COMPARATIVE STUDIES OF INFECTIOUS ANEMIAS IN RATS

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ABSTRACT

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By

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A syndrome of anemia with splenomegaly and nephritis accompanied by autoimmune factors was associated with infections of unrelated agents. It was called the "anemia-big spleen syndrome" by those relating it to immunoconglutinin (IK), and the "infectious anemia syndrome" by others relating it to cold-active hemagglutinin (CAH). This dissertation reports on a study of the immunopathologic factors associated with infectious anemia in rats resulting from infections of filterable agent of rat infectious anemia (RIA), *Trypanosoma lewisi*, *Babesia rodhaini* and *Plasmodium chabaudi*.

The agent of RIA was discovered as a contaminant of a *T. lewisi* infection. It was less than 0.20 micron in size and in mature rats it caused fatal hemolytic anemia with splenomegaly and glomerulonephritis associated with CAH. Weanling rats recovered after less severe disease. While the agent was not cultured or visualized, its size and the absence of other agents led to the suggestion that it was a virus. A new strain of *T. lewisi* was proven to be free of RIA agent. A less severe anemia with splenomegaly and glomerulonephritis accompanied by CAH was found to be associated with infection of this parasite. *B.*

rodhaini and P. chabaudi had been previously associated with infectious anemia.

Serum from rats made anemic by each of the infections contained elevated titers of both CAH and IK. It therefore appeared that the anemia-big spleen and infectious anemia syndromes were essentially the same disease.

Serum of rats recovered from infectious anemia contained IK but little or no CAH. When challenged with a heterologous agent they exhibited a nonspecific acquired resistance manifested by early anemia, reduced parasitemia, increased titers of IK and CAH and enhanced survival. While the resistance was associated with IK, it was also associated with CAH and antibody to soluble serum antigen (ABSA). Uninfected rats with autostimulated IK also exhibited the resistance after challenge.

Autostimulated rat IK, detected by agglutination of complement-fixed sensitized sheep erythrocytes, was IgM which reacted specifically with fixed C3. The IK levels reached peak values during the acute phase of infection. Chromatography of CAH-free hyperimmune rat globulin separated IK with the 19 S and ABSA with the 7 S fractions and indicated that CAH, IK and ABSA were different antibodies. The use of these fractions and the whole immune globulin to passively immunize rats infected with RIA agent and mice infected with P. chabaudi indicated that IK alone did not furnish protection, but animals given ABSA exhibited the early anemia, reduced parasitemia and prolonged survival. Whole globulin, containing both IK and ABSA, enhanced this effect of nonspecific acquired resistance.

Fluorescein conjugated ABSA and IK both reacted with erythrocytes of blood films and spleen impression slides from rats made anemic by each infection. ABSA was considered to react with soluble serum antigen (SA) which bound as a complex with its antibody to the cells. The IK reaction was believed to be with complement that had been fixed by the complex.

It appeared that complexes of SA and ABSA had roles in both anemia and nonspecific acquired resistance and the action might be enhanced by IK. In binding to blood cells or parasites the complexes could mimic opsonin to cause splenic sequestration or hemolytic crisis if optimal complement fixation by the complex was attained. The conglutinating activity of IK could enhance phagocytosis and sequestration. In animals that had IK and ABSA before SA was elaborated from infection, the complexes would form earlier and be removed from the circulation before they attained dangerous titers. Thus while parasites as well as blood cells were reduced by this nonspecific acquired resistance, it was suggested that the major protective function was primarily the early removal of immune complexes and keeping their titers below a critical threshold.

COMPARATIVE STUDIES OF INFECTIOUS ANEMIAS IN RATS

By

Santi Thoongsuwan

A DISSERTATION

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Dedicated

to

my wife, Mrs. Yanee Thoongsuwan

and

my family

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INTRODUCTION

In certain infectious diseases there exists a syndrome of anemia, splenomegaly and glomerulonephritis which is accompanied by the presence of auto-immune-like blood factors. Since the syndrome is more consistently associated with autoimmune factors such as cold-active hemagglutinin (CAH) than to the nature of the infectious agents, it was suggested that the disease was of an autoimmune nature and that it should be referred to as infectious anemia to differentiate it from the more classic forms of infectious diseases (Cox and Iturri, 1976). Thus infectious anemia may be associated with the infections of viral, rickettsial, or hemosporidian agents, but have as a common factor the association of CAH with the disease syndrome (Cox and Iturri, 1976; Cox et al., 1966; Oki and Miura, 1970; Schroeder et al., 1966).

Infectious anemias differ from the more classic infectious diseases in another respect. Their agents appear to be ineffective as immunizing agents and self curing immune responses to their infections are not conspicuous. It was well recognized by early workers that the acquired resistance of diseases such as malaria differed from the classic form of rejection of the infectious agent associated with sudden cuticular inflammation (allergic inflammation or hypersensitivity) that had been so clearly described by Jenner (1798). They therefore employed the term "premunition" to differentiate the acquired resistance of malaria from the classic form described by Jenner. Thus premunition is referred to as the immunity of chronic infection and has been found to be dependent upon the presence of a functional spleen (Sergent, 1963).

More recently it was recognized that premunition is in part a phenomenon of nonspecific acquired resistance (F. E. G. Cox, 1972; Cox and Milar, 1968; Ludford et al., 1969). Nonspecific resistance to infectious anemia was generated by immunization with soluble serum antigen (SA) from animals with acute babesial and malarial anemia (Corwin and Cox, 1969; Cox, 1966; Sibinovic et al., 1967). It has therefore been suggested that premunition might be a protective phenomenon active against infectious anemia rather than a resistance against a specific infectious agent (Cox and Milar, 1968).

Since CAH was found to be an autoantibody common to infectious anemia associated with a diversity of infectious agents, it is hypothesized that SA and other autoantigens might also be common to infectious anemia and that premunition might in part be a result of immunization with autoantigens. This hypothesis has been tested in studies of blood factors associated with infectious anemia mediated in rats by a filterable rat infectious anemia agent, babesial, trypanosomal and plasmodial infections. The results of these comparative studies are presented in this dissertation.

REVIEW OF LITERATURE

Definition of Infectious Anemia

The term "infectious anemia" does not appear in Dorlands, The American Illustrated Medical Dictionary, 20th Edition (1944). It was probably first used by Weinman (1944) in reference to anemia due to infections with *Bartonella* and related red cell parasites. Since it appeared that Weinman did recognize anemia as a syndrome associated with infections of various agents in man and animals, e.g., *Bartonella bacilliformis*, *Anaplasma marginale*, *Haemobartonella* spp. and *Eperythrozoon* spp., it is assumed that he had used the term in the sense presently intended. Specifically, infectious anemia refers to a syndrome consisting of anemia with splenomegaly and vascular-renal disease accompanied by autoimmune factors which may be associated with the infections of a variety of unrelated agents (Cox and Iturri, 1976). Thus the syndrome may be associated with the infections of viral, rickettsial or protozoan agents and regardless of the nature of the agent, the essential disease signs will be the same (Cox and Iturri, 1976; Cox et al., 1966; Ludford et al., 1969; McCluskey and Vassalli, 1971; Oki and Miura, 1970; Schroeder et al., 1966; Schroeder and Ristic, 1965).

In addition to being the causal agents for the disease syndrome, the agents of infectious anemia tend to share another common trait in that they appear to be incapable of immunizing their host during infection. Self curing immune responses are therefore not a prominent feature. Acquired resistance associated with these diseases differs from the more classic type demonstrated by Jenner (1798) and has been termed "premunition" to differentiate if from classic acquired immunity. Thus premunition, meaning literally forewarned, has been called infection immunity, or the immunity of chronic infection.

Premunition and Infectious Anemia

The major in depth treatise on the subject is that of Sergent (1963) which deals primarily with this protective phenomenon as it pertained in experimental and human malaria. It was pointed out that premunition did not produce a blood sterilizing immunity, but in many cases may be effective to the extent that parasitemia may be kept at subpatent levels for indefinite periods. Radical chemotherapeutic cures were soon followed by a complete loss of protection and the maintenance of protection was dependent upon the presence of the spleen.

More recent information has indicated that the protection may be of a nonspecific nature and may apply to infectious diseases other than malaria. Rodents that had recovered from babesial infections were resistant to malaria and vice versa (F. E. G. Cox, 1972; Cox and Milar, 1968). Ducklings that had recovered from viral duck infectious anemia were resistant to malarial infections that were lethal for normal ducks (Ludford et al., 1969, 1972). The presence of *Eperythrozoon coccoides* infections in mice conferred an enhanced resistance to rodent malarias (Ott et al., 1967; Peters, 1965). New Zealand Black (NZB) mice were much more resistant to rodent malaria than were the New Zealand White (NZW) mice, and after recovery from malaria the NZB mice did not show signs of autoimmune anemia and nephritis for more than a year (Greenwood et al., 1970; Greenwood and Voller, 1970a, b; Greenwood and Greenwood, 1971). Since it is now known that NZB mice carry congenital infections of Gross Lymphoma-Leukemia virus and maintain a persistent viremia (Mellors et al., 1969; Mellors and Huang, 1966), it is possible that while NZB mouse disease may be mediated by factors associated with the viremia, the nonspecific protection associated with these mice might be relatable to the nonspecific phenomenon of premunition. Nonspecific protection was demonstrated against malaria and babesiosis in animals after they had been immunized with soluble serum antigens found in the blood of animals with acute malaria or babesiosis (Corwin and Cox, 1969; Cox, 1966; Sibinovic et al., 1967, 1969). Thus it was indicated that the nonspecific resistance of premunition might be a phenomenon of immunization. The serum antigens associated with malaria and babesiosis were found not to be parasite antigen and the antigen associated with each of the infections were found to be similar if not identical in their serologic reactions. Evidence that these were not parasite antigens led to the suggestion that they might be modified host products or autoantigens (Soni and Cox, 1974, 1975a, b, c).

A concept that autoimmune factors relatable to anemia might have a protective function was advanced by Cox (1964) in a discussion of autoimmunity in malaria. It was pointed out that any immune mechanism, autoimmune or otherwise, that had a detrimental effect on erythrocytes might also be harmful to erythrocytic parasites. This overly simplistic concept was tested with the results that it was shown that antibody to normal rat or mouse erythrocytes had a marked deleterious effect on the infectivity of Plasmodium berghei parasites (Cox, 1969). Similar effects were demonstrated in rodent babesiosis and bovine anaplasmosis (McHardy, 1972, 1974).

As indicated by Sergent (1963), a functional spleen may be critical to the protection of premunition. Since this organ is richly endowed with cells that participate in the immune response and in immune phagocytosis, and is positioned to be exposed to antigens circulating in the blood, Taliaferro (1956) postulated that the primary penalty of splenectomy in malaria was the loss of the antibody response and the phagocytic activity that the organ contributed to protective immunity. Credibility was given this thought by the demonstration that intact animals responded somewhat better than splenectomized in generating antibody to intravenously administered toxoid (Taliaferro, 1956). However, it is probable that spleen function in premunition is more complex than was indicated by Taliaferro (1956).

In discussing hemolytic diseases, Dacie (1962) pointed out that antibody to erythrocytes in the form of cold-active hemagglutinin was frequently found associated with these diseases. He also pointed out that the spleen was one of the primary blood filters of the body and that one of its chief functions was the removal of effete erythrocytes from the blood. He suggested that this function might be enhanced by the action of autohemagglutinins. He speculated further suggesting that congenitally defective erythrocytes or those made defective by infectious agents might have functional antigenic sites that would stimulate production of autohemagglutinin and in turn enhance the removal of these cells from the blood. Thus the speculations of Dacie (1962) about a protective function for autoantibody against erythrocytes differed from those of Cox (1964) only in that Dacie did not recognize the potential importance of the spleen as a protective process in red cell infections.

These thoughts led to experiments in which it was found that cold-active hemagglutinin (CAH) was associated with anemia, splenomegaly and the phagocytosis of infected as well as uninfected erythrocytes by phagocytes of the spleen or bone marrow in animals with malaria, babesiosis and anaplasmosis. Since the anemia crisis associated with CAH was accompanied by marked reductions in parasitemia and recovery from malaria and babesiosis, it was suggested that this autoimmunologic process might be related to recovery (Cox et al., 1966; Schroeder et al., 1966). The importance of the spleen in recovery was indicated in that splenectomized bovines had more severe anaplasmosis than intact and often died showing signs of red water disease (Schroeder and Ristic, 1965).

The importance of the spleen in resistance to infectious anemia was clearly indicated in *Haemobartonella muris* infections of rats by Nelson (1970). Her rats, obtained from a commercial vendor, appeared to be healthy, but when they were subjected to splenectomy every one developed patent *H. muris* infection and each died within 2 weeks of hemolytic anemia. When *H. muris* free rats were infected they developed patent infections and in 6-8 days, they developed

moderately severe anemia from which they appeared to have recovered after 4 weeks. When these rats were given superimposed infections they again had patent infections with anemia but recovered as before. Splenectomy of these recovered rats invariably led to recrudescence of the infection and hemolytic anemia with death within 2 weeks. It was therefore clear that rats did not develop classic acquired immunity that protected them from repeated *H. muris* infection, and that such protection as did result from recovery from *H. muris* infection was totally dependent upon the presence of the spleen.

As indicated, agents of infectious anemia appear to be unable to stimulate classic acquired immunity during infection, and for this reason such agents have been referred to as compatible parasites (Cox and Iturri, 1976; Sprent, 1963). If, on the other hand, the antigenicity of the parasite for its host could be enhanced, it should follow that classic immunity should result from infection. In experiments in which the mouse parasite, *Plasmodium chabaudi*, was adapted to laboratory rats, the infections were first introduced into splenectomized rats using infected mouse blood. These rats ultimately developed *P. chabaudi* parasitemia on the sixth day after infection which remained patent for only 3 days. Blood of these rats was passed to fresh splenectomized rats which developed parasitemia after 4 days and then underwent spontaneous recovery. After the fourth passage in splenectomized rats spontaneous recovery from *P. chabaudi* malaria was still evident. It therefore appeared that mouse *P. chabaudi* parasites were potent antigen for rats and remained so for many generations of parasite reproduction. It was also clear that with these parasites the absence of a spleen was no major penalty to the development of protective immunity. Intact rats recovered from this partially adapted strain were highly resistant to *P. chabaudi* but were no more resistant than normal rats to challenge with *Babesia rodhaini*. Thus in this malaria the absence of a spleen imposed no marked penalty on the development of immunity, and the resulting immunity was specific in nature rather than the nonspecific kind associated with premunition (Musoke, 1973). It therefore indicated that when the agents of infectious anemia are sufficiently antigenic for their host, classic immunity rather than premunition will result from the infection.

Autoimmune Phenomena in Infectious Diseases

The development of the conception that disease may have an immunologic basis has been hampered by concepts that were more emotional than rational in basis. In spite of the demonstration that inflammation associated with the smallpox and cowpox was clearly related to acquired immunity by Jenner (1798), the concept of an allergic basis for disease was not enunciated until 1905 when von Pirquet published his ideas in Die Serumkrankheit (von Pirquet and Schick, 1905). It is only within recent years that von Pirquet's ideas have come to have some general acceptance and physicians, the descendants of the healers of the priesthood, still largely think in terms of casting out the evil spirits that are inflicted upon man for various transgressions (Magill, 1955).

The concept that disease could be a self destroying process from antibodies generated against ones own body was less acceptable. The concept of Ehrlich (1900) of "horror autotoxicus" was thus elevated to universal law rather than being kept in the realm of a valid generalization. In dealing with the growing evidence that autoimmune disease did exist, Burnett (1959) in his much quoted "clonal selection theory" is consistent with the law of "horror autotoxicus" in the idea that animals can normally only make antibody to their antigens that are sequestered from the immune system. Thus the incongruity of a concept that allowed autoimmune disease to be a part of infectious disease remains an absurdity for the scientific mind. One may have an infectious disease or one may have an autoimmune disease. One may not have both. The skepticism over an autoimmune basis for disease remains evident in the writings of modern authorities (Glynn and Holborow, 1971).

Partly through ignorance, and partly through bias, the nature of the syphilis associated Wassermann antibody has been ignored. Wassermann and associates first thought this antibody had been generated by the antigens of <u>Treponema pallidum</u> since it was detected with a "watery extract" of liver from a syphilitic foetus which was teeming with spirochaetes (Wassermann et al., 1906; Wassermann et al., 1906). However during the following year, Marie and Lavaditi (1907) found that "watery extracts" of normal liver would also react with the antibody. It might have been suspected at that time that Wassermann's antibody was an autoantibody; however, Ehrlich (1900) was not challenged. Since modern Wassermann antigen is an alcoholic extract of

lipids from beef heart, the dissociation of Wassermann antigen from those of *T. pallidum* is further indicated. Portnoy and Magnuson (1955) raised both Wassermann and treponema-immobilizing antibodies by injecting extracts of *T. pallidum* infected rabbit testicle into normal rabbits and concluded that the antibodies were not the same, but did not speculate on the nature of the Wassermann antibody.

The association of Wassermann antibody with diseases other than syphilis should have raised questions as to the nature of the antibody. Its association with yaws and pinta were accepted as valid since these were *Treponema* associated diseases. However, its association with other diseases was termed artifact and called "biological false positive" reactions (Wilson and Miles, 1964). Since the diseases with which "biological false positive" reactions are considerable in number, a partial listing of these diseases include malaria, kala-azar, leprosy, tuberculosis, rheumatic diseases and many others (Wilson and Miles, 1964).

As indicated, Wassermann antibody is associated with a number of diseases which are mediated by a variety of unrelated infectious agents. Kahn (1951) in studying reactions of his cardiolipin antigen found that with careful adjustments of electrolyte concentration, he could detect Wassermann-like antibody in the serum of many normal humans and animals. He therefore suggested that this was a normal or universal antibody, and that when cells that contained the lipid antigens were injured in a disease process, sufficient antibody for detection by conventional methods would result. In essence, Kahn was suggesting that Wassermann antibody was autoantibody.

During this era of great discoveries in immunology, 1900 to 1915, a phenomenon of the aggregation of cells treated with antibody and complemented by normal bovine serum attracted the attention of Ehrlich (Ehrlich and Morgenroth, 1900; Ehrlich and Sachs, 1902a, b) and Bordet (Bordet and Gay, 1906, 1908). Bordet (Bordet and Streng, 1909) termed this aggregation conglutination to distinguish it from the true agglutination which resulted from specific antibody. He termed the factor from bovine serum "coloide de boeuf" or "conglutinin" to differentiate it from agglutinating antibody. A colleague of Bordet, Streng (1909a, b) and Streng and Ryti (1923) proved that conglutinin was a nonantibody fraction of bovine globulin that reacted with complement that was bound to an antigen-antibody complex, but that it did not react with unactivated complement. In 1930 Streng found that a conglutinin-like factor could be stimulated in rabbits, which do not have natural conglutinin, by injecting them with complement adsorbed to blood cells or to bacteria, and called this antibody immunoconglutinin (Streng, 1930). Based on the fact that immunoconglutinin was stimulated by injections of fresh autologous serum absorbed on kaolin particles, the fact that its appearance was associated with complement depletion and its specific reactivity with fixed complement, Coombs (1959) and Commbs et al. (1961) suggested that it fulfilled the criteria for an autoantibody and that it should be associated with any event resulting in an intravascular antigen-antibody reaction. Thus immunoconglutinin should be raised by artificial stimulation, as well as infections with many infectious diseases. This appeared to be true since it has been associated with

antigenic stimuli (Ingram, 1962a, b) and many diseases mediated by a variety of unrelated agents such as in acute and chronic bacterial infections (Ingram, 1965a, b), in trypanosomiasis, typhoid fever, leishmaniasis, malaria and others (Woodruff, 1973; Woodruff et al., 1972).

The immunochemical and biological aspects of immunoconglutinins, of both human and rabbit origin, have been widely investigated. The great majority of the autostimulated immunoconglutinins are IgM antibodies (Bienenstock and Block, 1966; Lachmann, 1967). Immunoconglutinins that are detected by direct agglutination of complement-fixed cells are generally IgM antibodies, although immunoconglutinins of IgG, IgM and occasionally IgA classes have also been detected by specific antiglobulin technique (Henson, 1968). Lachmann and Thompson (1970) reported that human saliva regularly contains quite high levels of immunoconglutinin, this being antigenically IgA. Studies of the specificity of immunoconglutinins have been made, they reacted specifically against the bound C_3 (Lachmann, 1962; Lachmann and Coombs, 1965; Lachmann and Muller-Eberhard, 1968) and bound C_4 (Lachmann, 1966) components of complement. Mittal and Ingram (1969) showed that natural antibodies and immunoconglutinin, in the presence of complement, acted synergistically to increase bactericidal activity. Parappally and Ingram (1973) demonstrated that immunoconglutinin enhanced phagocytic activity of mouse peritoneal macrophages against bacterial cells _in vitro_. The amplifying effect of immunoconglutinin on complement fixation _in vivo_ was shown by Tedesco and coworkers, suggesting that this autoantibody potentiated the ability of complement to bring about red cell destruction (Tedesco et al., 1972).

Thomsen and Friedenreich in 1928 observed that erythrocytes that had been treated with bacterial extracts would detect hemagglutinins that could not be detected with normal blood cells (Friedenreich, 1928). Morton and Pickles (1947) and Wiener and Klatz (1951) found that Type "O" Ph-positive erythrocytes would be agglutinated by serum from persons suffering Rh disease. The belief that these workers were detecting incomplete antibody to Rh antigen was somewhat shaken with the finding that hemagglutinin was present in sera of persons with a variety of acquired hemolytic anemias (Dacie, 1962). Dacie (1962) suggested that this hemagglutinin was a naturally occurring autoantibody and functioned normally in the removal of effete blood cells from the circulation. In hemolytic diseases antigen was exposed after cells were damaged and the titers of the antibody would reach detectable levels. This hemagglutinin was found associated with the anemia of babesiosis, anaplasmosis and malaria (Cox et al., 1966; Schroeder et al., 1966; Schroeder and Ristic, 1965) and was subsequently found in the blood of animals suffering anemia from a variety of unrelated infectious diseases (Cox and Iturri, 1976). Soni and Cox (1975a) caused the hemagglutinin to be produced in normal chickens by injecting them with autologous trypsinized erythrocytes. Since anemia accompanying the appearance of the antibody, they concluded that it was an autoantibody and that in chickens, it was an anemia inducing factor.

In studying bone marrow of patients with systemic lupus erythematosus Hargreaves in 1948 (as cited by Humphrey and White, 1970) noted that many of the granulocytic cells had deformed nuclei and that vaguely outlined discs, that gave a positive Feulgen stain reaction, were frequently associated with the disease. It was subsequently found that these lupus erythematosus (LE) cells could be induced by treating normal polymorphonuclear cells with the serum of patients with systemic lupus erythematosus (SLE), and the inducing factor of the serum was identified as antibody of the IgG class (Holman, 1965). It appears that this autoantibody, termed lupus erythematosus (LE) factor, or anti-nuclear factor may consist of antibody to deosyribonucleic acid and to soluble extractable nuclear antigen of the cells. Stimulation of LE factor with purified fractions has not been achieved, except when they were injected as mixtures of complete Freund's adjuvent, which in itself may stimulate the antibody (Holman, 1972; Holman and Deicher, 1959). In addition to SLE, LE factor was associated with New Zealand Black mouse disease and with Aleutian Mink Disease, both of which are associated with congenital viral infections (Helyer and Howie, 1963; Holman, 1972; Howie and Helyer, 1968; Mellors and Huang, 1966). SLE had been reported as a rare and late developing sequelia of infection with BCG vaccine and the Vole _Mycobacterium_ used as prophylaxis for tuberculosis (Wilson and Miles, 1964). About 40% of rheumatoid arthritis cases have positive tests for LE factor (Holman, 1972).

A more ubiquitous autoantibody is recognized in the so called rheumatoid arthritis (RA) factor associated with rheumatic and other

chronic diseases, both idiopathic and infectious (Christian, 1971; Humphrey and White, 1970). The discovery of RA factor was based on the observation that sera of some persons, especially those with rheumatoid arthritis, would aggregate sheep erythrocytes treated minimally with anti-sheep red blood cell serum. It was subsequently found that semi-purified IgG absorbed to particles such as tanned cells, latex or bentonite particles would be aggregated in the same manner by these sera. Recovery of the factor from complexes of it and IgG coated particles indicated that it was usually globulin of the IgG class, but was often a mixture of IgM and IgG at a ratio of one molecule of IgM to 5 of IgG with a sedimentation coefficient of 22S. This mixture has been considered possibly as a complex of 19S antibody with 7S antigen (Metzger, 1967; Normansell and Stanworth, 1968). RA antibody appears to react with the Fc part of the IgG chain, which may in turn have been made reactive by the binding of the globulin to antigen or other particles (Milgrom and Witebsky, 1960). Thus in nature the factor may be stimulated by soluble complexes of IgG with antigen. The ability to generate this autoantibody may be under genetic control as was indicated by positive reactions within families, with or without signs of rheumatic diseases.

As with other autoimmune factors, RA factor has been associated with diseases associated with a variety of unrelated infectious agents such as malaria, kala azar, syphilis, tuberculosis, leprosy and bacterial endocarditis (Barrett-Connor, 1967; Christian, 1971).

Eaton (1939) found that serum of monkeys with acute *Plasmodium knowlesi* malaria would react with serum of persons recovered from vivax, falciparum or quartan malaria. The presence of the antigen in the serum of malarious monkeys was confirmed by Cox (1966) who also reported that injections of the globulin of the malarious monkeys into rats produced an anemia and afterwards these rats were resistant to *Plasmodium berghei* malaria. Sibinovic et al. (1967, 1969, 1967) detected antigen in the serum of dogs, horses and rodents with babesiosis that reacted with serum of rats recovered from *Babesia rodhaini* infection, dogs recovered from *Babesia canis* infection, and horses recovered from *Babesia equi* infection. Injection of globulin from dogs or rats with babesiosis caused anemia in the recipient animals and they were later resistant to infections with heterologous parasites. Cox et al. (1968) showed that the soluble antigens of serum from animals with acute malaria and babesiosis appeared to be identical in immunodiffusion tests, and Corwin and Cox (1969) found that immunization with serum containing this antigen would confer resistance to heterologous genera and species of haemosporidian parasites. Soni and Cox (1974, 1975a, b, c) confirmed the identity of the antigens associated with malaria and babesiosis, found these antigens not to react with antibody to purified parasite antigen and found that the antigen as a complex with its antibody were mediators of anemia and nephritis. In addition to malaria and babesiosis, this soluble serum antigen was found associated with *Eperythrozoon coccoides* infections of mice and *Haemobartonella muris* infections of rats (Cox and Iturri, 1976). Thus this soluble antigen of serum is rather ubiquitous in its association with infectious diseases and for this reason it was suggested that it might be an autoantigen (Cox and

Iturri, 1976). Antibody to this antigen might be another of the autoantibodies that are found associated with infectious diseases.

Antigen and Antibody Complexes in Diseases

The first observations on disease resulting from antigen and antibody alone were those recorded by von Pirquet and Schick (1905) in the monograph, "Die Serumkrankheit." From this it was clear that von Pirquet recognized that serum sickness was a result of antibody that had been stimulated by injections of horse serum and that the disease mimicked infectious diseases, both acute and chronic. Serum sickness became a model for the study of immunopathology, first as a local reaction in skin to study the effects on tissue of an immune animal of injected antigen as in the work of Arthus (1903, 1906), and then as a model for diseases resembling rheumatoid arthritis, lupus erythematosus, glomerulonephritis or polyarteritis (Dixon, 1971; Dixon et al., 1961; Udaka, 1971). It was established that the disease was associated with an antigen excess over antibody, which might best be achieved by intravascular inoculation of the antigen into nonimmune animals and watching the disease develop with the appearance of antibody, however similar results were achieved by the intravenous injection of nonprecipitating mixtures of antigen and antibody with antigen in excess (Germuth et al., 1967). The demonstration that immune complexes could be induced by the injection of autoantigens and that the appearance of these complexes was accompanied by signs of glomerulonephritis indicated that immune complex disease might be a functional mechanism in certain autoimmune diseases (Edgington et al., 1967). Experimental serum sickness has become a popular model for the study of the immunopathology of vascular-renal disease, and the role of complexes and complement fragments activated by complexes, in arteritis and glomerulonephritis have been well studied by McCluskey and Vassalli (1971) and Dixon (1971).

An additional aspect of immune complex disease has received less attention. Dixon (1966) suggested that soluble antigen-antibody complexes could bind nonspecifically with blood cells or other particles in blood, usually by means of the Fc chain of the antibody (Dixon et al., 1961). With the bound glubulin of the complex, the cells and particles will act as though they were opsoninized and will be sequestered in the spleen, or if conditions are correct, the cells or particles may activate complement and undergo intravascular lysis. Soni and Cox (1974, 1975b) verified this suggestion of Dixon (1966) by finding that injection of malarious globulin containing soluble serum antigen and its antibody into normal birds immediately resulted in anemia and that the antigen moiety of the complex could be detected on the surface of blood cells of the recipient birds by fluorescent antibody methods. Thus antigen-antibody complexes may be anemia inducing factors as well as the mediators of arteritis or glomerulonephritis.

An unexplored ramification of antigen-antibody complex activity is their role as determinants for autoimmunization. Immunoconglutinins are autoantibodies stimulated by altered C_3 and C_4 fragments of complement (Lachmann, 1962, 1966; Lachmann and Coombs, 1965). Similarly, rheumatoid arthritis factor is autoantibody to the Fc region of IgG that has been modified in some way when it forms a complex with antigen (Milgrom and Witebsky, 1960). It may be possible that complexes nonspecifically bound to erythrocytes may alter the cell membrane to the extent that the stroma antigens of Thomson and Friedenreich (Friedenreich, 1928) are expressed and therefore indirectly be responsible for the stimulation of autohemagglutinins. Similarly, the interaction of complexes with vascular endothelium or other cells may damage the cells to release the antigenic lipid that stimulate Wassermann antibody as was suggested by Kahn (1951). The fact that these autoantibodies might in turn form immune complexes with their antigens has received little literary consideration.

The Agents of Infectious Anemia

The agents of infectious anemia form a taxonomically diverse group which may be parasites of an almost equally diverse group of hosts, e.g., man, equines, canines, ovines, bovines, rodents, and avians. Thus viral infectious anemia has been a recognized disease in horses, ducks, and more recently, in laboratory rats. Agents of the rickettsia-like agents, *Bartonella*, *Anaplasma*, *Haemobartonella*, *Eperythrozoon* and perhaps others, may produce infectious anemia in man, swine, sheep, cattle, dogs, cats and rodents. Protozoan parasites of the *Plasmodium*, *Babesia*, and *Trypanosoma* groups are well known associates of the infectious anemia syndrome in man, various domestic animals and birds. Infections of agents listed have been associated with anemia and splenomegaly which were accompanied by a common autoimmune factor, cold-active hemagglutinin (Cox and Iturri, 1976; Cox et al., 1966; Oki and Miura, 1970; Schroeder et al., 1966; Schroeder and Ristic, 1965).

While the list of agents is incomplete, it clearly illustrates their taxonomic diversity. The infectious anemia syndrome may be associated with other infectious diseases, but with less frequency and in less dramatic ways. Anemia with splenomegaly and nephritis accompanied by autoimmune factors is commonly associated with Kala Azar, a generally fatal chronic disease which may result from infections of Leishmania donovani (Woodruff, 1973; Woodruff et al., 1972). In some cases of advanced syphilis hemoglobinuric anemia will be accompanied by the presence of autohemagglutinins (Donath and Landsteiner, 1904). Signs of jaundice or red water disease may be seen with considerable frequency in leptospirosis of man and animals (Wilson and Miles, 1964). During the acute viremic stages of Cytomegalovirus or Epstein-Barr virus, anemia with splenomegaly has been noted with some frequency (Jawetz et al., 1974). J. L. Soni (personal communication) observed anemia with splenomegaly and nephritis accompanied by cold-active hemagglutinin in chickens during an outbreak of Borrelia anserina infection at the Veterinary College at Jabalpur, India. Thus the number and diversity of agents that have been associated with the infectious anemia syndrome may be considerably greater than was indicated.

On the other hand the syndrome may be inconspicuous during the infections of the agents listed. Marchiafavia (1931) commented on the frequency with which he had seen patients whose blood was teeming with Plasmodium falciparum parasites who had not and did not later have symptoms relatable to pernicious malaria. Martin Young (personal communication) tells of calling Liberian or Panamanian children from their play to prick their fingers and then to find that the blood of these same children was teeming with parasites. Hackett (1937) protested that the Italians he found heavily infected with plasmodial parasites had few complaints that were relatable to malarial disease. J. L. Soni (personal communication) told of the frequency with which he had seen heavy infections of *Babesia bigemina* in Indian cattle that had no signs of the red water disease, which was associated with infection with this parasite during the Texas epizootic described by Smith and Kilborne (1893). Thus the absence of the infectious anemia syndrome during acute infection may be as much of a phenomenon as is its presence.

Agents of Infectious Anemia Used for Comparative Study

The objectives of the present research are to determine the nature of autoimmune-like factors which may be related to the pathogenesis and to the phenomenon of nonspecific acquired resistance in infectious anemias. It was therefore desirable that agents of taxonomic diversity which consistently induced the syndrome in a single host species be used for the study. As a host, Sprague-Dawley male rats were selected since much of the earlier works relating to anemia and nephritis from malarial, babesial and *Haemobartonella* infections had employed laboratory rats (Cox, 1966; Cox and Iturri, 1976; Cox et al., 1969; Schroeder et al., 1966; Sibinovic et al., 1967).

Two fortunate observations made from this laboratory provided two unrelated agents for the study. It was observed that a laboratory strain of *Trypanosoma lewisi* maintained at this laboratory produced unusually severe anemia during infections of rats. The trypanosome infection was eliminated by infecting rats that had been made immune by infection with a new strain of *T. lewisi* obtained from the American Type Culture Collection which is here designated as the ATC strain. These rats did not show trypanosome parasites in their blood, but on the seventh day after infection they showed signs of hemolytic anemia. Blood was transferred from these to fresh rats which in turn did not develop trypanosomal parasites, but developed hemolytic anemia and died within 8 days. This trypanosome free agent has been maintained by blood passage in rats, or as frozen infected blood in liquid nitrogen since June 1974. The agent passed a 0.2 micron filter and could not be cultured on media for bacteria, leptospira or mycoplasma. No organisms were detected by microscopic examination of blood of infected animals. The agent was not infective when inoculated into mature mice, and rats housed in the cage with infected rats did not become infected. While evidence is not complete, it is believed that this filterable replicating agent is a virus and that it can be handled with safety as a laboratory infection.

In rats the agent causes hemolytic anemia, splenomegaly and acute glomerulonephritis which are accompanied by the presence of high titers of cold-active hemagglutinin (CAH). Mature rats usually die of the disease within 8 to 10 days after inoculation. Young rats weighing 100 gm or less invariably recover and appear normal 30 days after infection. Reinfection of recovered rats results in reappearance of anemia from which the rats would recover. Studies of the scientific literature has not furnished information on an anemia inducing viral agent that is exclusively a parasite of rats. Since it fulfills the characteristics for infectious anemia, the disease has been referred to as rat infectious anemia (RIA). Details of the isolation of RIA virus and a description of the disease is presented elsewhere in this thesis (Thoongsuwan and Cox, 1976a).

During the isolation of RIA virus from its trypanosome companion, it was noted that rats infected with the new ATC strain of T. lewisi developed moderately severe anemia with splenomegaly, from which they did not completely recover until as late as 30 days after infection. An investigation of this infection was initiated since T. lewisi had been characterized as nonpathogenic for rats (Kudo, 1947; Soulsby, 1968). The study indicated that there was anemia with splenomegaly and mild glomerulonephritis accompanied by the presence of cold-active hemagglutinin (CAH) associated with the post-acute phase of T. lewisi infection of rats. The details of this study are reported elsewhere in this thesis (Thoongsuwan and Cox, 1976b).

These preliminary investigations have furnished two taxonomically unrelated agents, a filterable replicating agent presumed to be virus, and a hemoflagellate, T. lewisi, which may produce the infectious anemia syndrome in laboratory rats. For comparative purposes two haemosporidian parasites, Plasmodium chabaudi and Babesia rodhaini, were selected since previous study had indicated that the infectious anemia syndrome was associated with the infection of each in laboratory rats (Iturri and Cox, 1969; Musoke, 1973; Schroeder et al., 1966; Thoongsuwan, 1971; Thoongsuwan and Cox, 1973).

History and Taxonomy of the Agents Studied

Filterable Rat Infectious Anemia (RIA) Agent

As indicated, previous records of a viral agent of rats that caused disease signs resembling those of RIA were not found. However there was a single account of anemia resulting from the infection of newborn rats with Friends Murine Leukemia virus (Kuzumaki et al., 1974). In this work it was reported that the rats developed signs of leukemia and lymphomas in addition to anemia. Evidence of leukemia or tumors in rats infected with RIA agent was not found, and since RIA agent appeared not to be infective for mice, it was considered improbable that it could have been Friends virus (Thoongsuwan and Cox, 1976a). It was therefore assumed that RIA agent and RIA disease had not been previously described.

The other agents employed were protozoan parasites that had been known for some time and had been adequately classified by Levine (1961) whose taxonomic terminology will be followed.

Trypanosoma lewisi

Trypanosoma lewisi is believed to be the first trypanosome parasite found in mammalian blood (Levine, 1961). In classification it and the other trypanosomes are grouped in Class *Mastigasida*, the protozoans without pseudopodia, with one or more flagella and a vesicular nucleus. It is included along with those having one or two flagella in Order *Promastigorida* and with those with a single flagellum, a single nucleus, a kinetoplast and a parabasal body which given rise to the flagellum, in Family *Trypanosomatidae*. The genera of the family are exclusively parasitic, and in addition to Genus *Trypanosoma*, include other important parasites of vertebrates, Genus *Leishmania*. Other genera of the family, which are primarily parasites of arthropods and plants, must be mentioned since the trypanosomes may resemble them in form during the various stages of development in their life cycles (Levine, 1961).

Arthropod parasites of Genus *Leptomonas* may alternate between two forms: the first, a leptomonad stage, having a parabasal body located at the anterior pole of the cell and the flagellum attached to it emerging anteriorly without attachment to the ectoplasm by an undulating membrane, and secondly an unflagellated oval intracellular form resembling leishmanial parasites. Arthropod parasites of Genus *Crithidia* have a single form resembling the leptomonad except that the parabasal body is more centrally located and the flagellum emerges laterally and is attached to the ectoplasm as it proceeds anteriorally. In their cyclic development, trypanosomes may have leptomonal, crithidial, leishmanial or trypanosomal stages. In the latter form the parabasal body is posteriorally and the emerging flagellum is attached to the ectoplasm from the posterior to the anterior end by means of an undulating membrane (Kudo, 1947). This descriptive nomenclature appears to have caused confusion and modern texts refer to leishmanial forms as amastigotes, leptomonal forms as promastigotes, crithidial forms as epimastigotes and trypanosomal forms as trypomastigotes (Faust et al., 1975).

Among the trypanosomes in general, the trypanosomal forms are found as actively motile extracellular parasites of blood and the leishmanial forms are found as intracellular parasites of monocytes or the phagocytic histiocytes of various tissues. Leptomonal and crithidial stages are found as extracellular parasites of the foregut, the hindgut or the salivary glands of their arthropod vector hosts. The crithidial form of trypanosomes, which is infective for the vertebrate host, will be located in the mouth parts or salivary glands of arthropods infected with certain trypanosomes. Crithidial forms of others will be found in the hindgut and will be passed from the vector host in its feces. Thus parasites of some trypanosomes are passed to a new host by the bite of the blood sucking arthropod while others are passed by the contaminated feces being rubbed into wounds made by the feeding arthropod or by contamination of oral mucosa or the conjuctiva (Faust et al., 1975).

Other trypanosomes, perhaps as a result of adaptation, appear to have lost their biological requirement for an arthropod host. _Trypanosoma_ _evansi_, the agent for surra in equines or camels, is passed mechanically by the contaminated proboscis of blood feeding biting flies such as _Tabanus_ spp. or _Stomoxys_ spp., _Trypanosoma_ _equiperdum_, the agent of equine dourine or equine syphilis, is usually transmitted venerally (Levine, 1961).

Historically, the first trypanosome was described from the blood of salmon by Valentin in 1841 and from the blood of a frog by

Gluge in 1842. Lewis in 1878 described the first trypanosome from mammals in the blood of rats and Evans in 1881 was the first to relate trypanosomes to disease. Bruce in 1895 discovered the tsetse fly (Genus *Glossina*) as a host for African trypanosomes and in 1902 Dutton related *Trypanosoma gambiense* to the human disease, African sleeping sickness (Levine, 1961).

T. lewisi is distributed worldwide as a parasite of the common black rat, *Rattus rattus*, and the more common Norway rat, *Rattus norvegicus*, in which it appeared not to produce disease. It was readily adapted by blood passage to laboratory rats and has been referred to as nonpathogenic (Kudo, 1947; Soulsby, 1968). Its biological vector host, the rat flea *Ceratophyllus fasciatus*, becomes infected while taking blood from the infected rat. In the stomach of the flea, the parasite infects epithelial cells of the organ and multiplies. The emerging crithidial forms migrate to the hindgut where they may pass in the feces of the flea or may infect the epithelium of the gut. In feeding, fleas move freely from host to host and they defecate profusely. Thus the rat in grooming, or scratching could readily acquire infection from this ectoparasite (Kudo, 1947).

Since it is easily handled in the laboratory and is a potent immunogen for rats, *T. lewisi* has been used as a model for the study of trypanosomal immunity since as early as 1899 (Rabinowitsch and Kempner, 1899). The ATC strain used for this research was given to the American Type Culture Collection by Dr. Robert G. Yaeger of Tulane University School of Medicine, New Orleans, La. In observation of this parasite at this laboratory no evidence of RIA virus contamination has been found.

The haemosporiaian parasites, *Plasmodium chabaudi* and *Babesia rodhaini*, have been described in previous reviews (Musoke, 1973; Thoongsuwan, 1971).

Plasmodium chabaudi

The *P. chabaudi* strain was adapted from laboratory mice to rats by Musoke (1973). The strain had been used in previous investigations from this laboratory (Cox and Milar, 1968; Cox et al., 1968). It was provided by the late Dr. Elvio H. Sadun, then Director of the Department of Medical Zoology, Walter Reed Army Institute of Research, Washington, D.C., and was cleared of *Eperythrozoon coccoides* contamination by methods recommended by Thompson and Bayles (1966). When first adapted, this strain appeared to be a potent antigen and even splenectomized rats spontaneously recovered. With passages, the antigenicity appeared to have diminished, and after the 35th passage, all infected rats died during a hemolytic crisis (Musoke, 1973).

P. chabaudi was isolated from the blood of a tree rat, *Thallomys rutilans*, of the Central African Republic by Landau (1965), who was unable to find the natural vector mosquito (Landau and Chabaud, 1965). When it was transferred to laboratory mice *E. coccoides* contamination may have contributed to a false characteristic in that it was considered less lethal for mice than the previously described *Plasmodium berghei* and *Plasmodium vinckei*, which were also parasites of Central African tree rats (Ott et al., 1967). Elimination of the *E. coccoides* left *P. chabaudi* as lethal for mice as were the other

parasites, and recontamination resulted in mice being spared the lethal effects (Ott et al., 1967). Like P. vinckei, P. chabaudi infects mature erythrocytes with much greater frequency than does P. berghei, which preferentially infects reticulocytes, this characteristic remained evident after P. chabaudi had been adapted to rats (Musoke, 1973).

Malaria, the disease of human plasmodial infections, was described as a recognizable entity in Ebers Papyrus dating from 1500 B.C. The disease was related to bad air and mosquitoes in the Edwin Smith Surgical Papyrus dating from 1600 B.C. (Breasted, 1930; Halawani and Shawarby, 1957). Specific curative and suppressive drug, Quinine, was found in common use by South American Indians and was introduced as an antimalarial drug to Europe in 1640 (de Angelis, 1954). The etiologic agents for the malarial fever were not discovered until they were described in 1878 in the blood of French soldiers in Algeria by Laveran (1880, 1884). Remarkably, these observations of the three common species of human plasmodia were made without the aid of the oil immersion lens and the polychrome staining methods that are so heavily relied upon today. It remained for Ross (Ross, 1897, 1898) to discover the developmental steps of plasmodial parasites in mosquitoes employing parasites of the common magpies of India. The mosquito phase of human plasmodial parasites was reported at about the same time, by Grassi (1900) in Italy. Knowledge of the complete life cycle was furnished in 1944 by the observations of Huff and coworkers (Huff and Bloom, 1935; Huff and Coulston, 1944) on the exoerythrocytic schizogony cycle of the plasmodial parasites.

Babesia rodhaini

The strain of *B. rodhaini* used at this laboratory was obtained from the late Dr. Paul E. Thompson who was then with Parke Davis and Company, Ann Arbor, Michigan. It was used extensively as a model infection in laboratory rats (Cox and Milar, 1968; Cox et al., 1968; Iturri and Cox, 1969; Schroeder et al., 1966; Thoongsuwan and Cox, 1973).

B. rodhaini is another of the haemosporidian parasites found in tree rats of Central Africa. It was found in the blood of an arboreal rat, *Thamnomys surdaster surdaster* in the then Belgian Congo in 1950 and was adapted to laboratory mice by Van den Berghe and coworkers (1950). The arthropod vector was not discovered but was assumed to be an Ixodid tick. The parasite was adapted to laboratory rats by Beveridge (1953).

Early taxonomists had included Genera *Babesia* and *Plasmodium* within Order *Haemosporidia* of Class *Sporozoa*, Phylum *Protozoa* (Kudo, 1947). Rearrangement of the sporulating protozoans into new classes and subclasses as recommended by Levine (1961) has not contributed to clarity and some of the newer textbooks retain the older arrangement (Kudo, 1947; Brown, 1975). Since the life cycles and taxonomic relationships of a number of these parasites have not been elucidated, it is here preferred to leave the babesial and plasmodial parasites in the Order *Haemosporidia*.

Babesial parasites were first observed in the blood of African cattle suffering red water disease in 1888 by Babes (1888), and the parasites were given the name *Babesia* in his honor in 1893 by

Starcovici (1893). Hard ticks were identified as the arthropod vector by Smith and Kilbourne (1893) while they were working on the great outbreak of the disease in Texas. They also demonstrated that the parasites were passed from adult to young ticks via infected eggs. The developmental cycle of Babesia parasites in Ixodid ticks has not been completely elucidated.

Babesiosis is recognized as an important disease of many domestic and wild animals. It is of passing interest that the pet lioness of Mrs. Joy Adamson of Kenya, Elsa of her book, "Born Free," was reported to have died of babesiosis. Dr. C. G. D. Brown of Kenya has estimated that babesiosis, along with trypanosomiasis and East Coast Fever, caused by the tick borne Theilaria parva, may have killed as many as three million head of cattle in East Africa within a single year (personal communication). The recent discovery of several cases of human babesiosis has raised a concern that these parasites might also be a human health hazard in parts of the world (Anderson et al., 1974).

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Article 1

COMPARATIVE STUDIES OF INFECTIOUS ANAEMIAS IN RATS

I. HAEMOLYTIC ANAEMIA AND GLOMERULONEPHRITIS ASSOCIATED WITH HAEMAGGLUTININ IN RATS INFECTED WITH A FILTERABLE AGENT

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I. HAEMOLYTIC ANAEMIA AND GLOMERULONEPHRITIS ASSOCIATED WITH HAEMAGGLUTININ IN RATS INFECTED WITH A FILTERABLE AGENT[1,2]

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INTRODUCTION

It was observed that a laboratory strain of *Trypanosoma lewisi* used at this laboratory for teaching purposes, produced signs of haemolytic anaemia, often followed by death, in mature rats. Soulsby (1968) had characterized *T. lewisi* as a nonpathogenic trypanosome, and the disease signs presently reported differed dramatically from those previously observed (Cox, 1964). It was therefore, suspected that the disease signs observed in this infection might have been in part due to an occult companion agent, as was shown in the case of the laboratory strain of *Plasmodium lophurae* maintained at this laboratory (Ludford et al., 1969; 1972).

A long standing interest in the pathogenesis of infectious haemolytic anaemias prompted us to investigate this suspicion. The study resulted in finding that trypanosome free filtrate of the blood of rats infected with this strain of *T. lewisi* produced a haemolytic disease with death in mature rats within 5 to 10 days after inoculation. For more than a year, rats used to transfer the trypanosome-free agent at weekly intervals have all died of the disease.

Methods whereby the agent was isolated, our attempts to visualize and culture it and the disease produced by the agent are described in this communication.

MATERIALS AND METHODS

Experimental Animals:

Male Sprague-Dawley rats and Swiss mice used for the study were purchased from Spartan Research Animals Inc., Haslett, Michigan. Methods of animal care and experimental procedures were consistent with those promulgated by the Institute of Laboratory Animal Resources of the National Research Council.

Experimental Infections:

The agent was isolated from the blood of rats infected with a *Trypanosoma lewisi* strain that has been stored in liquid nitrogen in the Department of Microbiology and Public Health (MPH) of Michigan State University. The MPH strain was last used as an experimental infection in Dexamethasone treated rats by Patton and Clark (1968) who did not indicate its origin. A new strain was obtained from American Type Culture (ATC) Collection, 12301 Parklawn Dr., Rockville, Maryland. The MPH and ATC strains of *T. lewisi* were maintained by blood passage as described (Cox, 1964) or stored as glycerinated infected blood in liquid nitrogen.

For experimental infections, blood of etherized rats infected with *T. lewisi* was drawn by cardiac puncture and added 10 parts to 1 of heparinized saline (100 units sodium heparin/ml of 0.85% NaCl solution). After standing for 30 min at 5 C, the blood was centrifuged at 800 g for 10 min and the plasma removed. The sediment was washed 3 times by suspension in equal volumes of cold saline (0.85% NaCl solution) and centrifugation at 800 g for 10 min. After the final centrifugation the sediment was resuspended in saline and again sedimented by centrifugation. The sedimented cells and supernatant were then placed in a water bath at 37 C for 30 min to allow the trypanosomes to become active. The recovered supernatant contained active trypanosomes and a few blood cells. The number of trypanosomes per cu mm was determined microscopically using Sahli rbc pipettes, Hayems solution and a Neubauer hemocytometer counting chamber, following standard methods using for rbc counts. Infections were standardized to 1 X 10^6 trypanosomes per ml.

Detection and isolation of the companion agent of the MPH strain of *T. lewisi*

Six mature rats were inoculated intraperitoneally (IP) with 10^6 trypanosomes of the ATC strain and 6 others with the MPH strain of *T. lewisi*. Erythrocyte and parasite counts were made daily on all rats until death or recovery from the infections. The six rats recovered from ATC strain infection and 6 normal rats were each inoculated with 10^6 trypanosomes of the MPH strain. Blood for rbc and trypanosome counts were taken daily. The recovered rats remained trypanosome free but became acutely anaemic on the 7th day. Blood from these rats, diluted 1:100 was inoculated IP into normal rats. The passage rats remained free of trypanosomes and developed anaemia with haemoglobinuria on the 5th or 6th day after infection. The trypanosome-free agent (TFA) has been maintained by passage of 1 ml of a 1:100 dilution of blood from anaemic to fresh rats at 3-4 day intervals. It was also stored as infected glycerinated blood in liquid nitrogen, just as were the *T. lewisi* strains.

When TFA or trypanosomes were needed as experimental infections, rats were inoculated intraperitoneally with the stored blood. The infections were then maintained by blood passage as described (Cox, 1964).

Tests to identify TFA

Blood and urine of rats infected with TFA were examined by darkfield microscopy for spirochaetes. Wrights and Giemsa stained blood films were examined for haemosporidian parasites and for organisms resembling Haemobartonella. Blood of the anaemic rats was cultured for bacteria, leptospira and mycoplasma using media and procedures routinely employed in the diagnostic microbiology laboratory of the College of Veterinary Medicine of Michigan State University, as recommended (Carter, 1973). Blood from anaemic rats was cultured on three separate occasions using both aerobic and anaerobic methods.

A rat embryo cell culture line was initiated as recommended by Roberts and Carter (1972). These cultures were inoculated with blood from anaemic rats and observed daily for cytopathogenic effects. On the 7th day, supernatant of the cultures was transferred to freshly grown cells. Cells grown on cover glasses were fixed with methanol and stained with haematoxylin and eosin to be studied for inclusions and altered cell morphology (Roberts and Carter, 1972).

Cells from the 4th culture passage and cells from uninoculated cultures were recovered and disrupted by alternate freezing in methanol-dry ice mixture and thawing in a 37 C water bath. After centrifugation at 800 g for 15 min, 1 ml of the supernatant fluids was inoculated IP into rats. Red cell counts were made daily for 14 days on the 6 rats in each group. Three weeks after inoculation, each rat in each group was inoculated IP with 1 ml of a 1:100 dilution of blood from a rat infected with TFA. Red cell counts were again made daily until all of the rats were dead, or had recovered from anaemia.

Tests for infectivity of TFA for mice and for contact transmission to rats

Three normal rats and five mice were each inoculated with 1.0 ml of a 1:100 dilution of blood from rats with TFA infection. The rats were housed in the same cage with 3 normal rats. All animals were observed daily for 30 days. Red blood cell counts were made daily on all until death, or for 2 weeks. Weekly rbc counts were made on survivors after the second week. The uninoculated companion rats of the experiment were inoculated with blood from anaemic rats on the 30th day and daily blood counts were continued.

Fluorescent antibody tests of blood and spleens from infected rats for TFA

Young rats that had recovered from TFA infections were reinoculated with 1 ml of blood from anaemic rats. Three weeks later they were exsanguinated by cardiac puncture under ether anesthesia. The serum was recovered and the globulin precipitated twice at 50% of saturation with ammonium sulphate. The globulin was dialized against borate buffered saline, pH 8.4, ionic strength 0.175 until free of sulphate as determined by precipitate from added $BaCl_2$ solution. The globulin was then adjusted to 25 mg of protein per ml as determined by the methods of Lowry et al. (1951). It was then conjugated with fluorescein isothiocyanate (FITC) as recommended (Goldman, 1968). The conjugated globulin was filtered through a Sephadex G-25 column and absorbed with activated animal charcoal (Soni and Cox, 1975b). It was then absorbed with 1 ml of washed-packed buffy coat from blood of normal rats per 1 ml of the conjugate at 37 C for 2 hours. The conjugate was stored in small aliquots at -18 C.

Blood films and impression slides of the cut surface of spleen from normal rats and rats anaemic from TFA infection were fixed for 3 min in absolute methanol. The slides were then incubated with the FITC conjugated globulin diluted 1:4 with 0.01 M phosphate buffered 0.85% NaCl solution, pH 7.5 (PBS) at 37 C for 30 min. The slides were rinsed in 3 changes of PBS and a #1 cover glass was mounted with 90% glycerine in PBS. The slides were examined for fluorescent activity with a Zeiss Fluroscope. Photomicrographs were made with Kodak Tri-X film at an exposure time of 90 sec.

Filtration experiments

Whole blood from rats infected for 4 days with TFA was added to heparinized saline as described and subjected to freeze-thaw treatment using a dry ice and methanol mixture and thawing in a 37 C water bath. After two treatments the fluids were clarified by centrifugation at 800 g for 20 min. The supernatant was diluted 1:10 with saline and divided into equal portions. One portion was passed through a 0.20 micron Nalgaline filter unit (Nalge Sybron Corporation, Rochester, NY) and the other through a 0.45 micron membrane (Becton, Dickinson & Co., Oxnard, CA). Samples of each filtrate were inoculated to Myocoplasma medium obtained from Difco Laboratories, Detroit, Michigan and incubated for 7 days at 37 C in a high moisture and CO_2 atmosphere. The remaining filtrates were each diluted 1:10 and 1 ml of each was injected into each of 8 rats for the 0.45 micron filtrate and 8 for the 0.2 micron filtrate. RBC counts were made daily on each rat.

Tests for splenomegaly and erythrophagocytosis in rats infected with the MPH strains of T. lewisi and with TFA

In all seven normal mature rats, six mature rats used for passage of the MPH strain and eight used for TFA passage were autopsied and the spleen was measured in ml by volume displacement of saline in a graduate cylinder. Each spleen was cut transversely and impression smears were made of the cut surface. After the smears had air dried, they were fixed for 3 min in absolute methanol and stained for 30 min in Giemsa stain diluted 1:10 with 0.01 M phosphate buffer, pH 6.8. These preparations were examined for phagocytosis of erythrocytes and trypanosomes by splenic macrophages.

Smear preparations were made from bone marrow obtained by splitting the head of the femur. These were fixed and stained as described and also examined for erythrophagocytic activity.

Histologic study of kidneys from rats made anaemic by TFA infection

Kidneys were taken from 6 anaemic rats sacrificed for spleen studies. They were cut into small pieces 2-3 mm thick and fixed in 10% formalin. The tissues were cleared, dehydrated and embedded. Sections were cut at 4 microns and stained with hematoxylin and eosin (Luna, 1968). Kidney histopathology of the glomeruli and convoluted tubules was evaluated following criteria used for babesial and malarial nephritis (Iturri and Cox, 1969; Soni and Cox, 1974).

Photomicrographs of kidney, spleen and bone marrow were made with a Zeiss Fluroscope by tungsten filament illumination with a blud filter using Kodak High Speed Daylight Ektachrome film at an exposure of 0.25 sec.

Red cell and leucocyte counts, parasitaemia, and cold-active haemagglutinin (CAH) in rats inoculated with normal rat erythrocytes, and rats infected with TFA

Two groups of 14 young rats were used. The first group was inoculated with 10^9 normal rat blood cells per rat. Each rat of the second group was inoculated with 1 ml of a 1:100 dilution of blood from a rat infected with TFA. Six rats from each group furnished blood samples daily for wbc and rbc counts. The other eight in each group were divided into groups of four rats. These groups were bled by cardiac puncture on alternate days throughout the experiment to furnish daily serum samples to be tested for CAH as described (Thoongsuwan and Cox, 1973).

Rbc and wbc counts were made from blood drawn from the tip of the tail after it was snipped with scissors. Samples were taken with standard Sahli hemocytometer pipettes using Hayems fluid for rbc counts, and 2% glacial acetic acid as diluent for wbc counts. The counts were made microscopically using a Neubauer hemocytometer counting chamber. Blood films for differential leucocyte counts were stained with Wrights Stain.

Comparison of anaemia and mortality from TFA infections in old (300 gm) and young (100 gm) rats

A group of eight young rats and eight mature rats were each given the standard inoculum of TFA infected rat blood. Red cell counts were made daily on these rats until death or recovery.

EXPERIMENTAL RESULTS

Comparison of parasitaemia and anaemia in rats infected with the MPH and ATC strains of T. lewisi

The data from groups of six rats are shown, Table 1. Reductions in red cell counts of rats with MPH strain infections were noticed on day 6 or 7. On day 8 the average cell counts of these rats was less than 3 X 10^6 and most of the rats exhibited haemoglobinuria with urine the color of Port wine. Four of these rats were dead on day 9. The remaining 2 rats had recovered from both anaemia and parasitaemia by day 20. Reduction in cell counts of rats infected with the ATC strain were evident on day 7, and counts were lowest on day 9. These rats did not exhibit haemoglobinuria and all had recovered by day 24. Trypanosomal parasitaemia was lower in rats infected with MPH strain than it was in those given the ATC strain.

Parasitaemia and anaemia in normal rats and rats recovered from infection with the ATC strain after infection with the MPH strain of T. lewisi

The data from the 6 experimental and 6 control rats are presented, Table 2. Rats that had recovered from the ATC strain of T.

lewisi did not show trypanosomal parasitaemia throughout the experiment. However, all of the rats began to show anaemia on the 4th day which was severest on day 7. None of these rats died and all appeared to have recovered on day 14. The control rats had detectable trypanosomes in their blood on day 1. Parasitaemia was maximal on day 5 when anaemia was first evident. Blood cell counts fell rapidly and all died with signs of haemoglobinuria before the 8th day.

Attempts to detect and identify TFA

Study of blood from rats infected with TFA using darkfield illumination and study of Wright's or Giemsa stained blood films did not reveal the presence of spirochaetes, haemosporidian, or Haemobartonella. Repeated cultures of blood from anaemic rats for bacteria, leptospira and mycoplasma were negative.

Rat embryo cell cultures inoculated with blood and saline washings from minced spleen from anaemic rats did not reveal evidence of cytopathogenic effect or virus-like inclusions in cells through 5 subinoculations. Injection of supernatant from frozen-thawed cells inoculated with the 2nd and 4th passage culture supernatant did not produce anaemia in susceptible rats. Three weeks later when these rats were inoculated with TFA infected blood, they were as susceptible as rats inoculated with the supernatant from control cell cultures.

Results of tests for infectivity of filtrates of lysed blood from anaemic rats that had passed 0.45 and 0.2 micron millipore filters are shown, Table 3. Rats inoculated with material from the 0.45 micron filter all died of anaemia 7 days after injection, indicating that this filtrate was as infective as unfiltered blood. Signs of anaemia in rats injected with the filtrate from the 0.2 micron filter did not develop until the 11th day, but they too all died of haemolytic anaemia by the 13th day. Thus, it was indicated that TFA particle was less than 200 nm in diameter.

When tested with FITC conjugated globulin from rats hyperimmunized after recovery from TFA infection, blood leucocytes gave bright fluorescence. In some of the cells small dense areas gave intensive reactions. Cells of the spleen sinusoids reacted with "flare-like" fluorescence. No fluorescent activity was seen in blood and spleen preparations from normal rats, Figure 1.

Mice inoculated with diluted blood from rats made anaemic by TFA infection did not develop any signs of anaemia while control rats given the same material died with haemolytic anaemia within 8 days. Uninoculated rats housed in the cage with the infected rats did not develop signs of anaemia and appeared normal at the end of the observation period. These rats developed haemolytic anaemia and died within 8 days after they were finally inoculated with blood from anaemic rats.

Red blood cell counts, white blood cell counts, and titers of cold-active haemagglutinin in control rats injected with 10^9 normal rat cells, and rats inoculated with TFA

Red cell counts on the control rats increased over the 30 day period as the rats matured. The counts from rats infected with TFA fell sharply from the 4th through the 6th day. There were no deaths and all had recovered from anaemia by day 30. Rats infected with TFA had high titers of CAH from the 2nd through the 5th day. CAH was not detected in plasma samples from the control rats. Table 4.

Leucocyte counts were elevated on day 4 in the infected rats and remained so until day 14. Most of the leucocytosis appeared to be due to increases in the number of mononuclear cells, particularly monocytes. Leucocytes resembling those seen in myelogenous or lymphatic leukemia were not seen.

Comparison of anaemia and mortality in young and old rats infected with TFA

The data on rbc counts and mortality in this experiment are summarized, Table 5. Reductions in rbc counts occurred a day earlier in young rats than in mature, but did not drop as precipitously or as low. None of the young animals died, and all appeared to have recovered at the end of the experiment. The rapid reduction in rbc counts of the older rats was accompanied by haemoglobinuria with urine the color of Port wine. Five of these rats died on the 5th day and the remainder on the 6th.

Splenomegaly and erythrophagocytosis in rats infected with the MPH strain of T. lewisi and with TFA

The folumes of spleens from rats with the infections did not differ, however they were approximately 3 X those of normal rats, Table 6.

Phagocytosis of trypanosomes was not seen, however, erythrophagocytosis in the sinuses of the spleens from rats infected with MPH *T. lewisi* and with TFA was extensive, Figure 2. Phagocytosis of erythrocytes by bone marrow macrophagocytosis in rats infected with MPH _T. lewisi_ and TFA was as marked as in the spleen, Figure 3.

Histology of kidneys of rats infected with TFA

In kidneys from rats with anaemia from TFA infection changes in glomeruli varied from swelling of the glomerular tuft with moderate hypercellularity to nearly complete necrosis. The tuft was often swollen to the extent that it completely occupied Bowmans capsule, which had a thickened wall. The epithelium of the convoluted tubules was often swollen to the extent that the lumen of the tubules appeared to be no longer patent. In others there was necrosis of the epithelium. The basement membrane of tubules was usually swollen and interstitial edema was evident. Some tubules contained clear deposits resembling hyaline casts, Figure 4.

DISCUSSION

These experiments indicated that the haemolytic anaemia and mortality in rats infected with the MPH strain of _T. lewisi_ was due to an agent other than the trypanosome. The absence of bacteria, mycoplasma, spirochetes, _Haemosporidia_, or _Haemobartonella_-like organisms indicated that these agents were not involved. Infectivity of freeze-thaw lysed blood that had passed a 0.2 millimicron filter indicated that the particle size of the TFA was less than 200 nm.

Tests of blood films from anaemic rats with FITC conjugated globulin from hyperimmunized rats indicated that leucocytes might be infected. In some of these cells small areas of approximately 0.5 μ or less in size showed particularly intensive fluorescence. While it was considered possible that these small bodies might have been viral inclusions, we are not prepared to make such a conclusion at this time. Attempts to grow the agent in rat embryo cell cultures (and later in embryonated chicken eggs) were disappointing, as were also attempts to visualize the agent by electron-microscopic study of ultra thin sections of pelletized buffy coat from infected rat blood. Until the agent has been visualized or cultured, we would only suggest that it might be a virus. For convenience sake we have referred to the disease as rat infectious anaemia (RIA).

The origin of RIA agent is unknown. The MPH strain of T. lewisi was last used as an experimental infection in Dexamethasone treated rats (Patton and Clark, 1968). In examining their data it appeared that the parasitaemia in their untreated controls did not differ from previously observed data (Cox, 1964). We have given rats infected with the ATC strain of T. lewisi Dexamethasone treatments used by Patton and Clark (1968), but were unable to obtain similar results. More importantly, we were unable to detect signs of RIA agent after treatment of rats infected with the ATC strain. It therefore, appeared that the ATC strain, which had been donated to the American Type Culture Collection by Dr. Robert G. Yaeger of the Tulane University School of Medicine, was not contaminated with RIA agent (unpublished).

A review of the literature has revealed only one account of anaemia in rats associated with a filterable agent. Infection of neonatal rats with Friends lymphatic leukemia virus caused a runting syndrome associated with a high incidence of haemolytic anaemia which was accompanied by antibody to erythrocytes (Kuzumoki et al., 1974). While we did not infect neonatal rats, we have not seen signs of runting, lymphoma or chronic anaemia in young rats after infection with RIA agent.

In mature rats RIA was typically an acute haemolytic anaemia with haemoglobinuria and death within 5-8 days following inoculation. Anaemia was accompanied by a markedly enlarged spleen, extensive phagocytosis of erythrocytes by splenic and bone marrow macrophagocytes, and by high titers of CAH. These disease signs were accompanied by an acute glomerulonephritis involving both glomerular and tubuler elements of the kidney. Necrosis of glomeruli and tubuler epithelium was observed with considerable frequency. In young rats anaemia was less severe, haemoglobinuria was less evident, and they usually recovered within 3-4 weeks. Since recovered rats did not develop haemolytic anaemia following reinoculation, it can be assumed that they had an acquired resistance.

These observations add RIA virus, to the list of agents that produce anaemia with splenomegaly accompanied by autoantibody to erythrocytes (Cox and Iturri, 1976; Cox et al., 1966; Oki and Miura, 1970; Schroeder et al., 1966; Schroeder and Ristic, 1965). Since the agent appeared not to be infective for mice, it was not transmitted from infected to normal rats housed in the same cage, and the authors have suffered no ill effects after handling the agent for 2 years. It may serve as a safe and useful model for the study of viral anaemia.

SUMMARY

A replicating filterable agent isolated from the blood of rats infected with a strain of T. lewisi kept at this Department caused acute haemolytic anaemia, splenomegaly, glomerulonephritis and death within 5-8 days in mature rats. The disease was less severe in weanling rats which usually recovered within 3-4 weeks. The anaemia was accompanied by phagocytosis of erythrocytes by monocytes of the spleen and bone marrow and by high titres of cold-active haemagglutinin. In fluorescent antibody tests leucocytes from the blood of anaemic rats reacted strongly with globulin from hyperimmunized recovered rats, revealing the presence of small intranuclear inclusions in some cells. Filtrates of blood from anaemic rats passing a 0.20 micron filter caused anaemia and death of inoculated rats 3-4 days later than did blood filtrates passing a 0.45 micron millipore filter. Thus the particle size of the agent was smaller than 200 millimicrons. Attempts to grow the agent on rat embryo fibroblast cultures were unsuccessful; however, the failure to detect other infectious organisms allows the suggestion that the causal agent for this disease which we have called rat infectious anaemia, might be a virus.

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Table 1.--Mean number of red blood cells per mm^3 (RBC X 10^6), mean number of trypanosomes per mm^3 (T X 10^5) and standard error of the mean (S.E.) in blood of mature rats infected with the Microbiology and Public Health (MPH) and American Type Culture (ATC) strains of *Trypanosoma lewisi*.

Days	RBC X 10^6 ± S.E.		T X 10^5 ± S.E.	
	MPH strain	ATC strain	MPH strain	ATC strain
0	8.63 ± 0.29	8.33 ± 0.21	--	--
1	8.96 ± 0.23	8.92 ± 0.11	--	--
2	9.13 ± 0.34	8.73 ± 0.18	†	†
3	8.72 ± 0.26	8.53 ± 0.15	0.04 ± 0.01	0.03 ± 0.01
4	8.96 ± 0.26	9.14 ± 0.10	0.18 ± 0.03	0.41 ± 0.04**
5	8.36 ± 0.08	8.41 ± 0.17	0.72 ± 0.19	1.99 ± 0.44
6	7.91 ± 0.11	8.26 ± 0.25	1.36 ± 0.42	4.02 ± 0.91**
7	6.92 ± 0.38	7.55 ± 0.33	1.58 ± 0.49	4.62 ± 1.21**
8	2.98 ± 0.26	7.03 ± 0.41**	1.80 ± 0.59	4.24 ± 1.12
9	2.20 ± 0.05*	6.89 ± 0.38**	0.11 ± 0.01*	3.77 ± 1.25
10	3.20 ± 0.10	7.03 ± 0.46**	0.14 ± 0.04	2.44 ± 0.88
11	3.67 ± 0.16	7.15 ± 0.48**	0.16 ± 0.10	2.25 ± 0.73
12	5.52 ± 0.16	7.43 ± 0.39**	0.08 ± 0.02	2.31 ± 0.79
13	6.44 ± 0.18	8.17 ± 0.33**	0.06 ± 0.02	2.05 ± 0.83
15	6.93 ± 0.31	7.98 ± 0.39	0.02 ± 0.01	1.53 ± 0.64
17	7.26 ± 0.24	7.88 ± 0.31	0.08 ± 0.02	1.04 ± 0.73
20	8.67 ± 0.14	8.01 ± 0.41	--	0.36 ± 0.60
24	8.99 ± 0.07	9.28 ± 0.13	--	--

†Rare trypanosomes found.

*Four of 6 rats dead on 9th day. There was no mortality among the 6 rats of the group infected with the ATC strain.

**Significant at $P < 0.05$ or less (Students t).

Table 2.--Mean and standard error of the mean (S.E.) of red blood cells per mm^3 (RBC X 10^6) and the mean and S.E. of the number of trypanosomes per mm^3 (T X 10^5) in normal mature control rats, and mature rats recovered from infection with the American Type Culture (ATC) strain of *Trypanosoma lewisi*, after infection with the Microbiology and Public Health (MPH) strain of *T. lewisi*.

Days	Normal Rats		Recovered Rats	
	RBC X 10^6 ± S.E.	T X 10^5 ± S.E.	RBC X 10^6 ± S.E.	T X 10^5
0	9.96 ± 0.29	--	9.28 ± 0.35	---
1	9.58 ± 0.31	†	9.53 ± 0.28	---
2	9.52 ± 0.23	†	9.59 ± 0.30	---
3	10.19 ± 0.26	0.057 ± 0.01	9.73 ± 0.30	---
4	9.54 ± 0.33	0.567 ± 0.19	8.82 ± 0.20	---
5	7.11 ± 0.31	1.103 ± 0.39	7.73 ± 0.31	---
6	4.58*± 0.48	0.934*± 0.56	4.57 ± 0.83	---
7	2.54 ± 0.22	0.907 ± 0.65	2.98 ± 0.28	---
8			4.19 ± 0.32	---
10			5.54 ± 0.26	---
12			6.63 ± 0.16	---
14			8.57 ± 0.17	---

†Rare trypanosomes found.

*First mortality on day 6. Remainder of the 6 rats were dead on day 8. No mortality among the 6 recovered rats.

Table 3.--Mean number of red blood cells per mm^3 (RBC x 10^6) and standard error of the mean (S.E.) of 8 mature rats after inoculation with the supernatant of freeze-thaw treated whole blood of rats infected with TFA that had been passed through a 0.20 micron (200 nm) Millipore membrane, and 8 mature rats inoculated with the supernatant that passed through a 0.45 micron (450 nm) membrane.

Days	RBC X 10^6 ± S.E.	
	200 nm filtrate	450 nm filtrate
0	8.32 ± 0.09	8.43 ± 0.16
1	8.44 ± 0.10	8.56 ± 0.14
2	8.49 ± 0.09	8.65 ± 0.12
3	8.60 ± 0.12	8.64 ± 0.14
4	8.48 ± 0.12	8.40 ± 0.11
5	8.68 ± 0.13	8.57 ± 0.16
6	8.43 ± 0.12	6.88 ± 0.43
7	8.64 ± 0.14	2.77 ± 0.12*
8	8.73 ± 0.16	
9	8.63 ± 0.19	
10	8.24 ± 0.16	
11	5.39 ± 0.84	
12	3.04 ± 0.29**	

*Four of 8 rats in this group died on day 7, the remainder on day 8.

**Five of 8 rats in this group died on day 12, the remainder on day 13.

Table 4.--Mean and standard error of the mean (S.E.) of red blood cells per mm^3 (RBC X 10^6), mean number of trypanosomes per mm^3 (T X 10^5) and the mean titres of cold-active haemagglutinin (CAH) in blood of young rats injected with normal rat blood cell suspension (Group I), and rats infected with TFA (Group II).

Days	Group I			Group II		
	RBC X 10^6 ± S.E.	T X 10^5	CAH	RBC X 10^6 ± S.E.	T X 10^5	CAH
0	5.68 ± 0.16	---	---	5.93 ± 0.14	---	---
1	6.26 ± 0.27	---	---	6.32 ± 0.21	---	---
2	6.53 ± 0.25	---	---	6.73 ± 0.14	---	256*
3	6.79 ± 0.23	---	---	6.39 ± 0.19	---	256
4	6.83 ± 0.25	---	---	4.23 ± 0.43	---	256
5	7.05 ± 0.24	---	---	2.89 ± 0.08	---	256
6	7.35 ± 0.06	---	---	3.31 ± 0.23	---	---
7	7.18 ± 0.22	---	---	4.42 ± 0.18	---	---
9	7.21 ± 0.24	---	---	5.29 ± 0.18	---	---
11	7.26 ± 0.36	---	---	5.74 ± 0.17	---	---
14	7.44 ± 0.14	---	---	6.55 ± 0.13	---	---
17	7.64 ± 0.12	---	---	7.59 ± 0.16	---	---
20	7.86 ± 0.07	---	---	7.61 ± 0.24	---	---
24	7.75 ± 0.09	---	---	7.68 ± 0.13	---	---
30	7.95 ± 0.11	---	---	7.93 ± 0.12	---	---

*CAH titres were all 256 or higher.

Table 5.--Mean red blood cell counts (RBC X 10^6), ± standard error of the mean (S.E.) and mortality among 8 young rats and 8 mature rats after infection with TFA.

Days	Young Rats		Mature Rats	
	RBC X 10^6 ± S.E.	No. Dead	RBC X 10^6 ± S.E.	No. Dead
0	6.12 ± 0.18	0	9.54 ± 0.11	0
1	6.29 ± 0.09	0	9.23 ± 0.32	0
2	6.44 ± 0.11	0	9.86 ± 0.26	0
3	6.81 ± 0.14	0	9.47 ± 0.22	0
4	4.54 ± 0.33	0	9.34 ± 0.37	0
5	2.77 ± 0.42	0	7.27 ± 0.46	0
6	2.92 ± 0.12	0	2.61 ± 0.34	5
7	3.81 ± 0.23	0		8
9	4.63 ± 0.16	0		
11	5.76 ± 0.24	0		
14	6.48 ± 0.11	0		
17	7.13 ± 0.22	0		
20	7.62 ± 0.09	0		
24	7.96 ± 0.12	0		
30	8.14 ± 0.08	0		

Table 6.--Average spleen volume in ml of normal rats, rats infected with the Microbiology and Public Health (MPH) strain of *Trypanosoma lewisi*, and rats infected with TFA found as a companion of the MPH strain of *T. lewisi*.

	Number	Average Vol. in ml	Range
Normal rats	7	1.01	0.9 - 1.2
Infected with MPH strain	6	3.07	2.8 - 3.2
Infected with TFA	8	3.04	2.4 - 3.8

Fig. 1.--Photomicrographs of blood films and spleen impression slides from normal rats and rats with TFA infection after incubation with FITC conjugated globulin from rats hyperimmunized by TFA infections. A. Blood film from a normal rat (125X). B. Blood film from a rat with acute TFA infection (125X). Note the fluorescence of the cytoplasm of the polymorphonuclear (PMN) leucocytes, and the small intranuclear areas of fluorescence seen in some. C. PMN leucocytes showing fluorescence of intranuclear inclusions (1000X). D. Fluorescence of leucocytes in the spleen from a rat with acute TFA infection (125X).

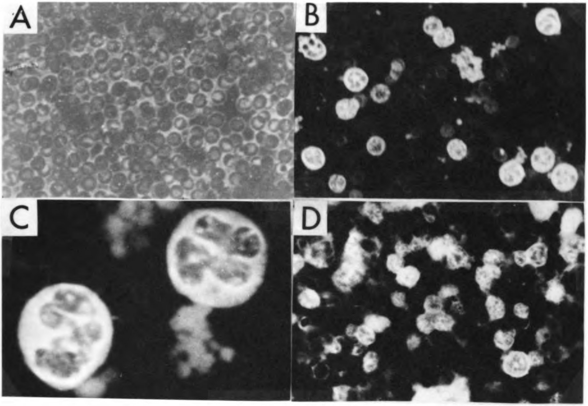

Figure 1

Fig. 2.--Photomicrographs of Giemsa stained spleen impression slides (1000X). A. Slide from normal rat spleen. B. Slide from the spleen of a rat infected with the ATC strain of *T. lewisi*. C. Slide from the spleen of a rat infected with the MPH strain of *T. lewisi*. D. Slide from the spleen of a rat infected with TFA. Phagocytosis of trypanosomes was not observed. Macrophagocytes engorged with erythrocytes were abundant in the preparations from rats infected with MPH *T. lewisi* and TFA.

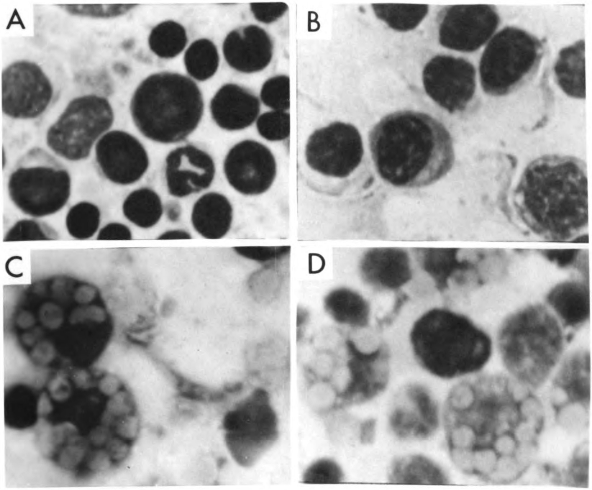

Figure 2

Fig. 3.--Photomicrographs of Giemsa stained bone marrow films (1000X). A. Preparation from a normal rat. B. Preparation from a rat infected with the ATC strain of *T. lewisi*. C and D. Preparations from a rat with TFA infection. Phagocytized trypanosomes were not seen, and macrophagocytes engorged with erythrocytes were abundant in preparations from TFA infected rats.

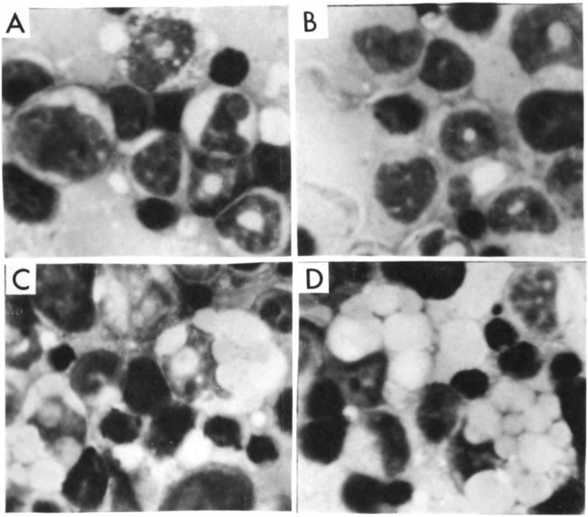

Figure 3

Fig. 4.--Photomicrographs of rat kidney sections cut at 4 µ and H and E stained (700X). A. View of glomerulus and adjacent convoluted tubules from a normal rat kidney. B-F. Views of sections from kidney of a rat with acute TFA infection. B. Note swelling and hypercellularity of the glomerular tuft, and that the lumen of the adjacent tubules are obliterated due to swelling of the epithelium. C. Note fat deposits adjacent to the tuft and the thickening of Bowman's membrane. D. Necrosis with the loss of structure of the capillary loop was a frequent finding. E. Desquamation of the tubular epithelium and masses resembling hyaline casts were found. F. Note interstitial edema and thickened basement membrane, found about the distal convoluted tubules.

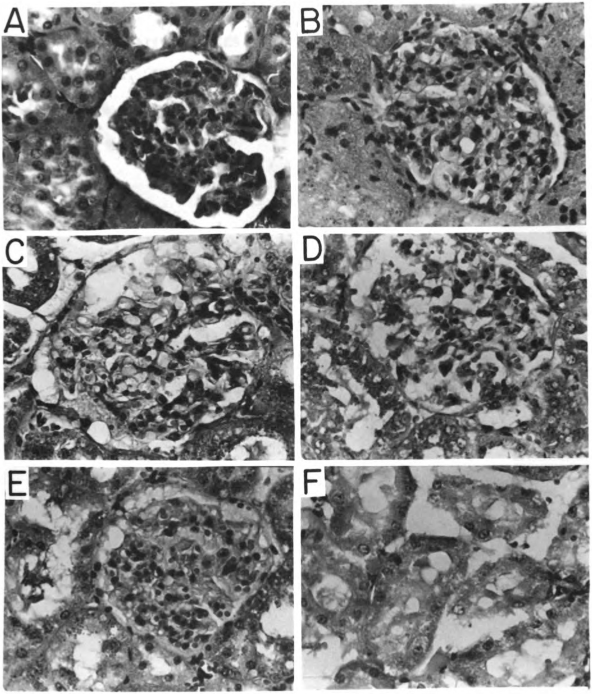

Figure 4

Article 2

COMPARATIVE STUDIES OF INFECTIOUS ANAEMIAS IN RATS

II. AUTOIMMUNE-LIKE ANAEMIA ASSOCIATED WITH *TRYPANOSOMA LEWISI* INFECTION

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COMPARATIVE STUDIES OF INFECTIOUS ANAEMIAS IN RATS

II. AUTOIMMUNE-LIKE ANAEMIA ASSOCIATED WITH *TRYPANOSOMA LEWISI* INFECTION[1,2]

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1 [1] This communication is from a thesis entitled "Comparative Studies of Infectious Anemias in Rats" submitted by the author in partial fulfillment of the requirements for the Ph.D. degree from Michigan State University. His participation in these studies was made possible by a Faculty Fellowship award from the Chulalongkorn University, Bangkok, Thailand.

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INTRODUCTION

A disease syndrome of anaemia with splenomegaly and vascular-renal disease accompanied by autoimmune-like blood factors may be associated with the infections of unrelated agents (Cox and Iturri, 1976; Cox et al., 1966; Iturri and Cox, 1969; Oki and Miura, 1970; Schroeder et al., 1966). The association of auto-antibody and the absence of a specific etiologic agent led to a suggestion that this infection-associated syndrome should be called infectious anaemia (Cox and Iturri, 1976).

The authors reported that a replicating filterable agent found as a companion of a laboratory strain of _Trypanosoma_ _lewisi_ in the blood of rats caused acute haemolytic anaemia, splenomegaly with erythrophagocytosis and acute glomerulonephritis which was accompanied by high titres of cold-active haemagglutinin (HA). Since other infectious agents were not detected and the companion agent passes a 0.2 micron filter, it was suggested that it might be virus. The disease resulting from infection was called rat infectious anaemia (RIA) (Thoongsuwan and Cox, 1977).

In the course of liberating RIA agent from its trypanosome companion it was noticed that the new (ATC) strain of _T._ _lewisi_ obtained from the American Type Culture Collection produced a moderately severe and persistent anaemia. It differed from the lethal haemolytic anaemia associated with infections of the contaminated strain and from RIA in that it was not haemolytic and none of the rats died. Since _T._ _lewisi_ had been characterized by Soulsby (1968) as "nonpathogenic" for mature rats, it was felt that infection of rats with this parasite deserved further investigation. It was found that there was a persistent anaemia with splenomegaly and signs of glomerulonephritis, accompanied by the presence of HA, associated with the late phase of infection with the ATC strain of *T. lewisi*. The details of these experiments are presented and discussed.

MATERIALS AND METHODS

Animals and infections

The strain and source of rats, the methods of animal care, the source and maintenance of the ATC strain of *T. lewisi*, and the methods for standardizing *T. lewisi* infections were described (Thoongsuwan and Cox, 1977).

Trypanosomal parasitaemia and anaemia in rats were determined by microscopic methods using Sahli haemocytometer pipettes with Hayem's diluting fluid and a Neubauer haemocytometer counting chamber as described (Thoongsuwan and Cox, 1977).

Tests for cold-active haemagglutinin (HA)

Rats were bled by cardiac puncture under ether anaesthesia. Three ml of blood was withdrawn and added to 0.3 ml of heparinized saline (100 units sodium heparin per ml of 0.85% NaCl). The plasma was harvested and stored at -18 C until the samples could be tested for HA with trypsinized rat erythrocytes as described (Thoongsuwan and Cox, 1973).

Studies for splenomegaly, erythrophagocytosis and glomerulonephritis

Experimental and control rats sacrificed for autopsy were exsanguinated by cardiac puncture under ether anaesthesia and the organs removed. Spleen volume was determined by fluid volume displacement as described (Soni and Cox, 1974). The organ was then cut transversely and impression slides were prepared, stained by the Giemsa method and examined for erythrophagocytosis (Thoongsuwan and Cox, 1977). The kidneys were cut into small pieces which were fixed in 10% formaldehyde. These were cleared, mounted, cut and stained with haematoxylin-eosin following standard procedures (Luna, 1968). Pathologic changes were evaluated by criteria of a swollen glomerular tuft, swelling of tubuler epithelium, edema of Bowman's membrane and tubular basement membrane. To obtain a numerical value for the severity of kidney damage (SKD), 100 randomly selected nephrons (glomerulus and adjacent convoluted tubules) were evaluated from 0 to 4 plus. The sum of the evaluations represented the SKD score for the animal (Iturri and Cox, 1969). Hypercellularity of glomeruli was used as a second parameter. Counts were made of the number of nuclei in the glomerular tuft (NGT) of 100 randomly selected glomeruli (Kibukamusoke and Hutt, 1967). Comparison of the means of the counts from infected and control rats were subjected to statistical analysis (students to test).

Microphotography

Photomicrographs of kidney sections and spleen impression slides were made with a Zeiss Fluroscope with tungsten filament illumination and a blue filter using Kodak High Speed Daylight Ectachrome film and an exposure time of 0.25 sec.

Erythrocyte and parasite counts and titers of cold-active haemagglutinin (HA) during the course of T. lewisi infection in rats

Two groups of 14 rats were used. Rats of the control group were each inoculated intraperitoneally (IP) with 1 x 10^9 normal rat blood cells. Rats of the 2nd group were each inoculated with 1 x 10^6 trypanosomes separated from infected rat blood as described (Thoongsuwan and Cox, 1976). Six rats from each group were used to furnish blood for rbc and trypanosome counts. The remaining 8 of each group were divided into groups of 4 rats. One group of 4 infected and 4 control rats were bled on days 0, 5, 9, 14 and 20, and the other groups of 4 were bled on days 3, 7, 11, 17 and 24 so that plasma samples from infected and control rats were taken at 2-day intervals without excessive stress on the animals. Plasma samples recovered from the blood were stored at -18 C until they could be tested for HA with trypsinized rat erythrocytes.

EXPERIMENTAL RESULTS

The means of rbc counts and trypanosome counts and the mean titres of the HA are presented graphically (Figure 1). As these rats were young, the rbc counts of the controls which were injected with normal rat erythrocytes, gradually increased from approximately 6 x 10^6/cu mm to near 8 x 10^6 at the end of the experiment. HA was detected first on day 5 near the time when a significant drop in the rbc counts was evident. Both HA and anaemia persisted throughout the period of chronic parasitaemia. Recovery from anaemia was associated with recovery from parasitaemia and the disappearance of HA from the blood of the rats. HA was not detected in plasma from any of the control rats of the experiment.

The spleens removed from rats with *T. lewisi* anaemia were engorged, firm and approximately three times the volume of spleens removed from normal rats and the kidneys were also enlarged. Urine collected from the rats did not show gross evidence of haemoglobinuria. In the impression slides, areas of red and white pulp were packed with erythrocytes. Trypanosomes were present but not in numbers greater than those seen in blood. Phagocytized erythrocytes but not trypanosomes were found occasionally in splenic macrophagocytes.

In sections of kidneys from rats with *T. lewisi* anaemia the glomerular tuft was swollen to the extent that Bowman's capsule was almost completely occupied. The number of mesengial nuclei of the tuft was increased from a normal of 40-50 to 70-80. Fibrinous adherence of the tuft to Bowman's membrane was evident and the membrane itself was thickened. The epithelium of convoluted tubules adjacent to glomeruli was swollen to the extent that the lumen of the tubules appeared to be closed (Figure 2). Abnormal numbers of hyaline casts were observed in the distal convoluted tubules. The means of the SKD scores and of NGT counts and mean spleen volumes for control and experimental rats are summarized in Table 1.

DISCUSSION

These experiments indicate that _T. lewisi_ is pathogenic for laboratory rats. During the post-acute phase of the infections rats suffer moderate to severe anaemia with splenomegaly and mild signs of glomerulonephritis accompanied by the presence of cold-active haemagglutinin detected with trypsinized rat erythrocytes. The onset of anaemia was sudden without visible signs of haemolysis. It then became chronic, persisting with gradual recovery for as long as parasitaemia and the haemagglutinin were detected. All rats appeared to have recovered from the disease by the 30th day after infection.

The signs of nephritis were primarily swelling of endothelial cells of the capillary loop, hypercellularity of the glomerular tuft, swelling of tubular epithelium, edema of basement membrane and extravasation of proteinaceous matter as indicated by the hyaline casts. Except that they were milder, these signs resembled those associated with anaemia in _Babesia rodhaini_ infections of rats, RIA and _Plasmodium gallinaceum_ infections of chickens, all of which were associated with the presence of cold-active haemagglutinin (Iturri and Cox, 1969; Soni and Cox, 1974; Thoongsuwan and Cox, 1977).

The relevance of cold-active haemagglutinin to the anaemia or nephritis associated with infections of rodents has not been indicated. However, its association with anaemia from infections with RIA agent, babesiosis, malaria, haemobartonellosis and eperythrozoonosis in rats or mice has been established (Cox and Iturri, 1976; Cox et al., 1966; Iturri and Cox, 1969; Oki and Miura, 1970; Schroeder et al., 1966; Thoongsuwan and Cox, 1977). Soni and Cox (1975) indicated that the agglutinin associated with anaemia of chicken malaria was autoantibody of the IgM class, and that it appeared to be an anaemia-inducing factor. We have stimulated production of cold-active haemagglutinin in rats by injections of freeze-thawed autologous blood cells, but as yet have not seen evidence of anaemia in rats from this autoimmunization (unpublished).

It appears that this rather simple test for cold-active haemagglutinin using compatible homologous erythrocytes treated with trypsin might be a useful indicator of suspected cases of autoimmune-like blood disorders.

SUMMARY

Anaemia with splenomegaly and signs of glomerulonephritis were found associated with the late acute and post-acute phase of _Trypanosoma lewisi_ infections of laboratory rats. The onset of the anaemia was associated with the peak of parasitaemia and the development of cold-active haemagglutinin (HA) for trypsinized rat erythrocytes. It persisted with gradual recovery for as long as the trypanosomes and HA were detected in the blood. Signs of glomerulonephritis consisted of hypercellularity of the glomerular tuft, swelling of vascular endothelium and tubular epithelium, thickening of Bowman's membrane and tubular basement membrane, and abnormal numbers of hyaline casts in the distal convoluted tubules. Residual damage to the kidneys was not evaluated.

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Table 1.--The means of spleen volumes, scores of kidney damage (SKD) and of the number of nuclei in the glomerular tuft (NGT) of 8 normal rats and 8 rats infected for 10 days with *Trypanosoma lewisi*.

	Infected	Control
Spleen volume (Range)	3.24 (2.8 - 3.7 ml)	1.0 (0.9 - 1.2 ml)
SKD ± S.E.	223.67 ± 19.1*	47.00 ± 3.06
NGT ± S.E.	76.89 ± 1.5*	52.43 ± 3.81

*Students t test, significant at $P > 0.01$.

Fig. 1.--Mean and standard error of the mean (indicated by vertical lines) for erythrocyte counts (RBC x 10^6), trypanosome counts (trypanosomes x 10^5) and titers of cold-active haemagglutinin (HA titer) in rats after infection with 1 x 10^6 *Trypanosoma lewisi* parasites and in control rats injected with 1 x 10^9 normal rat erythrocytes. Trypanosomes and HA were not found in blood of the control rats.

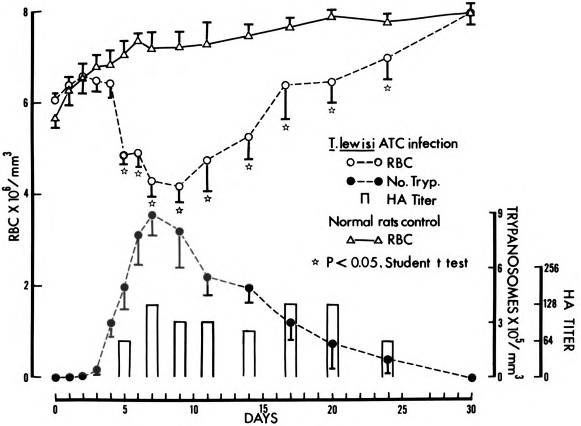
RBC X 10^6/mm^3
8
6
4
2
0
T. lewisi ATC infection
RBC
No. Tryp.
HA Titer
Normal rats control
RBC
P < 0.05, Student t test
TRYPANOSOMES X 10^5/mm^3
9
6
3
0
HA TITER
256
128
64
0
DAYS
0
5
10
15
20
25
30

Figure 1

Fig. 2.--Photomicrographs of sections of kidneys from rats infected for 10 days with Trypanosoma lewisi (A and B) and from a normal rat (C). In section A the glomerular tuft completely fills Bowman's space and the lumen of the adjacent convoluted tubules has been obliterated by swelling of the tubular epithelium; this nephron was given an SKD score of 4 plus. Approximately 100 nuclei may be counted in the glomerular tuft (NGT = 100). In contrast the nephrons seen in C received an SKD score of 0 and NGT count of approximately 45. Fibrinous adherence of the glomerular tuft to the thickened wall of Bowman's capsule. Thickening of the basement membrane of the tubules and some interstitial edema is evident (B). H and E stain 700 X.

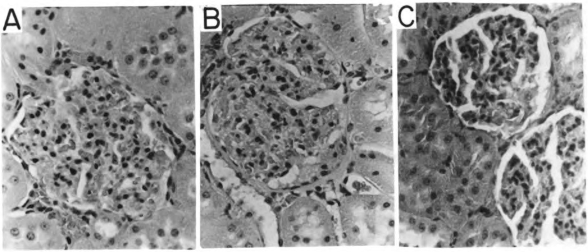

Figure 2

Article 3

COMPARATIVE STUDIES OF INFECTIOUS ANAEMIAS IN RATS

III. IMMUNOCONGLUTININ ASSOCIATED WITH NONSPECIFIC ACQUIRED RESISTANCE AND ANAEMIA

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COMPARATIVE STUDIES OF INFECTIOUS ANAEMIAS IN RATS

III. IMMUNOCONGLUTININ ASSOCIATED WITH NONSPECIFIC ACQUIRED RESISTANCE AND ANAEMIA[1,2]

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[1] This communication is from a thesis entitled "Comparative Studies of Infectious Anemias in Rats" submitted by the author in partial fulfillment of the requirements for the Ph.D. degree from Michigan State University. His participation in these studies was made possible by a Faculty Fellowship award from the Chulalongkorn University, Bangkok, Thailand.

[2] This communication is Journal Article No. from the Michigan Agricultural Experiment Station.

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INTRODUCTION

Past reports indicated that part of the resistance acquired by recovery from infectious anaemia was of a nonspecific nature. Rodents recovered from malaria were resistant to babesiosis and vice versa; mice with Eperythrozoon infections had an enhanced resistance to malaria, and ducks recovered from viral anaemia survived malarial infections that were lethal for controls (Cox, 1972; Cox and Milar, 1968; Ludford et al., 1969, 1972; Ott et al., 1967; Peters, 1965).

Diseases from each of these infections are similar in that each is manifested by anaemia and splenomegaly which is accompanied by cold-active haemagglutinin (CAH) (Cox and Iturri, 1976; Cox et al., 1966; Schroeder et al., 1966). This syndrome of anaemia accompanied by autoantibody to erythrocytes has since been recorded for rats infected with a filterable agent and for rats with Trypanosoma lewisi infection (Thoongsuwan and Cox, 1977a, 1977b). Another autoantibody, immunoconglutinin (IK) has been associated with anaemia in malaria and trypanosomiasis (Woodruff, 1973). Coombs et al. (1961) and Parappally et al. (1973) pointed out that the conglutination activity of IK might have a role in nonspecific acquired resistance. Following a similar line of thought in discussing autoimmunity in malaria, Cox (1964) suggested that autoantibody possessing a detrimental effect on erythrocytes might be deleterious to plasmodial parasites and thus contribute to resistance.

We have investigated this phenomenon of acquired nonspecific resistance associated with recovery from various infectious anaemias and have found it to be associated with IK.

MATERIALS AND METHODS

Experimental animals and infections

Male Sprague-Dawley rats weighing 100 to 180 gm obtained from Spartan Research Animals Inc., Haslett, Michigan, were used for all experiments. Animal care and experimental procedures were consistent with those promulgated by the Institute for Laboratory Animal Resources, National Research Council.

The present studies were made using rat infectious anaemia (RIA) agent, the American Type Culture (ATC) strain of *Trypanosoma lewisi*, *Babesia rodhaini* and *Plasmodium chabaudi*. RIA agent is a filterable agent associated with a strain of *T. lewisi* maintained at this laboratory. Filtration experiments indicated that its diameter was smaller than 0.20 micron. While it has not been grown in cell culture, failure to detect other infectious forms led us to suspect that it is a virus. In mature rats RIA agent produces acute haemolytic anaemia, splenomegaly, glomerulonephritis and death within 8 to 10 days. The disease is less severe in young rats and those weighing 100 gm or less usually survive. Anaemia was accompanied by high titres of cold-active haemagglutinin (Thoongsuwan and Cox, 1977a).

The ATC strain of *T. lewisi* was donated to the American Type Culture Collection by Dr. Robert G. Yaeger, Dept. of Tropical Medicine and Public Health, Tulane University, New Orleans, LA. We found that infections of rats with this strain caused a moderate anaemia with splenomegaly and nephritis which were accompanied by CAH. Our studies furnish no evidence that it was contaminated with RIA agent (Thoongsuwan and Cox, 1977b).

P. chabaudi was adapted to Sprague-Dawley rats by Musoke et al. (1977). Its origin and methods for maintenance have been previously described (Cox and Milar, 1968). The origin and methods for maintaining B. rodhaini have also been described (Thoongsuwan and Cox, 1973).

Standardized infections were induced by intraperitoneal injection of 1 ml of a 1:100 dilution of blood from a rat infected 4 days previously with RIA agent, 1 ml of a saline suspension of 1 x 10^6 trypanosomes from a rat infected for 5 days with T. lewisi, and 1 x 10^8 erythrocytes infected with B. rodhaini or P. chabaudi in 1 ml saline. All experimental infections were standardized by described methods (Cox, 1957; Cox and Milar, 1968; Thoongsuwan and Cox, 1977a).

Measurements of anaemia and parasitaemia

Blood obtained from the snipped tails of rats was drawn with a Sahli RBC hemocytometer pipette and diluted with Hayems solution. A Wrights stained blood film was prepared for determining the percentage of parasitized erythrocytes of rats infected with P. chabaudi or B. rodhaini (Cox, 1957). Red blood cells and trypanosome counts were made microscopically in a Neubauer hemocytometer counting chamber. Estimates of viraemia with RIA were not determined.

Autostimulation of immunoconglutinin in uninfected rats

Kaolin that had been washed with distilled water was ground, autoclaved, and dried. Six ml of blood was drawn from each rat and the recovered serum of each was stored at -18 C. One ml of serum of each rat was mixed with 10 mg of kaolin and incubated at 37 C for 10 min. The mixtures were then washed once, resuspended to original serum volume with 0.85% saline and each was mixed with an equal volume of Freund's complete adjuvant. A 1 ml injection was administered intramuscularly into each thigh of the donor rat. Two subsequent injections were given at weekly intervals using freshly prepared materials. One week after the last injection, they were bled for IK sera or infected for experimental purposes.

Titration of immunoconglutinin (IK)

IK from blood of experimental and control rats was titred by its conglutination of complement-fixed sensitized sheep red blood cells (SRBC) as described by Coombs et al. (1961) except that rats were used to generate anti-SRBC serum. Cooke microtiter methods were used to test serum for IK activity. Rats were given a single intraperitoneal injection of 1 ml of 2% suspension of washed SRBC and 9 days afterward the serum was recovered from blood drawn by cardiac puncture under ether anesthesia. The pooled serum was stored at -18 C. Optimal concentration of anti-SRBC giving least haemolysis and strong agglutination with IK positive serum was predetermined. Wells of microtiter plates were charged with 0.025 ml of 2-fold diluted sample, 0.025 ml of 1:10 heat inactivated horse serum, 0.025 ml of 0.5% complement-fixed sensitized SRBC and 0.05 ml of saline in that order. Negative controls for each sample consisted of sensitized SRBC treated with heat inactivated horse serum. The plates were covered with adhesive plastic film and mechanically shaken for 1 min before incubation at 37 C for 30 min. Final readings of the conglutination titre were made after storage overnight at 4 C.

Tests for cold-active haemagglutinin (CAH)

CAH was detected in plasma or serum with trypsinized rat erythrocytes as previously described (Thoongsuwan and Cox, 1973).

Tests for anaemia, nonspecific acquired resistance, CAH and IK in rats recovered from RIA, malaria, babesiosis and trypanosomiasis

Since blood induced infections were used in these experiments, control animals were injected intraperitoneally with 1 x 10^9 washed rat erythrocytes.

Rats recovered from RIA had been infected when young (100 gm) and recovery of anaemia was complete 4 weeks later (Thoongsuwan and Cox, 1977a). Rats infected with *T. lewisi* were not completely recovered from anaemia until 30 days after infection (Thoongsuwan and Cox, 1977b). Recovery of rats from *B. rodhaini* infection was facilitated by an intraperitoneal injection of 1 ml of 0.5% acriflavine hydrochloride 4 days after infection and recovery from anaemia was complete 3 weeks later. Rats recovered from RIA and *B. rodhaini* infections were each given a second injection of 1 ml of whole blood from rats with acute RIA or from rats with acute babesiosis. They were challenged ten days later.

Twenty-five RIA recovered and 25 control rats were challenged with *P. chabaudi*. Groups of 25 recovered and 25 control rats were challenged with *B. rodhaini*. Groups of 6 recovered and 6 control rats were challenged with *T. lewisi*. Challenge with RIA agent was made in 18 rats recovered from *B. rodhaini* and 16 recovered from *T. lewisi*, using a single control group of 16 rats.

Blood from 6 control and 6 experimental rats for RBC counts and staining was obtained daily commencing one day before challenge. Four animals of each group served as a source for plasma to be tested for CAH and IK activity. Samples of 3 ml were drawn on alternate days by cardiac puncture into 0.3 ml of heparinized saline. Mortality was recorded daily for each group.

Tests for anaemia, nonspecific acquired resistance, CAH and IK in rats autostimulated by injections with autologous serum absorbed on kaolin

Twelve normal rats were bled for serum samples before they were injected with autologous serum absorbed on kaolin as described. After autostimulation, blood for RBC counts and staining was taken on 6 experimental and 6 control rats. Blood was drawn for CAH and IK assays from 4 of the rats in each group. All rats were given 1 x 10^7 *B. rodhaini* infected rat blood cells the following day. RBC counts and blood films were taken daily and blood to be tested for CAH and IK activity was taken at 2 day intervals. Mortality for each group was recorded.

EXPERIMENTAL RESULTS

The relationships of immunoconglutinin and cold-active haemagglutinin in serum of rats with acute rat infectious anaemia (RIA), trypanosomiasis, babesiosis and malaria and in serum from rats recovered from each infection

Pools of serum taken from rats during acute infection and after recovery were tested for IK and CAH (Table 1). Rats with acute infections showed titres of IK ranging from 640 to 5120 compared to 20 in normal rat serum. After recovery IK titres ranged from 80 to 640. CAH was found in the serum pools from rats with acute infections but was not detected in serum of recovered rats.

Anaemia, immunoconglutinin, cold-active haemagglutinin and survival from RIA agent infection in rats recovered from T. lewisi and B. rodhaini infections

Rats recovered from *T. lewisi* and *B. rodhaini* were fully mature on day 30. Results of RBC counts, IK and CAH titres, and mortality are presented in Table 2.

Anaemia was evident 1 to 2 days earlier in rats recovered from *T. lewisi* and *B. rodhaini* infections than in control rats. Red cell counts fell more precipitously and were lower in the control animals than in recovered rats. This early anaemia was associated with a higher initial and a more rapid increase in IK titres after challenge than seen in the control rats. CAH was found in each group of challenged rats, but titres did not differ in the three groups. Fourteen of 18 rats recovered from *B. rodhaini* and 13 of 16 recovered from *T. lewisi* infections survived RIA agent infection and appeared to be normal 3 weeks later. None of the control rats were alive after day 9 and all exhibited haemoglobinuria at the time of death. Evidence of recrudescent *B. rodhaini* or *T. lewisi* infection was not found.

Protection from acute malaria and babesiosis in rats that had recovered from RIA

This experiment was performed with young rats (80-100 gm). These animals exhibited only moderate signs of anaemia with no haemoglobinuria following the first inoculation of RIA agent and less anaemia following the second injection. Data on parasitaemia, RBC counts, IK and CAH activity on these and control rats after challenge with *B. rodhaini* and *P. chabaudi* are presented in Tables 3 and 4.

After challenges, signs of anaemia developed earlier in the recovered rats than in the controls. The early reductions in RBC counts were accompanied by lower *P. chabaudi* and *B. rodhaini* parasitaemia. Early anaemia and reduced parasitaemia were associated with higher initial and more rapid increases in IK titres. CAH titres of control and experimental rats did not differ. When anaemia developed in the control rats the RBC counts fell more precipitously and remained lower than those observed in recovered rats. Thirteen of 25 rats challenged with *B. rodhaini* and 12 of 25 infected with *P. chabaudi* survived and appeared normal 3 weeks after infection. All 25 control rats for each infection died within 8 days.

Protection from trypanosomiasis and anaemia in rats recovered from acute RIA

The RBC and trypanosome counts from 6 RIA recovered rats and 6 control rats after challenge with *T. lewisi* are presented in Table 5. Additional rats for CAH and IK testing were not available. In both groups infections became patent and did not differ until day 5 when the trypanosome counts of the recovered rats were significantly lower than those seen in control rats. On the previous day the erythrocyte counts of the recovered rats were significantly lower than those of the controls. The trypanosomal counts of the recovered rats remained lower and reverted to negative earlier than those of the control group. The lower trypanosome counts of recovered animals were accompanied by lower erythrocyte counts from the 4th through the 16th day. Thereafter, the RIA rats recovered from anaemia more rapidly than did the controls.

Protection from babesiosis in rats with autostimulated immunoconglutinin

The RBC and parasitaemia counts of rats infected with 1×10^7 *B. rodhaini* infected cells are shown in Table 6. Both experimental and control rats developed parasitaemia at equal rates until the 5th day when both anaemia and reductions in parasitaemia became evident in the experimental group. The experimental rats were clear of parasitaemia 2 days earlier than the controls. Fifty percent of the stimulated rats and 25% of the control rats survived the challenging infection. On day 0 the stimulated rats had mean titres of 160 for IK and 117 for CAH. Throughout the experiment these titres rose more rapidly and were higher than those found in the controls.

DISCUSSION

These experiments confirmed and extended previous observations on nonspecific acquired resistance associated with recovery from anaemia-inducing infections. Enhanced resistance to *Plasmodium berghei* and to *P. chabaudi* has been associated with *Eperythrozoon coccoides* infections (Ott et al., 1967; Peters, 1965). The resistance of rodents recovered from malaria to babesiosis, and vice versa, observed by Cox and Milar (1968) was confirmed (Cox, 1972). Ludford et al. (1969, 1972) demonstrated that ducks recovered from viral anaemia had enhanced resistance to malaria. The present work has shown that this nonspecific acquired resistance can be stimulated in rats by infections of RIA agent and by *T. lewisi*. Further, it was shown that the resistance was functional against RIA agent and trypanosomal infections as well as against malaria and babesiosis.

The resistance was manifested as a reduction in parasitaemia which was accompanied by significant reductions in erythrocyte counts manifested on the 2nd or 3rd day after infection. The reduction in parasitaemia and early anaemia were associated with titres of IK that were higher than those seen in the control animals. The association of IK with nonspecific acquired resistance was further emphasized by the resistance of uninfected rats with autostimulated IK to *B. rodhaini* challenge.

This resistance was temporally associated with CAH, which was found in blood of rats during the course of each infection and in blood of uninfected rats with autostimulated IK. It was therefore not clear whether this resistance could be attributed to IK alone.

CAH was associated with acute *Babesia*, *Trypanosoma*, *Plasmodium*, *Haemobartonella* and RIA agent infections (Cox and Iturri, 1976; Cox et al., 1966; Schroeder et al., 1966; Thoongsuwan and Cox, 1973; 1977a; 1977b). In the present experiments CAH was stimulated in uninfected rats simultaneously with IK by injections of kaolin adsorbed rat serum in Freund's complete adjuvant. CAH had been stimulated in normal chickens by injection of disrupted autologous erythrocytes and was detected in chicken plasma with trypsinized human type "O" erythrocytes (Soni and Cox, 1975a). We stimulated CAH in rats by injections of disrupted autologous erythrocytes and demonstrated a reaction of the antibody with rat erythrocyte stroma (unpublished). CAH is therefore considered to be autoantibody to the erythrocyte stroma antigens (Friedenreich, 1928).

IK is autoantibody to fixed C3 and/or C4 (Lachmann, 1966; 1967; Lachmann and Muller-Eberhard, 1968). It has been associated with a number of infectious diseases involving anaemia and splenomegaly in man (Woodruff, 1973). We found IK associated with anaemia and splenomegaly from infections of RIA agent, _T. lewisi_, _P. chabaudi_ and _B. rodhaini_ infections in rats.

It is of interest that Woodruff (1973) speaks of the "anaemia-big spleen syndrome" associated with IK in infectious diseases, and _we_ speak of a syndrome of anaemia, splenomegaly and nephritis associated with CAH as the "infectious anaemia syndrome" (Cox and Iturri, 1976). From the present experiments it would appear that Professor Woodruff's and our syndrome may be the same.

Although the nature of the factors stimulating IK in these infections is unknown it seems reasonable to assume that it was stimulated by complement fixing immune complexes. However, such complexes were not sought in the present study. Candidates for IK stimulation might be a complex of stroma antigen and CAH. Parasite antigen and its antibody may also be present as soluble complex in the blood during acute infectious anaemias (Musoke et al., 1977; Soni and Cox, 1975a). Soni and Cox (1975b; 1975c) demonstrated that soluble complexes of soluble serum antigen (SA) and its antibody (ABSA) were present in the blood of malarious chickens, and that the complexes caused anaemia and nephritis when injected into normal birds. The presence of SA and ABSA was associated with anaemia in rats resulting from malaria, babesiosis and haemobartonellosis (Cox and Iturri, 1976; Cox et al., 1968; Soni and Cox, 1974; Thoongsuwan and Cox, 1973). Other factors may have formed immune complexes that fixed complement. Barrett-Connor (1967) reported a case of Plasmodium vivax malarial anaemia associated with positive tests for Wassermann antibody and for rheumatoid factor. We found Wassermann antibody and CAH associated with anaemia and glomerulonephritis in rodent babesiosis (Iturri and Cox, 1969). Complexes of any of these antibodies with their respective antigens could serve to stimulate IK.

While it is clear that IK was associated with this phenomenon of early anaemia and enhanced resistance, the presence of other immune bodies makes it unwise to suggest that IK alone contributed to resistance. Anaemia resulted from injections of SA into normal animals, and afterwards they had an enhanced resistance to heterologous species and genera of haemosporidian parasites (Cox, 1966; Sibinovic et al., 1967; Corwin and Cox, 1969). From the suggestion of Dixon (1966) we suspect that soluble complexes of SA and ABSA may have become bound to erythrocytes and/or parasites to mimic opsonin and cause these particles to be sequestered and phagocytized. Coombs et al. (1961) presented evidence that the conglutinating activity of IK with complement-fixed complexes of bacteria and antibody would enhance the clearing of bacteria and thus serve as a factor in nonspecific acquired resistance. It is possible that IK may play a similar role involving erythrocytes and parasites that have been coated with antigen-antibody complexes.

SUMMARY

Rats recovered from infectious anaemias had an acquired nonspecific resistance. Recovery from trypanosomal and babesial infections enhanced the resistance to infections with filterable rat infectious anaemia (RIA) agent, and recovery from RIA made rats more resistant to plasmodial, babesial and trypanosomal infections. The resistance was manifested by reduced parasitaemia which usually became evident on the 2nd or 3rd day of infection and which was accompanied by significant anaemia. Mortality from challenge of the recovered rats was usually less than those of the controls. Immunoconglutinin (IK) was detected in high titres in animals during acute stages of infection and remained present in lower amounts after recovery. After the recovered rats were challenged with a heterologous agent, the IK titres became elevated earlier and were usually higher than those of the controls. Uninfected rats with autostimulated IK also exhibited similar enhanced resistance to challenge. However, infections and autostimulation of IK also stimulated production of cold-active haemagglutinin (CAH). It was therefore not clear that the resistance could be attributed to IK alone.

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Table 1.--Immunoconglutinin (IK) and cold-active haemagglutinin (CAH) titres of pooled serum from 5 rats with acute and 5 recovered rats infected with rat infectious anaemia (RIA) agent, *Babesia rodhaini*, *Plasmodium chabaudi* and *Trypanosoma lewisi*.

Infection	IK		CAH	
	Acute	Recovered	Acute	Recovered
RIA	5120	320	512	0
Babesia rodhaini	2560	640	256	0
Plasmodium chabaudi	5120	80	256	0
Trypanosoma lewisi	640	80	128	0
Normal rats	20		0	

Table 2.--Mean ± standard error of erythrocyte counts per mm^3 (RBC x 10^6 ± S.E.), mean titres of immunoconglutinin (IK) and cold-active haemagglutinin (CAH) and mortality among control rats, recovered from infections of *Trypanosoma lewisi* and *Babesia rodhaini* after challenge with rat infectious anaemia agent.

Days Post Infection	Control Rats			Rats recovered from *T. lewisi*			Rats recovered from *B. rodhaini*		
	RBC x 10^6 ± S.E.	IK	CAH	RBC x 10^6 ± S.E.	IK	CAH	RBC x 10^6 ± S.E.	IK	CAH
0	8.41 ± 0.19	17	0	8.33 ± 0.22	53	0	8.27 ± 0.17	133	0
1	8.46 ± 0.18			8.69 ± 0.12			8.71 ± 0.17		
2	8.71 ± 0.19	13	75	8.44 ± 0.15	80	53	7.72 ± 0.28^b	133	96
3	8.72 ± 0.15			7.81 ± 0.16^a			7.15 ± 0.19^a		
4	8.59 ± 0.25	13	171	7.01 ± 0.22^a	120	213	5.70 ± 0.33^a	640	149
5	3.88 ± 0.79			5.38 ± 0.33			4.14 ± 0.59		
6	2.35 ± 0.25*	693	460	3.33 ± 0.46**	507	341	3.78 ± 0.63***	746	299
7				3.49 ± 0.47			3.51 ± 0.39		
8				4.10 ± 0.50	533	299	3.55 ± 0.78	2560	213
9				4.61 ± 0.47			3.89 ± 0.95		
10				5.40 ± 0.38	3413	171	4.27 ± 0.87	3480	171
11				5.60 ± 0.41			4.10 ± 0.65		

Table 2.--Continued.

Days Post Infection	Control Rats			Rats recovered from T. lewisi			Rats recovered from B. rodhaini		
	RBC x 10^6 ± S.E.	IK	CAH	RBC x 10^6 ± S.E.	IK	CAH	RBC x 10^6 ± S.E.	IK	CAH
12				6.38 ± 0.36	2133	171	5.05 ± 0.85	1280	107
13				6.70 ± 0.28			5.14 ± 0.61		
14				7.26 ± 0.27	1493	107	5.82 ± 0.57	1293	85
16				7.65 ± 0.29	320	3	6.47 ± 0.56	293	4
18				8.24 ± 0.12			7.48 ± 0.39		
20				8.57 ± 0.19	67	0	7.91 ± 0.31	73	0

*Four of the 16 rats died on day 6, the remainder on day 7.

**81.25% of the 16 rats survived.

***77.8% of the 18 rats survived.

[a,b] Significant at $P > 0.001$ and $P < 0.02$ respectively (Student t test).

Table 3.--Mean ± standard error of erythrocyte counts per mm^3 (RBC x 10^6 ± S.E.), the percentage of parasitized erythrocytes (% PE ± S.E.), mean titres of immunoconglutinin (IK) and cold-active haemagglutinin (CAH) and mortality among 25 control rats and 25 recovered from rat infectious anaemia (RIA) after challenge with Babesia rodhaini.

Days Post Infection	Control Rats				Rats recovered from RIA			
	RBC x 10^6 ± S.E.	% PE ± S.E.	IK	CAH	RBC x 10^6 ± S.E.	% PE ± S.E.	IK	CAH
0	8.59 ± 0.13	0	13	0	8.32 ± 0.09	0	107	0
1	8.77 ± 0.10	0.13 ± 0.02			8.59 ± 0.08	0.11 ± 0.01		
2	8.86 ± 0.11	1.11 ± 0.13	7	37	8.56 ± 0.07^c	0.66 ± 0.09^c	67	75
3	8.81 ± 0.12	5.78 ± 0.45			7.88 ± 0.11^a	1.88 ± 0.29^a		
4	8.15 ± 0.08	27.13 ± 2.84	107	85	7.26 ± 0.10^a	9.84 ± 2.44^a	587	149
5	3.49 ± 0.38	58.25 ± 2.19			5.28 ± 0.30^b	28.55 ± 5.78^a		
6	2.43 ± 0.29*	69.00 ± 1.22*	480*	213*	3.15 ± 0.29	35.40 ± 4.88^a	1920	256
7					3.05 ± 0.33**	31.74 ± 3.13**		
8					2.82 ± 0.25	4.70 ± 0.90	3840	341
9					2.97 ± 0.28	†		
10					3.67 ± 0.37	0	2133	29
12					4.96 ± 0.19	0		

Table 3.--Continued.

Days Post Infection	Control Rats				Rats recovered from RIA			
	RBC x 10^6 ± S.E.	% PE ± S.E.	IK	CAH	RBC x 10^6 ± S.E.	% PE ± S.E.	IK	CAH
14					4.97 ± 0.15	0	267	0
16					6.20 ± 0.35	0		
18					7.24 ± 0.26	0	160	0
20					7.72 ± 0.40	0		
22					8.76 ± 0.12	0		
24					8.70 ± 0.19	0	133	0

*100% mortality by day 7.

**48% mortality by day 7--52% survived.

[a,b,c]Significant at $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively (student t test).

Table 4.--Mean ± standard error of erythrocyte counts per mm^3 (RBC x 10^6 ± S.E.), % parasitized erythrocytes (% PE ± S.E.), mean titres of immunoconglutinin (IK) and cold-active haemagglutinin (CAH) and mortality among 25 control rats and 25 recovered from rat infectious anaemia (RIA) after challenge with *Plasmodium chabaudi*.

Days Post Infection	Control Rats				Rats recovered from RIA			
	RBC x 10^6 ± S.E.	% PE ± S.E.	IK	CAH	RBC x 10^6 ± S.E.	% PE ± S.E.	IK	CAH
0	8.51 ± 0.10	0	7	0	8.42 ± 0.12	0	147	0
1	8.80 ± 0.11	0.13 ± 0.02			8.66 ± 0.11	0.11 ± 0.01		
2	8.88 ± 0.18	1.25 ± 0.17	10	85	8.68 ± 0.13	0.79 ± 0.13	147	48
3	9.03 ± 0.08	6.59 ± 0.29			7.86 ± 0.11^a	2.65 ± 0.42^a		
4	8.14 ± 0.10	30.88 ± 1.23	853	171	7.26 ± 0.10^a	11.31 ± 1.87^a	1707	256
5	3.69 ± 0.26	63.75 ± 2.17			5.19 ± 0.23^a	29.13 ± 3.74^a		
6	2.90 ± 0.39*	68.00 ± 1.91*	960*	217*	3.35 ± 0.32	43.50 ± 3.43^a	7680	217
7					2.82 ± 0.24	31.60 ± 2.28		
8					2.13 ± 0.26**	10.00 ± 1.22**	5120**	128**
9					3.25 ± 0.27	1.06 ± 0.39		
10					4.47 ± 0.23	+***	3840	53
12					5.51 ± 0.20	0		

Table 4.--Continued.

Days Post Infection	Control Rats				Rats recovered from RIA			
	RBC x 10^6 ± S.E.	% PE ± S.E.	IK	CAH	RBC x 10^6 ± S.E.	% PE ± S.E.	IK	CAH
14					6.28 ± 0.22	0	853	0
16					6.35 ± 0.27	0		
18					7.20 ± 0.17	0	213	0
20					7.28 ± 0.17	0		
22					8.38 ± 0.15	0		
24					8.64 ± 0.20	0	107	0

*100% mortality by day 7.

**52% mortality by day 8--48% survived.

***Positive but uncountable.

[a] Significant at $P < 0.001$ (Student t test).

Table 5.--Mean number of red blood cells per mm^3 (RBC x 10^6), mean number of trypanosomes per mm^3 (T X 10^5) and standard error of the mean (S.E.) in blood of 6 control rats (Control) and 6 rats recovered from rat infectious anaemia (RIA-Recovered) after challenge with <u>Trypanosoma lewisi</u>.

Days	RBC x 10^6 ± S.E.		T x 10^5 ± S.E.	
	Control	RIA-Recovered	Control	RIA-Recovered
0	9.02 ± 0.10	8.93 ± 0.11	-*	-*
1	9.06 ± 0.06	9.43 ± 0.10	+**	+**
2	9.03 ± 0.09	9.01 ± 0.07	0.06 ± 0.01	0.02 ± 0.01
3	9.12 ± 0.15	8.65 ± 0.19	0.45 ± 0.05	0.31 ± 0.07
4	8.52 ± 0.14	7.68 ± 0.10[a]	2.14 ± 0.24	1.44 ± 0.38
5	7.38 ± 0.15	7.11 ± 0.18	5.62 ± 0.80	3.06 ± 0.80[d]
6	6.98 ± 0.26	6.48 ± 0.31	6.64 ± 0.49	3.91 ± 1.12[d]
7	6.90 ± 0.18	6.46 ± 0.13	7.10 ± 0.49	4.61 ± 1.26[d]
8	6.53 ± 0.17	6.23 ± 0.15	6.53 ± 0.48	3.82 ± 1.11[d]
9	7.00 ± 0.28	6.19 ± 0.19	6.63 ± 0.72	2.77 ± 0.86[b]
10	6.98 ± 0.28	6.13 ± 0.31	5.90 ± 0.79	1.37 ± 0.61[a]
12	6.97 ± 0.20	6.50 ± 0.42	5.23 ± 0.59	0.87 ± 0.43[a]
14	7.08 ± 0.23	6.93 ± 0.47	3.92 ± 0.61	0.85 ± 0.42[b]
16	6.99 ± 0.28	7.38 ± 0.39	3.90 ± 0.69	0.44 ± 0.30[a]
18	7.40 ± 0.19	8.01 ± 0.33	2.36 ± 0.58	-[c]
20	7.18 ± 0.27	8.59 ± 0.14[a]	1.27 ± 0.64	-
24	7.67 ± 0.28	9.31 ± 0.12[a]	0.49 ± 0.42	-
30	8.79 ± 0.15	9.23 ± 0.07	-	-

*No trypanosome found. **Rare trypanosomes found.

[a,b,c,d] Significant at $P < 0.001$, $P < 0.01$, $P < 0.02$ and $P < 0.05$ respectively (Student t test).

Table 6.--Mean ± standard error of the mean (S.E.) of red blood cells per mm^3 (RBC x 10^6), percentage of parasitized erythrocytes (% PE), mean titres of immunoconglutinin (IK) and cold-active haemagglutinin (CAH) of normal rats and rats immunized with autologous normal rat serum absorbed on kaolin (SAK) after Babesia rodhaini infection.

Days Post Infection	Normal Rats				SAK Immunized Rats†			
	RBC x 10^6 ± S.E.	% PE ± S.E.	IK	CAH	RBC x 10^6 ± S.E.	% PE ± S.E.	IK	CAH
0	8.51 ± 0.25	-	23.3	0	8.69 ± 0.18	-	160.0	117.3
1	8.49 ± 0.20	+			8.76 ± 0.18	+		
2	8.58 ± 0.16	0.04 ± 0.02	33.3	74.7	8.85 ± 0.22	0.06 ± 0.02	133.3	170.7
3	8.45 ± 0.16	0.34 ± 0.09			8.63 ± 0.13	0.32 ± 0.07		
4	8.69 ± 0.14	1.23 ± 0.32	40.0	107.0	8.62 ± 0.10	1.23 ± 0.19	186.7	213.3
5	8.61 ± 0.21	6.30 ± 1.83			8.01 ± 0.19	4.85 ± 1.07		
6	7.91 ± 0.29	23.00 ± 3.97	1706.7	207	6.91 ± 0.31^a	16.20 ± 3.27	2560.0	341.3
7	4.23 ± 0.57	42.76 ± 3.04			4.27 ± 0.53	24.05 ± 6.61^a		
8	2.44 ± 0.46*	19.28 ± 2.79	1066.7	341.3	3.93 ± 0.54**	1.73 ± 1.00^c	853.3	426.7
9	2.86 ± 0.60	2.33 ± 0.72			4.71 ± 0.90^a	-^{b}		
10	3.17 ± 0.53	+	853.3	106.7	5.16 ± 0.68^a	-	533.3	171
12	4.69 ± 0.49	-			6.09 ± 0.33	-		
14	6.86 ± 0.37	-	133.3	42.7	7.54 ± 0.29	-	106.7	53.3

*Nine out of 12 rats in this group died on day 8, 25% survived.

**Six out of 12 rats in this group died on day 8, 50% survived.

^{a,b,c}Significant at $P < 0.05$, $P < 0.02$, and $P < 0.001$ respectively (Student t test).

†Mean IK titre of rats before injection of SAK was 18.3. CAH was not detected.

Article 4

COMPARATIVE STUDIES OF INFECTIOUS ANAEMIAS IN RATS

IV. STUDIES OF THE NATURE OF IMMUNOCONGLUTININ ASSOCIATED WITH INFECTIOUS ANAEMIA OF RATS AND ITS ROLE IN NONSPECIFIC ACQUIRED RESISTANCE

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(To be submitted to Transactions of The Royal Society of Tropical Medicine and Hygiene, London, England)

COMPARATIVE STUDIES OF INFECTIOUS ANAEMIAS IN RATS

IV. STUDIES OF THE NATURE OF IMMUNOCONGLUTININ ASSOCIATED WITH INFECTIOUS ANAEMIA OF RATS AND ITS ROLE IN NONSPECIFIC ACQUIRED RESISTANCE[1,2]

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INTRODUCTION

Thoongsuwan et al. (1977) reported that immunoconglutinin (IK) was associated with anaemia in rats infected with the filterable agent of rat infectious anaemia (RIA), *Trypanosoma lewisi*, *Babesia rodhaini*, and *Plasmodium chabaudi*. Rats that had recovered from each of the infections maintained reduced but significant titres of this autoantibody. When the recovered rats were challenged with an agent other than the one used for initial infection, they exhibited a non-specific acquired resistance to the heterologous infection. This resistance was manifested 2 to 3 days after infection by significant reductions in parasitaemia which was accompanied by significant reductions in the erythrocyte counts. The recovered rats also had less mortality after challenge than the controls. After challenges, the IK titres of the recovered rats became elevated earlier and were generally higher than those of the controls. However, a role for IK in this resistance was unclear. Antibody to erythrocytes in the form of cold-active haemagglutinin (CAH) was also generated during the infections. An additional factor may have been involved. Soluble serum antigen (SA) and its antibody (ABSA) have been associated with anaemia and acquired resistance of unrelated infectious agents (Cox, 1966; Sibinovic et al., 1967, 1969; Cox et al., 1968; Cox and Iturri, 1967). Soni and Cox (1974, 1975a, 1975b, 1975c) demonstrated that CAH and complexes of SA and ABSA may have a causal relationship in anaemia and nephritis. Thus, factors other than IK may have contributed to the resistance.

We present the results of experiments designed to ascertain whether IK and/or ABSA contributed to the nonspecific acquired resistance of infectious anaemia.

MATERIALS AND METHODS

Experimental animals

Male Sprague-Dawley rats and white Swiss mice were obtained from Spartan Research Animals Inc., Haslett, Michigan. Methods for animal care and the experimental procedures were consistent with those promulgated by the National Institute for Laboratory Animal Resources of the National Research Council.

Experimental infections

Four taxonomically unrelated agents of infectious anaemia were used. The filterable agent of RIA was discovered and proven to cause infectious anaemia of rats at this laboratory (Thoongsuwan and Cox, 1977a). The American Type Culture (ATC) strain of *T. lewisi* was described (Thoongsuwan and Cox, 1977b). The *B. rodhaini* and *P. chabaudi* infections in rats were described by Thoongsuwan and Cox (1973) and Musoke et al. (1977). Standardized infections for each agent were prepared as described (Cox, 1957; Thoongsuwan and Cox, 1977a).

Preparation of serum fractions

Rats infected with *B. rodhaini* for 4-5 days were exsanguinated by cardiac puncture under ether anaesthesia. Serum was recovered from the clotted blood after setting overnight at 4 C and then stored at -18 C until used.

Rats that had recovered from *B. rodhaini* infection were hyperimmunized by 3 weekly intraperitoneal injections of 1 ml of whole heparinized blood from rats heavily infected with *B. rodhaini*. One week after the final injection the rats were exsanguinated by cardiac puncture under ether anaesthesia. Serum was recovered and stored as just described.

Serum collected from blood of rats with acute infection and from hyperimmunized rats was treated 3 to 4 times with ammonium sulfate at 50% saturation (50% SAS) until the precipitate was clear of haemoglobin discolouration. The recovered globulin was dialyzed at 4 C with repeated changes of borate buffered saline, pH 8.4, 0.175 ionic strength, until sulfate ion could no longer be detected with barium chloride solution. Each preparation was adjusted to its original serum volume.

Tests of the globulin fraction with trypsinized rat erythrocytes was performed as described by Thoongsuwan and Cox (1973) revealed the presence of CAH in globulin of rats with acute infection. CAH was removed by repeated absorptions at 4 C with trypsinized rat erythrocytes as described (Soni and Cox, 1974). The globulin fraction was tested for IK using methods modified (Thoongsuwan et al., 1977) from those of Coombs et al. (1961). ABSA was detected with SA from serum of rats with acute *P. chabaudi* or *B. rodhaini* infections by immunodiffusion in gel plates obtained from Research Products Division, Miles Laboratories Inc., Kankakee, Illinois.

The globulin fraction was pre-equilibrated with eluting buffer and subjected to gel filtration on Sephadex G200 column at 4 C using a gel bed of 5 x 90 cm and a flow rate of 24 ml/hr. The column was pre-calibrated using 2 ml of 1% solution of blue dextran as the marker for 19S peak. The globulin containing 26.5 mg/ml of protein was constituted to 3% sucrose and a 10 ml sample was underlayed onto the column. Fractions were eluted with 0.10 M pH 7.5 phosphate buffer in 0.85% NaCl solution and collected in 4 ml aliquots. Protein concentration was automatically monitored at 280 nm. Samples were stored at 4 C until used.

Pools of the 19S, 7S, and 4S fractions were concentrated in an Amicon untra filtration unit on a UM-10 membrane. After dialysis overnight in 0.01 M pH 7.5 phosphate buffer in 0.85% NaCl (PBS), the final protein concentration of each pool was determined as described (Lowry et al., 1951) and adjusted to 6 mg/ml for acute and 9 mg/ml for hyperimmune globulins. The samples were tested for ABSA with serum from rats with acute *B. rodhaini* and *P. chabaudi* infections.

Studies of the nature of IK stimulated in rats during B. rodhaini infection

A portion of the concentrated fraction pool that contained IK activity was treated with 2-mercaptoethanol (2-ME) as described (Chan and Deutsch, 1960). Treated and untreated portions were tested for IK reactivity against sheep erythrocytes sensitized with antibody (EA) that had been fixed with C14 (EAC14) and with C143 (EAC143) fragments of human complement. The EAC14 intermediate was prepared with optimally sensitized EA just as described by Linscott (1975).

The EAC143 intermediate was prepared from EAC14 intermediate with C2 that was purified following methods of Dias da Silva and Lepow (1967), and with C3 prepared by methods of Nilsson and Müller-Eberhard (1965). A suspension was made of 1.5 x 10^8 EAC14 cells/ml in sucrose-veronal buffered saline of 0.065 ionic strength, this 0.065 μ buffer was prepared as described by Rapp and Borsos (1963). Five ml of the suspension were reacted with 5 ml of C2 (50 effective molecules/ml) for 20 min at 30 C. The resultant EAC142 was washed 3 times with 0.065 μ buffer and adjusted to 5 x 10^8 cells/ml. One ml of EAC142 was mixed with 1 ml of C3 (1 mg/ml) and incubated for 90 min at 37 C to decay functional C2 activity, the preparation was then washed. The resulting EAC143, and EAC14 described above, were held at 0 C until tested for conglutinating activity with IK in Cooke microtiter plates as described (Thoongsuwan et al., 1977).

Fluorescein isothiocyanate (FITC) conjugation of globulin fractions

Globulin of rats containing IK stimulated by injections of autologous normal serum absorbed on kaolin as described by Thoongsuwan et al. (1977) was absorbed free of CAH as described (Soni and Cox, 1974). The 7S fraction of CAH-free globulin from rats hyperimmunized by *B. rodhaini* infections, containing ABSA and no IK, was prepared as described above. These preparations were conjugated with FITC, absorbed once with animal charcoal, and once with washed buffy coat cells of normal rats until unreactive with blood films or spleen impression slides of normal rats (Goldman, 1968; Thoongsuwan and Cox, 1977a).

Blood films and spleen impression slides prepared from rats with acute RIA, trypanosomiasis, babesiosis, malaria, and from normal rats were fixed with absolute methanol. They were incubated with a 1:10 dilution of the FITC conjugates at 37 C for 30 min, rinsed twice with PBS, and a coverglass was mounted with 90% glycerine in PBS. Fluorescent activity was studied with a Zeiss Fluoroscope equipped with an Osram HBO mercury lamp. Photomicrographs of the reactions were made with Kodak Tri X film at exposure times of 90-120 seconds (Thoongsuwan and Cox, 1977a).

Tests of IK, ABSA, and whole globulin containing both IK and ABSA for passive induction of nonspecific acquired resistance against RIA in rats and malaria in mice

Four groups of 16 mature rats were each inoculated with 1 ml of a 1:100 dilution of blood from a rat infected for 4 days with RIA agent (Thoongsuwan and Cox, 1977a). On following day one group of 16 rats was injected (IP) each with 1 ml of normal rat serum (NRS). A second group was given 1 ml of 19S globulin (9.0 mg of protein) from rats hyperimmunized by *B. rodhaini* infections. The third group each received 1 ml (9.0 mg protein) of the 7S fraction of globulin from the hyperimmunized rats. The fourth group was each given 1 ml (26.5 mg protein) of whole globulin from hyperimmunized rats. A group of 8 uninfected rats was each given 1 ml of the whole hyperimmune globulin. Erythrocyte counts were made daily on the 8 rats from each of the infected groups as well as the uninfected control rats. Mortality in each group was recorded.

The same hyperimmune rat globulin and its pooled fractions were used to perform the experiment in mice. Four groups of 10 mice were each infected with 2 x 10^5 mouse erythrocytes parasitized with *P. chabaudi* as described (Cox and Milar, 1968). These and a group of 10 uninfected mice were given experimental injections on day 1 and day 3 of the experiment. The control mice were each injected IP with 0.5 ml of whole globulin (13.25 mg of protein) on each day. The first group of 10 infected mice were given 2 injections of 0.5 ml of NRS. Two groups of 10 infected mice were given 0.5 ml injections (4.5 mg protein) of the 19S and 7S fraction of hyperimmune globulin respectively, and a fourth group of 10 infected mice were each given 0.5 ml of the whole globulin from the hyperimmune rats. Blood for erythrocyte counts and staining for parasite quantitation was obtained daily by snipping the tail of the mouse with scissors. Erythrocyte and parasite counts were made microscopically as described (Thoongsuwan and Cox, 1973). Mortality was observed and recorded daily.

EXPERIMENTAL RESULTS

Studies of serum of rats with acute babesiosis and serum of recovered and hyperimmunized rats

Figure 1 represents the results of Sephadex G-200 gel filtration profiles of globulin fractions from rats with acute *B. rodhaini* infection. IK activity was associated with the 19S peak showing a maximum titre of 5120. IK was not detected in the 7S or 4S fractions. CAH had been previously absorbed from the globulin prior to gel filtration and was not detected in the column fractions. ABSA was not detected by immunodiffusion assays in any of the column fractions.

The UV and IK profiles of a sample of globulin from hyperimmunized rats are shown in Figure 2. IK activity was again associated with the 19S peak although the titres were lower than those associated with acute infection. ABSA was detected in fractions representing the 7S peak. ABSA was not detected in individual 19S fractions or the concentrated 19S pool. Neither IK nor ABSA was detected in the 4S region (Figure 3).

IK activity as detected with cellular intermediates was shown in Figure 4. The immunoconglutination titre of the globulin with EAC143 was 1280. No activity was detected with EAC14. 2-ME treatment of the globulin fraction destroyed IK activity.

Tests on rats and mice passively immunized with 19S and 7S pools and with whole globulin of rats hyperimmunized by B. rodhaini infections for nonspecific acquired resistance

The results of erythrocyte counts of uninfected rats injected with whole immune globulin, and of rats infected with RIA agent that were injected with 19S pool (IK), 7S pool (ABSA) and whole globulin (IK and ABSA), are shown in Table 1. Injection of whole immune globulin appeared not to affect the erythrocyte counts of uninfected rats. The infected rats given the 19S (IK) pool developed anaemia at a rate that did not differ from infected rats given normal rat serum, all rats in each group died of RIA within 8 days. Infected rats given the 7S (ABSA) pool and whole hyperimmune globulin (IK and ABSA) both had erythrocyte counts that were significantly lower on days 3 and 4 than those of the infected rats given normal rat serum or 19S (IK) pool. On these same days the counts of the rats given whole globulin (IK and ABSA) were significantly lower than those given ABSA alone. Four of the 16 rats given ABSA alone and 7 of 16 given both IK and ABSA recovered from RIA.

The erythrocyte counts of uninfected mice injected with whole immune rat globulin and mice infected with _P_. _chabaudi_ and given injections of normal rat serum, IK, ABSA, or IK and ABSA are shown in Table 2a. The mean percentage of parasitized erythrocytes (% PE) of the infected mice are shown in Table 2b. The injection of hyperimmune whole globulin appeared to have no effect on the erythrocyte counts of uninfected mice. The development of anaemia and parasitaemia in infected mice injected with NRS or with 19S (IK) did not differ and none of the mice in these groups were alive on day 9. Infected mice given whole globulin had significantly lower erythrocyte counts than those given NRS from day 2 through day 6, and their counts were lower than were those of mice given ABSA alone from day 2 through day 4. After day 6 the erythrocyte counts of mice that were to recover from _P_. _chabaudi_ malaria or that would have prolonged survival, appeared to skew the mean of the erythrocyte counts. The % PE of mice given both IK and ABSA was lower than that for mice injected with NRS on day 4, and on day 5 through day 7, the % PE of the mice given ABSA alone was also lower than in the mice injected with NRS on day 5 through day 7. Longevity of 5 of 10 mice given ABSA was extended as was the longevity of mice given IK and ABSA. Three of the 10 mice in the latter group survived the malarial infection.

Immunofluorescent reactions of FITC conjugated IK and ABSA with blood films and spleen impression slides from rats with acute babesiosis, malaria, trypanosomiasis and RIA

The reaction of ABSA-free conjugated IK with blood and spleen slides from rats made anaemic by each infection are shown in Figure 5. The reaction of the 7S (ABSA) pool is shown in Figure 6. Neither of the conjugates gave fluorescent activity with blood films or spleen impression slides from normal rats. Both preparations reacted with erythrocytes from the peripheral circulation and with those sequestered in the engorged spleens from the rats made anaemic by each infection.

DISCUSSION

In a companion communication, it was shown that rats that had recovered from infectious anaemia initiated by one agent had an acquired nonspecific resistance to infectious anaemia initiated by heterologous agents. The resistance, manifested by early anaemia, early reductions in parasitaemia, and by enhanced survival of the recovered rats after challenge, was accompanied by early elevations in the pre-existing titres of IK. However, elevations in the titres of CAH were also observed after challenge in each infection and it was suggested that the blood of the recovered rats may have contained ABSA (Thoongsuwan et al., 1977).

We have studied the globulin (50% SAS fraction) of rats with acute babesiosis and of rats that had recovered and were then hyperimmunized by *B. rodhaini* infections. Finding all of the IK activity of globulin of rats with acute babesiosis and of the hyperimmunized rats confined to the 19S fraction and was susceptible to inactivation by 2-ME treatment. This evidence suggests that IK activity generated in rats during and after infection with B. rodhaini was associated with IgM. This result is supported by others (Lachmann, 1967; Lachmann and Coombs, 1965). Another supportive result shown here is the specific reaction of IK against fixed C3 as demonstrated previously by Lachmann (1962) and Lachmann and Coombs (1965). However, the failure of rat IK under these experimental conditions to react with fixed C4 is not supported by the result obtained with rabbit IK as reported by Lachmann (1966).

Since CAH had been removed from the globulins tested in these experiments, IK was distinctly identified with IgM and ABSA was found to be clearly associated with the 7S fraction, it was indicated that these antibodies are each distinct one from the other.

In the passive transfer experiments in which IK and ABSA in the nonspecific acquired resistance associated with recovery from infectious anaemia, there was no evidence that IK alone made a contribution. However, infected animals passively immunized with 7S (ABSA) did exhibit the resistance, and among infected animals given injection of whole globulin from hyperimmunized rats, the nonspecific resistance appeared to have been enhanced. The resistance was manifested as reduced parasitaemia accompanied by early anaemia and by reduction or delay in mortality of the passively immunized animals. Since this nonspecific resistance was induced by injection of immune factors generated by B. rodhaini infection and was effective against infection with filterable RIA agent and with infection of P. chabaudi, it is suggested that it was the result of humoral antibody lacking specificity for parasite antigen (S).

The reaction of FITC conjugated 7S (ABSA) with circulating blood cells and with cells sequestered in the spleen from anaemic rats indicated that the antigen for ABSA (SA) had been elaborated in each infection. Finding that SA was elaborated in acute RIA and trypanosomal infections as well as in acute malaria and babesiosis tends to support the suggestion of Cox and Iturri (1976) that SA is self-antigen. These experiments also indicated that SA was bound to the surface of circulating erythrocytes as well as those sequestered in the spleen. Soni and Cox (1975b) found that after injection of plasma containing SA and ABSA into normal chickens SA could be detected on the surface of erythrocytes of the injected birds with FITC conjugated ABSA. They postulated that soluble complexes of SA and ABSA became nonspecifically bound to erythrocytes and mimicked opsonin, causing these cells to be sequestered and phagocytized in the spleen and other filter organs as suggested by Dixon (1966). We interpret the reaction of ABSA observed in the present studies in the same way, i.e., the conjugate had reacted with the SA moiety of a SA-ABSA complex bound to the blood cells.

Reactions of IK-FITC conjugate with blood and spleen slides from rats made anaemic by each of the infections were also noted. These results suggest that complexes of SA and ABSA were bound to the cells. These complexes have apparently fixed C3 as indicated by positive reactivity with fluorescent IK. It is possible that FITC conjugated IK may be useful for indicating the presence of complement-fixed antigen-antibody complexes in vivo, but the reaction would have limited application in that it would tell one little about the offending antigen and antibody.

The absence of anaemia in uninfected rats or mice injected with immune globulin containing both IK and ABSA suggested that these autoantibodies of themselves had no effect. This was to be expected since these animals did not contain the antigen that could react with ABSA and, in turn, fixed the complement to react with IK. Similarly, it is suggested that no effect on the infected animals injected with IK alone could be seen until both SA and ABSA had been elaborated for forming complexes. On the other hand, ABSA injected into infected animals was present when SA was elaborated, and it is suggested that complexes were formed earlier by several days than they were in animals injected with NRS or IK alone.

Since the action of complexes binding to cells is apparently nonspecific, it is suggested that infected as well as uninfected cells, or even free parasites in the blood, would react with complexes which would subsequently be sequestered and phagocytized, or to be lysed if optimal complement fixation was attained. This postulated mechanism might account for the early anaemia and reduced parasitaemia seen in the recovered rats after challenge (Thoongsuwan et al., 1977) which might account for the early anaemia and reduced parasitaemia seen in the infected animals injected with globulin containing ABSA. The enhancement of this activity in the animals given both IK and ABSA could be attributed to the conglutinating action of IK reacting with complement-fixed complexes that were bound to cells or parasites.

In considering this autoantibody-associated phenomenon of nonspecific acquired resistance of infectious anaemia, it should be remembered that soluble complexes of SA and its antibody were implicated as major pathogenic factors (Soni and Cox, 1974; 1975a; 1975b; 1975c). It is suggested that a significant part of the resistance presently reported might be a result of early removal of antigen by the antibody which kept the concentration of soluble complexes at a sublethal level.

SUMMARY

Immunoconglutinin (IK) from rats with acute babesiosis and from recovered-hyperimmunized rats was shown, by agglutination of complement-fixed sensitized sheep erythrocytes, to be 19S and 2-mercaptoethanol sensitive Ig which reacted specifically with fixed C3 fragment of complement.

In addition to IK, blood of rats with acute babesiosis contained cold-active haemagglutinin (CAH) and soluble serum antigen (SA) and its antibody (ABSA). In hyperimmune rats, ABSA was associated with 7S Ig and CAH was not found. Thus CAH, IK and ABSA appeared to be distinct antibodies.

Rats infected with filterable rat infectious anaemia (RIA) agent and mice infected with _P. chabaudi_ were more resistant than controls after passive immunization with ABSA, but not with IK alone. The resistance was enhanced in animals given both ABSA and IK.

The reactions of fluorescein conjugated ABSA with blood films and spleen impression slides from rats with acute RIA, babesiosis,

malaria and trypanosomiasis indicated that SA had been elaborated in each infection and was bound to blood cells. Reactions of conjugated IK with these slides suggested the presence of a cell bound complement-fixing immune complex.

It is suggested that the cell bound complex mimicked opsonin causing the sequestration of erythrocytes and parasites, and that this action was enhanced by immunoconglutination. This mechanism could account for the early anaemia and reduced parasitaemia in the nonspecific acquired resistance associated with infectious anaemias.

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Table 1.--Mean erythrocyte counts per mm^3 ± standard error (RBC x 10^6 ± S.E.) of uninfected rats injected with whole globulin from rats hyperimmunized by Babesia rodhaini infections, and of rats infected with rat infectious anaemia agent which were then injected with normal rat serum (NRS), with 19S (IK) fraction of hyperimmune globulin, with 7S (ABSA) fraction of hyperimmune globulin and with whole hyperimmune globulin. Mean erythrocyte counts were made on 8 rats in each group. Mortality in groups of infected rats was based on deaths among 16 rats in each group.

Days	RBC x 10^6 ± S.E.				
	Control	Infected and injected with			
	Whole Globulin No infection	NRS*	19S*	7S**	Whole Globulin***
0	7.98 ± 0.49	8.23 ± 0.23	8.33 ± 0.18	8.38 ± 0.26	8.49 ± 0.22
1	8.26 ± 0.14	8.34 ± 0.18	8.45 ± 0.19	8.51 ± 0.32	8.66 ± 0.21
2	8.46 ± 0.24	8.18 ± 0.19	8.37 ± 0.22	8.52 ± 0.21	7.63 ± $0.24^{a,b}$
3	8.41 ± 0.18	8.56 ± 0.26	8.43 ± 0.34	7.73 ± 0.21^{a}	7.19 ± $0.28^{a,b}$
4	8.51 ± 0.29	8.47 ± 0.22	8.64 ± 0.16	7.51 ± 0.19^{a}	7.32 ± 0.21^{a}
5	8.42 ± 0.17	7.61 ± 0.32	7.76 ± 0.27	7.48 ± 0.31	7.17 ± 0.33
6	8.70 ± 0.20	3.81 ± 0.26	4.03 ± 0.37	4.33 ± 0.49	4.76 ± 0.58
7	8.58 ± 0.36	2.33 ± 0.18	2.02 ± 0.25	2.54 ± 0.50	3.10 ± 0.86
8	8.64 ± 0.19			2.61 ± 0.19	3.16 ± 0.59
9	8.75 ± 0.29			2.66 ± 0.24	2.92 ± 0.26

Table 1.--Continued.

Days	RBC x 10^6 ± S.E.				
	Control	Infected and injected with			
	Whole Globulin No infection	NRS*	19S*	7S**	Whole Globulin***
10	8.58 ± 0.17			2.89 ± 0.41	3.38 ± 0.31
12	8.72 ± 0.28			4.06 ± 0.23	4.47 ± 0.34
14	8.56 ± 0.18			5.68 ± 0.26	6.04 ± 0.35

*All died on day 8.

**Three rats died on day 8, nine on day 10, four survived.

***One rat died on day 7, eight on day 10, seven survived.

[a] Significant at $P < 0.05$ or less, over NRS group.

[b] Significant at $P < 0.05$ or less, over 7S group (Student t test).

Table 2A.--Mean erythrocyte counts per mm^3 ± standard error (RBC x 10^6 ± S.E.) of uninfected mice injected with whole globulin from rats hyperimmunized by *Babesia rodhaini* infections, and of mice infected with *Plasmodium chabaudi* which were then injected with normal rat serum (NRS), with 19S (IK) fraction of hyperimmune globulin, with 7S (ABSA) fraction of hyperimmune globulin and with whole hyperimmune globulin.

Mean percentage of parasitized erythrocytes ± standard error (% PE ± S.E.) of the mice in this experiment were recorded in Table 2B.

Days	RBC x 10^6 ± S.E.				
	Control	Infected and injected with			
	Whole Globulin No Infection	NRS*	19S*	7S**	Whole Globulin***
0	8.24 ± 0.22	8.46 ± 0.17	8.31 ± 0.11	8.29 ± 0.16	8.37 ± 0.09
1	8.21 ± 0.13	8.54 ± 0.14	8.38 ± 0.09	8.18 ± 0.21	8.40 ± 0.17
2	8.07 ± 0.18	8.22 ± 0.11	8.53 ± 0.18	8.13 ± 0.16	7.63 ± 0.23^{a,b}
3	8.28 ± 0.21	8.46 ± 0.19	8.16 ± 0.24	8.24 ± 0.18	7.44 ± 0.19^{a,b}
4	8.14 ± 0.12	8.57 ± 0.16	8.29 ± 0.21	8.12 ± 0.22	7.21 ± 0.21^{a,b}
5	8.39 ± 0.14	8.28 ± 0.19	8.04 ± 0.27	7.46 ± 0.29^{a}	7.02 ± 0.26^{a}
6	8.33 ± 0.20	7.51 ± 0.24	6.96 ± 0.38	7.04 ± 0.28	6.59 ± 0.32^{a}
7	8.21 ± 0.24	2.94 ± 0.36	3.17 ± 0.31	5.08 ± 0.43^{a}	5.82 ± 0.48^{a}
8	8.13 ± 0.32	1.27 ± 0.49	1.94 ± 0.47	3.34 ± 0.41^{a}	4.11 ± 0.72^{a}
9	8.28 ± 0.11			1.49 ± 0.25	2.78 ± 0.34^{b}

Table 2A.--Continued.

Days	RBC x 10^6 ± S.E.				
	Control	Infected and injected with			
	Whole Globulin No Infection	NRS*	19S*	7S**	Whole Globulin***
10	8.52 ± 0.28			1.31 ± 0.21	1.77 ± 0.26
11	8.37 ± 0.12			1.48 ± 0.22	1.86 ± 0.20
12	8.21 ± 0.17				3.17 ± 0.27
14	8.49 ± 0.18				3.84 ± 0.19

Table 2B.--Mean percentage of parasitized erythrocytes ± standard error (% PE ± S.E.) of mice in the same experiment as Table 2A.

Days	% PE ± S.E.				
	Control	Infected and injected with			
	Whole Globulin No Infection	NRS*	19S*	7S**	Whole Globulin***
0	-	-	-	-	-
1	-	+	+	+	+
2	-	0.06 ± 0.01	0.07 ± 0.02	0.07 ± 0.01	0.06 ± 0.01
3	-	0.31 ± 0.06	0.23 ± 0.05	0.26 ± 0.06	0.16 ± 0.04
4	-	1.06 ± 0.14	0.93 ± 0.12	0.89 ± 0.12	0.51 ± 0.09^{a}
5	-	10.08 ± 1.27	7.81 ± 1.53	3.52 ± 0.65^{a}	2.14 ± 0.48^{a}
6	-	38.88 ± 3.64	34.23 ± 3.27	22.76 ± 2.94^{a}	10.63 ± 2.06^{a,b}
7	-	61.49 ± 2.81	64.02 ± 1.93	49.38 ± 2.53^{a}	32.64 ± 3.89^{a,b}
8	-	65.52 ± 2.14	68.33 ± 2.39	61.36 ± 1.57	57.26 ± 3.38
9				50.41 ± 2.66	54.67 ± 3.19
10				25.67 ± 6.23	32.52 ± 4.86
11				12.53 ± 3.18	17.22 ± 3.74

Table 2B.--Continued.

Days	% PE ± S.E.				
	Control	Infected and injected with			
	Whole Globulin No Infection	NRS*	19S*	7S**	Whole Globulin***
12					1.85 ± 0.46
14					-

*Two of 10 mice in each group died on day 8, the remainders on day 9.

**Three mice died on day 8, two on day 9, the remainders on day 12.

***Two mice died on day 9, five on day 12, three survived.

[a] Significant at $P < 0.05$ or less--over NRS group.

[b] Significant at $P < 0.05$ or less--over 7S group (Student t test).

Fig. 1.--The optical density (O.D.) at 280 nm and the titres of immunoconglutinin (IK) in fraction samples after column chromatography with Sephadex G-200 of pooled, cold agglutinin-free, globulin from rats with acute *Babesia rodhaini* infection.

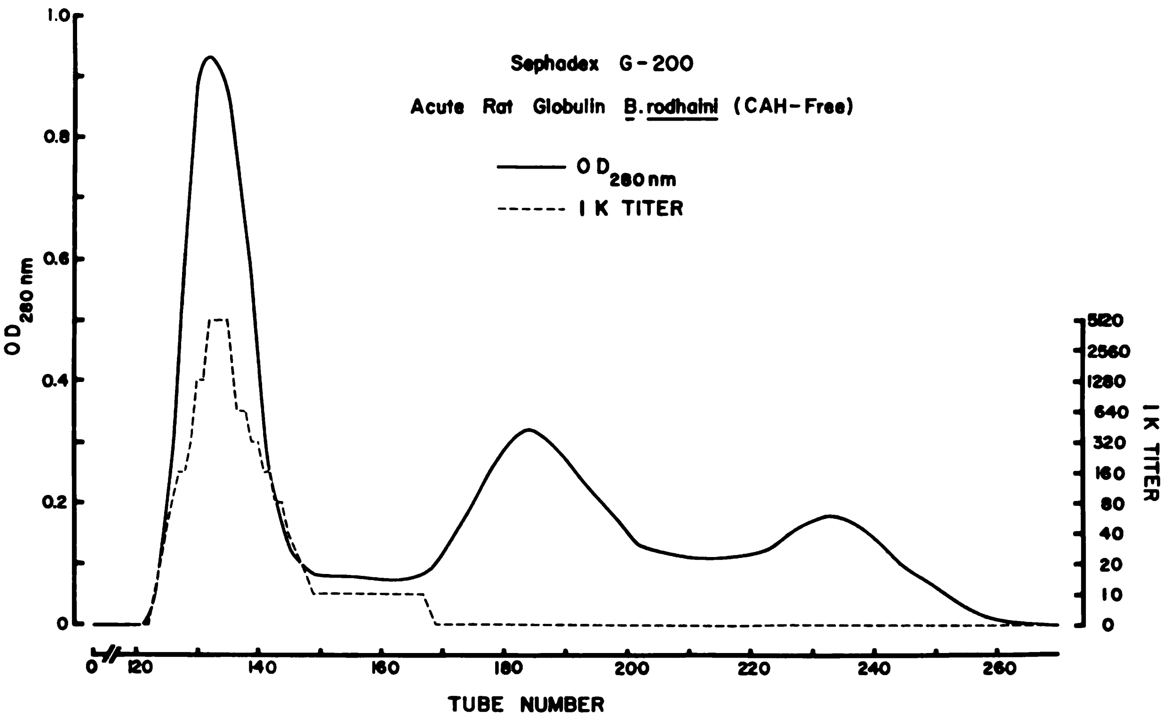

Sephadex G-200
Acute Rat Globulin B. rodhaini (CAH-Free)
OD 280nm
I K TITER
OD 280 nm
1.0
0.8
0.6
0.4
0.2
0
I K TITER
5120
2560
1280
640
320
160
80
40
20
10
0
0
120
140
160
180
200
220
240
260
TUBE NUMBER

Figure 1

Fig. 2.--The optical density (O.D.) at 280 nm, the titres of immunoconglutinin (IK) and the results of tests for antibody to serum antigen (ABSA) in fraction samples after column chromatography with Sephadex G-200 of pooled globulin from rats hyperimmunized by *Babesia rodhaini* infections.

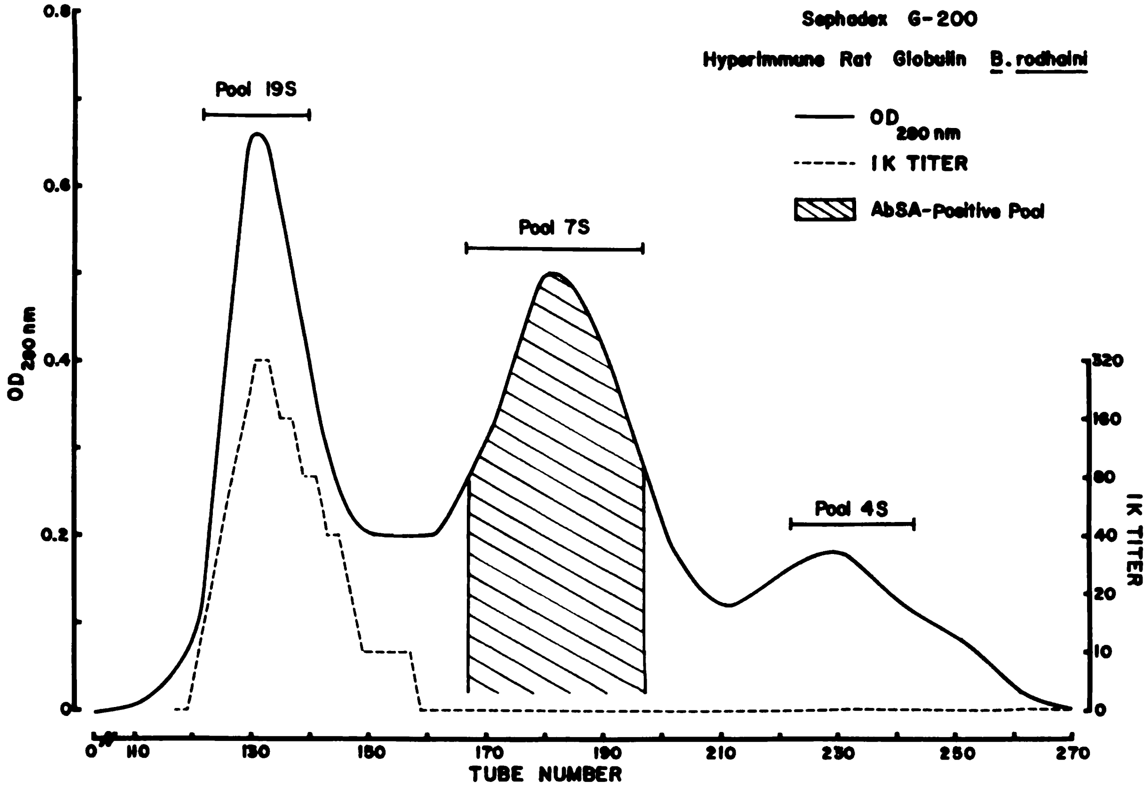

Sephadex G-200
Hyperimmune Rat Globulin B. rodhaini
OD 280 nm
IK TITER
AbSA-Positive Pool
Pool 19S
Pool 7S
Pool 4S
0.8
0.6
0.4
0.2
0
OD 280 nm
320
160
80
40
20
10
0
IK TITER
0
110
130
150
170
190
210
230
250
270
TUBE NUMBER

Figure 2

Fig. 3.--Photograph of immunodiffusion in gel test of 4S, 19S, and 7S fraction pools obtained by Sephadex G-200 column chromatography of globulin from rats hyperimmunized by *Babesia rodhaini* infections, whole globulin of the hyperimmunized rats (HIBr), whole globulin from rats hyperimmunized by *Plasmodium chabaudi* infections (HIPc) and normal rat globulin (N) with serum from rats with acute *B. rodhaini* infection (Br).

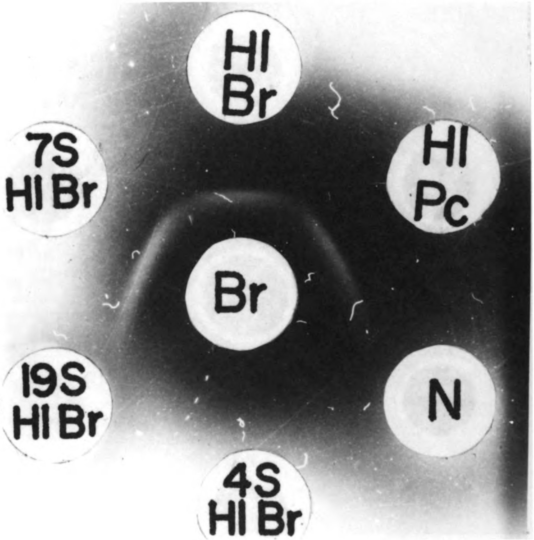


Figure 3

Fig. 4.--Reactions of immunoconglutinin (IK), from 19S fraction pools globulin from rats with acute Babesia rodhaini infection which had been absorbed free of cold-agglutinin, with sheep erythrocytes (E), sensitized sheep RBC (EA), sensitized sheep RBC fixed with C14 (EAC14) and C143 (EAC143) fragments of human complement.

2ME-IK = 2-mercaptoethanol treated IK.

Diluent control = No IK.

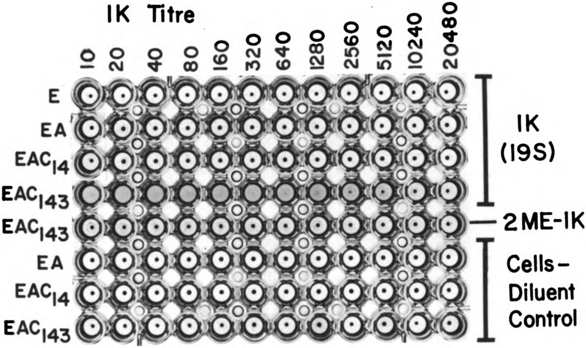
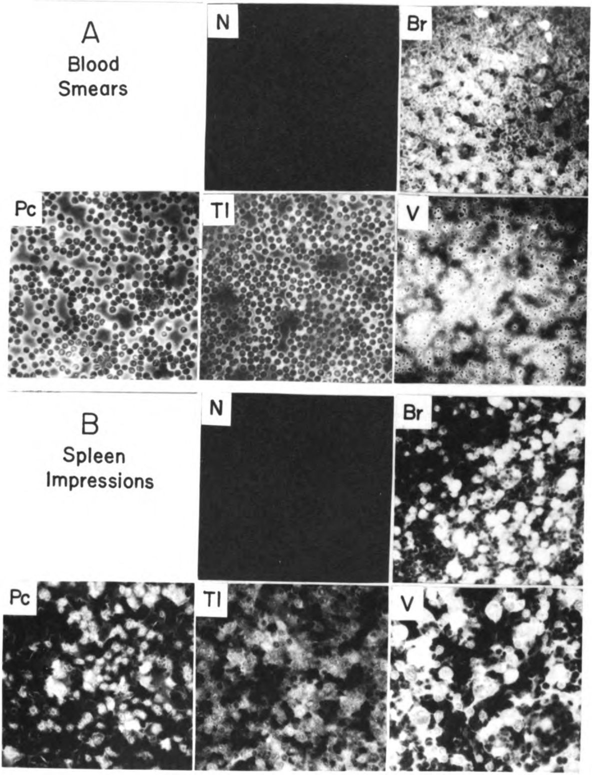

IK Titre
10
20
40
80
160
320
640
1280
2560
5120
10240
20480
E
EA
EAC14
EAC143
EAC143
EA
EAC14
EAC143
IK
(19S)
2ME-IK
Cells-
Diluent
Control

Figure 4

Fig. 5.--Photomicrographs of immunofluorescent reactions of fluorescein isothiocyanate conjugate of CAH-free IK globulin from rats immunized with autologous serum-absorbed kaolin.

A. With blood films from a normal rat (N), a rat with acute babesiosis (Br), a rat with acute malaria (Pc), a rat with acute trypanosomiasis (Tl), and a rat with acute rat infectious anaemia (V).

B. With spleen impression slides from a normal rat (N), a rat with acute babesiosis (Br), a rat with acute malaria (Pc), a rat with acute trypanosomiasis (Tl), and a rat with acute rat infectious anaemia (V).



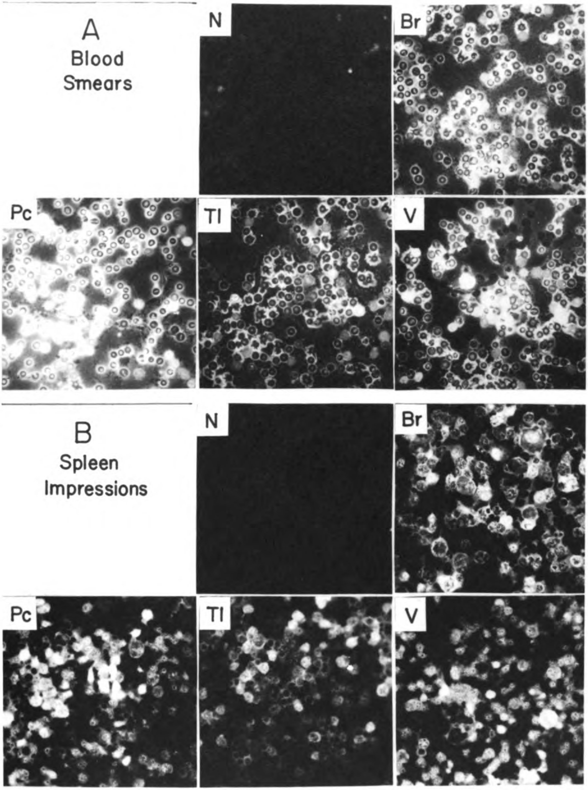
FA : Kaolin-Serum Induced IK

Figure 5

Fig. 6.--Photomicrographs of immunofluorescent reactions of fluorescein isothiocyanate conjugated 7S (ABSA) fraction of globulin from rats hyperimmunized by Babesia rodhaini infections.

A. With blood films from a normal rat (N), a rat with acute babesiosis (Br), a rat with acute malaria (Pc), a rat with acute trypanosomiasis (Tl), and a rat with acute rat infectious anaemia (V).

B. With spleen impression slides from a normal rat (N), a rat with acute babesiosis (Br), a rat with acute malaria (Pc), a rat with acute trypanosomiasis (Tl), and a rat with acute rat infectious anaemia (V).



FA : 7S Hyperimmune Rat <u>B</u>. <u>rodhaini</u>

Figure 6