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An Examination of the Red Blood Cell Alloantigentic System in the Chinese Hamster

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AN EXAMINATION OF THE RED BLOOD CELL ALLOANTIGENTIC SYSTEM IN THE CHINESE HAMSTER

by

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This research was done to determine whether any ABH-like blood group system was present in Chinese hamsters (Cricetulus griseus). The population of animals included 35 hamsters, 20 "normal" or outbred (10 males; 10 females) and 15 diabetic from The Upjohn Company's colony of inbred diabetic animals (8 males; 7 females; BE and BA lines). Animal sera and erythrocytes were compared by: hyperimmunization of rabbits (10 New Zealand White) with hamster erythrocytes; screening hamster erythrocytes and plasmas with human blood diagnostic typing antisera (Ortho Diagnostics), anti-H lectin, anti-A_1 lectin and human red cells of various types; absorption and elution of rabbit anti-hamster erythrocyte sera; gel diffusion of Ouchterlony; and electron microscopy. Results show that there are antigens present on hamster red blood cells which are specific for or at least cross-reactive with human A and B antigenic specificities, very possibly of the A_2 or A_2B subgroup. We conclude that Chinese hamsters show ABH-like specificities.

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This thesis is dedicated to my parents who helped me realize this educational achievement.

Kathryn Elliget Blashfield

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INTRODUCTION

Specificity is a characteristic of all serologic reactions which allows immunologic identification of hundreds of chemical configurations, so called antigens. Antigens may be considered as immunologic labels categorized according to the kind or type of specificity they confer. Some antigens are species specific. They have chemical configurations peculiar to a given species, such as the mouse. When such antigens are injected into a member of another species, as the rabbit, they elicit the production of specific antibodies that are capable of reacting with serum proteins or cells of all mice. It is possible to distinguish the tissue or body fluids of one species from another using these antigen-antibody reactions. The specificity of most interest to immunohematologists is that which confers individual specificity and allows one to distinguish individual members of the same species. These intraspecies differences are shown by the reaction of antibodies to antigens known as isoantigens or alloantigens.

The existence of alloantigens was first demonstrated in 1900 by Ehrlich and Morgenroth (1957) who cross-immunized goats with homologous red blood cells. Immunization was done by giving a single intraperitoneal injection of a large volume of water-laked blood. Laked blood was thought to be more stimulatory than uninjured erythrocytes of the same species. Whole homologous cells injected into animal's peritoneal cavities were assumed to be destroyed very slowly and thus absorption would be so gradual as to prevent the occurance of what they termed an "ictus

immunatoris." They found that injection of goats' blood into other goats resulted in the formation of isolysins but not autolysins, i.e., the serum of the injected animal never acquired the property of dissolving its own erythrocytes. Altogether they prepared thirteen isolytic sera and studied their characteristics by means of anti-isolytic serum. The results showed that they all differed from one another or that they represented different isolysins.

In the same year Landsteiner demonstrated differences among the red blood cells of individual human beings (Landsteiner, 1901). In his premiere observations he collected blood from himself and five of his colleagues and harvested the sera. Each serum was mixed with each of the donors' red cell suspensions and observed for a reaction. His results showed that some of the red cell suspensions were agglutinated by the sera of certain individuals while others were not. Landsteiner concluded that these reactions were attributable to the presence or absence of two agglutinable antigens, A and B, on the surface of red blood cells. Landsteiner's cells had neither of these antigens and therefore were classified as null or group O. He further concluded, since none of these experimental subjects had been previously immunized, that every individual must have natural antibodies in his serum directed against the antigen that is absent from his own cells. Thus, individuals belonging to group A would have anti-B serum antibodies, those belonging to blood group B would have anti-A serum antibodies and those belonging to blood group O would demonstrate both anti-A and anti-B serum antibodies.

The principals revealed by Landsteiner's early work, are that normal sera may agglutinate or hemolyze the erythrocytes of other in-

dividuals of the same species, and that on injection of red cells into an animal antibodies may be formed which by agglutination or hemolysis can differentiate the blood corpuscles of various individuals within a species. These observations were later extended to other animals.

In contradistinction to the seemingly simple scheme encountered with human blood, are the findings of Todd and White (1910). These authors studied the serum of cattle immunized against cattle plague using as antigen the blood of infected animals. Taking advantage of the isohemolytic properties of this sera they discovered a remarkable variety in cattle blood. These investigators fortuitously had at their disposal, at the Serum Institute, Cairo, about 100 animals used for the preparation of cattle plague serum. In the course of their immunization with cattle plague they had received large quantities of cattle blood under almost ideal conditions for the formation of isolysins. These cattle were immunized by a simultaneous protective inoculation of cattle plague immune serum and a small quantity of virulent cattle plague blood. After a short interval each animal was given an intramuscular injection of four liters of infectious virulent blood. These massive injections of four liters were repeated regularly every two months for as long as the animal was used for the production of serum. The cattle were bled for serum 14 days after the large injection of blood. On testing the fresh serum of the immunized cattle against the red blood cells of Cyprus and Egyptian cattle, practically no haemolytic action was observed. On addition of a little fresh guinea-pig serum, however, the immunized animal sera became powerfully haemolytic. The lack of haemolytic action of the fresh serum was obviously due to a requirement for foreign

complement activity. This requirement appears to have been overlooked by previous blood group researchers working with cattle (Frei, 1909).

Todd and White presented some very meaningful parameters which laid a firm foundation for those who were to follow in blood group research. They concluded that: antibodies are formed in the blood of an animal immunized against erythrocytes; the antibody formed is an isolysin and not an autolysin; the antibody is not activated by the complement present in the fresh serum of the homologous animal, but requires the addition of a foreign complement; immunized animal serum absorbed with an excess of red cells of another individual loses its power of haemolysing the red cells of this individual while retaining the ability to haemolyse the red cells of many others; and finally, that the red blood cells of any individual (excluding for the moment the question of heredity, which they had not yet considered) possesses characteristics which differentiate them from the red blood cells of any other individual even of the same species.

Using methods similar to those of Todd and White, von Dungern and Hirschfeld (1910) investigated the differences in red cells of dogs by means of iso-agglutinins. They came to the conclusion that dogs can be divided into several groups according to the agglutination of their corpuscles by antisera and concluded that the red blood corpuscles of all the dogs belonging to any one group have an identical chemical structure. Attempts were also made (von Dungern and Hirschfeld, 1910) using absorbed normal animal sera to uncover further differences in human blood cells in addition to the group distinction.

Again, by taking advantage of the isohaemolytic properties of

normal sera, a remarkable variety of blood cell reactions was found in chickens (Landsteiner and Miller, 1924). These experiments demonstrated that the blood cell antigenicity of almost every individual chicken was individualistic. This seemed to constitute a distinction between animal and human blood cell typing. Landsteiner, using pooled immune isoagglutinins from a number of immunized chickens, showed that sera absorbed with a single chicken's blood lost the ability to react with the cells used for absorption but reacted with the red cells of every chicken. Analogous results had been obtained by Todd and White with isolysins and cattle blood.

These reactions exhibited complete individual specificity and were not fundamentally different from those seen with human blood. They illustrated the existence of special properties possessed by single individuals. It was possible to demonstrate serologically defined factors in chickens, and cattle blood. In cattle about thirty factors were identified and their heredity control was later investigated (Ferguson et al., 1942).

By using various methods, numerous individual blood cell differences have been demonstrated in man. In particular, on immunizing rabbits with human red blood cells, it was possible to obtain, in addition to antibodies for the agglutinogens A and B, agglutinins for three other "factors," denoted as M, N and P (Landsteiner and Levine, 1928). These factors were found with equal frequency in all four major blood groups. The agglutinogens M and N provided a second instance of simple Mendelian inheritance in man. Landsteiner found their heredity to be determined by a pair of allelomorphic genes which give rise to three phenotypes M,

N and MN. He believed the genes to be located in a different pair of chromosomes from that governing the blood groups.

An additional property of human blood, Rh, was detected by means of immune sera prepared with the blood of rhesus monkeys (Landsteiner and Wiener, 1940). They showed that rabbit antiserum that had been raised by injecting rabbits with blood of the rhesus monkey agglutinated the red cells of 85% of Caucasians from New York. These individuals were termed Rh-positive and the remaining 15% were referred to as Rh-negative. The Rh factor was found to be inherited as a dominant Mendelian character. Wiener and Peters (1940) found the Rh factor to be clinically important as it could lead to the formation of isoantibodies upon blood transfusion or in mothers lacking the agglutinogen and bearing a child possessing it. Levine and colleagues (1941) found antibodies directed against erythrocytes to be the chief cause of erythroblastosis fetalis, a blood disease in new-born children.

By the early 40's it had been established that there were at least 72 different types of human blood cells, if one counts the two subgroups of A and AB (Wiener, 1943). This tabulation did not include the rare varities of A and N or subtypes of Rh.

Work comparing the isoagglutinins and relationships of group-specific structures in man and animals continued. After Landsteiner's discovery in 1900 of blood groups in man, investigations were conducted by Ottenberg and Friedmann (1911), Little (1929), Schermer and Otte (1953) and Tolle (1953) to detect blood groups in animals by using naturally occuring isoagglutinins as reagents. In these early years when the knowledge of the blood groups as well as specialized equipment were

relatively scarce, the observations made by these investigators have proved to be very interesting and must have required immense and laborious exertion.

By 1962 at least 10 genetic systems of blood groups were known in cattle (Srormont, 1962). Equine blood groups have been under investigation in several contries since the beginning of the nineteenth century. Podliachouk in 1957 isolated 13 specific agglutinins in equine sera corresponding to blood group factors A-M (Podliachouk, 1957). A few years later Stormont (1963) reported on the results of blood typing of horses with sixteen specifically different equine blood typing reagents. His work showed that six loci were involved in the genetic control of the sixteen blood group factors.

Danish researchers using isoimmunization of pigs found three red blood cell antigen factors, Ma, Mb and Mc (Nielsen, 1964). It was demonstrated that this M-system is comprised by four allele genes. Coupling studies showed that the M-system is not sex-linked hereditarily. Linnet-Jepson et al. (1958) showed independent segregation of the genes controlling haptoglobin, Gm groups and red cell blood groups in humans: ABO, MNS, Rh, P, Lewis, Lutheran and Duffy.

Domestic ducks were examined by Podliachouk (1964) and natural isoagglutinins demonstrated were rarely found. Using immunization and absorption techniques, he isolated 5 antibodies of different specificities. These antibodies determined 5 red cell antigens which he called, in order of their discovery, A, B, C, D and E.

The early investigators of red blood cell groups concentrated on either human beings or economically important domestic animals. Later,

research expanded to the use of other animals including rats, mice, monkeys and even fish.

Kuhns et al. (1969) discovered the presence of cross-reacting hemagglutinins in fresh water catfish and classified the fish into two distinct groups designated groups I and II. The agglutinogen in erythrocytes from group I individuals was human A-like in nature, based upon agglutination studies using lima bean lectin and human anti-A hemagglutinins. The hemagglutinins in group II sera were found to be highly cross-reactive with human erythrocytes, including A, and AB, which suggested a basis for interactions with group I red cells. The purpose of the studies of Kohns et al. was to determine blood group genetics and blood group linkage, particularly in embryonic development. Fresh water catfish were readily available for extensive embryologic studies and permitted the determination of blood group distribution in a more comprehensive fashion than possible in man. They found it intriguing that human zygotes were deficient in ABO blood group activity and speculated on additional study to determine the period in development at which the genetic information for ABO activity becomes expressed.

Hemagglutinins specific for the ABH blood group antigens have been found in the serum of the Australian fresh water catfish by Baldo and Boettcher (1970). Using ABH or Bombay erythrocytes, they demonstrated that in nine of twelve normal catfish sera samples, the greater part of the anti-human red cell activity was directed toward antigens of the ABH system and not to species antigens on the erythrocyte surface.

In 1925 (Landsteiner et al., 1925) marked differences were shown to exist between the blood cells of man and chimpanzee. Earlier investigators had shown by direct tests or with absorbed sera that differences between closely related species and individuals of the same species are often demonstrable by means of natural agglutinins. Some human sera had been shown to give strong agglutination of chimpanzee erythrocytes, irrespective of the blood groups. Landsteiner found the blood of anthropoid apes to contain isoagglutinogens and isoagglutinins indistinguishable from those present in human blood. He found that chimpanzees belong largely to group A, a few to O, and in orangutan and gibbons groups A, B or AB were recognized.

The work of Landsteiner and Miller (1924) was carried further by the investigations of Wiener and Moor-Jankowski (1943). They tested the saliva of monkeys demonstrating the presence of A and B blood group substances. One of their most important contributions to the ABO blood group research of apes and monkeys was the differentiation of A_1 and A_2 subgroups in chimpanzees. Schmitt in 1968 indicated that the rare dwarf chimpanzee, Pan paniscus, has blood that gives reactions serologically indistinguishable from human blood group A_1; while the common chimpanzee group A red cells were found to give reactions intermediate between those of human A_1 and A_2.

Of considerable interest is the observation of Schmitt on gelada monkeys. This species appeared to be unique in that the saliva of all 21 monkeys tested reacted as group O. However, this species was the only one in their study that did not adhere to Landsteiner's rule. The saliva in every case inhibited only anti-H, and not anti-A or

anti-B. The sera, however, did not contain both anti-A and anti-B, as required by Landsteiner's rule. Some monkeys had anti-A alone, others had only anti-B, and still others had neither. The investigators interpreted this observation as an example demonstrating the multiplicity of serological specificities characterizing the human agglutinogens A and B.

In human erythrocytes the A and B antigens occur as glycolipids (Kabat, 1955) regardless of the secretor status of the individual (Wiener et al., 1966). These antigens also occur as glycoproteins in erythrocyte membranes of secretors but not of non-secretors. Additionally, they are found in various body fluids of secretors. The distribution of these antigens was studied in two populations of vervet monkeys (Cercopithecus pygerythrus) by Downing et al. (1973). They tested for human-type ABO blood groups. Blood group antigens were found in saliva but not on the red cells. Anti-A and anti-B agglutinins in serum followed Landsteiner's rule. Individuals of groups A, B and AB, but not O, were found.

Downing et al. (1974) reported on the presence of blood group substances A, B, H, Le^a and Le^b in saliva and gastric juices of chalma baboons and in vervet monkeys. Moor-Jankowski and Socha (1978) made a comparative study of blood groups in rhesus, crab-eating, bonnet, pig-tailed and stump-tailed macaques and found significant similarities and differences among these species.

As information has accumulated on the inheritance of blood groups of nonhuman primates their value in genetic and taxonomic studies of red cell polymorphisms has been repeatedly demonstrated.

Differences in the distribution of blood group substances both human and simian types, among animal species have been shown to exist. ABO blood groups, or their homologues, can be found not only in man, anthropoid apes, Old and New World monkeys but in numerous other animals including rodents. The ubiquitous presence of the A-, B- and H-like substances is also reflected in the widespread occurence of "natural" antibodies and other substances, like lectins, with anti-A, anti-B or anti-H specificity. Thus, the presence of ABO blood group substances seem to cut across species lines.

Cohen (1962), in a summary of the blood groups observed in rabbits, reports only one system. Within this system 3 antigens denoted S, N and O of the system H_c could be determined by isoimmune sera. This H_c system is designated as the major blood group system of rabbits.

The first reports of erythrocyte antigenicity of the rat were carried out by Friedberger and Taslokwa (1928) using normal isohemagglutinins. They found four types of normal isohemagglutinins in wild rats. Burhoe (1947) later discovered antigen A by means of normal isoantibodies and an antigen M by immune isoantibodies. Both of these antigens were found to be inherited independently and form four blood groups: Am, A, M and O. Allelic antigens C-D identified by Owen (1948) were found to be codominant; the C-D locus was found in all tested rat strains. Owen also found antigens E and F to be controlled by solitary loci. It was possible to obtain antibodies in rabbits directed only against E by immunization with rat antigen E containing erythrocytes.

The A, B and H antigens are present not only in red cells of

most animal species but also in varying concentration in most of the other body tissues. The presence of these antigenic determinants in the secretions or tissues from a variety of species have been reported (Kabat, 1956). Hammarstrom et al. (1965) used a haemagglutination technique to find a large amount of A antigen in the colon, small intestine, and stomach of rats. Halpern et al. (1965) used a fluorescent antibody technique and noted blood group antigens in the colon of rats. In a study by Kent and Sanders (1965), the fluorescent antibody technique was used to demonstrate A antigenic determinants in tissue taken from several levels of the digestive tract of Sprague-Dawley rats. The water-soluble blood group A antigen was found only in mucin-containing cells or secretions which were identified with the periodic acid-Schiff's stain.

The occurance of some natural heteroagglutinins in mice has been described (Davidson and Stern, 1949; Brooke, 1965). Kirshbom and Hoecker (1963) reported the occurrence of agglutinins for human A erythrocytes in the sera of two different strains of mice. In strain C3H both sexes had the agglutinins; in strain C57BL/10 only the females had agglutinins and these could be absorbed by tissue of C57BL/10 males.

The sera of a large number of animal species agglutinate human erythrocytes including the mouse (Grubb, 1949; Kabat, 1956). Kirshbom (1965) presented data which characterize anti-A, anti-B and anti-O antibodies in mouse sera. He also described human agglutinins for mouse cells. Agglutinins for human erythrocyte types A, B and O were found in each mouse strain studied. Type A red cells were agglutina-

ted more often and more strongly than those of type B or O. Two types of anti-A agglutinins were found. One was sex-dependent and appeared only in female mice. The other appeared in both males and females.

Mouse heteroagglutinins have been shown to be 19S antibodies, like most human isoagglutinins, and also like human isoagglutinins they are not present at birth. They begin to appear in the first month and their titer gradually increases up to six months (Kirshbom and Hoecker, 1963). In a later study, Hoecker found mouse heteroantibodies against human group A red blood cells (Hoecker and Ramos, 1972).

Several species of animals have been found to possess a blood group system which demonstrates ABH characteristics. My purpose in doing this research was to investigate and define the blood group antigens of the Chinese hamster (Cricetulus griseus). Very little detailed work has been done with the blood group alloantigens of hamsters (Chinese, Jungerian, Syrian). Streilein (1979) has only recently been able to identify a single red blood alloantigenic system in the Syrian hamster. He states that the MHA strain contains an antigenic specificity expressed on its red cells which is not present with all of the other strains that he has worked with. He believes, however, that this antigen is also expressed on non-erythrocyte tissues as well. He and his colleagues have cross-immunized hamsters from different species (Chinese, Jungerian, Syrian) and produced hemagglutinating antibodies but present no evidence which suggests that these represent antigenic specificities uniquely expressed on red

blood cells. Streilein does not suggest that they are not unique to erythrocytes, he simply has not investigated the matter.

A recent report on 20 randomly selected Syrian hamsters (Pour, 1979) of the Eppley colony showed all of them to be of blood group O. All of these hamsters lacked anti-A and anti-B isoagglutinins in their plasma which makes them different from human type O individuals.

MATERIALS AND METHODS

Animals. Chinese hamsters (Cricetulus griseus) were acquired from two sources. Through the courtesy of The Upjohn Company, BE and AB line diabetic Chinese hamsters were obtained from their colony of genetic inbred animals. This colony was developed from breeding stock supplied by Dr. Yerganian from his colony maintained at the Children's Cancer Research Foundation, Boston, Massachusetts (Gerritsen and Dulin, 1966). Outbred non-diabetic Chinese hamsters were purchased from the Chick Line Company of Vineland, New Jersey. Fifteen diabetic and twenty non-diabetic or "normals" were used in this study. Both male and female animals were randomly selected.

Bleeding technique. For the collection of serum and blood cells, hamsters were repeatedly bled through the orbital sinus using a .32 mm heparinized capillary tube. Blood was collected into small test tubes containing .6 mg Na_2EDTA per .5 ml of blood. Plasma was separated by centrifugation at 3500 X g at 4 degrees Celsius for 1 minute, pipetted off and kept frozen at -20 degrees Celsius until used. The pelleted red cells were resuspended in .85% NaCl, ph 7.3 and washed 5 times in the same buffer by repeated pelleting at 3500 X g. The washed red cells were used for serological studies as a 2% suspension in pH 7.3 PBS (physiological buffered saline).

Hyperimmunization of rabbits with hamster erythrocytes. Ten New Zealand White male rabbits of approximately 1900 grams, obtained from a rabbit-breeding farm in Agusta, Michigan were used for the pre-

paration of antisera to individual hamster's erythrocytes. Packed, freshly drawn individual hamster red cells were washed three times with sterile PBS, pH 7.3 and mixed in a plastic .1 ml syringe with an equal volume of Freund's complete adjuvant containing 500 units of penicillin, 500 milligrams of streptomycin and 150 units of Fungizone. The solution was then homogenized. One tenth ml of the homogenate was injected into the drainage area of the right and left axial and inguinal lymph nodes of the recipient rabbit. As base line control prior to immunization, each animal was bled by cardiac puncture and 50 ml of heparinized blood collected. Following 3 weeks post-immunization, each animal was again bled, serologically tested for erythrocyte agglutinins and re-immunized. This booster dose was administered as previously described. One week following the booster dose the animals were bled and their serum titered against the homologous hamster red blood cells. The rabbit antisera were found to agglutinate the homologous hamster erythrocytes to a titer of 32 or greater. Each rabbit was then bled by cardiac puncture. Fifty ml of whole blood was allowed to clot and the serum was drawn away from the clot and frozen at -70 degrees Celsius.

Human erythrocytes and plasma. Human erythrocytes and plasma were obtained from several sources. Human blood types A, B, AB and O were secured from Mr. Chuck Lumbert, Chief of Blood Bank, Leila Hospital, Battle Creek, Michigan as outdated whole blood, packed red cells or plasma with permission from the Michigan Red Cross for use in research. Outdated human packed red blood cells of type AB were also obtained directly from the Michigan Red Cross Headquarters,

Lansing, Michigan, as mediated through Leila Hospital, with permission to use for research purposes. Whole blood samples of serological specificity A and O were donated on demand by various healthy individuals at Western Michigan University. These blood samples were drawn by Mr. David Duprey, Director of the Medical Technology Program, Mrs. Marjory Spradling, Professor and Medical Technologist, Karen Hart, Medical Technologist and various staff members at the Western Michigan University Student Health Center's Medical Laboratory. Whole blood samples were treated as previously described for hamster blood and plasma samples.

Blood diagnostic antisera. Blood grouping sera were obtained from both the Medical Technology Department, Western Michigan University, Kalamazoo, Michigan and the Medical Laboratory, Leila Hospital, Battle Creek, Michigan. Sera were of the following specificities: anti-A, anti-B, anti-A_1 lectin, anti-AB, anti-Rh (anti-D), anti-Le^a (anti-Lewis) (goat), and anti-Le_b (goat), all were Ortho products. Anti-H lectin (Ulex europaeus) was obtained from Miles Laboratories. Most antisera were used undiluted except for use in absorption studies where they were diluted to ease the absorption process.

Titration of agglutinins. The relative amount of antibody in any one test serum was determined by serial dilution. Red blood cells for titering were washed 3 times to remove all traces of plasma and red cell hemolysis. Red cells that required more than 5 washings to eliminate the evidence of hemolysis were unfit for use, and new samples were obtained. A 2% suspension of red cells was prepared in saline containing 20% albumin. A clean 1.0 ml pipette was used for

each transfer to avoid "carry over." Each of 12 tubes (10 X 75 mm) was labeled designating the serum dilution. To dilution tubes 1:2 through 1:2048 was added .1 ml of saline. To a blank tube and to the first tube containing saline was added .1 ml of the test serum. The contents of both tubes were thoroughly mixed using a Vortex mixer. Beginning with the first dilution (dilution 1:2) tube, .1 ml of the diluted serum was transferred to the following tube producing a dilution of 1:4. The transfers were continued through dilution 1:2048. The final .1 ml removed from the 1:2048 dilution was saved in case higher dilutions were needed. To each dilution tube, including the serum control, was added .1 ml of the 2% saline suspension of red cells. The mixture was well shaken and incubated at 37 degrees Celsius for 60 minutes. The tubes were then centrifuged (3500 rpm for 20 seconds in an Adam's Sero-Fuge) and observed for hemolysis or agglutination.

Direct blood grouping of hamster erythrocytes. Cross-agglutinations between the red cells and plasmas of each test animal were performed as a means of detecting isoagglutinins to blood group determinants. The cross-agglutination was performed under two test conditions: (1) in .85% saline and (2) a buffer of increased ionic strength (1.35% NaCl) containing 20% bovine serum albumin to reduce the zeta potential. Red cells were washed three times in saline and suspended to 2% in one of the two suspending media. Five-hundredths ml of test serum was added to a 10 X 75 mm test tube and .05 ml of the prepared 2% suspension of red cells. The mixture was shaken gently to mix and centrifuged (3500 rpm times 20 seconds with an

Adam's Sero-Fuge) to produce a well defined cell button. Following centrifugation the cell button was gently resuspended and observed for agglutination; macroscopically, by a 9X hand lens or under the low power objective of the microscope.

Detection of warm and cold agglutinins. Cross-agglutinations between the erythrocytes and sera of the test animals at various temperatures (25, 16 and 37 degrees Celsius) were performed as a means of identifying warm and cold agglutinins. Each cross consisted of three separate tests, one at each of the above temperatures. One-tenth ml of the test serum was put into each tube. Added to this was .05 ml of a 2% suspension of washed reagent cells. Pre-washing of the reagent cells is necessary. The contents were mixed thoroughly and centrifuged at 3500 rpm for 20 seconds (Adam's Sero-Fuge). After centrifugation the cell buttons were gently resuspended and observed for hemolysis and/or agglutination. The tubes were then incubated at their respective temperatures for 1 hour. They were again centrifuged and observed for hemolysis and/or agglutination both macroscopically and microscopically. Fifteen hamster sera were tested in this manner.

Anti-H lectin (Ulex europaeus). Anti-H lectin was used to define group O and the subgroups of A. Known human red cells of group O were used as control cells. A fresh 2% suspension of each hamster's red cells was prepared. To a 10 X 75 mm test tube was added .05 ml of anti-H lectin and .05 ml of the saline-suspended red cells. The contents were mixed thoroughly and centrifuged at 3500 rpm for 20 seconds using an Adam's Sero-Fuge to produce a well defined red cell button. After centrifugation the cell button was gently resuspended

and observed macroscopically for agglutination. If the reaction appeared to be negative macroscopically, a 9X hand magnifying glass was used or one drop of the suspension placed on a slide and observed microscopically at 10X for weak agglutination.

Anti-A_1 lectin (Dolichos biflorus). This lectin was used specifically for detection of A_1 cells. Five- hundredths ml of A_1 lectin was placed in a 10 X 75 mm test tube and to it added .05 ml of a 2% saline suspension of hamster erythrocytes. These were mixed thoroughly and centrifuged at 3500 rpm for 20 seconds in an Adam's Sero-Fuge to produce a well defined red cell button. After centrifugation the red cell button was gently resuspended and observed for agglutination. If macroscopic examination was negative, a 9X magnifying glass was used or microscopic examination at 10X. All hamster red cells were tested for A_1 antigen.

Absorption studies. Absorption studies were done to remove anti-A and/or anti-B (or other minor antibodies) from sera containing antibody concentrations suitable for reagent use. The ability of cells to remove an antigen from serum or eluates is indicative of the presence of that antigen on the cell. In most cases a single absorption was sufficient to remove the isohemagglutinins. In a few cases absorption had to be repeated once or twice. The completeness of the absorptions was always monitored. Absorption was carried out in the cold at 4 degrees Celsius and at 37 degrees Celsius. Two temperatures were used because the serologic behavior of antibodies at different temperatures can be used as an indicator of the specificity of mixed agglutinins. Naturally occurring IgM antibodies react best with sa-

line-suspended red cells at 16 degrees Celsius and room temperature. Immune IgM antibodies are best demonstrated at 37 degrees Celsius. The IgG agglutinins react best with albumin and antiglobulin tests or with enzyme-modified red cells. For absorption of high-titered antibodies, diluted serum was used. For weak antibodies the volume of undiluted serum was greater than the volume of red blood cells. The usual volume was one part undiluted serum to one volume washed, hard-packed cells. Selected red blood cells for absorption were washed at least three times with isotonic saline. The supernatant saline after the last washing was completely removed to avoid dilution of the test serum. Because absorption is directly related to the degree of serum-cell contact, a stoppered, large-bore tube was used and placed on its side. Packed red cells and serum were thoroughly mixed and incubated at either 4 or 37 degrees Celsius for 30 to 60 minutes. To insure maximum exposure of cell antigens to antibodies, the tubes were frequently agitated. Centrifugation was carried out in the cold (3500 rpm for 45 seconds) to prevent elution of antibody from red blood cells. Serum was removed immediately after centrifugation. The absorbed serum was tested for complete antibody removal against a freshly prepared suspension of the red blood cells used previously for absorption. If the absorbed serum still showed the presence of agglutinins, the procedure was repeated with fresh aliquots of washed, packed cells until no microscopic evidence of agglutination was discernable. If an eluate was to be made, the original pelleted cells were saved with their absorbed antibody. The following specific absorptions were carried out: anti-AB blood

grouping sera (Ortho Diagnostics Inc., Raritan, New Jersey) at a 1:10 dilution and a 1:20 dilution was absorbed with known human type AB red cells and reacted with the erythrocytes of each individual hamster to check for agglutination or hemolysis; a 1:8 dilution of anti-A blood grouping sera (Ortho Diagnostics) was absorbed with known human red cells of type A and reacted with each hamster's red cells; a 1:8 dilution of anti-B blood grouping sera (Ortho Diagnostics) was absorbed with known human red cells of type B and tested against the erythrocytes of each individual hamster for hemolysis or agglutination; plasma from a human blood group O individual (containing anti-A and anti-B agglutinins) was absorbed with human erythrocytes of types A, B, and AB and then reacted with the red blood cells of each hamster under study; the rabbit anti-hamster sera were absorbed with A, B, and AB human red cells and the absorbed sera tested with each hamster's erythrocytes; rabbit anti-hamster red cell-sera were absorbed with human red cells of type AB and the absorbed sera then tested with known human erythrocytes of types A, B, AB, and O; rabbit anti-hamster red cell-sera were absorbed with human red cells of types A, B, AB, and O and the absorbed sera reacted against human erythrocytes of the same four serologic specificities; pooled rabbit antiserum was absorbed with the erythrocytes of one hamster and the absorbed serum reacted with the erythrocytes of every hamster. In each of the above absorption studies heat inactivation was used (56 degrees Celsius for one-half hour) to destroy complement activity of the rabbit sera. Direct typings were carried out in a medium containing 30% bovine serum albumin.

Elution studies. The method of Rubin (Rose and Friedman, 1976) for elution of antibodies from the surface of antigen was used with slight modification. Cells were washed four times in large volumes of saline. The last wash was kept and tested with the eluate to insure that the red cells were washed free of serum and not contaminated with diluted serum antibody. Twice the volume of ether and half the volume of saline were added to washed packed red cells and mixed thoroughly for 1 minute. This mixture was incubated at 37 degrees Celsius for 30 minutes. After incubation, the mixture was centrifuged at 1000 rpm for 10 minutes. The bottom layer was separated off and placed in a labeled, unstoppered test tube and incubated at 37 degrees Celsius for 30 minutes to allow the excess ether to evaporate. If after one-half hour the ether was not evaporated, the solution was bubbled with compressed air to remove the remaining ether. This separated fraction contained the eluted antibody and was tested with reagent cells. Specific elutions carried out include the following: rabbit antisera absorbed with human AB erythrocytes were reacted with their homologous hamster red cells, the eluate from this complex was tested against the red cells of each hamster to see if the agglutinins remain; rabbit antisera were reacted with human AB red cells, the antibody from this reaction was eluted off and tested with all of the hamster's erythrocytes. All rabbit sera used in these studies were heat inactivated at 56 degrees Celsius for one-half hour. The agglutinations were carried out in the presence of 30% bovine serum albumin.

Mercaptoethanol cleavage of immunoglobulins. This technique

was used to identify the rabbit immunoglobulin class produced against hamster red cells. A stock solution of 1 molar 2-mercaptoethanol was prepared by adding 92.2 ml pH 7.4 phosphate-buffered saline to 7.8 ml of 2-mercaptoethanol (2-ME). A working solution (.2 M 2-mercaptoethanol) was made by adding one part 1 M 2-ME to nine parts pH 7.4 phosphate-buffered saline. First, equal volumes of the serum under examination and .2 M 2-ME were mixed. Equal volumes of the serum under test and pH 7.4 phosphate-buffered saline were mixed for serum control. The mixtures were incubated at 37 degrees Celsius for 15 minutes. The 2-ME serum and the control serum were placed in separate dialysis sacs and dialyzed against cool running tap water for 45 minutes. They were then dialyzed against two changes of 500 ml buffered saline (PBS, pH 7.4) for 1 hour each. The dialyzed sera were then put into labeled tubes and tested for serological activity. Three rabbit antisera were treated in this manner and then tested with their homologous hamster red blood cells. As an additional control, anti-A blood grouping serum (undiluted) was treated with 2-ME and then reacted with fresh, washed human red blood cells of type A.

Double diffusion (gel diffusion of Ouchterlony). This technique was used to detect the presence and number of antibodies in test sera. Gels were prepared by dissolving 4 g of Nobels agar in 100 ml of .15 M phosphate buffer pH 7.1 (Solution I: Na_2HPO_4 - $2H_2O$ 26.7 g, distilled water to 1 liter. Solution II: KH_2PO_4 20.41 g, distilled water to 1 liter. Mix in proportion solution I 67 ml; solution II 33 ml) and 300 ml distilled water. Ten milligrams of sodium azide was added as a preservative. The mixture was heated to 100 degrees Celsius to

dissolve the agar and allowed to cool to 45 degrees Celsius. Agar was then dispensed in 2 ml aliquots into a level 30 mm plastic petri plate and allowed to gel. Two 3 mm diameter wells were cut into the agar 10 mm apart (measured from the center of each well). The wells were then filled with either 10 ul of antiserum or antigen. The gel was covered in a moist chamber and allowed to stand at room temperature for 2-3 days. Immune precipitates will be visible as lines in the gel located between the antigen-antibody wells. The number of bands represent the least number of antigen-antibody complexes present. This procedure was done to test each hamster serum against its homologous rabbit anti-hamster erythrocyte serum.

Protein A. Protein A was obtained as a lyophilized preparation from Pharmacia Fine Chemicals. In order to microscopically monitor the Fc-Protein A reaction, the Protein A in these studies was labeled with fluorescein isothiocyanate. The conjugate contained approximately 6 fluorescein isothiocyanate (FITC) groups per molecule of Protein A. Fractions of erythrocytes were reacted with specific reagents including antibody mixtures. Following a 40 minute reaction at 37 degrees Celsius, the red blood cells were washed 3 times with phosphate buffer. The washed red blood cells were then suspended in a predetermined dilution of FITC-Protein A and reacted at 37 degrees Celsius for 30 minutes. The red blood cells were again washed 3 times with phosphate buffer, mounted under a coverslip in the same buffer and observed at an excitation illumination of 490 nm in a Nikon UV light microscope.

Electron microscopy. Cationized ferritin was obtained from

Miles Laboratories Inc., Elkhart, Indiana. The material was prepared according to the procedure of D. Danon et al. (1972). In order to visualize the negatively charged cell membranes of the erythrocyte cell surface for electron microscopy, the following procedure was carried out. The commercial cationized ferritin solution was diluted .1 ml to .5 ml with Veronal HCl buffered saline pH 7.2. This working solution was used to resuspend a pellet of red blood cells (after they had been fixed for 1 hour at 4 degrees Celsius with 1.5% paraformaldehyde and .5% glutaraldehyde the cells were washed 2X with PBS then exposed to ferritin). The suspension was gently shaken every 10 minutes for 30 minutes at ambient temperature. The red blood cells were then washed 3 times in PBS further fixed in 3% glutaraldehyde at 4 degrees Celsius for 1 hour, washed 2X in phosphate buffer and post-fixed for 1 hour at ambient temperature in 1% OsO_4 at room temperature. The cells were then washed 2X in phosphate buffer, dehydrated in alcohol and embedded in Polyscience 812 substitute embedding material. Sections were prepared with glass knives on an LKB III Ultramicrotome. Silver to gray sections were collected on 200 mesh copper grids, stained with uranyl acetate and lead tartrate. Observations were made at 80 kV with a Siemens IA electron microscope.

Additional observations on cationized ferritin stained and unstained control cells were made by a pseudoreplication technique according to the following procedure. Erythrocytes, after treatment as aforementioned or without treatment, were fixed in 3% glutaraldehyde in phosphate buffer for 60 minutes at ambient temperature. During fixation the cells were forced on to a coverslip by a force

of 500 X g. Following glutaraldehyde fixation the coverslip-cells were washed in phosphate buffer 2X then post-fixed in a stationary position in 1% OsO_4 in phosphate buffer for 1 hour at ambient temperature. Following post-fixation the cells were washed 2X in phosphate buffer, dehydrated in alcohols to 100% ethanol then critical point dried with liquid CO_2. The dehydrated cells were shadowcasted with platinum carbon, rotary coated with carbon then digested with 25% KOH at 60 degrees Celsius for 24 to 48 hours. Released sheets of replica were picked up on a piece of fine copper wire, transferred to .1 N HCl, and three baths of distilled water. The replicas were then mounted on 75 X 300 mesh nickle grids and viewed by means of the electron microscope.

RESULTS AND DISCUSSION

Titration of agglutinins. Determining the relative titer of antibody in a serum is done by testing serial dilutions of the test serum against the respective antigen. The titer of an antibody is expressed as the reciprocal of the highest dilution in which a 1+ agglutination reaction is recorded. When reading these results one must consider that tubes showing less than a 1+ reaction, i.e., ± reactions, are not significant. Table 1 indicates that all of the rabbit anti-hamster erythrocyte sera agglutinates the homologous hamster red blood cells to a titer of 32, and agglutinates human red cells of types A, B, O, and AB to a titer of 2, 1, 0 and 1 respectively.

Table 2 shows titration results after the rabbit antisera had been absorbed with the erythrocytes of one hamster. Results show that absorption of the rabbit serum with erythrocytes of one hamster causes a reduction in agglutination of other hamster's red cells from a titer of 32 to 4 and of human A, B, O and AB red cells to a titer of 0.

Zero titers for the reactions with human erythrocytes mean that the antigens on the hamster red cell have removed from the serum all agglutinins that might be specific for or cross-reactive with human blood group antigens.

One might expect an agglutination titer against donor cells to reach a level of 256 with a good antigen. It is possible that: (1) the concentration of erythrocytes used for immunization was too low,

Table 1

Titration of Rabbit Anti-hamster Erythrocyte Sera

Against Erythrocytes of Various Specificities

Test Cells	Number of Sera Reacted	Reciprocal of the Highest Dilution Showing Agglutination
Homologous Hamster Cells	10	32
Human Group A	10	2
Human Group B	10	Undiluted
Human Group AB	10	0
Human Group O	10	Undiluted

Table 2

Titration of Rabbit Anti-hamster Erythrocyte Sera Absorbed With A Single Hamster's Erythrocytes and Tested Against Erythrocytes of Various Specificities

Test Cells	Number of Sera Reacted	Reciprocal of the Highest Dilution Showing Agglutination
Hamster Red Cells[a]	10	4
Human Group A	10	0
Human Group B	10	0
Human Group AB	10	0
Human Group O	10	0

[a]The absorbed sera were tested against the erythrocytes of each donor hamster.

(2) the concentration of antigens per erythrocyte was too few, (3) the antigens on the erythrocytes were not accessable, (4) the rabbit failed to recognize and react against the erythrocyte antigens or (5) our course of immunization was a bit too little. If we assume that 32 is the titer against surface determinants of the hamster red blood cells, then essentially no antibody concentration developed against major human blood group substances. There is the possibility that type A substance or a type of A substance may be a minor component of the antigenic material of the hamster red blood cell.

Once this rabbit antisera has been absorbed by homologous hamster red blood cells, the expected reduction in the homologous titer as well as a concomitant obliteration of the reactions to the human red blood cells of types A, AB, and B are observed. This is indicative of the presence of a minor antigenic component present in both human A, AB and B cells and this hamster's red blood cells. This component may be related to the A, AB and B blood group substance or be a separate entity that is represented on these cells. It is probably not a structural protein component of the red blood cell as the type O cells failed to elicit a reaction.

Since the antibodies in the immunized rabbits were raised against whole hamster red blood cells, one might expect potential antibodies against surface glycoproteins, structural proteins and possibly absorbed serum proteins.

Direct blood grouping of hamster erythrocytes. Cross-agglutinations between the red cells and plasmas of all hamsters studied indicate that there are no isoantibodies detectable in the sera of Chinese

hamsters when the suspending medium is normal saline (.85% NaCl). Even the two lines of highly inbred diabetic hamsters failed to show agglutination with outbred animals. Based solely on this test, no conclusions should be drawn as to the absence or presence of isoantibodies to hamster erythrocyte antigens. For agglutination to occur more than a simple red cell suspension in normal saline may be required. For instance, both anti-A IgG and anti-B IgG antibodies of blood group O humans agglutinate in simple saline-suspended group A and group B cells. In contrast, the anti-Rh (anti-D) antibodies require albumin, enzyme modified red cells, or antiglobulin molecules to complete the agglutination reaction. In general, the agglutination reaction is greatly influenced by several factors including the number and distribution of the antigenic determinants on the cellular membranes and the physical structure of the antibodies. The human red blood cell is a biconcave disk, 7.5 micrometers in diameter, with a porous cellular membrane that is structured into alternate ridges and troughs where the various blood group antigenic determinants are distributed. The group A and group B sites are located on the crests of the ridges. Below these primary blood group antigens are the Rh (D) antigen sites. On the floors of the troughs are the Duffy antigenic reactive sites. In fact, even lowering the zeta potential does not influence the agglutination reaction of the Duffy antibodies since only the indirect antiglobulin test will demonstrate the presence of the Duffy antigen-antibody complex. The Duffy antigenic sites are located deep in the depressions and the distances are increased beyond the spans of the short IgG or IgA antibody mole-

cules. Since we do not know the location or characteristics of erythrocyte antigenic receptor sites, or the classes of corresponding immunoglobulin molecules in the Chinese hamster, then we must, of necessity, manipulate test conditions in order to accomodate the specificities of Chinese hamster serology. Table 3 shows results obtained after reduction of the zeta potential to enhance agglutination. Red cells carry a negative charge and repel each other so aggregation is prevented. This negative charge results from the carboxyl groups of the N-acetylneuraminic acid or sialic acid found in the glycoproteins of the red cells. When red cells are suspended in plasma or normal saline the cations contained in the suspending medium are attracted to the cell membrane which causes a dense double layer of ions about the red cell surface. The outer edge of the double layer is called the surface of shear or slipping plane. Because the negative charges at the surface of shear exceed the positive charges, an electrical potential exists between the red cells called the zeta potential. Under normal conditions the distance between suspended red cells is 1000 A. Measurements of the various classes of immunoglobulins show the IgG and IgA antibodies to be 240 A to 250 A in length. The IgM antibodies are from 950 A to 1000 A in length. Evidently, the IgM antibody has the size to span the normal distance between red cells while the other two cannot. For the agglutination involving the IgG or IgA antibody to go to completion, this distance must be reduced by reducing the zeta potential. The electrostatic forces should be reduced to optimum levels so that the shorter IgG and IgA molecules can span the erythro-

Table 3

Cross-agglutinations, in the Presence of 30% Bovine Serum Albumin, between 15 Hamsters

Normal-hamster Erythrocytes	Normal-hamster Plasma										Diabetic-hamster Plasma				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	H	0	0	H	0	0	H	H	0	0	H	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	H	H	0	0	H	0	0	0	0	0	H	H	0	0
7	H	0	0	H	H	H	0	0	0	H	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	H
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 3 Continued

Diabetic-hamster Erythrocytes	Normal-hamster Plasma										Diabetic-hamster Plasma				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
11	0	H	H	H	0	0	H	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Note. "H" indicates hemolysis.

cyte to erythrocyte gap. Reduction of the zeta potential can be accomplished by (1) lessening the charge on the red cells, (2) raising the dielectric constant with colloid media or (3) increasing the ionic strength of the electrolytes. Care must be taken not to induce spontaneous agglutination of non-sensitized cells by reducing the distance below 150 A. Results in Table 3 show that by reducing the zeta potential we have revealed the presence of some isoantibodies to red cell determinants, probably of the IgG or IgA class. No agglutination was observed either macroscopically or microscopically. However, hemolysis was the consistent response, indicating complement activity. These data indicate that there are blood group differences between the normal and diabetic Chinese hamsters regarding isoantibodies to red cell determinants.

A screening procedure was done to examine the thermal amplitude of the antibody-red cell interaction. Warm-reacting antibodies are maximally reactive at normal physiologic temperatures of 37 degrees Celsius. Cold-reacting antibodies bind with their target cells at temperatures below 31 degrees Celsius and sometimes as low as 4 degrees Celsius. These antibodies dissociate from the erythrocyte when the temperature is raised. Cold reacting antibodies are directed against polysaccharide antigens and are usually IgM, whereas protein antigens are usually reactive at warm temperatures and are of the IgG type. A range of temperatures from 4 to 37 degrees Celsius was used to reveal or identify these potential antibodies. All cross-agglutinations were carried out in the presence of bovine serum albumin. Table 4 shows that there was no agglutination or hemolysis

Table 4

Detection of Warm and Cold Agglutinins in the Sera of 15 Experimental Animals

Animal Sera of	Temperatures of Reactivity in Degrees Celsius			
	4	16	25	37
10 Normal Hamsters	0	0	9[a]	0
5 Diabetic Hamsters	0	0	4[b]	0

Note. Each cross-agglutination i.e., every combination between the erythrocytes and plasma of each hamster, was performed four times--once at each temperature.

[a]Nine out of 10 hamsters reacted at that temperature.

[b]Four out of 5 hamsters reacted at that temperature.

at temperatures other than 25 degrees Celsius (room temperature). This information indicates a possible protein antigen reacting with an IgG type antibody. Hemolysis as opposed to agglutination was the observed reaction. Agglutination is the most conspicuous serologic effect of erythrocyte-antibody interactions, however, hemolysis does occur under certain in vitro conditions. In human blood group testing, hemolysis is not considered a very dependable reaction because it is not observed with regularity and because of its dependence upon complement. ABH antibodies will sometimes coat cells without causing agglutination and they are usually of the IgG type. Although hemolysis is not a preferred effect, it should be considered as the result of a specific antigen-antibody reaction in these studies.

There are several factors to consider when evaluating the absent or very weak reactions observed between the erythrocytes and sera of Chinese hamsters. There are two distinct phases of the reaction when a suspension of antigens is mixed with specific agglutinins. The first phase is the immediate attachment of antibodies to the antigenic determinant. The second phase involves the clumping or agglutination where the sensitized particles are aggregated into visible clumps. The second phase could require from several minutes to several hours. Perhaps not enough time was allowed for the second phase of the agglutination phenomenon. If isoantibodies to blood group substances are present, they could be attaching to the red cell antigenic determinant and a too-short incubation time could be inhibiting a visible clumping or agglutination. It is possible that a several-hour incubation time is required for a firm

agglutination to occur.

Another consideration is that when dealing with antigenic cross-reactivity it may be better to look for cross-reactivity at 0 degrees Celsius (Trentin, 1967). Because the reactive portion is often a single sugar residue reacting with only a portion of the determinant of the cross-reacting antibody, thermal agitation is disturbing and many cross-reactions are seriously diminished at room temperature or at 37 degrees Celsius. Trentin suggests that it may be advisable to wait 6 or 7 days before taking readings, as small reactions may become visible very slowly.

Additional factors to consider which affect the agglutination reaction are electrostatic forces, ionic activity of the electrolyte, pH, agitation, gravitational force (rate of centrifugation) and sedimentation. For example, gentle agitation during incubation may enhance some agglutination reactions in saline while inhibiting the reaction that requires the addition of antiglobulin serum. Centrifugation must be optimum as it could lead to false positive results. Few reactive sites may also be a factor in failure of agglutination. In some instances it is possible for agglutination to be blocked because IgG antibodies may loop and attach both combining sites to the same red blood cell, if the antigenic reactive sites are poorly spaced. If by chance the agglutinating antibodies are of the IgA class (monomer), they would require specific anti-IgA antiglobulin to affect agglutination. If the antibody concentration and number of reactive sites are low, the delicate agglutinates of IgG and IgA could be easily broken or shaken apart. IgM antibody-red cell combinations

are more firm and would not break as easily.

The formation of isoantibodies has been theorized to be dependent upon the gut fluora of the animal. This theory explores the idea that early in evolution there may have been bacteria present in the gut which expressed antigenic specificities A and B on their cell wall. The presence of these specific substances would then have caused the formation of circulating isoantibodies specific to them. If this theory were to hold for the hamster, then it would appear that there existed no such bacteria in the ontogeny of the Chinese hamster thus explaining the absence of isoagglutinins to blood group specificities.

The prevalent theory of the early years of blood group research was that "natural" occuring blood group antibodies were hereditary (Kabat, 1956). Also, several researchers have proposed that the human anti-blood group A and B isoagglutinins result from exogenous stimuli and represent cross-reacting antibodies (Trentin, 1970) and that this is the predominant if not the only cause of the "naturally" occuring isoagglutinins anti-A and anti-B in humans.

Trentin also discusses an experiment which upholds the relationship of microbes to blood group substances. Chickens of many races form human anti-B blood group antibodies; these antibodies had been regarded as gene products. He set out to test this assumption by experimenting with germ-free chickens as well as chickens reared under ordinary conditions from the same hatch. It was found that germ-free chickens possessed no anti-blood group B-specific antibodies 45 days after hatching. Chickens raised under normal conditions

showed about the same titer as normal humans. The feeding of live blood group B-active Escherichia coli produced an extremely high titer in the germ-free chickens. The feeding of bacteria having no blood group activity did not lead to the formation of blood group antibodies.

One should also consider the fact that this species of hamster was brought to this country in the form of only a few breeding pairs. If blood groups are rare in this species, then a selection of a single type could have been possible. Additionally, since these are colony animals, all breeding pairs may have been selected from an inbred colony even though they were taken wild. Further, inbreeding may have selected against all of the different blood group antigenic types if there were indeed any to begin with. The only solution for this problem is to go to the "wild" and randomly pick from the hamster society for serological blood group typing.

A_1 lectin. All animals were negative for the A_1 antigen. It is possible to distinguish group A_1 cells from group A_2 cells by means of the specific phytohemagglutinin, Dolichos biflorus. Lectins, although not antibodies, have a chemical configuration complementary to polysaccharide blood group antigens and can cause specific clumping of erythrocytes. Renkonen (1948) was first to use them as blood group reagents. He believed that these substances in plants function metabolically as carbohydrate carriers. An A_1 lectin of this type is prepared from the seeds of Dolichos biflorus and is commercially available.

A_1 cells have three times as much A determinant as A_2 cells while

the reverse is true for the amount of H substance. Recently Mohn (1977) described experiments in which sonicated erythrocyte stromata from A_1 and A_2 individuals were tested with human immune anti-A serum by double diffusion in agar gels. He found a precipitin band common to both the A_1 and A_2 preparations which corresponds to the A_{common} subgroup. In addition, he found a second precipitin band that was evident only with the A_1 stroma, an indication of the A_1 antigen. These studies show that there are definite qualitative as well as quantitative differences between the A_1 and A_2 subgroups.

Negative results obtained with the hamster erythrocytes indicate that either A_2 or A_2B cells may be present because they both would disperse into a smoothe cell suspension when reacted with the A_1 lectin. A_1 or A_1B cells on the other hand would present a solid agglutinate. Indeed, each cell button (each hamster) dispersed into a smoothe cell suspension and showed no evidence of even weak agglutination upon microscopic examination. The possibility cannot be overlooked, however, that the A_1 antigen, if present on Chinese hamster erythrocytes, has not reached its full characteristics. In humans, the distinction between A_1 and A_2 cells can only be made with the cells of adults because the A_1 antigen does not attain its full *in vitro* serologic characteristics until after the first year of life. Hamsters may follow this pattern of development or retain their immature characteristics permanently.

Anti-H lectin. The anti-H lectin was used in this study to define the subgroups of A or to define group O. The *Ulex europaeus* extract reacts with H substance; it agglutinates cells that have H

antigenic activity in a manner proportional to the amount of H substance present. The H gene activity is fundamental to the expression of human ABO genes. If a person is homozygous hh, the ABO genotype makes no difference, or, the phenotype will be red blood cells that fail to react with anti-A, anti-B, or anti-H. However, a negative reaction with the anti-H lectin does not indicate that the individual is the amorph, hh, or expresses no H activity. There are several interpretations to results obtained from use of this lectin. One must critically read the agglutination results microscopically. Group O and A_2 red cells will present solid agglutinates. Group A_{1-2} are moderately agglutinated with anti-H lectins while group B red cells have reactions that vary from strong positive to negative. All hamster's erythrocytes showed negative results when tested with the anti-H lectin. Negative reactions can mean one of three possibilities: (1) A_1 or A_1B antigens are present which disperse easily into smoothe cell suspensions; (2) O_h or Bombay cells are present which are not agglutinated with the anti-H lectin; or (3) group A_2B cells are present which may not react with the anti-H lectin. These results correspond to the negative A_1 lectin results in that they indicate the possibility of an A_2 subgroup of the A antigen.

Before reporting the results of various absorption and elution studies, we will present data from some preliminary tests that were performed to screen Chinese hamster red cells and plasmas for human-like antigenic determinants and/or agglutinins.

The erythrocytes from a group of 20 hamsters, 10 normal and 10 diabetic (BE line) were tested against 6 commercially prepared human

antisera (Ortho Diagnostics) specific for human blood group substances A, B, AB, Rh, Le^a and Le^b. Table 5 shows the agglutination reactions from these crosses. The results indicate the presence of antigenic determinants on the hamster erythrocytes reactive to human anti-A and anti-B antibodies. There were no reactions to human Rh substance. The results of the reactions to the Lewis substances present a contradiction based upon human blood group reactivity, and necessitates further discussion. The Lewis system in humans is primarily a system free in the body fluids. The Le^a and Le^b antigens that comprise the system are common components of saliva and plasma. These antigens are passively absorbed by the red cells as glycosphingolipids from the plasma but not from the saliva. Thus, these antigens are different from most other red cell antigens. The three phenotypes which are produced from this system, Le^{a+b-}, Le^{a-b+} and Le^{a-b-}, are inherited from a single pair of allelic genes, Le and le. Although genetically independent of the Hh, Sese (Secretor system), and ABH genes, an interrelationship exists. The Sese genes eventually determine which Lewis antigen will be demonstrated in the saliva and on the red cells when the individual is a Lewis secretor. Generally, Lewis secretors of the Le^a substance are non-secretors (sese) of the ABH substances and secretors (SeSe, Sese) of the ABH substances secrete Le^b substance in the saliva and plasma. The results of the hamster erythrocyte-Lewis system do not definitively show that they are or are not secretors of ABH substance. Because there are more high-affinity reactions in the hamster anti-Le^a column than in the anti-Le^b column, there is a greater probability of

Table 5

Hamster Erythrocytes Reacted Against Human Blood Typing Sera

Red Cells of	Anti-A	Anti-B	Anti-AB	Anti-Rh	Anti-Le[a]	Anti-Le[b]
Normal Hamsters						
1	3+	2+	4+	0	2+	1+
2	3+	2+	4+	0	2+	1+
3	3+	2+	4+	0	1+	1+
4	3+	2+	4+	0	1+	2+
5	3+	2+	4+	0	3+	2+
6	3+	2+	4+	0	2+	2+
7	3+	2+	4+	0	2+	2+
8	3+	2+	4+	0	2+	2+
9	3+	2+	4+	0	2+	2+
10	3+	2+	4+	0	2+	2+

Table 5 Continued

Red Cells of	Anti-A	Anti-B	Anti-AB	Anti-Rh	Anti-Le^a	Anti-Le^b
Diabetic Hamsters						
71	3+	2+	4+	0	2+	1+
52	3+	2+	4+	0	2+	1+
72	3+	2+	4+	0	1+	1+
73	3+	2+	4+	0	0	2+
76	3+	2+	4+	0	2+	1+
13	3+	2+	4+	0	2+	2+
14	3+	2+	4+	0	2+	2+
15	3+	2+	4+	0	2+	2+
19	3+	2+	4+	0	3+	2+
24	3+	2+	4+	0	2+	1+

Note. 4+ = solid agglutinate; 3+ = a clump that will break easily; 2+ = agglutination barely visible macroscopically; 1+ = microscopic examination necessary to see aggregates; 0 = no agglutination

their being non-secretors of the ABH substances. Occasionally small amounts of the ABH substance can be found in the saliva of the non-secretor (sese) and small amounts of the Le^a substance is found in the saliva or plasma of the ABH secretors (Se). In group A and B humans there is a lesser amount of Le^b substance in the saliva and on the red cells. This general trend can also be seen in our hamster test results. One plausable explanation for the presence of an Le^{a+b+} phenotype in these hamsters' red blood cells is the fact that red blood cells from newborn human infants give unreliable reactions with anti-Le^a and are usually negative with anti-Le^b. However, in subjects who are destined to be Le^{a-b+}, the red blood cells go through an intermediate Le^{a+b+} stage during the first few years of life. All of the hamsters tested were in their first or second year of life. It is possible that the hamsters may be destined to be Le^b and therefore secretors of the ABH substances. Alternatively, the hamster may not follow the human example for Le^a-Le^b and continue to show both Le^a and Le^b on their red blood cells. The serum from each hamster was tested for the presence of agglutinins anti-A and anti-B using various human red cell types. The results (see Table 6) show that two hamsters have low concentrations of antibodies to H substance and eight show either anti-A or anti-B activity in their serum. The agglutination reactions were all weak indicating little specific antibody or that they were all low affinity, cross-reactive antibodies. Also, the sera of the hamsters could contain low affinity antibodies to protein antigens other than blood group antigens. Since there were positive reactions to A, B, or AB human red cells,

Table 6

Hamster Sera Tested Against Human Erythrocytes for Detection of Anti-A or Anti-B Agglutinins

Serum from Hamster No.	Human Erythrocytes of Types			
	O	A	B	AB
1	0	0	0	0
2	0	0	1+	±
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
8	0	0	1+	0
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0
12	0	0	2+	±
13	1+	2+	2+	2+
14	0	0	0	0
15	0	0	0	0
16	0	0	0	0
17	0	1+	0	0
18	0	0	0	0
19	0	1+	0	0
20	1+	2+	1+	1+

Table 6 Continued

Serum from Hamster No.	Human Erythrocytes of Types			
	O	A	B	AB
21	0	1+	0	0
22	0	0	0	0

Note. Hamster numbers 18 through 22 are diabetic (from the Be line).

the indication is that there are antibodies present in hamster sera which are directed against human ABH blood group substances.

Immune sera prepared in rabbits against Chinese hamster red blood cells were tested for the presence of antibodies to the donor red blood cells, other hamster red blood cells and to different human erythrocyte types. Table 7 shows results of crosses between the post-immunization antisera and erythrocytes of different types. Each rabbit serum showed a 4+ agglutination reaction with the red cells of the donor hamster as well as with cells from other hamsters. Understandably this method did not single-out any individual antigen blood group type in these hamsters because of the immunization with whole erythrocytes. Antibodies were likely formed to numerous antigenic characteristics on the hamster's red blood cells. The strong 4+ reaction results from these multiple antibodies reacting with multiple antigenic determinants including any blood group determinants that may be present.

The rabbit immune sera reacted with human erythrocytes of antigenic specificities of types A and B to varying degrees. These data indicate that there are either immune or naturally occuring agglutinins to human blood group types in the rabbit sera. By diluting the rabbit antisera 1:8 (Table 8), the majority of these antibodies' reactions were eliminated. A 1:8 dilution of the antisera had no affect on the agglutination reaction to hamster erythrocytes.

When the above results are compared to data in Table 9, it can be seen that the majority of agglutinins formed to human blood group antigens, post-immunization, are of the immune or induced antibody

Table 7

Post-immunization Rabbit Antisera

vs. Various Red Cell Types

Type of Red Cell	Agglutination Reaction of Rabbit Sera									
	1	2	3	4	5	6	7	8	9	10
Hamster[a]	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Human-A	3+	3+	4+	2+	2+	4+	4+	0	0	0
Human-B	4+	4+	4+	3+	2+	2+	0	0	0	0
Human-AB	4+	4+	2+	0	0	4+	0	0	0	3+
Human-O	0	0	0	0	0	0	0	0	0	0

[a]A 4+ reaction occured between every combination of rabbit antisera and hamster red cell suspension; all 35 hamsters were tested.

Table 8

Post-immunization Rabbit Antisera Diluted 1:8

vs. Various Red Cell Types

Type of Red Cell	Agglutination Reaction of Diluted Rabbit Sera									
	1	2	3	4	5	6	7	8	9	10
Hamster[a]	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Human-A	0	0	2+	0	0	0	0	0	0	0
Human-B	0	0	0	0	0	0	0	0	0	0
Human-AB	2+	2+	2+	0	0	0	1+	0	0	0
Human-O	0	0	0	0	0	0	0	0	0	0

[a]A 4+ reaction occured between every combination of rabbit antisera and hamster red cell suspension.

type. Pre-immunization rabbit sera show a few weak agglutination reactions to human red cells, with the exception of rabbit No. 3 whose serum has high affinity antibodies to human blood group substances A and B. When each pre-immunization rabbit serum was tested against each hamster's red blood cells (see Tables 10 and 11), it could be seen that there exist low affinity reactive substances (agglutinins) to unknown determinants present on hamster erythrocyte surfaces. Following a 1:8 dilution of the rabbit sera, these naturally occuring or immune agglutinins were removed.

Absorption studies. Commercial blood grouping sera (Ortho) was absorbed with various human erythrocytes to determine if agglutination of the hamster erythrocytes was caused by the specific blood group agglutinins or by other components of the typing sera. Table 12 shows that there is no agglutination reaction between hamster red cells and blood-group typing sera after A and B antibodies have been completely removed. This indicates that the antibodies present in the human typing serum are reacting with A- and/or B-like antigenic determinants present on the hamster erythrocyte surface. An interpretation of these findings is that the antigens present on the hamster erythrocyte surface could be classified as A-like, B-like or AB-like. It is not possible to distinguish, however, the subgroups of A or B using anti-AB typing sera. Special techniques are required for this determination, i.e., lectins.

When serum from normal human blood group O donors was tested with hamster erythrocytes agglutination occurs. The data in Table 13 shows that the complex of antigen-antibody forms a solid agglu-

Table 10

Pre-immunization Rabbit Sera

vs. Hamster Erythrocytes

Red Cells of Hamster No.	Agglutination Reaction Against Rabbit Sera									
	1	2	3	4	5	6	7	8	9	10
1	2+	0	2+	2+	1+	2+	2+	2+	0	2+
2	2+	2+	2+	2+	2+	2+	0	2+	2+	2+
3	2+	1+	2+	2+	0	0	2+	2+	2+	2+
4	1+	0	2+	2+	2+	2+	1+	1+	1+	1+
5	2+	0	2+	2+	2+	1+	2+	1+	1+	1+
6	1+	0	2+	1+	1+	2+	1+	1+	1+	1+
7	1+	0	2+	2+	2+	2+	2+	2+	2+	2+
8	2+	0	0	1+	1+	1+	2+	1+	0	1+
9	1+	2+	1+	2+	2+	2+	2+	2+	2+	2+
10	0	1+	1+	1+	2+	1+	0	1+	1+	1+

Table 11

Pre-immunization Rabbit Sera Diluted 1:8

vs. Hamster Erythrocytes

Red Cells of Hamster No.	Agglutination Reaction Against Rabbit Sera Diluted 1:8									
	1	2	3	4	5	6	7	8	9	10
1	0	0	±	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	±	0	0	0	0	0	±	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	±	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0

Table 12

Reaction of Absorbed Human Blood Grouping Serum (Ortho Diagnostics) with Individual Chinese Hamster's Erythrocytes

Serum	Erythrocytes	
	No. of Hamsters Reacting/No. Tested	Agglutination Reaction
Anti-AB Blood Grouping Serum (1:8 Dilution) Absorbed with Human AB Erythrocytes	0/35	0
Anti-AB Blood Grouping Serum (Diluted 1:8) - Control	35/35	4+
Anti-A Blood Grouping Serum (Undiluted) Absorbed with Human A Erythrocytes	0/35	0
Anti-B Blood Grouping Serum (Undiluted) Absorbed with Human B Cells	0/35	0

Note. 4+, solid agglutinate; controls for the last two were negative for the reaction between absorbed serum vs. the red cells used to absorb the serum

Table 13

Reaction of Chinese Hamster Erythrocytes with the Serum of Blood Group O Individuals

Serum	Erythrocytes	
	No. of Hamsters Reacting/No. Tested	Agglutination Reaction
Undiluted Serum from Blood Group O Individuals (Anti-A, Anti-B Antibodies)	35/35	4+
Serum from Blood Group O Individuals (Diluted 1:10)	35/35	1+

tinate. When this group O serum was diluted 1:10, it still retained the ability to agglutinate. As with the previous data, these indicate that the antigens present on hamster erythrocytes have A- and/or B-like characteristics.

Absorption studies on the rabbit anti-hamster erythrocyte serum served to (1) specifically remove reactivity directed against cells of known specificity, thus helping to identify antibody specificity (2) to remove antibodies from serum of one specificity that might by reason of titer, steric hindrance or other factors, mask a second antibody reaction and (3) to remove antibodies from a mixture of antibodies for later partial purification.

When the rabbit anti-hamster sera was absorbed with AB human red blood cells, a sharp reduction in titer to A, AB and B cells occured, Table 14. As would be expected, the AB antibodies or agglutinins were essentially all removed. However, some reactivity remained to A and B type cells. It is likely that the concentration of antigenic determinants on the AB cells were sufficiently concentrated to reduce the serum titer to a level below agglutinability but with the more densely saturated A or B cells the remaining antibodies were sufficient to cause an agglutination reaction. Alternately, all of the major A - B antibodies may have been removed and the reactions observed were due to minor antigen-antibody interactions which could now be expressed. Another possibility is that the absorption by AB cells removed inhibitors that presented weak affinity antibodies from previously reacting. In any event, there appeared to be specific agglutinins in the rabbit anti-hamster ery-

Table 14

Rabbit Antisera to Hamster Erythrocytes Absorbed with Human Type AB Cells and Tested Against Human Cells of Various Specificities

Absorbed Rabbit Antisera	Agglutination of Human Erythrocyte Type			
	A	B	AB	O
1	3+/1+[a]	4+/±	4+/0	0/0
2	3+/1+	4+/±	4+/0	0/0
3	4+/1+	4+/±	2+/0	0/±
4	2+/±	3+/±	0/0	0/±
5	2+/1+	2+/±	0/±	0/±
6	4+/±	2+/0	4+/0	0/±
7	4+/±	0/0	0/0	0/0
8	0/1+	0/±	0/0	0/0
9	0/1+	0/0	0/0	0/0
10	0/1+	0/±	3+/±	0/±

[a]Unabsorbed reaction/absorbed reaction

throcyte serum directed against human A and B substances.

Interestingly, when these same rabbit antisera were sequentially absorbed with human type A, B, AB and O cells all reactivity to human cell types was removed, however, the sera retained their high agglutinability to all hamster cells tested, Table 15.

Elution studies. Immune serum from each rabbit immunized against hamster erythrocytes was absorbed with AB human red cells. The absorbed antisera were then tested with the homologous hamster red cells. Agglutination was observed as a 2+ reaction. The antibodies from these agglutinated hamster cells were eluted off and then reacted with the erythrocytes of a number of other hamsters. Table 16 shows the results of this elution-agglutination study. These data indicate that the agglutinins which remain after absorption with AB red cells are not of A or B specificity. The eluates were reacted with human AB red cells, as a control, and gave a zero agglutination reaction in each case. The control indicates that no anti-A or anti-B antibodies remain in the eluates. Weak reactions occured with test cells and could be explained as being antibodies to minor blood group systems, subgroups of A or B, or antibodies to the many protein moities that can be assumed to be present on hamster erythrocyte surfaces.

The antiserum of each of the ten rabbits was reacted with human red cells of type AB. The antibody was then eluted from the red cells and the eluate tested with each of the donor hamster's erythrocytes. In each case (each of the ten rabbit antisera against each hamster's red cell suspension) the eluate gave a 2+ agglutination reaction. These results indicate that antigenic determinants on hamster erythro-

Table 15

Rabbit Antisera to Hamster Erythrocytes Sequentially Absorbed with Human Type A, AB, B and O Red Blood Cells

Absorbed Rabbit Antiserum	Agglutination Reactions with Human RBC's				Agglutination Reactions with Various Hamster RBC's	
	A	B	AB	O	Reacted/ Tested[a]	Degree of Agglutination
1	0	0	0	0	35/35	4+
2	0	0	0	0	35/35	4+
3	0	0	0	0	35/35	4+
4	0	0	0	0	35/35	4+
5	0	0	0	0	35/35	4+
6	0	0	0	0	35/35	4+
7	0	0	0	0	35/35	4+
8	0	0	0	0	35/35	4+
9	0	0	0	0	35/35	4+
10	0	0	0	0	35/35	4+

[a]No. of hamsters that reacted/No. of hamsters tested

Table 16

Agglutination Reaction of Eluate from Hamster Erythrocytes Agglutinated by Human AB-Cell-Absorbed Rabbit Immune Sera

Eluates	No. Reacting/No. Tested[a]	Degree of Agglutination
1	35/35	6 of 1+ 29 of ±
2	34/35	12 of 1+ 22 of ±
3	32/35	3 of 1+ 29 of ±
4	33/35	8 of 1+ 25 of ±
5	34/35	3 of 1+ 31 of ±
6	32/35	10 of 1+ 22 of ±
7	35/35	4 of 1+ 31 of ±
8	34/35	6 of 1+ 28 of ±
9	30/35	2 of 1+ 28 of ±
10	35/35	3 of 1+ 32 of ±

Note. The eluate was obtained from the reaction between rabbit antisera, absorbed with human AB red cells, and the homologous hamster's red cells; there were ten rabbits.

[a]No. of hamsters that reacted/Total No. of hamsters tested

cytes have A- and/or B-like characteristics. This data agrees with that found when hamster red cells were tested with commercial (Ortho Diagnostics) blood grouping sera anti-A and anti-B (Table 5).

2-Mercaptoethanol cleavage of immunoglobulins. Beta-mercaptoethanol (a sulfhydryl compound) is a denaturing agent which is used to distinguish IgM molecules from IgG molecules. It acts by cleaving the disulfide bonds and splitting 19S IgM molecules into 7S subunits which are unable to agglutinate red blood cells. Immunoglobulin G molecules are unaffected by sulfhydryl compounds such as 2-ME. Table 17 shows the results obtained in an effort to identify the class of antibody which was produced by rabbits against hamster erythrocytes. Controls included rabbit sera treated only with PBS and an additional control using human blood grouping serum of the anti-A type. The later control was used because it is known that antibodies to blood group antigens are of the IgM class. If 2-ME serum and control serum have equal activity, which these results illustrate, the antibody is of the IgG class. After having given a second booster dose of erythrocyte antigen, it is not surprising that we see IgG-class molecules.

Double diffusion (gel diffusion of Ouchterlony). The blood group antigens are found in most body fluids and on most organ tissues. Double diffusion in gel was performed to check for the presence of rabbit antibodies to hamster red cell proteins present in plasma, specifically blood group antigens. Each rabbit antiserum was ran against its homologous hamster's serum. Results showed no bands of precipitation between any of the ten combinations indicating that blood group specificities are either not present in the hamster's sera or that, if

Table 17

Effect of Beta Mercaptoethanol (ME) Treatment on Antisera Agglutination Reactions

	Degree of Agglutination of Cells			
	Human Group A	Hamster 1	Hamster 2	Hamster 3
ME Treated				
Rabbit 2 Sera	0	4+	4+	4+
Rabbit 3 Sera	2+	4+	4+	4+
Rabbit 4 Sera	0	4+	4+	4+
Anti-A Human Sera	0	ND	ND	ND
PBS Treated				
Rabbit 2 Sera	0	4+	4+	4+
Rabbit 3 Sera	4+	4+	4+	4+
Rabbit 4 Sera	0	4+	4+	4+

present, are in too low a concentration to be detected by this method.

The test also confirmed that the washing procedure used for the original erythrocyte inoculum removed all or most of the adhering serum proteins. Should serum proteins, such as globulins or albumins remain on the erythrocyte inoculum, one would expect to see significant precipitates formed.

These data emphasize that the agglutinating antibodies observed in the rabbit antisera are not simply reacting with surface absorbed serum proteins but are likely directed towards structural elements of the erythrocyte.

Protein A. Protein A is isolated from the Cowan I staphylococcus strain. It is a receptor-specific protein that recognized the Fc region of immunoglobulin G (IgG) from a variety of species. In interacting with IgG-type antibodies it does not impair antigen binding. It has been found to bind to the Fc fragment of human IgG of subgroups 1, 2 and 4 and to IgG from a number of animals including rat, mouse, guinea pig, rabbit and goat.

Because Protein A reacts with IgG-type antibodies from many species without affecting antigen binding, it is a valuable tool in cell studies to determine surface binding or distribution of immunoglobulins. For example, lymphoid cells bearing surface IgG form rosettes with erythrocytes coated with Protein A.

The results of the Protein A studies were positive in that they were successful in determining the presence of IgG-type immunoglobulins on the erythrocytes of hamsters. This indicates that there is a specific binding.

Electron microscopy. The surfaces of most animal cells are negatively charged. The negativity of diverse tissues and blood cells is attributable to the ionized carboxyl group of sialic acid. The sialic acids occupy peripheral, terminal positions in the oligosaccharide portions of the glycoproteins and glycolipids that make up a large component of the coat material of the cell surface.

Numerous reagents such as the trivalent cation lanthanum (La^{3+}), colloidal iron, thorium, gold sols, alcian blue and cationized ferritin have been used to specifically stain for the negativity (sialic acid) of the cell surface.

Cationized ferritin has several advantages over other reagents, namely it is intensely electron dense, has a distinct structure and has less tendency toward anomalous binding.

Figures 1-10 show the results of the cationized ferritin and pseudoreplication studies. Normal hamster, diabetic hamster and control human cells were included in this study. The discussion for each is with the description for each photomicrograph.

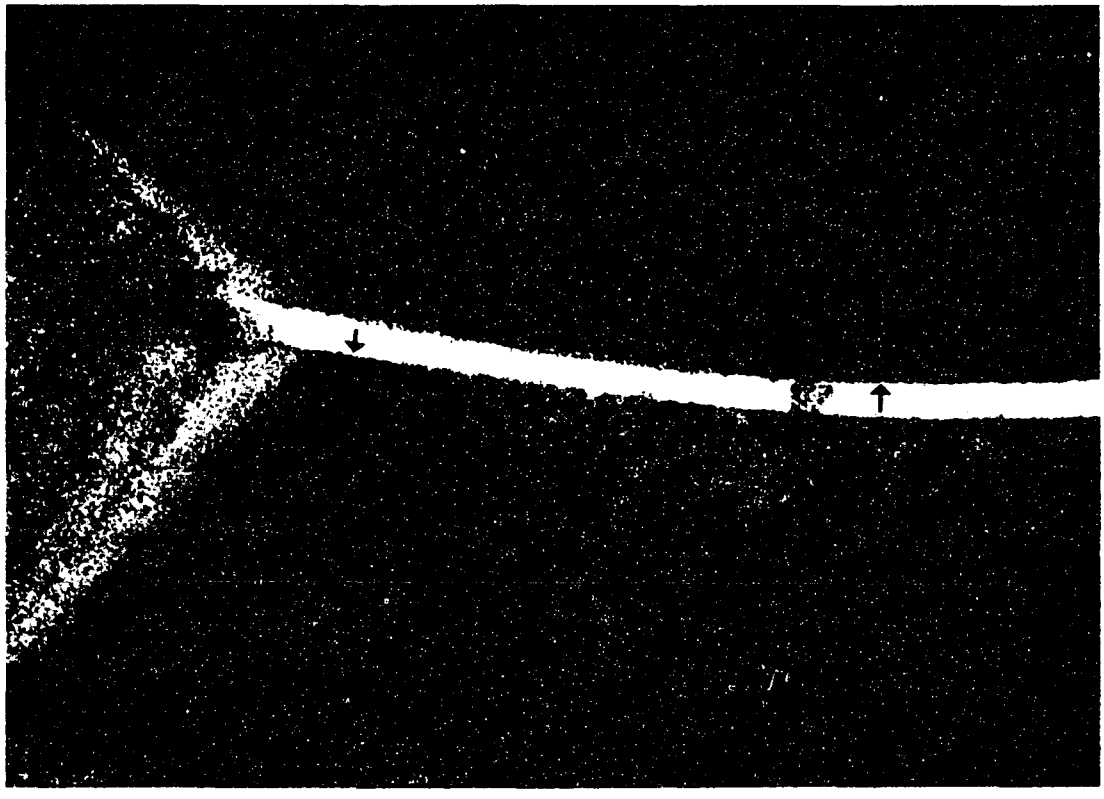

Figure 1. Normal, human type O erythrocytes stained with a polycationic derivative of ferritin to label the negative charges on the cell surface. Individual ferritin aggregates can be easily seen at the position of the arrows. Normal human erythrocytes displayed a thin layer of binding, usually a few nanometers thick and spread evenly on the cell surface. Since sialic acid accounts for a large component of the cationic binding, and these residues occupy a terminal position on the glycoproteins prominent in blood group antigens, the staining distribution is an indication of antigen dispersement.

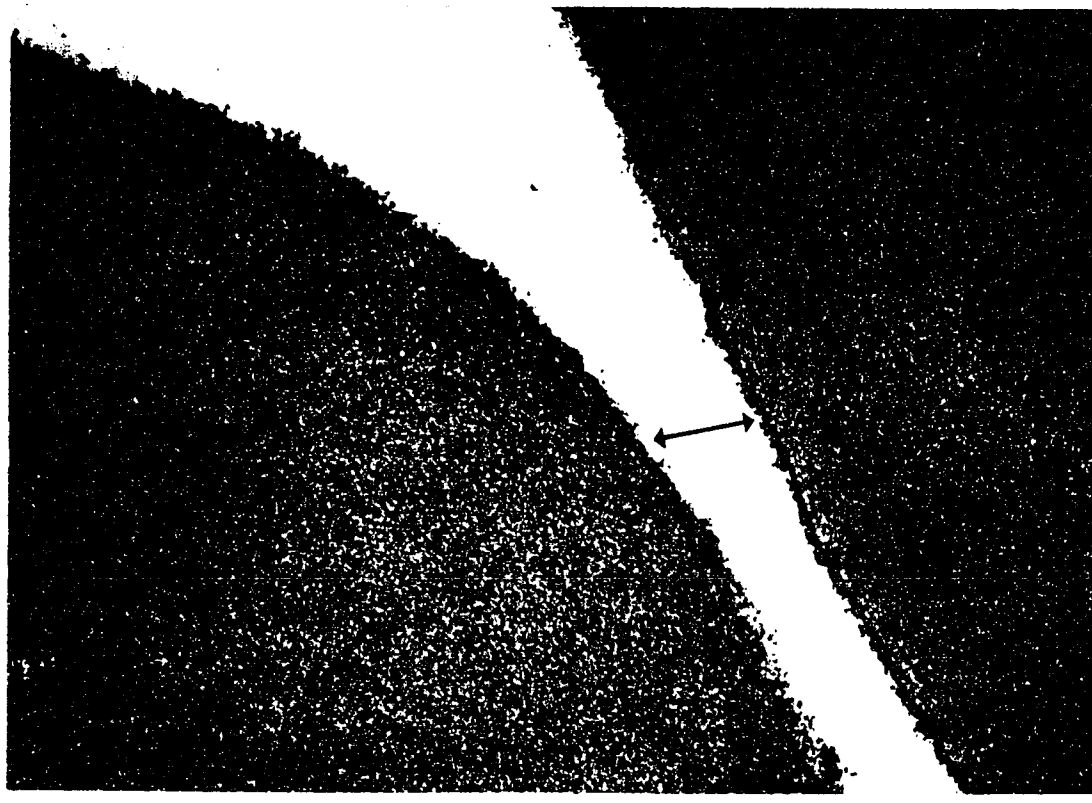

Figure 2. Normal Chinese hamster erythrocytes stained with cationized ferritin displaying a pattern of negative charges less uniform than that of human erythrocytes. The depth of the charged layer in the Chinese hamster appears to be at least twice that of the human, indicative of either longer oligosaccharides on the glycoproteins or greater numbers of sialic acid residues.

Figure 3. Erythrocytes from an individual of an inbred line of Chinese hamsters which display frequent diabetic symptoms. This particular animal was judged diabetic, however, at the time of sampling no glucose was found in the urine; therefore, we considered it "prediabetic" or intermittent diabetic. The cationized ferritin staining of the erythrocytes revealed an anionized layer of non-uniform depth. As can be seen at the arrows, frequent tufts of surface staining material project several nanometers. This was a characteristic of all erythrocytes of this animal and another similar subject.

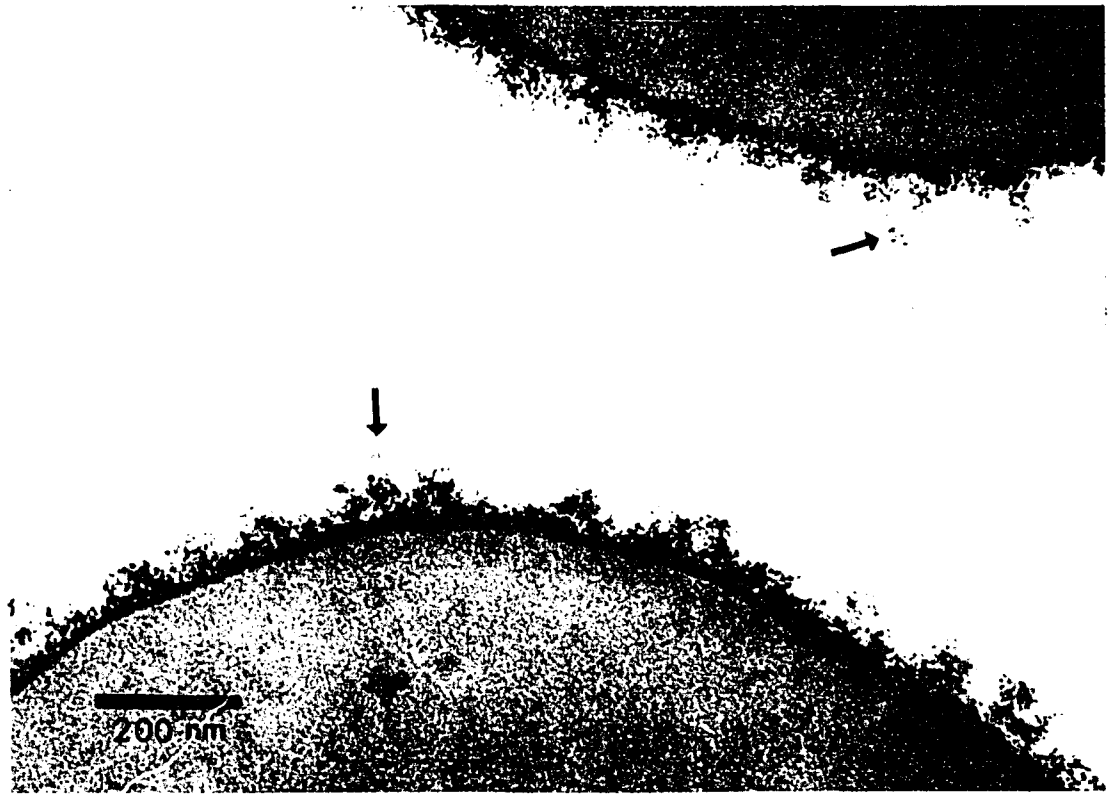

Figure 4. Cationized staining of the erythrocyte membrane of a line of Chinese hamsters demonstrating frank symptoms of diabetes, namely quantities of glucose in the urine. The erythrocyte staining produced a coronal effect with long branch-like tufts of anionic substances vividly decorated with ferritin. This layer of staining measured from a few nm to nearly 100 nm.

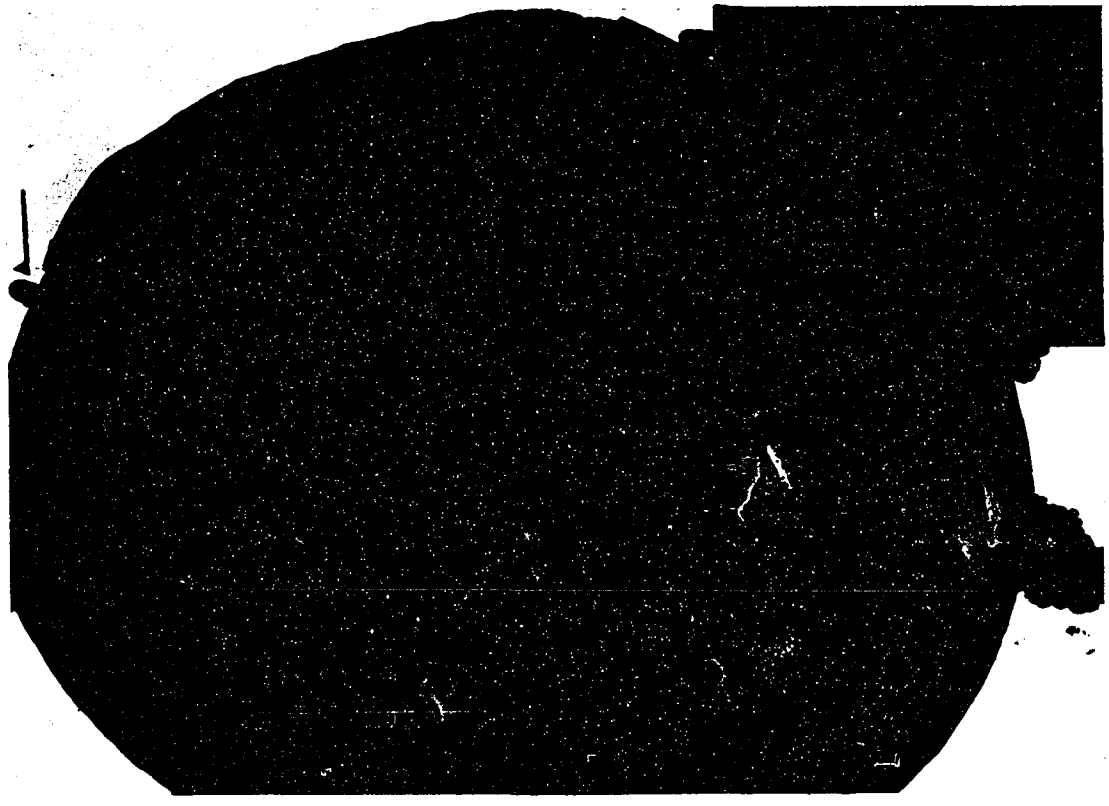

Figure 5. Pseudoreplica of the erythrocytes of a normal Chinese hamster. Characteristically the cells show microcrenations or protrusions of the cell membrane as shown at the half arrow. The surface of this erythrocyte is shown in higher magnification (74,000X) in the insert. The bleb shown at the arrow denotes the area of higher power examination. The erythrocyte membrane reveals no striking topographical feature except that of minor surface irregularity.

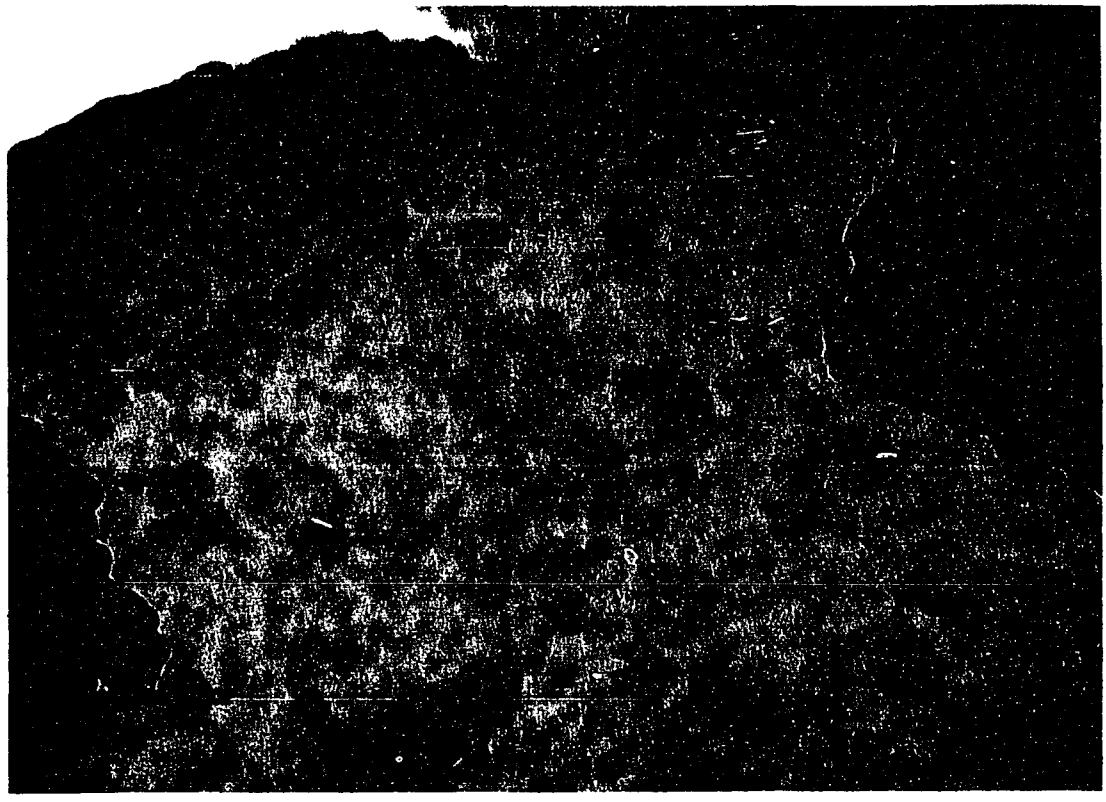

Figure 6. Pseudoreplica of the erythrocytes of a normal Chinese hamster previously stained with cationized ferritin. At a magnification of 92,000 times, one can discern accumulations of ferritin irregularly over the surface. This procedure reveals a pattern of ferritin staining (anionic material) previously indicated by thin sections of embedded erythrocytes.

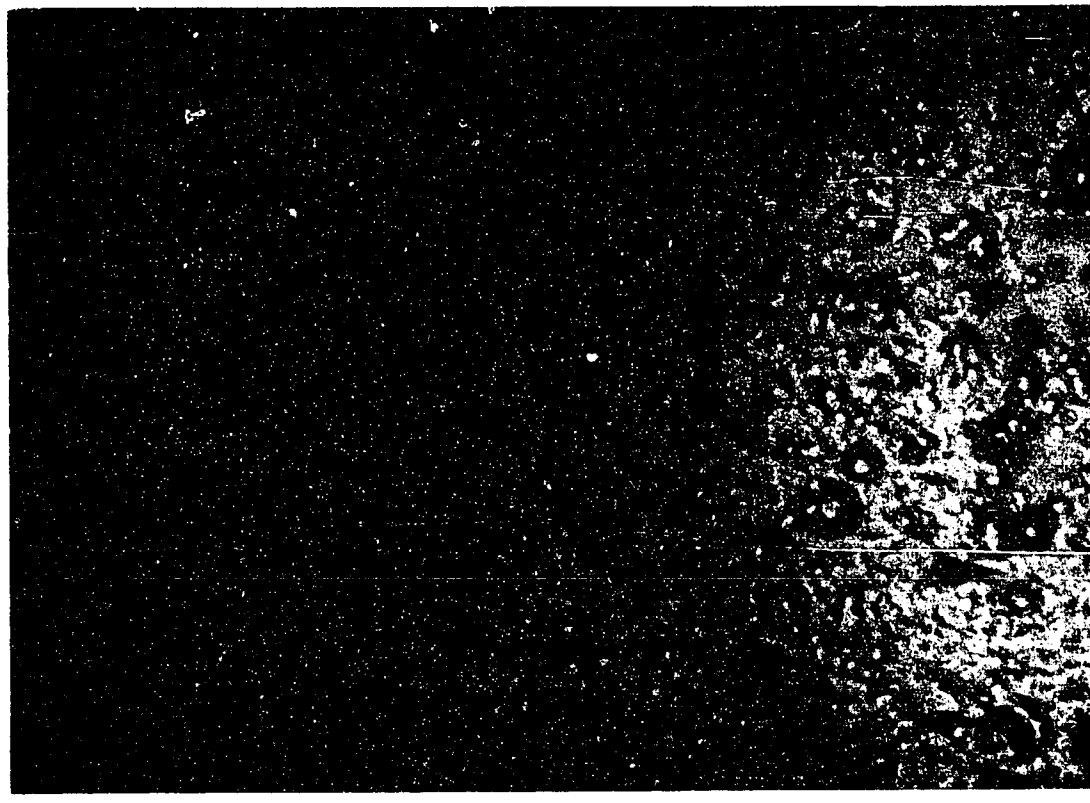

Figure 7. Pseudoreplica of cationized ferritin, stained erythrocytes from a diabetic Chinese hamster. Patterns of irregular accumulations of ferritin stained material can be seen (at the short arrows) unevenly distributed over the cell surface. Irregular patterns of lesser or no staining (long arrow) can also be seen. These patterns of staining are in agreement with that revealed by thin sectioning.

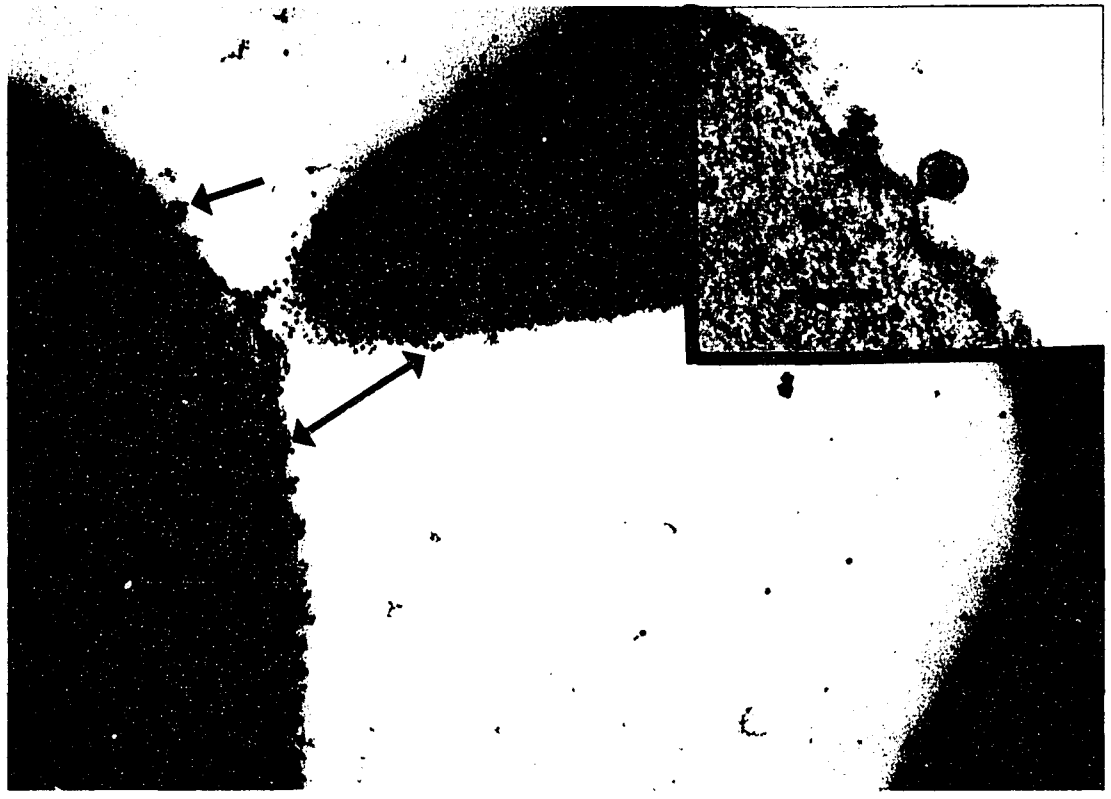

Figure 8. Cationized ferritin stained section of a "prediabetic" hamster erythrocyte. The cell surface displays the previously encountered irregular staining as shown at the double arrow. An unexpected observation at this 92,000X was the presence of several particles, indicated by the single arrow, which had the physical appearance of viruses. Typically, these particles were attached or seemingly attached to the erythrocyte surface; however, numerous free particles were observed as were particles within the erythrocyte substance. As shown in the insert at 260,000X, the particle is a single walled structure containing a densely stained, centrally located core. The particle in cross-section appears to present a 5 regular-sided structure of 27 to 28 nm in diameter. No significance is attributed to these particles, however, they were found only in this "pre-diabetic" animal.

Figure 9. Normal Chinese hamster erythrocytes exposed sequentially to high concentrations of homologous rabbit anti-erythrocyte serum followed by cationized ferritin. These particular cells, following the antibody exposure, exhibited a dense layer of cation-staining over the cell membrane. There appeared to be no interference due to previous antibody binding.

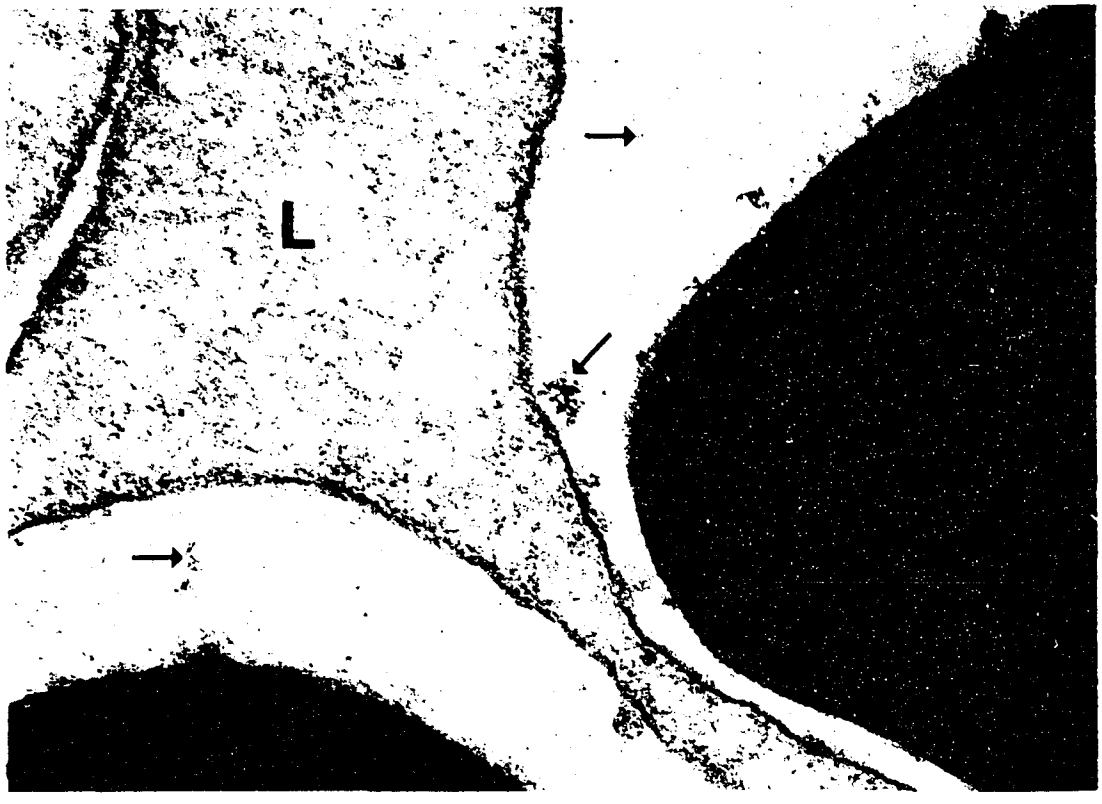

Figure 10. Another normal Chinese hamster's erythrocytes exposed first to homologous rabbit anti-erythrocyte serum followed by cationized ferritin. Although complement was reduced in these samples, obvious lysis of many cells occured. The degree of staining with ferritin is reduced and ferritin appears to have been removed from the membranes into the surrounding medium.

SUMMARY

Reactions of rabbit anti-hamster erythrocyte sera, human blood typing sera, lectins and absorption-elution studies indicate that there may be a minor component on hamster erythrocytes related to A and/or B human blood group substances. Cross-agglutination of hamster sera and erythrocytes in the presence of 30% bovine serum albumin revealed the presence of isoantibodies to red cell determinants. These hamster sera isoagglutinins were warm-reacting antibodies and of the IgG type. The use of specific lectins indicated that the blood group substances on Chinese hamster erythrocytes, which react with human anti-A and/or anti-B substances, may very possibly be of the A_2 or A_2B subgroup. Additionally, some Chinese hamster sera show low-affinity reacting agglutinins to human ABH substances. Through absorption and elution of rabbit anti-hamster erythrocyte sera and human typing sera, we again see that there are antigens present on hamster red blood cells which are specific for or at least cross-reactive with human A and B antigenic specificities. Protein A, used as a specific cell marker, revealed the presence of IgG-antigen complexes on the Chinese hamster erythrocyte surface comparable to human control red blood cells following agglutination by human or rabbit typing sera. The hamster erythrocyte glycocalyx was shown to bind cationized ferritin particles equally as well as control human erythrocytes, although in a much denser surface configuration, as demonstrated by electron microscopic examination.

The above studies have given no obvious or cencrete evidence leading to differences between the normal and diabetic Chinese hamsters used for examination.

We can elucidate from this study that the Chinese hamster can now be included among the many animal species that show human ABH-like blood group specificities.

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