INTRASPECIES AND INTERSPECIES TRANSFERS OF DELAYED TYPE HYPERSENSITIVITY TO GUINEA PIGS AND THEIR PERITONEAL EXUDATE CELLS WITH RNA EXTRACTED FROM LYMPHOID TISSUES FROM GUINEA PIGS AND CATTLE

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

SHANG WANG

1973

ABSTRACT

INTRASPECIES AND INTERSPECIES TRANSFERS OF DELAYED TYPE HYPERSENSITIVITY TO GUINEA PIGS AND THEIR PERITONEAL EXUDATE CELLS WITH RNA EXTRACTED FROM LYMPHOID TISSUES FROM GUINEA PIGS AND CATTLE

by

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Ribonucleic acid extracts were made from normal (N-RNA) and BCG-infected (S-RNA) guinea pigs and cattle. The RNA was extracted from the spleen and lymph nodes of guinea pigs and from the lymph nodes of cattle. Only RNA extracts with $OD_{260}/OD_{280} = 2$ were used. The concentration was adjusted on the basis that 1 mg RNA/ml $OD_{260} = 20$. To allow passive sensitization, 10^8 PEC in 2 ml medium and 500 ug RNA in 0.1 ml were mixed with gentle agitation and incubated at 37°C in 5% CO_2 for 60 min. Cells were centrifuged and washed twice. The criterion of passive tuberculin sensitization by RNA in vitro of normal guinea pig peritoneal exudate cells (PEC) was the inhibition of PEC migration radially or from capillary tubes by 10 ug tuberculin (PPD)/ml.

Both guinea pig and bovine S-RNA conferred sensitivity in vitro to normal guinea pig PEC; N-RNA did not. This was interpreted as intraspecies and interspecies in vitro passive sensitization.

Normal guinea pigs were inoculated intraperitoneally with their own (autologous), washed PEC after incubation with guinea pig S-RNA. Migration of peritoneal cells collected 25 days later was inhibited by PPD. This was interpreted as intraspecies in vivo passive sensitization.

Normal guinea pigs were inoculated intraperitoneally with autologous, washed PEC after incubation with bovine S-RNA or bovine N-RNA. Three days later, 10 TU PPD injected intradermally elicited 18-25 mm reactions (delayed type only) on guinea pigs whose cells had been incubated with bovine S-RNA; none on guinea pigs whose cells had been incubated with bovine N-RNA. From the same guinea pigs nine days after autologous cell injections, there was inhibition of migration in vitro by PPD of PEC from guinea pigs whose cells had been incubated with bovine S-RNA; little or none with bovine N-RNA. The transfer of PPD sensitivity to guinea pigs and to their PEC by inoculation of autologous washed PEC incubated with Bovine S-RNA was interpreted as interspecies in vivo passive sensitization.

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Shang Bosco Wang

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1973

ACKNOWLEDGEMENTS

The author expresses his sincere appreciation to Dr. Virginia H. Mallmann for her guidance and to the group of the Tuberculosis Project at Michigan State University for their assistance throughout this study.

Particular gratitude is also extended to my parents for their continuous encouragement to me in my education.

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INTRODUCTION

A tuberculin reaction has been the classical example of delayed type sensitivity. Its immunologic status and role in tuberculo-immunity and pathogenesis has been controversial. It can not be transferred in serum with antibody-mediated immunity and sensitivity. The classic work by Landsteiner and Chase (88) nearly thirty years ago demonstrated that immunologically-specific sensitivity can be transferred to man and guinea pigs with homologous white cells, later by Lawrence (89) by transfer factor extracted from white cells. Very few cells were required and the passively acquired sensitivity persisted too long for transfer factor to be antibodies. It was not inactivated by DNase, RNase or trypsin.

It is reasonably well established now that delayed sensitivity is mediated by thymus-dependent small lymphocytes. When delayed sensitivity has been induced, the sensitive lymphocyte possesses immunologically specific receptor sites. The tuberculin-sensitive lymphocyte reacts with tuberculin and releases effector molecules. The effector molecules are not immunoglobulins and, with exception of transfer factor, probably are not immunologically specific in their action. They may attract, activate or immobilize macrophages, thereby contributing to the "localizing" immunity of tubercle formation or a delayed type skin reaction. The effector molecules do not passively confer long-term delayed sensitivity in vivo. They produce effects on cells detectable in vitro, referred to as in vitro correlates of delayed sensitivity, such as the migration-inhibition factor (MIF). The immunologic role of macrophages is more controversial.

There are numerous reports on the transfer of antibody-producing capacity by RNA and RNA-antigen complexes extracted from lymph nodes, spleen,

liver and peritoneal exudate cells. There have been fewer transfer of delayed sensitivity. The RNA was extracted from lymph nodes, thoracic duct lymphocytes or peritoneal exudate cells.

This is a report of intraspecies and interspecies transfer of tuberculin sensitivity with RNA extract from lymphoid cells. The sensitivity was detected by inhibition of migration in vitro of normal guinea pig peritoneal exudate cells and by intradermal tests of guinea pigs. The RNA extracts were made from lymph nodes and spleens of guinea pigs and from lymph nodes of cattle infected with viable, attenuated Mycobacterium bovis strain Bacille de Calmette-Guerin (BCG).

LITERATURE REVIEW

The genus Mycobacterium includes a variety of organisms ranging from the pathogenic species such as M. tuberculosis to the saprophytic nonpathogenic species such as M. phlei. The three species of the genus Mycobacterium primarily responsible for tuberculosis of mammals and birds are M. tuberculosis, M. bovis, and M. avium for which man, cattle and chickens are the primary but not exclusive hosts, respectively. As the incidence of tuberculosis due to the three species has decreased, the incidence of disease and sensitivity due to the anonymous mycobacteria has been recognized in man (140) and in cattle (108). Cross reacting antigens and other similarities contribute to difficulties in differentiating among the infecting species. The common properties linking these organisms include their acid-fastness and the similarity of the basic structure of peptoglycans, mycosides, and mycolic acids of their cell walls, and the degree of homology found around their DNA. There are antigenic determinants common to some or all, but relatively few specific for a species (138).

The pathogenic mycobacteria are facultative intracellular parasites. They survive or even multiply within macrophages generally capable of killing many other bacteria. Other facultative intracellular parasites include species of the genera Salmonella, Pasteurella, Brucella and Listeria, and some fungi and protozoan. The number which survive or are destroyed are dependent on such factors as on the number and virulence of the organisms, the portal of entry and the species and condition of the animal infected (65, 66). The facultative intracellular parasites are able to survive substances in or from phagocytic cells, including the hydrolytic enzymes in the lysozymes. The surviving intracellular bacilli may multiply

and may kill the phagocytic cells by which they were ingested. When delayed type sensitivity and a increased macrophage activity, now called cell mediated immunity, developes, the infection may be limited and granulomata or tubercles formed (130, 131, 135).

Killed mycobacterial cells induce delayed sensitivity which wanes with time, and there is less increased phagocytic activity than with viable attenuated cells (173, 174, 175). Attenuated strains of mycobacteria have been selected by serial passage through unnatural hosts or on artificial culture media. The Bacille de Calmette-Guerin (BCG), is a strain of M. bovis attenuated by serial culture on bile-containing media in 1909. This strain has been and is still used to immunize humans against tuberculosis. It is also used extensively in experimental animals to study mechanisms of delayed sensitivity and cellular immunity.

The consistent immune response in tuberculosis is not the production of circulating antibody, nor that the immunity and sensitivity can be passively transferred with serum. The most consistent response is the development of delayed type sensitivity to tuberculin which can be transferred by lymphoid cells, transfer factor or peritoneal exudate which contains macrophages and some lymphocytes (16, 24, 27, 71, 88, 116, 118, 147, 158, 159, 177, 178).

The role of macrophages in the induction of delayed sensitivity has not been conclusively resolved. Macrophages in the liver, spleen, lymph nodes and peritoneum readily ingest and digest the effete host cells and molecules and most foreign proteins and cells (115). However, some foreign materials may be retained for weeks or months within macrophages (70). Labelled bovine serum albumin persisted in macrophages in the spleen and liver phagocytes of rabbits for periods of up to 130 days (22). Although

macrophages can readily digest may antigens, they are probably incapable of producing antibody gamma globulin (120, 128).

Macrophages may be activated by some factors which are produced by lymphocytes during blast cell formation in response to antigens, or non-specific stimulation (11, 33, 72, 112, 117, 122, 125, 146) and release of molecules (61) such as migration inhibitory factor (11, 12, 15, 17, 38, 39, 41, 42) macrophage aggregation factor (95), chemotactic factor (172), skin reactive factor (164), blastogenic factor (43, 166), transfer factor (92), lymph node permeability factor (145, 180, 181), monocytogenic hormone (182), cytotoxic factor (44, 63, 64, 139), interferon (67, 179) and complement (126).

Activation causes many changes in macrophages. Some of the changes include the size of cell (10, 29), its process (23), Golgi apparatus (30), the number of lysosomes (23, 29, 30), lysosomal enzymes (29, 35, 73), mitochondrial enzymes (10, 34, 35), phagocytosis (10, 18, 35, 36, 69, 74, 75, 79, 101, 149, 159, 162) and mitotic rate (84). Some changes may contribute to the ability of macrophages to destroy bacteria, others may not.

The number of defense cells, especially macrophages, increases in a tuberculous lesion or delayed sensitivity reaction. In the delayed hypersensitivity reaction, circulating monocytes (macrophages) emigrate from the blood stream into the granulomatous lesion where they may proliferate (86, 167, 168, 169). In a host in which delayed hypersensitivity has developed, the tubercle bacilli or thin products will cause local macrophage activation, proliferation, and immunity to occur at a faster rate and to a greater degree than in normal host (52, 105, 106). The monocytogenic humoral factor (182) from lymph nodes apparently causes the bone marrow to produce or release macrophages. It may also contribute to local macrophage accumulation, activation, and proliferation. After inoculation of tubercle

bacilli, macrophages not only are directly or indirectly sensitized by the bacilli and its tuberculin-like products, they also contribute to immunity by an increasing ability to destroy the bacilli (101).

Whether the macrophage responds in an immunologically specific manner after the induction of delayed sensitivity and cell mediated immunity is controversial. Macrophage cytophilic immunoglobulins and non-immunoglobulin specific receptor sites on macrophages have been reasonably well established. There is as clear evidence for adherence of soluble immune complexes to macrophages (4). Whether any of these are a critical part of delayed sensitivity has not been resolved. It is stated in the review (163) on macrophage cytophilic antibodies that "while evidence accumulates that some form of cell-bound antigen specific material plays a role in delayed hypersensitivity, there is little to suggest that it is identical to serum macrophage-cytophilic antibody." In regard to cell mediated immunity, it states:

> "This is manifested by an increase in the phagocytic and bactericidal abilities of macrophages and usually arises as the result of infection by an organism capable of intracellular multiplication such as Mycobacterium tuberculosis or Listeria monocytogenes. Infection with such an organism stimulates the appearance of a new population of small lymphocytes. These cells migrate rapidly to the site of infection, where, after interaction with antigen, they are able to influence the surrounding macrophages so that they are more capable of combatting the invading organism. The induction of this lymphocyte population and its further interaction with antigen are immunologically specific. However, the modification of macrophages as a result of this interaction is nonspecific. Stimulated macrophages exhibit enhanced phagocytosis and destruction of any antigen presented to them. For this reason it is improbable that a macrophage-cytophilic antibody mediates these changes in macrophage behavior."

Cellular immunity such as the acquired cellular resistance in tuberculosis, was defined by Dannenberg in 1968 (36) as a state in which macrophages have been activated, proliferated, and possess an increased capacity to destroy tubercle bacilli. Initially, the blood is the major source of macrophages in these lesions (56, 86, 150, 151, 152, 167, 168, 169) but, as chronicity is established, local proliferation may become more important (86, 87, 152). A lymph node permeability factor (145, 180, 181) is present in lymphocytes or macrophages or both in the lesion site of inflammation of tuberculosis. This factor attracts more cells to the area and increases the blood supply which provides more oxygen and nutrients for cells (100, 101, 102). If inflammation is severe, the fibrin formed tends to fix the bacilli locally (100, 101).

The following is from a recent report by Dannenberg et al. (37):

> "The following concept of the pathogenesis of tuberculosis is proposed. Large numbers of macrophages accumulate at the site of the bacilli, which stimulate these macrophages to develop high levels of microbicidins and digestive enzymes. DH and interaction with lymphocytes are intimately involved in the local antimicrobial immunity so developed. In a typical lesion such as local immunity does not develop rapidly enough to be effective immediately, and the macrophages are killed by the high concentration of bacilli and their tuberculin-like products. New macrophages enter and distribute the bacillary load among them. These cells can develop microbicidins and enzymes even more quickly than the initial cells, because of the presence of increasing hypersensitivity in the host and probably other, as yet unknown, factors. If this second group of macrophages dies before the bacilli are eliminated or inhibited, a third group of macrophages ingests them. The sequence continues through subsequent groups. Because of the ever increasing speed of developing cellular immunity, one of these groups will succeed in overcoming the bacilli. Then the lesion will regress and heal."

The lymphocytes which become sensitive to tuberculin (11, 33, 117, 125) can passively transfer delayed hypersensitivity to a normal host (16, 24, 27, 28, 62, 88, 90, 97, 98, 99, 113, 118, 177, 178). Both macrophage activation and macrophage proliferation may be direct effects of tuberculin, or more probably indirect effects mediated by factors (listed previously) released from sensitive lymphocytes.

Cellular immunity is a combination of both immunological specific factors and nonspecific factors. The major effect observed is mainly nonspecific in nature, because such immune macrophages have an increased capacity to destroy and inhibit many types of bacteria (46, 104, 105, 107). Cellular immunity is an immunological mechanism specifically engendered and recalled, but is nonspecific in effect. The systemic protection afforded by cellular immunity is relatively minor but it is sufficient to increase the resistance of the host against a variety of unrelated microorganisms (104, 105, 106, 107).

A characteristic of delayed hypersensitivity in man is the delayed skin reaction. When an antigen is injected intradermally into a previously sensitized animal, the typical delayed reaction begins to appear after four hours, may reach a peak at twenty-four hours, and may fade after forty-eight hours. It is grossly characterized by induration, erythyma, and occasionally necrosis. The histology of the delayed reaction has been studied by numerous investigators (28, 49, 56, 86, 87, 113, 136, 137, 170, 171). These observations suggest that tubercle bacilli and their components in the local lesion stimulate both the development of epithelioid cells and an increase in their enzymes, and that this increase is associated with bacillary destruction.

There are two reasons for long duration of the hypersensitivity to tuberculin. The first is its immunological nature. Some lymphoid cells have the "memory" for the antigen to which they previously reacted. The second reason is that the bacilli or their antigens may persist in the caseous center of "closed" lesions (114, 133, 154). The occasional escape of bacilli or their products from the lesions maintains hypersensitivity to tuberculin.

Landsteiner and Chase (88) found that viable blood leukocytes were effective in the transfer of the ability to develop a delayed sensitivity skin reaction to tuberculin and to streptococcal antigens. Lawrence (89) has transferred the sensitivity with extracts of leukocytes prepared by freezing and thawing or by osmotic lysis and named the active material from blood leukocytes transfer factor. It can be inactivated by heating at 56°C for 30 min. It is dialyzable and its molecular weight is estimated at less than 10,000. It is not immunoglobulin. It is resistant to RNase. It passively confers cutaneous reactivity and converts normal lymphocytes in vitro and in vivo to antigen-responsive state. The sensitivity is immunologically specific. Transfer factor differs mechanistically in two ways from the effector molecules of the in vitro correlates from sensitive lymphocytes: transfer factor is mechanically or chemically extracted from cells without antigen present and the factor then confers an ability to the recipient to respond to only the appropriate antigen; the effector molecules are elaborated from sensitive lymphocytes by contact with the antigen to which they are sensitive and the effector molecules nonspecifically affect other cells or their activities.

One of the widely used tests of the in vitro correlates to detect delayed sensitivity is the MIF test, the inhibition of migration of macrophages

on a glass surface. It is elaborated by the sensitized lymphocytes when stimulated by specific antigen. With certain exceptions such as phytohemagglutinin (PHA) (85, 121) and conconavalin A (8, 127, 156, 157), the lymphocytes produce them in vitro only, or at least more of them, when stimulated by the same antigen as that with which active sensitization of lymphocytes was induced in vivo. Rich and Lewis observed nearly 40 years ago (134) that the migration of splenic or lymphocytic cells would be inhibited if these cells from sensitized animals were placed with specific antigen in vitro. George and Vaughan developed a more quantitative system (57) to study this in 1962. By modifications of the technique, Bloom and Bennett and as well as some other investigators (11, 12, 15, 38, 39, 41, 42) have indicated that cellular immunity involves both a sensitized lymphocyte population and a normal macrophage population. They (36) estimated that one lymphocyte is sufficient to affect indirectly the migration of about 1,000 macrophages. The MIF is heat stable at 56°C for 30 min, and a nondialyzable macromolecule of an estimated molecular weight between 35,000 and 55,000. It is generally accepted that MIF and the other in vitro correlates are probably not immunoglobulins, DNA or RNA.

It is well accepted that antibody-mediated immunity and sensitivity can be transferred by RNAs and/or antigen-RNA complexes. These molecules are a part of the immune system but their functions at the cellular level are not yet defined (2). The RNAs have been extracted from a variety of cells from a variety of species of animals. The transfer of the immune capacity has been tested in vivo and in vitro. The kinds of antigen have been quite varied. It has not been clearly resolved that the systems for different antigens and different cells are the same. The literature on the passive transfer of antibody production by RNA extracts will be reviewed first, then on delayed sensitivity and cell mediate immunity.

Sterzl and Hrubesova (155) first reported that a ribonucleoprotein extract (RNA) from the spleens of rabbits injected two days before with Salmonella paratyphi B gave rise to antibody production in adult rabbits and in 5-day old rabbits. The latter was evidence that more than only antigen had been transferred. The 5-day old rabbits did not respond to antigen only. The RNA extracts passively conferred the ability to produce antibodies. Subsequently, this was confirmed in X-irradiated rabbits (53).

In 1957 Garvey and Campbell (55) found S^{35} labelled albumin complexed with RNA extracted from liver cells after rabbits were injected with the albumin. The complex was 200 times more effective in guinea pigs than the original antigen. The dissociated complexes were not immunogenic. A secondary response was enhanced by the antigen-RNA complexes in rabbit lymph node cultures (68) and by RNA extracts from mouse peritoneal exudate cells in syngenic mice (5, 6). The antigen-nucleoprotein complexes behaved as a "super antigen" and appeared to associate readily with soluble RNA (141). These findings promoted the suggestion that the RNA was RNA-antigen complexes transferred from antigen stimulated macrophages to antibody producing cells, thereby initiating an immune response.

The immunologic active RNA may be antigen-free(48, 77, 78, 80, 119, 142). Sharp (148) and Fishman (48) presented suggestive evidence that RNA is passed from a macrophage to a lymphocyte by means of cytoplasmic appendages. Fishman has described the synthesis of T2 bacteriophage antibody by lymph node cells in vitro when normal cells are cultured with macrophages (47) or RNA from macrophages (48) which have been incubated with T2 phages. The response is sensitive to ribonuclease. Weiss and Fishman (176) have also suggested that the active RNA is of higher molecular weight because the RNA from rabbit macrophages, obtained from the MAK II (16S) and MAK III

(23 to 28S) of MAK column (109, 153) must be active in stimulating anti-T2 antibody synthesis.

Several investigators have shown that RNA related to antibody production has many characteristics of messenger RNA and that the mRNA is coming from antigen stimulated lymphocytes rather than macrophages (78).

It has been suggested that antibody synthesis is dependent on the production of a species of RNA (103). This RNA is inhibited by actinomycin D (165)and it is relative unstable to turnover(93). The light and heavy chains of the gamma globulin molecule are synthesized on separate classes of polysomes (7, 143). Since these chains are different sizes it is probable that there is unique messenger RNA code for each type of chain.

Cohen and workers (25, 26) used RNA extract from the spleens of mice immunized with sheep erythocytes to convert a small portion of the spleen cells of normal mice to cells forming hemolytic antibody. The extract was sensitive to RNase and insensitive to pronase, trypsin and amylase. The most active RNAs sedimented in the 8-12S region of sucrose gradient, suggesting a molecular weight of approximately 1-3 x 10^5, too small to serve as an intact messenger RNA for antibody globulin which has a minimum weight of 160,000(45). It is still possible that the smaller RNA may represent a portion of a larger RNA which has broken down during isolation.

Friedman et al. (54) found that the RNA obtained from T2-infected macrophages contained antigenic components of the bacteriophage which could be detected by complement fixation. Askonas and Rhodes (5, 6) also demonstrated RNA extracted from mouse peritoneal cells after the uptake of I^{131} hemocyanin caused the production of antibody directed against hemocyanin injected in normal mice. The RNA preparation contained significant amounts of radioactivity reflecting the presence of antigen.

Gottlieb et al. (58) reported that RNAs derived from two sets of peritoneal exudate cells (PEC) exposed to R17 and T2 phage annealed to DNA. The ability of the bound RNA to elicit antibody formation against T2 was measured. The two RNAs recovered from cells infected with either antigen were identical. Thus, the RNAs were not messenger RNAs.

In the review of antigen-RNA interactions by Gottlieb and Schwartz (60) a comparison is given between crude or bulk-RNA extracts and a minor component, an RNA-protein complex, designated as RNP. The latter was unaffected by DNase. It could be extracted from macrophages whether exposed to antigens or not. It was immunogenic only if the macrophages had been exposed to antigen. The RNP fraction was equal in immunogenicity to that of crude or bulk RNA extracts. Only fragments of antigen bound strongly to the RNP molecule and antigen-RNP complexes could not be dissociated by the methods to dissociate antigen-RNA complexes. The table given by Gottlieb and Schwartz is as follows:

CHARACTERISTICS OF ANTIGEN-RNP AND ANTIGEN-RNA INTERACTIONS

Criteria	Antigen-RNP	Antigen-RNA
Source of RNA	Unique to macrophages	Any cell homogenate
Favored by Mg^{2+} ion	No	Yes
Abolished by EDTA	No	Yes
Abolished by hot phenol	No	Yes
Location in RNA profile on polyacrylamide gel electrophoresis	Only on RNP molecule	Broadly dispersed
Antigen in complex	Always fragmented	Native

The ribonucleoprotein complex does not contain the base sequence information sufficient to code for antibody globulin. The ribonucleoprotein complex has an S_{20} of 1.8, suggesting a molecular weight of about 12,000, of which

approximately 28% is protein (59) and a relatively high guanine-cytosine content of 58% (58). The small size would exclude a messenger role for this RNA, since it is too small to code for either the light (molecular weight 25,000) or the heavy (molecular weight 50,000) chains of gamma globulin.

Therefore, there appears to be two different types of immunogenically active RNA from macrophages. The immunogenicity of macrophage RNA has been attributed to the presence of an antigen component derived from antigenic processing (5, 6, 54, 58), as well as to unique informational properties of the macrophage RNA (1, 26, 48). Thus, there are probably two different cell types. They have been summarized by Bishop and Gottlieb (14) in the following table:

	Antigen-RNA complex	RNA specifying allotypic determinants
Type of globulin produced	7S, 19S(?)	19S only
Sensitivity to RNAse	1 : 15	1 : 75
Antigen present	yes	no
Sensitivity to actinomycin	no	yes
Location in sucrose gradient	heavy fractions	light fractions
Contains messenger RNA	no	possibly

Five ways in which RNA may affect the immune response of cells are as follows:

1. The information for synthesizing immunoglobulin is transferred by information RNA (iRNA) which is free of antigen (48, 77, 78, 80, 119, 142). Some investigators have suggested that this kind of iRNA is messenger RNA (mRNA) (78).

2. RNA may be serving a protective role. Friedman and some other investigators (54, 58) have found that RNA preparations contain antigen or fragments of antigen. The complex is resistant to low concentrations of RNase and trypsin and it may preserve antigenic determinants as they are transported through the circulation to immunocompetent areas such as spleen and lymph nodes.

3. RNA may serve as an adjuvant . Braun et al. (19) have demonstrated that polynucleotides and oligonucleotides can serve as nonspecific stimulators of the immune response and amplify the response to antigenic stimuli. Youmans (183) has also indicated that mycobacterial RNA may function as an adjuvant for induction of delayed hypersensitivity.

4. RNA may be an "activator" or "derepressor." Braun suggested that the macrophage combines a processed antigen with a nonspecific RNA which is assumed to be capable of turning on all of stem cells into antibody forming lymphocytes (19, 20).

5. Bishop and Gottlieb (14) have suggested that it is possible that ribonucleoprotein complex is not respoinsible for induction of the immune response, but may serve to regulate the amount of antigen available to the definitive antibody producing cell.

The reports on the passive transfer of delayed sensitivity and cellular immunity by RNA extracts are few. Mannick and Egdahl (110) passively conferred sensitivity to rabbit lymph node cells in vitro with RNA extracts from lymph nodes from rabbits sensitized by skin homografts. The in vitro cells elicited a positive reaction in homograft donors, not in nondonors. No sensitivity was transferred by extracts from nonsensitive rabbit lymph nodes.

Fong et al. (50, 51) have reported that cultured macrophages from rabbits immunized with BCG were resistant to the effects of virulent tubercle bacilli, and brucella but that normal macrophages were susceptible. This resistance could be transferred by the microsome fraction derived from the immunized cells or by RNA extracted from this fraction. The ability to

transfer resistance was abolished by ribonuclease.

Incubation of normal rabbit lymph node or spleen cells with RNA obtained from lymph nodes draining the site of a rejected homograft apparently converted cells into a state of transplantation immunity, as detected by their ability to elicit a positive skin reaction in recipients (110) or to cause a more rapid rejection of a skin transplant in recipients (111). The uptake of RNA by host cells, mostly histiocytes, is probably a requirement for induction of cellular resistance (3, 51, 110, 111).

The migration of human macrophages from lymph node tissue cultures from tuberculosis patients is inhibited by the purified protein derivative (PPD) of tubercle bacilli. When nonsensitive cells were incubated with an RNA extracted from lymph nodes of human donors sensitive to PPD, histoplasmin, or both, the migration of these cells was specifically inhibited by the respective antigen (39, 160, 161). The migration-inhibition effect could be transferred to normal C57Bl/6J spleen cells by RNA extracted from lymph nodes and spleens of immunized mice which had rejected sarcoma-1 tumors (94).

The conversion of nonsensitive cells to sensitive cells by an RNA extract is highly sensitive to ribonuclease. Although it has not been conclusively excluded that the conversion to a state of delayed hypersensitivity may be the result of active immunization by antigen or antigen fragments the possibility exists that the active RNA may be a messenger mechanism which confers sensitivity. This phenomenon has also been found in vitro in guinea pigs (81) and rabbits (9). In addition, RNA from the lymph nodes of guinea pigs sensitive to PPD, brain antigen, DNP-oligolysine and E-DNP-oligolysine conferred on nonsensitive guinea pigs lymphoid cells the ability to transfer to nonsensitive guinea pigs (82) cells the ability

to have a delayed hypersensitivity skin reaction to the appropriate antigen. Nonsensitive guinea pigs peritoneal exudate cells were incubated in vitro with RNA extracts from a rhesus monkey with delayed hypersensitivity to PPD only, or PPD and coccidioidin. The peritoneal exudate cells were inhibited in their migration from capillary tubes according to the presence of the appropriate antigen. Conversion of nonsensitive guinea pig peritoneal exudate cells were sensitized with monkey RNA extracts (123). The growth of tumor isograft in inbred mice is inhibited by intraperitoneal injections of syngeneic spleen incubated in vitro, with RNA extracted from guinea pig immunized with the same mouse tumor (132).

Paque and Dray have also indicated that RNA extracted from primate lymphoid tissue of rhesus monkeys with skin-test sensitivity to keyhole limpet hemocyanin (KLH), PPD or coccidioidin can convert nonsensitive human peripheral leukocytes to sensitive leukocytes. In the presence of specific antigen the sensitive leukocytes release MIF which inhibited the migration of nonsensitive guinea pig macrophages (124).

The purpose of this research reported in this thesis to passively sensitize normal guinea pig cells in vitro with RNA extracts from BCG-infected guinea pigs and cattle and then to attempt to passively sensitize normal guinea pigs by injection of autologous cells incubated in vitro with guinea pig and bovine-RNA. If this were possible, it would then be feasible to use the model to study the role of the donor RNA in passive sensitization and to develop a test from lymphoid tissue to aid in detection of bovine tuberculosis.

Sensitization of guinea pigs. Random-bred albino guinea pigs from a closed colony since 1961 were inoculated subcutaneously in the cervical area with viable attenuated Mycobacterium bovis strain Bacillus de Calmette-Guerin (BCG) in Freund's incomplete adjuvant (Difco Laboratories, Detroit Michigan). Viable BCG were suspended at an optical density reading of 0.15 at 620 nm, approximately 10^8 cells/ml. Each guinea pigs was inoculated with approximately 1×10^7 cells in 0.5 ml of adjuvant.

Sensitization of cattle. Tuberculin negative, 6 to 9 month-old steers from a tuberculosis-free herd were inoculated subcutaneously in the left brisket with 0.5 mgm wet weight (10 mgm/ml) viable BCG in 0.5 ml incomplete Freund's adjuvant. After euthanization approximately 5 months later tissues were removed and stored at -70°C until RNA extraction. Prior to euthanization, the steers were tuberculin sensitive by caudal and cervical tests, and in vitro tests. Control steers were negative.

RNA extraction. Lymph nodes and spleens of BCG-infected and normal guinea pigs, and lymph nodes of BCG-infected and normal cattle were excised and if the RNA extractions were not made immediately, stored at -70°C until they were to be used. The RNA extraction was by a modification of the procedure described by Scherrer and Darnell (144). Tissues were homogenized with a Waring blender at 4°C in distilled phenol containing 0.1% 8-hydroxyquinoline (J.T. Baker Chemical Co., Phillipsburg, N.J.) saturated with 0.01 M sodium acetate (J.T. Baker Chemical Co., Phillipsburg, N.J.), 0.5% sodium dodecyl sulfate (K & K Laboratories, Inc., Plainview, N.J.), and 4 ug/ml of polyvinyl sulfate (Eastman Organic Chemicals, Rochester, N.Y.). An equal volume of sodium acetate used to saturate phenol was added to the homogenate with 0.5 mg of bentonite (Fisher Scientific Co., Fairlawn, N.J.)

per ml. Particular care was taken to use 10-12 ml of the aqueous phenol extraction buffer and an equal volume of sodium acetate containing 0.5 mg of bentonite/ml for each gram of tissue used. This mixture was heated to 60°C with intermittent stirring for 10 min and rapidly cooled to 10°C in an ice water bath. The phenol and aqueous phases were separated by centrifugation at 40,000 X g at 4°C for 10 min. The aqueous phase containing RNA was carefully removed with a pipet. An equal amount of fresh buffer saturated phenol was added to the aqueous phase and the extraction procedure repeated three times or until the OD_{260}/OD_{280} ratio 2. After the last extraction, the aqueous phase was precipitated with 95% ethanol and stored in the freezer overnight. The RNA precipitate was centrifuged at 40,000 X g at 4°C for 15 min. The pellet was dissolved in 0.3 M sodium acetate (pH 5.1) and treated with 10 ug deoxyribonuclease (DNase, Worthington Biochemical Corporation, Freehold, N.J.) in 0.1 ml of aqueous RNA extracts for 30 min at 37°C. Deoxyribonuclease was removed by re-extraction of treated RNA with phenol and by alcohol reprecipitation of the RNA extracted from phenol acetate buffer as described above. The RNA was reprecipitated at least three times with three parts of 95% ethanol at -70°C and stored for use as a pellet under ethanol at -70°C. The RNA preparations used had an OD_{260}/OD_{280} ratio of 2.0.

Prestimulation and collection of peritoneal exudate cells (PEC). Random bred albino guinea pigs were inoculated intraperitoneally through the linea alba with 5 ml sterile light mineral oil (E.H. Sargent and Co.). Four days later, the animals were inoculated intraperitoneally with 40 ml of Hank's Balanced Salt Solution (BSS) (Microbiological Associates Inc., Bethesda, Maryland) containing 6 units of heparin (Scientific Products, Evanston, Illinois) per ml. The peritoneal exudate was removed aseptically with a

sterile syringe and centrifuged 500 X g for 10 min at 4°C. The sedimented PEC were washed and centrifuged three times in cold BSS without heparin. They were resuspended in TC 199 medium (Microbiological Associates Inc., Bethesda, Maryland).

Passive sensitization of guinea pig PEC in vitro by RNA extracts. The RNA extracts were dissolved in a minimum of BSS (0.3-1.0 ml) and the concentration was adjusted on the basis that 1 mg of RNA/ml has an OD_{260} of 20. The RNA, 500 ug in 0.1 ml BSS was added to washed PEC, 10^8 cells in 2 ml of TC 199 medium. The mixture was incubated 60 minutes at 37°C in a 5% carbon dioxide incubator with intermittent shaking. Washed PEC were incubated with BSS as controls. After incubation, the RNA treated and RNA untreated PEC were centrifuged at 140 X g for 10 min. The supernatant fluid was discarded. The peritoneal exudate cells were resuspended in TC 199 medium containing 0.2% L-glutamine (Grand Island Biological Co., Grand Island, N.Y. 14072) and 10% inactivated fetal bovine serum (Microbiological Associates Inc., Bethesda, Maryland) and 5% inactivated guinea pig serum (Grand Island Biological Co.). The cells were used immediately in capillary tube or spot tests for migration-inhibition.

Passive sensitization of guinea pigs by injection of autologous washed PEC after incubation in vitro with RNA. The RNA extracts were dissolved in a minimum of BSS (0.3-1.0 ml) and the concentrations adjusted on the basis of 1 gm of RNA/ml yields an OD_{260} of 20. The PEC from each guinea pig were suspended in BSS and mixed with RNA extracts after adjusting the concentration of peritoneal exudate cells to 10^8 cells/500 ug of RNA. The mixtures were incubated at 37°C in 5% CO_2 for 60 minutes with gentle shaking, centrifuged and the cells washed twice with BSS.

Cells were resuspended, 10^8/ml BSS, and each guinea pig was inoculated intraperitoneally with its own (autologous) cells only. The guinea pigs had not been skin tested before receiving their own RNA-treated cells. The closed colony has had no natural sensitivity during the past 12 years.

Capillary tube test for migration-inhibition. Capillary tubes (0.5-0.9 x 75 mm) were filled with PEC suspensions, plugged at one end with sterile paraffin and centrifuged at 55 X g for 15 min. At the interphase of medium and cells, the capillary tubes were cut by diamond point, placed in a sterile petri dish and anchored with sterile paraffin. Two ml medium TC 199 containing 0.2% of L-glutamine, 5% guinea pig serum and 10% of fetal bovine serum with or without 10 ug PPD/ml (Parke-Davis, Detroit, Michigan) was added to each petri dish. They were incubated at 37°C in 5% carbon dioxide for 24 hrs. After incubation, the dishes were placed on a projection microscope and the areas of cell migration (migration index) was calculated by the methods of George and Vaughn (1962) using the formula:

$$\text{M.I.} = \frac{\text{Average area of migration with antigen}}{\text{Average area of migration without antigen}} \times 100$$

Spot test for migration-inhibition. The spot test was developed and modified in our laboratory (129) as follows:

When the cells were ready to be tested, they were suspended in TC 199 at approximately 10^8 cells/ml, and drawn into a ½ ml syringe with a 25 gauge needle. One drop was placed at the center of each cover slip in a petri dish and incubated at room temperature 15 minutes. The cover slips were rinsed carefully in BSS to remove nonadherent cells and placed in a small, plastic petri dish (35 x 10 mm). To each petri dish, 2.0 ml of TC 199 with 2% L-glutamine, 5% guinea pig serum and 10% fetal bovine serum with or without 10 ug PPD (Parke-Davis)/ml.

The vertical and horizontal diameters of each spot was measured under the light microscope 10X objective with an ocular micrometer and the average recorded. The chambers were incubated at 37°C, 5% CO_2 for 24 hours. The diameters were measured, and the increase in average diameter recorded as migration. The migration index was calculated as for the capillary test.

Skin tests. Guinea pigs were skin tested with 0.1 ml containing 10 TU PPD-S (Parke Davis). The sites of inoculation were observed 15 minutes, 2, 24, 48 and 72 hours later. The size of the reactions was recorded in mm of erythema and/or edema.

Passive sensitization tests. Tests were made to determine if passive sensitization occurred under the following circumstances:

1. Pooled RNA from BCG-infected guinea pigs to normal guinea pig PEC in vitro measured by MIF tests (Table 1).

2. Individual guinea pig RNA from BCG-infected guinea pigs which were sensitive to PPD in vitro prior to extraction (Table 2) to normal guinea pig PEC in vitro measured by MIF tests (Table 3).

3. Autologous cells after incubation with RNA from BCG-infected guinea pigs to normal guinea pig in vivo measured by MIF tests 25 days later (Table 4).

4. Bovine RNA from BCG-infected cattle which were sensitive to PPD in vitro prior to extraction (Table 5) to (a) normal guinea pig PEC in vitro as measured by MIF (Tables 6-12); (b) to normal guinea pigs in vivo by their autologous cells as measured by MIF nine days later (Tables 13-16) and by skin tests three days later (Table 17).

RESULTS

Passive transfer of delayed sensitivity in vitro and in vivo to normal guinea pig PEC by RNA extracted from BCG-infected guinea pigs. The results from five capillary tube tests on PEC from each test group of guinea pigs to detect in vitro transfer of sensitivity are given in Table 1. The average of migration of PEC incubated in BSS and no PPD was 65.0 ± 1.4 and represented a hundred percent migration (100.0 ± 2.2). The migration of the PEC, which had been incubated previously with RNA extracted from a BCG-infected guinea pig (S-RNA) was inhibited by PPD (M.I. = 23.5 ± 2.2); there was little or no inhibition without PPD (M.I. = 95.4 ± 1.4). The migration of PEC incubated previously with the RNA which had been extracted from the spleen and lymph nodes of a normal guinea pig (N-RNA) was not inhibited by PPD (M.I. = 91.3 ± 3.7) or without PPD (M.I. = 92.3 ± 2.2). The migration of normal PEC was not inhibited by PPD (M.I. = 96.1 ± 3.9).

The migration of PEC from two BCG-infected guinea pigs, prior to extraction of the RNA above, was inhibited by PPD (M.I. = 48.3 ± 0.80, and M.I. = 36.9 ± 1.8, Table 2). The migration of normal PEC was inhibited by PPD when previously incubated with RNA from either guinea pig (M.I. = 53.6 ± 1.6 and M.I. = 38.3 ± 1.3, Table 3); the migration was not inhibited without PPD (M.I. = 102.4 ± 1.1; M.I. 96.1 ± 1.4). The migration of PEC previously incubated with BSS without RNA as controls was not inhibited by PPD (M.I. = 103.4 ± 1.5) or without PPD (M.I. = 100 ± 0.8).

The migration of PEC from a guinea pig which 25 days previously had received its own cells incubated in vitro with the RNA extracts from BCG-infected guinea pigs was inhibited by PPD (M.I. = 44.5 ± 2.9) and not without (M.I. = 100 ± 1.8, Table 4).

TABLE 1. Migration inhibition tests of normal guinea pig peritoneal exudate cells (PEC), after incubation with RNA extracts from spleens and lymph nodes of BCG-infected guinea pigs (S-RNA) or normal guinea pigs (N-RNA) cultured with and without Purified Protein Derivatives (PPD).

Capillary tube	BSS[a]	BSS + PPD	N-RNA	N-RNA + PPD	S-RNA	S-RNA + PPD
1	61	60	62	60	64	15
2	65	65	58	62	60	12
3	63	*103[b]	56	*5	61	12
4	67	*100	60	52.5	*7	14
5	69	ND[c]	64	63	63	CON[d]
Average (with all value)	65.0±1.4	82.0±11.3	60.0±1.4	48.5±11.0	51.0±11.0	15.3±1.4
Migration Index (%)	100.0±2.2	126.1±17.4	92.3±2.2	74.6±17.0	78.4±16.9	23.5±2.2
Average (without outlying value)	65.0±1.4	62.5±2.5	60.0±1.4	59.4±2.4	62.0±0.9	15.3±1.4
Migration Index (%)	100.0±2.2	96.1±3.9	92.3±2.2	91.3±3.7	95.4±1.4	23.5±2.2

[a] Hanks Balanced Salt Solution

[b] *outlying value

[c] Not done

[d] Contaminated

TABLE 2. Migration inhibition tests of peritoneal exudate cells (PEC) from BCG-infected guinea pigs prior to ribonucleic acid extraction from spleen and lymph nodes.

Guinea Pig #1 (GP-I)

Capillary tube	BSS[a]	BSS with PPD
1	58	30
2	61	29
3	63	28
4	56	28
5	ND[b]	CON[c]
Average	59.5±4.9	28.8±0.5
Migration Index (%)	100.0±8.3	48.3±0.8

Guinea Pig #2 (GP-II)

Capillary tube	BSS	BSS with PPD
1	30	12
2	33	12
3	33	10
4	25	9
5	28	11
6	ND	12
Average	29.8±1.4	11.0±0.5
Migration Index (%)	100.0±4.6	36.9±1.8

[a] Hanks Balanced Salt Solution

[b] Not done

[c] Contaminated

TABLE 3. Migration inhibition tests of peritoneal exudate cells (PEC) from a normal guinea pig after incubation in vitro with RNA extracts from the spleens and lymph nodes from BCG-infected guinea pigs (GP-I or GP-II, TABLE 2) cultured with and without Purified Protein Derivatives (PPD).

Capillary tube	PEC + BSS[a]	PEC + BSS + PPD	PEC + RNA(GP-I)	PEC + RNA(GP-I) + PPD	PEC + RNA(GP-II)	PEC + RNA(GP-II) + PPD
1	94	101	97	52	93	38
2	97	98	98	48	88	39
3	96	95	100	ND[b]	94	35
4	94	100	95	53	91	34
Average	95.3±0.8	98.5±1.4	97.5±1.0	51.0±1.5	91.5±1.3	36.5±1.2
Migration Index (%)	100.0±0.8	103.4±1.5	102.4±1.1	53.6±1.6	96.1±1.4	38.3±1.3

[a] Hanks Balanced Salt Solution

[b] Not done

TABLE 4. Migration inhibition tests of peritoneal exudate cells (PEC) from a normal guinea pig (GP-III) injected 25 days previously with autologous PEC after incubation with RNA extracts from the spleens and lymph nodes of BCG-infected guinea pigs (GP-I and GP-II) cultured with and without Purified Protein Derivatives (PPD).

Capillary tube	PEC without antigen	PEC with PPD
1	50	25
2	47	26
3	50	20
4	52	19
5	48	20
Average	49.4±0.9	22.0±1.4
Migration Index (%)	100.0±1.8	44.5±2.9

Passive transfer of delayed sensitivity to normal guinea pig PEC in vitro with RNA extracts from BCG-infected steers. Prior to RNA extractions from bovine lymph nodes, the migration of blood leucocytes from steers #36 and #51 infected with BCG were inhibited by 10 ug of a tuberculo-protein (138) (M.I. = 19.5 ± 9.5 and 20.0 ± 4.0), the migration of blood leucocytes from a normal steer was not inhibited (M.I. = 100.0 ± 4.8, Table 5).

The M.I. of PEC from normal guinea pigs previously incubated with BSS or RNA from a normal steer (NB-56-RNA) with or without PPD was 91.6 ± 3.0 and 100.0 ± 12.4 respectively for GP-56-1 (Table 6); 100.5 ± 4.8 and 100.0 ± 3.2 respectively for GP-56-2 (Table 7). The M.I. with the cells incubated with RNA from a normal steer (NB-56-RNA) with and without PPD were 99.1 ± 6.2 and 91.9 ± 6.7 for GP-56-1 (Table 6); 86.7 ± 7.1 and 92.5 ± 11.2 for GP-56-2 (Table 7).

The migration of PEC previously incubated with RNA extracted from BCG-infected steers, SB-36-RNA and SB-51-RNA, was inhibited by PPD. The results are given in Table 8 and Table 9 for SB-36-RNA and in Table 10 and 11 for SB-51-RNA. The migration of PEC from a normal guinea pig (GP-36-1) previously incubated with SB-36-RNA were inhibited by PPD (M.I. = 53.6 ± 3.1, Table 8) and was not inhibited without PPD (M.I. = 96.1 ± 1.3). The migration of PEC from another guinea pig (GP-36-2), previously incubated with SB-36-RNA, was inhibited with PPD (M.I. = 5.1 ± 1.8); not without PPD (M.I. = 97.6 ± 2.7, Table 9).

The migration of PEC from GP-51-1 previously incubated with SB-51-RNA was inhibited with PPD and not without PPD (M.I. = 4.8 ± 2.1 and M.I. = 102.9 ± 3.9, respectively, Table 10). The migration of PEC from another normal GP (GP-51-2) previously incubated with SB-51-RNA was inhibited with PPD (M.I = 4.7 ± 1.4); not without PPD (M.I. = 117.1 ± 2.5, Table 11).

TABLE 5. Migration inhibition tests of leucocytes from a normal steer (#56) and two BCG-infected steers (#36 and #51) prior to ribonucleic acid extractions of lymph nodes.
(Tests reported by Jane Walsh)

Spot Chamber	Leucocytes from #56						Leucocytes from #36						Leucocytes from #51					
	No Ag			B-24[a]			No Ag			B-24			No Ag			B-24		
	0 hr	24 hrs	DIFF[b]	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF	0 hr	24 hr	DIFF
1	420	530	110	400	540	140	330	420	90	380	390	10	370	510	130	360	380	20
2	320	420	100	380	440	60	320	440	120	460	490	30	340	460	120	390	420	30
Average			105.0±5.0			100.0±12.7			105.0±15.0			20.0±10.0			125.0±5.0			25.0±5.
Migration Index (%)			100.0±4.8			95.2±12.1			100.0±14.3			19.5±9.5			100.0±4.0			20.0±4.

[a] A tuberculo-protein extracted from BCG

[b] Increase in diameter of the spot during incubation

TABLE 6. Migration inhibition test of normal guinea pig (GP-56-1) peritoneal exudate cells (PEC) incubated with the RNA extracts from the lymph node of a normal steer (NB-56-RNA) cultured with and without Purified Protein Derivatives (PPD).

Spot Chamber	BSS[a] 0 hr	BSS[a] 24 hrs	BSS[a] DIFF[b]	BSS + PPD 0 hr	BSS + PPD 24 hrs	BSS + PPD DIFF	NB-56-RNA 0 hr	NB-56-RNA 24 hrs	NB-56-RNA DIFF	NB-56-RNA + PPD 0 hr	NB-56-RNA + PPD 24 hrs	NB-56-RNA + PPD DIFF
1	164.5	194.5	30	117	138	21	162	180	18	141.5	168	26.5
2	176	CON[c]	ND[d]	150	172	22	172	195.5	23.5	176	202.5	26.5
3	127.5	147.5	20	167.5	CON	ND	147	168.5	21.5	145	165.5	20.5
4	141.5	164	22.5	163	186.5	23.5	138	164	26	169	191.5	22.5
Average		24.2±3.0			22.2±0.7			22.3±1.7			24.0±1.5	
Migration Index (%)		100.0±12.4			91.6±3.0			91.9±6.7			99.1±6.2	

[a] Hanks Balanced Salt Solution

[b] Increase in diameter of the spot during incubation

[c] Contaminated

[d] Not done

TABLE 7. Migration inhibition tests of normal guinea pig (GP-56-2) peritoneal exudate cells (PEC) incubated with the RNA extracts from the lymph node of a normal steer (NB-56-RNA cultured with and without Purified Protein Derivatives (PPD).

Spot	BSS[a]			BSS + PPD			NB-56-RNA			NB-56-RNA + PPD		
Chamber	0 hr	24 hrs	DIFF[b]	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF
1	152.5	CON[c]	ND[d]	147	169	22	173.5	201.5	28	177.5	198.5	21
2	146.5	175.5	24	131.5	159	25	166	181	15	174	196.5	22.5
3	155	177.5	22.5	171.5	191.5	21	146.5	168	21.5	175	CON	ND
4	136.5	CON	ND	146.5	172	25.5	138.5	160	21.5	168	185	17
Average			23.3±0.8			23.4±1.1			21.5±2.6			20.2±1.6
Migration Index (%)			100.0±3.2			100.5±4.8			92.5±11.2			86.7±7.1

[a] Hanks Balanced Salt Solution

[b] Increase in diameter of the spot during incubation

[c] Contaminated

[d] Not done

TABLE 8. Migration inhibition tests of normal guinea pig (GP-36-1) peritoneal exudate cells (PEC) incubated with the RNA extracts from the lymph nodes of a normal steer(NB-56-RNA) or BCG-infected steer (SB-36-RNA) cultured with and without Purified Protein Derivatives (PPD).

Capillary tube	NB-56-RNA No Ag	NB-56-RNA + PPD	SB-36-RNA No Ag	SB-36-RNA + PPD
1	51	53	52	25
2	53	52	50	25
3	50	49	*5[a]	30
4	50	50	48	31
5	53	ND[b]	49	CON[c]
6	54	ND	50	CON
Average (with all values)	51.8±0.7	51.0±0.9	42.3±7.5	27.8±1.6
Migration Index (%)	100.0±1.4	98.4±1.8	81.7±14.5	53.6±3.1
Average (without outlying value)	51.8±0.7	51.0±0.9	49.8±0.7	27.8±1.6
Migration Index (%)	100.0±1.4	98.4±1.8	96.1±1.3	53.6±3.1

[a] *outlying value

[b] Not done

[c] Contaminated

TABLE 9. Migration inhibition tests of normal guinea pig (GP-36-2) peritoneal exudate cells (PEC) incubated in vitro with the RNA extracts from the lymph nodes of normal steer (NB-56-RNA) or BCG-infected steer (SB-36-RNA) cultured with and without Purified Protein Derivatives (PPD).

Spot Chamber	NB-56-RNA 0 hr	NB-56-RNA 24 hrs	NB-56-RNA DIFF[a]	NB-56-RNA + PPD 0 hr	NB-56-RNA + PPD 24 hrs	NB-56-RNA + PPD DIFF	SB-36-RNA 0 hr	SB-36-RNA 24 hrs	SB-36-RNA DIFF	SB-36-RNA + PPD 0 hr	SB-36-RNA + PPD 24 hrs	SB-36-RNA + PPD DIFF
1	142.5	152	19.5	156.5	176	19.5	152.5	170.5	18	161.5	162	0.5
2	162.5	184	21.5	146.5	168.5	22	162.5	181.5	19	185	185	0
3	152	170.5	18.5	156.5	175.5	19	165	184	19	185	187	2
4	156	174	18	157.5	176.5	19	205	CON[b]	ND[c]	193.5	195	1.5
5	169	189.5	20.5	155	173.5	18.5	165	185.5	20.5	162.5	163.5	1
Average			19.6±0.6			19.6±0.6			19.1±0.5			1.0±0.4
Migration Index (%)			100.0±3.3			100.0±3.2			97.6±2.7			5.1±1.8

[a] Increase in diameter of the spot during incubation

[b] Contaminated

[c] Not done

TABLE 10. Migration inhibition tests of normal guinea pig (GP-51-1) peritoneal exudate cells (PEC) incubated in vitro with the RNA extracts from the lymph nodes of normal steer (NB-56-RNA) or BCG-infected steer (SB-51-RNA)cultured with and without Purified Protein Derivatives (PPD).

Spot	NB-56-RNA			NB-56-RNA + PPD			SB-51-RNA			SB-51-RNA + PPD		
Chamber	0 hr	24 hrs	DIFF[a]	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF
1	168.5	190.5	22	137.5	168	30	211	236.5	25.5	202.5	203.5	1
2	119	150	31	ND[b]	ND	ND	216.5	247.5	31	180	182.5	2.5
3	173	204	31	147.5	188	40.5	197.5	227.5	30	215	215.5	0.5
Average			28.0±3.0			35.3±5.3			28.8±1.1			1.3±0.6
Migration Index (%)			100.0±10.7			125.9±18.8			102.9±3.9			4.8±2.1

[a] Increase in diameter of the spot during incubation

[b] Not done

TABLE 11. Migration inhibition tests of normal guinea pig (GP-51-2) peritoneal exudate cells (PEC) incubated in vitro with the RNA extracts from the lymph nodes of normal steer (NB-56-RNA) or BCG-infected steer (SB-51-RNA) cultured with and without Purified Protein Derivatives (PPD).

Spot	NB-56-RNA			NB-56-RNA + PPD			SB-51-RNA			SB-51-RNA + PPD		
Chamber	0 hr	24 hrs	DIFF[a]	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF
1	153.5	177.5	24	154.5	178	23.5	166	193.5	27.5	159.5	161.5	2
2	136.5	151	14.5	147	177	30	215	CON[b]	ND[c]	178	179	1
3	165	185.5	20.5	143	175.5	32.5	161	189	28	147.5	148.5	1
4	199.5	232	32.5	193	CON	ND	154.5	183.5	29	146	147.5	1.5
5	164.5	191	26.5	173.5	CON	ND	136.5	162.5	26	172.5	172.5	0
Average			23.6±3.0			28.7±2.7			27.6±0.6			1.1±0.3
Migration Index (%)			100.0±12.8			121.5±11.4			117.1±2.5			4.7±1.4

[a] Increase in diameter of the spot during incubation

[b] Contaminated

[c] Not done

The results from Tables 6-11 are summarized in Table 12. There was passive sensitization of normal guinea pig PEC in vitro by RNA extracted from lymph nodes of BCG-infected steers.

Passive sensitization in vivo of normal guinea pigs by inoculation of autologous PEC after incubation in vitro with RNA from BCG-infected steers. The migration of PEC from two guinea pigs (GP-56-1 and GP-56-2) nine days after they had received autologous PEC after incubation in vitro with RNA from a normal steer (NB-56-RNA) was not inhibited with or without PPD. The M.I. indices were 111.4 $\pm$ 5.0, 100.0 $\pm$ 4.3 and 87.6 $\pm$ 7.1, 100.0 $\pm$ 4.9, respectively, (Table 13).

The migration of PEC from two guinea pigs (GP-36-1 and GP-36-2)nine days after they received autologous cells after incubation in vitro with RNA from a BCG-infected steer (SB-36-RNA) was inhibited by PPD (M.I. = 9.6 $\pm$ 3.1 and M.I. = 15.6 $\pm$ 1.8) and not inhibited by PPD (M.I. = 100.0 $\pm$ 4.1 and 100.0 $\pm$ 9.0, Table 14).

The migration of PEC from two guinea pigs (GP-51-1 and GP-51-2) nine days after they had received autologous cells after incubation in vitro with RNA from a BCG-infected steer (SB-51-RNA) was inhibited by PPD (M.I. = 16.0 $\pm$ 2.9 and M.I. = 20.8 $\pm$ 3.7) and not inhibited by PPD (M.I. = 100.0 $\pm$ 4.3 and 100.0 $\pm$ 3.2, Table 15.

The results of Tables 13, 14 and 15 are summarized in Table 16. The guinea pigs were passively sensitized in vivo, as detected by migration - inhibition tests, by RNA extracted from lymph nodes from BCG-infected cattle, not from a normal steer.

Skin tests with 10 TU PPD three days after guinea pigs received autologous cells incubated with RNA from normal bovine RNA elicited no detectable

TABLE 12. Summary of TABLES 6-11 of migration tests to detect passive sensitization of normal guinea pig PEC in vitro by RNA extracts from lymph nodes of a normal steer (NB-56-RNA) or from BCG-infected steers (SB-36-RNA or SB-51-RNA).

Guinea Pig	Migration Average		Migration Index(%)
	PEC without PPD	PEC with PPD	
GP-56-1	22.3 ± 1.7	24.0 ± 1.5	99.1 ± 6.2*[a]
GP-56-2	21.5 ± 6.6	20.2 ± 1.6	86.7 ± 7.1*
GP-36-1	49.8 ± 0.7	27.8 ± 1.6	53.6 ± 3.1**[b]
GP-36-2	19.1 ± 0.5	1.0 ± 0.4	5.1 ± 1.8**
GP-51-1	28.8 ± 1.1	1.3 ± 0.6	4.8 ± 2.1**
GP-51-2	27.6 ± 0.6	1.1 ± 0.3	4.7 ± 1.4**

[a]The migration index was the percentage difference between the migration of the PEC incubated with BSS and with PPD.

[b]The migration index was the percentage difference between the migration of PEC incubated with NB-56-RNA and with SB-36-RNA or SB-51-RNA.

TABLE 13. Migration inhibition tests of peritoneal exudate cells (PEC) from normal guinea pigs (GP-56-1 and GP-56-2) nine days after intraperitoneal injection of autologous PEC incubated previously with the RNA from normal steer (NB-56-RNA) cultured with and without Purified Protein Derivatives (PPD).

Spot Chamber	GP-56-1			GP-56-1 + PPD			GP-56-2			GP-56-2 + PPD		
	0 hr	24 hrs	DIFF[a]	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF
1	325	346	21	294	318	24	275	299.5	24.5	225.5	248	22.5
2	312.5	334	21.5	ND[b]	ND	ND	304	324.5	20.5	320	339.5	19.5
3	395	413.5	18.5	315	337	22	250	274	24	ND	ND	ND
4	390	408	18	325	345	20	278.5	298.5	20	295	310	15
5	328.5	346	17.5	299	319	20	271	290.5	19.5	326	345	19
Average			19.3±0.8			21.5±1.0			21.7±1.1			19.0±1.5
Migration Index (%)			100.0±4.3			111.4±5.0			100.0±4.9			87.6±7.1

[a] Increase in diameter of the spot during incubation

[b] Not done

TABLE 14. Migration inhibition tests of peritoneal exudate cells (PEC) from normal guinea pigs (GP-36-1 and GP-36-2) nine days after intraperitoneal injection of autologous PEC incubated previously with the RNA of BCG-infected steer (SB-36-RNA) cultured with and without Purified Protein Derivatives (PPD).

Spot	GP-36-1			GP-36-1 + PPD			GP-36-2			GP-36-2 + PPD		
Chamber	0 hr	24 hrs	DIFF[a]	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF
1	336	355	19	292.5	295	2.5	220	246	26	240	243	3
2	295	315	20	281	282.5	1.5	221	240	19	240	242	2
3	321	344	23	269	271	2	ND[b]	ND	ND	242.5	246.5	4
4	299	320	21	ND	ND	ND	222	240.5	18.5	253.5	256.5	3
5	ND	ND	ND	309	311	2	265	283.5	18.5	261.5	265.5	4
Average			20.8±0.9			2.0±0.7			20.5±1.8			3.2±0.4
Migration Index (%)			100.0±4.1			9.6±3.1			100.0±9.0			15.6±1.8

[a] Increase in diameter of the spot during incubation

[b] Not done

TABLE 15. Migration inhibition tests of peritoneal exudate cells (PEC) from normal guinea pigs (GP-51-1 and GP-51-2) nine days after intraperitoneal injection of autologous PEC incubated previously with the RNA of BCG-infected steer (SB-51-RNA) cultured with and without Purified Protein Derivatives (PPD).

Spot Chamber	GP-51-1			GP-51-1 + PPD			GP-51-2			GP-51-2 + PPD		
	0 hr	24 hrs	DIFF[a]	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF	0 hr	24 hrs	DIFF
1	295	318	23	278	282	4	249.5	271.5	22	272.5	279	6.5
2	289.5	311.5	22	297	301.5	4.5	312.5	336.5	24	257	260	3
3	282	302.5	20.5	302.5	306	3.5	237	258.5	21.5	280	286	6
4	293.5	313	19.5	300	303.5	3.5	234.5	255	20.5	256	258.5	2.5
5	307	325	18	301.5	302.5	1	241.5	261.5	20	273	277.5	4.5
Average			20.6±0.9			3.3±0.6			21.6±0.7			4.5±0.8
Migration Index (%)			100.0±4.3			16.0±2.9			100.0±3.2			20.8±3.7

[a]Increase in diameter of the spot during incubation

TABLE 16. Summary of TABLES 13, 14 and 15 of migration tests to detect passive sensitization of normal guinea pig PEC in vivo by RNA extracts from lymph nodes of a normal steer (NB-56-RNA) or from BCG-infected steers (SB-36-RNA or SB-51-RNA).

Guinea Pig	Migration Average		Migration Index(%)
	PEC without PPD	PEC with PPD	
GP-56-1	19.3 ± 0.8	21.5 ± 1.0	111.4 ± 5.0
GP-56-2	21.7 ± 1.1	19.0 ± 1.5	87.6 ± 7.1
GP-36-1	20.8 ± 0.9	2.0 ± 0.7	9.6 ± 3.1
GP-36-2	20.5 ± 1.8	3.2 ± 0.4	15.6 ± 1.8
GP-51-1	20.6 ± 0.9	3.3 ± 0.6	16.0 ± 2.9
GP-51-2	21.6 ± 0.7	4.5 ± 0.8	20.8 ± 3.7

reactions. All guinea pigs which received autologous cells after incubation with RNA from BCG-infected steers had 18-25 mm reactions at the site of inoculation of 10 TU of PPD at 24, 48 and 72 hours. There were no detectable reactions 15 minutes or 2 hours after the PPD injection. The results are given in Table 17.

The results of the in vitro and in vivo passive sensitizations of guinea pigs by bovine RNA are summarized in Table 18.

TABLE 17. Results of skin tests[a] of guinea pigs three days after intraperitoneal injection of autologous PEC incubated with the RNA from normal steer (NB-56-RNA) or BCG-infected steer (SB-36-RNA and SB-51-RNA) observed at 24 hrs, 48 hrs and 72 hrs.

Guinea pig	24 hrs			48 hrs			72 hrs		
	Edema region (mm)	Erythema region (mm)	Thickness	Edema region (mm)	Erythema region (mm)	Thickness	Edema region (mm)	Erythema region (mm)	Thickness
GP-56-1	NR[b]	NR	NR	NR	NR	NR	NR	NR	NR
GP-36-1	18	18	2X	20	19	2X	14	14	2X
GP-51-1	20	20	3X	20	16	4X	14	14	2X
GP-56-2	5	5	1X	NR	NR	NR	NR	NR	NR
GP-36-2	25	25(16)[c]	3X	25	20(16)	3X	20	16(9)	2X
GP-51-2	25	25(12)	4X	25	22(14)	4X	20	20(9)	2X

[a] Skin tested with 10 TU PPD (Parke-Davis)

[b] No detectable Reaction.

[c] ()With a deeper red region at the center of erythema region

TABLE 18. Summary of TABLES 12, 16 and 17 of migration inhibition and skin tests to detect passive sensitization in vitro and in vivo of normal guinea pigs by RNA extracts from lymph nodes from a normal steer (NB-56-RNA) and from two BCG-sensitized steers (SB-36-RNA and SB-51-RNA).

	In vitro sensitization	In vivo sensitization by injection of passively sensitized autologous cells	
Guinea Pig	Migration of PEC	Migration of PEC	Skin Reaction
GP-56-1	99.1 ± 6.2	111.4 ± 5.0	none
GP-56-2	86.7 ± 7.1	87.6 ± 7.1	none
GP-36-1	53.6 ± 3.1	9.6 ± 3.1	20 mm
GP-36-2	5.1 ± 1.8	15.6 ± 1.8	25 mm
GP-51-1	4.8 ± 2.1	16.0 ± 2.9	20 mm
GP-51-2	4.7 ± 1.4	20.8 ± 3.7	25 mm

DISCUSSION

The purpose of this research was to passively sensitize guinea pigs and their PEC to PPD with homologous RNA and to determine if bovine RNA would passively sensitize normal guinea pigs and their PEC. If so, it could provide a model to study the kind and role of RNA in delayed sensitivity reactions and a means of detecting tuberculous herds by tests of lymph nodes collected at abattoirs at the time of slaughter.

The results obtained by passive sensitization with homologous RNA were similar to the results reported by Jureziz et al. (81) of the inhibition by antigen of migration in vitro of PEC from normal guinea pigs after incubation with RNA extracted from the spleens and lymph nodes of sensitive guinea pigs. The migration of PEC was inhibited by PPD after incubation with RNA from a donor infected with viable BCG, not after incubation with RNA extracts from normal guinea pigs. Jureziz et al. used a pool of lymph nodes and spleens from randomly bred guinea pigs for RNA extractions and tested cells from single guinea pigs to which the results shown in Table 1 are similar. The results of transfers with separate RNA extracts from lymph nodes and spleens of two guinea pigs infected with BCG are given in Table 3. There was inhibition of migration of normal PEC by PPD after passive sensitization by each of the RNA extracts. The amount of inhibition of PEC from the donor prior to transfer (Table 2) was greater by GP-II than GP-I. The number of animals is too few to draw any conclusions regarding the relative amount of sensitivity transferred.

The RNA extracts used by Jureziz et al. (82) were from syngenic guinea pigs, and incubated with syngenic but not autologous lymphoid cells, which were injected into guinea pigs of the same syngenic strain. Passive

sensitization required epinephrine. They suggested it increased the phagocytic capacity of lymphoid cells. In our experiments to transfer sensitivity in vivo, normal guinea pigs received washed autologous PEC after incubation in vitro with RNA. Because the PEC contain predominantly macrophages, no epinephrine was used.

The use of autologous cells for passive sensitization in vivo made it unnecessary to have syngenic strains to avoid histoincompatibility. It did create some operational difficulties because with RNA the animal can not be killed to obtain the PEC which are incubated with RNA and returned to the animal. The care required during the collection usually resulted in too few cells to make adequate tests by the capillary tube test for MIF. The radial migration test requires fewer cells than the capillary test and results from previous studies (108, 129) with bovine leucocytes indicated the "spot" test was equally as good or better than the capillary test.

There was passive sensitization 25 days after injection of autologous PEC cells when they had been incubated with the RNAs from BCG-infected guinea pigs (Table 4). This indicated that "immune-RNA" would transfer sensitivity not only to normal exudate cells in vitro, but that washed cells after incubation with the RNA also transferred sensitivity to normal guinea pigs. This was interpreted as an in vivo intraspecies passive sensitization.

In vitro and in vivo interspecies transfer also occurred. There was some inhibition of normal PEC from guinea pigs in vitro by RNA from the normal steer (NB-56-RNA) (Tables 6 and 7). Therefore, cells with NB-56-RNA were used as controls (Tables 8, 9, 10 and 11) when determining the M.I. of PEC incubated with RNA from BCG-infected steers. This was indicated in Table 12 which summarized the results.

When guinea pigs were inoculated with washed autologous cells after incubation with RNA there were strong skin reactions to PPD three days later (Table 17) and inhibition of migration by PPD of their PEC nine days later (Tables 13-16) when the RNA was from BCG-infected cattle. What effect the skin test at three days may have had on the subsequent M.I. test at nine days was considered. There is an unresolved, long term controversy as to the effect of tuberculin testing an actively sensitized animal. It may increase, decrease or not affect subsequent skin tests or serologic tests. Little or nothing has been reported on the effect on passively sensitized animals. In our laboratory, we have found that skin testing cattle infected with BCG decreases the M.I. 3-20 days after the skin test. Therefore, it seemed probable that in a passively sensitized animal, a skin test would more probably cause a decrease than an increase in M.I. Because there is a chance of the losing the guinea pig when collecting PEC, we elected to skin test first. It is possible that the skin test could activate lymphocytes and macrophages other than locally at the site of the test and cause the cells in the PEC to be more active in vitro. It might decrease the number of responsive cells in the peritoneal cavity, or have no effect. This should be determined.

Many reports have indicated that immunogenic RNA is sensitive to RNase (76, 110, 160, 161). RNase-treated extracts do not confer sensitivity on normal cell populations, suggesting that intact RNA has some role in the transfer (161). It is of great importance therefore, in the whole process of RNA extraction to prevent the degradation of RNA. Little biological activity in RNA preparation is lost if stored at -20°C or less, either in alcohol or in aqueous solution because low temperature decreases the enzymatic activities of RNase (2). In this experiment, samples were stored under -70°C

and extractions made at 4°C except in the steps of denaturing protein by hot phenol at 56-60°C for 10 min. High concentration of RNA may compensate for small losses of RNA by RNase. Most investigators use 100-1000 ug of RNA/10^7-10^8 cells in a volume of 0.1-5.0 ml (2). We used 500 ug of RNA/10^8 cells in 2 ml of medium.

Several inhibitors of RNase have been used in the phenol mixtures. Polyvinyl sulfate (PVS) has been reported to be an inhibitor of RNase by Bernfield et al. (13). Addition of 8-hydroxyquinoline to the phenol improved the yield of RNA, decreased the protein contamination, and lessened but did not eliminate, the action of RNase (83). Bentonite can absorb RNase on its surface but it must be removed immediately because RNase absorbed on bentonite still has some enzymatic activities. The possible contamination of DNA can be eliminated by use of DNase.

The RNA preparation in this experiment was extracted by hot phenol at least three times or until the OD_{260}/OD_{280} ratio equal to or greater than 2. Some of the initial attempts at extraction were unsuccessful but no difficulty was encountered thereafter. Under this condition, DNA and the protein are presumably removed (144). However, slight amount of contaminations have been reported. Herscowitz and Stelos (76) found that RNA preparations having an OD_{260}/OD_{280} ratio of 2 contained 2 to 10 ug of protein/800 ug of RNA, as measured by the method of Lowry et al. (96) using bovine serum albumin as standard, and 8 to 12 ug of DNA/800 ug of RNA, as measured by the diphenylamine reaction (21) using slamon sperm DNA. These contaminations are presumed to be negligible.

Redistilled phenol was saturated with sodium acetate which prevents phenol from crystallizing under 4°C. Phenol saturated in buffer probably is a better denaturing agent of protein than phenol alone. Although phenol may

extract RNA, the subsequent purification can result in degradation of RNA and destroy its activity. Significant amounts of active RNA may be lost in the phenol-buffer interphase during extraction (2).

Autologous peritoneal cells were incubated in vitro with RNA and then washed before injection into the same guinea pig. The interaction of PEC with RNA before injection was believed to increase the probability of passive sensitization by the RNA. The cells were washed to remove free RNA. It was presumed that RNA was incorporated by some of the PEC cells but no tests were made to determine if RNA were only adsorbed to the surface of the PEC cells. Such studies will be necessary to precisely determine the roles of RNA and the PEC in the passive transfer. The need of epinephrine to increase phagocytosis by lymphoid cells does imply the need of phagocytosis for the passive transfer (82).

Peritoneal exudate cells are very susceptible to specific inhibition of migration in vitro and it has been suggested that migration inhibition is the one of the best methods to correlate delayed hypersensitivity (57). There are some difficulties such as an insufficient number of peritoneal exudate cells to run capillary tube test; partial stacking of cells rather than monolayer migration; variable diameters of capillary tubes, the eveness of the cut and closeness to the glass when immobilized with paraffin; lack of uniformity of the glass surface on which cell migrate; an air bubble at the mouth of capillary tube; unequal pH charges in each chamber during incubation. The "spot" test lessens some of the problems but not all. In the author's opinion, it is simpler, more convenient, and more reproducible than capillary tube technique but no comparative tests were made in this study.

The slight inhibitory effect on PEC by N-RNA without PPD or by PPD without RNA is probably due to non-specific release of effector molecules from lymphocytes (124) or the RNA extracts may contain small amounts of phenol or alcohol toxic for PEC.

Whether transfer factor occurs in bovine cells has not been determined. It has been found in a number of species. The following differences between transfer factor and RNA extract do not exclude that passive sensitization in vivo was by transfer factor: RNA extracts are inactivated by RNase sensitivity, transfer factor is not; RNA is nondialyzable and has a sedimentation value between 8S to 12S suggesting a molecular weight greater than 80,000 (160); transfer factor is dialyzable and molecular weight is less than 10,000 (92). However, the hot phenol extraction of RNA probably excludes the transfer factor.

The degree of specificity of sensitivity transferred has not been determined yet. The "immune-RNA" from either guinea pigs or steers did passively transfer delayed hypersensitivity to guinea pigs and their peritoneal exudate cells from normal guinea pig. It provides a model for further study of the role of RNA in delayed sensitivity reactions and the possibility of an in vitro test to detect tuberculosis.

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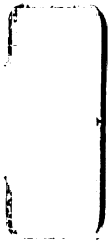

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