals sensitized with sheep erythrocytes, sensitized normal lymphocytes in vitro.

The classic work by Fishman and Adler (1965) showed that the antibody produced by normal lymphocytes, treated with IRNA, was found first to be IgM - allotype of the donor RNA (Fishman, 1966) - and then later was of the IgG type, possessing the allotype of the recipient cell type. Bell and Dray (1971) have also shown that IRNA treated lymphocytes produce IgG and IgM antibodies of foreign heavy and light chains.

Transfer of cell mediated immunity correlates have received as much success as the humoral immune transfers. Mannick and Egdahl (1962) were the first to demonstrate that rejection of allografts could be enhanced by prior treatment with specific IRNA. Normal animals receiving injections of the allograft antigen, but previously injected with IRNA to the allograft antigen, were able to reject the graft much quicker than did normal control animals.
There have also been numerous reports of the in vivo transfer of delayed type hypersensitivity in animals receiving IRNA immunization. Jureziz (1968) transferred MIF production and delayed type hypersensitivity with direct injections of IRNA, while Scheetz (1971) showed these same factors in normal animals immunized with normal lymphocytes treated with tuberculin IRNA. Paque and Dray (1970) have also demonstrated RNA mediated in vivo transfer of cellular immunity by measuring these same factors.

The tumor antigen system has received the most attention in IRNA studies. This may be due to the fact that the most promising aspect of the study of IRNA is the eventual prevention and delay of tumor in humans, and thus these models, using tumor antigen, are the most likely to be funded. The advantages of using IRNA as an agent against tumors have been reviewed in a paper by deKernon and Pilch (1975). Firstly, there would be no need to subject the patient to the tumor antigen challenge itself - a procedure which would be, at best, extremely dangerous. The patient would also not receive blocking or suppressive factors which might be transferred if whole cell preparations from similar tumor individuals were used. There would be no graft versus
host reaction, and, as IRNA itself is not a strong antigen, there would be minimal possibility of adverse systemic and local hypersensitivity.

The founding work in this area was carried out by Alexander (1967). The protocol was designed so that mice would receive immunizing doses of a lymphoblastic leukemia, and after the mice were shown to be immune, IRNA was extracted from the spleens. In vitro treatment of normal lymphocytes with the IRNA was responsible for turning the normal lymphocytes into "killer" cells against the specific tumor line as measured by an in vitro cytotoxicity assay. Kennedy (1969) showed that IRNA was also able to transfer anti-tumor activity in vivo. The RNA used in this experiment was extracted from mice which had received immunizing doses of a BP8 tumor line. Normal mice were given direct injections of the IRNA and then were subsequently challenged with the same tumor. Significant delay and prevention of the tumor growth was noted which was dependent upon the RNA and, in addition, the effect was found to be dependent upon the amount of RNA used to immunize the animals. Using similar protocols, Ramming and Pilch (1971) induced protection in vivo against
a C3H carcinoma by first immunizing animals with IRNA preparations. Normal RNA injections did not delay growth of the tumor.

More recently, Schalger and Dray (1975, 1976) have shown regression of a line 10 diethylnitrosamine induced tumor in guinea pigs receiving a lethal dose of the antigen subcutaneously, and then injected 5 days later, at the same site, with a mixture of normal peritoneal exudate cells, antigen-specific IRNA, and the tumor antigen itself. The effect was found to be dependent upon the presence of the IRNA and upon the specificity of the antigen used to immunize the IRNA donor animals. The IRNA could be of either syngeneic or xenogeneic origin, thus satisfying one of the critical criteria for the IRNA therapy in general - that being the source of the tumor specific IRNA be of xenogeneic origin. Delay of IRNA therapy by 12 days resulted in unchecked growth of the tumor. Injection of the lethal dose of tumor antigen on both sides of the guinea pig, followed by injection of the IRNA at only one of the sites, resulted in the regression of tumor at both of the sites, thus suggesting that the activity produced by the IRNA was systemic.

Although it is known that responses to histocompati-
bility antigens can be transferred with IRNA from properly immunized animals (Kern, 1976a), recent observations have confirmed that the protective immunity to tumor challenge afforded by IRNA is due to tumor specific antigens and is not involved with histocompatibility antigens. Ramming and Pitch (1970) showed in a guinea pig system that if a syngeneic tumor cell line were used to generate syngeneic IRNA in these animals, the IRNA was able to transfer tumor specific immunity to normal lymphocytes which was responsible for subsequent demonstration of dytolysis of the tumor in vitro. In this same system, these investigators (1971) showed that xenogeneic tumor RNA could transfer responses against the tumor only if the recipient cell used was syngeneic with respect to the line of origin of the tumor cell.

Kern, et al. (1976b) studied similar phenomena in a human tumor situation. Patient's tumor (gastric carcinoma, malignant melanoma, and cancer of the breast) were injected into sheep. The splenic, xenogeneic IRNA was extracted and used to convert normal human peripheral blood lymphocytes into cytotoxic killers against the tumor antigen in vitro. In this same work, IRNA from spleens of sheep immunized
with normal fibroblast, autologous to the tumor line, was able to transfer tumor cytotoxic capabilities to normal allogeneic lymphocytes. The authors claim that the normal transplantation antigens associated with both the tumor and the fibroblast cells were responsible for the killing. In fact, if lymphocytes autologous to the tumor line were used as recipients of the normal fibroblast IRNA, no cytotoxicity was observed. In this same line of thought, Kern (1976) showed that IRNA extracted from the peripheral blood of cured cancer patients (allogeneic IRNA) could transfer cytotoxicity to normal human peripheral blood cells, but only if the tumors of the IRNA donors and the tumors used in the \textit{in vitro} assay were of the same histological type.

There have been few reports, however, where cancer patients have been cured by an immunotherapy protocol using IRNA. One promising work by deKernon and Pilch (1975) uses an immunotherapy program similar to that advocated by Dodd (1973). Patients' tumor antigens were extracted and used to immunize rams. The IRNA extracted from the spleens of these animals was used to treat the patient's own peripheral blood lymphocytes, which were reinjected back into the patient. Although the report points to some success in
holding tumor load in check, perhaps the greatest interest of the work is that there were no reported complications due to the repeated administration of the IRNA.

Biochemical studies of IRNA, in relation to the transfer of immunity is greatly needed. Whether the IRNA is a superantigen (Friedman, 1963), a messenger RNA (Bilello, 1976), or a non-specific augmentor for the response to antigen (Braun, 1972) is still a matter of controversy. Several investigators agree that the active molecule of RNA is of a low molecular weight, as reported by Dodd and Scheetz (1973). There have also been numerous studies indicating that the molecule has a poly-A tail associated with it (Green, 1976). Unpublished data in this laboratory by Greenup show preliminary evidence that disassociation of this poly-A "species" of RNA from other molecules enhances the transfer of sensitivity as measured by cytotoxic responses against tumor. It will be interesting when similar studies are conducted identifying different molecular "species" of RNA and associating them with various functions and the origins of those functions.

If IRNA is to be used in any preventative of therapeutic sense, it is important to understand all of the possible reactions induced by IRNA. The aim is the
development of a safer and more efficient means with which to carry out an immunotherapeutic protocol with IRNA for any disease. This laboratory has been undergoing such an in vitro investigation for many years. Scheetz (1972) described dose response patterns in the tuberculin antigen system. Varying amounts of IRNA were used to treat groups of normal lymphocytes which were then stimulated with PPD and measurements for antibody and MIF production were made. Only certain amounts of IRNA were able to transfer antigen sensitivity. Evans (1976) has shown similar dose patterns in the 4198 tumor antigen system, while Pennline (1976) has further defined this system, characterizing and distinguishing the effects of treating lymphocytes with either TL-RNA (RNA from tumor bearing animals) or IL-RNA (RNA from animals immune to the tumor). Blocking antibody, as well as cytotoxic antibody, was transferred to normal lymphocytes by the TL-RNA. IL-RNA transferred only cytotoxic antibody. This same investigator has defined an antigen free system by which to measure cytotoxic and blocking antibody.

This work has lead to the development of the present study, which further characterizes the dose response patterns utilizing an antigen free system. The results assign
antigen, unprocessed by macrophages or any other system, the role of suppressing the immune function of IRNA sensitized lymphocytes when presented in the proper concentration. No similar function has been given to antigen in the literature. These observations raise questions as to the nature of the different cell populations which are involved with the antigenic stimulation leading to the immune state, the nature of the control of these populations, and the state of the antigen responsible for either induction or suppression of the immune response.

The control of the immune response, either in a helper or suppressor function, has been suggested as the homeostatic balance between various cell populations in the immune system (White, 1973). Although the literature can only be speculative as to the nature of the control or identification of the various cell populations responsible, it is quite clear that the balance is maintained under precisely defined conditions. The greater part of studies are concerned with the suppression of the immune response, perhaps since it is widely held that development of most pathological conditions of the immune system is due to the absence of, or due to a non-effective, immune response. These investigations are conflicting when compared with one another and the
numerous hypotheses as to the nature and origin of the suppression are unable to generate conclusive supporting evidence. Perhaps this is so since suppression could be operative at any point along the sequence of events leading to the evident immune response. Further, the operation of these suppressor mechanisms may differ. This sequence will briefly be reviewed before the actual suppressor mechanisms are discussed.

The first cell population in that sequence actually to come in contact with the antigen is widely believed to be the macrophage and thereby it has been assigned an intermediary role between the antigen and the lymphocyte system (Unanue, 1975a). Indeed the macrophage is the primary cell found at the site of a delayed type hypersensitivity and is of prime importance in affording protection against bacterial infections (Unanue, 1975b). Assigned the role of processing the antigen, the macrophage either brings antigenic determinants of hapten or carrier sites to B and T cells (Fishman and Adler, 1963) or is involved in actual cellular interactions and even the release of pharmacological mediators (Grey, 1972). Some of these mediators are cytophilic for T cells (Unanue, 1972). What is reasonably certain is that the main
characteristic of the macrophage - phagocytosis - is necessary for both its immunological afferent and efferent functions.

**In vitro** studies by Rosenthal (1975) show that antigen stimulation of sensitized lymphocytes, as measured by blastogenesis assays, is dependent upon the presence of the macrophage. Incubation of the macrophage with antigen results in binding of the antigen to the surface. Apparently, the antigen which is macrophage bound, and which eventually comes in contact with the lymphocyte, is not fully metabolized, leading to the speculation (Unanue, 1975a) that the percentage of the total antigen introduced into a host which actually comes in contact with the lymphocytes is very low. There is good evidence by Rosenthal that the "signal" which is required to stimulate a sensitized cell is dependent upon close cellular contact with the macrophage (which was previously incubating with the antigen) since stimulation was dependent upon cellular concentrations and there was no apparent soluble mediator from incubating macrophages which was able to duplicate the effects found when using whole macrophages. In the absence of antigen, macrophages were found to be able to bind, although weakly and reversibly, to the surface of lymphocytes. Rosenthal speculates that this
type to binding might facilitate the presentation of antigen to lymphocytes by macrophages. Binding when antigen is present is irreversible. In either case, it is important that the histocompatibility groups of the two cell populations be the same. Apparently non-antigen binding does not involve immunoglobulin (Lipskey, 1975), while the nature of the interaction in the presence of antigen is still unclear. According to some evidence, the lymphocytes may assume the role of controller of the interaction between the macrophage and the antigen as it can be shown that T cell products attach themselves to part of the antigen, and thus inhibit phagocytosis. The products form either IgM-antigen or IgT-antigen complexes (Phodes, 1973), both of which react with the membrane of macrophages, again limiting or controlling the processing of antigen.

Assignment of these, and other functions, to T and B lymphocytes and their subpopulations has generated intense investigation. The issue is apparently complicated by the intricate interactions of these populations, the only part of which is measurable being the "net" effect of these relationships (Clayman, 1966). Studies involving the measurement of humoral responses in a number of antigen
systems have indicated that some antigens require that T cells as well as B cells be operative in order that antibody be produced. Clayman (1966), was among the first to observe this phenomenon working in the erythrocyte antigen system. Working with the hapten, DNP, Benacerraf (1967) showed that antibody responses by B cells were dependent upon the presence of cells responding to a carrier antigen. These cells immune to the carrier were found to be T lymphocytes (Raff, 1970b) and are now referred to as helper T cells. Responses to antigens requiring such helper cells are referred to as T-dependent responses (Mitchell and Miller, 1968) and tolerization of helper T cells will lead to suppression of the B cell response (Gershon, 1968). There is also evidence for the existence of B cells which are T-independent and which can be directly tolerized (Gershon, 1970).

The thymic derived population has also been divided into subpopulations, each of which is assigned different immunological function. There are at least five T subpopulations which have been reviewed by Bigley (1975). T<sub>1</sub> cells are non-immunocompetent lymphocytes found in fetal liver, while T<sub>2</sub> lymphocytes are immunocompetent and represent those cells which carry out the immunosurveillance
function. T3 lymphocytes have specific antigen receptors, produce lymphokines and are connected with inflammatory reactions. T4 cells induce nonspecific bone marrow cells to mediate cellular mediated immunity while T5 lymphocytes are the short lived helper cells.

The macrophage, B lymphocytes, and the five T lymphocyte subpopulations hold close associations with one another and interact either directly or through soluble mediators. These interactions have been extensively reviewed by Greaves (1973). At best, knowledge of these associations is incomplete and confusing. In particular, studies which deal with those suppressive interactions are most difficult.

The conception of suppression has evolved into two major themes; central and peripheral control as first described by Medawar (1956). Central control involves antigen or antigen-antibody complexes which bind to the sensitized T or B lymphocyte in such a fashion to shut them off. Two hypothetical molecular models have been proposed by Bretchner and Cohen (1970) and by Lederberg (1959) describing this phenomenon and will be discussed below. Peripheral control involves the interaction between sensitized lymphocytes and either a suppressing cell population itself or products of that suppressing cell.
The nature of the mechanisms involving peripheral control is still unclear. The most popular model involves antibody mediated suppression. The antibody might assume the role of a feedback inhibitor (Uhr and Moller, 1968), it may compete with B receptors for the antigenic determinants (Brody, 1967), divert the whole immunogen from contact with B cells, or finally it may induce tolerization of the B cell (Diener and Feldman, 1972) or the T cell by antibody-antigen complexes. Apparently there is some confusion as to whether antigen-antibody complexes effect peripheral or central control. The role of antibody in the peripheral suppression control has received much attention in the tumor antigen system. Kaliss (1969) showed that by passive administration of anti-tumor antibody in mice, growth of tumor could be "enhanced". Thus the term "enhancing" factors is used to demote either antibody, or antibody-antigen complexes (Hellstrom and Hellstrom, 1970).

T cells themselves have been shown by several investigators to suppress B cell function effectively. Gershon and Kondo (1971) showed that the phenomenon of antigen competition was dependent upon the population of thymic derived cells. Animals which had been thymectomized did not
demonstrate antigen competition. In 1972 Gershon further
illustrated the suppressive nature of T cells by showing
that normal thymocytes suppressed the responses of educated
cells when the two populations were mixed in an in vitro
culture. This investigator could find no soluble mediator.
However other workers (Ranney and Steenberg, 1976) demon­
strated a soluble factor with a molecular weight of less
than 1000 which effects normal lymphocytes in culture in
the same manner as shown by Gershon. Splenic lymphocytes
from tumor bearing mice have also been shown to be suppres­
sive for normal spleen cell or lymph node cellular prepara­
tions (Pope, et al., 1976). Suppression was measured by a
reduced response to mitogen. Lymph node cells from the
tumor bearing mice had no effect on lymphocytes from normal
mouse spleens or lymph nodes. Elimination of this popula­
tion of suppressor T cells by in vivo administration of
specific antiserum resulted in an increased production of
IgM antibody against pneumococcal antigen type III, but had
an inhibitory effect on the production of IgG (Barker and
Stashak, 1970). Suppression of allotypic antibody forma­
tion in mice can be mediated by anti-allotypic T cells
(Jacobenson, et al., 1972). Very interestingly, T cells
serve to inhibit the release of anti-erythrocyte autoantibody by B cells in NZB mice (Allison, 1971) and the author suggests that in old age, as the T cell control is lost, the animals develop haemolytic anaemia.

Central control of the sensitized cell involves the interaction with the cellular surface of antigen or antigen complexes. The manner of presentation of the antigen is crucial (note the role of macrophages previously described) as two hypothetical models by Bretchner and Cohen and Lederberg described. Each theory relies on the concept of antigen recognition receptor sites. After combination with antigen, there are generated signals which direct either induction or suppression of the immune state. Lederberg's model attempts to describe a situation whereby, "the immature antibody-forming cell is hypersensitive to an antigen-antibody combination: it will be suppressed if it encounters the homologous antigen at this time". As the cell matures, or if a secondary response develops, the effect of antigen is lost. Bretchner's model, which is more complex, involves the sensitized cell which is able to be suppressed or induced into antibody production. Neither state is a prerequisite for the other. Whether the cell is suppressed or not
depends upon presentation of the antigen, and thus an individual cell might find itself in a flux between these two states. The model continues by asserting that tolerance is induced by recognition of only one antigenic determinant while expression is dependent on the recognition of two determinants. The second determinant represents the carrier molecule or the helper function of T cells previously described. Control of B or T lymphocytes is the same. The interaction of the antibody recognition site on sensitized cells with antigen results in conformational changes of receptor sites, some of which signal suppression while others signal induction. Control of the immune response by antigen would thus involve a number of conditions, such as state and concentration of the antigen. There is much work to support either of the two above hypothesis.

The fact is that it has been known for many years that tolerance can be induced in animals by initial injection of antigen at birth, with repeated injections of the same antigen at 4 to 6 months (Smith, 1958). As the antigen concentration lowers, tolerance is lost. This and other similar work at the time was largely instigated by a review by Campbell (1957) on the persistance of antigen in vivo and
its effect on the induction of the immune response or tolerization and its effect on the development of pathology. In the next fifteen years there was much work describing the tolerant state, particularly the concept of low and high zone tolerance (Mitchison 1964). Most of this work can be found reviewed by Greaves (1973).

More recently Gershon, Gery and Wakeman (1974) have shown that animals immunized with large doses of either gamma globulin, serum albumin, or erythrocytes, demonstrate a suppressed PHA response, but that this state of suppression lasts only one week following the immunization. The authors conclude that when concentrations of antigen were too great the signal received by the lymphocytes was to "turn off". In time, the in vivo concentration lessened and the cells "turned on". In this same work, presence of antigen in cultures of lymphocytes, which were being measured for PHA responses, enhanced the suppression. The amount of suppression was proportional to the amount of antigen used to immunize the animals, the amount of antigen used in culture, the number of lymphocytes in culture, and the amount of PHA used to stimulate the cells.

Eardley (1976) has investigated the nature of generating
T suppressor cells in vitro. A relationship was noted between the amount of antigen and the number of lymphocytes, used in the Mishell-Dutton in vitro sensitization experiments, necessary to generate suppression. There was also a ratio of antigen to lymphocytes which initiated helper function and enhanced immunological responsiveness. Pike and Nossal (1976) also demonstrated the requirement for the optimal quantities of antigen for induction of the production of antibody by sensitized lymphocytes. Both of these last two experiments support the results of this thesis where it will be shown that a relationship exists between the amount of RNA used to sensitize lymphocytes, or the concentration of sensitized lymphocytes themselves, and the concentration of antigen which is necessary for the production of immune factors. Other relationships between antigen and RNA or sensitized cells will be discussed as suppressive.

Thus the trend in the study of tolerance and/or suppressive of immunity was begun in the in vivo situation and developed and characterized most fully in vitro. These investigations have at all times attempted to describe the relationship between antigen and the control of the immune response. But the implication of antigen playing a
suppressing role, as a natural function, has largely gone unnoticed in the literature. Nor has any hypothesis, describing cellular responses which develop early as a result of antigen exposure leading to establishment of the immune state, or more importantly, the development of immune pathology, incorporated this knowledge.
Materials and Methods

**Sheep Erythrocyte**: Fresh, pooled, sheep blood was supplied by the Animal Facility of the Department of Microbiology, The Ohio State University, and stored in sterile vacutainer bottles containing ACD solution to prevent coagulation. Before using the erythrocytes for immunization or *in vitro* assays, they were washed three times in Seligman's Balanced Salt Solution (pH-7.0).

**Mycobacterium bovis** (BCG): Tice strain of *Mycobacterium bovis* (BCG) was supplied by the Culture Collection of the Department of Microbiology. The organisms were obtained on agar slants and were transferred to 250 ml culture flasks containing 100 ml's of either Middlebrook-Dubos Bacto Broth medium of Difco 799H medium, each with Tween 80 in order to prevent clumping of cells. Organisms to be used for the extraction of ribosomes were grown in pellicle form by culturing in a modified Prauskauen and Beck medium (Youmans and Youmans, 1975).
4198 and 4198V Polyoma Murine Fibrosarcoma: The 4198 tumor cell line was initially developed by Ting and Law (1965) from the normal C3H 4094 cell. The related 4198V line, also developed by Ting (1970), carries on its surface eight times the amount of antigen than does the 4198. Both cell lines could be cultured in vitro in RPMI-1640 medium with 10% fetal calf serum in an atmosphere of 5% CO₂. The 4198V cell was used in the in vitro cytotoxicity assays while the 4198 line was used to immunize animals, challenge suspected immune animals, or to transfer tumor load in vivo.

Immunization of IRNA Donor Animals: The three antigen systems described were used to immunize animals which would be used as the source of IRNA. CD-1 mice were immunized intraperitoneally with a single injection of 3x10⁹ sheep erythrocytes; outbred guinea pigs received a single injection of 1x10⁷ 4198 tumor cells intramuscularly. All of the animals were sacrificed on day 14 past immunization and their spleens collected and frozen for IRNA extraction, C3H mice demonstrated palpable tumor at this time. One group of CD-1 mice received a second injection of sheep erythrocytes intraperitoneally on day 14. These mice were considered donors of immune RNA from a secondary immune response and
were sacrificed 7 days after the second injection of red blood cells when their spleens were collected and frozen. All IRNA donor animals were tested for appropriate responses (antibody, MIF and skin tests) at the time of spleen collection to insure that an adequate immune state existed.

**IRNA Extraction:** IRNA from spleens of immune animals was extracted using a phenol-water two phase system. This procedure was first developed by Thor (1971), and later redesigned and modified for experimentation in this laboratory by Scheetz (1972).

Splenic tissue was surgically removed from animals and immediately processed or stored in a -70°C freezer. This procedure minimized the effect of ribonucleases. Careful cleaning and heating of all apparatus used in this extraction procedure, as well as the use of polyvinyl sulfate (PVS), bentonite, and sodium dodecyl sulfate (SDS), as reagents in the extraction buffer, designed to remove ribonucleases, proved to be essential for the most efficient yield of IRNA per gram tissue weight and for the maintenance of a biologically active preparation of IRNA.

Frozen tissue (up to 2.0 g) was first placed into 90% phenol, saturated with extraction buffer, and homogenized
in a Virtis Homogenizer for 5 minutes at a speed of 5000 rpm's at 4°C. Ten milliliters of the buffer saturated phenol was added and homogenization was continued for an additional 5 minutes at 10,000 rpm's. The tissue was then homogenized for a third 5 minute interval, with another 10 ml's of phenol added, and with an increased speed to 15,000 rpm's. Thirty milliliters of extraction buffer (containing sodium acetate, 8-hydroxyquinone, sodium dodecyl sulfate and PVS) were added, along with 2 mg of bentonite, followed by an additional 10 minutes of homogenization.

This suspension was carefully pipetted into a 250 ml erlenmeyer flask equipped with a stopper through which a thermometer had been secured. Immediately the flask was submerged into a 60°C water bath and carefully swirled so that the temperature of the suspension would reach a temperature of 55°C in exactly 10 minutes. This step has proved to be critical for maximum separation of DNA from the RNA and maintenance of the structure of the RNA itself. The heated flask was quickly plunged into an ice bath so that the temperature would be reduced to 0°C in as short a time period (2 minutes) as possible in order that the phenol phase separate from the water phase. The suspension was then centrifuged at 40,000 X g (18,000 rpm) for 3 minutes.
at 4 C. The aqueous phase was collected, taking care to avoid contamination with the interfacial material or with the phenol phase, both of which were reextracted with one half of their volume of buffer plus 2 mg of bentonite, heat treated, cooled and centrifuged as before. The aqueous layers were combined, and this preparation was treated with half volumes of 90% phenol and 2 mg bentonite and, again, heated, cooled, and centrifuged as before. The aqueous layer was extracted once more, and the final aqueous phase was brought to 0.3 M with addition of 2.5 M acetate buffer. Three volumes of ethanol, distilled, were used to precipitate the RNA. After storage at -20 C for 24 hours, the RNA was centrifuged at 18,000 rpm's at a -20 C for 15 minutes. At this time a preliminary quantitation was performed, as described below, and the RNA reprecipitated with 3 volumes of ethanol. These precipitations served to purify the preparation. After another 24 hour storage period, the RNA was centrifuged as before, quantitated, and distributed in one milliliter volumes at a concentration of 1 mg/ml in freezing vials. In this state the RNA could be stored for several months at -70 C, or up to two weeks at -20 C. For longer periods of storage, the RNA was lyophilized and stored at either temperature.
**Spectrophotometric analysis:** Optical density readings on a Gilford Model 2400 spectrophotometer measured the absorbance of light at 230, 260, and 280 nm. RNA has the property of maximally absorbing light at the 260 nm wavelength, while proteins (specifically phenylalanine, tryptophane, and tyrosine) absorb at 230 and 280 nm, and DNA absorbs at 260 nm along with the RNA. Initial analysis of the purity of the RNA preparations was obtained by calculation of the ratios of the optical density at 260/280nm and at 260/230 nm. A ratio of 2.0 or more indicated reasonably pure RNA, although these ratios are not a measure of the structural integrity of the molecule as even degraded RNA can have a ratio of 2.0. Sucrose density gradients must be used to determine the integrity (see below). For quantitation purposes, it was determined that a 1 mg/ml solution would absorb 24 O.D. units at 260 nm.

**Chemical evaluation:** In order to assay for the presence of contaminating protein and DNA material, orcinol and diphenylamine assays, respectively, were performed as described by Williams and Chase (1968), Volume II. An RNA preparation consisting of less than 5% of either DNA or protein was considered optimal for biological activity.
Sucrose Density Gradient Evaluation: In order to evaluate the potential for transference of biological activity, a profile of the centrifugation of RNA through a sucrose gradient was obtained. In Beckman 4 X 5/8 tubes, a gradient of sucrose ranging from 6-21% was linearly formed by the ISCO Model 180 Gradient Former. Sigma crystalline, Grade I, sucrose was used and was assumed to be ribonuclease free. Fifty to one-hundred ugs of the IRNA was layered on the surface of the gradient, followed by centrifugation in a Beckman Model L-2 ultracentrifuge at 116,000 X g in a SW27 swinging bucket rotor for 18 hours at 4 C.

Using an ISCO Model 180 density gradient fractionator, the sucrose gradient was pushed out of the tube by injecting a 50% sucrose solution through the bottom of the tube. The effluent was carried through the flow cell of an ISCO Model 222 spectrophotometer and the absorbancy read at 254 nm.

Sephadex Fractionation: Preparative samples of fractionated IRNA used to study further the relationship between size of the molecule and biological activity, were obtained utilizing Sephadex G-200. The gel was swelled in 0.01 M acetate buffer (pH-5.0) with PVS and .02% sodium azide. Pharmacia columns were employed with adapters able to support an ascending
flow. Column effluents were run through a Gilford Model 2400 Spectrophotometer (absorbance of light at 260 nm.) and the fraction collected in a Buchler Fractomat. The process of the fractionation was followed on a chart recorder.

An estimation of void volume was made with blue dextran and the amount of IRNA used in the actual fractionation procedure was from 2-5 mgs. As the fractions were collected, they were adjusted to 0.3 M and precipitated with ethanol overnight as previously described. Fractions were quantitated and stored at -70 C.

Polyacrylamide Gel Electrophoresis: This procedure was used in conjunction with the sucrose density gradient described to analyze the molecular integrity of the IRNA. With this technique, it is possible to evaluate RNA more closely due to the fact that this assay is able to separate the IRNA into more peaks or divisions. This will become extremely useful when investigations of different species of IRNA, each with specific functions, becomes possible.

The stock reagents used in this assay include: acrylamide-N, N', -Methylenebisacrylamide, 20%; tris-borate-EDTA Buffer, pH 8.3 (Peacock's Buffer); DMAPN (6.4%) used as a catalyst; 3-dimethylaminopropionitrile; ammonium persulfate
(1.6%); agarose grade A. Initially, 0.2 g of the agarose was dissolved in 20.9 ml's of distilled water by boiling, until the solution was clear, in an erlenmeyer flask over a magnetic stirrer hot plate. The flask was equipped with a reflux condensor with cold water running through it in order to prevent excessive evaporation of the agar. When the solution was clear, the hot plate was adjusted to medium heat and the agar was allowed to reflux for an additional 15 minutes. During this time, 6 ml's of acrylamide-BIS, 2.5 ml's of DMAPN, and 2.5 ml's of Peacock's Buffer (10X) were combined in a small flask and heated in a water bath set at 38-40 C. The agarose, after the 15 minute interval, was cooled in this same bath and held at that temperature. The agar was constantly swirled during this cooling period so that isolated pockets of cooling agar did not develop. In this manner the agar was prevented from hardening. The combined reagents described above were added to the agarose and the entire mixture swirled. The ammonium persulfate was added (0.6 ml) and gently swirled into the solution. Quickly, as the persulfate will chemically begin to "set" the agarose, the suspension was pipetted into special gel tubes, pretreated with Photo-Flo (Kodak) at a dilution of 1:200. This pre-treatment enables easy sliding of the gels out of the tubes.
after the electrophoresis. The tubes should be filled up to 1/2 inch from the top. This is best performed using a pasteur pipette and a rubber bulb. After the gel begins to set, approximately 5 minutes, the top is sealed with distilled water. This step also insures a level edge at the top of the gel after setting is complete. The bottoms of the tubes have been sealed by virtue of a special rack with rubber plugs, into which the tubes tightly fit. The gel is allowed to polymerize for 2-24 hours at room temperature.

**Electrophoresis:** An electrophoresis gel apparatus, complete with a mount for the gel tubes, and a reservoir for buffer, was connected to a power source so that the negative pole was at the top and the positive pole was at the bottom. The reservoir was filled with iX Peacock's buffer. With the tubes in place, a current was supplied so that each tube received approximately 2-3 mA for 50-60 minutes. The temperature of the chamber was reduced to 4 C during this time. The IRNA to be analyzed was diluted in a 40% sucrose solution made with 0.001 M Na₂EDTA so that the final concentration of RNA was between 200 and 250 ugs per ml. Each gel received 100 uls of this suspension so that each received roughly 25 ugs of IRNA. A
solution of Brom Phenol Blue was also diluted in sucrose, and added to one of the tubes to serve as a marker with which to monitor the progression of the IRNA. A current of 1 mA per tube was applied for 10 minutes in order to allow even entrance of the IRNA into the gel. The current was then increased to 2 mA per tube for the duration of the run. The IRNA moves to the positive pole.

**Staining the Gels:** At the end of the run, the buffer was discarded and the tubes removed. Under water, and using water pressure supplied with a small rubber bulb over the end of the tube, the gels were slid out of the tubes. Each gel was placed in a larger plastic tube, with holes cut in the sides and appropriate markings on the caps, in order to maintain the order of the gels during staining. The plastic tubes were placed in a plastic tray with a tight fitting cover and were flooded with Stains All (Eastman 2718), a dye which stains RNA bluish purple, DNA blue, and protein pink or red. A stock solution of Stains All was made at a concentration of 0.1% in 100% formamide. This was accomplished by dissolving 0.1 g of the stain in 100 ml's of formamide for 4-6 hours with a magnetic stirrer. The pH was adjusted to 7.4-7.6 with HCl. The working stain was
made by combining 10 ml's of the stock solution with 90 ml's of formamide and 100 ml's of water. This yielded a 50% formamide solution. The pH was adjusted to 7.5 and the stain was immediately poured into the plastic tray. The gels were exposed to the dye, in the dark and on a rocker table, for 1 hour. The gels were rinsed in running water until bands were apparent and then scanned at 750 nm wavelength on a Gilford scanner.

**In Vitro Sensitization with IRNA:** Normal splenic lymphocytes from mice (CD-1 and C3H) and from guinea pigs were collected by isopaque-ficoll gradient separation using RPMI 1640 medium, without fetal calf serum and with Hepes buffer to minimize clumping of cells. The medium was used at a pH of 7.2 for cells to be cultured and at a pH of 8.3 for cells to be used directly in the Jerne Plaque assay as experimentation suggested a pH factor to be critical in this test. The cells were suspended at a concentration of $2 \times 10^6$ lymphocytes per milliliter and distributed in two milliliter volumes in screw cap, 15 X 100, culture tubes (a total of 4 million cells). The lymphocytes were treated with 50 ugs of DEAE-Dextran per 4 million cells prior to treatment with the IRNA. The IRNA was added in
quantities ranging from 25 to 1000 ugs depending on the experiment. The cells were incubated at 37 C for 30 minutes with frequent agitation, after which time they were washed several times in RPMI with 10% fetal calf serum and 1X pen strep, and 1X glutamine. The lymphocytes were suspended in a final volume of 2 ml's of medium. Those cells, erythrocytes, or PPD at this time. All cells were incubated at 37 C with 5% CO₂ for 24 hours. Supernatants were collected, and the cells were resuspended in fresh medium. After an additional 24 hour incubation, the second set of supernatants were collected and added to the first set. All of the supernatants were kept at -20 C until they were used in either the indirect migration inhibition assay, or in the erythrocyte lytic assay. Cells which were employed directly into the Jerne Plaque assay were originally suspended at 8 million cells per ml before treatment with the IRNA. After incubation with the IRNA, the cells were washed and used in the Jerne Plaque assay.

**Jerne Plaque Hemolysis in Gel:** Lymphocytes from normal animals separated from red blood cells by isopaque ficol and treated with IRNA as previously described, were measured
for antibody synthesis using a modification (Yamada, 1969) of the Jerne Plaque assay. A 1.4% agarose suspension (grade B-Calbiochem) was prepared in distilled water by boiling. The agar suspension was then held at 49 C in a water bath, to which was added 1 part 2X MEM Salt Solution, and 0.2 parts fetal calf serum which had been previously heat inactivated and absorbed with sheep erythrocytes. This agar medium suspension was then aliquoted into 0.6 ml quantities and held at 49 C. A 2% suspension of sheep erythrocytes was prepared and equilibrated at 37 C, as well as the tubes holding the IRNA treated lymphocytes. Three tenths of the erythrocyte suspension was added to the lymphocytes (the total 8 million cells were suspended in 0.1 ml) and this mixture of red blood cells and lymphocytes was added to the agar-medium suspension. The suspension was quickly dropped from a height of 60 cm onto sterile petri dishes so that each plate held 7 agar drops. The lips of the dishes were equipped with moistened Whatman filter paper discs (#9) and the plates were incubated for 3-9 hours at 37 C with 5% CO₂. At this time two drops of a 1:10 dilution of guinea pig complement (absorbed three times with sheep erythrocytes in the cold) was added to each agar
drop. The plagues which developed were the result of IgM antibody. To detect IgG antibody forming lymphocytes, a 1:30 dilution of rabbit anti-IgG serum (previously absorbed with sheep erythrocytes) was made in the 1:10 dilution of complement and added to the agar drops (2 drops each). Calculation of plaque forming cells (PFC's) was made by determining the number of lymphocytes per agar drop and the average number of plaques per drop. The ratio of PFC's per lymphocytes was determined and expressed as the number of PFC's per $10^6$ or $10^7$ lymphocytes. This number reflects the IgM producing cells (effected by complement alone). Calculation of PFC's for IgG antibody was made by subtracting PFC's effected by complement alone from the number of PFC's effected by complement with the rabbit anti-mouse IgG added.

**Migration Inhibition Assay:** This assay was previously described by Scheetz (1972), and is an indirect measurement of the production of MIF by lymphocytes treated with IRNA. Supernatants from IRNA treated lymphocytes were collected as previously described. Special chambers designed in this laboratory were used, housing 8 capillary tubes in supporting glass slides. The capillary tubes were
filled with normal peritoneal exudate cells, taken from the peritoneum cavity of a normal guinea pig injected with thioglycollate broth 48-72 hours previously. The peritoneal exudate cells were suspended to 3-4 x 10^7 cells per ml before filling the capillary tubes, which were then centrifuged at 2000 rpm's for 10 minutes. If any erythrocytes were detected the cell suspension was treated with 0.14 M NH₄Cl made up in distilled water. The capillary tubes, after centrifugation, held a cell pack of approximately 3 mm. The tubes were etched with a diamond point pencil at the interface of the cells and the medium and broken, exposing the cell pack. Quickly the tubes were secured into the chamber with wax. The supernatants were introduced into the chambers with a 5 ml syringe and the entire chamber was sealed with wax. The chambers were incubated at 37 C for 24 hours. At this time the migration of the peritoneal exudate cells was projected or photographed and traced on paper. The area of migration was then determined by planimetry. Percent migration inhibition was determined by the following formula:

\[
\% \text{MI} = 100 - \frac{\text{Area of Test Supernatants}}{\text{Area of Control Supernatants}} \times 100
\]
Control supernatants were either supernatants taken from normal lymphocytes without IRNA treatment and incubating with and without antigen, or normal lymphocytes treated with normal RNA (from normal lymphocytes) and incubated with or without antigen.

Complement Dependent Cytolysis: This assay was especially designed to detect the formation of antibody by lymphocytes incubating without antigen stimulation, whether the lymphocytes were sensitized in vitro with IRNA or in the normal in vivo situation. Cytotoxicity plates (Falcon) which had wells housing 0.4 ml each were used. Into each well was distributed 100 ul of a $3 \times 10^6$ sheep erythrocyte suspension so that each well received $3 \times 10^5$ red blood cells. Seventy five ul of the test and control supernatants (the same as those used in the MIF assay) were added to the wells and the plate were incubated at 37 C for 3 hours. Fifty ul of a 1:10 dilution of complement (absorbed with sheep erythrocytes) was delivered into the wells. To detect IgG production, the rabbit anti-mouse IgG serum was diluted 1:30 in the complement and added to the wells. The plates were reincubated for a total of 24 hours. The number of red blood cells in each of the wells was counted using a
hemocytometer and the percent lysis was calculated as follows:

\[
\% \text{ Lysis} = \frac{\text{Test Number of Erythrocytes}}{\text{Control Number of Erythrocytes}} \times 100
\]

Controls included supernatants from normal lymphocytes, not treated with IRNA, incubated with or without antigen.

**Lymphoblast Transformation Assay:** IRNA treated lymphocytes were used directly in measurements of the ability of these cells to undergo blast transformation. Falcon cytotoxicity plates, with wells housing 0.4 ml each, were used to incubate the lymphocytes. Each well received \(3 \times 10^5\) lymphocytes suspended in 100 ul volumes. The lymphocytes were either not exposed to any antigen, or they were incubated with concentrations of sheep erythrocytes ranging from \(3 \times 10^5\) - \(3 \times 10^8\) cells per well. Controls included normal lymphocytes, incubated with or without antigen, and a row of erythrocytes alone to measure uptake of thymidine by the red blood cells. The plates were incubated for 3-5 days at 37 C and 5% CO\(_2\). Twenty-four hours prior to the termination of the incubation period, 2 mC of thymidine (specific activity 6.7 C/mM - New England nuclear) was
added to each well. At the end of the incubation period the cells were collected and washed on fiberglass filter paper utilizing the MASH unit. The filter discs were dried and placed in scintillation cocktail fluid in 1 dram sized vials and stored at 4 C. The emittance of radiation, indicative of incorporation of the thymidine by cells undergoing blast transformation, was measured on a Packard TriCarb Liquid Scintillation Spectrophotometer. The stimulation index was determined by the ratio of the incorporation of control cells to that of the test lymphocytes.

**Microcytotoxicity Assay:** This assay measures the ability of lymphocytes to directly attack and kill the 4198V (in this case) antigen target cell. Initially, the target cell is seeded onto Falcon microcytotoxicity plates (#3034) with wells holding 10 ul. Each well is seeded so that there are between 75 and 100 target cells. Incubation at 37 C is necessary so that the tumor cells can attach to the bottom of the well. The plates are then washed several times at the end of a 24 hour incubation period. This is best accomplished by sharply "snapping" the plate back and forward. Washed, lymphocytes are then added to each well so that the ratio of the lymphocytes to the target cell is at
least 100:1. The plates are further incubated for 48 hours. During this time, tumor cells, killed by the action of the lymphocytes, round up and detach from the plates. The plates were again washed, and the cells fixed with a mixture of 50% acetone and 50% alcohol. After thirty minutes, the remaining tumor cells were stained with methylene blue and counted by direct microscopic observation.

Ribosomal RNA Extraction: The following extraction procedure is one derived from that described by Youmans and Youmans (1965) and from that developed in this laboratory by Troendly (1967). The procedure produced an RNA segment (70S) coupled with protein.

Mycobacterium bovis (BCG, Tice stain): was grown in pellicle form as previously described. In pellicle form, the mass of organisms obtained was maximal, producing the greatest amounts of ribosomal RNA. Each extraction required 10 flasks containing the pellicle growth. After drying the organisms under negative pressure, the total weight of the organisms used in each extraction was approximately 5 grams. The dried organisms were washed with phosphate buffer saline (0.01 M, pH of 7.0, containing 0.45 g NaH₂PO₄, 2.4 g Na₂HPO₄
H₂O, and 8.5 g NaCl).

Three volumes of a sucrose medium (0.44 M sucrose, 0.25% SDS, 3 X 10⁻² M MgCl₂, 2 X 10⁻⁴ phosphate buffer, at a pH of 7.0) were added to Waring blender. The SDS precipitates at 4 C so the medium is added to the blender before adding the BCG organisms. The washed, and dried BCG was then added and spun in the blender at a high speed in order to break up aggregations of cells. This step was absolutely necessary to maximize the yield obtained. The evenly suspended bacteria were then transferred to a pre-cooled nitrogen bomb and the cells were broken apart with 900 lbs nitrogen gas pressure. This was accomplished by equilibrating the cells at the high pressure for 30 minutes, with constant stirring, and then subjecting the cells to normal air pressure, whereby the cells explode. The fraction was immediately collected in a slush of the sucrose medium and held in a salt and ice bath. The suspension was centrifuged at 26,000 X g for 15 minutes, and the supernatant collected and centrifuged again for 10 minutes at 46,99 X g. The top 80% of this supernatant was collected and centrifuged at 144,000 X g for 3 hours. The precipitate was considered to be the ribosomal fraction. This pellet was suspended in 0.01 M phosphate buffer, with
10^{-4} MgCl_2 to preserve the integrity of the ribosomes. The pellet was suspended at a concentration of 50 mg (wet weight) per ml and transferred to a flask. To this suspension were added equal volumes of a 0.5% SDS suspension so that the final concentration of SDS was .25%. The ribosomes were transferred into tall test tubes and left overnight at 4°C. The next day the suspension was centrifuged at 36,000 Xg for 20 minutes to get rid of the precipitated SDS and the supernatant was centrifuged for 3 hours at 144,000 X g. The final precipitate was suspended in the phosphate buffer with 10^{-4} MgCl_2 and quantitated spectrophotometrically.

In vivo sensitization with IRNA: Attempts to sensitize animals in vivo have led to minimal success. In this investigation an attempt was made to transfer sensitivity for sheep erythrocytes to normal C3H mice by either direct injections of IRNA or by immunization with IRNA treated lymphocytes. Normal C3H lymphoid cells were collected and treated with IRNA as previously described. Each recipient mouse received 8 million lymphocytes on day 1 and another 8 million treated cells on day 9. A second group of mice received normal lymphocytes, not treated with IRNA, and a third set received direct injections of IRNA (the same
amount used to treat the lymphocytes). At this time the mice were sacrificed and their splenic lymphocytes tested for the production of antibody using the Jerne plaque assay. A separate experiment was designed whereby animals received the same immunization protocol as described, including all three sets of mice, but the animals were challenged with 1 ml of a 5% suspension of erythrocytes 18 hours before their spleen cells were to be removed and tested for antibody production in the Jerne plaque assay.
Results

Conditions by which normal lymphocytes have been sensitized in vitro by immune RNA vary according to the literature, and with them the experimental results reported by the individual investigators. Therefore, there is no apparent "law" governing the transfer of immunity by IRNA and the characterization of the mechanisms involved becomes impossible. Thus this investigation has been aimed at discerning conditions and basic mechanisms by which transfer of antigenic sensitivity by IRNA is accomplished, which in turn may be helpful in discerning the normal regulation and advancement of the immune state in vivo.

This report investigates handling of immune RNA during sensitization and conditions under which that transfer of sensitivity might be maximized. Once these conditions had been standardized, an attempt was made to arrive at characterizations of the transfer of sensitivity and to investigate the control of the transferred response.
Conditions for Transfer of Sensitivity in Vitro

When immune RNA is extracted from spleens of animals there is always the danger of degradation by enzymes. For this reason, after the IRNA had been aliquoted in its final state, lyophilized, and frozen, it was carefully analyzed for degradation using sucrose density gradients. A standard graph obtained from this procedure is seen in Figure 1. There are three peaks which have been divided according to their sedimentation properties. The 0–8S peak represents small fragments of RNA, including those that build up as a result of degradation. If the height of this peak is less than half as high as the peak obtained for the 8–18S size, then the IRNA is assumed to be intact and has the best chance to transfer biological activity. Any RNA which does not meet these standards is not used. In Figure 2 the results obtained from analysis of IRNA on polyacrylamide gel electrophoresis is seen. The characteristic three peak profile is seen with the 0–8S peak the smallest. This technique can be used to separate the IRNA into more than three fractions depending on the concentration of agar used in the procedure of making the gels. This analysis may prove valuable with the need to demonstrate and identify different
Figure 1: Sucrose density gradient analysis of immune RNA.
Figure 2: Polyacrylamide Gel Electrophoresis analysis of immune RNA.
ABSORBANCE (570)
molecular species of IRNA which transfer different biological activity.

Once the IRNA had been successfully obtained, including biochemical analysis for DNA and protein contamination of not more than 5%, attention was turned to the actual procedure of the transfer of antigen sensitivity. The first question to be analyzed was how many lymphocytes could be maximally sensitized with any given amount of RNA. The procedure in this laboratory has usually been to treat lymphocytes on the basis of a quantity of IRNA per 4 million lymphocytes as described by Scheetz (1972). Since a concentration of 100 ugs BCG IRNA was already known to effect some production of MIF by normal lymphocytes with exposure to PPD, this dosage was used to treat varying concentrations of lymphocytes. As shown in Figure 3, a maximum transfer of immunity was established if the number of cells used was, as initially described by Scheetz, 4 million. Treatment of varying numbers of lymphocytes with other dosages of IRNA was not done.

In addition to the treatment of 4 million cells with IRNA, 50 ugs of DEAE-Dextran was used to enhance the uptake of the nucleic acid. This procedure was initially described in reports indicating that this substance affected the
Figure 3: Production of migration inhibition factor by varying concentrations of normal guinea pig splenic lymphocytes treated with 100 ug of guinea pig tuberculin IRNA.
ability of lymphocytes to take up viral RNA (Duc-Nguyen, 1968). Using the same system and conditions as that used to establish optimum cell number, the concentration of DEAE-Dextran was varied with constant number of lymphocytes (4 million) and a constant amount of IRNA (100 ugs). Figure 4 illustrates that 50 ugs of DEAE-Dextran effected optimal conditions for transfer of antigen sensitivity. One important experimental observation was that RNA was precipitated by DEAE-Dextran (prepared in acetate buffer at a concentration of 1 mg per ml) if the two were simply mixed together in equal proportions. With this knowledge, plus the belief that DEAE-Dextran is able to bind to the membranes of cells (Doch, Bishop, 1968), it might be assumed that there exists a definite proportion between lymphocytes, DEAE-Dextran, and RNA which effects maximal transfer of sensitivity to antigen.

If lymphocytes were treated with IRNA and the supernatant collected and saved after the 30 minute incubation, the question was posed whether there would be RNA remaining in that supernatant which would be able to effect transfer of sensitivity to another 4 million lymphocytes. When this experiment was performed it was found that the first group
Figure 4: The effect on the production of MIF when varying the amount of DEAE-Dextran used to treat groups of 4 million normal guinea pig splenic lymphocytes sensitized with 100 μg of guinea pig tuberculin IRNA.
of lymphocytes demonstrated antigen sensitivity, however, the second set of cell receiving the supernatant from the first set was not antigen sensitive (Figure 5). When sucrose density profiles were used to analyze the supernatant it was found that the IRNA was completely in the degraded form (Figure 6A). IRNA was also found to be degraded if incubated in RPMI medium even if there were no lymphocytes present (Figure 6B). Apparently, the procedure with which we were transferring immunity was not adequate to secure the integrity of the IRNA during the 30 minute span.

The supporting medium (RPMI-1640) was made with the chemical Diphenyl-aminoethyl, a compound which has been reported to bind nuclease (Wiener, 1972). Figure 6C shows that IRNA incubated in this medium, without lymphocytes, was not degraded. Unfortunately, at the concentration of DEP used the lymphocyte viability was affected to such a degree that use of the chemical was not feasible. However, use of DEP in the extraction procedure and sensitization offers a promising technique and one which ought to be more fully investigated.

Work in this laboratory has shown that the viability and membrane integrity of lymphocytes is affected by changes
Figure 5: Production of MIF by A) normal guinea pig splenic lymphocytes, B) normal guinea pig splenic lymphocytes treated with 100 ug of guinea pig tuberculin IRNA, and C) normal guinea pig splenic lymphocytes treated with supernatants from group B cells.
Figure 6: Sucrose density gradient analysis of A) IRNA incubated in RPMI 1640 with lymphocytes, B) IRNA incubated in RPMI 1640 without lymphocytes, and C) IRNA incubated with RPMI 1640 with DEP and no lymphocytes.
in pH of the suspending medium. Since treatment with RNA of normal lymphocytes generally leaves the medium acid, the effect of pH on the transfer of humoral and cellular immunity by IRNA was investigated. Table I shows the effect of pH on the capacity of sheep erythrocyte RNA to effect transfer of normal lymphocytes into plaque forming cells (PFC's). As indicated, both IgM and IgG PFC's were maximal among IRNA treated lymphocytes when the pH of the medium was 7.4. If the pH varied from this point, fewer PFC's were detected. Prior to these studies the technique used resulted in a medium for plaque estimation which was more acid, and transfer of immunity as measured by the Jerne Plaque assay had been met with varying success. Whether this is a true indication of the effect of pH on the transfer of humoral immunity or just a reflection of the technique is not known. However, the capacity of lymphocytes from an antigen-sensitized animal to produce plaque forming cells was not affected by variations in pH. Further, the transfer of cellular immunity by IRNA, which has always been easier to demonstrate than PFC's, has not been shown to be affected by changes in pH of the medium during incubation (Table II).
Table I

Effect of pH on the Ability of IRNA\(^1\) to Transfer Sensitivity To Normal Lymphocytes\(^2\) As Measured by Antibody Production

<table>
<thead>
<tr>
<th>pH</th>
<th>PFC's/10^6</th>
<th>IgM</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
<td>1.0</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>7.4</td>
<td>50.0</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>7.9</td>
<td>2.5</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>8.3</td>
<td>1.2</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)IRNA extracted from spleens of CD-1 mice immunized with a single injection of sheep erythrocytes (3 x 10^9 cells).

\(^2\)Normal splenic guinea pig spleens treated with 25 ugs of IRNA per 4 million cells.
Table II

Effect of pH on the Ability of IRNA$^1$ to Transfer Sensitivity To Normal Lymphocytes$^2$ as Measured by MIF Production

<table>
<thead>
<tr>
<th>pH</th>
<th>% Migration Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>32%</td>
</tr>
<tr>
<td>6.0</td>
<td>35%</td>
</tr>
<tr>
<td>7.1</td>
<td>36%</td>
</tr>
<tr>
<td>8.2</td>
<td>35%</td>
</tr>
</tbody>
</table>

$^1$Sheep erythrocyte IRNA extracted from spleens of CD-1 mice.

$^2$Normal splenic guinea pig spleens treated with 25 ugs of IRNA per 4 million lymphocytes.
Characterization of the Transfer of Antigen Sensitivity

The sheep erythrocyte antigen system (SRBC) was used in this investigation as the primary tool with which to monitor capabilities of IRNA to transfer humoral and cellular sensitivity to normal lymphocytes. With this system, a comparison was also made between the characteristics of IRNA treated lymphocytes and those lymphocytes sensitized in vivo by the injection of antigen in the usual fashion. The tuberculin and tumor antigen systems serve to substantiate those patterns found with the sheep red blood cell system, perhaps indicating a commoness of these patterns produced by IRNA of different specificities.

The sheep erythrocyte system: Figure 7 illustrates the dose response pattern of treating groups of 4 million normal guinea pig splenic lymphocytes with varying amounts of IRNA extracted from guinea pigs immunized with a single dose of red blood cells and sacrificed 14 days after that immunization (primary immune response). The treated cells were cultured and measurements were made for the production of MIF. If no antigen was introduced into the culture, MIF production was equal regardless of the amount of IRNA treatment. When these same cells were exposed to $3 \times 10^8$ sheep erythrocytes,
Figure 7: Production of MIF by normal guinea pig splenic lymphocytes treated with guinea pig 14 day primary sheep erythrocyte IRNA; cultured with (●; 3 X 10^8 erythrocytes) and without (○) antigen exposure.
MIF production was measured only by those cells treated with 25 ugs of IRNA. All other cells treated with larger amounts of IRNA were suppressed from producing this immune factor. As shown in Figure 8, similar patterns could be found if the IRNA used to treat the lymphocytes was extracted from spleens of guinea pigs immunized in the same fashion as previously described but sacrificed 21 days after immunization. In this instance larger doses of IRNA transferred less MIF production than IRNA from 14 day IRNA but the effects of antigen were similar - mainly suppressive, except with cells treated with 25 ugs RNA, and even in this case, MIF production was not increased. In each of the two situations described, the IRNA was extracted from animals undergoing a primary immune response. Figure 9 shows that IRNA, from lymphocytes of an animal sacrificed 7 days past immunization, did not confer an MIF response to normal lymphocytes. A comparison between these results obtained with the IRNA and the production of MIF by the IRNA donor lymphocytes themself show that guinea pig lymphocytes, 7 days after immunization, did not produce MIF when exposed to antigen, while lymphocytes 14 and 21 days past immunization did. The responses of these IRNA donor lymphocytes not exposed to antigen was not done.
Figure 8: Production of MIF by normal guinea pig splenic lymphocytes treated with guinea pig 21 day primary sheep erythrocyte RNA; cultured with (●; 3 X 10⁶ erythrocytes) and without (○) antigen exposure.
Figure 9: Production of MIF by normal guinea pig splenic lymphocytes treated with guinea pig 7 day primary sheep erythrocyte IRMA; cultured with (●; 3 × 10⁶ erythrocytes) and without (○) antigen exposure
Fourteen day primary immune IRNA extracted from CD-1 mice immunized with sheep erythrocytes conferred sensitivity to normal C3H mouse lymphocytes as measured by IgM lysis of sheep erythrocytes. As shown (Figure 10) the same pattern of expression and suppression developed as that seen for MIF production in the guinea pig system. Those IRNA treated cells not exposed to antigen exhibited equal quantities of IgM production at all levels of IRNA treatment, while, upon antigen exposure, the principal reaction was suppression. As seen in the MIF assay, there was an optimal proportion of antigen, IRNA dose, and lymphocyte number, which would demonstrate expression of immune factors (IgM). Here actual antigen stimulation of antibody was seen by cells treated with 25-50 ugs IRNA. Again, those cells not exposed to antigen produced equal amounts of antibody regardless of the amount IRNA used to treat the cells. This antigenic suppression of IgM antibody was not seen with IgG. Measurement of IgG lysis of red blood cells was possible by the IRNA treated cells with or without antigen exposure (Figure 11). The amount of IgG produced was independent of the amount of IRNA used to treat the cells, and no suppression was noted by antigen. Due to the manner of
Figure 10: Production of IgM antibody by normal C3H mouse splenic lymphocytes treated with 14 day CD-1 mouse primary sheep erythrocyte IRNA; cultured with (●; $3 \times 10^6$ erythrocytes) and without (○) antigen exposure.
Figure 11: Production of IgG antibody by normal C3H mouse splenic lymphocytes treated with 14 day CD-1 mouse primary sheep erythrocyte RNA; cultured with (●; $3 \times 10^6$ erythrocytes) and without (○) antigen exposure.
the calculations used to obtain values of IgG lysis, in experiments where large amounts of IgM was measured, it appears as if IgG production has been decreased or inhibited, however, in our judgment, this is not the case.

Preliminary investigation has shown that the antigen dependent suppression seen with MIF or IgM production effected by the 14 day primary IRNA is specific for the antigen used in sensitizing IRNA donor animals. Lymphocytes treated with 200 ugs of sheep erythrocyte IRNA and exposed to either 50 ugs PPD or $3 \times 10^8$ human group 0 red blood cells, demonstrated 40-50% migration inhibition, and 50-70% lysis of sheep erythrocytes, as measured by IgM lysis.

In order to relate these phenomena to normally sensitized cells, animals with an ongoing primary response to sheep erythrocytes (7 days past immunization) were sacrificed and their spleen cells cultured with and without antigen exposure. As shown in Table III these cells caused 90% lysis if they were cultured without antigen. Upon exposure to $3 \times 10^8$ erythrocytes, these cells did not produce IgM antibody, similar to the antigen suppression of IRNA treated cells. Again, these same sensitized cells treated with 50 or 400 ugs of primary immune RNA produced IgM antibody
Table III

Effect of IRNA and Antigen on the Production of IgM Antibody by Splenic Lymphocytes From C3H Mice Immunized
With Sheep Erythrocytes

<table>
<thead>
<tr>
<th>Percent Lysis</th>
<th>Cultured Without Antigen</th>
<th>Cultured With Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitized Lymphocyte</td>
<td>90%</td>
<td>-6%</td>
</tr>
<tr>
<td>Sensitized Lymphocyte Treated with 50 ugs Primary IRNA</td>
<td>90%</td>
<td>1%</td>
</tr>
<tr>
<td>Sensitized Lymphocyte Treated with 400 ugs Primary IRNA</td>
<td>93%</td>
<td>2%</td>
</tr>
<tr>
<td>Sensitized Lymphocyte Treated with 50 ugs Secondary IRNA</td>
<td>85%</td>
<td>5%</td>
</tr>
<tr>
<td>Sensitized Lymphocyte Treated with 400 ugs Secondary IRNA</td>
<td>15%</td>
<td>8%</td>
</tr>
</tbody>
</table>

1Splenic lymphocytes collected 7 days following an intraperitoneal injection of $3 \times 10^9$ sheep erythrocytes.

2$3 \times 10^8$ sheep erythrocytes

3IRNA extracted from spleens of CD-1 mice immunized 14 days with sheep erythrocytes.

4IRNA extracted from spleens of CD-1 mice receiving three injections, each one week apart, of sheep erythrocytes.
actively, which however was completely suppressed by antigen. Similarly, primarily sensitized lymphocytes treated with 50 ugs of secondary IRNA (IRNA extracted from spleens of mice undergoing a secondary immune response from two or more injections of antigen), showed 85% lysis of sheep red blood cells without antigen exposure, but were completely suppressed by antigen exposure. If the cells were treated with 400 ugs of the secondary IRNA, this larger dose was suppressive with or without antigen. A suppressive mechanism against sensitized lymphocytes during a primary response was also seen with normal RNA (RNA extracted from the spleens of normal guinea pigs). As shown in Table IV, IgM production by these lymphocytes was suppressed by treatment with normal RNA if those cells were taken from the immunized animal at least 7 days after immunization. There was no effect on IgG production at any time.

Thus, IRNA from lymphocytes obtained from mice and guinea pigs during the primary response to sheep erythrocytes readily transferred to normal lymphocytes the capacity to produce IgM antibody and MIF in doses from 25 to 1000 ugs, no quantitative difference being noted which related to size of dosage. However, such IRNA also transferred an antigen
Table IV

The Effect of Normal RNA on Splenic Plaque Forming Cells
From Animals Sensitized In Vivo With
Sheep Erythrocytes

<table>
<thead>
<tr>
<th>Days After Immunization&lt;sup&gt;1&lt;/sup&gt;</th>
<th>PFC's/10&lt;sup&gt;6&lt;/sup&gt; Untreated Cells</th>
<th>PFC's/10&lt;sup&gt;6&lt;/sup&gt; Normal RNA Treated Cells&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>4</td>
<td>900</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>95</td>
<td>420</td>
</tr>
<tr>
<td>10</td>
<td>80</td>
<td>500</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>700</td>
</tr>
<tr>
<td>14</td>
<td>ND&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1000</td>
</tr>
</tbody>
</table>

<sup>1</sup>CD-1 mice were immunized with a single intraperitoneal injection of 3 X 10<sup>9</sup> sheep erythrocytes.

<sup>2</sup>Lymphocytes were treated with 50 and 500 ugs of guinea pig normal RNA per 4 million cells.

<sup>3</sup>Not Done.
dependent suppressor RNA so that production of these factors was completely or partially suppressed in the presence of antigen. This antigen dependent suppression was specific and was shown to completely inhibit IgM production by lymphocytes directly from an animal during a primary response to sheep erythrocytes, indicating that the antigen dependent suppressor RNA equally suppresses immunized as well as IRNA treated normal lymphocytes. It is not suppressive for IgG antibody. It is apparently present in normal RNA since such RNA inhibited the primary response of IgM antibody by sensitized lymphocytes.

Further evidence indicating that IRNA treated cells, with antigen exposure, will be suppressed from production of immune factors except in strictly defined proportions between IRNA and antigen, is found when these IRNA treated cells are employed directly in the Jerne Plaque assay to measure PFC's. In Figure 12 it is seen that the major response to antigen exposure in this assay is suppression while the demonstration of PFC's was determined by a critical proportion between 25 and 50 ugs IRNA and the concentration of antigen used (2%). Such suppression of response has not been seen in the Jerne Plaque assay measuring antibody
Figure 12: Detection of plaque forming cells by normal CD-1 splenic lymphocytes treated with CD-1 14 day primary sheep erythrocyte IRNA.
production by normally sensitized cells from primary immune animals. However, since normally sensitized cells from primary immune animals can be suppressed with antigen exposure when in culture, it might be expected that the proportion of antigen to lymphocytes designed in this assay was originally described by Jerne in order to achieve maximum demonstration of response. In preliminary experimentation with normally sensitized cells it was found that by increasing the amount of antigen used in the Jerne assay 2 to 3 fold, that the demonstration of PFC's could be decreased from one third to one half the number demonstrated in the original experimental design. It is clear that lymphocytes sensitized in vivo as a result of antigen injection are not as acutely affected by alterations in antigen concentrations as are IRNA treated lymphocytes.

Additional experiments were conducted to test the effect of varying the amount of antigen (number of sheep erythrocytes) on the responses of either IRNA or normally sensitized cells in culture. In Figure 13 the effect of varying amounts of antigen on normal guinea pig lymphocytes treated with either 25 or 200 ugs of IRNA, measured by production of MIF, can be seen. The cells treated with 200
Figure 13: Production of MIF by normal splenic guinea pig lymphocytes treated with 25 (•) and 200 (○) ug of CD-1 14 day primary sheep erythrocyte IRNA, and cultured with varying concentrations of sheep erythrocytes.
ugs of IRNA represent the population of cells inhibited from production of MIF by exposure to $3 \times 10^8$ sheep erythrocytes. As seen, if the amount of antigen was reduced to $3 \times 10^6$ red blood cells the lymphocytes produced MIF. Further reduction of antigen however suppressed the cells again. The population of lymphocytes treated with 25 ugs of IRNA was not affected by antigen treatment at any level, however, those cells treated with $3 \times 10^4$ erythrocytes apparently showed a maximal response. It might be argued that lymphocytes treated with 25 ugs were suppressed only by very large doses of antigen, while those treated with 200 ugs were suppressed by antigen amounts above and below an optimal.

When lymphocytes extracted from spleens of normally sensitized mice undergoing a primary immune response were cultured with varying amounts of antigen (Figure 14) the same effects on IgM production were observed as with the lymphocytes treated with 200 ugs IRNA as seen by MIF production. Cells exposed to low amounts of antigen, $3 \times 10^4$ to $3 \times 10^6$ erythrocytes, demonstrated IgM antibody production as measured by lysis, while $3 \times 10^7$ and $3 \times 10^8$ erythrocyte exposures were mainly suppressive. Viability of the lymphocytes after 48 hours culturing was 60–70%.
Figure 14: Production of IgM (○) and IgG (●) antibody by splenic lymphocytes from CD-1 mice immunized at 7 days with sheep erythrocyte, cultured with varying amounts of sheep red blood cell antigen.
Measurement of IgG production indicated that levels of antigen exposure which were suppressive for IgM production, did not affect IgG production. Levels of antigen exposure whereby IgM was produced masked the measurement of the production of IgG and thus made quantitation impossible. However, it can be concluded that at least at high antigen exposure, the lymphocytes producing IgG escaped suppressive control by antigen. If the experimental design were altered, demonstration of IgG production at low levels of antigen exposure might be possible. These lymphocytes cultured without antigen demonstrated 96% lysis as measured for IgM production. Again, the experimental procedure did not allow quantitation of IgG. The antigen dependent suppression of IgM production was specific for the sheep erythrocyte antigen since sheep red blood cell sensitized lymphocytes incubated with human erythrocytes (3 X 10^8 cells) demonstrated 97% lysis.

Preliminary investigation using IRNA from the spleens of animals undergoing a secondary immune response demonstrated MIF dose response curves different from those obtained with IRNA from primary immune sources (Figure 15). As shown, if the cells were treated with varying amounts of IRNA and
Figure 15: Production of MIF by normal guinea pig splenic lymphocytes treated with CD-1 mouse secondary sheep erythrocyte IRNA; cultured with (●; $3 \times 10^8$ erythrocytes) and without (○) antigen exposure.
exposed to antigen, the amount of suppression was negligible. Maximal MIF response occurred if the amount of IRNA used was 20 ugs. As previously described in the primary immune system, the amount of MIF production by cells not exposed to antigen was the same regardless of the amount of IRNA used to treat the cells.

Figure 16 shows the result of exposing lymphocytes, extracted from spleens of animals undergoing a secondary immune response to sheep erythrocytes, to varying amounts of antigen. Neither production of IgM or IgG was affected by the presence of antigen (as seen in the primary immune system, the production of IgM was affected by antigen, secondary IRNA, and normal RNA).

Measurements for lymphoblastogenesis by IRNA treated normal lymphocytes has resulted in failure (Deckers, 1976). Since the assays described previously in this report have demonstrated a requirement for an optimal proportion between antigen and IRNA treated cells, it was hoped that this was the reason for the previous failures. An amount of IRNA was chosen, 25 ugs, which had previously demonstrated minimal suppression with antigen exposure. Either normal guinea pig splenic lymphocytes (Table V) or normal
Figure 16: Production of IgM (○) and IgG (●) antibody by splenic lymphocytes from CD-1 mice undergoing a secondary immune response to sheep erythrocytes.
Table V

Measurement of Lymphoblast Transformation Factor In Guinea Pig Splenic Lymphocytes Treated With IRNA\(^1\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antigen Exposure</th>
<th>SI(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 ugs</td>
<td>4 X 10^5</td>
<td>1.25</td>
</tr>
<tr>
<td>25 ugs</td>
<td>4 X 10^6</td>
<td>1.30</td>
</tr>
<tr>
<td>25 ugs</td>
<td>4 X 10^7</td>
<td>1.46</td>
</tr>
<tr>
<td>25 ugs</td>
<td>None</td>
<td>.82</td>
</tr>
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</table>

\(^1\)IRNA extracted from spleens of CD-1 mice immunized with a single injection of sheep erythrocytes.

\(^2\)Stimulation Index: Measured 5 days after IRNA and antigen treatment and calculated on the basis of incorporation by lymphocytes without IRNA treatment and with antigen exposure.
C3H splenic lymphocytes (Table VI) were treated with this amount of IRNA and cultured with and without antigen exposure of varying concentrations. As shown, there was a minimal response by either of these cell populations after either three (mouse cells) or five (guinea pig) days incubation. The ratio of incorporation of thymidine for test lymphocytes to that for control never reached an index of 2 which is generally agreed upon as the minimum positive index. Cells not exposed to antigen likewise did not show stimulation index levels above 2 which would indicate that the antigen did not, at least, suppress the responses of the IRNA treated cells (since the index ratios of non-antigen stimulated and antigen stimulated cells were very similar).

The in vitro work described has illustrated that IRNA transferred specific activity to normal lymphocytes and that the response measured is dependent on the presence and concentration of antigen. These data would be most useful if they would aid in the transfer of specific immunity by IRNA in the in vivo condition, this being the main objective of any immunotherapeutic program using IRNA. To this end therefore, preliminary work was performed in the sheep erythrocyte
Table VI

Measurement of Lymphoblast Transformation Factor in C3H Splenic Lymphocytes Treated with IRNA\(^1\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antigen Exposure</th>
<th>St(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 ugs</td>
<td>3 X 10(^2)</td>
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<td>25 ugs</td>
<td>3 X 10(^4)</td>
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<td>25 ugs</td>
<td>3 X 10(^6)</td>
<td>1.88</td>
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<td>25 ugs</td>
<td>3 X 10(^7)</td>
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</tr>
<tr>
<td>25 ugs</td>
<td>None</td>
<td>1.40</td>
</tr>
</tbody>
</table>

\(^1\)IRNA extracted from spleens of CD-1 mice immunized with a single injection of sheep erythrocytes.

\(^2\)Stimulation Index: Measured 3 days post IRNA treatment and antigen exposure and calculated on the basis of incorporation by lymphocytes without IRNA treatment and with antigen exposure.
system in an attempt to convert normal C3H mice into sensitized animals by virtue of IRNA sensitized cells or by direct injection of IRNA. C3H mice received either $8 \times 10^6$ sheep erythrocyte IRNA treated lymphocytes (50 ugs per 4 million cells) or 100 ugs of IRNA directly injected, on days 0 and 7. Two days after the final injection of RNA, the animals were sacrificed and their splenic lymphocytes extracted. Measurements of PFC's were made using the Jerne Plaque technique. Control mice were those not receiving IRNA or IRNA treated lymphocytes. As Table VII shows, the responses of these mice immunized with IRNA were only slightly higher than measurements made from normal spleens of untreated animals. Interestingly, the Jerne Plaque assay, used to measure the responses, was shown to be an assay by which detection of sensitivity of IRNA treated lymphocytes was very difficult. The responses of the mice receiving the IRNA immunizations were not tested in any antigen free system. Mice receiving the same IRNA immunization were immunized, 65 days following the second treatment with IRNA, with a single injection of sheep erythrocytes and their spleens collected 18 hours after this immunization. This experiment was performed in order to assess any change
Table VII

In Vivo Sensitization of C3H Mice With IRNA\(^1\) Treated Lymphocytes

<table>
<thead>
<tr>
<th>Immunization(^2)</th>
<th>PFC's/10(^6)</th>
<th>IgM</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H Lymphocytes (untreated)</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C3H Lymphocytes Treated with IRNA(^3)</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IRNA(^4)</td>
<td>19</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^1\)IRNA extracted from spleens of CD-1 mice immunized 14 days previously with a single injection of 3 X 10\(^9\) erythrocytes.

\(^2\)C3H mice received IRNA and lymphocytes (8 X 10\(^6\)) on day 0 and on day 9 after which their spleens were removed and the cells tested for PFC's.

\(^3\)50 ugs of IRNA per 4 million cells (200 ugs total).

\(^4\)200 ugs I.P.
in the immune system in the IRNA treated mice. Although the data are minimal, Table VIII indicates that the mice receiving the IRNA treated lymphocytes were the only ones to respond to the antigen after the short time period, perhaps suggesting sensitization by the earlier treatment. It would be interesting, in light of the knowledge of suppression of response with antigen, to measure suppression of response in vivo with these IRNA treated mice several days following immunization.

The BCG, 4198, and 4198V Antigen Systems: Characterization of IRNA transfer of antigen sensitivity to normal lymphocytes may best be determined utilizing a simple antigen system such as the sheep erythrocyte system described. In order to examine the facts learned with sheep red blood cell IRNA in other antigen systems, the 4198 polyoma induced fibrosarcoma, and BCG (Tice strain) were employed. These two antigens represent causative agents of two diseases and, to whose control and eventual cure, IRNA may play a role.

4198 RNA was first extracted from the lymphocytes of spleens of mice bearing tumors (TL-RNA). This TL-RNA was then used to treat normal guinea pig lymphocytes, which were
Table VIII

Production of Antibody by Splenic Lymphocytes of C3H Mice Previously Immunized with Sheep Erythrocyte IRNA\(^1\) and Challenged with Sheep Erythrocytes\(^2\).

<table>
<thead>
<tr>
<th></th>
<th>PFC's/10(^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
</tr>
<tr>
<td>Lymphocytes from Normal C3H Mice</td>
<td>0.3</td>
</tr>
<tr>
<td>Lymphocytes from C3H Mice Immunized</td>
<td></td>
</tr>
<tr>
<td>With IRNA Treated Lymphocytes(^3)</td>
<td>7.1</td>
</tr>
<tr>
<td>Lymphocytes from C3H Mice Immunized</td>
<td></td>
</tr>
<tr>
<td>With IRNA(^4)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

\(^1\)IRNA extracted from spleens of CD-1 mice immunized 14 days with a single injection of sheep erythrocytes.

\(^2\)C3H mice were immunized with IRNA 65 days previous to an intraperitoneal injection of 3 X 10\(^9\) sheep erythrocytes. Mice were sacrificed 18 hours after challenge.

\(^3\)Two injections consisting of 8 million C3H lymphocytes treated with 100 ugs of CD-1 sheep erythrocyte IRNA 9 days apart.

\(^4\)Two injections of 100 ugs CD-1 sheep erythrocyte IRNA each, 9 days apart.
then cultured with and without antigen exposure as previously described. Upon measurement of the MIF produced, the same pattern of dose response was seen as that described in the sheep erythrocyte antigen system (Figure 17). If the cells were exposed to tumor antigen, the only TL-RNA concentration which demonstrated MIF production was 100 ugs. Any dosage of TL-RNA above and below that suppressed the response. Cells not cultured with antigen demonstrated equal production of MIF at any dose of TL-RNA. The amount of MIF measured with an optimal amount of TL-RNA (100 ugs) with antigen exposure was not higher than that seen without antigen exposure, and in fact was measured somewhat lower than that seen by cells not exposed to antigen.

IRNA extracted from animals sensitized with one injection of BCG was considered to be primary BCG-RNA, as that described in the sheep erythrocyte system. When used to treat lymphocytes from guinea pigs in varying amounts (Figure 18) the dose response pattern resembled that seen in both the tumor and red blood systems. Lymphocytes not treated with antigen after IRNA treatment demonstrated equal amounts of MIF production regardless of the amount of IRNA used, while those cells receiving 50 ugs of PPD in culture demonstrated
Figure 17: Production of MIF by normal splenic guinea pig lymphocytes treated with tumor lymphocyte RNA, with (●; 1 X 10⁶ tumor cells) and without (○) antigen exposure.
Figure 18: Production of MIF by normal guinea pig lymphocytes treated with guinea pig tuberculin RNA with (●; 50 μg PPD) and without (○) antigen exposure.
suppression of response with all doses of IRNA except 50 ugs IRNA per 4 million lymphocytes. In all of the dose responses investigated in this and the other two antigen systems, the range of IRNA with antigen exposure which would result in stimulation of the cells to produce immune factors was very narrow.

By fractionating the immune RNA with Sephadex G-200 it was possible to conclude that the size of the molecular species of IRNA which was causing the antigen induced expression and suppression was in the same size range as that which induced responses without antigen, and indeed the species of IRNA may be the same. In Figure 19 the 6-12S fraction, previously described by Scheetz, et al. (1972) as the active fraction, was used to transfer tuberculin sensitivity to normal guinea pig lymphocytes. These cells were cultured with 50 ugs of PPD. MIF was produced by normal lymphocytes treated with 2 ugs of the fraction in the presence of antigen, while other amounts of Fraction III were suppressive. The entire dose pattern seen is identical to that observed with the use of whole IRNA in this and the other two antigen systems. Important to note is that 25 times less IRNA was required to transfer measured responses, and that both
Figure 19: Production of MIF by normal splenic guinea pig lymphocytes treated with Fraction III of guinea pig tuberculin IRNA, with antigen exposure (50 ug PPD).
UGS rRNA FRACTION III (6–12s)
the expressive and suppressive capabilities of IRNA were transferred by this fraction.

The BCG antigen system has been of particular interest in the immunologic study of tumor immunity since it has been shown that in the human and animal systems, immunization with BCG has led to delay (Sokal, et al., 1974) and in some cases regression (Morton, et al., 1974) of tumors. Likewise, ribosomal RNA antigens from BCG have conferred a degree of non-specific resistance against virulent *Mycobacterium tuberculosis* (Youmans and Youmans, 1965). Preliminary investigation was made on the effect of a ribosomal RNA antigen, extracted from BCG on the growth of tumor in C3H mice challenged with the 4198 fibrosarcoma. Initially it was found that mice immunized with a single injection of BCG demonstrated cytotoxic splenic lymphocytes for the 4198 tumor antigen (Table IX). Lymphocytes from specifically immunized mice to the fibrosarcoma demonstrated 50% cytotoxicity *in vitro* while those lymphocytes from mice immunized with BCG demonstrated 35% cytotoxicity. When mice were immunized with the ribosomal RNA vaccine 21 days prior to tumor challenge, and compared with mice not receiving the ribosomal RNA, it was found that there was a delay of growth
Table IX

In Vitro Cytotoxicity of 4198V Tumor Cells by Splenic Lymphocytes From C3H Mice Immune to 4198 Tumor and BCG.

<table>
<thead>
<tr>
<th>Animals</th>
<th>% Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>---</td>
</tr>
<tr>
<td>Tumor Immune¹</td>
<td>55%</td>
</tr>
<tr>
<td>BCG Immune²</td>
<td>35%</td>
</tr>
</tbody>
</table>

¹Mice immunized with three injection of 4198 tumor cells (5 X 10⁵ each) at weekly intervals and splenic cells collected at 30 days.

²Mice immunized with 1 X 10⁷ viable organisms and splenic cells collected 21 days after injection.
of tumor for 16 days in 50% of those mice tested (Table X). It is not certain how BCG or ribosomal vaccine is able to demonstrate delay and in some cases protection against tumor antigens and throughout these investigations efforts were made to show differences in the immune state of animals immunized with BCG. In very preliminary evidence shown in Table XI, it was found that when mice were immunized with BCG, followed by immunization with sheep erythrocytes, their splenic lymphocytes demonstrated a complement independent lytic action (ADCC) against the sheep erythrocytes, not seen in mice immunized with either BCG or sheep erythrocytes alone. The BCG immunization did not have any effect on the complement dependent lytic ability of lymphocytes from the mice receiving both the BCG and the sheep antigens.
Table X

Delay of Tumor Growth in C3H Mice Immunized With Ribosomal RNA Extracted From *Mycobacterium bovis* (BCG)

<table>
<thead>
<tr>
<th>Tumor Growth After Challenge&lt;sup&gt;1&lt;/sup&gt; Days</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
<th>18</th>
<th>20</th>
<th>22</th>
<th>24</th>
<th>28</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA Immunized&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>1/4</td>
<td>1/4</td>
<td>1/4</td>
<td>2/4</td>
<td>2/4</td>
<td>4/4</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>C3H mice were challenged with $5 \times 10^5$ 4198 polyoma tumor cells I.M.

<sup>2</sup>C3H mice were immunized with 100 ugs of ribosomal RNA 21 days prior to tumor challenge.
Table XI

Effect of BCG Immunization on the Immune Response to Sheep Erythrocytes in C3H Mice

<table>
<thead>
<tr>
<th>Animals</th>
<th>C' Independent Lysis</th>
<th>PFC's/10^6</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BCG Immunized^1</td>
<td>1%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SRBC Immunized^2</td>
<td>1%</td>
<td>100</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>BCG and SRBC Immunized^3</td>
<td>90%</td>
<td>110</td>
<td>494</td>
<td></td>
</tr>
</tbody>
</table>

^1C3H mice immunized I.M. with 1 X 10^7 viable organisms and the spleens removed at 14 days.

^2C3H mice immunized with 3 X 10^9 RBC's and the spleens removed at 14 days.

^3C3H mice immunized with 1 X 10^7 BCG organisms I.M. and reimmunized with sheep erythrocytes 14 days later. Spleens removed 28 days past the BCG injection.
Discussion

Immune RNA from sensitized splenic lymphocytes from mice or guinea pigs undergoing a primary response to sheep red blood cells transferred to normal lymphocytes (syngeneic or xenogeneic with respect to donor lymphocytes) the capacity to produce lytic antibody and MIF over a wide range of dosage. The amount of antibody of lymphokine produced, and seemingly the rate of production, was the same regardless of the dose of IRNA in the absence of antigen. However, the predominant response of these IRNA treated cells to antigen was the suppression of the production of both products partially or entirely. It is obvious from the data that the response to antigen is dose-dependent, since a given number of lymphocytes treated with different amounts of IRNA may vary from complete to partial suppression, to seemingly no effect on the amount produced as compared with that produced in the absence of antigen, all the way to actual stimulation of the response, with the same dosage of antigen. Thus, to obtain positive production of these agents, 4 million lymphocytes treated
with 200 ugs IRNA responded with the maximal production of MIF only to $3 \times 10^6$ sheep erythrocyte exposure, while $3 \times 10^4$, $3 \times 10^8$, and $3 \times 10^{10}$ sheep red blood cells were definitely suppressive. In the same figure, the response was maximal by the same number of lymphocytes treated with 25 ugs of the same IRNA in the presence of $3 \times 10^4$ erythrocytes, while other doses of sheep erythrocytes being mildly or markedly suppressive. An excellent example of this IRNA-antigen dosage relationship is the demonstration of plaque-forming cells (Figure 12), which was either usually negative or inconsistent until it was realized that the test itself required this dosage adjustment. As can be seen the test is essentially negative with the technique and numbers of erythrocytes used except with lymphocytes treated with 25 to 50 ug IRNA.

Studies with IRNA from lymphocytes of animals undergoing a secondary immune response, as well as experiments measuring responses of the lymphocytes themselves, indicate that the suppressive control for the production of IgM and MIF by antigen has been largely reduced, or even eliminated, and that production of IgG, as seen in the primary immune responses, was not under antigen control. Thus it is
possible that after the "shift" between the primary and secondary responses the animal would be able to produce larger amounts of immune factors, and indeed, this quantitative elevation can be measured in secondary responses which are also detectable for longer periods of time. By virtue of the fact that these demonstrations were shown, not only with IRNA sensitized lymphocytes but also by lymphocytes sensitized in vivo by antigen, it can be concluded that the antigen-dependent suppression described is not merely an experimental artifact seen with the transfer of sensitivity with IRNA. Moreover, there is some evidence to support the hypothesis that IRNA is acting as a molecular mediator to control sensitized lymphocytes in vivo by suppression, a phenomenon described in this report in vitro (Tables III and IV).

Indeed, the intent of these studies in the sheep red blood cell system had been to take advantage of IRNA as a molecular tool with which to study control suppressor mechanisms. What aspects of those immune mechanisms studied were dictated largely by results of preceding work investigating transfer of antigen sensitivity to normal guinea pig lymphocytes with either tumor lymphocyte RNA (TL-RNA, Figure
17) or BCG-IRNA (Figure 18). The production of MIF was used as a measure of positive response. The peculiar dose response patterns described in the sheep erythrocyte system was first described in these models. Unexpectantly, if these cells were cultured without antigen the amount of MIF production found was independent of the concentration of BCG-IRNA or TL-RNA used to treat the lymphocytes. Even more surprising, if antigen were added to the culture, radical changes of those patterns occurred. MIF production by TL-RNA treated lymphocytes, exposed to $1 \times 10^6$ tumor cells, was evident only by those cells treated with 50 ug of TL-RNA. Further, BCG-RNA treated cells produced MIF only with 50 ug IRNA when exposed to PPD. It was interesting that the crude Sephadex fraction of BCG-IRNA, when used to sensitize normal lymphocytes exposed to antigen in culture, produced similar dose response patterns (Figure 19), indicating that the informational RNAs directing both the expression and antigen-dependent suppression are found in the same sized molecule, and may even be the same molecule. The size (6-12S) of the active fraction III probably is an indication of the size requirement for passage into the lymphocyte. Important to note is that transfer of sensitivity with Fraction III
required 25 times less RNA material than did the whole preparation which effects transfer of sensitivity is small.

It was regarded important to improve the understanding of those mechanisms leading up to the point of the antigen-dependent suppression seen in these two systems, as well as characterization of the phenomenon itself. The chance of a successful immunotherapy program against cancer, such as IRNA offers, is dependent upon this knowledge. With these perspectives in mind, study of the well defined sheep erythrocyte responses was begun, some of the results of which have been reviewed early in this discussion. The tuberculin antigen system, more complicated than the sheep erythrocyte antigen model since the nature of protective immunity to tuberculosis is still a matter of controversy, was not chosen for these investigations.

Thus, from all of the work described, it is clear that primary immune sheep erythrocyte RNA, BCG-IRNA, and TL-RNA, all evoke similar response patterns when using each respective RNA to treat normal lymphocytes. The initial conclusion of these results was that antigen is playing a controlling suppressor function of these IRNA sensitized cells in all three systems. Studies of primary immune lymphocytes from
animals immunized with sheep erythrocytes demonstrated the antigen-dependent phenomenon as well. Only secondary immune responses, and the production of IgG in the primary immune system, seemed not to be under the influence of the antigen suppression. As will be described, these secondary responses are similar to those found in tumor immune animal models (Pennline, 1976).

These experiments have thus unveiled interesting possibilities of the relationship between antigen, its concentration and nature of presentation to the lymphocyte, and the controlling factors in the in vivo immune response. This becomes particularly important in relation to cancer as it is widely believed that the growth characteristics and the tumor load are critical factors in determining whether an efficient immune response ensues, and thus pathology never becomes evident or regression of tumor is found, or whether a perverse form of the immune state develops leading to tumor. Immunology critically needs a testable, universal, hypothesis to explain early immunological responses against antigen which lead to either this normal or pathological immune state.

Such a hypothesis, based on this report, might suggest
that in certain instances where there is a persistence of antigen, such as in tuberculosis and tumor, IgM and at least the MIF correlate for cellular immunity would be limited, affected by the antigen-dependent suppression due to antigen overload (non-optimal proportions for expression). The primary cell affected would be the IgM producing lymphocyte, the result of which could be the delay or inhibition of the shift into the secondary response. Thus there would be a prolongation of the primary immune response. The amount of immune factors produced would be kept to some level which lymphocytes are capable of in the absence of antigen or by an adjustment of the amount of antigen (Figure 13). This could account for what has been referred to as enhancing factors in tumor situations and to provide a basis in fact for the suggestion by Prehn (1971) that the original event in the immunologic response to a tumor is enhancement of the tumor. The mechanism of this could be this positive response to antigen accompanying any primary response—suppression or limitation, but not elimination of known measureable responses. This response, and thus too the information RNA, is probably "acquired" or activated
during the early response since the only suppression found to be transferred by normal cell RNA suppressed or inhibited only IgM production at any given time. In the case of cells treated with IRNA from tumor bearing animals it is probably an RNA different from the RNA responsible for "blocking" factors since Pennline (1976) has reported that these substances are produced by cells treated with such RNA in the absence of antigen. Likewise, a difference is suggested between RNA from lymphocytes from tumor bearing mice and RNA from mice artificially immunized by tumor removal or several injections of tumor cells since, while both induce production of cytotoxicity and ADCC, RNA from mice resistant to tumor does not transfer production of "blocking" factors to treated cells.

In the tumor situation, immunity or protection is possible by complete tumor removal or "spaced" injections of tumor cells artificially. Using the hypothesis advanced here, "baseline" suppressed cytotoxicity can be reversed by removal of antigen, leaving only lymphocytes not subject to suppressor perhaps due to the lack of appropriate receptors or elimination or depression of cell populations producing suppressor RNA. In the production of artificial immunity
it can be postulated that essentially the same reversal of primary response suppressor RNA can be accomplished by this type of antigen presentation, or that repeated application of antigen somehow keeps this phase of the primary response from becoming permanently established. Strangely, either removal of the tubercle bacilli by chemotherapy or by repeated BCG injections, results in the establishment of the complete immune state.

It is interesting in this regard that unpublished data by Pennline in this laboratory indicate that the cytotoxicity of lymphocytes sensitized to a mouse fibrosarcoma can be doubled to practically 100% killing by treatment with either TL-RNA or IL-RNA indicating a complete reversal of "baseline" suppression. Quite possibly the appropriate model for immunotherapy of cancer would be the creation and reversal of the "baseline" suppressed primary state.

A protocol based on this hypothesis essentially means that the IRNA sensitized lymphocytes would have to become established in the host's immune system and operate independently of the antigen suppressed population of lymphocytes. It would therefore be of great advantage if the IRNA sensitized lymphocytes underwent blastogenesis. However, no
lymphoblastic activity could be measured with the primary sheep erythrocyte IRNA treated cells (Tables V and VI). An initial speculation might be that the RNA had not become incorporated into the genome of the recipient cells, perhaps due to the absence of the RNA transference necessary, or lack of the appropriate mechanisms, and thus the RNA would not be carried throughout new generations of lymphocytes. There are complicating experimental factors - the time interval for incubation of lymphocytes and that necessary for the actual uptake of the thymidine - which would result in false negative data. This could be particularly important in view of the fact that seemingly very few lymphocytes actually take up the IRNA as shown in the Jerne Plaque assay (Figure 12). Another consideration is that only primary IRNA was used in these experiments. As described, secondary IRNA did not possess the information for antigen-dependent suppression, and perhaps if this "species" of RNA was used, successful measurements of lymphoblastic activity would be made.

The fact that the primary IRNA sensitized lymphocytes did not undergo blastogenesis might be important when considering the fact that mice immunized with these IRNA sensitized
lymphocytes did not demonstrate antibody forming cells in their splenic lymphocytes (Table III). Other investigators in this laboratory (Evans, 1976) have shown that there is minimal measurable cytotoxic activity in splenic populations of animals injected with lymphocytes treated with tumor immune RNA. There have been, however, reports of transfer of delayed type hypersensitivity, as measured by skin tests, in guinea pigs immunized with IRNA specific for tuberculin (Scheetz, et al., 1972) and for tumor antigen (Paque and Dray, 1970).

There is some question as to whether the demonstration of antigen-specific responses in animals receiving IRNA immunotherapy against tumor is necessary in order to establish a protective state. For instance, unpublished data from this laboratory have shown that animals, which have received IL-RNA sensitized lymphocytes but whose splenic lymphocytes themselves are not cytotoxic, are indeed protected to some degree from tumor challenge. Moreover, in vivo correlates of immunity have not been shown to parallel protection in tumor situations or even in tuberculosis, thus suggesting that the mode of protection against either pathology is speculative. In this same respect, this
report has also shown that animals immunized with a ribosomal RNA fraction from BCG organisms are also protected from tumor challenge, while Youmans and Youmans (1974) have shown that injections of the whole organism will likewise protect against tumor challenge. Each of these immunizing agents have been called non-specific enhancers of the immune system, although Borsos and Rapp (1973) have demonstrated cross-specificity of antigens on the surface of BCG and melanoma.

The fact is no one knows by what mechanism BCG or the ribosomal vaccine is able to effect protection against tumor. This report (Table XI) has shown that BCG, injected 21 days prior to immunization of sheep erythrocytes in C3H mice, will alter the response to the sheep erythrocytes. The mice developed complement independent cytolytic mechanism against erythrocytes, much resembling the ADCC cytotoxicity by lymphocytes against tumor antigen. Whether these findings are connected with the "altered" protective state against tumor associated with IRNA, BCG, or ribosomal RNA immunizations, is not known, but it is very necessary that the study of an immunotherapeutic program against tumor, or any other immunological disease state consider all of the possible mechanism whereby that protective state might be
transferred. Perhaps it is the inhibition of the antigen-dependent suppressed state which is important.

Thus, this report has repeatedly stressed that one of the best ways to understand those complex immune mechanisms associated with the tumor, or tuberculosis situation is to study the control of the immune system with respect to its diverse constituents. Likewise, it might be necessary and advantageous to study the different "species" of RNA. For instance, it is not clear whether the reactions of IRNA treated cells with and without antigen and the dosage relationship between IRNA and antigen are the result of different "species" of RNA molecules or if these relationships are aspects of one type of RNA. It would seem that different biological responses are the product or what can be regarded as biological "species" of RNA from different cell populations. Since sheep erythrocytes are a thymus dependent antigen, and we have measured IgM antibody and MIF, it is intriguing to postulate involvement of T-helper, T-suppressor, and B cell RNA as well. If a system of interacting RNA "species" does exist, then elimination of suppressor information would result in higher antibody production, an effect already demonstrated with the splenic lymphocytes themselves.
(Gershon, 1971). More interesting, it may be possible to isolate an RNA fraction, information from which is responsible for the antigen-dependent suppression. If removal of T suppressor lymphocytes from a cellular preparation resulted in the elimination of the antigen-dependent suppression, then most likely information for suppressive mechanisms would be found in the same RNA "species". Alternatively, the antigen-dependent suppression might be considered as a control mechanism operating from the time of antigen exposure, in vivo, and up to the point of the sensitization of the lymphocytes, including the generation of T suppressor cells, and thus distinguishing it from known suppressor mechanisms. Since suppression of antigen up until now has largely gone unnoticed it seems reasonable that the antigen-dependent suppression phenomenon is separate from known suppressor control mechanisms. The contribution of this knowledge may be significant in answering questions of the relationship between antigen and immunological control, so evidently important in the tumor situation.
Summary

Demonstration of the transfer of antigen sensitivity to normal mouse and guinea pig splenic lymphocytes *in vitro* was shown using IRNA extracted from the spleens of guinea pigs and CD-1 mice sensitized with a murine polyoma induced fibrosarcoma, BCG, and sheep erythrocytes. The primary concern was to demonstrate conditions by which the transfer of sensitivity could be maximized. These investigations made use of BCG-IRNA. It was found that there was an optimal proportion between the number of lymphocytes to be sensitized, the amount of IRNA used, and the amount of DEAE-Dextran, which was necessary to affect the maximal amount of MIF production. In each assay a standard amount of PPD antigen was used to stimulate the sensitized lymphocytes. These results were applied to the TL-RNA with similar results. At the time of these investigations the suppressive effect of antigen was not known.

The development of an assay to measure production of MIF and antibody by lymphocytes treated with BCG or TL-RNA
without antigen exposure resulted in dose response curves which were radically different from those found with antigen exposed IRNA sensitized cells. Without antigen, production of MIF by the IRNA sensitized cells was equal regardless of the amount of IRNA in the sensitization procedure, thus eliminating the requirement of the optimal proportion described in earlier studies.

Further investigation into the nature of this phenomenon antigen induced suppression was performed in the well defined sheep erythrocyte system in order to apply the information towards the understanding of the immunological pathology of the tumor and tuberculosis states. Both the antigen independent and the antigen-dependent suppressor response were repeated by treating normal splenic lymphocytes with IRNA extracted from the spleens of animals undergoing a primary immune response to sheep erythrocytes. Only the production of IgG antibody was independent of the suppression by antigen. These responses were also demonstrated by lymphocytes taken from the spleens of animals sensitized by injections of sheep erythrocytes. Exposing these sensitized lymphocytes, as well as the IRNA sensitized lymphocytes, with varying amounts of antigen concentration demonstrated that
there was an amount of antigen which allowed production of the immune factors. The range of antigen concentration which allowed production was greater for the lymphocytes sensitized in vivo by antigen injection than it was for the IRNA sensitized cells. Transfer of in vivo immunity in mice by injection with either the primary IRNA, or lymphocytes treated with the primary IRNA, was a failure, as were measurements of lymphoblast transformation by lymphocytes sensitized with the same IRNA. IRNA extracted from spleens of animals undergoing a secondary immune response to sheep erythrocytes failed to demonstrate the antigen-dependent suppression when used to sensitize normal lymphocytes.

In a somewhat unrelated study designed to compare the BCG and tumor antigen system, ribosomal RNA, extracted from BCG organisms, induced protective immunity in C3H mice when injected intraperitoneally 21 days prior to challenge with the polyoma fibrosarcoma tumor cells. Splenic lymphocytes from C3H mice immunized with BCG were cytotoxic for the tumor cells in vitro perhaps indicating cross reactivity between the BCG and the tumor cells. There was also shown some evidence that BCG altered the response of C3H mice to sheep erythrocytes if the BCG were injected 21 days prior to the erythrocyte injection.
Bibliography


Bell, C., Dray, S. 1971 Conversion of non-immune rabbit spleen cells from an immunized rabbit to produce IgM and IgG antibody of foreign heavy chain allotype. Journal of Immunology. 107:83.


BiLello, P., Fishman, M., Koch, G. 1976 Evidence that immune RNA is messenger RNA. Cellular Immunology. 23:309.


Diener, E., Feldman, M. 1972 Relationship between antigen and antibody induced suppression in immunity. Transplantation Review. 8:76.


Evans, S. 1976 Immunological properties of anti-tumor "immune" RNA. Ph.D. Dissertation. The Ohio State University. Columbus, Ohio.


Fishman, M. 1966 Antibody formation initiated in vitro. II. Antibody formation and allotypic specificity directed by RNA. Journal of Immunology. 97:554.


Gershon, R., Kondo, K. 1970 Cell interaction in the induction of tolerance; the role of thymic lymphocytes. Immunology. 8:723.


Kennedy, C.T.C., Cater, D., Hartvert, F. 1969 Protection of C3H mice against BP8 tumor by RNA extracted from lymphocytes and spleens of specifically sensitized mice. ACTA Pathology Microbiology Scand. 77:196.


Pennline, K., Evans, S., Dodd, M.C. 1976 Distinct immunological properties of RNA extracted from lymphocytes of tumor immune (IL-RNA) and tumor bearing (TL-RNA) mice. Abstract. The 13th annual meeting of the Reticuloendothelial Society. pg. 32a, #62.


Scheetz, M.E. II. 1972 The transfer of tuberculin sensitivity and cytotoxicity against tumor cells to human peripheral blood lymphocytes using xenogenic RNA. Ph.D. Dissertation. The Ohio State University. Columbus, Ohio.


Schlager, S., Dray, S. 1976 Complete regression of a guinea pig hepatocarcinoma by immunotherapy with "tumor-immune" RNA or antibody to fibrin fragment E. Israel Journal of Medical Science. 12:344.


Ting, R., Law, L. 1965 The role of thymus in transplantation resistance induced by polyoma virus. Journal of the National Cancer Institute. 34:521.


White, R.G. 1973 Immunopotentiation by Mycobacterium in complete Freund type adjuvant as the failure of normal immunological homeostasis. Immunopotentiation. Elsevier. pg. 47.


Yamada, H., Yamada, A. 1969 Antibody formation against 2,4-dinitrophenyl-hapten at the cellular level. Journal of Immunology. 103:357.
