ANTIGENIC VARIANTS OF THE HAEMOSPORIDIAN PARASITE, BABESIA RODHAINI, SELECTED BY IN VITRO TREATMENT WITH IMMUNE GLOBULIN

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ABSTRACT

ANTIGENIC VARIANTS OF THE HAEMOSPORIDIAN PARASITE, *BABESIA RODHAINI*, SELECTED BY *IN VITRO* TREATMENT WITH IMMUNE GLOBULIN

By

Santi Thoongsuwan

Relapse of latent infections is in part a result of biological variation of the parasite induced by the acquired immunity of the infected host. However, among infections with haemosporidian parasites such as *Plasmodium* or *Babesia* spp., the immune responses in infections are generally poor and ineffectual, and a significant part of the acquired immunity is mediated by the non-specific serum antigens associated with acute infections. Earlier work on relapse mechanisms of plasmodial infections indicates that variation which occurs in the parasites of relapsed infections are not affected by immunity induced by the parental or original strain. However, it is not clear which of the immunogenic substances of the parasite are selected or whether the changes are more quantitative than qualitative. In this study experiments to answer these questions will be performed using the haemosporidian

parasite, Babesia rodhaini and rats and mice as the host animals.

Rat erythrocytes infected with the original (O) strain of B. rodhaini were treated in vitro with immune globulin from rats that had recovered from acute infection and were then inoculated intraperitoneally into mice. The blood of these mice was then examined for parasites that had survived the treatment. Test mice that developed high parasitemia were bled and the infections were passed to normal rats. Parasitized blood of these rats was then treated with the immune globulin and surviving parasites were again recovered from the test mice. With repeated treatment and recovery a strain of parasites, designated as the immunity resistant-1 (IR-1) strain, was recovered from test mice. This strain was not affected by treatment with the immune globulin mediated by the original (O) strain of the parasites.

Immune globulin from rats that had recovered from the "immunity resistant-1" (IR-1) strain was effective in treatment of the IR-1 strain but had no effect on the O strain parasites. Continued treatment of the IR-1 strain with homologous immune globulin and recovery of surviving parasites by intraperitoneal inoculation of mice resulted in selection of a second immunity resistant strain (IR-2). Treatment of the latter strain with immune globulin mediated by either the O or the IR-1 strains had no effect.

Treatment of the O, IR-1 or IR-2 strains with an immune globulin prepared from rats recovered from IR-2 strain infection had no effect.

When mice infected with each of the three strains were tested for serum antigen production it was found that each strain caused their host animals to elaborate the non-specific antigen(s) during acute infection. Antibody to non-specific antigens was detected in each of the immune globulins prepared from serum of the recovered rats. Ouchterlony tests indicated that the serum antigens elaborated during infection with each strain were similar if not identical and quantitative changes in the amount of antigens were not detected. This observation was in part confirmed in an experiment in which rats that had recovered from the O and IR-1 strains were challenged with parasites of the homologous and heterologous strains. Recovered rats were equally resistant to each of the challenging infections.

From the results of these experiments it appears that in addition to the acquired resistance mediated by the non-specific antigens found in serum of animals with acute B. rodhaini infections, there is an immunogenic response to strain specific antigens which may be associated with the parasite particle. It was indicated that there were three antigen serotypes of this strain specific nature, the one manifested by the parasites of the O

strain, the one expressed by parasites of the IR-1 strain and the absence of such an antigen in the parasites of the IR-2 strain.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

INTRODUCTION

Earlier work indicated that immunologic mechanisms were selective in the relapse of plasmodial infections. Plasmodium berghei infections of mice were induced into states of latency by properly timed intervention in the course of infection with Atabrine treatment. These mice were found to be resistant to challenge with inoculations of P. berghei parasites that were 100% lethal for normal mice, however none of the treated mice was sterilized of the parasites by the treatment. Transient recrudescences of parasitemia occurred frequently and fatal relapses with high parasitemia occurred as long as four months after induced latency (Cox, 1957, 1958). Parasites from mice with severe relapses were established in normal mice for passage as relapse strains (RS). When they were used to challenge mice that had been infected with the original strain (OS) and given latency-inducing treatment, the challenging RS infections produced significantly higher mortality than did challenge with the OS parasites. Reciprocal experiments using mice infected with RS parasites and given latency-inducing treatment could not be

accomplished since the RS infections did not develop latent infections after treatment.

Further work indicated that the failure of the RS infected mice to develop latent infections was not a result of acquired drug resistance. Further, it was found that the diseases produced by the OS and RS strains in normal mice differed. With OS parasites mice suffered a marked anemia with reduced parasitemia and 30-50% mortality on or about the eighth or tenth day of infection. This anemia-parasitemia crisis was inconspicuous in mice infected with RS parasites. From this work it was concluded that variation or selection of a new antigen for the RS parasites had not been demonstrated. From the data and the behavior of the RS parasites in normal mice it appeared more likely that they had lost a part of the immunogenic properties manifested by the OS parasites (Cox, 1959; 1962).

Further elucidation of this phenomenon was furnished by the evidence that acquired immunity to Plasmodium infection was in part a result of responses to non-specific antigen which was elaborated or liberated into the serum of animals with acute malaria. These immunogenic non-specific antigens were found first in the sera of monkeys with acute Plasmodium knowlesi. They reacted in serologic tests with serum of birds, rodents, and human volunteers that had recovered from malarial infections. When the antigens were injected into rats, they developed resistance to challenge

with P. berghei (Cox, 1966). Similar serum antigens were found to be present in serum of horses, dogs, and rats during the acute stages of babesiosis. (Sibinovic et al., 1967a; 1967b and 1969). The non-specific serum antigens of acute malaria and acute babesiosis were subsequently shown to be similar, if not identical, in both serologic and immunogenic properties (Cox, Milar and Patterson, 1968; Corwin and Cox, 1969).

In a study of cross protection immunization it was shown that mice recovered from Plasmodium chabaudi infection were resistant to challenge with Babesia rodhaini but were not resistant to P. berghei challenge. In the latter instance the P. berghei parasites were retarded in growth for a period but then grew in the immunized mice as rapidly as they did in control animals. In these experiments it was suspected that the P. berghei parasites had undergone variation in the immune mice and that the variant strain did not respond to the non-specific immunity mediated by P. chabaudi infection (Cox and Milar, 1968). The experiments to test this hypothesis were done with ducks that had been immunized with serum antigen by challenging them with Plasmodium lophurae. By repeated passage of P. lophurae in immune ducks two strains that differed from the original strain in their response to immunity were selected. The first strain, which was as lethal for immune ducks as it was for normal birds, was termed an immunity resistant (IR)

strain. The other, which was always suppressed by immunity was termed susceptible (IS) strain. Since both the IR and IS strains were equally virulent for normal ducks it appeared that the only variation occurring in the selective process was the difference in the responses of the strains to serum antigen induced immunity. It was suggested that the IR strain was not affected by serum antigen induced immunity because it did not cause the antigen to be elaborated during acute infection (Corwin et al., 1970).

At that time it was impossible to test this hypothesis with the duck-P. lophurae system because of Duck Infectious Anemia Virus contamination of all avian plasmodial infections maintained at this laboratory (Ludford et al., 1971). Since B. rodhaini infections of rats also cauded the elaboration of immunogenic serum antigen, an attempt was made to see if the hypothesis could be tested with the B. rodhaini-rat and mouse system.

The details of these experiments are reported in this thesis.

REVIEW OF LITERATURE

The Haemosporidian Parasites

Infectious agents of the Haemosporidia all are characterized by a stage of life cycle that lives in the erythrocytes of the host animal. They are grouped under the protozoan class Sporozoa, the group of parasites which have, or is presumed to have, two or more reproductive cycles. The sexual life cycle (sporogony) takes place in the body of a blood sucking arthropod, and the asexual (schizogony) cycle occurs in the body of an intermediate host. These relationships for selected genera of hemosporidian parasites are summarized in Tables 1 and 2.

Babesia

Babesia species multiply asexually within the host erythrocytes by division into two or more daughter cells. Babes (1888) was first to observe the Babesia parasites in the blood of African cattle with signs of hemoglubinuria and named the parasites Haematococcus bovis. Starcovici (1893) renamed the parasites Babesia bovis. Smith and Kilborne (1893) recognized the role of ticks as biological

Table 1. Taxonomic relationship of Haemosporidian parasites under study.

Phylum	Class	Order	Family	Genus	Type Species
Protozoa					
	Sporozoa				
		Haemosporidia			
			Plasmodiidae	Plasmodium	P. vivax
			Haemoproteidae	Haemoproteus	H. columbae
				Leucocytozoon	L. simondi
			Theileridae	Theileria	T. parva
			Babesiidae	Babesia	B. bigemina

Table 2. Important parasites of the genera Plasmodium and Babesia, with their vertebrate and invertebrate hosts.

Genus	Example Species	Vertebrate Host	Invertebrate Host
Plasmodum	P. vivax	Man	Anopheles spp.
	P. falciparum	Man	" "
	P. malariae	Man	" "
	P. ovale	Man	" "
	P. knowlesi	Monkey	Anopheles Hackeri
	P. cynomolgi	Monkey	Anopheles spp.
	P. gallinaceum	Bird (chicken)	Mansonia crassipes Aedes aegypti
	P. lophurae	Bird (ducks)	? Aedes or Culex
	P. berghei	Rodents (rat)	Anopheles dureni
	P. chabaudi	Rodents (mouse)	Anopheles spp.
Babesia	B. bigemina	Cattle	Boophilus spp.
	B. canis	Dog	Rhipicephalus sanguineus
	B. gibsoni	Dog	Rhipicephalus sanguineus
	B. ovis	Sheep	Rhipicephalus bursa
	B. motasi	Sheep	"
	B. caballi	Equine	R. sanguineus
	B. equi	Equine	R. sanguineus
	B. rodhaini	Rodents (mouse, rat)	?

vectors of the causative agent of this disease, and demonstrated passage of the parasites from tick to tick by transovarial means.

Although the Babesia parasites have been referred to by various synonyms, the term "Babesia" has been held as valid for the current classification (Bergey, 1957; Neitz, 1956; Levine, 1961).

Transmissions of babesiosis in nature is accomplished by hard ticks belonging to the family Ixodiidae and genera Ixodes, Boophilus, Dermacenter, Haemaphysalis, Rhipicephalus, and Hyalomma. Blood sucking flies such as Stomoxys and Tabanus have been implicated as mechanical vectors (Abramov, 1952; Neitz, 1956).

Morphologically Babesia parasites are generally pyriform to round or oval in shape. Locomotion is by flexion or gliding. Budding forms or daughter cells are produced by binary fission or schizogony. Levine (1961) concluded this reproduction was asexual.

Babesia rodhaini

Babesia rodhaini is a protozoan parasite of rodents isolated from the blood of the Congo tree rat (Thamnomys surdaster surdaster) and adapted to white mice (Van den Berghe et al., 1950). The arthropod vector was not described; however, it is assumed to be a hard tick. Rodhain (1950) observed that infection of white mice, splenectomized

cotton rats and syrian hamster with B. rodhaini was generally mild. However, repeated passage of B. rodhaini in white mice yielded a strain which produced infections with mortality rates of 80 to 90 percent occurring within 8 to 11 days (Colas-Belcour and Vervent, 1953; Beveridge, 1953; Goodwin and Richards, 1960). Beveridge (1953) established the organism in 6 week-old white rats by repeated passage of infected blood through mice and splenectomized rats. Schroeder et al. (1966) showed that the infected white rats frequently had severe hemoglobinurea and anemia.

Wright's stain gave the organism the appearance of single blue ring with pink laterally situative nucleus. Small pear shaped and amoeboid forms were occasionally observed. The organisms appeared to multiply by budding or fission (Van den Berghe et al., 1950; Beveridge, 1953).

Flewett and Fulton (1959) and also Rudzinska and Trager (1960; 1962) studied the ultrathin sections of B. rodhaini infected cells. The parasites were found within the cells, not on the surface, and were similar to Plasmodium except that vacuoles containing hematin were absent. The digestion of hemoglobin within the vacuole appeared to be complete as no pigment was formed. Penetration of the cell occurred without leakage of hemoglobin.

Rickard (1970) observed that B. rodhaini infected rat erythrocytes exhibited rates of oxygen uptake and

glycolysis far higher than normal. However, the ratio of oxygen uptake to glucose utilization was not greater than that reported for normal erythrocytes, and less than that reported for erythrocytes infected with Plasmodium species which suggested a greater dependence of B. rodhaini on anaerobic metabolism. The major pathway of glucose metabolism in B. rodhaini was therefore considered to be via anaerobic glycolysis to lactate.

Plasmodium gallinaceum Brumpt, 1935

This important parasite of the domestic hen was probably first seen by Dr. Broussias in 1910 at Nhatrang in Indo-China and has since served as a much used model for the study of malarial disease and for the study of plasmodial parasites. Brumpt (1935) described the organism under the name Plasmodium gallinaceum. The natural vector of P. gallinaceum was found by Niles et al. (1965) to be mosquitoes of the Mansonia crassipes species; however, a large number of mosquitoes species are able to transmit the parasite experimentally. P. gallinaceum is more oligoxenous than any malarial parasite and can develop in Aedes, Culex, Armigeres, Culiseta, Mansonia and Anopheles genera of mosquitoes. Aedes aegypti is a mosquito commonly used for laboratory experiments since 100% readily become infected.

Primary exoerythrocytic schizogony of P. gallinaceum takes place chiefly in macrophages of the skin after

introduction of sporozoites, and two generations of tissue-inhabiting parasites are required before the parasite becomes capable of infecting red blood cells. These two stages are called cryptozoites and metacryptozoites, respectively.

McGhee (1949) showed that sporozoites of P. gallinaceum develop in lymphoid-macrophage system of 12-16 day old chick embryos. Dubin et al. (1949) showed that sporozoites will also grow in tissue cultures of embryonic spleen cells in macrophages producing sometimes as many as twelve parasites in a single cell.

Huff et al. (1960) studied the development of exoerythrocytic schizonts in tissue culture in regard to the nucleus and cytoplasm. The nucleus divides rapidly and the process is complete in 3 or 4 minutes. Meyer and Musacchio (1963) maintained the exoerythrocytic stages of P. gallinaceum in tissue cultures for 4 years, and found little diminution in its virulence, or ability to invade the blood after this interval.

Schizogony in blood occurs fairly synchronously, especially after intravenous inoculation or after injection of few parasites; the cycle was shown by Giovannola (1938) to last for 36 hours. The parasitemia mounts day by day until there are more parasites than corpuscles. Intensity of infection depends upon age of the birds. After the acute phase, the infection subsides to a low level, to

recrudesce later at longer or shorter intervals. Parasites probably do not disappear completely from the system.

Barretto and de Freitas (1945) showed that P. gallinaceum caused a mortality rate of 100% in young chicks, less than 250 Gm. in weight, of 87% in chickens weighing 300-350 Gm. and 45% in fowls weighing 1,000 Gm. Adult birds usually survive both in nature and laboratory.

Ingram et al. (1961) have shown that sporozoites of P. gallinaceum have greater immunogenic properties than the blood stages of the parasite, when injected into the naturally resistant rabbit. Corradetti et al. (1964) demonstrated that comparative study with fluorescence of sporozoites, only the homologous systems gave positive results.

Kielmann et al. (1970a;b) demonstrated the use of P. gallinaceum parasite as an antigen for the detection of malarial antibodies in human sera by means of fluorescent antibody technique. The authors showed that there were no differences in antibody titers measured with the use of antigens from different P. gallinaceum strains and, with Plasmodium falciparum and Plasmodium cynomolgi bastianelli parasites.

Relapse Mechanisms

Relapse or recrudescence of chronic or latent infections has come to be considered an immunologic phenomenon. Since the early work of Novy and Knapp (1906) indicated

that relapse strains of the spirochetes of relapsing fever had antigens that differed from those of the original strain, relapse has been considered to be a result of antigenic variation induced or selected by immunity mediated by original infection. Meleney (1928a; 1928b) substantiated this early work and demonstrated that as many as nine antigenic strains could be selected from an original strain by repeated passage of the strain under the selective force of immunity. He was able to show that immunity for each of his strains was specific.

In other cases of relapse this kind of antigenic variation was not as evident as it was in relapsing fever. The antigens of Rickettsia prowazeki isolated in the relapse of classic typhus (Brills disease) were reported to be essentially the same as those of the reference strain kept in the laboratories at the School of Hygiene at Harvard University (Plotz, 1943). The only change in serologic activity noted was that the presence of agglutinins for Proteus "O" strain found in serum of persons recently recovered from primary attack were absent or not increased during the recrudescence of infection (Zinsser, 1934).

In viral infections variations have been demonstrated in the antigenic complexes of the organisms isolated in outbreaks that occur in different years. However, these variations are more quantitative than qualitative since there is not an appearance of new antigens. These variations

occur typically in outbreaks of influenza and are reviewed by Davenport et al. (1953).

Among the parasitic protozoan infections, variation among trypanosomal parasites was first demonstrated by Ehrlich and Ritz(1914) when they reported that the parasites of recrudencent infections did not respond to immunity induced by the original strain of the parasite. Antigenic variation induced in cultures of free living ciliates by treatment with antisera has become a popular device for geneticists, who use protozoa as the experimental model. However, in spite of relapse being an inherent trait of protozoan infections, little study of the phenomenon has been reported. Recrudescences are uniquely prominent among the erythrotropic parasites of the group Haemosporidia. The parasites can be frequently found in blood during chronic infection and recrudescence with disease can occur repeatedly for long periods after initial infection. Relapse of quartan malarial infection, Plasmodium malariae, has been reported 36 years after initial infection (Spitler, 1948).

Cox (1959) was first to indicate that there was an immunologic basis for relapse in plasmodial infections. By properly timed intervention in the course of Plasmodium berghei infections of mice with antimalarial treatment it was found that the mice would recover from acute infection and developed chronic or latent infections. While these mice were resistant to challenging infection, they did not

undergo self-cure and recrudescences of parasitemia occurred with frequency. Some mice developed high parasitemia and died as late as three months after treatment (Cox, 1957; 1958). Parasites taken from these relapsed infections and established as relapse strains did not respond to immunity mediated by the original strain. When attempts were made to induce infections with the relapse strains into latent infections, it was found that they had apparently lost their immunogenic properties. Relapse strain infections did not develop into latent states. In untreated infections the parasitemia-anemia crisis which was conspicuous in infection with the original strain was absent in infections with relapse strains. From these experiments it was concluded that antigenic variation in the classic sense had not been demonstrated. Rather, it appeared that the relapse strains had lost their immunogenic antigens and therefore they could neither respond to immunity induced by the original strain nor immunize mice (Cox, 1959; 1962).

Brown and Brown (1965) found that monkey erythrocytes bearing mature parasites of relapsed P. knowlesi infections were not agglutinated by sera of monkeys recovered from parent strain infection. Neither would sera of monkeys recovered from relapse strain infection agglutinate cells bearing the mature parasites of the parent strain. Latency or recovery was also noted to be difficult to induce in monkeys infected with relapse strain parasites. The

agglutination of cells bearing P. knowlesi schizonts was considered to be a species specific phenomenon since Eaton (1938) had found that these cells were agglutinated by serum of monkeys recovered from P. knowlesi but not by serum of monkeys recovered from Plasmodium inui.

Non-Specific Serum Antigens

The presence of soluble antigens in the serum of monkeys with acute infections of Plasmodium knowlesi was reported by Eaton (1939). The same soluble antigens when injected into normal monkeys caused the formation of complement-fixing antibodies which reacted against the antigen as did those antibodies gound in serum of convalescent animals. No protective or agglutinating antibodies were elicited by them. An apparent correlation was reported between the degree of parasitemia and the resulting titer of complement-fixing antibodies in convalescent serum. Precipitating antigens was reported in serum of chicks infected with P. lophurae (Torry and Kahn, 1949).

Cox (1966) detected non-specific antigens in serum of monkeys with acute P. knowlesi that reacted in serologic tests with serum of human volunteers recovered from P. falciparum, rats recovered from P. berghei, hens recovered from P. gallinaceum and monkeys recovered from P. knowlesi. When injected into rats, the non-specific antigens induced immunity to P. berghei infection.

Antigen in the serum of horses with acute infections of a mixture of Babesia caballi and Babesia equi was demonstrated in gel precipitation test with serum of clinically recovered horses (Sibinovic et al., 1965); similar antigens were found in the serum of dogs with acute Babesia canis and rats with acute B. rodhaini (Sibinovic et. al., 1967a). They suggested that serum antigens were probably the significant immunizing substances associated with acute babesiosis. The antigens were present during the period of acute parasitemia and were not detected after parasites had disappeared from the peripheral blood (Sibinovic et al., 1967b).

A study was made of some of the physical, chemical and serologic properties of antigens from serum of horses infected with B. equi, B. caballi; dog with B. canis; and rats with B. rodhaini. These antigens were isolated by molecular sieving and concentrated by ultracentrifugation. Further fractionation of these materials revealed two different components with serum antigen properties. Antigen A was associated with the gamma globulins while antigen B was associated with the beta serum fraction. Each of the antigens reacted with serum of animals recovered from each of the three infections, but not with serum of uninfected animals. In gel diffusion test, the A antigens from each host showed lines of identity as did the B antigens. However, the tests showed that A and B were neither similar nor identical. It was also shown that only the B antigens

from dogs and rats were immunogenic. Physical and chemical studies denoted that both antigens were of complex structures containing peptides, lipids, phosphatides and polysaccharides. The antigens could not be distinguished from normal host substances.

Cox et al. (1968) showed that there were serologic cross reactions between the serum antigens of rats infected with Plasmodium berghei, of rats with B. rodhaini and of ducks with Plasmodium lophurae and the recovered serum from each of the three infections. Mice recovered from Plasmodium chabaudi were shown to resist challenge with B. rodhaini; rats recovered from P. berghei were resistant to B. rodhaini challenge (Cox and Milar, 1968).

Corwin and Cox (1969) reported their finding on the immunogenic activities of the non-specific serum antigens of ahaemosporidian infection: P. lophurae and Plasmodium "spartani" in ducks, P. gallinaceum in chickens and B. rodhaini in rats. It was concluded that the immunity of these infections was mediated by identical or similar substances since the serum of each of the acute malarial and babesial infections immunized ducks against P. lophurae.

Hemagglutinins for Trypsin-Treated Erythrocytes

The literature concerning the use of enzyme-altered erythrocytes as tools for studying cellular surface antigens was reviewed by Springer (1963). He pointed out that the

Thomson-Friedenreich phenomenon (Friedenreich, 1928) was the earliest evidence indicating certain bacterial or viral enzyme might alter the erythrocytes so that they became agglutinated by previously undetected antibodies. The enzyme has since been identified as neuraminidase. Injection of bacterial enzyme into guinea pigs was able to alter guinea pig's erythrocytes *in vivo*, and the antibodies developed which reacted with autologous erythrocytes (Ejby-Paulson, 1954a;b).

Proteolytic enzymes such as trypsin, papain and ficin were substituted for bacterial enzymes for the purpose of altering erythrocytes (Wiener and Klatz, 1951; Morton and Pickles, 1947; 1951). The proteolytic enzyme-altered erythrocytes were then used in a hemmagglutination test to detect incomplete Rh antibodies.

Henningson (1949) and Mabry *et al.* (1956a;b) demonstrated that antisera from patients with acquired hemolytic anemia reacted with enzyme-treated erythrocytes. Dodd *et al.*, (1953), Smith *et al.*, (1954) and Wallace *et al.*, (1955) showed that hemagglutinins developed in rabbits after injection of autologous trypsin-treated erythrocytes which reacted *in vitro* with the treated erythrocytes. The serum of human cases of acute infectious mononucleosis and serum of calves with acute *Anaplasma* were shown to react with antigenic sites uncovered by trypsin treatment of bovine erythrocytes (Burnett and Anderson, 1946; Stone and Miller,

1955; Tomsick and Bauman-Grace, 1960; Mann and Ristic, 1963a;b).

Cox, Schroeder and Ristic (1966) found the hemagglutinins for autologous and homologous trypsin-treated erythrocytes in the serum of rats infected with P. berghei. The titer of agglutinin was found to be correlated better with the severity of anemia and intensity of erythrocytic destruction by phagocytes of the spleen and bone marrow than was the number of parasites in the circulation. Schroeder and Ristic (1965a;b) studied the relationship of the autoimmune process to anemia in bovine anaplasmosis and showed that the hemagglutinin for trypsin-treated erythrocytes appeared about the time of initial parasitemia and persisted throughout the period of anemia. It was also found that in B. rodhaini infections of rats the titer of hemagglutinin was correlated better with removal of erythrocytes from the circulation than was parasitemia (Schroeder, Cox and Ristic, 1966).

Mechanisms of Anemia in Erythrocytic Infections

Early workers believed that the anemia which was a common feature of erythrocytic infections was due to direct lysis of infected cells by escaping parasites. However, Oliver-Gonzales (1944) found intravascular agglutination of erythrocytes followed by extensive intravascular hemolysis

of both parasitized and unparasitized blood cells in cases of Plasmodium falciparum infections. The phagocytosis of normal red blood cells in the spleen of patients with black water fever was found by Christophers and Bentley (1908). These observations suggested that some mechanisms other than parasitic lysis were destroying red blood cells in malarial infections.

Zuckerman (1964) reviewed the subject of auto-immunization and other types of indirect damage to host cells as factors in anemia in certain protozoan diseases. Circulating hemolysin and erythrophagocytosis by circulating leucocytes and by tissue macrophages were reported by Wright et al. (1952).

Taliaferro and Mulligan (1937) suggested that the immunity response to malarial infection was the phagocytic response and was due to acquired immunity following exposure to infection. Taliaferro (1949) and Thomson (1933) proposed a cellular basis for immunity in human, bird and animal malaria. The basis was found on observations of the extensive erythrophagocytosis of both infected and uninfected erythrocytes in the blood stream, spleen, liver and bone marrow of malarial infected animals.

Zuckerman (1958; 1960) concluded that development of anemia, a positive Coombs test and demonstration of erythrophagocytosis during the course of rodent malarial infections were compatible with the idea that rats had

become immune to their own erythrocytes. The removal of uninfected immature erythrocytes from the blood stream of ducklings during the course of drug suppressed Plasmodium lophurae infection was considered to be a result of auto-immunization (McGhee, 1964).

Cox (1966) found that globulins from monkeys with acute Plasmodium knowlesi injected into normal rats produced anemia followed by resistance to Plasmodium berghei challenge. Corwin and McGhee (1966) observed a similar syndrome when acute plasma from P. lophurae infected ducklings was injected into normal ducklings. These observations suggested that there were substances free in the serum during acute infection which produced both anemia and immunity.

Cox et al. (1966) showed the anemia in P. berghei infected rats was accompanied by extensive phagocytosis of both normal and infected erythrocytes in the spleen and the bone marrow. Agglutinins for trypsin-treated homologous erythrocytes were also present.

Working with B. rodhaini, Schroeder et al. (1966) found that severity of anemia was not commensurate with the parasitemia and that macrophages were phagocytizing both normal and parasitized erythrocytes in spleen and bone marrow. They also demonstrated the association of hemagglutinins for trypsin-treated autologous and heterologous erythrocytes from animals with anemia at the onset of

erythrophagocytosis by the spleen and bone marrow. It has been suggested that anemia of other erythrocytic infections might also be mediated by similar mechanisms, since similar findings of autoimmune activity have been shown in several different parasitic systems; i.e., Haemobartonella muris infections of rats and Eperythrozoon coccoides infections of mice (Cox, H.W.: Personal communication).

MATERIALS AND METHOD

Parasites

Babesia rodhaini "original (O) strain" was obtained from Dr. Paul E. Thompson, Parke Davis and Company, Ann Arbor, Michigan. The strain was maintained by serial passage at twice weekly intervals in both white Swiss mice and Wistar rats.

Plasmodium gallinaceum was obtained from Dr. Julius P. Kreier, Faculty of Molecular and Cellular Biology, Ohio State University, Columbus, Ohio. It was maintained by serial passage at 4 day intervals in white leghorn roosters.

Experimental Animals

For experiments with *B. rodhaini*, female white Swiss mice, certified as *Eperythrozoan* free, of about 20 to 30 grams body weight were obtained from Carworth Farms, Portage, Michigan, and male white Wistar rats, certified as *Haemobartonella* free, weighing about 150 to 200 grams were obtained from Harlan Industries, Cumberland, Indiana.

White leghorn cockerels were obtained from Rainbow Trail Hatcheries, St. Louis, Michigan, and were reared in departmental animal rooms to be used for *P. gallinaceum* experiments.

Methods of Infections

1. The experimental animals were injected by the intraperitoneal route in the case of rats and mice, and intravenously in roosters.

2. For collecting blood for routine experimental procedures in rats and mice, heparinized 0.85% saline (100 units- heparin[1] per ml.) was used in 10% by volume of blood collected. In chickens 0.78% saline with heparin was used.

3. The infecting dose of B. rodhaini employed was $1x10^8$ and $1x10^9$ parasitized erythrocytes for mice and rats, respectively, which were washed twice with saline. The infecting dose of P. gallinaceum for chickens was $1x10^8$ parasitized cells. Infecting inocula were standardized by described methods (Cox, 1957, 1958).

Haematological Techniques

1. Blood for preparing films and red blood cell counts was obtained by snipping the tip of the tail in case of mice and rats and from the leg vein of the roosters. The blood films were stained by Wright's stain. The percentage of parasitized erythrocytes (P.E.) was estimated by looking at 20 fields of 100 RBC each.

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[1]Heparin Sodium U.S.P. 175 Unit per mg., General Biochemicals, Chagrin Falls, Ohio.

Red blood cell counts were made either with a hemocytometer or a Coulter Model B. counter. Blood to be counted with a hemocytometer was collected in red blood cell hemocytometer pipettes and diluted with Hayem's solution. Counts were made microscopically using a Spencer hemocytometer counting chamber. Blood to be counted with the Coulter counter was collected in 10 mm^3 amounts in Sahli pipettes and diluted in saline solution.

2. Blood for serological experiments and transfer was collected by cardiac puncture from chickens and rats and by an incision of the brachial artery of mice. All animals were anesthetized with ether prior to bleeding.

Plasma for serological tests were collected by centrifugation of heparinized blood at 800xG in the International centrifuge size 2 for 10 minutes. Serum was obtained from whole blood after standing overnight at 4°C and centrifugation at 800xG for 10 minutes. Both plasma and serum were stored at -20°C. Plasma was clarified of fibrin by centrifugation at 2,000xG for 15 minutes in the International high-speed refrigerated centrifuge model HR-1.

Preparation of Antigens

Serum-Antigen(s)

Acutely infected animals with approximately 60% parasitized erythrocytes (P.E.) were bled and plasma or

serum was collected. The serum or plasma was tested for serum antigen by means of the tube bentonite flocculation test which is described below. Either plasma or serum was used for antigen. Antigen-bearing globulins were prepared by the same means as used for immune globulins, which is described below.

Parasitic Antigen Slides

The parasitic antigen used in indirect fluorescent antibody (IFA) technique was prepared by making thin smears of infected rat blood on clean slides. Rats infected with B. rodhaini were bled when about 15 to 25% of the erythrocytes were parasitized, and the blood was mixed with equal volume of Alsever's solution. It was centrifuged at 800xG for 10 minutes and the supernatant fluid was discarded. The cells were then washed 3 times with 20 volumes of phosphate buffered saline pH 7.2 and centrifugation as above. The packed washed cells were suspended in a small amount of phosphate buffered saline and were used to prepare the films which were allowed to air-dry. The prepared slides were packed in soft tissue paper, wrapped in tin-foil and kept at -20°C in a well closed container. Before use, the slides were transferred to 4°C for one hour and then room temperature for another hour.

Preparation of Immune Globulin(s)

A group of animals was infected lightly; i.e., about $1x10^6$ P.E. of B. rodhaini per rat. The recovered animals were reinfected with heavily parasitized blood of the same strain. Two weeks later the survivors were bled and immune serum was collected. Pools of the serum were tested for antibody to serum antigen by means of tube bentonite flocculation test described below. The immune globulin(s) was obtained using cold ammonium sulfate precipitation. Sixty ml. of saturated ammonium sulfate aqueous solution was slowly added to 40 ml of serum at 4°C and stirred for 20 minutes. The globulin(s) fraction was separated by centrifugation 10,000xG in a refrigerated centrifuge and dissolved in a small volume of distilled water. The dissolved globulin was homogenized and dialyzed overnight at 4°C against 0.04 M phosphate buffered saline pH 7.2. The protein concentration was measured spectrophotometrically at 280 millimicrons wavelength in the Coleman spectophotometer model 124. The preparation was stored at -20°C. Plasma or serum of rats with acute B. rodhaini or chickens with P. gallinaceum infections was prepared similarly and designated as acute globulin(s) for serum antigen.

In Vitro Neutralization and In Vivo Test

For testing effects of immune globulins on parasitized erythrocytes an immunologic device employed in studying immunity to Trypanosoma spp. was used (Rabinowitsch and Kempner, 1899; Laveran and Mesnil, 1901; Cox, 1964a,b, 1969). Trypanosomal parasites normally passed quickly into the blood stream after intraperitoneal inoculation. However, if the parasites were treated with immune serum, or were inoculated into immune animals, they did not pass into the blood but remained in the peritoneal cavity.

The B. rodhaini infected rats with about 60% P.E. at the mature stage of the parasites were bled into heparinized saline. The blood was washed twice with saline and the volume was standardized at $1x10^9$ P.E. per ml with phosphate buffered saline (PBS) pH 7.2. To each volume of suspension was added 4 volumes of immune globulin preparation standardized at 5 mg of protein per ml. For the two controls, PBS and normal rat globulin were used instead of immune rat globulin. To these mixtures normal rat serum was added to a final concentration of 10% by volume. After incubation for one hour at 37°C with gentle shaking, the cells were washed with PBS to remove excess globulin. Aliquots of the treated cells containing $1x10^8$ parasitized erythrocytes (P.E.) suspended in PBS were injected intraperitoneally into each test mouse. The percentage of P.E.

for each mouse was determined at 0, 30, 60, 120 minutes and at two hour intervals for 8 hours and then daily until death or recovery.

Selection of the B. rodhaini Strains

Selection of Immunity Resistant-1 (IR-1) Strain

By means of "the *in vitro* neutralization and *in vivo* test" method described, the *B. rodhaini* "original" (O) strain was neutralized with immune globulin from rats recovered from "O" strain *B. rodhaini* infection. None of the recipient mice had significant parasitemia until after the 4th day. Some of the mice had transient parasitemia and recovered, others developed high parasitemia and died. Parasitized blood was drawn from a moribund mouse and passed to a normal rat. When this rat developed parasitemia of about 60% P.E., it was exsanguinated and the infected cells were treated as described. After 21 treatments and passages the strain was not affected by immune globulin mediated by the "O" strain. This strain was designated as immunity resistant-1 (IR-1) strain.

Selection of Immunity Resistant-2 (IR-2) Strain

Normal rats were lightly infected with the IR-1 strain of parasites, and after recovery were given a second

large inoculation of the parasitized blood cells from the same strain. These rats were exsanguinated two weeks later and immune globulin was recovered as described. This globulin was used to treat the IR-1 parasitized erythrocytes which were then inoculated into test mice as described for selection of the IR-1 strain. Parasitized blood was drawn from a moribund mouse and passed to normal rat. The infected cells from this rat were treated again. After 4 treatments, this strain of parasites was no longer affected by immune globulin mediated by the IR-1 strain of parasites and was designated as the immunity resistant-2 (IR-2) strain.

Serological Tests

Haemagglutination Test (H.A.) with Trypsin-Treated Erythrocytes

Plasma samples from mice with acute B. rodhaini infection were tested for haemagglutination using the technique of Morton and Pickles (1947) as modified by Mann and Ristic (1963). The trypsin solution was prepared by adding 0.25 Gm. of trypsin[2] to 100 ml of 0.85% saline and filtered through Whatman No. 2 filter paper to clarify the solution. Erythrocytes were obtained from normal mice by brachial incision. The blood was collected in heparinized saline

[2]Trypsin 1: 250, General Biochemical, Laboratory Park, Chagrin Falls, Ohio.

and the plasma was removed after centrifugation at 800xG for 10 minutes. The cells were washed twice in saline; five-tenths of a ml of the packed-washed cells were suspended in 4.5 ml of 0.25% trypsin solution and incubated at 37°C for 20 minutes. The trypsin-treated cells were washed twice with saline and centrifuged at 800xG for 10 minutes. A 2% suspension of the treated RBC was prepared in 0.85% saline. Serial two fold dilutions of the plasma samples to be tested were prepared to give a final test volume of 0.2 ml. To each dilution was added an equal volume of 2% suspension of trypsinized RBC. Incubation was carried out at 22°C for 4 hours and a reading was made. They were stored overnight at 4°C and read again the following morning.

A positive test was represented by an aggregate of cells which did not break up readily on mild agitation. Aggregates that remained intact after agitation were graded as 4+. Others were classified as 3+, 2+, and 1+, depending on the nature of aggregate after agitation. Tests in which the cells resuspended smoothly were read as negative. When interpreting the results a 1+ was invariably classified as doubtful or negative since trypsinized RBC tend to be very sensitive to agglutination.

Tube Bentonite Flocculation Tests for Serum Antigen and for Antibody to Serum Antigen

Preparation of the Bentonite Stock Suspension

Serum or plasma from control and experimental animals was tested for serum antigen or antibody to serum antigen using a Tube Bentonite Flocculation Test (TBFT) modified from methods described (Cox et al., 1968). One and three-tenths Gm. of Bentonite[3] were pulverized with a pestle and mortar. The powder was suspended in 300 ml of distilled water; the suspension was homogenized in a Waring blender for 2 minutes and allowed to stand for 5 minutes before it was re-homogenized for another 2 minutes. The blended mixture was poured into a large graduated cylinder and diluted to 1500 ml by adding distilled water. The suspension was allowed to stand at room temperature for one hour and was then centrifuged for 15 minutes at 800xG. The supernatant fluid so obtained was re-centrifuged at the same speed for 15 minutes. The sediment from this centrifugation was resuspended in 300 ml of distilled water and homogenized in the blender for 1 minute. This stock bentonite suspension was kept in a flask covered with parafilm at 4°C for future use.

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[3]Crude Bentonite, trade name Volclay Bentonite, obtained from American Colloid Company, Skokie, Illinois.

Sensitization of Bentonite Particles

Sensitization of the bentonite suspension was accomplished by adding 10 ml of stock bentonite suspension to 0.1 ml of properly diluted acute infection or immune globulins. After storage overnight at 4°C, one ml of 0.1% aqueous methylene blue was added to the mixture and mixed in an electric shaker. The mixture was then centrifuged at 800xG for 5 minutes, the supernatant fluid was discarded and the sediment was resuspended in 10 ml of PBS pH 7.2, mixed again in the shaker and centrifuged as before. After a second washing the sediment was resuspended in 10 ml of distilled water and 0.2 ml of 0.5% aqueous bovine serum albumin solution were added. The suspension sensitized with immune globulin was used to test for serum antigen and the suspension treated with acute infection globulin was used to test for antibody to serum antigen.

Standardization of Acute and Recovered Globulins

To determine the optimum concentration to be used in the experiments, each immune and each acute infection globulin prepared from 60% cold ammonium sulfate precipitation was titrated. A block titration was done by using concentrations of 1:500, 1:1000, 1:2000, 1:4000 and 1:8000 of the globulins in PBS to sensitize the bentonite particles. They were titrated each against the other globulin prepared

as serial two-fold dilution from 1:4 to 1:8182 with PBS in 0.2 ml volumes by adding 0.1 ml of sensitized bentonite preparation to each tube of each globulin dilution. The processes were carried out as described below in the serum testing procedure. This block titration of the reagents gave optimum reactions for chicken-P. gallinaceum globulins when diluted 1:8000 and for rat-B. rodhaini globulins when diluted 1:2000. Each of the reagents was then tested against known positive and against normal sera from chickens, rats, and mice to determine the specificity.

Tube Bentonite Flocculation Test

Serial two-fold dilutions of the sera, or plasma to be tested were prepared in PBS, starting at 1:4 to 1:4096. The final test volume of each dilution was 0.2 ml. To this was added 0.1 ml of sensitized bentonite mixture. The mixtures were agitated on a rotary shaker at 120 oscillations per minute for 20 minutes at 22°C. The test was read and graded as follows:

4+ :Button-like aggregate of particles which remained intact after vigorous shaking.

3+ :Aggregate disrupted in the form of bigger flakes.

2+ :Aggregate disrupted in the form of small size flakes.

1+ :Aggregate on shaking disrupted in the form of fine granules.

- :Aggregate became completely agranular.

For a positive control, the known positive serum from chicken-P. gallinaceum system was used. Tests of normal rat serum and normal chicken plasma were also added as controls.

Gel Diffusion Test

Ouchterlony (1953, 1968) double gel diffusion slide tests were used as one of the serologic tests for the experiments. The gel diffusion agar was prepared from 0.85 Gm of refined grade Ionagar No. 2[4] dissolved, by heating, in 100 ml of barbital solution ionicity 0.15, pH 7.4. This solution was prepared by adding 6.98 Gm sodium barbital[5], 6.0 Gm sodium chloride and 2.7 ml 1 N hydrochloric acid to distilled water and brought up to a volume of 1000 ml (Crowle, 1961). About 7 ml was spread on 5 x 7.5 cm. acid-alcohol cleaned slides and allowed to harden before wells were drilled. The wells were of either 5 or 6 mm. diameter and 8 mm. apart from the central well as well as from other peripheral wells. The wells were then filled with reactants and refilled 2 or 3 times within the first 6 hours. The slides were incubated at 22°C. The precipitin lines were clearly visible at about 48 to 72 hours. After the precipitin lines were clearly visible the slides were then

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[4]Ionager No. 2, Colab Laboratories, Inc., Box 66, Chicago Heights, Illinois.

[5]Barbital Sodium, Merck & Co., Inc., Rabway, N.J.

stained as described by Carpenter (1965) and Crowle (1961). The agar slides containing lines of precipitate were immersed in saline for several hours to remove untreated protein. Changing the saline several times was needed during this period. A piece of filter paper was saturated with distilled water and placed over the agar so that no air bubbles were trapped beneath the paper. The paper was removed after the agar and paper dried. The slides were stained by immersion for 15 to 30 minutes in 0.05% azocarmine B[6] in 2% acetic acid. They were then washed with 3-4 changes of 2% acetic acid until the excess stain was removed.

Indirect Fluorescent Antibody (IFA) Studies of the Antigens of the O, the IR-1 and the IR-2 Strains of B. rodhaini Parasites

Antigen slides for the IFA test were prepared as described by Voller (1964) from the blood of rats with parasitemia at 15 to 25% P.E. for each of the strains. Slides prepared in the same manner from the blood of uninfected rats were used as the control. Slides taken from -20°C storage were transferred to a 4°C refrigerator for one hour and were then held at room temperature for one hour. The slides were then fixed in acetone for 10 minutes

[6]Azocarmine B, Pharmaceutical Laboratories, National Aniline Division, Allied Chemical and Dye Corporation, 40 Rector Street, New York, N.Y.

and then allowed to air dry. Test areas were marked with a diamond marker on the reverse side of the smear and the immune globulin to be used in tests was applied to the test area. The slides were incubated at 37°C for 30 minutes and then washed twice for 10 minutes in PBS, pH 7.2. After the slides had air dried, the anti-serum to rat globulin conjugated with fluorescein[7] was added to the test areas and the slides were incubated at 37°C for 30 minutes. The slides were then washed twice for 10 minutes in PBS and allowed to air dry. Fluorescent antibody mounting fluid[8] was added to the slide and the test areas were covered with a cover glass.

The slides were examined with an A.O. Spencer Fluorolume Model 645 Illuminator equipped with an HBO Osram 200 watt mercury lamp attached to an A.O. Fluorescent binocular microscope. Light was controlled with a Schott G G-9, 20 mm barrier filter and a Schott B G-12, 3 mm excitor filter. Microphotographs were taken with Kodak Tri-X 35 mm film at 2 minute exposures.

Immune globulins mediated by the 3 strains of B. rodhaini were each adsorbed twice with bentonite sensitized globulin of rats with serum antigen mediated by acute B. rodhaini infection or with bentonite sensitized with

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[7]Rabbit Anti-Rat Globulin Serum Fluorescein Conjugated, Preserved with 0.01% Merthiolate, Sylvana, Millburn, N.J.

[8]Bacto FA Mounting Fluid, Difco Laboratories, Detroit, Michigan

globulin bearing serum antigen mediated by acute P. gallinaceum infections in attempts to remove antibody to the non-specific serum antigen present in the immune globulins. Ten ml of bentonite suspension was mixed with 1.0 ml of the globulin and processed as described for the tube bentonite flocculation test, except that the distilled water was not added after the last washing and the globulin to be adsorbed was added directly to the sensitized bentonite sediment. The mixtures were incubated at 22°C for 1 hour with shaking. After each adsorption the mixtures were centrifuged at 800xG for 10 minutes and the supernatant globulin recovered.

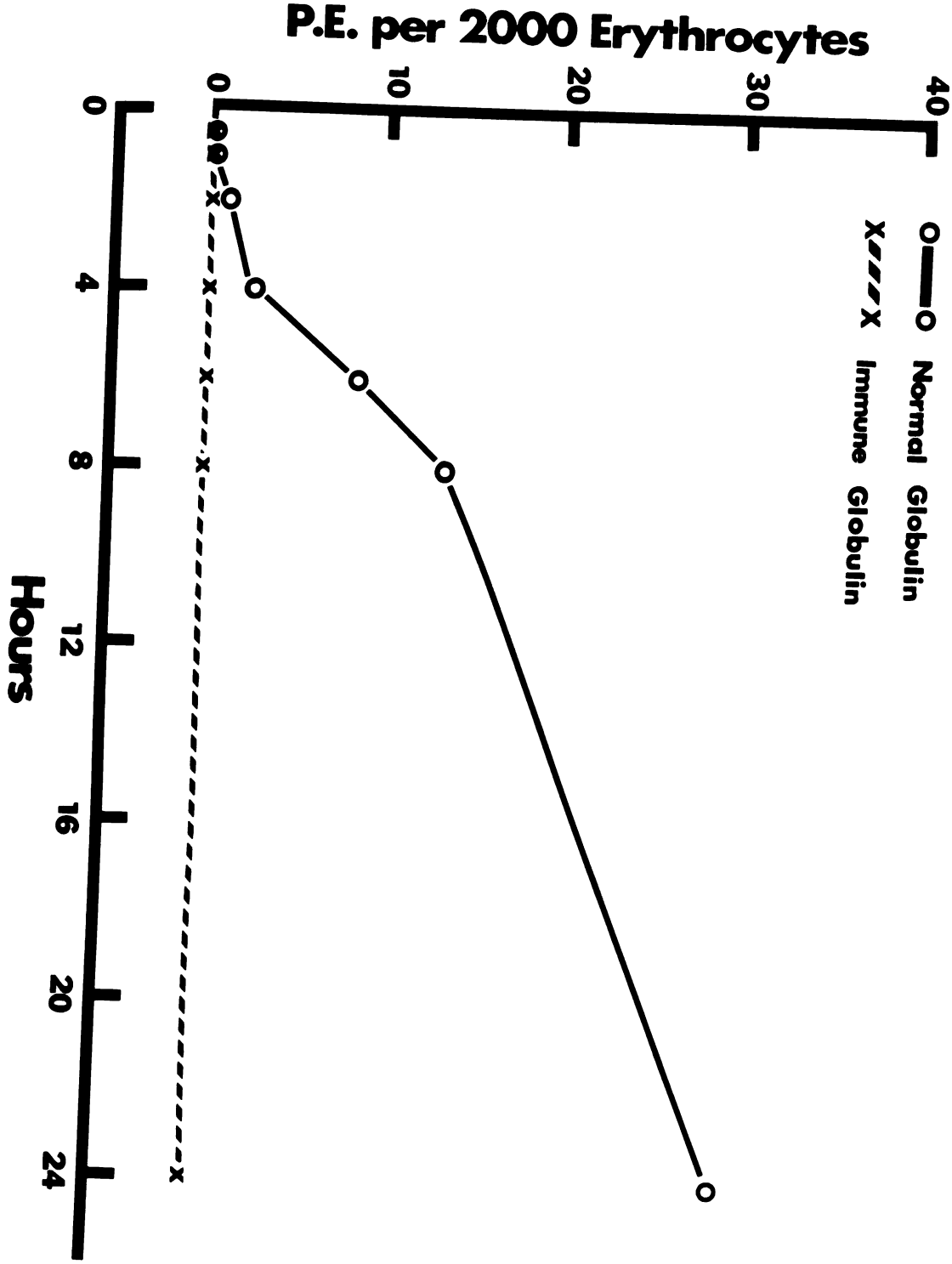
EXPERIMENTS AND RESULTS

The Effect of Treatment with Hyperimmune Globulin on the Passage of Babesia rodhaini Infected Erythrocytes from the Peritoneal Cavity into the Blood Stream of Normal Mice

A preliminary experiment was performed to determine whether or not immune globulin mediated by B. rodhaini infection would affect the passage of parasitized erythrocytes from the peritoneal cavity into the blood stream. One aliquot of infected erythrocytes was treated with immune globulin and the others were incubated with normal rat globulin or phosphate buffered saline (PBS) as described. After the infected cells were washed, each aliquot was divided into 4 equal portions which were inoculated intraperitoneally into 4 test mice.

The rate at which rat erythrocytes infected with B. rodhaini appeared in the peripheral blood circulation of normal mice after peritoneal inoculation is shown, Figure 1. Infected cells incubated at 37°C for one hour with globulin of rats that had been hyperimmunized by repeated infections of B. rodhaini were not evident in the blood of test mice 24 hours after inoculation, while cells incubated with normal rat globulin were detected within an hour, just as were cells treated with the PBS-normal rat serum control.

Figure 1. The effect of treatment with immune globulin at 37°C for one hour on the passage of Babesia rodhaini infected rat erythrocytes into the blood circulation of mice after intraperitoneal inoculation.

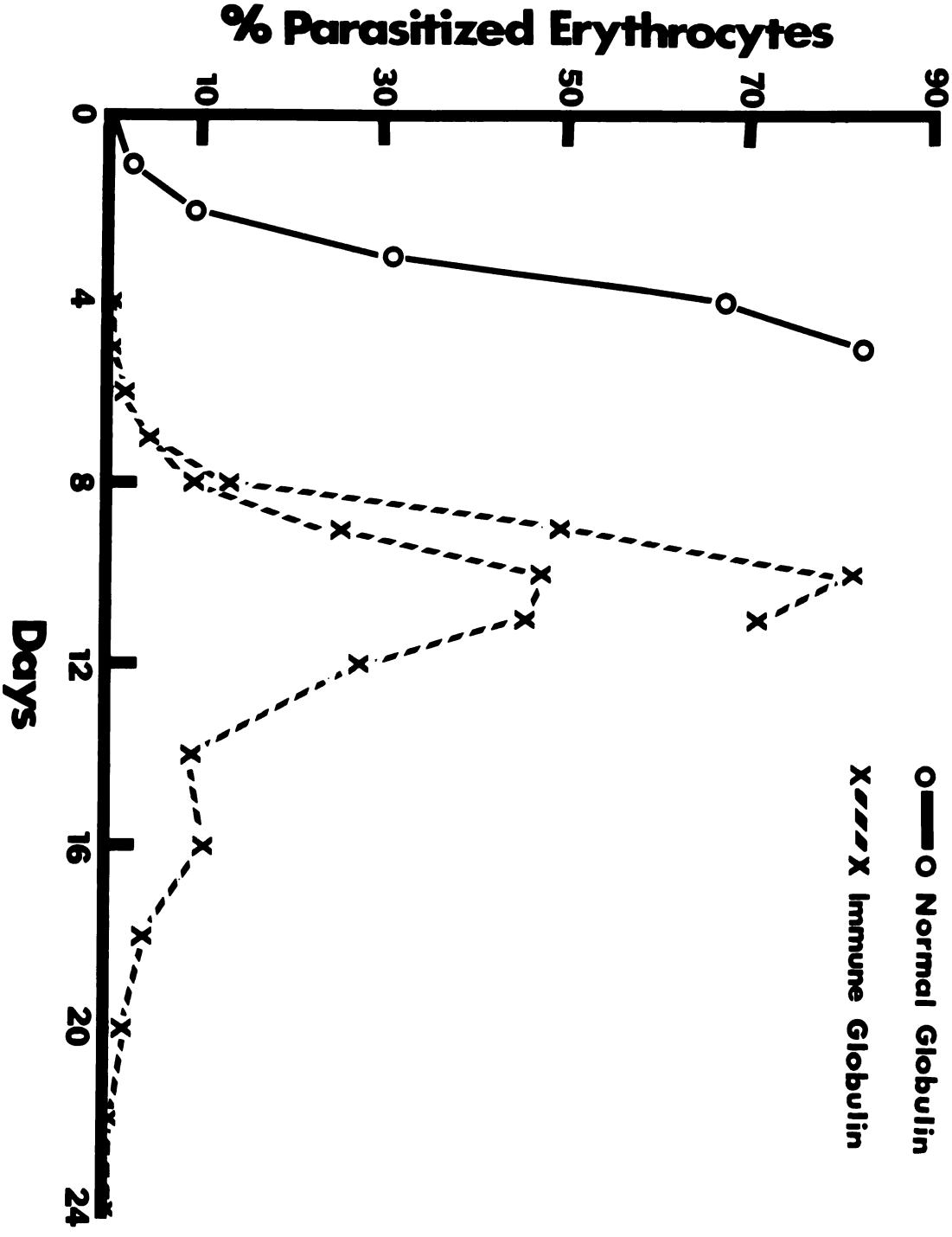

P.E. per 2000 Erythrocytes
0
10
20
30
40
Hours
0
4
8
12
16
20
24
O—O Normal Globulin
X---X Immune Globulin

The Selection of the Immunity Resistant-1 (IR-1) Strain of B. rodhaini

In a second experiment, portions of infected rat cells were treated as described with immune globulin, normal rat globulin and PBS. These infected cells were tested in mice as in the preliminary experiment using 4 mice to test cells treated with immune globulin, 4 to test cells treated with normal rat globulin and 4 to test the cells incubated with PBS.

The cells treated with normal rat globulin and PBS passed quickly from the peritoneal cavity into the blood of the test mice. The infected cells treated with immune globulin did not appear in the blood until after the 4th day. Thereafter, 2 of the mice had transient parasitemia and recovered while the other 2 developed high parasitemia and died, Figure 2. Blood from a moribund mouse with high parasitemia that had been a recipient of the immune globulin treated cells was taken and transferred to a normal rat. This passage strain was designated as immunity resistant-1 (IR-1) strain of B. rodhaini. The infected blood of this rat was taken and treated as before and inoculated into test mice. This process was repeated until on the 21st treatment, the cells infected with selected strain were not affected by treatment with the immune globulin and passed from the peritoneal cavity into the blood of the recipient mice as readily as did the cells that had been treated with normal rat globulin.

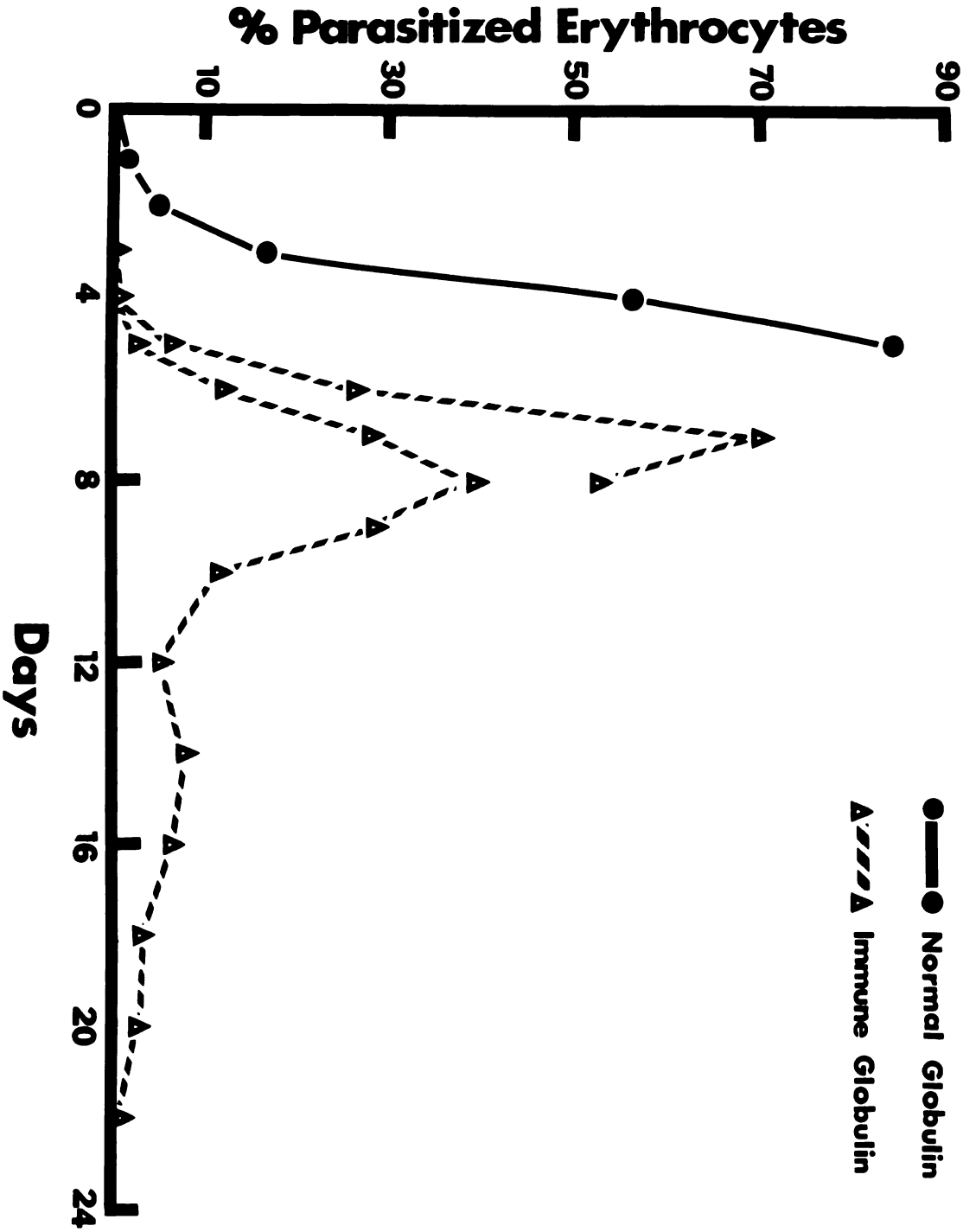
Figure 2. The course of infection of Babesia rodhaini treated in vitro with immune globulin and inoculated intraperitoneally into mice. Development of patent infection and emergence of an immunity resistant-1 (IR-1) strain of parasites.


% Parasitized Erythrocytes
0
10
30
50
70
90
Days
4
8
12
16
20
24
O Normal Globulin
X Immune Globulin

Selection of Immunity Resistant-2 (IR-2) Strain of B. rodhaini

Hyperimmune globulin was prepared from rats that had recovered from infection with the IR-1 strain of B. rodhaini. This globulin was incubated at 37°C for 1 hour with rat red blood cells infected with the IR-1 parasites and the washed cells were inoculated intraperitoneally into a group of 8 test mice just as in the 1st and 2nd experiments. Parasitized cells treated with the immune globulin did not appear in the circulation of the recipient mice until after the 4th day after inoculation. Thereafter 3 of the mice had transient parasitemia and recovered while the other 5 had high parasitemia and died. Blood from one of the 5 mice with high parasitemia was transferred to a normal rat and given the designation of immunity resistant-2 (IR-2) strain of B. rodhaini, Figure 3. The infected blood cells of this rat were incubated at 37°C for one hour with the immune globulin mediated by the IR-1 infection and after the cells were washed, they were inoculated intraperitoneally into test mice. One of these mice that had high parasitemia after the 4th day post-inoculation was bled and the cells used to repeat the selective process. After 4 treatments and passages the selected strain treated with IR-1 strain immune globulin passed from the peritoneal cavity into the blood of recipient mice as readily as did the cells that had been treated with normal rat globulin.

Figure 3. The course of infection of the IR-1 strain of Babesia rodhaini after in vitro incubation with immune globulin mediated by IR-1 strain infection and intraperitoneal inoculation into mice. The development of patent infection and the emergence of the second immunity resistant (IR-2) strain.

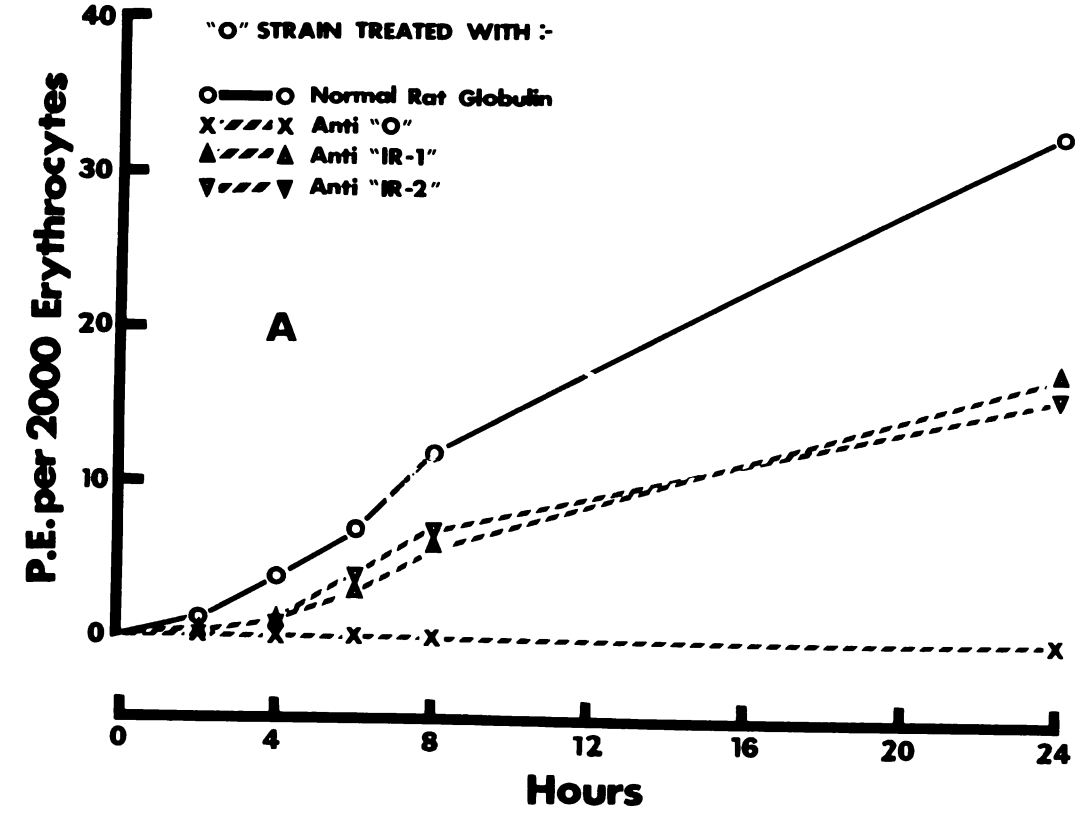

% Parasitized Erythrocytes
10
30
50
70
90
0
4
8
12
16
20
24
Days
Normal Globulin
Immune Globulin

The Testing of the O Strain, the IR-1 Strain and the IR-2 Strain of B. rodhaini Parasites for Strain Specific Antigens

An immune globulin was prepared by repeated infection of rats with the IR-2 strain of parasites. This globulin and the immune globulins mediated by infections of the O and the IR-1 strains of B. rodhaini were used in experiments in which the O, the IR-1 and the IR-2 strains of parasites were treated as above with the homologous and the heterologous immune globulins. After washing, the treated cells were inoculated intraperitoneally into groups of 6 test mice. The rate that the treated cells passed from the peritoneal cavity of the test mice into their blood streams are shown, Figures 4a, 5a, and 6a. In these experiments parasites of the O and the IR-1 strains treated with their homologous immune globulins were inhibited in passage from the peritoneal cavity into the blood for more than 24 hours. The parasitized cells treated with heterologous immune globulins appeared in the blood of the test mice about as rapidly as did cells treated with globulin of normal rats. Cells infected with the IR-2 strain of parasites were not markedly affected by treatment with the homologous immune globulin mediated by IR-2 infection. Neither did this immune globulin have any effect when it was used to treat the O and the IR-1 parasites. Infected cells of each strain treated with this globulin passed from the

Figure 4a. The effects of incubation at 37°C for one hour with immune globulins mediated by the O strain (Anti-O), the IR-1 strain (Anti-IR-1) and the IR-2 strain (Anti-IR-2) of Babesia rodhaini and with normal rat globulin on the passage of O strain infected cells into the blood of mice after intraperitoneal inoculation.

Figure 4b. The course of O strain Babesia rodhaini infection inoculated intraperitoneally into mice after in vitro treatment with immune globulins and normal rat globulin as shown in Figure 4a.


"O" STRAIN TREATED WITH :-
Normal Rat Globulin
Anti "O"
Anti "IR-1"
Anti "IR-2"
A
P.E. per 2000 Erythrocytes
40
30
20
10
0
0
4
8
12
16
20
24
Hours

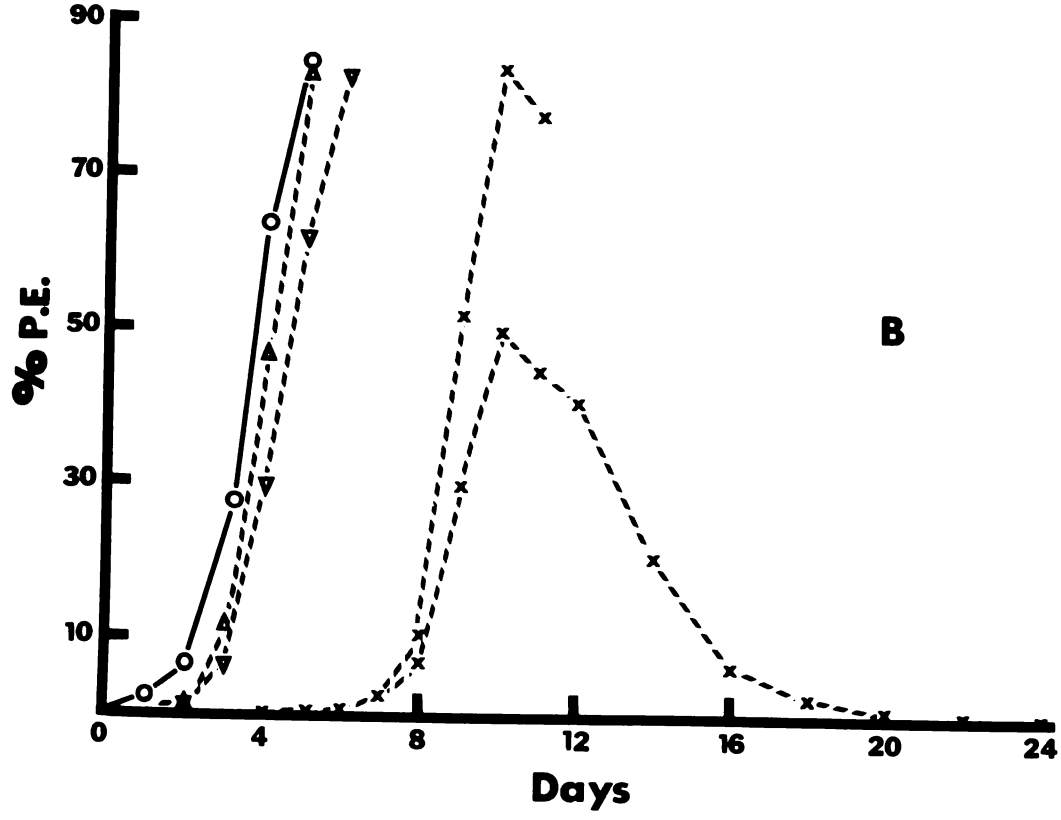
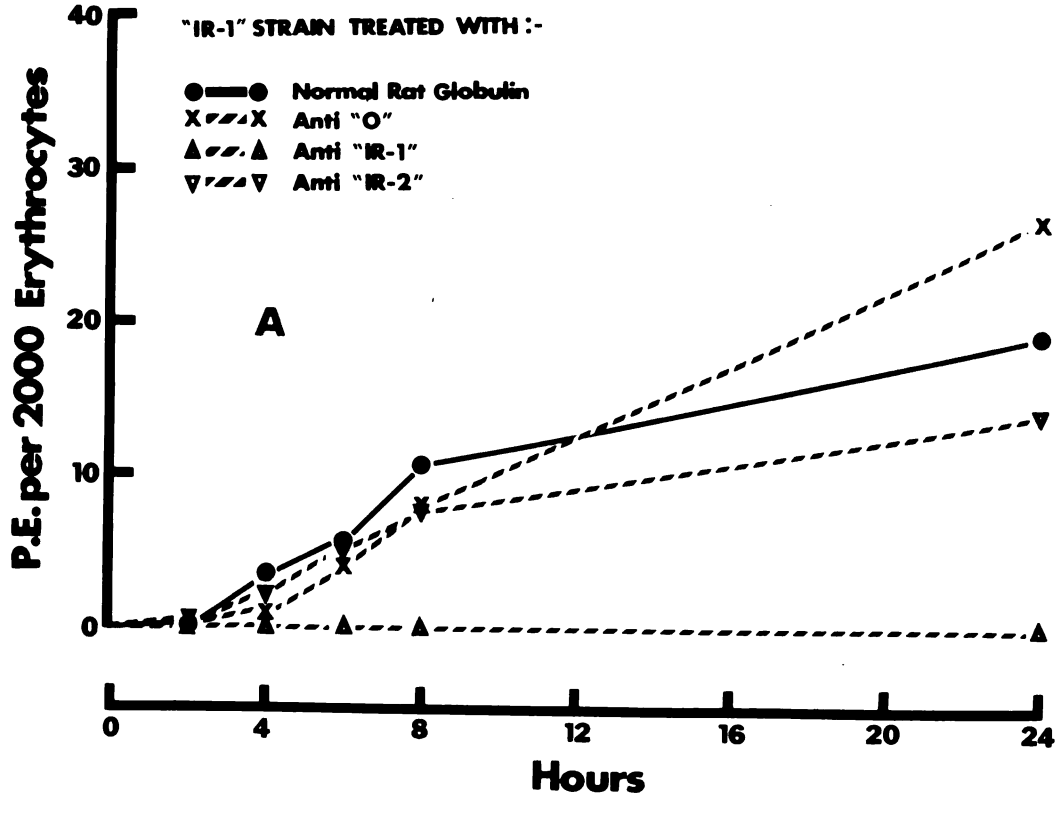

B
% P.E.
90
70
50
30
10
0
4
8
12
16
20
24
Days

Figure 5a. The effects of incubation at 37°C for one hour with immune globulins mediated by the O strain (Anti-O), the IR-1 strain (Anti-IR-1) and the IR-2 strain (Anti-IR-2) of Babesia rodhaini and with normal rat globulin on the passage of IR-1 strain infected cells into the blood of mice after intraperitoneal inoculation.

Figure 5b. The course of IR-1 strain Babesia rodhaini infection inoculated intraperitoneally into mice after in vitro treatment with immune globulins and normal rat globulin as shown in Figure 5a.


"IR-1" STRAIN TREATED WITH :-
Normal Rat Globulin
Anti "O"
Anti "IR-1"
Anti "IR-2"
A
P.E. per 2000 Erythrocytes
40
30
20
10
0
0
4
8
12
16
20
24
Hours

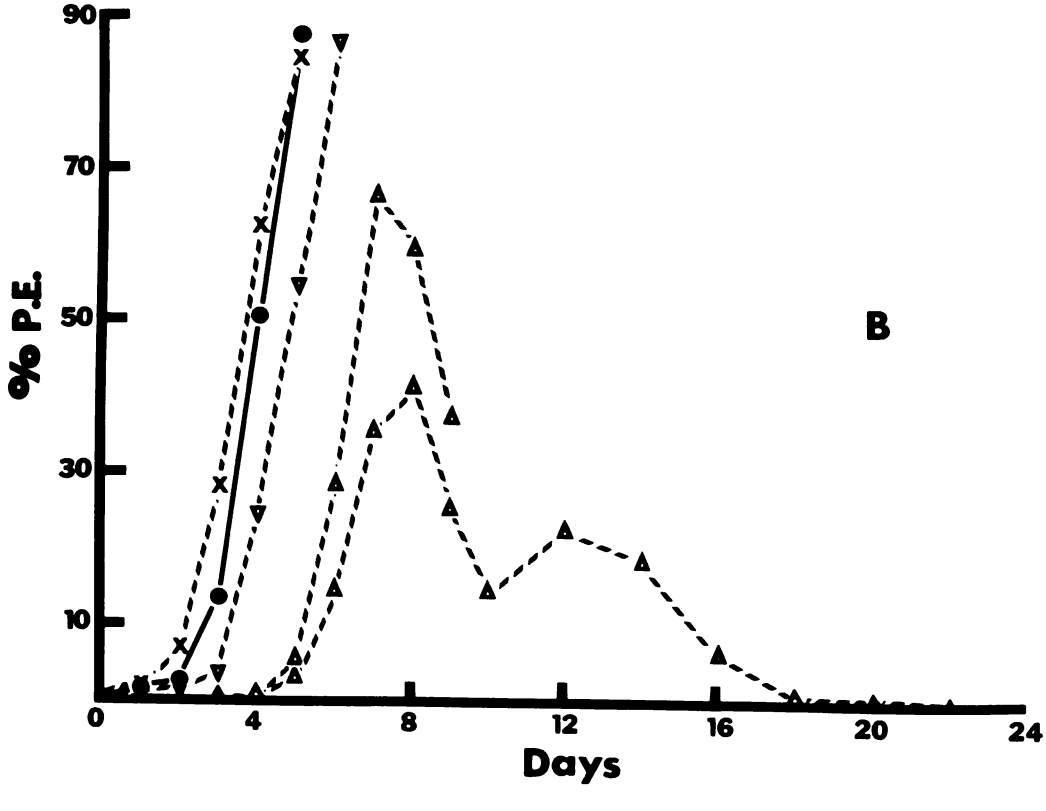
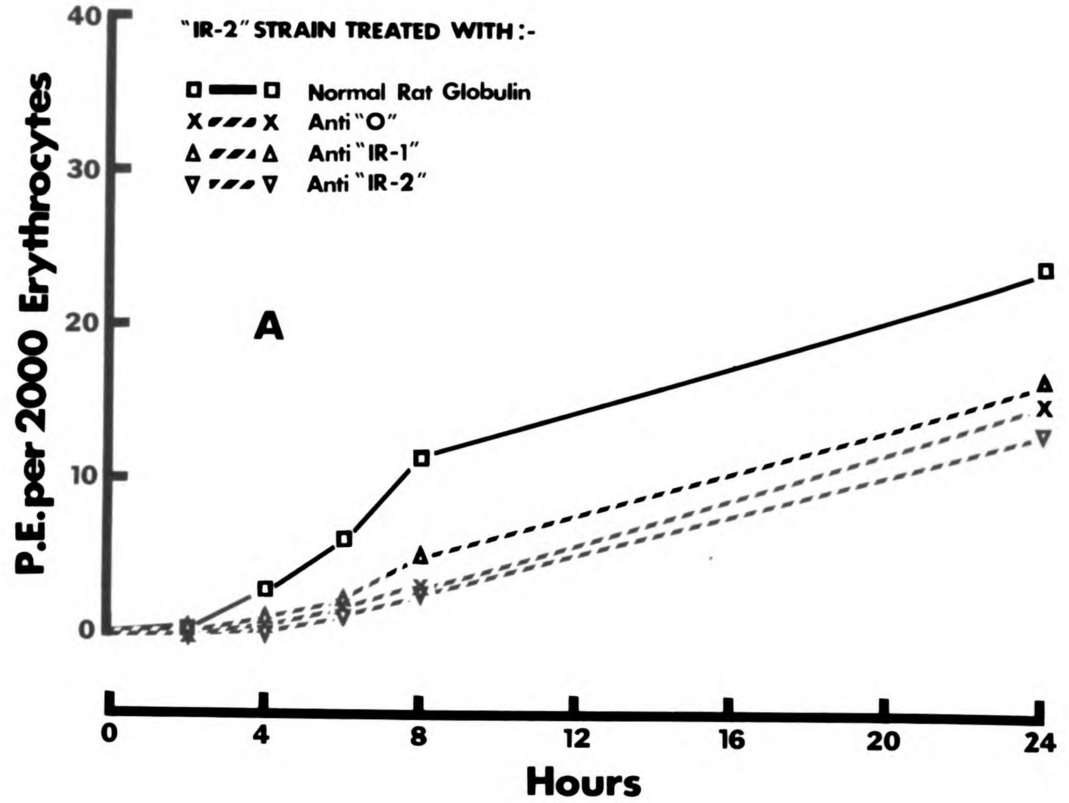

B
% P.E.
90
70
50
30
10
0
4
8
12
16
20
24
Days

Figure 6a. The effects of incubation at 37°C for one hour with immune globulins mediated by the O strain (Anti-O), the IR-1 strain (Anti-IR-1) and the IR-2 strain (Anti-IR-2) of Babesia rodhaini and with normal rat globulin on the passage of IR-2 strain infected cells into the blood of mice after intraperitoneal inoculation.

Figure 6b. The course of IR-2 strain Babesia rodhaini infection inoculated intraperitoneally into mice after in vitro treatment with immune globulins and normal rat globulin as shown in Figure 6a.


"IR-2" STRAIN TREATED WITH:-
Normal Rat Globulin
Anti "O"
Anti "IR-1"
Anti "IR-2"
A
P.E. per 2000 Erythrocytes
40
30
20
10
0
0
4
8
12
16
20
24
Hours

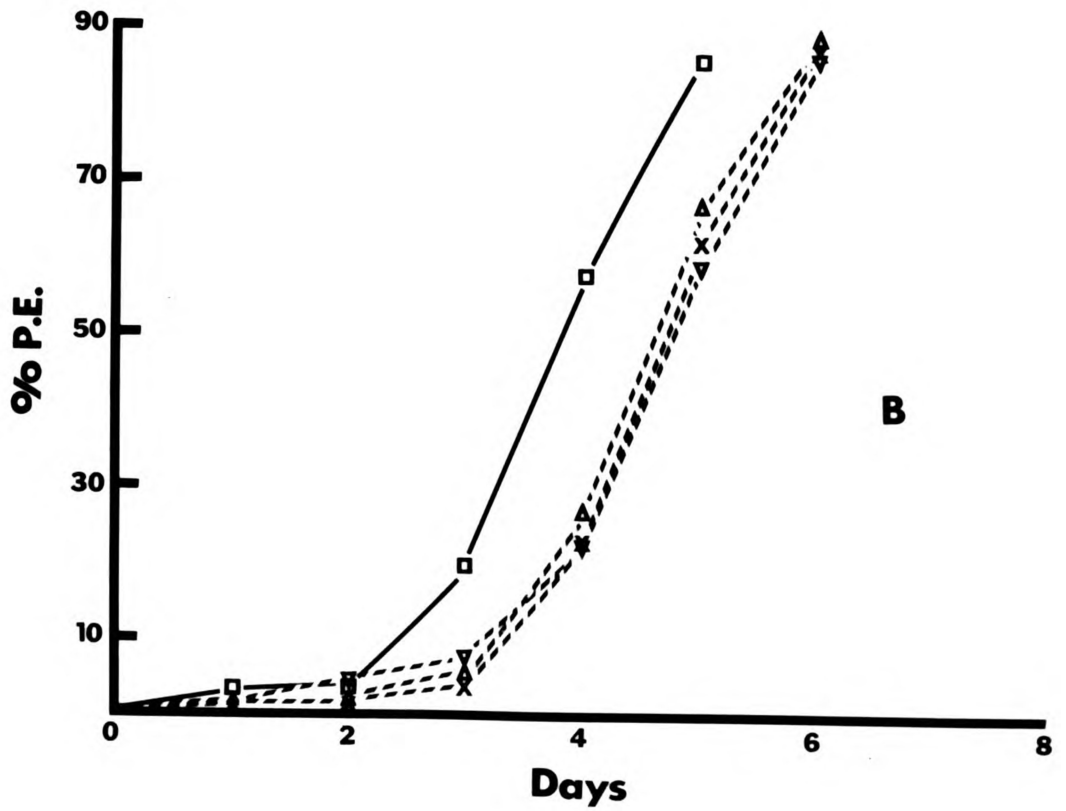

% P.E.
90
70
50
30
10
0
2
4
6
8
Days
B

Table 3. Summary of experiments in which rat erythrocytes infected with the original (O) strain, the immunity resistant-1 (IR-1) strain and the immunity resistant-2 (IR-2) strain of Babesia rodhaini were treated each with its homologous and the heterologous immune globulins.

	No. mice used	No. mice showing parasitemia within 24 hours	No. mice survived	% surviving
O strain treated with				
PBS	22	22	0	0
Normal rat globulin	22	22	0	0
Anti-O	22	0	9	40.91
Anti-IR-1	18	18	0	0
Anti-IR-2	6	6	0	0
IR-1 strain treated with				
PBS	18	18	0	0
Normal rat globulin	18	18	0	0
Anti-O	18	18	0	0
Anti-IR-1	18	0	7	38.89
Anti-IR-2	6	6	0	0
IR-2 strain treated with				
PBS	6	6	0	0
Normal rat globulin	6	6	0	0
Anti-O	6	6	0	0
Anti-IR-1	6	6	0	0
Anti-IR-2	6	6	0	0

peritoneal cavity into the blood about as readily as did cells treated with normal rat globulins.

The parasites of the O and IR-1 strains treated with homologous immune globulins were not evident in the blood of the test mice until after 4 days. Thereafter, in each case 4 of the mice inoculated with infected cells treated with homologous immune globulin developed high parasitemia and died while 2 mice from each group developed transient parasitemia and recovered. In cases of treatment with heterologous immune globulins, all of the test mice in each group developed high parasitemia and died at the same rate as did mice inoculated with cells treated with globulin from normal rats. The IR-2 strain was not affected by treatment with immune globulins mediated by any of the strains. All of the test mice in each group developed parasitemia during the 1st day and died before the 7th day, Figures 4b, 5b, and 6b. The data from all of the experiments in which each of the selected strains were tested with homologous and heterologous immune globulins are summarized in Table 3.

Tests for Strain Specific Immunity in Rats Recovered from the O and the IR-1 Strains of B. rodhaini

Rats that had been given light infections of the O and IR-1 strains of B. rodhaini and had recovered were given both homologous and heterologous challenging infections with

each strain of parasites. Four recovered rats were used for each challenge and 4 normal rats were used as the control for each challenging infection. The data obtained from this experiment are shown in Table 4. Rats recovered from both strains were equally resistant to homologous and heterologous challenging infections. Both the O and the IR-1 parasites appeared to be equally virulent for normal rats since none of the controls were alive after the 6th day of the experiment. Rats recovered from the IR-2 strain and challenged with the IR-2 parasites were not included in this experiment since this strain had not been selected at the time.

Tests of the IR-2, the IR-1 and the O Strains of B. rodhaini in Mice. Observations on Parasitemia, Anemia, Agglutinins for Trypsinized Erythrocytes, Non-specific Serum Antigen and Antibody to Serum Antigen Production

Three groups of mice, 25 each, were infected with the O, the IR-1 and the IR-2 strains of B. rodhaini, respectively. Each day thereafter 3 mice from each group were brought to examination and necropsy. A blood smear was prepared for determination of the percentage of P.E. for each mouse, a red blood cell count was made on each and each mouse was exsanguinated and the plasma collected to be tested for cold haemagglutinin for trypsinized mouse erythrocytes, for non-specific serum antigen and for antibody to the serum antigen. The means of the data from the

Table 4. Percentage parasitized erythrocytes (%P.E.) in rats recovered from infection with the original (O) strain and the immunity resistant-1 (IR-1) strain of Babesia rodhaini which were challenged with the parasites of homologous and heterologous strains.

Rat No.	Recovered From	Challenged With	% P.E. on day after challenging -1	1	2	3	4	5	6	7	8	10	12
1	Normal rat	O strain	0	0.2	1.2	5.5	22	53	76	D**			
2	" "	" "	0	0.3	2.0	14	43	77	D				
3	" "	" "	0	0.2	1.5	7.1	30	58	84	D			
4	" "	" "	0	0.3	1.7	7.0	31	64	87	D			
		Mean	0	0.25	1.6	8.4	31.5	63	82.3				
5	Normal rat	IR-1 strain	0	0.4	2.4	6.9	33	78	D				
6	" "	" "	0	0.1	1.8	5.2	23	57	79				
7	" "	" "	0	0.2	1.6	6.4	28	59	84	D			
8	" "	" "	0	0.5	2.2	7.0	36	74	D				
		Mean	0	0.3	2.0	6.4	30	67	81.5				
9	O strain	O strain	0	0	0	0	0	0	0	0	0	0	0
10	" "	" "	0	0	0	0	0	0	0	0	0	0	0
11	" "	" "	0	0	0	0	0	0	0	0	0	0	0
12	" "	" "	0	0	0	0	0	0	0	0	0	0	0
		Mean	0	0	0	0	0	0	0	0	0	0	0
13	O strain	IR-1 strain	0	+*	+	0.2	0.3	+	0	0	0	0	0
14	" "	" "	0	+	+	0.1	0	0	0	0	0	0	0
15	" "	" "	0	+	0	0	0	0	0	0	0	0	0
16	" "	" "	0	0	+	0	0	0	0	0	0	0	0
		Mean	0	+	+	0.075	.075	+	0	0	0	0	0
17	IR-1 strain	O strain	0	0	0	0	0	0	0	0	0	0	0
18	" "	" "	0	0	0	0	0	0	0	0	0	0	0
19	" "	" "	0	0	0	0	0	0	0	0	0	0	0
20	" "	" "	0	0	0	0	0	0	0	0	0	0	0
		Mean	0	0	0	0	0	0	0	0	0	0	0
21	IR-1 strain	IR-1 strain	0	+	0	0	0	0		0	0	0	0
22	" "	" "	0	0	0	0	0	0		0	0	0	0
23	" "	" "	0	0	0	0	0	0		0	0	0	0
24	" "	" "	0	+	0	0	0	0		0	0	0	0
		Mean	0	+	0	0	0	0		0	0	0	0

**Dead.

*Positive but uncountable.

Table 5. The mean percentage of parasitized erythrocytes (% P.E.), the mean red blood cell counts (RBC x 10^6 per c. mm.), the mean of the titers of agglutinins for trypsinized erythrocytes (HA titer), and the means of the titers of serum antigen (Ag. titer) and antibody to serum antigen (Ab. titer) in groups of 3 mice brought to necropsy daily after infection with the original (O) strain, the immunity resistant-1 (IR-1) strain and the immunity resistant-2 (IR-2) strain of Babesia rodhaini.

B. rodhaini	No. Mice Necropsies	Day After Infection	% P.E.	RBC x10^6 per c. mm.	HA titer **	Tube Bentonite Flocculation Tests (TBF)	
						Ag. titer*	Ab. titer*
O strain	3	1	1.6	9.06	0	0	0
"	3	2	7.4	8.60	8	0	0
"	3	3	26	8.81	8	0	0
"	3	4	61.5	6.44	16	8	4
"	3	5	83.3	1.57	32	16	16
IR-1 strain	3	1	1.2	8.87	0	0	0
"	3	2	5.8	9.02	4	8	4
"	3	3	17.3	8.71	32	16	8
"	3	4	56.7	5.55	64	16	32
"	3	5	84	1.31	32	0	256
IR-2 strain	3	1	1.9	8.78	0	0	0
"	3	2	7.8	8.25	4	8	8
"	3	3	28.3	8.62	64	16	8
"	3	4	66	6.18	64	128	64
"	3	5	86.7	1.29	32	0	128
Normal Mice Control	5	-	0	8.81	0	0	0

*Titers expressed as reciprocals of the TBF test titers.

**Titers expressed as reciprocals of the HA test titers.

examinations and tests are presented in Table 5. Both parasitemia and anemia developed at nearly equal rates in all mice. Haemagglutinins were detected on the second day of infection but did not appear to be correlated with the development of anemia. Serum antigen was detected on the second day of the experiment in mice infected with the IR-1 and IR-2 strains but was not evident until the 4th day in mice infected with the O strain. Similarly, mice infected with the IR-1 and IR-2 strains had higher titers of antibody to serum antigen which were detected earlier than did mice infected with the O strain of B. rodhaini.

Ouchterlony Double Gel Diffusion Slide Test of Serum Antigens Elaborated by Acute Infections of the O Strain, the IR-1 Strain and the IR-2 Strain of B. rodhaini in Rats

In order to determine whether or not there had been alterations induced in the non-specific serum antigens elaborated during the stages of acute infection with B. rodhaini by the selective treatments applied in these experiments, serum from rats with acute infections of each strain were tested in Ouchterlony double get diffusion slide tests against each of the immune globulins mediated by each strain. In the tests serum from an acute infection was tested against the immune globulins and against normal rat globulin. Each of the immune globulins was tested in reciprocal tests against each of the acute infection rat serum and normal

rat serum. The results of these tests are pictured in Figure 7. In all of the tests, serum of acute infection from each of the strains reacted with each of the immune globulins and showed the precipitin lines of identical antigens. Similarly, the tests of the immune globulins mediated by each strain showed the lines of identical antibody when they were tested against each of the acute infection sera.

Indirect Fluorescent Antibody (IFA) Studies of Parasites of the O, the IR-1 and the IR-2 Strains of B. rodhaini

Attempts to demonstrate the antigens and antibodies of a strain specific nature that resulted from the selective processes of these experiments by immunofluorescent methods failed. The results of these experiments indicated that the immune globulins mediated by each strain combined with parasites of each strain but did not react with control antigen slides prepared from uninfected rat blood. Variation in immunofluorescent activity in either homologous or heterologous reactions was not evident when the test slides were stained with anti-rat globulin serum conjugated with fluorescein, Figure 8.

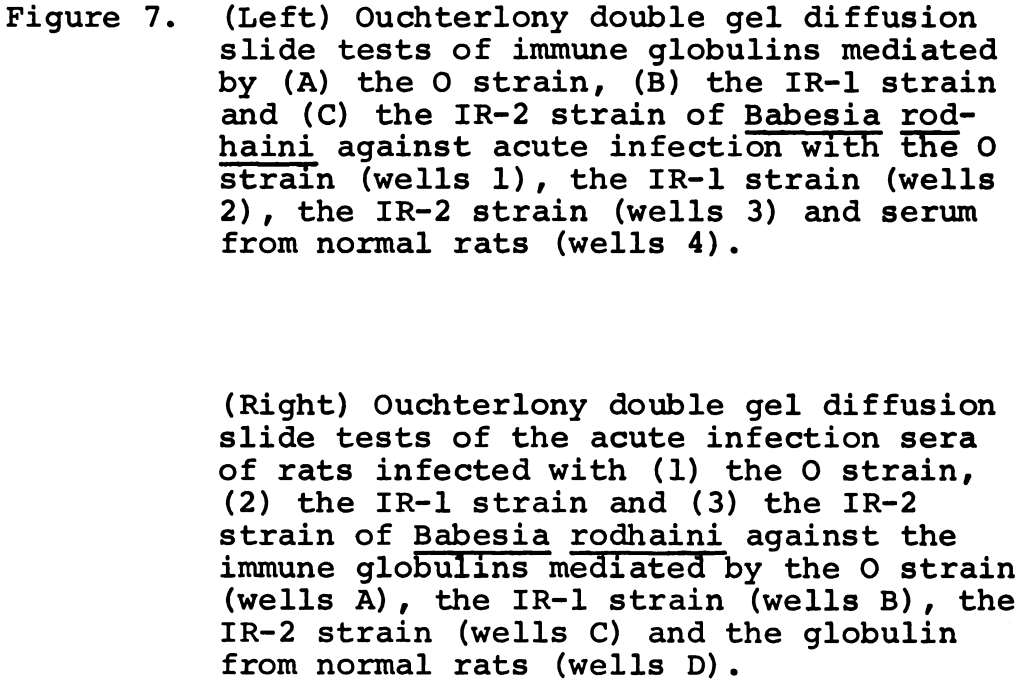
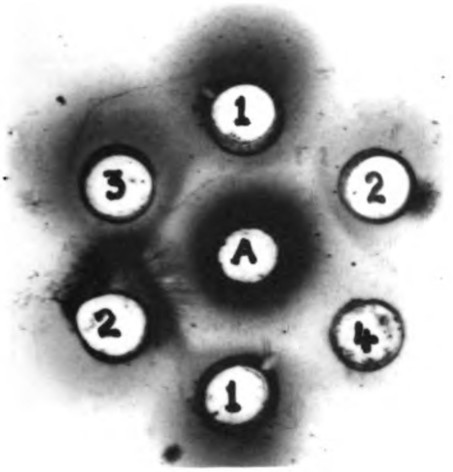


Figure 7. (Left) Ouchterlony double gel diffusion slide tests of immune globulins mediated by (A) the O strain, (B) the IR-1 strain and (C) the IR-2 strain of Babesia rodhaini against acute infection with the O strain (wells 1), the IR-1 strain (wells 2), the IR-2 strain (wells 3) and serum from normal rats (wells 4).

(Right) Ouchterlony double gel diffusion slide tests of the acute infection sera of rats infected with (1) the O strain, (2) the IR-1 strain and (3) the IR-2 strain of Babesia rodhaini against the immune globulins mediated by the O strain (wells A), the IR-1 strain (wells B), the IR-2 strain (wells C) and the globulin from normal rats (wells D).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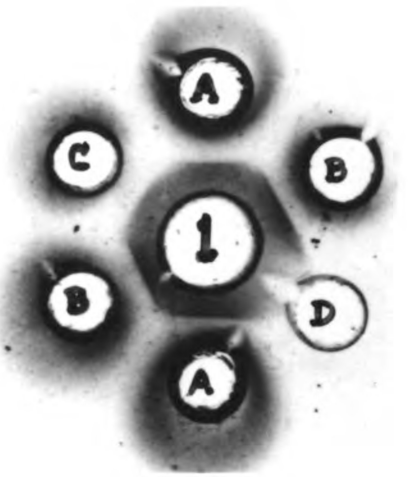
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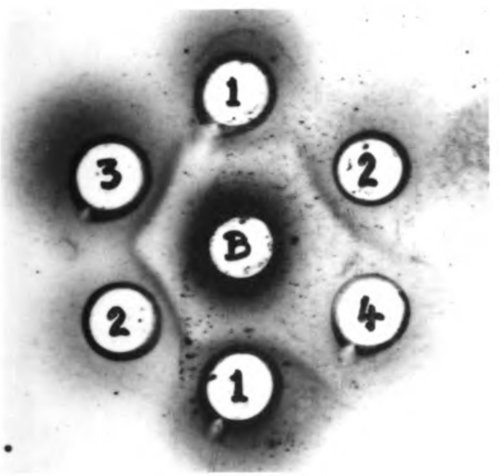
D
A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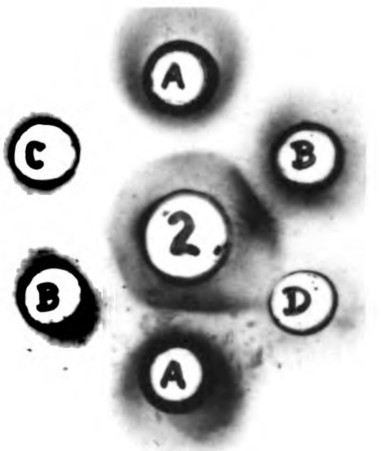
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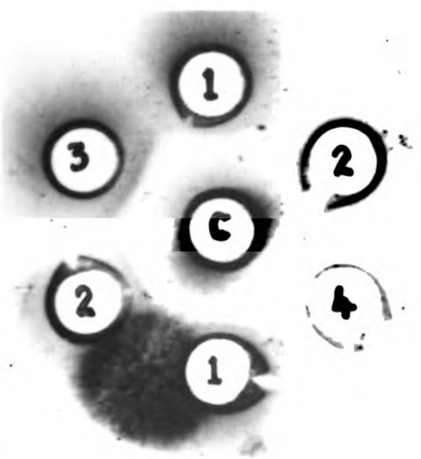
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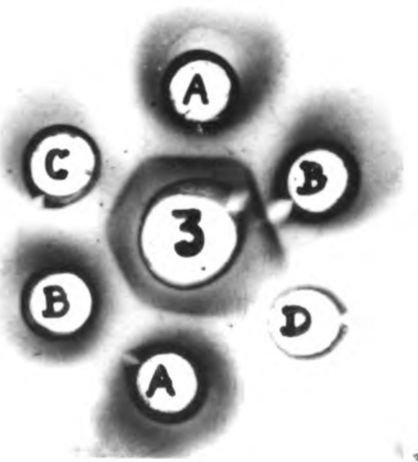
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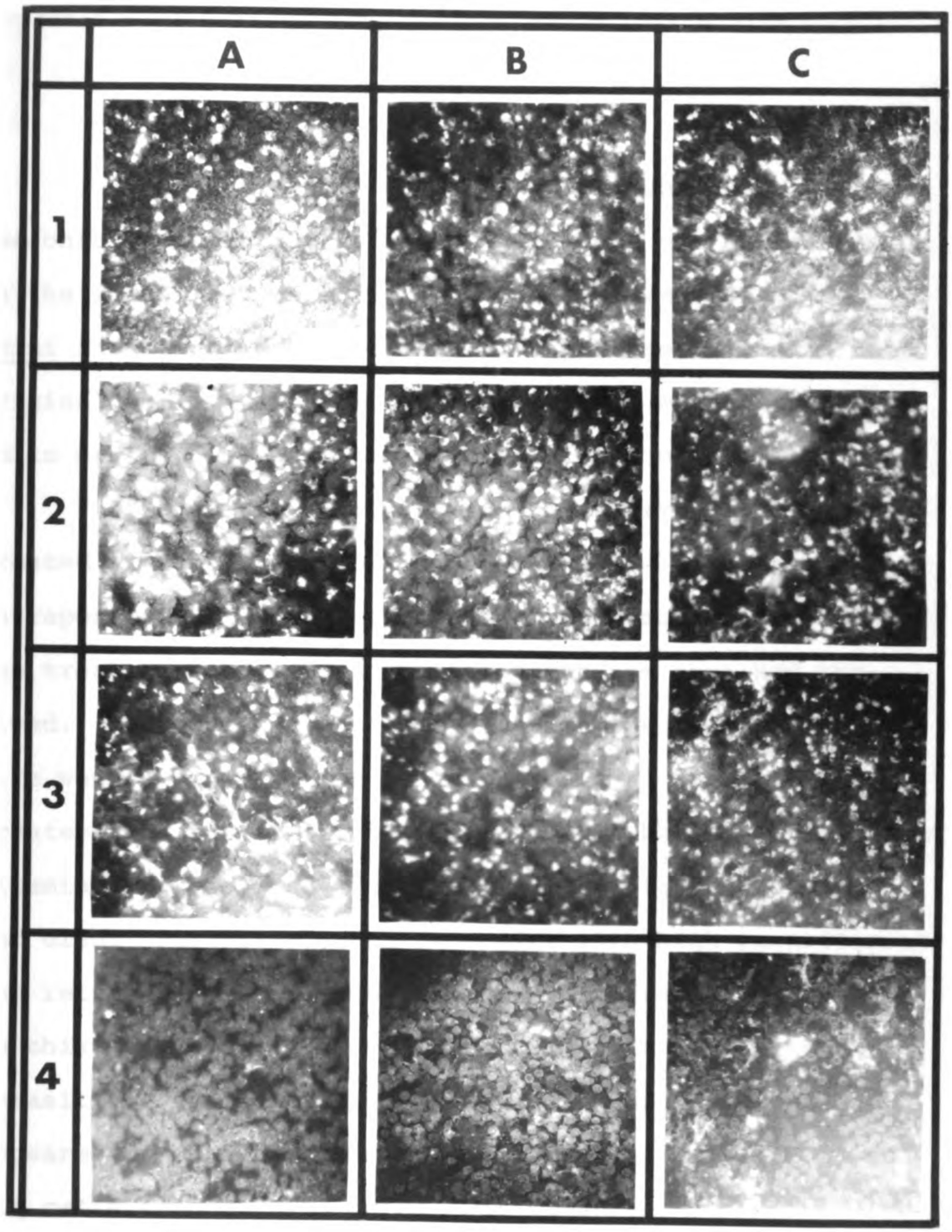
Figure 8. Indirect immunofluorescent reactions of antigen slides prepared from rat blood cells infected with (1) the O strain, (2) the IR-1 strain, (3) the IR-2 strain of Babesia rodhaini and (4) from normal rat red blood cells. After they had been reacted in homologous and heterologous tests with immune globulins mediated by (A) the O strain, (B) the IR-1 strain and (C) the IR-2 strain and then stained with anti-rat globulin serum conjugated with fluorescein. (X 225)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DISCUSSION

From the results of these experiments it seems reasonably clear that antigens which were not manifested in the immune response to the original strain of B. rodhaini infection were expressed after the parasites of the original strain had been treated in vitro with immune globulin mediated by original strain infection.

Original (O) strain parasitized erythrocytes were treated with immune globulin and the cells were inoculated intraperitoneally (IP) into mice to determine the effect of the treatment and to recover the parasites that had survived. The appearance of parasites in the blood of the mice was retarded for as long as 4 days as a result of the treatement. Thereafter some of the mice had transient parasitemia and recovered, others developed high parasitemia and died. Parasites taken from mice with high parasitemia and returned to rats were repeatedly treated and recovered in this manner until on the 21st time of treatment the parasitized cells were not affected by the treatment and appeared in the peripheral blood circulation as quicly as did cells treated with normal rat globulin.

This strain of parasites, designated as immunity resistant-1 (IR-1), was used to prepare immune globulin by infecting rats and then repeatedly reinfecting them after recovery. When the IR-1 strain was treated with this immune globulin and then inoculated IP into mice the parasites did not appear in the circulation of the recipient animals, whereas O strain parasites treated with this globulin were evident one hour post-inoculation.

After 4 treatments of the IR-1 strain with its immune globulin a second immunity resistant strain designated IR-2 was derived that did not respond to treatment with either of the previously prepared immune globulins. Immune globulin prepared against the IR-2 strain did not restrict passage of IR-2 infected erythrocytes from the peritoneal cavity into the blood nor did it have any effect on either the O or the IR-1 strains. It was therefore evident that strain specific immunogenic antigen was apparently not manifested by the IR-2 parasites.

Effects of these strain specific immunogenic antigens were not manifested when rats were immunized by infections with the O and the IR-1 strains and then given homologous and heterologous strain challenging infections. All of the rats, regardless of the strain used in immunization, were apparently equally resistant to each of the strains. In this experiment with rats it seems probable that the non-specific immunity of the rats was due in a

major part to immunization with the non-specific serum antigens of acute haemosporidian infections (Cox, 1966; Sibinovic *et al.*, 1967a, 1967b; Cox, Milar and Patterson, 1968; Cox and Milar, 1968; Corwin and Cox, 1969). Since these non-specific antigens of serum of animals with acute malaria or babesiosis are also elaborated in the acute stages of other infectious anemias, such as acute *Haemobartonella muris* infections of rats, *Eperythrozoon coccoides* infections of mice and in acute infection with duck infectious anemia virus (DIAV) of ducks, it seems unlikely that they are antigens associated with the parasite particles (Cox, H.W., Personal communication; Ludford *et al.*, 1969).

In explaining the role of these non-specific antigens in immunity it is postulated that they are elaborated by the infected cells and that antibody to the antigens reacts with the parasitized cells but not with the parasites per se. This postulate has basis in experimental evidence. Eaton (1938, 1939) found that monkey erythrocytes bearing mature schizonts of *P. knowlesi* were agglutinated by serum of human beings recovered from malaria or serum of monkeys recovered from *P. knowlesi*, but that cells bearing young merozoites of the parasite were agglutinated by neither of the sera. Sibinovic *et al.* (1969) found that red cells of either dogs or rats bearing mature budding forms of *B. canis* of *B. rodhaini* were agglutinated by sera of either rats or dogs that had recovered from babesiosis. Again, cells

bearing young forms were not agglutinated. When cells with mature parasites were reacted with fluorescent antibody to serum antigen, the whole cell showed fluorescent activity, but cells with young parasites showed only minimal activity adjacent to the parasite.

It has been suggested that cells infected with erythrotropic parasites elaborate non-specific antigens in the manner that cells infected with tumor viruses elaborate tumor antigens which are related to neither the host nor virus species, but which are each related one to another (Good and Finstad, 1968; Corwin et al., 1970). Protective immunity mediated by non-specific antigens would therefore be something of an artifact since it had an indirect rather than a direct effect on the parasite.

The results of these experiments indicated that the capability of the selected strains to elaborate non-specific antigen had not been impaired by the treatments. It was also indicated that serum antigens elaborated by each of the strains were similar, if not identical. Mice infected with each strain had detectable antigen and agglutinin for trypsin treated erythrocytes during acute infection. Ouchterlony gel diffusion studies of acute serum and immune serum globulins mediated by each strain indicated that the antigens present in serum of diseased animals were identical.

It therefore seems that the variant antigens selected in these experiments were associated with the parasite

particles and were strain specific. It appears that there were two strain specific antigens that had a role in immunogenesis. The work demonstrated that the dominant antigen of the O strain and of the IR-1 strain was strain specific and immunogenic. The absence of effects from treatment with immune globulins mediated by the IR-2 strain on any of the strains would seem to indicate that selection may have depleted this strain of strain specific immunogenic antigen and that immunity mediated by this strain was probably due to the elaboration of non-specific serum antigens only.

Serologic identification of the strain specific antigens was not demonstrated in this research. Attempts to prepare antigens of a specific nature from parasites liberated from red blood cells as reported by Sibinovic et al., (1966) failed. Attempts to demonstrate the specific antibody to these antigens by indirect fluorescent antibody techniques also failed. The results of the experiments indicated only that the immune globulins reacted with the parasites of each strain in antigen slides prepared from rat blood infected with each strain and did not react with control antigen slides prepared from the blood of uninfected rats. In both homologous and heterologous tests of the immune globulins with the 3 strains of parasites, fluorescent activity from the indirect staining method was of equal intensity.

In retrospect it now seems that direct immunofluorescent methods might have been more useful in attempting to achieve the present objectives. However, even with this method, results might have been obscured by the several idiopathic immune responses of acute B. rodhaini infection and much care would have to be exercised in adsorbing the immune globulins to remove all antibodies except those that reacted with the strain specific antigens.

However, it is pointed out that the phenomenon of peritoneal localization of antibody treated parasites has been shown to be an exquisitely sensitive immunologic device. The phenomenon was first reported by Rabinowitsch and Kempner (1899) when they observed that Trypanosoma lewisi parasites treated with serum of recovered rats failed to appear in the blood circulation after intraperitoneal inoculation whereas parasites treated with normal serum could be easily detected after a few minutes. This observation was confirmed by Laveran and Mesnil (1901). Their further study indicated that the phenomenon occurred with equal efficiency when the parasites were simply inoculated into the peritoneal cavity of the immune rats. Laveran used this device extensively to study acquired immunity and the innate immunity of alien hosts to trypanosomes (Laveran and Petit, 1909).

It is remarkable that such a useful and simple serologic device did not attract more attention. It was

rediscovered when it was found that Trypanosoma cruzi parasites inoculated intraperitoneally into recovered mice did not appear in the peripheral circulation, but were detected within two hours after inoculation into normal mice. The work of Rabinowitsch and Kempner (1899) and Laveran and Mesnil (1901) was confirmed. In addition the device of treating T. lewisi parasites with immune serum and inoculating the treated parasites intraperitoneally into mice was employed. It was also shown that passivley immunized mice also effectively prevented passage of the parasites into the circulation. Since T. lewisi is not a natural parasite of mice, the fate of the parasites in the circulation of normal mice was followed and it was found that little or no effect was observed until the trypanosomes suddenly began to lyse about 48 hours after inoculation. Thus observations of Laveran and Petit (1909) on the fate of parasites inoculated into alien hosts were in part confirmed (Cox, 1964a).

When erythrocytes infected with P. berghei were injected into the peritoneal cavity of immune mice the localization phenomenon was not observed. These cells passed into the circulation of immune mice as readily as they did when they were inoculated into normal mice. However, the parasitized cells were cleared from the circulation with remarkable rapidity after 24 hours. When the parasites were liberated from the hosts' red blood cells prior to inoculation, the result was essentially the same.

The parasites passed quickly into the circulation of immune mice, but were quickly removed from the blood after about 24 hours. However, when mice that had been immunized with normal rat erythrocytes were inoculated with P. berghei infected cells from rats the phenomenon was effective and the parasitized red cells were excluded from the circulation for as long as 5 days. It was therefore suggested that the immunity to malarial parasites differed in some fundamental way from the acquired immunity to trypanosomal parasites (Cox, 1964b). It was subsequently found that immunity to normal red blood cells would serve as an effective protective mechanism when the cells of the same species harboring P. berghei parasites were inoculated IP into the otherwise susceptible animals. It was suggested that immunity directed at the infected cell but not at the intracellular parasite might be a functional mechanism in malaria. In this instance, immunity afforded by antibody to normal red cells seemed about as effective as the immunity afforded by immunization with non-specific serum antigens (Cox, 1969; Corwin and Cox, 1969).

In the present work it was something of a surprise that the peritoneal localization phenomenon could be demonstrated with B. rodhaini parasites treated with immune globulin. It is pointed out, however, that the immune globulin used in the present work was from rats that had been repeatedly reinfected with large inocula of blood heavily

infected with B. rodhaini. Thus the present work differs from that of Cox (1964b) in that the mice and rats tested had only recovered from a single or only 2 P. berghei infections prior to inoculation. It remains to be seen whether or not this phenomenon can be applied to plasmodial infections and to determine whether antigenic variation can be induced as was done in the present work.

Earlier studies of relapse of P. berghei infections of mice indicated that antigenic variation of the kind observed in the present work had not occurred. While parasites isolated from mice with relapsed infections (relapse strains) were not affected by immunity induced by parent strain infection, it was found that the relapse parasites were not immunogenic; i.e., infection with these parasites was not as readily induced into latency as were infections with the parent strain. In normal mice the relapse strains appeared to be not as virulent as the parent strain. The crisis involving a sudden loss of blood cells, parasites, and high mortality which was conspicuous in the parent strain was negligible in relapse strain infections. On the basis of that information it was suggested that relapse strains of P. berghei may have lost antigens that had been functional in both immunity and in pathogenesis (Cox, 1959, 1962). It has since been learned that the appearance of antibody to non-specific serum antigen was prominent in the serum of animals just prior to this anemia-parasitemia

crisis (Cox, H.W., Personal communication). It was therefore thought that relapse might have been mediated by loss of the ability of the parasites to cause these non-specific antigens to be elaborated (Cox and Milar, 1968). This idea has been tested in ducks immunized with non-specific serum antigens. By repeated passage of P. lophurae parasites in the immunized ducks a strain of P. lophurae was derived that did not respond at all to the non-specific immunity (Corwin et al., 1970). This immunity resistant strain could not be tested to determine whether or not it caused serum antigen to be elaborated because of contamination with DIAV which also causes the antigens to be elaborated during acute infection (Ludford et al., 1969; Cox, H.W., Personal communication). It had been postulated that this mechanism involving the loss of the ability to cause non-specific serum antigens to be elaborated might be elucidated in the present experiments; however, this did not prove to be the case.

In other work on the relapse mechanisms of malaria it was found that monkey erythrocytes bearing mature parasites of relapsed P. knowlesi infections were not agglutinated by serum of monkeys recovered from parent strain infection. Neither would serum of monkeys recovered from relapse strain infection agglutinate cells bearing the mature parasites of the parent strain. It was also noted that latency or recovery was difficult to induce in monkeys infected

with relapse strain parasites. The agglutination of cells bearing P. knowlesi schizonts was considered to be a species specific phenomenon since Eaton (1938) had found that these cells were agglutinated by serum from monkeys recovered from P. knowlesi but not by serum of monkeys recovered from Plasmodium inui (Brown and Brown, 1965).

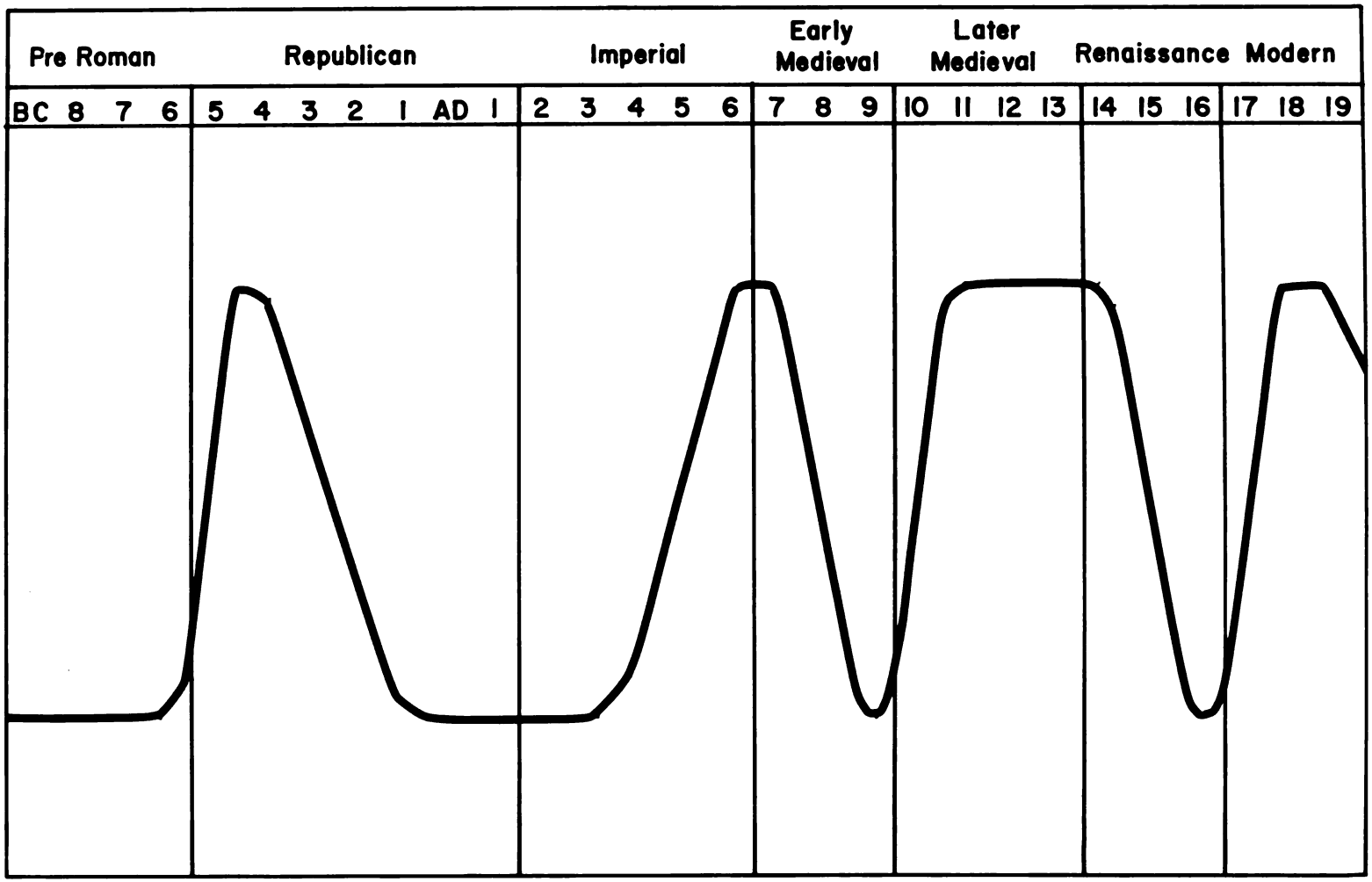
It was unfortunate that Brown and co-workers had not studied the work of Eaton and others more closely. Eaton (1939) reported that cells bearing schizonts of P. knowlesi were agglutinated by serum of persons recovered from human malarial infections as well as by the serum of monkeys recovered from P. knowlesi. After consideration, it would seem likely that Brown et al. had seen variation in plasmodial parasites similar to that reported earlier by Cox (1959, 1962).

A question unanswered by the present research is the matter of the reversion of the selected strains to the original. As mentioned above, it seems from these data that the original strain was a heterologous population of the various antigenic or unantigenic strains. If this is true, it would seem likely that both the IR-1 and IR-2 strains would tend to revert to the original strain and that antigenic parasites would reappear in the IR-2 strain once the selective force was no longer applied. Reversion to a heterologous population is an aspect of this work that was not investigated.

Another variable among infections with haemosporidian parasites is the variation in the severity of disease from one period of history to another. This kind of variation was illustrated vividly by the historical investigations of Scott (1939), and was presented in graphic form in his history book. That drawing has been borrowed to illustrate the point, Figure 9. The Pontine marshes, south of Rome, have been during recorded history at times a place of pestulance unfit for human habitation and at other times the garden and bread basket of Rome. If one considers a moment, it must be realized that the Roman Compagna was not free of mosquitoes and malarial infections for the long periods in which there were no malarial fevers. The parasites and their hosts must have been living together under some state of compatible relationship through the years of low endemicity only to eventually end the treaty and become inhospitable during the epidemic years of the killing malarial fevers. That the diseases in question were malaria is not doubted.

> It is not clear when the intermittents (fevers) first appeared in Ancient Italy. The worship of FEBRIS as a goddess in Rome was very ancient but she may have represented the great fevers which were mostly typhus and typhoid. It seems likely that the intermittents were introduced about 200 BC. The earliest written statements which appear to indicate fever with clearly marked periodicity are bits of dialogue found in Plautus (184 BC) and Terence (159 BC). . . (Russell, West and Manwell, 1946.)

Figure 9. Endemicity and epidemicity of malarial (intermittent) fevers in the Roman Compagna (Pontine Marshes, just south of Rome) during the period of 800 BC to 1900 AD. (Borrowed from Scott, H.H., 1939, A History of Tropical Medicine. Edward Arnold and Co., London.)



Graph of decline and increase in Malaria in the Roman Campagna

from Scott: History of Tropical Medicine vol. I, p. 137.

Marchiafava (1931) wrote very emphatically of the marked changes that he had observed in the severity of Plasmodium falciparum malaria during his life time of study (1880-1930's) and insisted that pernicious malaria had all but disappeared from the Roman Compagna. Hackett (1937) wrote at great length about the amount of malarial infection transmission and of the benign nature of malarial disease in Italy from 1920 to 1935. Foy et al. (1935, 1948) in studies of historical malaria in Greece found that references to black water fever or hemoglobinuria would disappear from medical records for periods as long as 50-100 years, only to reappear and be listed as common causes of death for many consecutive years.

For the proponents of the concept of an immunologic basis for disease, there is something to be said for evidence from the present research that parasites can be selected that are not antigenic in their native host. Since repeated infection with the IR-2 variant did not produce immune globulin that responded in the manner that the globulins induced by the IR-1 or the O strain of B. rodhaini, it might well be assumed that the IR-2 strain may have lost antigens that were of significance in both protective immunity and in the pathogenic processes of babesiosis. If this strain had also been selected to the extent that it would have not produced, or would not cause to be produced, the non-specific serum antigens of acute

infection, it could be imagined that the IR-2 strain of B. rodhaini should have been nearly bland and would have produced neither immunity nor disease in infection. Such infections without disease have been observed in Babesia bigemina infections of cattle from Australia (Johnston, L. A. Y.; Personal communication with H. W. Cox). They were also descirbed for P. falciparum infections where the blood of the patient was teeming with parasites and the patient was free of all signs of illness (Marchiafava, 1931). It is possible that the present work has furnished insight into this unusual phenomenon.

SUMMARY

By means of *in vitro* treatment with immune globulins from rats recovered from *Babesia rodhaini* infections, three serologic types of *B. rodhaini* parasites were separated from the passage strain of parasites maintained at this laboratory. The first serotype was the one expressed in the immune globulin of rats recovered from the "original" (O) passage strain which differed from the second type which was expressed in the immune globulin mediated by the "immunity resistant-1" (IR-1) strain. The third serotype, the "immunity resistant-2" (IR-2) strain, was characterized by the apparent absence of strain-specific immunogenic antigens of the kind manifested by the O and IR-1 strains.

Serologic tests performed on mice infected with each strain and homologous and heterologous challenges of rats recovered from the O and IR-1 strains confirmed that a part of the acquired immunity mediated by *B. rodhaini* infections was the response of the host to non-specific serum antigen(s) which were elaborated in serum during the acute disease stage of haemosporidian infections. It was also demonstrated by the experiments that these antigens had not been changed qualitatively or quantitatively by the selective treatments.

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