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STUDIES ON THE CELLULAR SITE OF ACTION OF RNA-ANTIGEN COMPLEXES DERIVED FROM MOUSE MACROPHAGES

by

Sandra LaVelle White

A dissertaion submitted in partial fulfillment
of the requirements for the degree of
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(Microbiology)
in The University of Michigan
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ABBREVIATIONS

ASC	Adherent Spleen Cells
B	Bone Marrow Derived Cells
BGG	Bovine Gamma Globulin
BME	Eagle's Basal Medium
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
EALC	Equine Anti-Lambda Chain Serum
EAKC	Equine Anti-Kappa Chain Serum
GAGC	Goat Anti-Gamma Chain Serum
GAMC	Goat Anti-Mu Chain Serum
HA	Hemagglutination titer
HBSS	Hanks Balanced Salt Solution
HGG	Human Gamma Globulin
IP	Intraperitoneally
IV	Intravenously
n-RNA	RNA Extracted From Normal Macrophages
NASC	Nonadherent Spleen Cells
NRS	Normal Rabbit Serum
PBS	Phosphate Buffered Saline

PEC	Peritoneal Exudate Cells
PFC	Plaque Forming Cells
Poly A:U	Polyadenylic, Polyuridylic Acid Complexes
PPD	Purified Protein Derivative
R	Rads
RFC	Rosette Forming Cells
RNA	Ribonucleic Acid
RNA:HGG	RNA Extracted From Macrophages Sensitized To HGG
SE	Standard Error Of Arithmetic Mean
SRBC	Sheep Red Blood Cells
T	Thymus Derived Lymphocytes

INTRODUCTION

Definition of the cell types involved and their individual role in the synthesis of antibody continues to be a central problem in immunology. An optimal immune response to most antigens usually needs interaction or collaboration among three types of cells, the macrophage, the T lymphocyte derived from the thymus, and the B lymphocyte derived from bone marrow in mammals or the bursa of Fabricus in chickens (1-2). Recently, it has been shown that normal spleen cells can be separated into two sub-populations, one which adheres to glass (thought to be macrophages) and one consisting of nonadherent lymphoid cells. On exposure to antigen (sheep red blood cells), neither population alone was capable of responding with antibody synthesis, but recombination of the cells endowed them with the capability of inducing an immune response (3-5). Following this key discovery further studies utilizing buoyant density centrigugation to fractionate the normal spleen population have shown that the nonadherent cell population was composed of two cell types and the adherent cell population exhibited the characteristics of a macrophage on the gradient (6,7). In support of the

latter fact, it was shown that anti-macrophage serum abrogated the additive role of the adherent population (8).

Studies utilizing irradiated mice as recipients of limiting numbers of thymus cells or bone marrow cells have shown that the actual antibody forming cells are of bone marrow origin (9,10). The cell derived from or influenced by the thymus "cooperates" with bone marrow-derived precursors of antibody forming cells to initiate the humoral response. The first direct evidence for this T and B cell interaction was provided by Claman and co-workers on studies of the humoral response to SRBC (11). Reconstitution of lethally irradiated mice with either spleen, bone marrow, or thymus plus bone marrow cells from normal or immune syngeneic donors, followed by antigenic challenge with SRBC clearly showed a dissociation of the capability for antibody synthesis among the various cell populations. Support for this interpretation, that the marrow population contained "effector cells" capable of producing antibody, but only in the presence of "auxiliary cells", was forthcoming in subsequent studies carried out in several other laboratories (12-14).

Though it is generally agreed by most investigators that the macrophage also is required for the <u>in vitro</u> response to SRBC, there is no general agreement on the mode of action or function of this cell. Evidence has been

presented that the macrophage facilitates cell-to-cell interaction, whether by antigen concentration or other means (15-18). Recently, Feldman et al. have favored the macrophage functioning as a transporter of T cell receptor-antigen complexes to B cells (19,20). There has been equally supportive evidence that the macrophage functions by providing a source of nutrients or special factors which in some manner facilitate in vitro lymphocyte growth (5,21-24) or lymphocyte-antigen interaction (25). The question of whether the role of the macrophage is to "process" or handle antigen in an appropiate way, by degrading large molecules to smaller more immunogenic forms (5,26,27) or producing an immunogenic nucleic acid moiety also has received much attention (28-30).

There has been considerable interest, in recent years, in identification and characterization of various RNAs in cells involved in the immune response. A major finding has been that certain RNA extracts from macrophages and spleen cell preparations appear to be free of antigen and yet are able to induce antibody formation. The antibody produced in these instances was of the same allotypic specificity as the donor RNA though the recipients of the RNA were allotypically different rabbits (31,32). These experiments suggest a messenger-type function in the TNA extract, the significance of which is not known.

However, most of the reported immunogenic RNAs have been found to have fragments of antigen complexed to the RNA (33-36).

Previous studies by Johnson and Johnson (37) have shown that a 4S RNA-rich fraction, isolated by phenol extractions of antigenically stimulated peritoneal exudate cells (PEC) was capable of inducing rosette forming cells and antibody formation to BGG on injection into Balb mice. The antigen contamination of this RNA, as determined isotopically was below 10^{-11}g. It could not be concluded from these studies which cell type the RNA-rich fraction stimulated or whether antibody synthesis resulted from trace amounts of antigen combined with a nucleic acid acting as an adjuvant (38) or whether the RNA itself was the induced mediator.

The experiments described herein were designed to help clarify the role of the macrophage in antibody synthesis by obtaining information on these two questions. The data are interpreted in support of the hypothesis that when normal macrophages are incubated with antigen a minute amount of antigen remains attached to the RNA, and that this RNA-antigen complex is capable of stimulating directly the bone marrow derived cell in the nonadherent population.

HISTORICAL

Near the turn of the century, Metchnikoff (39) discussed the concepts of immunology current at that time. He reviewed some of the earliest reports on phagocytosis by mammalian leukocytes, published by Grawitz in 1877 and 1881, and also discussed his own experiments on macrophages which began during the same decade. In these earlier works Metchnikoff was able to show that materials were digested within phagocytes of various species (40) and that engulfment of anthrax bacilli by phagocytes of vertebrate animals led to, in his words, "a desperate struggle" between bacilli and the ameboid cells (41). He was struck by the similarities between leukocytes in inflammatory exudates of higher animals and phagocytic ameboid cells of the echinoderms, and hypothesized that cells of this type had the important function in body defense of ingesting and digesting extraneous material. The principal tenet of "the theory of phagocytes", formulated by Metchnikoff, was that phagocytes were solely responsible for immunity to harmful agents.

The ubiquitous phagocytic cells that we know as macrophages vary in many of their characteristics, depending on their location, physiological state, and function. Although Metchnikoff appreciated many of their potentialities nearly a century ago, most other early investigators regarded macrophages primarily as scavenger cells. Research in many fields has made it increasingly obvious that macrophages do, in fact, have a multitude of important functions above and beyond their ability to scavenge and dispose of effete cells and extraneous matter.

Structure, Source and Maturation of the Macrophage

Peritoneal macrophages are easily obtained from experimental animals and hence have been studied more extensively than other macrophages. North and Mackaness (42) examined normal peritoneal macrophages from non-immunized mice by electron microscopy. These macrophages were shown to have characteristics typical of peritoneal macrophages of many species. The cytoplasm is enclosed by a unit membrane, approximately 80 Å thick, with many protuberances and invaginations indicating a high degree of activity. The peripheral cytoplasm in the numerous cellular processes is finely granular and usually

lacks the structure seen in the rest of the cytoplasm, i.e. membranes of endoplasmic reticulum with and without attached ribosomes, free ribosomes, often arranged in polysomes, and cylindrical mitochondria. Cytoplasmic vesicles are of three main types: small pinocytic vesicles, various sized organelles containing a fine granular material, and larger denser vacuoles. The nucleus is oval with one or more indentations. Other investigators have reported similar observations on the ultrastructure of mouse peritoneal macrophages (44).

The ultrastructure of peritoneal macrophages of mice that have survived an initial infection with Listeria monocytogenes, termed "immune macrophages", was compared with normal macrophages (45). The cytoplasmic membrane of immune macrophages is smoother and has fewer protuberances and invaginations than the membrane of nonimmunized macrophages. Compared with nonimmune peritoneal macrophages the cytoplasm of immune macrophages is less dense and appears to be highly hydrated. It contains fewer vesicles and a very extensive Golgi apparatus. It was suggested that the differences seen between immune and nonimmune cells was probably the result of the emergence in immune mice of a

new population of peritoneal macrophages which are in the process of differentiation.

Peritoneal macrophages from nonimmunized hamsters are similar to those of the mouse, as evidenced by the electron microscopy studies of Dumont and Sheldon (46). Mouse and hamster peritoneal macrophages are not appreciably different in morphology from other mammalian and avian peritoneal macrophages, e.g. rat and chicken (47); rabbit (48) and guinea pig (49).

The sources and maturation of macrophages have been subjects of much conjecture due mainly to the lack of availability of tools for studying these problems. However, in recent years, techniques for radioactive labeling and tracing of cell division and migration have provided better approaches.

Experiments using radiation chimeras have provided evidence that the bone marrow is a major source of macrophage precursors. Indeed it has been suggested that bone marrow stem cells serve as the sole primary origin of macrophages (50). Balner (51) transplanted mouse bone marrow cells into lethally X-irradiated recipients. Cytotoxicity tests conducted with specific alloantisera

demonstrated that at 6 weeks all of the free peritoneal macrophages in the resulting chimeras were derived from the transferred bone marrow cells. In similar experiments, Goodman (52) showed that at various times, ranging from several months to over a year after transfer of bone marrow, virtually all of the peritoneal macrophages in the recipients were of donor origin.

Other experimental evidence likewise strongly supports the thesis, (reported in various studies of Maximov in the 1930's), that cells from the bone marrow serve as the principal progenitors of macrophages. For example, parabiosis experiments have demonstrated conclusively that many exudative macrophages are derived from blood borne cells (53). The common circulation between two parabiotic rats was interrupted long enough to label cells in one of the partners with a pulse of tritiated thymidine. Subsequently radioautography showed that a substantial number of macrophages in induced inflammatory exudates of the unlabeled partner were labeled, and hence were blood borne, presumably monocytes from bone marrow. Studies with labeled cells further indicated that precursors of macrophages proliferate rapidly and continuously in areas distant from the inflammatory site, and release their progeny into the circulation. The extent and characteristics of labeling of peritoneal exudate cells in rats given tritiated thymidine also indicated that a population of morphologically unidentified bone marrow cells provides

precursors of peritoneal macrophages (54).

Still other evidence which supports the concept that bone marrow cells serve as principal progenitors of macrophages, as well as other cells found in inflammatory lesions has come from work of Spector and Willoughby (55). These investigators transfused lymph node, thymus, or bone marrow cells into rats treated with 600 R of X-irradiation. It was found that, whereas rats restored with lymph node or thymus cells lacked the capacity to mount a cellular inflammatory response, animals which received bone marrow cells regained this capacity. Macrophages in other locations as the lungs (56) and liver (57) have also been shown to be derived from cells in the bone marrow.

Other potential sources of macrophages include mobilization and proliferation of existing tissue macrophages (58,59) and migration of Kupffer cells into the lung (60).

The Fate of Antigen in Peritoneal Macrophages

Several studies on the fate of labeled antigen in mouse peritoneal exudate cells have been reported recently. Askonas et al, (61) studied the persistence and degradation of protein antigens in cells from mice previously injected with peptone. Intraperitoneal

injection of 1 ug of ^{131}I-labeled hemocyanin resulted in a maximum of about 0.4% being taken up by the cells within 2 hr. Of this amount 90% was degraded and lost from the cells withing 2 hr. The residual 10% had a much longer half-life, some presisting for weeks. Extracts of the cells showed that within 5 hr. after antigen injection, only a small proportion of material was degraded to a size which was no longer acid-precipitable.

Kolsch and Mitchison (62) also studied in some detail the intracellular distribution of labeled antigens (heat denatured BSA and synthetic polypeptides) in peritoneal cells. Antigen was labeled with ^{131}I or ^{125}I to enable "pulse and chase" experiments. At various times after exposure to the antigen, the cells were homogenized, subcellular fractions isolated, and then analyzed by isopycnic centrifugation in sucrose gradients. Cells from normal and X-irradiated animals were equally effective in taking up the labeled antigens. 90% of the cell-associated antigen was degraded within 3 hr. and when cells from normal animals were used the residual 10% of radioactivity was retained for at least 8 hr. The newly phagocytosed antigen was located mainly in 1.0 gm cm^{-3}

density fraction whereas the retained antigen was in a fraction banding at a density of 1.26 gm cm^{-3}. X-irradiated animals appeared to be deficient in their ability to retain antigen in this latter compartment. These results confirmed earlier finding of Unanue who had used keyhole limpet hemocyanin and found 90% of the antigen to be degraded and 10% retained (63).

Interest in the nature of the retained antigen led to further experiments. Either macrophages were exposed to labeled antigen in vitro for varying lengths of time or labeled antigen was injected into the peritoneum, and the cells recovered from the cavity after a limited time period and maintained in tissue culture. Some of the persisting antigen was found to be retained on the plasma membrane of the cells. This was demonstrated by (1) electron microscopic radioautography; (2) the ability to release antigen from the cell by brief treatment with trypsin; (3) ability of cells containing antigen to absorb either Fab' or $F(ab)_2$ fragments prepared from purified preparations of specific antibody (63,66). Askonas and Jaroskova removed cell-associated antigen (hemocyanin) by exposing cells to ethylenediaminetetracetate (EDTA).

The removed protein, when examined by polyacrylamide gel electrophoresis, was present largely as the monomer with only a small amount in degradation products (66). These investigators also demonstrated essentially undegraded antigen inside the cell and showed that the cells containing antigen would slowly release antigen possibly by a process of exocytosis or because of membrane turnover (67,68).

The Fate of Antigen in the Macrophages of Lymph Nodes

With antigen tracing studies, two main areas or sites in lymph nodes where antigen becomes trapped have been identified: one is associated with typical macrophages situated in medullary areas or perifollicular zones of lymph nodes, or perifollicular or marginal zones of spleen; the second is associated with the lymphoid follicles. The characteristics of antigen trapping in these two areas appear to differ (69-71). The experimental antigens most frequently used include albumins, hemocyanins Salmonella flagellin, immunoglobulin, ferritin, and synthetic polypeptides.

Antigens enter into a lymph node via afferent lymphatics and are readily taken up by the macrophages of

the medulla and perifollicular areas. Studies on the distribution of flagellin-^{125}I in lymph nodes showed it to be in association with macrophages and usually in intracellular vesicles or in phagolysosomes (72). The macrophages responsible for this uptake showed morphological characteristics in general similar to macrophages isolated from the peritoneal cavity. Of further interest are the findings that macrophages in tolerant animals take up antigen to the same extent as macrophages from normal animals (69). Uptake of antigen by macrophages was not reduced by X-irradiation (73) but was enhanced if the antigen was bound to antibody in an immune complex (14).

The Fate of Antigen in Lymphoid Follicles

The fate of the antigen in the follicles seems to be quite different from its fate in the medullary and follicular macrophages. Antigen must be bound with antibody in order to be localized in the lymphoid follicles (69,74-76). Ultrastructural examination has shown most of the antigen in the follicles to be between closely packed cells on what appear to be membranes of reticular cells with dendritic processes (77). These cells have certain characteristics of macrophages. Although they are not actively phagocytic, they have the capacity to phagocytize materials under certain circumstances (71). Antigen

localized in the follicles persists for prolonged periods of time primarily, due to the stickiness of antibody molecules in this area whereas most of the antigen associated with the extra-follicular macrophages is eliminated. Also follicular antigen trapping appears to be radiosensitive (70,78).

The association between uptake of antigen by extra-follicular macrophages or follicular cells and the immune process is not known. However, we do know that extra-follicular macrophages handle antigen in a way very similar to that of peritoneal macrophages (7,80); we also know that some antigen bound to transplanted peritoneal macrophages is immunogenic (81-83). The logical assumption is that antigen bound to extra-follicular macrophages of the spleen and lymph nodes, should be available for immune recognition for some time.

Immunogenicity of Macrophage Bound Antigens In Vivo

A system employed to study the immunogenicity of macrophage bound antigens consists of isolating macrophages containing the antigen and transplanting them to syngeneic hosts which are observed for their immune responses (27, 30,82). The macrophages are obtained mainly from the

peritoneal cavities of mice or rats after injection of as irritating or inflammatory substance such as thioglycollate or proteose peptone broth. The peritoneal macrophages so obtained are well differentiated cells which originated from a pool of cells in the bone marrow (84). The evidence is limited and conflicting on whether the immunogenicity of antigen varies depending on the source of macrophages to which it is bound. The immunogenicity of bacteriophage T4 bound to Kuppfer cells was compared to that bound to peritoneal macrophages. When the bacteriophage was bound to the Kuppfer cells it showed a reduced immunogenicity as compared to T4 bound to peritoneal macrophages (85). In contrast to these findings, Howard (86) claimed that BSA was of comparable immunogenicity when bound to isolated Kuppfer cells, alveolar macrophages or peritoneal macrophages.

In the last six years, several characteristics of the immune response to antigens transferred in live macrophages have been studied. It is clear that immunocompetent lymphocytes must be present in order to interact with antigen and initiate the immune response. Transfer of macrophages into tolerant recipients (30) or into X-irradiated recipients (81,87-89) does not lead to an immune response. These type of studies clearly emphasize the

fact that macrophages are not involved in antibody synthesis. Earlier studies have shown that antigen on cells from allogeneic (30) or xenogeneic donors (81) or dead macrophages (30) induce smaller responses than antigen bound to living syngeneic cells. More recently, Rosenthal and co-workers (18,90) have demonstrated that in guinea pigs the macrophages must be both metabolically intact and histocompatible with the lymphocytes.

It is well established that antigen bound to live macrophages is immunogenic and can induce primary immune responses (28,82,83,91) and prime for a secondary challenge (81,91). The extent of the immune response depends on the amount of antigen transferred in or on the cell (92). Macrophage bound antigen was also found to be more effective than free antigen in inducing delayed hypersensitivity in rats (93) and guinea pigs (94-96).

The degree of enhancement of immunogenicity offered an antigen molecule by its binding to macrophages appears to be dependent on the degree of uptake of the antigen by macrophages. Generally, those antigens taken up poorly by tissue macrophages (e.g. albumins) induce a much higher immune response if they are transferred bound to macrophages rather then being given as free antigen (30,81,91). In contrast, if the immune response of free

or macrophage-bound antigen is compared using antigens which *in vivo* are taken up readily by macrophages, one finds free antigen induces a much higher response. This has been demonstrated using foreign red blood cells (89) and keyhole limpet hemocyanin (81). These findings are readily explainable when one considers that large particulate antigens are readily trapped by lymph node and spleen macrophages and therefore are suitably situated for triggering immune states. Thus, it is logical that when these antigens are transferred bound to macrophages their concentration in lymphoid tissues is not necessarily enhanced. In the case of antigens not readily phagocytized by macrophages, transfer of antigen bound to macrophages leads to a stronger immune state by concentrating more antigen in the lymphoid tissue.

The Role of the Macrophage in the In Vitro Antibody Response

The two *in vitro* systems most frequently used in recent years include the antibody-forming cell (PFC) response to foreign red cells (usually SRBC) or the proliferating cell response to soluble antigens or allogeneic cells. An antibody-forming cell response can be readily obtained using the method of Marbrook (97) or Mishell and Dutton (98). In the former method, cells are placed in

a dialysis bag which is immersed in a flask with tissue culture medium. In the Mishell-Dutton system cells are cultured in dishes that are rocked for several days; the medium which contains fetal calf serum, is supplemented daily. With both systems the immune response is measured by the numbers of PFC present 4-5 days after the culture is initiated.

There is general agreement that the primary anti-RBC response requires the presence of at least three types of interacting cells. Two of these cells are lymphoid and correspond to the T and B lymphocytes identified in vivo; the remaining cell has all the characteristics of a macrophage and is best identified by its property of adherence to glass surfaces. The evidence that the third type of cell is a macrophage is as follows: (1) it has phagocytic properties; (2) the cell adheres to glass or plastic surfaces; (3) it has the classic morphological criteria of macrophages; (4) it is radioresistant; (5) it does not synthesize antibodies; (6) it is affected by treatments which normally block the reticuloendothelial system (RES) in vivo; and finally (7) it is affected by specific anti-macrophage serum (5,99).

Mosier (3) was the first to demonstrate that an adherent cell population was required in the above described

system. He was able to culture mouse spleen cells on plastic dishes for 30 minutes and separate the cells into those adhering to the dishes (approximately 10%) and those not adhering (approximately 90%). Neither of the populations responded independently to SRBC but recombination of both populations reconstituted the response to SRBC. Subsequently, these studies were confirmed and expanded.

Close examination of the above system by Mosier showed that during the incubation period cell clusters occurred (15). About 10% of these clusters contained most of the antibody-forming cells in the cell population, and many clusters contained one or more large monocytic cells together with several lymphocytes. The prevention of clustering by mechanical means or by excess antibody to the antigen blocked the immune response. Results of a similar nature were reported using adherent and nonadherent mouse spleen cells (100). Pierce and Benacerraf (16) showed that the formation of cell clusters was necessary for the first 24-48 hr during which time lymphoid cells became "activated" and thereafter developed into antibody-forming cells independent of antigen and macrophages. Further analysis of these clusters by McIntyre and Pierce has shown them to be composed mainly of macrophages and T cells. As most PFC were not within a cluster but were single, it was suggested that the

B cell must freely associate with and dissociate from the cluster (17). Hartman et al. (4) separated adhering and nonadhering cells from mouse spleens using glass beads or plastic surfaces. When small numbers of adhering cells were added cooperation was evident. Precursor cells, as expected, were present predominantly in the nonadhering cell population.

Using two different antigens, RBC and polymerized flagellin, Shortman, Diener, and colleagues (7,101) examined the requirement for macrophages in cell culture systems. Three procedures for obtaining subpopulations of mouse spleen cells were used: equilibrium centrifugation in gradients of bovine serum albumin; filtration through glass beads with separation according to cell size; and filtration through glass bead columns. In the last case, the adhering cells could be recovered from the column. Marked differences were found between the behavior patterns of the two antigens. Practically all cells which phagocytized the SRBC were classified as macrophages and were found to band in the less dense regions of the gradient. In contrast, cells which phagocytized the polymerized flagellin were polymorphonuclear cells and were in denser regions of the gradient. The ability of the different fractions to initiate an antibody response either in vitro

or *in vivo* was tested. It was found that these two antigens had different cell requirements based on which fractions of the gradient were able to cooperate to give an immune response. These studies were confirmed and extended by Haskill, using cell populations separated on glass columns (7).

Utilizing adherent and nonadherent spleen cells from different strains of mice and assaying for plaque forming cells (PFC) in the presence of anti H-2 serum, Hartman (4) showed conclusively that the nonadherent population conatined progenitors of PFC.

Adherent cells obtained from mice up to 48 hr after they had received 250 to 1000R *in vitro* were as effective in culture as adherent cells from nonirradiated mice (102, 21,6). However, 3 days after X-irradiation the mouse spleens were depleted of adherent cells (103).

From the cited works it can be seen that SRBC have been the antigen of choice in most *in vitro* studies. The general agreement amongst investigators is that the macrophage is required for an *in vitro* response to SRBC. However, various investigators have different theories on how the macrophage functions.

The Macrophage as an Antigen Processor

One of the important areas of concern is the form in

which antigen in presented to the lymphocyte. Delineation of this problem will naturally help in elucidating the role of the macrophage. In Mosier's original work, the impression was given that most SRBC were phagocytized by the macrophages. Hence, the inference was that immune recognition must have followed interiorization of the antigen. Recently it has been shown that ammonium chloride treatment of adherent cells exposed to SRBC abolishes their capacity to support in vitro responses of lymphocytes (104). The ammonium chloride presumably lysed the non-phagocytized SRBC. It was shown that the inability of macrophages to support an immune response after treatment was not due to the effect of the ammonium chloride on the adherent cells. If SRBC were added to the macrophages after treatment they were fully capable of responding. This study would thus indicate that the in vitro response to SRBC is directed toward extracellular antigen and not that phagocytized. Shortman and Palmer (5) have presented evidence that SRBC must be solubilized before they can interact with macrophages. These workers incubated 24 hr macrophage culture fluid with SRBC, after several hours the SRBC were centrifuged and discarded. The resulting supernatant was able to stimulate nonadherent cells for an anti-SRBC response. SRBC by themselves were not stimulatory. From these data they inferred

that macrophages probably generate factors which in turn liberate antigen from SRBC. Along these lines, Feldman and Palmer (26) have claimed that macrophages are not required for the in vitro immune response to a soluble extract of SRBC. Their results imply that SRBC must be degraded to a small size in order to trigger lymphocytes for antibody synthesis.

Recent findings by Calkins (25) suggest that the macrophage elaborates a soluble factor which then probably acts on antigen. He found that adherent and nonadherent cells need not come in contact for the production of an in vitro immune response. It was necessary, for the antigen to be in contact with the nonadherent population, although not with the adherent population.

Much of the work today on the role of the macrophage is being done in vitro. Gorczynski et al. (103) have correlated an adherent cell in vitro with spleen macrophages operational in vivo. They found that spleen cell suspensions devoid of adherent cells 3 days after X-irradiation of mice did not support in vitro responses by lymphocytes; similiarly suspensions of lymphocytes (depleted of macrophages) transplanted into mice irradiated 3 days previously did not form antibody in vivo. A similar correlation was found when mice were injected with large doses of horse red

cells; such mice did not make antibodies when immunized to SRBC (unless replenished with macrophages) nor did the spleens contain active adherent cells for *in vitro* responses to SRBC. Cells isolated from normal spleens were capable of restoring the capacity for both *in vivo* and *in vitro* responses. These cells, presumably macrophages, had identical physical properties when separated on density gradients or velocity sedimentation. These experiments strongly suggest that the role of the macrophage *in vitro* may be similar if not identical to their role *in vivo*.

The Macrophage as a Facilitater of Cellular Interactions

The possibility that the macrophage might serve as a cell that facilitates cellular interactions has received much support. Physical approximation between lymphocytes and macrophages has been observed during cultivation of these cells (15,105-107). Although the exact nature of these cellular associations remains obscure, their occurrence both *in vitro* and *in vivo*, often in relationship to defined functional events, suggests physiologic significance. In vitro induction of primary antibody responses (5,16), as well as antigen-mediated *in vitro* proliferation of immune lymphocytes (105,108,109), has been observed to involve direct physical contact between lymphocytes and

antigen-containing macrophages. Anatomic approximation thus may facilitate functional cooperation between macrophages and lymphocytes during the expression of an immune response. Recent findings of Feldman (19) give support to these concepts. He investigated the requirement for macrophages in thymus-dependent antibody responses in vitro. Utilizing macrophage deficient populations from mice primed to dinitrophenylated (DNP)-fowl gamma globulin (FGG) he demonstrated that these cells were unable to respond to DNP-FGG. DNP-reactive B cells were present however, as they were fully able to respond to macrophage-independent and thymus-independent DNP-flagellin. The capacity for a thymus-dependent anti-DNP response was restored by adding PEC treated with anti-theta serum. These findings were thought to suggest that antibody responses to protein antigens involving T and B lymphocyte collaboration also require the presence of macrophages.

In support also of the concept that the macrophage serves as a cell which facilitates cellular interaction are the findings that macrophages possess a unique ability to recognize and bind lymphocytes and thymocytes by a mechanism distinguishable from other known macrophage receptors (18). It is thought that the macrophage-lymphocyte interaction is mediated by a macrophage-binding site that is

either identical or closely linked to histocompatibility antigens (110).

The Macrophage as a Source of Growth Enhancing Conditions for Lymphocytes

Evidence that macrophages supply factors which enhance the growth of lymphocytes comes from experimental results of several investigators. Dutton and collaborators (21,22) found that culture fluids of adherent spleen cells or better, of peritoneal macrophages allow nonadherent spleen cells to respond to antigen. The chemical properties of the factor in question have not been determined. Hoffman and Dutton postulated that the factor might be an enzyme necessary for SRBC processing, an antibody-like molecule or a growth-conditioned media-type factor.

Chen and Hirsch (25) suggest in studies on the effects of mercaptoethanol and peritoneal macrophages on *in vitro* antibody formation that they exhibit some sort of trophic influence on the medium and/or on the lymphoid cells. This effect consequently results in markedly improved lymphocyte viability as well as increased numbers of cells producing antibody to SRBC. The presence of mercaptoethanol abrogated the need for macrophages in antibody production by lymphoid cells. It has been shown that mercaptoethanol will completely reverse the effect of

high O_2 in normal or macrophage depleted cultures. A 24-hr. culture supernate of peritoneal exudate cells also protected cultures against high O_2 (111). Thus in vitro, one role of macrophages may be to provide optimal conditions for survival of immunocompetent cells via production of reducing compounds.

In line with these findings are the studies which show that the macrophage releases into cultures a low molecular weight factor which is able to enhance or suppress DNA synthesis in lymphoid cells. Whether one gets enhancement or suppression is governed by the macrophage-lymphocyte ratio (112).

Antigen-RNA Complexes

The ability to transfer immunity using ribonucleoprotein extracts was first described by Sterzl and Hrubesova (113). A ribonucleoprotein preparation, obtained from the spleens of adult rabbits, injected with Salmonella paratyphi B two days before sacrifice, led to the production of specific antibody against this organism. In addition, such an effect could be demonstrated in 5-day-old rabbits which normally failed to respond to Salmonella antigens, thus ruling out passive transfer of antigen alone. In some manner, the ribonucleoprotein material

conferred immunocompetence on the young rabbits. Similar studies by Friedman (114), using lymph node nucleoproteins from rabbits immunized with Shigella paradysenteriae, demonstrated the formation of specific agglutinins in normal and sub-lethal X-irradiated adult recipients after injection of the nucleoprotein extract.

Experiments carried out by Garvey and Campbell (115) on the nature and fate of retained antigen also pointed to a role for nucleic acid-protein complexes in the immune system. Rabbits injected with ^{35}S-labeled sulfanilate-azo-bovine serum albumin (^{35}S-BSA) were found to retain a small percentage of the antigen in the liver for prolonged periods of time. Much of this material was present in degraded form and was associated with RNA (116). These antigen-RNA complexes were shown to be potent immunogens by injecting them into guinea pigs and assaying three weeks later for in vitro anaphylaxis. The liver complexes were found to be up to 200 times more effective in sensitization to ^{35}S-BSA than the original antigen; however, when the complex was dissociated, neither the antigen fragment nor the RNA was immunogenic. Later studies (117), using an in vitro assay system which tested the ability to induce a secondary response in primed rabbit lymph node cultures, also demonstrated the

increased potency of antigen-RNA complexes over an equivalent amount of antigen alone. The source of these complexes is not clear. Garvey (118) believes that they come from the liver parenchymal cells while others believe the Kupffer cells are a more likely source (119).

Similar conclusions on the enhanced immunogenicity of antigen-RNA complexes were reached by Askonas and Rhodes (120). In studies utilizing RNA from macrophages which had taken up ^{125}I-labeled hemocyanin, they not only demonstrated that about 1.01% of the total label taken up by the cells was found associated with the RNA, but also showed that the RNA-antigen complex was 20 times more effective than an equivalent amount of antigen alone. As a result of such studies the term "super antigen" has come to be applied to these complexes.

An entirely <u>in vitro</u> system was first studied by Fishman and Adler (33,34). They found that lymphocytes responded immunologically to extracts of peritoneal exudate cells which had been exposed to T2 bacteriophage for 30 min <u>in vitro</u>. The experimental system consisted of culturing lymph node cells (33) or, alternatively, of placing lymph node cells in diffusion chambers and implanting them into X-irradiated rats (34). The filtrate from PEC exposed to T2 phage induced neutralizing antibodies within

8 days of culture. The neutralizing antibody produced by the lymph node cells was specific for T2 phage in that cultures did not contain neutralizing antibodies to T1 or T5 phage. Although prior exposure of these cells to T2 phage could not be ruled out, direct addition of T2 bacteriophage to lymph node cell cultures did not result in antibody formation. These extracts were ribonuclease (RNase) sensitive and sedimented as a low molecular weight RNA. It was experiments of this nature which led many investigators to feel that in order for an antigen to be immunogenic it must first be "processed" by macrophages.

Michael and Kuwatch (121) concluded that the immunogenicity of the RNA extracted from phage exposed macrophages was attributable to endotoxin in the bacteriophage preparation. However, recent reports demonstrate that RNA-antigen preparations from spleen cells exposed to bacteriophage 81a, can induce specific antibody formation against phage in primed mice (35, 122). Bacteriophage 81a infects Streptococci fecalis, which is a gram-positive organism, thus there is no possibility of endotoxin contamination. Subsequently, several laboratories have demonstrated that the immunologic activity of the preparations is due to the antigen in the extract.

The original reports of Fishman and Adler (34) indicated that the RNA preparations containing the ability to induce antibody were free of antigen. However, the use of relatively insensitive methods for detection of protein antigens and in view of the findings of others, it is to be questioned whether the preparations were actually antigen free. Indeed, Friedman et al. (123), Askonas and Rhodes (120) and others (126-128) have demonstrated that the RNA fractions contained antigen and/or fragments of antigen.

In a direct test carried out by Gottlieb et al. (125), using a system analogous to Fishman and Adler combined with nucleic acid hybridization studies, it was demonstrated that the RNA found in sensitized macrophages was the same as in normal macrophages. Also, if two sets of cells were exposed to two different antigens, the two RNA species recovered from cells infected with either antigen were identical. These results were interpreted as indications that the RNA species was not messenger RNA since it was not unique.

Subsequent studies carried out (129) showed that all of the immunological activity of the RNA preparations (assayed six days after addition of RNA to spleen tissue cultures) could be accounted for by the antigenic fragments associated with a particular RNA species. This was

initially shown by subjecting the bulk RNA preparation to density gradient centrifugation in cesium sulfate solution. RNA preparations derived from macrophages were found to contain an RNA-protein complex having a banding density of 1.58g/cc, intermediate between RNA (ρ = 1.66-1.68) and protein (ρ = 1.28-1.40). The minor component of the complex was sensitive to pronase and RNases but was unaffected by DNase. It contained all of the immunogenic activity, as compared to bulk RNA and had an S value of 28.

These experimental results differ somewhat from the later observations of Fishman and Adler (130), who found two immunogenic fractions in their RNA preparations. In disagreement with Gottlieb's findings also are those of Roelants and Goodman (126). In their exploration of the chemical nature of RNA-antigen complexes they found them to be of a 4-5S size and to be the only type of RNA synthesized after macrophage exposure to antigen.

Roelants and Goodman (131) have been the main exponents of the theory that RNA-antigen complexes are <u>in vitro</u> artifacts. They found that many antigens taken up by macrophages could complex with RNA - the common denominator of these antigens was the presence of charged groups in the molecule. Uncharged antigens, although immunogenic <u>in vivo</u>, were not found to be associated with RNA from

macrophages. Mixing RNA with antigen *in vitro* did not result in complex formation (126). In further studies (131) they showed the binding of RNA and antigen to be strongly dependent on Mg salts. They proposed that antigen chelated to Mg could bind and aggregate a transfer-type RNA. Recent experiments of Roelants *et al*. (132) clearly indicate that *in vitro* complexing of antigen to RNA of other cells like HeLa or even bacteria (*Escherichi coli*) occur. These complexes formed with RNA of other non-macrophage cells were highly immunogenic.

There are, of course, findings which are in direct opposition to those discussed above. Fishman and Adler (133) utilizing a cell free system have shown that RNA from normal cells (which must be functional in the immune response, e.g. spleen, liver) combined with solubilized T2 phage plus cell sap and incubated for 30-60 min at 37 C produced RNA-antigen complexes. Gottlieb (119) has offered a series of technical objections to the conclusions reached by Roelants *et al*.

RNA and Transfer of Humoral Immunity

Some observations on RNA from macrophages are not explicable on the basis of an RNA-antigen complex. Several workers have isolated RNAs, capable of transferring immune

phenomena, which they feel are free of any antigenic component. Mitsuhashi and his collaborators (134-136) extracted RNA from the peritoneal exudate cells of mice immunized with Salmonella flagella. Injections of this RNA into nonimmune mice led to the production of specific antibody as detected by an immunocyto-adherence assay. The activity of this preparation was abolished by RNase but was not affected by DNase, pronase, trypsin or anti-flagella mouse serum. Furthermore, serial transfer of the peritoneal exudate cell RNA through six mice did not alter its immunogenic activity. Johnson and Johnson (37) exposed mouse peritoneal exudate cells to BGG in vitro and demonstrated the ability of phenol extracted RNA from these cells to induce hemagglutinating antibody titers in syngeneic mice. If the BGG was labeled with ^{131}I no antigen was detected, the level of detectability set the antigen concentration at less than 10^{-11}g. In addition, pronase treatment enhanced the immunogenicity. Jachertz and Drescher (137) have extracted an "informational" RNA from a cell-free system. This RNA was capable of inducing specific antibody in recipient nonimmune guinea pigs. Its activity was abolished by RNase, and only minute amounts of RNA (0.8 ug) were required per recipient animal.

Some of the most convincing data for an "informational" RNA has come from Dray and collaborators. In early experiments Adler et al. (31) extracted RNA from peritoneal exudate cells exposed to phage of rabbits of a given allotype. This RNA was mixed with lymphocytes from rabitts of a different allotype. Some of the IgM antibody molecules carried the allotypic specificities of the donor. Bell and Dray have extended these studies considerably (138-142). Their data show that nonimmunized rabbits, homozygous for the b4 and b5 light-chain allotype, when injected with RNA extracts of lymphoid tissues from immunized rabbits, homozygous for the b5 and b4 allotype, possessed some IgM and IgG immunoglobins with the foreign allelic light chain allotype characteristic of the RNA donor (140). The RNA conversion of lymphoid cells to synthesize IgM and IgG of foreign, allelic light-chain allotype (in vivo) when assayed 18-20 hr after the RNA injection was qualitatively in accord with the data obtained for the RNA conversion of lymphoid cells in vitro (138,139). They have recently demonstrated the presence of these foreign light-chain allotype antibodies to be present in the serum up to 37 days after immunization (141,142).

Clearly, the evidence of the cited experiments suggests that immune RNAs other than those involving

RNA-antigen complexes exist.

RNA and Transfer of Cellular Immunity

Several workers have demonstrated the transfer of cellular immunity by various RNAs. Mannick and Egdahl (143) exposed normal rabbit lymph node cells *in vitro* to RNA extracted from lymph nodes of rabbits immunized by skin homografts. The normal cells became specifically sensitized as shown by their ability to elicit a positive skin reaction when injected in donors of the skin homografts, but not when injected into other rabbits. Only RNA from immune lymph nodes was effective, and this RNA was RNase sensitive. Later studies demonstrated that the RNA sensitized cells were able to bring about a second set rejection of transplanted skin (144). Fong *et al.* (145) showed that ribosomal RNA preparations from peritoneal exudate cells of BCG-immunized rabbits, injected into normal recipients, enabled the recipients' macrophages to become resistant to infection by the tubercle bacillus. Subsequent experiments demonstrated that these cells were also resistant to *Brucella* (146). These reports suggest that the RNA preparations are conferring antimicrobioal cellular immunity on macrophages.

Ramming and Pilch demonstrated that immunity to murine tumor isografts could be transferred to previously untreated mice by the intraperitoneal administration of syngeneic spleen cells pre-incubated with RNA extracted from the lymph nodes and spleens of guinea pigs immunized with the particular mouse tumor being treated (147-149).

Alexander et al. (150) demonstrated that crude RNA extracts from tumor-immunized rat or sheep thoracic duct lymphocytes could specifically retard the growth of the same primary tumor when injected back into the original donor. Purer RNA preparations were less efficient (151). It is not certain whether this was due to loss of the immunogenic component by the RNA or loss of protection against ribonucleases in the serum.

The use of immune RNA in the macrophage migration inhibition system, an in vitro correlate of delayed hypersensitivity has been investigated by several people. Thor (152) extracted RNA from lymph nodes of PPD or histoplasmin-sensitive humans and found it was able to confer on nonsensitized cells the ability to manifest migration inhibition when the cells were incubated with the specific antigen. The activity of the preparation was inhibited by treatment with small amounts of RNase but not with DNase or trypsin.

Paque and Dray (153) have reported recently that PEC from normal guinea pigs, incubated with RNA extracted from PPD and coccidiodin skin test-sensitive monkeys, were inhibited in their migration in the presence of specific antigen. They have extended these studies to show that RNA extracted from primate lymphoid tissue can convert nonsensitive human peripheral leukocytes to sensitive leukocytes (154).

Over 20 years ago it was discovered that a dialyzable fraction of leukocytes derived from tuberculin-sensitive donors had the capacity to confer their sensitivity on nontuberculin-sensitive individuals. This topic has been well reviewed by Lawrence (155) who made the original observation and named the dialyzable fraction "transfer factor." Of great interest in the last year has been the suggestion that there might exist a relationship between transfer factor and RNA-antigen complexes (156). Available information suggests that transfer factor is a complex of small molecular weight peptides associated with oligoribonucleotides (157). Gottlieb (119) has labeled crude preparations of transfer factor and submitted them to density gradient analysis in cesium sulphate solution. He found that one fraction banded as a peptide and the other banded between RNA and protein, very similar to the

ribonucleoprotein components he has reported (125). There are many differences between transfer factor and various RNA extracts which have been reported to transfer cellular immunity but these possible new beginnings, which suggest a relationship between the two, appear promising.

Use of Adjuvants to Magnify Events During Immune Induction

There are a variety of agents which enhance the immune response (158). In recent years much attention has been focused on the selectivity of these adjuvants relative to their target cell for action and also on their ability to magnify cellular events of the immune response. The two adjuvants which I will be mainly concerned with in this review are endotoxin and oligonucleotides.

The possibility that nucleic acids might in actuality stimulate the immune response was first noted in studies which were attempting to elucidate the adjuvant action of endotoxin (159). It was noticed that endotoxin was cytotoxic to the small lymphocytes which were located perifollicularly near antibody-forming cells. Consequently, it was postulated that as a result of cell-killing by endotoxin, nucleic acids were released and these nucleic acids were in fact stimulating the immune response (159). Support and extension of this hypothesis has come from many sources (38,160,161).

Polynucleotides can stimulate primary as well as secondary responses, i.e. 19S antibodies as well as 7S antibodies (37,162,163). In addition it has been demonstrated that poly A:U, given without specific antigen, but in conjunction with a modifier of lymphocyte permeability, such as diluted anti-lymphocyte serum, could evoke significant booster-type responses to previously experienced antigenic stimuli. In this respect, poly A:U, when combined with modifiers of lymphocyte permeability, can mimic some of the apparently nonspecific stimulatory efforts of bacterial endotoxins (165).

Polynucleotides not only enhance antibody formation in normally responding animals, but can also stimulate responses under conditions in which the responses are weak or impaired. Thus it has been shown that poly A:U is able to restore the capacity for an immune response to SRBC of neonatally thymectomized mice (166). Polynucleotides can also neutralize the damaging effects of X-irradiation on antibody formation (167) and can assist in the production of normal responses in genetically low responder strains (168).

Several cell populations have been reported to be targets of stimulation by polynucleotides. Johnson and Johnson (37) have given a direct demonstration of the

effects on members of the macrophage population. Increased antibody titers were observed in mice which received macrophages exposed *in vitro* to antigen and poly A:U as opposed to macrophages which were only exposed to antigen. Addition of poly A:U to macrophages incubated with antigen was associated with an increase in uridine uptake and resulting RNA synthesis which could be correlated with increased antibody synthesis.

Cone and Johnson (166) reconstituted lethally irradiated mice with poly A:U, graded numbers of T cells and constant numbers of B cells or with the reverse combination of cells. They found the elevation in anti-SRBC rosette forming cells (RFC) was inversely proportional to the number of T cells injected. Poly A:U was not able to enhance the immune response of irradiated mice when injected with graded numbers of B cells and a constant number of T cells. This evidence suggested that the adjuvant action of poly A:U was mediated through an effect on T cells. Subsequently, they reported direct evidence in support of a stimulatory effect of poly A:U on T cells. No effect on B cells was observed (169). Hamaoka and Katz (170) analyzed the adjuvant action of poly A:U in a system measuring the humoral immune response to hapten-carrier conjugates in mice and reported findings similar to Cone

and Johnson. They found that T cells had to be present for poly A:U to manifest its effects and presented evidence for poly A:U exerting its adjuvant action on a small number of unimmunized T lymphocytes. Support in favor of these findings has been forthcoming from several other laboratories (171,172).

Endotoxin (LPS) has been shown to be a modest or poor stimulant for human blood leucocytes (173,174), mouse thymocytes or mouse lymph node cells (175) but a very potent one for mouse spleen cells (174). Gery et al.(176) have shown that thymus independent B lymphocytes play the main role in the transformation response of mouse spleen cultures to LPS. Karyotypic analysis of spleen cultures from mouse chimeras with T_6T_6 thymus grafts showed that only 1.2% of the LPS-stimulated cells were of thymus origin.

Andersson et al. (177) cultured normal spleen cells with an optimally stimulating dose of LPS and evaluated protein synthesis by the incorporation of tritium labeled leucine which was added to the cultures. LPS-treated spleen cell cultures produced elevated levels of 19S proteins as compared to non-LPS treated cultures. The fact that LPS, in vitro is mitogenic on the B-cell population of lymphocytes has been reported from many other laboratories also (178-181). The relationship between the

in vitro mitogenic activity of LPS and its *in vivo* effects is unclear, and data are still too scanty to make a valid extrapolation. However, just as the mitogenic effect of LPS appears to be a property of the lipid A moiety, so too, many of the *in vivo* effects are also elicited by lipid A (182).

MATERIALS AND METHODS

Experimental Animals

Balb/Aj mice were inbred in our laboratory. The history of this strain has been documented (183). All experimental mice were from this colony and were 9-12 weeks old when used except for thymectomized mice which were 4-5 weeks old. Mice serving as donors of macrophages were 4-9 months of age and were obtained from our colony and two additional sources: (1) Inbred Balb mice obtained from a colony maintained by Dr. Richard Porter and Miss Elizabeth Dusseau, School of Public Health, The University of Michigan; (2) $Balb/_{DUB}$ obtained from Flow Research Laboratories, Dublin, Virginia. In experiments employing neonatally thymectomized mice, animals of both sexes were used. In all other experiments either male or female mice were used. New Zealand white rabbits, approzimately 1-2 kg were obtained from a local supplier.

Antigens

HGG, Fraction II was obtained from Miles Pentex Laboratories, Kanakee, Illinois (lots 31,33,37). Chromatographically purified 7S HGG (lot T-4529) was obtained from Mann Research, New York, N.Y. Mice received 0.5ml IP of a 1mg/ml solution in Eagle's Basal Medium (BME).

Antigen was used as indicated in the in vitro incubation system.

Antisera

Specific goat anti-mouse gamma globulin (lot GMG02) and goat anti-mouse IgM (lot GMM01) sera were obtained from Meloy Laboratories; Springfield, Va. Monovalent goat anti-human IgG (γ-chain specific, lot 204F043) and goat anti-human IgM (μ-chain specific, lot 205F052) were obtained from Kallestad Laboratories Inc., Chasks, Minn. Rabbit anti-bovine serum albumin (BSA) was obtained from Miles Pentex Laboratories, Kanakee, Illinois (lot 14).

Rabbit anti-HGG serum was prepared in this laboratory. Rabbits were injected intramuscularly weekly, for 4 weeks with 0.25 ml of a 10mg/ml suspension of HGG in 0.15M NaCl and Freunds complete adjuvant (FCA). One week after the 4th injection (week 5) the rabbits were bled intracardially (IC). Subsequently the rabbits were challenged subcutaneously (SC) with 1 mg of HGG on weeks 6 and 8. One week after each challenge the rabbits were bled IC (10ml/bleeding). Serum from all bleedings was pooled, absorbed with SRBC and inactivated at 56°C for 30 minutes prior to use. The serum had a hemagglutination titer of 1:71360. Anti-C_3HeB/Fe theta serum was prepared in AKR

mice according to the method described by Reif and Allen (184).

Homoribopolynucleotides

Polyadenylic acid (poly A) potassium salt (lots 11-57-301, 66, 69) and polyuridylic acid (poly U) ammonium salt (lots 11-64-308, 77) were purchased from Miles Laboratories, Elkhart, Indiana. Each polymer was stored at -20°C at a concentration of 3mg/ml in phosphate buffered saline (PBS) pH 7.2. Polymers were complexed *in vitro* by mixing equal volumes of the two stock solutions for 10 minutes at room temperature. Mice received 150ug of each polymer, or a total of 300ugms in 0.5ml of diluent. Poly A:U was used as indicated in the *in vitro* incubation system.

Endotoxin

Serratia marcesans endotoxin, SMTCA-AA, (prepared by the Boivin procedure by Dr. A. Nowotny of Temple University, Philadelphia, Pa.) was always incubated with cells at the following concentration: 10ug endotoxin/# of cells to be injected into one mouse. This ratio was established based on the dosage (10µg/mouse) found to be effective for *in vivo* adjuvant activity (204).

Enzymes

Nuclease free pronase (lot 023032) B grade was obtained from Calbiochem, Los Angeles, Calif. It was self digested for 1 hr at 37°C. Proteolytic activity was verified by solubilization of HGG, as measured by loss in capacity to be precipitated by anti-HGG.

Irradiation

Balb/Aj mice 9-12 weeks old were exposed to 800R whole-body irradiation at a distance of 71cm from an X-ray source delivering 66.7R/min, (kilovolts = 250; filter = 0.5mm Cu+1mm Al).

Peritoneal Exudate Cell Isolation

Macrophages (used synonymously with PEC) were isolated by a modification of the method of Gallily and Feldman (27). Mice were injected with 3.0 ml of thioglycollate medium IP. Five days later the cells in the peritoneal cavity were washed out with 10.0 ml of Hanks balanced salt solution (HBSS), pH 7.2, containing penicillin, streptomycin and heparin (100 units, 50 ug, and 5 USP/ml respectively). Purification was achieved by centrifugation at 900 rpm for 10 min, removal by aspiration of the supernatant fluid and top 1-2 mm packed cells, resuspension of the pellet in 10 ml HBSS, pH 7.2, and repetition of the centrifugation

2 times. Finally, the pellet was suspended in 5 parts 0.2% NaCl for 20 seconds to lyse the red cells. The cells were mixed for 20 seconds, immediately diluted 10-fold with BME and counted in a hemocytometer. The cell population was routinely greater than 95% macrophages and contained approximately 1% lymphocytes (determined by differential counts).

In Vitro Incubation of Macrophages with Antigen

Incubation with antigen was carried out for 2 hr in a 37°C shaking water bath, in screw cap 50ml Erlenmeyer flasks. Cells were suspended at a concentration of 5×10^7 cells/ml and incubated with antigen at a cell:antigen ratio of 2.5×10^7 cells:0.5 mg HGG. After incubation cells were washed 2 times with BME and centrifuged for 20 min at 1000 rpm. The washed packed cell pellet was frozen in an acetone-dry ice bath and stored at -20°C for later RNA extraction. Macrophages to be used for normal RNA were treated as above except, after harvesting and washing, the cells were frozen and stored rather than being incubated with antigen.

Isolation of RNA from Macrophages

Purified macrophages which had been accumulated and stored at -20°C were thawed in 2 volumes of buffer (0.15 M NaCl, 0.1 mM EDTA containing 10^{-3} M $MgCl_2$ and 0.5% SDS).

The cells were then homogenized for 20 strokes in a glass homogenizer. An equal volume of freshly distilled water saturated phenol (equal volumes of phenol and warm water mixed and allowed to separate for 60-70 min at room temperature, phenol then removed) was added and the mixture shaken for 10 min at 25°C and stirred 3 min at 63°C. The aqueous phase was removed after centrifugation at 1000 rpm for 15 min. An equal volume of chloroform-isoamyl alcohol (24:1 v/v) was added to deproteinize the aqueous phase and after shaking for 10 minutes the mixture was centrifuged at 1000 rpm for 10 minutes. This deproteinization was repeated 3 times. The resulting aqueous phase was treated with 1 mg self-digested pronase/ml of nucleic acid for 60 min at 25°C. The pronase treated nucleic acid was deproteinized again with chloroform-isoamyl alcohol and finally precipitated with cold absolute ethanol. Precipitation was allowed to proceed for 24 hrs at -20°C followed by centrifugation at 17,000 rpm for 30 min. The pellet was resuspended in a small quantity of buffer (0.2 M LiCl, 0.02 M Tris pH 7.8, 10^{-3} M $MgCl_2$) and concentrations determined by absorption at 260 mu ($A_{260}^{1\ cm/1\%}$=230).

Cell Preparations

Spleens were removed aseptically from mice, extraneous

fascia removed and spleens rinsed 2 times in sterile HBSS (penicillin, 100 units/ml and streptomycin 50 ug/ml). Spleens were sectioned along their midline and gently teased with a scapel. The resulting cell suspension was aspirated several times through a syringe, passed through an 80-mesh screen, washed 3 times in HBSS and then subjected to an osmotic shock treatment with 5 volumes of 0.2% NaCl for 20 seconds, then immediately diluted 10-fold with BME and centrifuged. The cells were suspended in BME with 5%-20% calf serum containing penicillin and streptomycin, to a concentration of 10^7 viable cells/ml. Adherent cells were separated from spleen cell preparations by three 45 min culture periods in Falcon plastic petri dishes. The nonadhering cells were decanted after each incubation period into fresh petri dishes. After the final incubation period the nonadhering cells were decanted, centrifuged and resuspended in fresh BME, 5-20% calf serum at a concentration of 1-1.5 X 10^7 cells/ml.

Thymus lymphocytes were obtained from 4-5 week old male or female Balb/Aj mice. Mice were killed by cervical dislocation, thymic lobes removed and minced in cold HBSS (penicillin-100 units/ml). Cell suspensions prepared in this manner were 90-98% viable as determined by trypan blue dye exclusion.

Bone marrow cells were prepared from 6-9 month old male or female Balb/Aj mice. Animals were killed by cervical dislocation and the hind legs removed. Muscle and skin was removed from the femurs and tibae and the epiphyses were snipped. A 26 gauge needle attached to a syringe containing cold HBSS was inserted into the bone and the marrow plug was flushed out. The extruded plugs were dispersed by aspiration with a syringe and a 21 gauge needle and the cells were washed 3 times in HBSS and suspended to a concentration of 10^7 viable cells/ml. Suspensions prepared in this manner yielded 87-95% viable cells as determined by trypan blue dye exclusion.

In Vitro Incubation Procedures

Spleen cells, prepared as described above, were suspended to appropriate concentrations in BME containing 2mM L-glutamine, 5-20% calf serum, penicillin 100 units/ml and streptomycin 50ug/ml. Appropriate concentrations of antigen, RNA, poly A:U and antisera were added, depending on the individual experiment. In cases where antiserum was used, it was added last.

Thymus or bone marrow cells were suspended to appropriate concentrations in BME (2mM L-glutamine, penicillin and streptomycin as described above). In experiments where

thymus and bone marrow cells were incubated together it was at a ratio of 4×10^7 thymus cells/10^7 bone marrow cells/ml of BME.

Five ml of the cell suspensions plus various experimental additives were put into 50 ml screw capped Erlenmeyer flasks. A gas mixture of 95% air, 5% CO_2 was introduced into each flask when needed. The suspensions were incubated at 37°C in a shaking water bath for 1.5 hr. After incubation the cells were washed 2 times with 100 volumes of HBSS, resuspended in HBSS and injected IV in 0.5 ml volumes.

Neonatal Thymectomy

Thymectomy was performed by a modification of the method of Sjodin (185). Mice, 6-8 days old, were anesthetized by exposure to ether for 1-1.5 min. A midline incision was made from the level of the mandible to the first rib. Each thymic lobe was removed by aspiration with a pasteur pipette attached to a suction system. The incision was sutured and the mice warmed under a light until they were fully recovered from the anesthesia.

Mice were checked for thymic remnants prior to removal of their spleen for assay. Approximately 5% of the animals had thymic remnants that could be visualized at 25 x magnification using a dissecting microscope.

Isotopic Labeling Procedure

Balb/$_{Aj}$ mice, 9-11 wks of age were lethally irradiated and 24 hr later reconstituted with various populations of treated or non-treated cells. Five days after reconstitution mice received 8 uCi IP of ^{3}H-methylthymidine (15.7 Ci/mM, Amercham-Searle, Arlington Heights, Ill.). Three hr afterwards, all mice were killed as quickly as possible by cervical dislocation, spleens removed and each spleen placed in 5 ml of cold 0.15 M NaCl. Each spleen was homogenized in a glass homogenizer and without allowing the suspension to settle, triplicate samples (0.5 ml/sample) were taken and spotted onto Whatman glass fiber discs. The discs were then washed with 10 ml each of cold 0.15 M NaCl and 10% TCA followed by 2 ml washes of 95 % ethanol then absolute ethanol. The disc were dried either overnight in a 37°C warm room or for 10 min under as infrared light. Dried discs were placed in scintillation vials, and 10 ml toluene-PPO-POPOP poured into the vials. Samples were counted on either a Beckman Liquid Scintillation Counter or New England Nuclear Liquid Scintillation Counter.

Anti-Theta Treatment of Bone Marrow Cells

Anti-theta treatment of bone marrow cells to obtain a pure B cell population was carried out according to the

procedure of Neiderhuber (186). Bone marrow cells were obtained as described earlier. After the cells were washed 3 times they were suspended in 1:50 diluted anti-theta serum at a concentration of 10^7 cells/ml of anti-theta. (The dilution of anti-theta serum had been determined previously by a ^{51}Cr release cytoxicity study which showed a 1:400 dilution to yield 50% ^{51}Cr release. To insure maximum cytotoxicity a dilution 8 times more concentrated than that shown to give 50% ^{51}Cr release was used). The cells plus anti-theta were incubated in a 50 ml screw cap centrifuge tube, at an angle, for 30 min at 37° C. The tube was shaken every 10 min. The cells were centrifuged and washed once in HBSS after which the washed pellet was suspended, at a concentration of 10^7 cells/ml, in 1:6 diluted guinea pig complement (previously absorbed with syngeneic spleen cells and agarose). The cells were incubated under the same conditions with the guinea pig complement as with the anti-theta serum. After 30 min incubation at 37°C the cells were washed three times in HBSS after which they were counted. According to trypan blue exclusion the cells were 97% viable.

Absorption of Guinea Pig Complement (GPC')

Complement, either reconstituted or from freshly bled guinea pigs was used. Ten ml of GPC' was added to 240 mg

of agarose and the mixture incubated at 4 C for 30 min with frequent shakings. The suspension was centrifuged for 15 min at 8000 rpm in a Sorvall RC 2-B after which the GPC' was decanted and the centrifugation repeated. To the agarose absorbed GPC' was added 1 ml of 0.1 M EDTA in distilled water to preserve complement activity during spleen cell absorption. The GPC' was then added to 1 ml of packed syngeneic spleen cells (prewashed once). The absorption was allowed to proceed for 30 min at 4 C with frequent shakings. Following the absorption the suspension was centrifuged for 20 min at 8000 rpm and the GPC' decanted. To 9 ml of the freshly absorbed GPC' was added 1 ml of 0.1 M $CaCl_2$ to replace ions previously chelated by EDTA. The GPC' was immediately aliquoted and stored at -70 C. All complement was thawed only once before use.

Determination of Antibody

Passive Rosette Assay. Nine days after reconstitution, the immune response to HGG was measured by the rosette assay (187, 188) as adapted for protein antigens by Stout (189). Spleens from each experimental group were pooled, dispersed and washed in PBS, pH 7.2. SRBC were washed 3 times in PBS, pH 7.2, and resuspended to a 50% concentration. To 3 ml HGG (20mg/ml) and 0.1 ml 50% SRBC, 0.5 ml

(100 mg/ml) ECDI[1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl] obtained from Ott Chemical Co., Muskegon, Michigan, was added. The mixture was incubated 50-70 min at 4°C, with slow stirring on a magnetic mixer. The cells were then centrifuged at 1500 rpm for 10 min, washed twice in 1.0% NRS in PBS, pH 7.2, and finally resuspended to a concentration of 5%. A tenth of a ml of a 5% SRBC suspension was added to 1 ml of a 5-10 x 10^6 spleen cells/ml suspension in a 51 x 60 mm tube. The tubes were incubated at a 45 angle for 1 hr and overnight at 4°C. Rosette forming cells were enumereted by counting the spleen cell-SRBC mixture in a hemocytometer at 200X magnification. Approximately 20,000 to 80,000 spleen cells were counted, depending on individual experiments. In all experiments using irradiated mice rosettes were determined on non-irradiated mice (injected only with saline) as a positive control of the technique.

Determination of Plaque Forming Cells. The number of hemolytic plaques in agar (PFC) was determined by the method of Jerne (190) as modified by Golub (191). A volume of 0.05 ml of the cell suspension and 0.05 ml of a 14% suspension of HGG conjugated SRBC were added to 0.5 ml of 0.5% agarose solution. The agarose solution was kept at 49°C in a water bath prior to the addition of the cells. The mixture was immediately layered on glass slides.

After 1 hr of incubation at 37°C PFC were developed by incubating slides for 3 hr at 37°C on special racks in the presence of GPC' diluted 1:10.

Passive Hemagglutination. Mice were anesthetized with ether and the axillary vessels were severed. Blood was collected by capillary pipettes. Equal volumes of blood were obtained from all mice in a group and pooled. Serum was stored at -20°C until used. Serum titers were determined under identical conditions.

Antibody content in the serum was determined by the passive hemagglutination test utilizing a modification of the method of Boyden (192). SRBC were washed twice in 0.15M NaCl, once in PBS pH 7.2. This 2.5% suspension was mixed with four volumes of PBS pH 6.4 containing 3.0 mg antigen /ml of 2.5% SRBC suspension. This mixture was incubated with stirring, for 20 minutes at room temperature. Cells were washed once in PBS pH 7.2 and once in 1:250 NRS in PBS pH 7.2 and resuspended to 2.5% in 1:250 NRS. All dilutions of serum were made in 1:50 NRS and PBS pH 7.2. Each serological tube contained a final volume of 0.5 ml and to this was added 0.05 ml of the SRBC suspension. After mixing, the suspensions were incubated for 2 hr at room temperature (25°C). Tubes giving a ragged pattern or diffuse settling of cells in the entire bottom of the tube

were scored as positive agglutinations. The titers were recorded as the reciprocal of the highest dilution of antiserum giving agglutination.

Statistical Methods

The variance (s^2) for experimental groups was calculated as follows:

$$s^2 = \frac{n(\sum_{i=1}^{n} x^2) - (\sum_{i=1}^{n} x)^2}{n(n-1)}$$

where n= number of identically treated groups in different experiments or the number of ratios, resulting from pairing identically treated control and experimental groups in repeated experiments.

x= experimental number of RFC/10^6 spleen cells obtained by pooling spleens in one group or ratio obtained when RFC/10^6 spleen cells of the control group is compared with the experimental group.

The standard error of the arithmetic mean (SE) was

calculated by:

$$SE = \frac{s}{\sqrt{n}}$$

where s = the square root of the variance

n = number of experimental groups (treated the same in various repeated experiments).

The differences between experimental groups were determined as follows: The hypothesis tested stated that the RFC response of mice undergoing treatment A was similar to that of mice undergoing treatment B, i.e. $H: \mu_A - \mu_B = 0$ where μ = arithmetic mean of the number of RFC in repeated groups of various experiments. When the Student paired t test was used the hypothesis tested stated that the ratio of the RFC response of mice undergoing treatment A compared to mice undergoing treatment B was equal to 1 where μ = arithmetic mean of the ratios of RFC in the compared groups in various experiments. The Student t test formula for two populations was used to determine the probability of this hypothesis.

$$t = \frac{\bar{x}_A - \bar{x}_B}{s\sqrt{\frac{1}{n_A} + \frac{1}{n_B}}}$$

where n = number of identically treated groups in various experiments or number of ratios, resulting from pairing identically treated control and experimental groups in repeated experiments.

$\bar{x}$ = arithmetic mean of RFC/10^6 spleen cells of repeated experimental groups or of ratios obtained by pairing control to experimental groups in repeated experiments.

s = square root of the variance in each of the set of ratios being evaluated or the set of results from repeated experiments being evaluated.

The t value was located in standard statistical tables and the p value with which the hypothesis could be negated was established. P values of 0.01 or less were considered highly significant. Experiments reported in this dissertation were repeated at least once except for experiments appearing in table 6, which were not repeated.

RESULTS

In earlier studies carried out in this laboratory (37) it was demonstrated that macrophages treated with bovine gamma globulin were able to induce an enhanced immune response as compared to BGG alone. When RNA was extracted from such macrophages it was capable of inducing antibody formation to BGG, as measured by hemagglutination and RFC. Initial studies utilizing radioisotopic labeling could not demonstrate antigen as a contaminant of the RNA preparation. As the absence or presence of antigen was important in understanding the mechanism of action of these RNA-rich extracts, an in depth examination of this question was undertaken in this thesis study. HGG was chosen as a more advantageous antigen system for study, inasmuch as monospecific antisera were available against the various chains of this molecule. Thus, if any antigen were present and functional its characterization would be made easier.

Effect of In Vitro Incubation with Macrophages on the Immunogenicity of HGG

Prior to investigating the presence or absence of antigen in RNA preparations it first was necessary to

demonstrate that the immunogenicity of HGG could in fact be enhanced by exposure to PEC and that RNA extracted from such cells could induce antibody formation to HGG. The optimal concentration of antigen for antibody induction *in vivo* was determined by injecting 3 different antigen concentrations IP into groups of 3 mice each. On days 6, 8, and 11 after injection of HGG the mice were bled, sera from groups of 3 mice were pooled and antibody titers determined by passive hemagglutination. Peak titers of 1:640 on day 8 were observed with 0.5 mg and 0.25 mg whereas the 1.0 mg dosage titered at 1:80 on the same day. Five-tenths milligram was selected as the preferred dosage of HGG. The experimental procedure involved exposure of macrophages to the antigen *in vitro* followed by use of the exposed macrophages as the source of antigenic stimulation.

The results of incubating HGG with macrophages at a ratio of 0.5 mg HGG/2.5 x 10^7 macrophages for 2 hr at 37°C can be seen in table 1. Such macrophages gave significantly higher HA titers on days 7 and 9 than HGG injected alone. When poly A:U was added to the incubation mixture a 1 tube higher titer was observed. When RNA was extracted from macrophages which had been exposed to HGG, this RNA was biologically active and could induce antibody formation.

As can be seen in table 2, on day 4, 0.1 mg of RNA was able to induce rosette forming cells to almost the same extent as HGG alone. Previously (37) it had been shown that there was a maximum antigen contamination of 10^{-11} g Thus table 1 and 2 confirm the previous work in this laboratory (37).

Demonstration of the Presence of Antigen in Macrophage-RNA Preparations

If antigen were indeed a contaminant of the macrophage-RNA moiety and consequently responsible for its antigenic specificity, then antiserum against the antigen should be able to abrogate the antibody inducing ability of the RNA (127). Initially attempts were made to incubate the RNA with anti-HGG serum, then inject the RNA into mice. However the presumed activity of serum nucleases destroyed all RNA activity, as measured by loss of the RNAs ability to induce RFC to HGG. The presumed adverse effects of serum nucleases will be discussed later. Several different methods were used to try and circumvent this problem. Anti-HGG was complexed onto polyacrylamide gel and macrophage-RNA samples passed through with hopes that any RNA:antigen moieties would be retained and RNA molecules would pass through. However, there was a great deal of non-specific absorption and the overall loss in RNA material was too great to warrant use of the technique for multiple

TABLE 1

INCREASED ANTIBODY SYNTHESIS INDUCED BY EXPOSURE OF ANTIGEN TO MACROPHAGES

Experimental Group	Reciprocal HA Titer Day 7	Day 9
HGG only	640	160
Macrophages + HGG	5120	1280
Macrophages + HGG + Poly A:U	10240	2560

Dosage: HGG - 0.5mg/mouse; poly A:U - 150ug each; Mice received 1.5 x 10^7 macrophages IP (incubated at a ratio of 0.5mg HGG/2.5 x 10^7 macrophages/300 ug poly A:U). Irradiated mice (800R) were bled on days 7 and 9, the serum pooled and titered by passive hemagglutination. Each group contained 3 mice.

TABLE 2

Induction of Antibody Formation by

RNA Isolated from Macrophages

Experimental Group	RFC/10^6 Spleen Cells Day 4	Day 6
NaCl only	600	1600
HGG only	6600	4000
Macrophage RNA	4600	4300
Macrophage RNA + poly A;U	8600	6700

Dosage: HGG - 0.5mg/mouse; macrophage RNA - 0.1mg/mouse; poly A:U - 150 ug of each. Irradiated mice (800R) received above materials IP. Spleens were removed on day 4 and 6, pooled and assayed for RFC. Each group represents data obtained from 3 mice.

studies. In other studies the gamma globulins were precipitated from the anti-HGG serum and purified on DEAE-cellulose. These purified globulins were then used instead of the whole serum in an attempt to block the effects of the serum nucleases. The IgG fraction of the rabbit anti-HGG serum failed to work also in that factors were still present which inactivated the RNA. However, employing the culture system of Mosier (3), which utilized adherent and nonadherent cells, a more suitable system for studying this question was found.

Nonadherent spleen cells (NASC) obtained as outlined in Materials and Methods were mixed with either RNA alone, or RNA from macrophages incubated with HGG(RNA:HGG) or RNA:HGG plus NRS or rabbit anti-HGG serum (10^7 NASC/0.1 mg RNA/0.4 ml serum). Following incubation and several cell washings, 10^7 NASC were injected into mice which had been lethally irradiated (800R) 24 hrs earlier. Nine days after reconstitution the mice were sacrificed, their spleens removed and assayed for RFC and/or PFC. In table 3 it can be seen in the 3 experiments done, that whenever rabbit anti-HGG was incubated along with the RNA:HGG and NASC, the effect of the RNA:HGG was abrogated. This loss of ability to induce antibody formation by the RNA:HGG was not due to serum nucleases as when NRS was

TABLE 3

Neutralization of Macrophage-RNA by

Rabbit Anti-HGG Serum

Experimental Group	RFC/10^6 Spleen Cells Expt. 1	Expt 2	Expt 3
A) Non-irrad control	1000	2000	900
B) NASC+n-RNA	3200	8200	2800
C) NASC+RNA:HGG	8100	20,600	6500
D) NASC+RNA:HGG+NRS	7100	15,400	5600
E) NASC+RNA:HGG +Rabbit anti-HGG	* 2300	6600	3100

n-RNA = RNA from normal macrophages.
RNA:HGG = RNA from macrophages exposed to HGG in vitro.
NASC = Nonadherent spleen cells.

Dosage: 10^7 NASC/0.1mg RNA/.4ml serum (anti-HGG HA titer = 1:71360) incubated 1.5 hr at 37°C. Irradiated mice (800R) received 10^7 NASC IV Spleens were removed on day 6, pooled and assayed for RFC. Each group represents data obtained from 3-5 mice.

* When ratios between groups E and groups D for all 3 experiments were evaluated collectively by Student's paired t test, group E results, together, differed from group D at p= .05.

added there was no decrease in antibody formation.

In all of the following experiments it can be observed that when mice were irradiated and reconstituted with cells treated with n-RNA the RFC response was much higher than in the non-irradiated controls. This increase in RFC response has been attributed to the RNA acting as an adjuvant (163) on the background response to HGG. Thus n-RNA treated cells are the control group to which experimental groups are always compared. The non-irradiated group serves only as a control on the technique and the overall background response of the mice used for the experiment.

Because of the many presumed variables involved in this system (e.g. variation in amount of antigen actually conjugated to the SRBC; potency of individual batch of RNA, number of NASC which homed to spleen), it was difficult to compare results from one experiment to another. Others have also reported a variance of absolute numbers of PFC in different experiments because of different efficiencies of hapten coupling (193). Consequently, groups could only be compared within an individual experiment. Though there is great variability in the absolute numbers of RFC from experiment to experiment, the relative differences between groups are of the same magnitude. Thus, using Student's t test and comparing group E, which received NASC which had been incubated with RNA:HGG + rabbit anti-HGG, with group D,

which received NASC incubated with RNA:HGG + NRS, group E was found to be significantly lower than group D at a p value of 0.05. If compared with group C it was significantly lower at a p value of 0.05.

In several experiments spleens were assayed for RFC and PFC to ascertain whether these two assays for antibody forming cells gave comparable results. In most cases, as exemplified in figure 1, the results were comparable but the plaque assay was much less sensitive, giving results about 100-fold less than RFC. In several other experiments however, RFC and PFC differed greatly. Possible explanations for this will be offered later.

Thus, the results of these initial experiments were interpreted as evidence that a fragment of antigen was complexed to the RNA.

Studies on the Nature of the Antigen Complexed to Macrophage-RNA

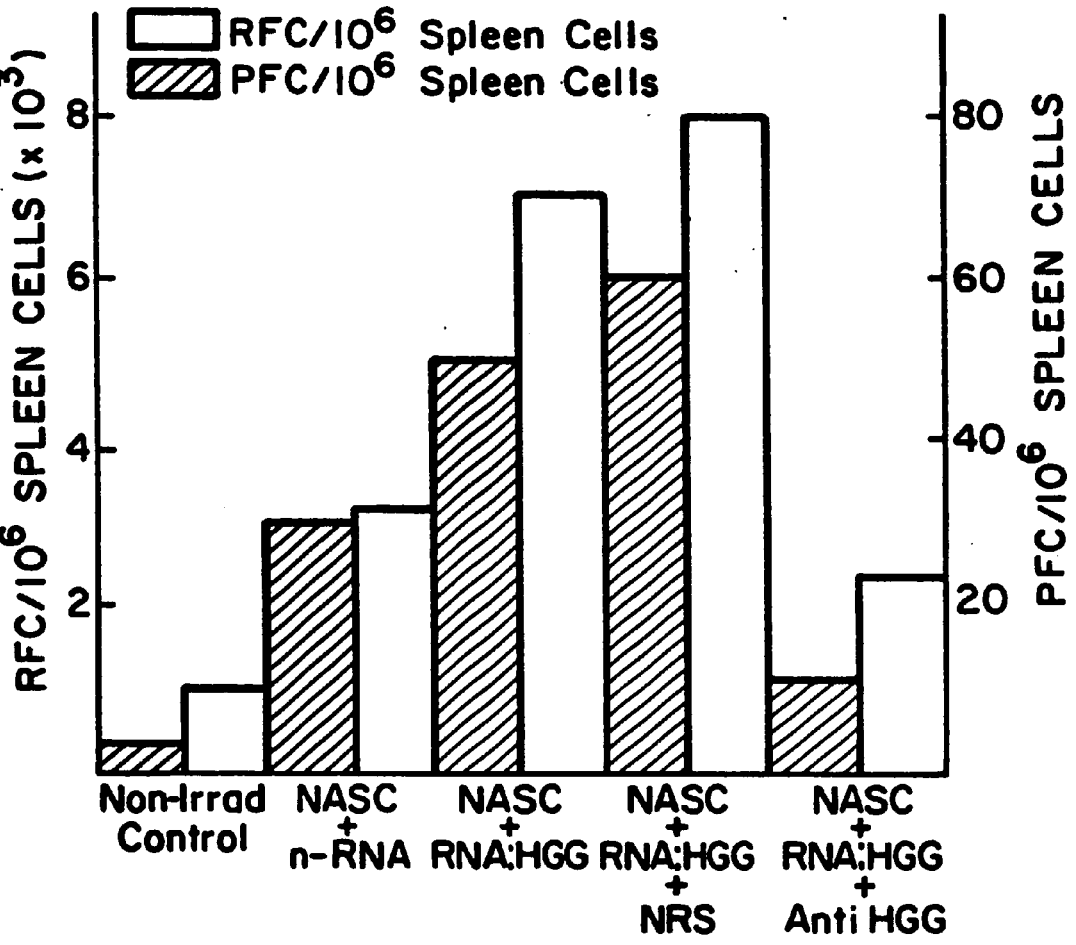
With the foregoing workable system and data to indicate the presence of antigen in the RNA preparations, studies were initiated which would give more insight into the nature of the antigen or fragment of antigen complexed to the RNA. Though the earlier studies had given evidence that the ability of macrophage-RNA was not due to serum

Figure 1

CORRELATION BETWEEN RFC AND PFC

IN LETHALLY IRRADIATED MICE

RECONSTITUTED WITH NASC



Irradiated mice (800R) received 10^7 NASC iv which had been incubated in vitro [10^7 NASC/0.1mg RNA/.4ml serum (HA titer = 1:71360)] for 1.5 hr at 37°C. Spleens were removed on day 9, pooled and assayed for RFC and PFC.

nucleases it was not conclusively shown that this effect was specific for HGG. Thus, prior to study of the nature of the antigen fragment, the specificity of the effect of rabbit anti-HGG serum was determined.

The same experimental design was used as discussed above. Lethally irradiated mice were reconstituted 24 hrs after irradiation with 10^7 NASC which had been exposed *in vitro* to RNA:HGG with or without rabbit anti-HGG or rabbit anti-BSA serum. Nine days after reconstitution the mice were sacrificed, and their spleens were removed, pooled and assayed for RFC. The results of this experiment can be seen in table 4. Several groups had to be omitted in one of the experiments due to shortage of RNA; but, both experiments gave meaningful results. When mice were injected with NASC which had been exposed to RNA:HGG + anti-HGG, their ability to produce antibody to HGG was reduced to that of mice receiving NASC exposed to RNA from normal macrophages (n-RNA), 3100 RFC/10^6 spleen cells versus 2800 RFC/10^6 spleen cells, and significantly lower than mice receiving cells incubated with RNA:HGG. In contrast, when mice received NASC exposed to RNA:HGG + anti-BSA serum their ability to induce rosette formation to HGG was basically unaffected, as the number of rosettes was the same as in mice receiving cells exposed to RNA:HGG

TABLE 4

SPECIFICITY OF RABBIT ANTI-HGG SERUM FOR RNA:HGG

Experimental Group	RFC/10^6 Spleen Cells	
	Expt 1	Expt 2
A) Non-irrad Control	900	700
B) NASC+n-RNA	2800	4200
C) NASC+RNA:HGG	6500	ND
D) NASC+RNA:HGG+NRS	5600	ND
E) NASC+RNA:HGG+Anti-HGG	3100	5600
F) NASC+RNA:HGG+Anti-BSA	* 5600	11,900

Irradiated mice (800R) were injected IV with 10^7 NASC which had been exposed *in vitro* to RNA:HGG ± antiserum (10^7 NASC/0.1mgRNA/0.4ml anti-serum) 24 hrs after irradiation. Nine days after reconstitution mice were sacrificed, spleens removed, pooled and assayed for RFC. Each group represents data obtained from 3-5 mice.

Anti-BSA = Rabbit anti-bovine serum albumin (HA titer greater than 1:142,720)

ND = Not done

* When the ratios between group F and group E (or B) in Expt 1&2 were compared collectively by Student's paired t test group F data, collectively, differed from group E or B at $p = .05$.

only. This can be seen by comparing groups C and F. In experiment 2, though group C was not done, the RFC response of NASC exposed to RNA:HGG + anti-BSA (11,900 RFC/10^6 spleen cells) was much higher than the response observed with NASC + n-RNA (4200 RFC/10^6 spleen cells) or NASC + RNA:HGG + anti-HGG (5600 RFC/10^6 spleen cells). These results confirmed the earlier findings which suggested that HGG was complexed to the macrophage-RNA and offered evidence for specificity.

Several investigators have reported the presence of fragments of antigen which remained on macrophage membranes and were antigenic (82,83). Also, the presence <u>in vivo</u> of fragments of antigen associated with oligoribonucleopeptides in spleen and lymph nodes has been reported (194,195). Thus, I considered the possibility that the antigen present in the RNA preparation might be only a part of the gamma globulin molecule. To investigate this possibility, studies reported in table 5 were done. NASC were exposed <u>in vitro</u> to RNA:HGG. If at the time of this exposure, NRS or goat anti-human IgM (absorbed so that it was μ chain specific) was also included in the mixture, the spleen cells, when injected into lethally irradiated mice were able to induce antibody formation to HGG. This can be seen by looking at groups C and E which do not differ significantly. On the other hand, if the

TABLE 5

EFFECT OF VARIOUS MONOSPECIFIC ANTISERUM ON THE ANTIBODY INDUCING CAPABILITY OF RNA:HGG

Experimental Group	RFC/10^6 Spleen Cells	*P value
A) Non-irrad control	650 ± 100	-
B) NASC+n-RNA	3500 ± 80	.01<p<.05
C) NASC+RNA:HGG+NRS	9950 ± 2500	-
D) NASC+RNA:HGG+GAGC	4650 ± 290	.01<p<.05
E) NASC+RNA:HGG+GAMC	8750 ± 2480	n.s.

GAGC = Goat anti-IgG (gamma chain specific) serum
GAMC = Goat anti-IgM (mu chain specific) serum

SE = Standard error of arithmetic mean

n.s. = not significant

* All groups compared to group C

Irradiated mice (800R) were injected with 10^7 NASC IV, which had been exposed *in vitro* to RNA:HGG ± antiserum (10^7 NASC/0.1mg RNA/0.4ml antiserum). Spleens were removed on day 9, pooled and assayed for RFC. Data represents the arithmetic mean of results of two experiments, 3 mice/group.

NASC were incubated with RNA:HGG in the presence of goat anti-human IgG (absorbed so that it was monospecific for δ chain), the ability of these cells to induce antibody formation to HGG in vivo was reduced. From the table it can be seen that group D differs significantly from group C at a p value of 0.05.

Preliminary studies utilizing antiserum specific for either kappa or lambda chain in the same type of experimental design as described above suggest that at least part of the antigen fragment on the RNA:HGG is kappa chain. Evidence for this can be seen in table 6. When mice were reconstituted with NASC treated with RNA-HGG + anti-kappa chain serum, their ability to produce RFC was three-fold lower than mice receiving cells exposed to RNA:HGG + NRS. In contrast, cells treated with anti-lambda chain serum showed only a slight decrease in RFC. This can be seen in table 6 where cells treated with RNA:HGG + NRS had 8100 RFC/10^6 spleen cells whereas NASC treated with anti-lambda serum had 6100 RFC/10^6 spleen cells. These are preliminary results and confirming studies relative to the identification of light chain fragments on the RNA:HGG are necessary. However, these data are interpreted as evidence that the fragment of antigen on the RNA:HGG consists of at least part of the gamma heavy chain and kappa light chain. Further studies are needed to determine if the gamma and kappa chains are present in their entirety and

TABLE 6

EFFECT OF ANTI-KAPPA AND ANTI-LAMBDA CHAIN ANTISERUM ON THE ANTIBODY INDUCING CAPABILITY OF RNA:HGG

Experimental Group	RFC/10^6 Spleen Cells
A) Non-irrad control	1000
B) NASC+n-RNA	3800
C) NASC+RNA:HGG+NRS	8100
D) NASC+RNA:HGG+EALC	6100
E) NASC+RNA:HGG+EAKC	2600

EALC = Equine antiserum to human lambda chain
EAKC = Equine antiserum to human kappa chain

Lethally irradiated mice (800R) were injected IV with 10^7 NASC. Cells were treated as in table 5. Spleens were removed on day 9 and assayed for RFC. Each group represents 3 mice.

to ascertain whether lambda chains are definitely absent.

Although, in view of the above data, it was unlikely that a contaminant protein other than gamma globulin was responsible for the antigenicity, additional supportive data were gained from the following experiment. This experiment was designed the same as those reported earlier in tables 4 and 5 but when the spleens were assayed for RFC, red blood cells were used which were conjugated with fraction II HGG (the usual assay) and also RBC which were conjugated with chromatographically pure 7S globulin. It can be seen in table 7 that approximately 75% of RFC which were resolved using fraction II HGG were directly responsive to 7S globulin also.

Cellular Requirement for Antibody Formation to HGG in Reconstituted Lethally Irradiated Mice

Johnson and Johnson (37) had demonstrated that antigen activated macrophages were capable of stimulating nonadherent cells to produce antibody. However, Meiss and Fishman (128) reported that in their system RNA extracted from T2 phage-activated macrophages required both adherent and nonadherent cells for antibody formation. Consequently, it was important to extend the earlier studies done in

TABLE 7

COMPARISON BETWEEN RFC DETECTED BY PURIFIED HGG(7S) VERSUS IMPURE HGG(FRACTION II)

Experimental Group	RFC/10^6 Spleen Cells +Fraction II	*7S	%RFC to 7S Globulin
Non-irrad control	1000	700	70
NASC+n-RNA	4000	3200	80
NASC+RNA:HGG	8400	7400	88
NASC+RNA:HGG +anti-HGG	3700	2100	57

+ SRBC conjuated with fraction II HGG used to assay RFC

* SRBC conjugated with chromatographically pure 7S gamma globulin used to assay for RFC.

Irradiated mice (800R) received 10^7 NASC treated as in table 4. Spleens removed on day 9, pooled and assayed for RFC using SRBC conjugated with fraction II HGG or chromatographically pure 7S gamma globulin.

this laboratory by investigating whether RNA extracted from macrophages exposed to HGG *in vitro* was able to induce antibody production in nonadherent spleen cells *in vivo*.

Both adherent cells and nonadherent cells were needed for antibody production to HGG in my control system, as seen in table 8. When RNA extracted from macrophages exposed *in vitro* to HGG (RNA:HGG) was incubated with either nonadherent cells or a mixture of nonadherent and adherent cells for 1.5 hr at 37 C and the cells injected subsequently into lethally irradiated (800R) syngeneic mice it was seen, table 9, that the adherent cell population was not needed by the RNA:HGG complex for induction of an antibody response. The antibody response observed was specific for the antigen exposed to the macrophage as RNA extracted from macrophages that had never been exposed to antigen produced a significantly lower response. It was concluded that macrophage exposure to HGG caused the formation of an RNA-rich antigen fraction which, on isolation, was immunogenic and abrogated the need for the macrophage (adherent cell) in initiating an antibody response.

Induction of Antibody Formation in T and B Cells by RNA:HGG

Collaboration between T and B lymphocytes occurs in

TABLE 8

CELLULAR REQUIREMENTS FOR

ANTIBODY RESPONSE TO HGG

Experimental Group	RFC/10^6 Spleen Cells Expt 1	Expt 2	Expt 3
Non-irrad Control	900	600	1100
NASC + HGG	2200	4400	3200
NASC + ASC + HGG	*6400	14,700	5300

NASC = nonadherent spleen cells
ASC = adherent spleen cells

Irradiated mice (800R) received 10^7 NASC or NASC+ ASC incubated in vitro 1.5 hrs with HGG (10^7 cells/0.5mg HGG). Cells were injected IV in a volume of 0.5 ml. Nine days after reconstitution, mice were sacrificed, spleens removed and assayed for RFC. Each group represents data pooled from 4-5 mice.

* When ratios between NASC+ASC+HGG groups and NASC+HGG groups in all three experiments were compared by Student's paired t test, the NASC+ASC+HGG data collectively differed from the collective data of the NASC+HGG groups at a p value of .05.

TABLE 9

ABILITY OF MACROPHAGE-RNA TO REPLACE

NEED FOR ADHERENT CELL

Experimental Group	RFC/10^6 Spleen Cells Expt 1	Expt 2	Expt 3
Non-irrad control	100	1000	400
NASC + n-RNA	2500	3200	1600
NASC + RNA:HGG	*7100	7100	3800

Irradiated mice (800R) received 10^7 NASC (treated as in table 4). Nine days after reconstitution mice were sacrificed and spleens assayed for RFC. Each group represents data of spleens pooled from 4-5 mice.

* When ratios between group 3 and 2 (Expt 1-3) were evaluated collectively by Student's paired t test, group 3 results differ significantly from group 2 results at p=.01.

the induction of antibody formation to many antigens, including heterologous erythrocytes (196, 197), serum proteins (198) and hapten-protein conjugates (199). Feldman (19) in a recently reported paper gave data supportive of his proposed hypothesis that macrophages are required for antibody responses to protein antigens involving T and B cell collaboration. According to him, the macrophage acquired receptors from T cells, which then bind antigen and the macrophage transports the antigen to the B cell. In contrast to this, Seeger and Oppenheim (94) have shown that macrophage-bound antigen directly activated the T cell. Since my data showed that RNA:HGG could induce antibody formation in NASC and that it abrogated the need for the adherent cell, it was important to investigate the interaction between RNA:HGG and T and B cells. This interest was enhanced by Unanue's report (200) on the inability of macrophage-bound antigen to replace the need for the T cell in antibody production and also by the mixed reports referred to above on the interaction of macrophage-bound antigen with T and B cells.

Thus, the following questions were proposed:

1) Can RNA extracted from antigen exposed macrophages (RNA:HGG) induce antibody formation in T and B cells? i.e. is the macrophage needed for the interaction of

RNA:HGG with T and B cells and resulting antibody formation?

2) If RNA:HGG can replace the need for the intact macrophage, on which lymphoid cell population does it manifest its action?

To obtain answers to the first question, mice were subjected to lethal irradiation and 24 hrs later thay were reconstituted with T and B cells which had been incubated in vitro with either n-RNA or RNA:HGG. Table 10 shows that when the cells used for reconstitution had been exposed to RNA:HGG they induced a significantly higher antibody response to HGG, as measured by RFC, than cells exposed to n-RNA. It can be seen that cells incubated with n-RNA gave a significantly higher response than the non-irradiated controls which received nothing. However it was observed in other experiments (not herein reported) that reconstitution with T and B cells alone could give results equal to T + B + n-RNA. A possible explanation for this will be discussed later. These initial studies indicated that RNA:HGG was able to induce T and B cells to produce antibody to HGG. Confirmation of these studies can be found in ensuing tables, as the n-RNA and RNA:HGG treated cells served as controls for the studies reported below.

TABLE 10

EFFECT OF RNA:HGG ON T AND B CELLS

Experimental Group	RFC/10^6 Spleen Cells Expt 1	Expt 2
Non-irrad control	800	400
T + B + n-RNA	3200	1500
T + B + RNA:HGG	*6100	3600

Irradiated mice (800R) received $4x10^7$ cells + 10^7 B cells which had been incubated in vitro with RNA ($4x10^7$ T cells + 10^7 B cells/0.1mg RNA/ml BME). Cells were injected IV in 0.5ml volumes. Nine days after reconstitution mice were sacrificed and spleens in each group assayed for RFC. Each group represents data of spleens pooled from 4-5 mice.

T = thymus derived lymphocytes
B = bone marrow derived lymphocytes

* When ratios between group 3 and 2 (Expt 1&2) were evaluated collectively by Student's paired t test, group 3 results together differ from the collective data of group 2 at a p value of .05.

Several different approaches were used to try and determine which population of lymphoid cells interacted with the RNA:HGG. The experimental design was to inject mice, which had been lethally irradiated (800R) 24 hrs previously with either T or B syngeneic cells which had been incubated in vitro with RNA:HGG. On the following day (day 2) or several days later (day 3 or 4) the population which had not been injected on day 1 was injected. The rationale behind this was that this procedure would limit only one lymphoid population to RNA exposure inasmuch as any RNA:HGG which might have been retained on the membranes of RNA:HGG exposed cells should have been destroyed by serum nucleases within 24 hrs. In experiment 2, table 11, the RNA:HGG exposed cells were treated with RNase (.25mg/.1mg RNA:HGG in incubation mixture) prior to being injected into the mice. This added to the assurance that no RNA would be carried over. In all cases cells were washed (2-3x) before being injected. Thus, if any of the experimental groups treated as above induced an antibody response similar in magnitude to mice receiving both T and B cells incubated with RNA:HGG, then it could be inferred that it was only necessary for the RNA:HGG to interact with that particular lymphoid cell population.

When the T cell population was exposed to RNA:HGG and B cells were added later, in 6 out of 7 experiments (the one

TABLE 11: Irradiated mice (800R) received either $4x10^7$ thymus (T) cells plus 10^7 bone marrow (B) cells, which had been incubated in vitro with n-RNA or RNA:HGG ($4x10^7$ T cells/10^7 B cells/0.1mgRNA/ml BME) or they received only one population of the cells which had been exposed to RNA:HGG (10^7 B cells/0.1mg RNA or $4x10^7$ T cells/0.1mgRNA) and on a subsequent day (2, 3, or 4) received the population which was not exposed to RNA:HGG. Spleens were removed on day 10, pooled and assayed for RFC and PFC. Each group represents 3-5 mice.

* Cells treated with RNAse after 1.5 hr incubation with RNA:HGG

\+ ND = not done

** All mice died before spleens were removed on day 9.

TABLE 11

EFFECT OF RECONSTITUTING LETHALLY IRRADIATED MICE WITH POPULATIONS OF T AND B CELLS EXPOSED SEPARATELY TO RNA:HGG

Expt. #	Inoculum (in vitro incubation mixture) Day 1	Day 2	Day 3	Day 4	Per 10^6 Spleen Cells RFC	PFC($\times 10^2$)
1	Non-irrad mice	--	--	--	800	+ND
	T+B+n-RNA	--	--	--	3200	ND
	T+B+RNA:HGG	--	--	--	6100	ND
	T+RNA:HGG	B	--	--	2100	ND
	B+RNA:HGG	T	--	--	5500	ND
2	Non-irrad mice	--	--	--	1800	100
	T+B+n-RNA	--	--	--	2700	200
	T+B+RNA:HGG	--	--	--	5200	400
	*T+RNA:HGG	B	--	--	7500	500
	*B+RNA:HGG	T	--	--	15,000	1000
3	Non-irrad mice	--	--	--	3000	100
	T+B+n-RNA	--	--	--	12,000	600
	T+B+RNA:HGG	--	--	--	20,500	4100
	T+RNA:HGG	--	B	--	10,500	500
	T+RNA:HGG	--	--	B	11,800	400
	B+RNA:HGG	--	T	--	**	
	B+RNA:HGG	--	--	T	**	

exception being experiment 2 in table 11), there was basically no increase in RFC over the controls, as evidenced in table 11. However, when B cells were incubated with the RNA:HGG, followed by non-exposed T cells, the level of antibody induced was about equal to or greater than groups receiving T and B cells exposed together to RNA:HGG. Though the RFC in these groups did not differ significantly from the n-RNA control group the observed increases did suggest that possibly B cells could be induced to produce antibody directly by RNA:HGG.

In both experiments 2 and 3, PFC were determined as well as RFC. Though the PFC were of a much lower order of magnitude they basically followed the pattern of the RFC. This, however, did not always hold true as in some experiments the PFC results were quite different from the RFC.

Induction of Antibody Formation in B Cells by RNA:HGG

As a result of studies reported in the previous section, the question as to whether RNA:HGG could directly induce B cells alone to produce antibody was studied in greater detail as follows. Lethally irradiated mice (800R) were injected IV 24 hrs after irradiation, with 4×10^7 T + 10^7 B cells which had been incubated *in vitro* with either normal RNA (n-RNA) or RNA from macrophages sensitized to HGG (RNA:HGG)

or they received only 10^7 B cells and no T cells were given at all. Hence, if antibody production could be found in mice receiving only B cells it would strongly suggest that RNA:HGG was acting directly on B cells. Results of these studies can be seen in table 12. When B cells alone were exposed to RNA:HGG and then injected into lethally irradiated syngeneic mice the antibody response to HGG was about two and a half times greater than the response in mice which received T and B cells exposed to n-RNA. It should also be noted that in experiment 3 when mice received B cells which had been exposed to HGG, the RFC response of 4200 RFC/10^6 spleen cells was the same as that in the control group which received normal RNA (n-RNA) treated cells. However, here again mice which received B cells treated with RNA:HGG produced a response greater than two times that of the control group. In experiment 1 and 3 which included groups receiving T and B cells exposed to RNA:HGG the RFC response was not significant. In both cases the response of mice receiving B cells treated with RNA:HGG slightly exceeded that of mice receiving T and B cells treated with RNA:HGG.

TABLE 12

ABILITY OF RNA:HGG TO INDUCE ANTI-HGG ANTIBODY FORMATION IN B CELLS DIRECTLY

Experimental Group	RFC/10^6 Spleen Cells Expt 1	Expt 2	Expt 3
Non-irrad Control	1100	700	900
T+B+n-RNA	2700	3900	4300
T+B+RNA:HGG	4000	ND	8100
B+RNA:HGG	*6800	9700	9300
B+HGG	ND	ND	**4200

ND = not done

Irradiated mice (800R) received $4x10^7$ T cells + 10^7 B cells IV, exposed in vitro to RNA (0.1mg) for 1.5 hr, or they received only 10^7 B cells previously incubated with RNA:HGG (10^7 B cells/0.5mg HGG/ml). Nine days after reconstitution, spleens were removed, pooled and assayed for RFC. Each group represents 3-4 mice.

* When ratios between B+RNA:HGG groups and T+B+n-RNA groups were evaluated together (Expts 1-3) by Student's paired t test, the data from the B+RNA:HGG groups together differed from the T+B+RNA:HGG group at $p<.01$.

** In experiments not reported in this thesis it was observed that T+B cells alone injected into irradiated mice resulted in RFC counts greatly exceeding non-irrad controls. Examples are listed below:

	Expt A	Expt B	Expt C
Non-irrad control	700	1000	500
T + B cells only	2900	4000	3900

This should be considered when evaluating the B+HGG group.

Ability of RNA:HGG to Induce Anti-HGG Antibody in Thymectomized Irradiated Mice Restored with Anti-Theta Treated B Cells

From the preceding data, it was evident that RNA extracted from macrophages sensitized to HGG (RNA:HGG) was able to induce B cells to form rosettes in the presence of fewer T cells than required for antibody formation when HGG was the antigen. This was shown in table 12 where B cells plus HGG gave a RFC response equal to the control whereas B+RNA:HGG groups gave a significant response over the controls. It can not be said conclusively that RNA:HGG substituted for the role of the T cells as it is known that a small population of T cells is sequestered in bone marrow (200-202) and this bone marrow was the source of B cells. Consequently definitive evidence for RNA:HGG being a T-independent antigen would need further proof.

Accordingly, mice 6-8 days old were thymectomized. Four weeks after thymectomy these mice were irradiated (800R) and 24 hrs later they were reconstituted with either T and B cells or B cells alone. In both cases cells were incubated with RNA:HGG. The B cells used in all cases were treated with anti-theta serum and complement to destroy any T cells which might be present in the bone marrow cell preparation. Mice receiving B cells which had been incubated with RNA:HGG produced a significantly-higher antibody response than mice receiving B cells exposed

to n-RNA, as can be seen in table 13. Also, as was the case in earlier studies the HGG was not able to stimulate B cells to produce antibody. This can be seen if group 2 is compared with group 3 which received n-RNA treated cells. In both experiments, mice receiving B cells exposed to n-RNA gave higher responses than mice in B + HGG groups. If T cells were added to the incubation mixture they seemed to suppress the antibody response. This has been noted in several other experiments. Unfortunately, all mice died in this group in experiment 1. The cause for this cannot be explained but a possible low level infection is suspected. Due to the deaths in this group, experiment 1 in itself cannot stand alone, but in conjunction with the repeat and other experiments it can be interpreted to mean that the complexing of a RNA-rich fraction from macrophages to HGG converts HGG from a T-cell dependent antigen to a T-cell independent antigen.

Effect of Adjuvants on the Ability of RNA:HGG to Induce Antibody Formation in T and B Cells

Previous studies have shown that the homoribopolymer complex of poly A:U manifests its adjuvant action by acting on antigen reactive cells of thymus origin (166). Likewise it is well established that endotoxin (LPS) acts on

TABLE 13

ANTIBODY FORMATION IN THYMECTOMIZED IRRADIATED MICE RECONSTITUTED WITH ANTI-THETA TREATED BONE MARROW CELLS

Inoculum (incubated in vitro)	RFC/10^6 Spleen Cells Expt 1	Expt 2
(non irrad control)	800	500
B+HGG	2800	1300
B+n-RNA	3900	3300
B+RNA:HGG	+ 8500	8000
B+T+RNA:HGG	*	4400

+ When the ratios between B+RNA:HGG groups and B+n-RNA groups were evaluated collectively by Student's t test, the results of the B+RNA:HGG groups together, differed from the data of the B+n-RNA groups at p=.05. (Collectively=Expts 1 and 2).

* All mice died before day 9 probably as a result of an infection in the cage.

Thymectomized, irradiated (800R) mice received 10^7 B cells (anti-theta + C' treated) which were incubated in vitro (1.5 hr) with RNA (10^7 B cells/0.1mg RNA/ml) or they received $4x10^7$ B cells exposed to RNA under same conditions as B cells alone (10^7 B cells/0.1mg RNA/ml). Nine days after reconstitution mice were sacrificed, spleens removed, and assayed for RFC. Each figure represents pooled data from 3-4 mice/group.

B cells and is considered to be mitogenic to lymphoid cells of bone marrow origin (180, 181). Consequently, I speculated that if RNA:HGG did not need the T cell, then poly A:U should not have an effect on the antibody inducing ability of RNA:HGG. In contrast, if RNA:HGG was acting on the B cell then there was a good possibility that LPS would enhance the response. As may be seen in table 14, the number of RFC in mice which received cells incubated with RNA:HGG and poly A:U failed to show any meaningful increase over mice receiving cells which were not incubated with poly A:U. In contrast, when mice received cells which had been incubated with RNA:HGG and endotoxin as seen in table 15, larger increases in RFC were observed in all 3 experiments as compared to mice receiving cells exposed only to RNA:HGG. The increases seen with poly A:U treated cells were extremely small and it should be noted that the maximum increase seen with poly A:U, (1300 RFC) in experiment 1 of table 14 is about one-half the minimum increase seen with endotoxin (2500RFC) in experiment 1 of table 15. Also, in experiment 3 table 15, both poly A:U and endotoxin were used in the same experiment. Here again, poly A:U treated cells showed an increase of 200 RFC (only 3%) over mice receiving RNA:HGG

TABLE 14

EFFECT OF POLY A:U ON THE ABILITY OF

RNA:HGG TO INDUCE ANTIBODY FORMATION

Cell Mixture Injected	RFC/10^6 Spleen Cells Expt 1	Expt 2	Expt 3
(non-irrad control)	400	700	500
T+B+n-RNA+Poly A:U	1800	3000	ND
T+B+RNA:HGG	3600	6100	4200
T+B+RNA:HGG+Poly A:U	4900	6700	3400

Poly A:U = Polyadenylic-polyuridylic acid
ND = not done

Concentrations for incubation: $4x10^7$ T cells/10^7 B cells/ 0.1mg RNA/300ug Poly A:U Irradiated mice (800R) received 0.5ml of cell suspension IV containing $4x10^7$ T cells + 10 B cells, previously incubated with specificed additives in proportions specified above. Nine days after reconstitution, spleens were removed and assayed for RFC. Each group represents data pooled from 3-5 mice.

TABLE 15

EFFECT OF ENDOTOXIN ON THE ANTIBODY INDUCING ABILITY OF RNA:HGG

Cell Mixture Injected	RFC/10^6 Spleen Cells Expt 1	Expt 2	Expt 3
Non-irrad mice	1100	900	1100
T+B only	3400	ND	ND
T+B+n-RNA	ND	ND	5100
T+B+RNA:HGG	7300	6500	10,200
T+B+RNA:HGG+Endotoxin	9800	13,600	15,300
T+B+RNA:HGG+Poly A:U	ND	ND	10,400

ND = not done

Poly A:U = polyadenylic:polyuridylic acid

Concentrations for incubation: $4x10^7$ T cells/10^7 B cells/ 0.1mg RNA/300 ug Poly A:U or 10 ug endotoxin/ml. Irradiated mice received 0.5ml of cells IV containing $4x10^7$ T cells and 10^7 B cells previously incubated with specified additives in proportions above specified. Nine days after reconstitution spleens were removed and assayed for RFC. Each group represents data pooled from 3 mice.

treated cells, whereas RNA:HGG + endotoxin treated cells showed an increase of 5,100 RFC (50%) over the same group. Only in experiment 2 can a statistically significant increase be seen when endotoxin is used. As seen in table 15, the antibody response of mice receiving cells exposed to RNA:HGG was doubled by exposing the cells to endotoxin. These studies have been interpreted as supportive of the thesis that RNA:HGG acts on B cells.

Effect of RNA:HGG on DNA Synthesis in B Cells

All studies to this point indicated that RNA:HGG was functioning as a T-independent antigen. In view of the recent findings of Coutinho and Möller (203) showing that many thymus independent antigens possessed mitogenic properties, studies were designed to test whether RNA:HGG was mitogenic to B cells. The increase in DNA synthesis, as measured by ^{3}H-thymidine uptake was taken as an indicator of mitogenic activity.

Mice were irradiated as usual and reconstituted with B or T cell populations which had been exposed to RNA:HGG. Five days after reconstitution the mice were injected IP with 8 μCi of ^{3}H-thymidine, 3 hr later the spleens were removed and assayed for radioactivity. The five day period was chosen, as it has been found that

maximum antibody synthesis is occurring at this time (204). The radioactive pulse was allowed to proceed for 3 hrs as this longer pulse cut down on the error factor observed with shorter pulses (204). Each individual mouse spleen was assayed for radioactivity and triplicate samples were done on each. The results of these experiments are reported in table 16. As the RNA was always slightly toxic to cells, mice which received cells incubated with RNA, in all probability received slightly fewer cells than in other groups. Cells routinely were counted prior to in vitro incubation and this count was considered constant throughout the rest of the manipulation. To compensate for possible cell loss or death in mice receiving RNA, cells were always incubated at a concentration equal to the number of cells required to inject all mice in the group plus cells were added for one extra inoculum. (e.g. if 3 mice were to receive 10^7 cells apiece, then 4×10^7 cells were incubated and injected into 3 mice). Considering these facts when analyzing table 16, groups receiving n-RNA treated cells must serve as a control for groups receiving RNA:HGG treated cells. The B cell only or T cell only groups can only serve as controls for mice which received cells exposed to HGG. In all three experiments mice which received B cells incubated with

TABLE 16

EFFECT OF RNA:HGG ON DNA SYNTHESIS IN T AND B CELLS

Cell Mixture Injected	^{3}HT Uptake CPM (mean of triplicate samples)± SE Expt 1	Expt 2	Expt 3	Expt 4
Non-irrad control	1200± 363	6000±228	1443±130	3533±704
B cells only	ND	4700±140	ND	3974±916
B + HGG	ND	3300±725	ND	4889±512
B + n-RNA	1100± 115	650±289	ND	6186±778
B + RNA:HGG	13,300±1306	5350±409	1332±610	13,798±415
T cells only	ND	ND	51± 12	444± 34
T + HGG	ND	ND	94± 5	544±109
T + n-RNA	ND	ND	84± 20	296± 34
T + RNA:HGG	ND	ND	54± 14	230± 5

SE = standard error

Irradiated mice received either 10^7 B cells or $4x10^7$ T cells IV which had previously been incubated with either 0.5mg HGG or 0.1mg RNA. Five days afterwards they received 8 μCi of ^{3}H-thymidine IP. Three hours later all spleens were removed and assayed for radioactivity. Each figure represents the mean of data from 3 mice.

RNA:HGG showed a significantly greater uptake of ^{3}H-thymidine over mice receiving cells treated with n-RNA. In contrast if groups which received B cells only or B cells + HGG are compared in experiments 2 and 4, no difference was observed between the two in ^{3}H-thymidine uptake. This suggests that in keeping with its known thymus dependency HGG alone in contrast to RNA:HGG, does not stimulate the B cells to increased DNA synthesis. Experiments 3 and 4, in table 16 demonstrate the effects on DNA synthesis in T cells. A look at all T cell groups in both of these experiments show that RNA:HGG had no effect. Likewise HGG alone did not stimulate the T cells. In experiment 3 and 4 both B cell and T cell groups were exposed to RNA:HGG. In both cases the response of B cells was significantly greater than the response of T cells.

DISCUSSION

Many investigations have demonstrated the requirement for cell interactions and/or cooperation among mouse spleen cells for successful generation of immune responses to heterologous erythrocytes (196,197,205), hapten-carrier conjugates (199) and serum proteins (198) *in vivo* and *in vitro*. Physical approximation between lymphocytes and macrophages has been observed during cultivation of these cells (105-109), as well as when fixed preparations of lymphoid organs were examined (206, 207). The recent findings of Lipsky and Rosenthal (18) have served to elucidate the nature of these interactions. They demonstrated that macrophages possessed a unique ability to recognize and bind lymphocytes and thymocytes by receptors which were distinguishable from others known on macrophages. The subsequent findings of Shevach and Rosenthal (90,110) implicated a binding site on the macrophage, which was either identical or closely linked to histocompatibility antigens, as the mediator of macrophage-lymphocyte interaction. Thus, these investigators hypothesized that, since macrophage-lymphocyte interaction occurred in the absence of relevant antigen, an early event in the immune

response might involve antigen-independent reversible binding of lymphocytes to macrophages by means of a specific cellular recognition mechanism. Consequently, they proposed that only when macrophages had previously taken up antigen and retained it in immunogenic form would this initial binding step serve to select specifically immunocompetent lymphocytes and promote their proliferation. There have also emerged in the literature other proposals for various roles of the macrophage in these interactions. Amongst these have been that the macrophage: (a) serves as a source of nutrients or special factors which enhance in vitro growth (22-24); (b) creates, with T cell assistance, a correct matrix of antigenic determinants on its surface for proper B-cell activation (19); (c) "processes" or handles antigen in an appropriate way by degrading large molecules to smaller more immunogenic forms (26-28); (d) produces an immunogenic nucleic acid moiety (136-137).

Earlier studies in this laboratory had shown that the ability of RNA to induce an immune response was RNase sensitive (37) and that pronase increased the immunogenicity of the RNA. Whether or not the pronase acted by ridding the RNA of proteins interfering with cell interactions is not known. In the results reported herein it can be seen that the addition of anti-HGG serum to the nonadherent

spleen cell suspension, being incubated with RNA from HGG sensitized macrophages (RNA:HGG), depressed the ability of the RNA:HGG to induce antibody formation in the nonadherent spleen cells. Since normal rabbit serum had no effect in the same system it can be inferred that the loss of antibody inducing ability was due to a neutralizing effect by the anti-HGG serum on the RNA and not to nucleases present in the serum. The specificity of the effect of the anti-HGG serum was further supported by the demonstration that antiserum to a non-cross reacting antigen (BSA) had no effect on the RNA:HGG. The presence of antigen, albeit a very small amount in the RNA moiety is thus implicated. That antiserum to human kappa chains and human gamma chains decreased the ability of RNA:HGG to induce antibody formation indicated that the RNA:HGG was composed of both heavy and light chains. Whether an entire immunoglobulin molecule is present or only a fragment cannot be ascertained from data obtained in these studies. However, the data indicate that HGG was present in the RNA moiety and represented by whole or partial gamma heavy chains and kappa light chains. Since the antibody inducing ability of the RNA is also RNase sensitive (37), then both RNA and antigen are necessary to initiate antibody formation. The amount of antigen associated with the RNA is too small to initiate

an antibody response on its own. Therefore, the RNA must in some way serve as an adjuvant for the antigen. It is interesting to note in this connection that polynucleotides have been shown to stimulate antibody formation to as little as 1 nanogram of bovine gamma globulin (208).

The demonstration that the adherent cell is needed for an antibody response to HGG in lethally irradiated reconstituted mice is in agreement with the findings of others who have shown the need for the adherent cell in the in vitro antibody response. The RFC count in mice receiving only NASC incubated with HGG was significantly higher than in mice which received no irradiation. However, it has been observed in other experiments (not reported in this thesis) that just the manipulation of irradiating the mice and reconstituting with nonadherent spleen cells only resulted in RFC equal in number to those found when HGG was incubated with the cells. Thus the high response seen in mice receiving NASC previously exposed to HGG was not significantly higher than NASC alone.

There are several possible explanations. It is well established that nucleic acids serve as enhancers of immune responses (162,163). Dead cells, resulting from irradiation disintegrate and thus release nucleic acids into the mouse. These nucleic acids could very easily be

enhancing the background response observed in non-irradiated controls. Another possible explanation can be found in the recent reports of Schmidtke and Dixon (209). They showed that the surface adhesiveness of macrophages is increased after irradiation. It is conceivable that at the time of assay these macrophages, sequestered in the spleen are accounting for the non-specific increase in the number of rosettes. The plaque forming assay would have helped to resolve this problem as the stickiness of macrophages should not have had any effect on the results. Attempts to use this assay for antibody forming cells was not as fruitful as had been hoped. The PFC response was very low, approximately 0.01 of the RFC response. Also it could not be demonstrated consistently that the RFC results and PFC results were parallel. For these reasons I continued using the RFC response though it didn't always agree with the PFC results and I don't feel that this fact weakens the strength of my data. Though Harris et. al (210) in earlier works has demonstrated that PFC and RFC are the same cells, more recent reports using refined microtechniques give contrasting findings. Kurata (211), utilizing the Cunningham method (212) for plaque formation, included in his spleen suspension rosettes recently formed. The location of the rosettes were marked then the SRBC and

complement (C') were added. He found that the RFC did not form plaques and thus concluded that in vitro RFC included very few if any PFC. Donnelly (212), utilizing a similar type of system also demonstrated that only a small number of cells formed rosettes and plaques.

As evidenced in most of the data it was quite difficult to obtain results whose absolute values were similar in replicate experiments. This has been attributed to the fact that the results were dependent on so many various variables in the experimental system and resulting assay. The batch of SRBC available at the time and the consequent efficiency of conjugation of HGG to the SRBC, the immunogenicity of the particular batch of RNA:HGG used, the number of spleen cells or T and B cells which homed to the spleen after reconstitution; all of these were factors to be considered in the final number of RFC obtained. Pasanen (193) in studies with PFC has reported a variance of absolute numbers of PFC in different experiments as a result of different efficiencies of hapten coupling. Throughout the studies conducted for this thesis it was found that approximately 70-75% of the experiments would yield positive results. The negative responses were attributed to the various variables discussed above, with the immunogenicity of the particular RNA being one of the most important factors.

Though great differences could be observed between various experimental groups within one experiment, it was difficult to demonstrate statistical significance utilizing the standard Student t test because of the great variance in absolute numbers of RFC from experiment to experiment. Consultation with a representative of the Biostatistics staff in the School of Public Health resulted in the recommendation that the paired Student t test be used. Since the variation from experiment to experiment was great but the relative differences in RFC between groups within various experiments were fairly constant, statistics were performed on the ratios between groups in experiments rather than on the absolute numbers of RFC in various experiments.

The findings that the RNA from macrophages incubated with HGG (RNA:HGG) is able to induce nonadherent spleen cells to produce antibody, thus overriding the need for the adherent cell is in disagreement with those reported by Meiss and Fishman (128). They found that T2 phage-RNA needed both the adherent cell and the nonadherent cell for antibody to T2 phage to be produced in vitro. Adler and Shaefer (213,214) also recently reported the necessity for glass adherent cell in the inductive phase of the

primary *in vitro* response of spleen cells to intact T2 phage. They postulated that the function of the adherent cell was solubilization of antigen and feeding of the RNA to lymphoid cells. The role of solubilization of antigen has been attributed to macrophages by others also (5). The reason for the difference in my observations from those of Adler *et al.* cannot be explained, however there are several factors which should be considered.

The experimental system used in their laboratory was different from that which I used. Both Adler and Schaefer studied antibody formation in an *in vitro* system and used a particulate antigen. In contrast, I was using a soluble antigen and looking at *in vivo* antibody production. It is possible that the macrophage handles particulate and soluble antigen in a slightly different manner. Though Shaefer *et al.* reported the need for the adherent cell for T2 phage-RNA to induce an antibody response, they also found that detergent solubilized antigen did not require the adherent cell (214). The reasons for the differences observed in adherent cell requirements were not explained. Recent *in vitro* studies have been interpreted as indicating that the critical factor in B cell activation by both thymus dependent and thymus independent antigens is presentation of antigenic determinants in a multivalent

configuration of suitable epitope density (215, 216). One postulate to explain this difference is that antigen in a molecular form which does not require further solubilization or assistance in obtaining proper antigenic determinant presentation to the B cell will not need the macrophage. It is possible that RNA:HGG and the detergent solubilized T2 phage of Adler *et al*. fit such specifications and don't need the macrophage, whereas the T2 phage-RNA does not meet these specifications.

Another possible explanation for the lack of agreement of my data with that of Adler and Shaefer lies in the fact that their system was *in vitro* and mine *in vivo*. It is known that X-irradiation does not kill all adherent cells *in vivo* (217); therefore the RNA could conceivably activate the macrophages present resulting in a model *in vivo* which actually equates with the *in vitro* model of Adler and Shaefer. Evans and Alexander (218) have demonstrated that double stranded RNA, when exposed to macrophages for 24 hrs can activate the macrophages.

The fact that RNA:HGG could induce antibody formation in NASC led to a closer analysis of lymphoid cells and how they might interact with RNA:HGG. The ability to demonstrate significant levels of antibody formation by T and

B cells exposed to RNA:HGG was not as striking or consistent as when NASC were used. However it was noted that T and B cell suspensions were much more susceptible to the toxic effects (e.g. cell lysis) of RNA than NASC. Whether this accounted for any differences in effect cannot be said. However, when B cells alone were exposed to RNA:HGG and injected into mice, significant antibody responses were observed. In many cases a greater response was elicited by RNA:HGG treated B cells than by mice which received T and B cells. This led to the speculation that possibly the T cell was suppressing the response. There have been reports of T cells suppressing primary and secondary responses (219); however, it isn't known whether these suppressor cells are different from helper T cells or not. Yet this does not obscure the major finding in this thesis that RNA:HGG stimulates B cells directly to produce antibody in the absence of macrophages and T cells.

Utilization of thymectomized, irradiated mice as recipients of B cells which were treated with anti-theta serum and complement gave conclusive evidence that in the absence of T cells, RNA:HGG was able to induce antibody formation in B cells. This could not be accomplished by HGG alone. These data were interpreted to mean that a RNA rich moiety obtained from macrophages exposed to HGG was

able to convert the antigenic fragment complexed to it from a thymus dependent antigen to a thymus-independent antigen.

Recent findings on B cell activation offer several possible interpretations for my findings. There have been proposals that B cell activation may require two separate signals (220), signal one being either a T cell product (221-223) or a factor released by the macrophage (24,224, 225) and the second signal being supplied by antigen. Recently, it has been proposed that the non-specific signal for B cell activation is provided by the interaction of the complement component C3b with the B cell surface (225). These investigators suggest that the product of C3 activation might serve as an additional signal on a complement receptor bearing cell. Hadding et al. (226) have shown that dextran sulphate, a polyanion which has been shown to be mitogenic for B cells, activated C3 proactivator and C3. Thus he suggests that dextran sulphate might manifest its mitogenic activity through activation of C3. Consequently it is also conceivable that the RNA portion of the RNA:HGG might activate C3 and in this indirect way still serve as the second nonspecific signal for the B cell.

On the other hand, Möller (227) has presented convincing evidence for only one signal being required. In

his system, mitogens supplied the only signal with antigen serving to focus that signal on the specific B cell. In view of the fact that I was able to demonstrate that RNA:HGG had mitogenic properties to B cells, a similarity can be seen to exist between these results and Möller's demonstration that lipopolysaccharides (LPS) can convert heterologous red cells into thymus-independent antigens (179). Also to be considered are the findings of Möller et al. demonstrating that many of the T-independent antigens are endowed with such properties because they possess within one molecule both the non-specific mitogenic signal for B cells and the specific antigenic focuser (203).

Very pertinent to the interpretation of data presented in this thesis are the recent reports of mitogenic properties of polyanions and polynucleotides (228-230). Diamanstein et al. (228) showed that dextran sulphate, a polyanion, was able to stimulate DNA synthesis in spleen cells from thymectomized, lethally irradiated and bone marrow protected mice. They later extended these studies and demonstrated that various polyanions, including poly A:U and poly I:C showed a correlation between their ability to increase DNA synthesis and enhance antibody formation (229).

In the vein of these above discussed findings, I

interpret the data presented in this thesis as follows. An RNA-rich fraction isolated from HGG sensitized macrohages has complexed to it a fragment of the HGG. This RNA endows the HGG with the ability to directly activate the B cell to produce anti-HGG antibody. I propose that this activation is achieved by the RNA supplying the one signal for B cell activation by interacting with the mitogen receptor on B cells and the HGG fragment supplies the specific localization for this signal.

Further studies are necessary to determine which, if either of the above proposed mechanisms can actually explain the ability of RNA:HGG to directly activate B cells to produce antibody.

As indicated in the literature and reviewed in the history section of this thesis, there are many roles proposed for the macrophage, suggesting that the macrophage is multipotential and probably functions in several interrelated ways in the immune response. Formation of RNA:antigen complexes by macrophages appears to be one of the multiple ways in which macrophages can serve the body as a major defensive unit.

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