STUDIES OF BOVINE BLOOD CELL SURFACES

Thesis by

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ABBREVIATIONS

Con A:	Concanavalin A
EM:	Electronmicroscopy
GARWS:	goat-anti-rabbit-whole serum
H-2:	histocompatibility-2 system; major histocompatibility system of the mouse
Hcy:	Busycon caniculatum hemocyanin
Hcy/unit area:	hemocyanin per unit area
i.d.:	intradermally
IEP:	immunoelectrophoresis
MEM:	minimal essential medium
PHA:	phytohemagglutinin
PBS:	phosphate buffered saline, pH 7.2
RABI:	rabbit-anti-bovine-immunoglobulin
RABI-Hcy:	rabbit-anti-bovine-immunoglobulin glutaraldehyde coupled to hemocyanin
RABWS:	rabbit-anti-bovine-whole-serum
RAH:	rabbit-anti-hemocyanin
TEM:	transmission electron microscopy
Tla:	thymus leukemia antigen
s.c.:	subcutaneous
SEM:	scanning electron microscopy
WGA:	wheat germ agglutinin

ABSTRACT

The cell surface expression of genetically defined bovine red cell antigens has been studied by electron microscopic and serological techniques. Electron microscopic cell surface localization of specific antigens on bovine red cells has been achieved with the use of an indirect labeling reagent: hemocyanin glutaraldehyde coupled to rabbit-anti-bovine-immunoglobulin (Hcy-RABI). When the specific antigenic sites on the cell surface are combined with their corresponding antibodies, the secondary application of Hcy-RABI serves to visualize these sites for electron microscopy.

Serological dosage reagents for the Z antigen are known to differentiate Z homozygotes (Z/Z) from heterozygotes (Z/-) in terms of the kinetics of complement mediated hemolysis. In the present study, cells homozygous for the Z antigen and saturated with anti-Z antibody were found to take up approximately twice as much Hcy-RABI as cells heterozygous for Z; cells negative for Z showed only background labeling values. Cells possessing the J antigen, a soluble serum substance secondarily absorbed to the red cell surface, were also examined for their quantitative uptake of hemocyanin. Many intergrades of J positive cells exist, ranging from cells which require large amounts of anti-J antibody for complement mediated lysis to cells which are lysed by minute quantities of specific antibody. The quantity of label taken up by a sampling of cells was found to be inversely related to the amount of antibody necessary to lyse those cells.

Sequential double labeling studies were conducted to characterize the cell surface steric configurations of antigens whose genes reside in 1) the same blood group system; 2) different blood group systems; and 3) cis versus trans conformations within a system. In no case was steric hindrance found. This result indicates that each antigenic determinant examined is spacially distant from others; it suggests that the determinants may be coded for by distinct genes, and that the antigens labeled are not a series of determinants on a common backbone macromolecule. Sequential double labeling of one set of antigens gave a value which was twice the sum of the two single label values. This phenomenon was noted only for one particular pair of antigens, and only on cells treated initially with one of the antisera. The increased uptake of the second antibody was highly specific. This observation suggests that new antigenic sites are revealed in the presence of bound antibody directed against another specificity, on cells labeled for this particular pair of antigens.

Concanavalin A (Con A) binding experiments on trypsinized and nontrypsinized cells strongly indicate that the Con A receptor and the A antigen are molecular unique cell surface entities. Trypsinization of A positive cells caused increased binding of anti-A, accompanied by clustering of the A antigen sites and of the intramembranous particles seen in freeze-fracture experiments. These phenomena were accompanied by cell agglutination.

Using bovine red cell blood typing reagents in a leukocyte microcytotoxicity system, bovine leukocytes were found to have specific surface antigens. In this preliminary study there is no obvious association between leukocyte antigens and red cell antigens of any individual animal. The leukocyte and erythrocyte antigenic systems appear to be distinct from each other.

TABLE OF CONTENTS

INTRODUCTION

The purposes of the studies reported in this thesis are to investigate 1) the relationship between gene dosage and antigenic expression on the cell surface; 2) the topological distribution of specificities on the cell surface in relation to the genetic configurations that control them; 3) the effects of enzyme treatment on expression of cell surface antigens and on Concanavalin A binding; and 4) the identification of bovine leukocyte surface antigens.

Bovine red blood cell antigens provide an excellent system in which to study the relationship between the genetic definition of antigens and antigen expression on the cell surface. The genetics and serology of these antigens are well documented and their variety is vast. Animals whose red cells are genetically and serologically defined are available. Monospecific antibody reagents directed against the red cell antigens are obtainable, and have been tested exhaustively for specificity and activity. At present over 60 bovine red cell antigenic factors are known. These factors fall into 11 genetic systems as determined by parentage studies (Stormont, 1962).

Bovine red cells are easy to handle, are stable at normal refrigerator temperature for 4 to 6 weeks after collection, and are easily used in immunological tests with specific antisera to detect their surface antigens. In addition, these cells are readily usable for electron microscopic localization of the surface antigens. The great array of surface antigens, the availability of monospecific antisera, and the background of genetic definition of these cells, make them an

obvious choice for studying the cell surface expression of genetically defined antigens. The experiments described in this thesis have been designed to correlate serological observations with electron microscopic localization phenomena. This approach has enabled the author to quantitate and map the distribution of bovine red cell surface antigens in relation to the serological activity of specific antisera and the genetic composition of the cells.

Comprehensive introductions to the specific subjects of individual chapters are contained in the introductory sections of the chapters. The remainder of this general thesis introduction presents brief backgrounds on bovine red cell antigens and electron microscopic cell surface labeling techniques. The following background section on bovine blood groups includes various interesting and unsolved problems involving the organization of the genetic material coding for the antigens. The remainder of this thesis attempts to answer some of the questions raised in the following introductory section.

Bovine blood group antigens

The first polymorphic bovine red cell antigens were described by Ferguson (1941) who obtained blood typing antibodies by immunizing dams with whole blood from their daughters. Seven red cell specificities were described, and their inheritance demonstrated. The hemolytic assay and the absorption procedures used to test and characterize the antisera were also described.

Within the next few years additional bovine red cell antigens were found (Ferguson et al., 1942), and rabbit heteroimmune antisera were introduced into bovine blood typing. The antigens continued to appear as dominant factors (Ferguson et al., 1942) and there was no evidence of gene interaction affecting their expression. The concept of "unit" reagents, i.e., antisera detecting single specificities, was introduced; and the antigens were described as being either coded for by closely linked genes or by a series of multiple alleles (Ferguson et al., 1942; Stormont and Cumley, 1943). The use of bovine red cell typing to determine parentage was pointed out (Stormont and Cumley, 1943).

The nomenclature and serology became more complicated as subtypes of various factors were encountered (Stormont, 1950). For example, it was found that the isoimmune antiserum detecting the O factor could be fractionated by absorbing with various sets of bovine red cells to leave O_1, O_2, and O_3 subsets of antibodies. Absorption with O_1 cells removed all antibody from the anti-O antiserum; absorption with O_2 cells left a population of antibodies which still reacted with O_1 cells; and absorption with O_3 cells left antibodies which reacted with O_1 and O_2 cells. Similar subtype-sets were found for antisera directed against X, U, and E′ specificities. It was also noted (Stormont et al., 1945, 1951; Stormont, 1950) that certain factors did not appear in all possible combinations. For example, in the grouping BGK, K was never found without B and G, but B or G could occur alone or with each other. This observation furthered the

hypothesis that multiple alleles rather than closely linked genes were probably the basis of coding for the antigens (Stormont et al., 1945, 1951). This genetic concept will be discussed later at length.

Stormont (1950) also pointed out that certain of the antigens were present together with greater frequencies than one would expect from random combinations. Stormont, Owen, and Irwin (1951) developed the idea that certain of the bovine red cell specificities were associated as blocks or complexes. Stormont (1955) introduced the term "phenogroup" to denote the "blocks of blood factors that are inherited en masse." The B complex took form as being composed of 38 blood factors which were expressed as 80 phenogroups consisting of particular combinations of the single factors. The C system included 7 blood factors which were found in 22 different groups (Stormont et al., 1951). It was also noted that while some phenogroups were constant from breed to breed, others differed tremendously among Holstein-Friesian, Guernsey, Jersey, and Brown Swiss cattle (Owen et al., 1947; Stormont et al., 1951).

The bovine red cell antigens thus far noted in this discussion are cell membrane bound specificities. The J antigen, in contrast, is a soluble serum substance which secondarily adsorbs to the red cell surface (Stormont, 1949, 1954). Normal antibodies directed against the J antigen are present in the sera of many cows who lack the J antigen. Higher titered J reagents are produced by immunizing "J negative rabbits" (rabbits whose sera do not inhibit the lysis of J positive bovine red cells in the presence of specific anti-J antibody)

with human A red cells. Such rabbits produce antibodies which cross-react strongly with bovine J cells (Stormont, 1960).

At present, the 11 genetic blood group systems shown in Table 1 have been identified in domestic cattle. They range in complexity from the 36 factor, 300 phenogroup B complex (Stormont et al., 1951; Stormont, 1962) to the single-factor Z system (Stormont, 1952). The C system (Stormont et al., 1951, Stormont, 1960) is also moderately complex, consisting of 9 blood factors (see Table 1) which have been found in 35 phenogroups. Less complex systems include the F-V system (Stormont et al., 1951; Owen et al., 1958; Stormont, 1962), the A system (Stormont, 1962), the J system (Stormont, 1949, 1960; Stone and Irwin, 1954), the single-factor L system (Stormont, 1962), the M system (Stormont, 1962), the N system (Stormont, 1962), the S system (Stormont et al., 1961), and the R′-S′ system (Stormont, 1962). Table 2 shows a sampling of the phenogroups found on bovine red cells.

As alluded to previously, the organization of the genes coding for the bovine red cell blood group antigens is not established (Stormont et al., 1945, 1951; Stormont, 1955, 1972). There are, of course, a number of clearly independent genetic loci, each concerned with the control of a distinct set of phenogroups. The blocks of factors within such a set, inherited together, have posed problems for interpretation. Since the original observations on these factor associations were made, it has continued to be perplexing whether each specificity is

Table 1

Bovine Blood Group Systems

	Systems										
	B	C	F-V	A	J	L	M	N	S	Z	R′-S′
Specificities	B_1	C_1	F	A_1	J	L	M_1	N	S	Z	R′
	B_2	C_2	V	A_2			M_2		U	ZZ	S′
	G	E		D					U′		
	I_1	R_1		H					H′		
	I_2	W		Z′					H′H′		
	K	R_2									
	O_1	X_1									
	O_2	X_2									
	O_3	L′									
	O_X										
	P										
	Q										
	T_1										
	T_2										
	Y_1										
	Y_2										
	A′										
	B′										
	D′										
	E_1′										

Table 1 (Continued)

	Systems										
	B	C	F-V	A	J	L	M	N	S	Z	R′-S′
Specificities	E_2'										
	E_3'										
	F′										
	G′										
	I′										
	J′										
	K′										
	O′										
	Y′										
	Q′										
	G″										

After Stormont, 1962.

Table 2

Sampling of Bovine Red Cell Phenogroups

in the B and C Systems

	Code	Phenogroup
B system phenogroups	b	--
	B12	$BGKO_XE'_2F'\ O'$
	B28	$BGKO_2Y_1A'E'_3G'K'Y'B'O'$
	B50	$O_1Y_1E'_3G'$
	B54	$O_1T_1E'_3F'\ K'$
	B62	$O_3J'K'O'$
	B67	PY_2
	B77	O_XY_1D'
	B91	Q
	B158	GO_XO'
C system phenogroups		C_1
		C_1E
		C_1X_1
		C_1ER
		C_1WX_2
		C_2EWX_2
		WX_2L'
		RWX_2
		C_2ERX_1

After Stormont, 1962.

coded for by a separate gene, the genes tightly linked, or whether one gene codes for a molecule which has numerous antigenic specificities (Ferguson et al., 1942; Stormont and Cumley, 1943; Stormont et al., 1945, 1951; Stormont, 1950, 1955; Owen, 1959, 1960). Indirect evidence suggests that series of multiple alleles exist which code for the various phenogroups, rather than sets of closely linked genes each coding for a particular unit specificity. For example, the situation previously described for the BGK antigens suggests that the following alleles exist: $\underline{B}^{b}$, $\underline{B}^{B}$, $\underline{B}^{G}$, and $\underline{B}^{BGK}$. If these antigens were coded for by a series of closely linked genes, each gene specifying one factor, instead of multiple alleles, one would expect to find K occurring in the absence of B and G. But this expression has never been found (Stormont et al., 1945, 1951; Stormont, 1950, 1955, 1972; Owen, 1959, 1960). Also, it is difficult to explain subtypes (i.e., O_1, O_2, and O_3; E′, etc.) in terms of series of closely linked genes, while multiple alleles with cross-reactive products could readily account for the observed serology (Stormont, 1950, 1955; Stormont et al., 1951; Owen, 1959, 1960). A classic example has been known for a long time in the human ABO system. Cells of type A_1 individuals react with anti-A and anti-A_1 reagents, while cells of type A_2 individuals react only with anti-A. This reaction pattern is readily explained by postulating that alleles $\underline{A_1}$ and $\underline{A_2}$ code for glycosyl transferases that have slightly different activities. The immunodominant sugar terminating the polysaccharide sidechains in A_1 is

n-acetylgalactosamine; the same sugar characterizes A_2, but some of the side chains remain incomplete under the influence of the less efficient A_2 allele (Schachter et al., 1973). The ultimate products of the allelic series are thus cross-reactive but distinct from each other. If two closely linked genes coded for the A_1-A_2-O serotype series, one would then expect individuals reactive with only anti-A_1. Such individuals have not been found.

Understanding the A_1-A_2-O system in terms of cross-reactive, related antigens controlled by multiple alleles is promoted by the accepted terminology. The relationships can be listed as:

	anti-A	anti-A_1
A_1	+	+
A_2	+	o
O	o	o

The factor terminology in the bovine systems is less explicit; to take the B-K relationship as a simple example, the observed reactions are:

	anti-B	anti-K
BK	+	+
B	+	o
b	o	o

It should be noted that this pattern of reactions is the same as for A_1-A_2-O; a different symbolism would represent the reactions as:

	anti-B	anti-B_1
B_1	+	+
B_2	+	o
b	o	o

For this reason, as well as brevity of representation, workers with the phenogroup system of cattle have adopted a standard symbolism; allele B_{28} in the B system, for example, codes for the phenogroup encompassing factors $BGKO_2Y_1A'E_3'G'K'Y'B'O'$. This symbolism is intended to be inexplicit in the sense that it does not attempt to distinguish between cross-reactive factors controlled by allelic cistrons and the products of tightly linked cistrons. It seems very likely that both of these bases of genetic and serological variation are present in so complex a set of specificities of the B phenogroups of cattle.

Certain blood factors never occur when other factors are present; some factors exclude others. For example, E'_1 never occurs with J', and B never occurs with E'_1. This might be taken to imply that B - E'_1 - J' form a set of alleles. But J' is also found in other sets. Therefore, if one were to postulate that linked loci code for these antigens, there must be more than one locus coding for the J' antigen. (Actually, the number turns out on this hypothesis to be 22 loci.) It is difficult to accept that that many J' loci would be evolutionarily conserved as separate identical genes. Stormont (1955) concluded that the observed data fit the model of multiple alleles and that each phenogroup is probably coded for by its own allele.

The model of linked genes is also difficult to support in the absence of a large amount of recombination data. Out of 30,000 Dutch cattle blood typed, 57 apparent recombinants or mutations were found in the B system (Bouw and Fiorentini, 1970). Including data collected on American domestic cattle, Stormont (1963) estimated that the "recombination frequency" in the bovine B complex is 10^{-3}. However, many of the possible B factor combinations, given the 36 factors known in the B system, were not found among the recombinants. These data again suggest a smaller number of genes than the number of B system blood factors. Indeed, if we suppose that the recombinants are intragenic, then we can say that the bovine B system is a single gene, multiple allele system (Stormont, 1972).

The complexity of reactions that one observes in bovine red cell typing can well be due largely to the cross-reactive nature of the antisera used (Owen, 1959, 1960). Owen has described the blood group factors as symbols which correspond with fractions of heterogeneous antibody populations. Landsteiner demonstrated many years ago that antibodies will cross-react with various antigens that are similar to each other (see Landsteiner, 1936). Even antibodies directed against a simple hapten appear serologically complex if other similar haptens are reacted with the antiserum. The concept of a blood typing reagent as detecting a "unit" antigen is an inaccurate approach to describing that reagent. The apparent unity depends to a large extent on how many similar antigens the antiserum is tested against. As

Landsteiner said (1936): "Thus to a mosaic of serological reactions there need not always correspond a coordinate mosaic of chemical structures in the antigen." For example, the F-V system of domestic cattle was thought to be a two factor system in which F and V are simple allelic alternatives and FF cells give a stronger reaction to the anti-F reagent than do FV cells. However, a V_2 factor was found in the American bison (Owen et al., 1958). Absorption of a bovine anti-V reagent with bison V_2 cells leaves an antibody fraction which still reacts with bovine V red cells. Therefore, the bovine V reagent, previously thought to be a "unit" reagent, has been fractionated into a V_2 subtype which reacts with bison cells and a V_1 subtype that reacts exclusively with V bison cells. Furthermore, the V_2 subtype shares something in common with F, because bison-cattle hybrids which are genotypically FV_2 demonstrate a dosage reaction as if they were FF. Animals of genotype FV_1, on the other hand, display a single dose of F.

In all of these examples, and particularly in the subtyping relationships, there is a large amount of cross-reactivity which indicates series of closely related but different specificities. A model for gene organization which would be consistent with the cross-reactivities would be a series of allelic genes which code for macromolecules which have similar properties, but which also have individual properties as recognized by antibodies (Stormont, 1950; Owen, 1959, 1960).

This problem of linked genes versus multiple alleles is also found in the human Rh or CDE system. Similar to the BGK situation, c, e, and f have only been found in the combinations cef, ce, c, e, and the absence of all 3 factors (Sanger et al., 1953). Again, it is difficult to understand the serology in terms of separate genes coding for unit antigens, but easy to understand the system in terms of cross-reactivity of sets of allelic products.

Electron microscopic labeling

Various labeling techniques have been developed to localize antigens and receptor molecules on cell surfaces. Specific antibodies (Coons, 1956) and plant lectins (Nicolson and Singer, 1971) have been covalently and non-covalently coupled to molecules which are detectable at the light (Coons, 1956), transmission electron (Singer, 1959), or scanning electron (Brown, 1974) microscope levels. Alternatively, antibodies have been covalently bound to enzymes which deposit a light- or electron-visible reaction product at the site of the enzyme following addition of the enzyme substrate (Nakane and Pierce, 1966; Avrameas and Uriel, 1966).

Indirect labeling procedures have been developed in which the light- or electron-visible marker molecule or enzyme is covalently bound to an anti-immunoglobulin (Andres et al., 1967; Avrameas, 1969b). This reagent can then be used with a specific immunoglobulin in a sandwich technique. The indirect labeling technique is useful when the specific antibody is limited in quantity.

At the light microscope level, antibodies have been flourescinated (Coons, 1956), coupled to horse-radish peroxidase (Avrameas, 1969, 1970), coupled to latex beads (A. Senyei, personal communication), and radioactively tagged for autoradiography (Berenbaum, 1959).

At the transmission electron microscope (TEM) level, several of the light microscope techniques are also usable. The specific peroxidase reaction can be controlled by addition of substrate and hydrogen peroxide for use at the TEM level (Avrameas, 1969, 1970, 1971, 1972). EM autoradiography with specific antibodies is also possible (Pierce et al., 1964). In addition to the above localization methods, other molecules have been developed for use in the TEM. Singer (1959; Singer and Schick, 1961) conjugated specific antibodies to horse-spleen ferritin using m-xylylene diisocyanate. The ferritin is visible in the TEM by virtue of its iron electron dense core. Various viruses, including southern bean mosaic virus (SBMV; Hammerling et al., 1969) and tobacco mosaic virus (TMV; Aoki et al., 1971) have been used in indirect labeling procedures on serial sections (Aoki et al., 1971) and on surface replicas (Koo et al., 1973). Smith and Revel (1972) have used hemocyanin from the snail Busycon caniculatum on cell surface replicas to localize Concanavalin A receptor sites. Hemocyanin has also been used in thin sectioning studies for antigen localization (Karnovsky et al., 1972).

At the scanning electron microscope (SEM) level, hemocyanin (Brown, 1974), various viruses (Aoki et al., 1971), and latex spheres

(Molday et al., 1974) are resolvable for labeling purposes.

Hammerling et al. (1968) have pointed out that chemical covalent coupling of antibodies to marker molecules frequently reduces the antigen binding activity of the specific antibody. Also, in the chemical cross-linking of antibody and marker molecules it is difficult to control the number of antibodies bound per marker, and vice versa. Therefore, purification of the labeled antibody reagent is usually necessary. To overcome these difficulties these authors (Hammerling et al., 1968; Aoki et al., 1969) developed a technique in which hybrid antibodies are prepared having one combining site for the surface antigen under study, and a second combining site for ferritin. Hybrid antibodies were prepared following the dissociation and reassociation procedures of Nisonoff et al., (1960) and Nisonoff and Rivers (1961). Using hybrid antibodies for labeling eliminates the necessity to couple antibody and the electron dense marker molecules chemically, and also provides for a one-to-one correspondence of antibody to labeling molecule.

A further refinement of the hybrid labeling technique to allow for specific double labeling with two different marker molecules has been developed (Lamm et al., 1972). In this method, the surface antigen is labeled with a specific antibody coupled to a hapten. The marker molecule is bridged to the hapten-coupled antibody via a hybrid antibody consisting of anti-marker molecule and anti-hapten binding sites. The original hybrid antibody method can also be used with

different marker molecules in the direct method (Hammerling et al., 1969; Aoki et al., 1971). However, in the indirect method one must use sequential labeling and there is the possibility of some dissociation of the first hybrid antibody with specific immunoglobulin and its replacement with the second hybrid antibody applied.

To localize the cell surface distribution of a particular antigen or other receptor on a cell surface it is necessary to prepare the cells so that the surface can be seen under TEM conditions. Serial sectioning techniques are difficult to use in mapping the cell surface distribution of antigens unless the sections are serially reconstructed into three-dimensional models (Stackpole et al., 1971). Scanning electron microscopy provides an adequate visualization of the cell surface, but does not allow for the resolution obtainable in the TEM. Nicolson and Singer (1971) have enabled large surfaces or red blood cell membranes to be visible under the TEM by lysing the cells at an air-water interface and spreading the membranes on EM grids. Artifacts of the lysis procedure, however, may give inaccurate labeling patterns. TEM on labeled cell surface replicas (Smith and Revel, 1972) provides good resolution and exposure of a large area of the cell surface on intact cells.

Because of the fluidity of membranes (Frye and Edidin, 1970; Edidin and Weiss, 1972; Edidin and Fambrough, 1973; Edidin, 1974), the attachment of a bivalent reagent may induce artificial aggregation of surface molecules (Unanue et al., 1972; also see discussion in

chapter III of this thesis). Many investigators have therefore used monovalent antibody fragments (Fab fragments) rather than intact bivalent antibodies for cell surface labeling (Unanue et al., 1972). Alternatively, prefixation of the target cells, or incubation of the cells below the transition temperature of the membranes, is adequate to inhibit ligand-induced redistribution of cell surface macromolecules in the presence of a bivalent reagent (Unanue and Karnovsky, 1973; Rosenblith et al., 1974; see the more extensive discussion in Chapter III of this thesis).

The phenomenon of "capping", or the polar redistribution of surface molecules induced by binding specific bivalent antibody (Taylor et al., 1971) has been found by fluorescence labeling for many cells and surface antigens (Taylor et al., 1971; Unanue et al., 1972; Kourilsky et al., 1972). Some antigens, however, such as the mouse H-2 antigens, will cap, but only by an indirect anti-immunoglobulin procedure. In a direct labeling procedure, the H-2 sites form only isolated aggregates on the cell surface (Unanue et al., 1972). All of the capping studies indicate that the surface membranes of particular cells are fluid, that antigenic determinants can be redistributed by attachment of bivalent reagents, and that the redistribution can be blocked by low temperature, metabolic inhibitors, or prefixation (Unanue and Karnovsky, 1973).

The surface membranes of mature red cells, however, do not display capping or redistribution of antigenic determinants following incubation with a bivalent reagent (Smith and Revel, 1972).

In order to localize the surface antigens on bovine red cells, certain conditions were required. An electron dense marker molecule was needed which was small enough that steric hindrance of the marker molecules was not significant. For the quantitative studies, TEM was chosen over SEM because of the higher resolution in TEM. A method of visualizing large areas of the cell surface was necessary, so that antigen distribution and quantitation could be readily determined. A labeling procedure in which the cells were kept intact and viable was required such that antigen distribution was altered as little as possible during the labeling procedure. Finally, a specific EM labeling reagent was needed which could be used to label many different bovine red cell specificities, so that numerous experiments could be done on different antigens without having to prepare a new labeling reagent for each experiment.

For the above reasons it was decided to use Busycon caniculatum hemocyanin (Smith and Revel, 1972) covalently coupled with glutaraldehyde to rabbit-anti-bovine-immunoglobulin (Hcy-RABI). This was a multipurpose labeling reagent and could be used with any bovine antiserum to label bovine red cells specifically. The Hcy-RABI was purified by column chromatography and tested immunologically for antigen binding activity. Early in the work, a hybrid antibody was prepared for labeling purposes. This molecule had one combining site for bovine immunoglobulin and a second binding site for hemocyanin. Preparation of the hybrid antibody in quantity, and retention of the

antigen binding activities of the purified hybrid, however, were not adequate for EM labeling purposes (S. Rosenberg, unpublished results).

The labeling procedure employed utilized intact red cells. The labeled cells were visualized for TEM by preparation of carbon replicas of the cell surfaces which enabled large sections of the cell surfaces to be examined.

MATERIALS AND METHODS

Cells

Bovine blood was generously supplied by Dr. Clyde Stormont of the University of California at Davis (UCD). Cows were bled from the jugular vein into an anticoagulent buffer, Alsever's solution (isotonic 0.03 M citrate - 0.115 M dextrose, pH 7.2). The whole blood in Alsever's solution was stored at 4 °C and was used until six weeks post-bleeding.

Whole blood for the bovine leukocyte study was collected from the jugular vein into 100 × 16 mm Vacutainer tubes (Becton-Dickenson and Co., Rutherford, N.J.) containing 14 mg. powdered EDTA. Samples were stored at 4 °C and were usually tested within 7 days of collection. Cells from cows of the UCD and the California State Polytechnic Institute at Pomona (Cal Poly) herds were tested for leukocyte antigens.

The red cells were prepared for use in all electron microscope (EM) experiments by washing with at least a 5-fold excess of 0.1 M phosphate, 0.85% saline, pH 7.2 buffer (PBS) four times. Fresh cells were prepared in the morning and were used the same day. For serological experiments, washed red cells were suspended in either PBS or 0.85% unbuffered saline.

Phenotyping and genotyping for the UCD animals were done by Dr. Stormont's laboratory. The records of the genotypes of these animals are kept on file at the Serology Laboratory, UCD. Phenotyping of the Cal Poly cows was done by the author, using the lytic assay procedure with reagents provided by Dr. Stormont.

Busycon caniculatum hemocyanin (Hcy)

Hemocyanin was isolated from the snail Busycon caniculatum. The snail's shell was broken over the area of the heart with a hammer. The heart was slit and the hemolymph allowed to empty into a beaker. Approximately 50-100 ml of hemolymph was obtained from each large Busycon. The hemolymph was then either filtered through Whatman No. 1 filter paper or centrifuged at low speed to remove the shell pieces and other debris. The hemocyanin was purified by either pelleting at 57,000 × g for 30 minutes in a Beckman L-2 ultracentrifuge and then resuspending in PBS, or by dialyzing exhaustively against PBS. Sometimes the hemocyanin was chromatographed on a 2.5 × 80 cm Agarose A 1.5 column.

The purified protein solution was forced through a 0.45 μ Millipore filter and stored at 4 °C in a sterile tube.

Concanavalin A (Con A)

Purified Con A was generously supplied by Dr. S. Brown. It had been purified according to her procedure (Brown, 1974).

Bovine antisera

All bovine antisera were kindly supplied by Dr. Clyde Stormont. These reagents are used for commercial red cell typing. The prefix "C" or "ST" before an antiserum number indicates the reagent is an isoimmune antiserum; the prefix "R" indicates the serum is a rabbit heteroimmune antiserum.

Anti-386 antiserum is a bovine antiserum prepared by Dr. Stormont by immunization of a cow with washed bovine red cells and leukocytes from a donor whose cells differ from the recipient's red cells only in the B complex.

Bovine IgG and IgM

Anti-J (ST 48) IgG was precipitated according to the procedure of Campbell et al. (1970) by adding saturated ammonium sulfate solution to 4 ml of whole antiserum to a final concentration of 33% saturated ammonium sulfate. The precipitate was resuspended in 0.017 M phosphate buffer, pH 6.3, dialyzed against that buffer, and chromatographed on a 10 × 1 cm DEAE-cellulose column equilibrated with the phosphate buffer according to the protocol of Blakeslee et al. (1971). Under these conditions the IgG passes through and most other serum substances remain on the column. The fractions were monitored at 280 mμ for protein and the appropriate fractions pooled and concentrated. This partially purified IgG was then applied to a 2.5 × 80 cm Sephadex G-200 column equilibrated with PBS, pH 7.2. Immunoelectrophoresis (IEP) and gel diffusion patterns were examined following each purification step to assay for purity.

Anti-J (ST 48) IgM was isolated according to the procedure of Campbell et al. (1970). Four mls of anti-J whole serum was added dropwise to 80 mls of a 2% boric acid solution. The precipitate was washed, resuspended in PBS and dialyzed against PBS at 4 °C. The partially purified IgM was applied to a 1.5 × 80 cm Agarose A 0.5

column. IEP's and gel diffusion patterns were examined at each step of the purification procedure to assay for purity.

Purified anti-J IgG and IgM were each concentrated (Amicon cell; XM-50 membrane) to the original serum volume (4 mls in each case) and stored at -20°C.

Rabbit antisera

Rabbit-anti-bovine-immunoglobulin (RABI): Rabbit R72 was immunized with 0.75 mg bovine IgG plus 0.75 mg bovine IgM purified from bovine normal serum as described above. The immunoglobulins were suspended in 0.5 ml of PBS, homogenized with 0.5 ml of complete Freund's adjuvant, and injected intradermally (i.d.) and/or subcutaneously (s.c.) at 4 - 10 sites on the rabbit's back. Five weeks later, R72 was desensitized with an intraperitoneal injection of 0.75 mg IgG plus 0.75 mg IgM in 0.5 ml PBS. Two days later, the same immunoglobulin dose was given intravenously, and after one week the rabbit was ear bled for 60 ml of blood. R72 was boosted several times by intraperitoneal injection followed 2 days later by an intravenous injection, followed one week later by bleeding. Immunoglobulin doses were the same as in the primary immunization schedule.

Rabbit-anti-hemocyanin (RAH): Rabbits were immunized with Busycon caniculatum hemocyanin (Hcy) by intravenous injections in the marginal ear vein of 0.5 ml of a 2 mg/ml solution of Hcy in PBS every other day for 7 injections. Rabbits were bled on days 7 and 8 following the last injection. Animals were boosted with 0.5 ml of a 2 mg/ml solution of Hcy per the schedule for the immunoglobulin boosts.

Goat-anti-rabbit-whole-serum (GARWS) and rabbit-anti-bovine-whole serum (RABWS) were purchased from Miles Laboratory (Kankakee, Ill.).

Rabbit-anti-bovine-immunoglobulin - hemocyanin reagent (RABI-Hcy)

Isolation of RABI: RABI was isolated from whole R72 immune rabbit serum by the immunoabsorbent technique of Avrameas and Ternynck (1969). Purified bovine IgG (150 mg) and IgM (50 mg) were polymerized with gluteraldehyde in the presence of lysozyme (800 mg; Sigma Chemical Co., St. Louis, Mo.). The washed, insoluble immunoabsorbent was stored at 4 °C in PBS with 0.01% azide. Specific RABI was prepared according to the procedure of Avrameas and Ternynck (1969), by absorbing whole RABI with the bovine immunoglobulin immunoabsorbent at pH 7.2 and eluting the specific antibody at pH 2.8. The isolated specific RABI was neutralized, dialyzed exhaustively against PBS, concentrated (Amicon cell, XM-50 membrane), and stored at -20 °C. Figures 1A and 1B show an immunoelectrophoresis slide and a gel diffusion pattern of the immunoabsorbent purified specific RABI as tested against bovine IgG, bovine IgM, GARWS, and RABWS.

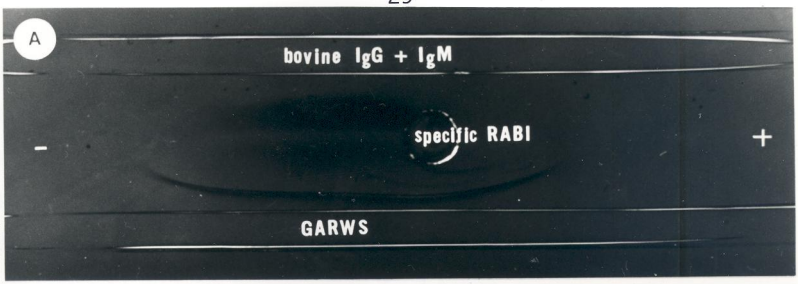
Conjugation of RABI and hemocyanin: RABI and hemocyanin were coupled according to the procedure of Avrameas (1969) with the following modifications: RABI was concentrated to 10 mg/ml (Amicon cell, XM-50 membrane) in PBS and hemocyanin to 70 mg/ml in PBS. 0.22 ml of a 0.5% solution of glutaraldehyde in PBS was added while vortexing vigorously to 1 ml of RABI plus 1 ml of hemocyanin. The

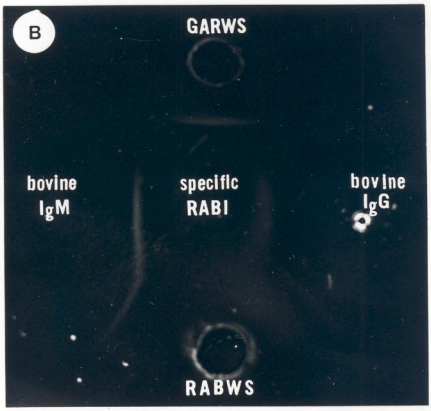
Figure 1

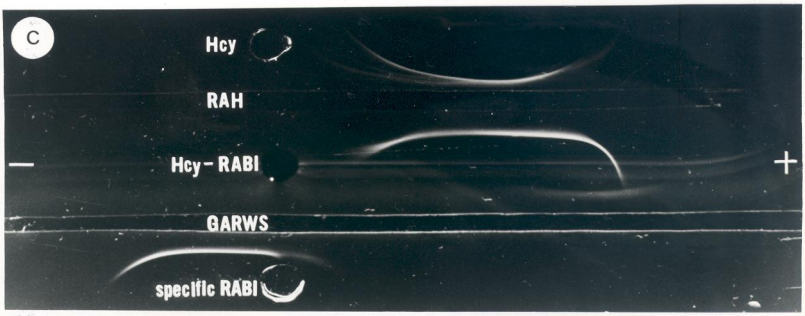
A. Immunoelectrophoresis slide of immunoabsorbant purified RABI diffused against bovine IgG and GARWS.

B. Gel diffusion pattern of purified specific RABI diffused against GARWS, bovine IgG, RABWS, and bovine IgM.

C. Immunoelectrophoresis slide of purified RABI-Hcy labeling reagent demonstrating covalent coupling of hemocyanin and RABI.


A
bovine IgG + IgM
specific RABI
-
+
GARWS


B
GARWS
bovine IgM
specific RABI
bovine IgG
RABWS


C
Hcy
RAH
—
Hcy - RABI
+
GARWS
specific RABI

glutaraldehyde plus protein solution was allowed to stand at room temperature for 45 minutes without agitation. 0.2 ml of a 2 M glycine solution in PBS was then added. Following a 15 minute incubation of the mixture at room temperature, the unpurified RABI-Hcy was dialyzed against PBS at 4°C. Three 1 liter dialyses were performed, changing the dialysis buffer every 2 - 3 hours.

Purification of RABI-Hcy: The coupled RABI-Hcy was purified by chromatographing on a 1.5 × 80 cm Agarose A 1.5 column equilibrated with PBS. The coupled RABI-Hcy came out in the break-through volume along with Hcy-Hcy polymers and free Hcy. Free RABI and RABI polymers chromatographed in the included volume. The appropriate fractions were pooled and concentrated (Amicon cell, XM-50 membrane).

The purified RABI-Hcy was forced through a 0.45 μ Millipore filter and stored in 1 ml aliquots in sterile tubes at 4°C. The concentration of the coupled labeling reagent was 12.5 mg protein/ml. Figure 1C shows an immunoelectrophoresis slide of the purified RABI-Hcy tested against RAH and GARWS. Note the altered mobility of the RABI in the RABI-Hcy conjugate, demonstrating the coupling of RABI and hemocyanin.

Cell labeling

Bovine red cells were washed 4 times and resuspended at a concentration of 6% by volume, in PBS made 0.01 M in magnesium and 0.001 M in calcium. Several drops of the red cell suspension

were applied to an ethanol cleaned glass coverslip for 10 minutes at room temperature. The coverslips were then washed by dipping 10 times in a succession of 3 beakers each filled with 25 ml of PBS. All additional washes were performed in the same manner. A monolayer of red cells adheres to the coverslip, and all further procedures were done with the coverslip-mounted cells. The cells were then prefixed with 0.1% glutaraldehyde in cacodylate buffer, pH 7.2 for 15 minutes at 4°C and washed with PBS as usual. Depending on the particular experiment the cells were then reacted with the desired labeling reagents. For example, cells being studied for the Z antigen would be treated first with a bovine anti-Z antiserum, washed, and incubated with RABI-Hcy. Cells labeled with Con A would be treated first with Con A, washed, then treated with hemocyanin. All bovine antisera were diluted with equal parts of PBS for labeling. Con A was used at 100 ug/ml. Hemocyanin was diluted to 1 mg/ml. RABI-Hcy was used at a concentration of approximately 12.5 mg protein/ml. Following the application of each reagent, the cells were washed with PBS.

After appropriate labeling, the cells were fixed with 2.5% glutaraldehyde in cacodylate buffer, pH 7.2 for 15 minutes at 4°C, followed by fixation in 2% osmium tetroxide for 15 minutes at 4°C. The samples were then dehydrated sequentially through 40%, 50%, 60%, 70%, 80%, 90%, 95%, and 100% ethanol. After remaining in

100% ethanol for a minimum of 15 minutes with at least one alcohol change, the samples were critical point dried from freon (Cohen et al., 1968).

Sequential double labeling technique

Samples labeled for two antigens were handled as usual except that, following the RABI-Hcy incubation, the cells were washed through PBS and another labeling cycle was done with a second antiserum. The remaining reagent treatments and fixation were as in the single label experiments. Cells labeled first with anti-R_2 antiserum, then RABI-Hcy, followed by anti-X_2 antiserum, followed by RABI-Hcy, and finally fixed, are labeled in the R_2, X_2 direction. Cells labeled first with anti-X_2, RABI-Hcy, anti-R_2, RABI-Hcy are labeled in the X_2, R_2 direction.

Preparation of cells for electron microscopy

The dried cells were rotary shadowed at an angle of 8° with 8 cm of 80% platinum, 20% palladium wire, diameter 0.008 inches, and carbon coated at a 90° angle.

The carbon replicas were removed from the glass coverslip as described by Smith and Revel (1972) by flotation on hydroflouric acid, followed by 3 water washes. The organic material was digested away from under the carbon replica by flotation on Clorox for approximately 45 minutes. The free replicas were then washed with distilled water and mounted on 300 mesh copper grids.

The grids were examined in a Phillips 201 transmission electron microscope operated at 60 kv.

Freeze-cleavage procedure

Red cells to be freeze-fractured were treated with the desired reagents as described previously, washed, and then fixed as a suspension in 2.5% glutaraldehyde in cacolylate buffer, pH 7.2, for one half to one hour. The fixed cells were washed 3 times with 25% glycerol in PBS and pelleted in the glycerol-PBS mixture. The fixed cells were allowed to sit in the glycerol-PBS mixture for a minimum of one hour prior to being freeze-fractured. For those experiments carried out at 4 °C, all procedures were done in an ice bath with buffers kept at 4 °C. Test tubes, antisera, and fixative were precooled on ice.

For freeze-cleavage, small drops of the fixed red cell pellet were rapidly frozen in freon 22, applied to a stage at liquid nitrogen temperature, and freeze-fractured in a Balzars instrument. The freeze-fractured surfaces were platinum-palladium shadowed at an incident angle of 45°, and carbon replicated in the Balzars instrument. The replicas were removed, mounted on grids, and examined as described previously in this thesis.

Lytic assay

Bovine antisera were assayed for the presence of hemolytic antibody by the procedure of Ferguson (1941). Tests were read qualitatively and scored negative, ±, trace, 1, 2, 3, or 4, with negative indicating no hemolysis, and 4 complete lysis. In most tests,

antisera were assayed at successive 2-fold dilutions. The titer of an antiserum was taken as the highest 2-fold dilution which still gave significant hemolysis (trace). Rabbit normal serum (RNS), neat, served as the complement source. Rabbits were bled at regular intervals for normal serum and the serum stored in 0.5 ml aliquots at -70 °C. RNS stored longer than 4 months was not used for complement. In all experiments reported here, normal serum from only 2 rabbits was used.

Antiserum absorption procedure

Absorptions were performed by mixing 1 ml of diluted antiserum (1:1 with PBS) with 1 ml aliquots of packed, washed red cells of the desired absorbing capacity. Exhaustive absorptions required 4 successive steps, centrifuging down the cells and transferring the supernatant to fresh cells at each step. Some antisera were absorbed and tested lytically following each red cell aliquot absorption. In these cases (e.g., the anti-R_2 and anti-X_2 sera), several drops of antiserum were removed for testing following each absorption and the remainder carried on to the next absorption.

For the J (ST 48) absorptions, a 1:1 dilution of ST 48 in PBS was absorbed as above with the following modifications: Cells plus antiserum were incubated at 23 °C for 10 minutes followed by a 45 minute incubation at 4 °C before centrifuging and transferring the supernatant to an aliquot of fresh cells.

Agglutination procedure for cells and antisera

Red cell agglutination was performed in microtiter plates (Cooke Engineering Co., Alexandria, Va.). 25 µl of antiserum was diluted sequentially in 25 µl of diluent (usually 1% RNS) using 25 µl dilution loops (Cooke Engineering Co.). 25 µl of a 2% suspension of red cells was added to each well. The tests were scored and photographed approximately 4 hours after being set up. Usually the plates were shaken twice during the 4 hour incubation.

Indirect agglutination (Coombs test)

The agglutination procedure was as described above except the red cells were precoated with antiserum before being placed in the test wells. 25 µl of packed, washed red cells were incubated for 15 minutes with 150 µl of antiserum diluted 1:1 with PBS. Following sensitization, the coated red cells were washed 3 times with excess PBS at 4°C and resuspended to a 2% suspension in PBS. RABI antiserum was diluted in the agglutination test as described above.

Agglutination procedure for cells and concanavalin A

Red cells were scored for agglutination according to the procedure of Hines (1971), except that the tests were run in microtiter plates and 25 µl of Con A and 25 µl of a 2% suspension of red cells were incubated per test well.

Cell trypsinization

Cells were trypsinized according to the procedure of Hines et al. (1972) with the following modifications: One ml of packed, washed red cells was incubated in a 37°C water bath for 15 minutes

with 5 ml of a 0.1% trypsin solution in PBS. Trypsinized cells were washed 4 times with excess PBS.

Immunoelectrophoresis and gel diffusion

Immunoelectrophoresis (IEP) was performed in 1% agar using 0.09 M veronal buffer, pH 8.6. Slides were electrophoresed at constant current, 6 ma per slide, for 2.5 hours. Substances were allowed to diffuse for 16 - 24 hours for both IEP's and gel diffusions. Slides were either photographed with back indirect lighting, or were dried and stained with Buffalo blue-black.

Electron microscope data analysis

Counting: For each experiment 10 - 18 labeled cells from widely separate areas on a grid were photographed. The negatives were printed as 8 × 8 inch photographs and negative and photograph magnifications were always constant. Hemocyanin molecules were counted in a unit area measured out by a plexiglass square randomly placed on the cell surface, and expressed as Hcy/unit area ("mean labeling value"). Within a related set of experiments a constant size area was always counted; however, some sets of experiments were counted with different sized plexiglass squares. Therefore, unless indicated, comparisons in labeling values should not be made from experiment to experiment. Wherever possible, photographs were taken, coded, and counted without knowledge of the particular experiment.

Statistical methods: The mean of each experiment was calculated by summing the hemocyanin counts for each photograph and

dividing by the total number of photographs counted. The standard error was determined by the following formula:

$$\frac{\sqrt{\Sigma(\bar{x} - x_i)^2}}{\text{total } x_i\text{'s}}$$

The standard error calculation was performed on either an Olivetti programmable calculator or on the Caltech PDP-10 computer using standard error programs kindly written by Dr. R. C. Rosenberg. Significance was measured by the value of

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(SE_1)^2 + (SE_2)^2}}$$

Values for p were calculated to the nearest 0.01.

Isolation of bovine peripheral leukocytes.

Blood samples were diluted with an equal volume of Alsever's solution, centrifuged, and the buffy coat containing the leukocytes removed and diluted with an equal volume of minimal essential medium, Earle's salts (MEM, Gibco). Peripheral leukocytes were isolated from this suspension by centrifugation in Ficoll-Isopaque gradients (Thorsby and Bratlie, 1970). Isolated leukocytes (including platelets) were washed 3 times with excess cold MEM and counted in a hemocytometer.

Leukocyte microcytotoxicity assay

Amos' microcytotoxicity assay (Amos et al., 1969) was modified slightly for use in the bovine leukocyte system as follows: MEM was

used as diluent. In the one-step assay, target leukocytes at a concentration of 2×10^6 cells per ml were incubated at room temperature with dilutions of antiserum for 30 minutes prior to addition of complement. Target cells, antiserum, and complement were then incubated at 37°C for 1 hour. In the Amos two-step assay (Amos et al., 1969) the cell concentrations were as above. Antiserum plus cells were incubated for 15 minutes at room temperature. An approximately 15 microliter drop of diluent was then added per test well and the plates were flicked and complement added to each test well. The plates were then incubated at 37°C for 1 hour. In both the two-step and one-step tests, target cell killing was recorded as per cent dead cells. Tests were scored using a Zeiss inverted phase microscope.

Rabbit normal serum, used unabsorbed at a 1:10 dilution, served as the complement source.

Serum and complement controls for all reagents tested ranged from 3 to 15% dead cells, with most control values at about 10% killing.

CHAPTER I

GENE DOSAGE AND ANTIGENIC EXPRESSION ON THE CELL SURFACE OF BOVINE RED CELLS

Introduction

If one allele codes for one product, one would expect an individual homozygous for that allele to have twice as much of that specific product as a heterozygote. Several attempts have been made to correlate the zygosity of individuals with the quantitative expression of gene products. Various enzyme assays are quantitative, and heterozygous individuals can be differentiated from homozygous individuals (Knox and Messinger, 1958; Beutler et al., 1964; Schneider et al., 1968; Lieberman, 1969; Beutler and Kuhl, 1970; Schulman et al., 1970).

The quantitative expression of blood cell antigens has also been studied. Early experiments of this kind were reported by Owen (1948 and 1960) and described the zygosity effects displayed on rat red cells. Dealing with the C and D rat red cell antigens, he found that an anti-C reagent displayed the same titer with CC and CD cells, but that the mean clump size of the agglutinated cells differed considerably. CC cells showed clump sizes almost exactly twice as large as CD cells. The same observation was made for DD and CD cells with an anti-D reagent. Owen also noted that homozygous cells absorbed approximately twice as much antibody as heterozygous cells for the particular antigens under study. At appropriate reagent dilutions the zygosity of a set of cells could be predicted from their quantitative agglutination clump size.

Agglutination assays of human red cells for the Duffy antigens have revealed a bimodal distribution of unrelated individuals typed for

the Fy^a antigen (Race et al., 1953). Two doses of the $\underline{Fy^a}$ allele produce more Fy^a antigen than does a single dose. Similar results were also found in the Kidd system using the anti-globulin test (Crawford et al., 1961). Most anti-Jk^a sera reacted more strongly with Jk^a homozygotes than with Jk^a heterozygotes. A marked zygosity effect is also found in the MN system with MM individuals reacting more strongly in an indirect anti-globulin test with anti-M than MN individuals; cells of NN individuals do not react at all (Race and Sanger, 1962). The effect for the s allele is so strong that one can predict the S genotype of an individual from his reaction with an anti-s antiserum (Levine et al., 1951; Race and Sanger, 1962). In cows there is a distinct effect in the F-V system as detected by red cell hemolysis. Cells from individuals homozygous for the F antigen (FF) are lysed more rapidly by anti-F than cells from heterozygous (FV) individuals (Stormont, 1952).

The above examples have all been demonstrated indirectly by serological reactions, i.e., agglutination or hemolysis. More direct measurements of antigen quantity have also been performed. Using ^{131}I and ^{125}I labeled anti-Rh_0(D) antibody, Masouredis (1960, 1964; Masouredis et al., 1960, 1962, 1964) found a bimodal distribution of bound anti-Rh_0(D). The correlation, however, was not complete because there were cases of Rh_0(D) positive cells which failed to pick up the antibody. Additional studies by Masouredis (1967) were conducted on families and it was found that within a family the father had either twice as much, or

the same quantity of ^{125}I labeled anti-Rh_0(D) uptake as his children, with some exceptions. However, the zygosity correlation with the quantitative expression of the Rh_0(D) antigen only held up within families, as one would expect based on the serological complexity of the Rh antigens and antisera.

The Z system of cattle is a good system in which to study the correlation of zygosity and antigenic expression because it is a single factor system uncomplicated by obvious antigenic complexity. (Stormont, 1952). Animals are homozygous (Z/Z), heterozygous (Z/-), or negative (-/-) for the Z antigen. Bovine antisera are available which in a complement-mediated lytic assay can distinguish homozygous cells from heterozygous cells and from cells negative for the Z antigen. These reagents enable positive Z genotype identification.

The J substance of cattle is a soluble serum substance which secondarily adsorbs to the red cell surface (Stormont, 1949). The antigen is a protein-associated glycolipid (Slomiany and Horowitz, 1971; Slomiany et al., 1973). The Lewis substance of humans (Sneath and Sneath, 1955) and the R substance of sheep (Rasmusen, 1962) are also serum antigens which secondarily coat red cells, and which behave similarly to the J substance of cattle.

Three phenotypes have been described for cows according to how much J antigen their cells and serum possess. J^{cs} animals have J substance both on their cells and in their serum; J^{s} cows are reported to have J antigen only in their serum; and j^{a} animals lack J in their

serum and on their red cells, as measured by complement-mediated red cell lysis and by inhibition of hemolysis (Stone and Irwin, 1954).

J negative cells can be coated in vitro with J substance by incubating the j^a cells with plasma from J positive animals (Stormont, 1949). The in vivo coating of J negative cells by J substance is demonstrated in twin cattle experiments. Fraternal twinning is frequently found in cows, and the twins usually have identical blood types, while full sibs which are not twins rarely have the same cellular antigens (Owen, 1945). Using a differential lysis technique Owen (1945 and 1946) demonstrated that those sets of twins with identical blood types have a mixture of erythrocytes in their circulation. The mosaicism is the result of the exchanging of the hematopoetic stem cells between the twins via vascular anastomoses formed during embryogenesis. In the case of the soluble J antigen, however, Stormont (1949) showed that in twins which displayed red cell mosaicism it was not infrequent to find the J antigen on all of the red cells of one twin, while the cells of the co-twin completely lacked the J antigen. The soluble J substance is therefore produced by tissues distinct from those which produce red cells, and can coat any bovine red cells in vivo.

Some bovine red cells display a very low J titer and require large amounts of anti-J antibody to lyse them, while other cells have high J titers and require only minute amounts of antibody for cellular lysis (Stormont, 1949). This kind of variation is probably the result of the action of multiple alleles at the J locus (Conneally et al., 1962).

In this report, direct EM labeling techniques have been used to study quantitatively the cell surface expression of the Z and J antigens. Individuals homozygous (Z/Z) for the Z gene are found to display approximately twice as much antigen on their red cell surfaces as individuals heterozygous (Z/-) for the gene. Negative (-/-) animals show only background levels. The J system reflects the relationship between the quantitative expression in the serum of a soluble glycoprotein and its secondary quantitative absorption on the red cell membrane. It is found that the J titer of a cell corresponds to the quantity of J antigen present on that cell's membrane. During the course of this study, evidence was obtained for an additional antigen on a population of J negative cells which does not display lytic or direct hemagglutinating activity, but is revealed in EM studies and can be serologically detected by an indirect or Coombs reaction (Coombs et al., 1945) with anti-immunoglobulin.

Materials and Methods

The Z and J antigens were visualized for electron microscopy according to the labeling procedures detailed in the general Materials and Methods section of this thesis. Cells were prepared, hemocyanin molecules counted, and significance tests applied as described. Cell populations from 11 different animals were prepared for electron microscopy. Of these 11 animals, the genotypes of 4 were known at the time the labeling was done (#1475, #1499, #1502, and #1559), while the genotypes of the remaining 7 animals were unknown (coded samples

#1, #2, #3, #4, #5, #7, and #8). Most experiments were repeated a minimum of 2 times, although only one set of values is reported because all results were consistent within experimental error.

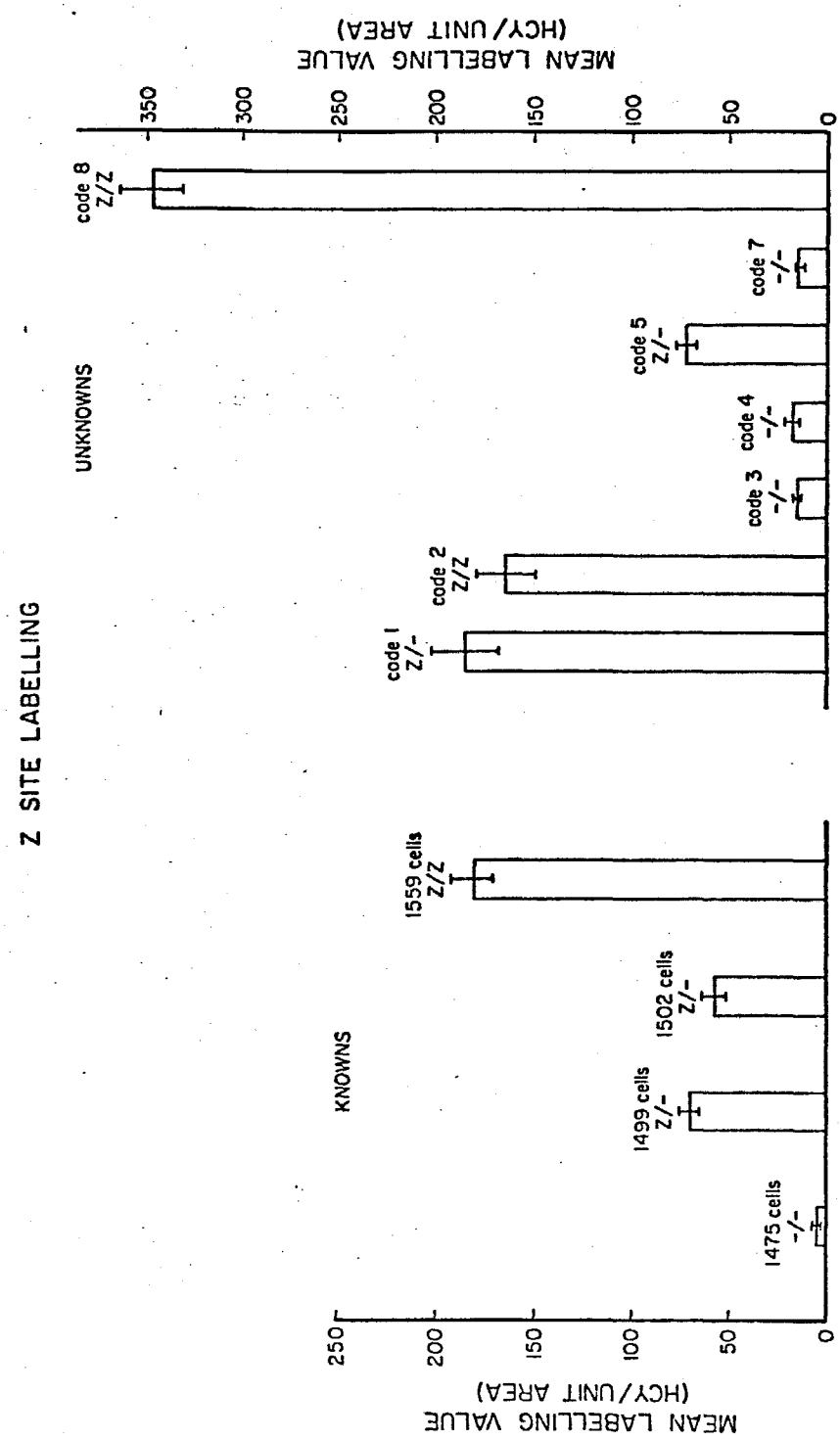
Results

The mean labeling values and standard errors for cells labeled for the Z antigen are graphed in figure 1. The cells of known genotype are plotted on the left side of the figure, and the cells of unknown genotype on the right side. The ordinate is the mean labeling value or the number of hemocyanin molecules counted per arbitrary unit area of cell surface (see Materials and Methods).

The labeling values fall into 4 classes: 1) those cells with less than 8 hemocyanins per unit area; 2) those cells with 57 through 69 hemocyanins; 3) those cells with 164 through 183 hemocyanins; and 4) a cell with 346 hemocyanins. The mean labeling values for these classes are all significantly different from each other ($p < 0.01$). Those cells with less than 8 antigenic sites are genotypically negative for the Z antigen. The cells clustering around 60 sites are heterozygous for the Z specificity. Of the cell populations displaying approximately 170 sites per unit area, two are homozygous for the Z antigen, while one is heterozygous. The high labeling cell population is from a cow homozygous for Z.

Excluding code #1 and code #8 samples, cells heterozygous for the Z antigen display approximately half as much antigen as homozygous cells, while the cells negative for the Z gene show only background levels

Figure 1. Mean EM labeling values for the Z antigen on eleven cell populations.


Z SITE LABELLING
MEAN LABELLING VALUE
(HCY/UNIT AREA)
0
50
100
150
200
250
KNOWNS
1475 cells
-/-
1499 cells
Z/-
1502 cells
Z/-
1559 cells
Z/Z
UNKNOWNS
code 1
Z/-
code 2
Z/Z
code 3
-/-
code 4
-/-
code 5
Z/-
code 7
-/-
code 8
Z/Z
MEAN LABELLING VALUE
(HCY/UNIT AREA)
0
50
100
150
200
250
300
350

of label. The two cell populations which do not fit this scheme, code #1 and code #8, show more label on their surfaces than one would expect from their Z genotypes. These two cell populations will be dealt with in the discussion section.

Figure 2 shows typical electronmicrographs of replicas of bovine red cells labeled for the Z antigen. Figure 2A is of a homozygous (Z/Z) cell, figure 2B of a heterozygous (Z/-) cell, and figure 2C of a cell negative for Z (-/-).

EM binding studies were performed to determine the number of J antigenic sites per cell on red cells from 9 different animals. These cells have J titers ranging from 0 to 1024. The J titer refers to the reciprocal of the highest antiserum dilution (in a series of doubling dilutions) which will lyse the cells in the presence of rabbit complement. Therefore those cells with a J titer of 128 require a higher antibody concentration to lyse them than those cells with a J titer of 1024.

Table 1 shows the mean labeling values and the corresponding J titers of cells from 9 animals. At the time of hemocyanin labeling the J titers of 5 of these cells were unknown (#1566, #1513, #1570, #1441, and #1428), while the J titers of the remaining 4 cells (#1475, #1499, #1559, and #1502) were known. The labeling values fall into 3 classes: 1) those cells with 10 or fewer hemocyanins per unit area; 2) those cells with 38 to 51 hemocyanins; and 3) those cells with 69 or 102 hemocyanins. The cells with 10 or less hemocyanins are genotypically negative for the J antigen. The cells displaying 38 to 51 hemocyanins show low J titers,

Figure 2

(A) Electronmicrograph of a replica of a homozygous (Z/Z) cell labeled with anti-Z (ST 54), RABI-Hcy.

(B) Electronmicrograph of a replica of a heterozygous (Z/-) cell labeled with anti-Z (St 54), RABI-Hcy.

(C) Electronmicrograph of a replica of a Z negative (-/-) cell labeled with anti-Z (ST 54), RABI-Hcy.

Magnification: 18,500×.

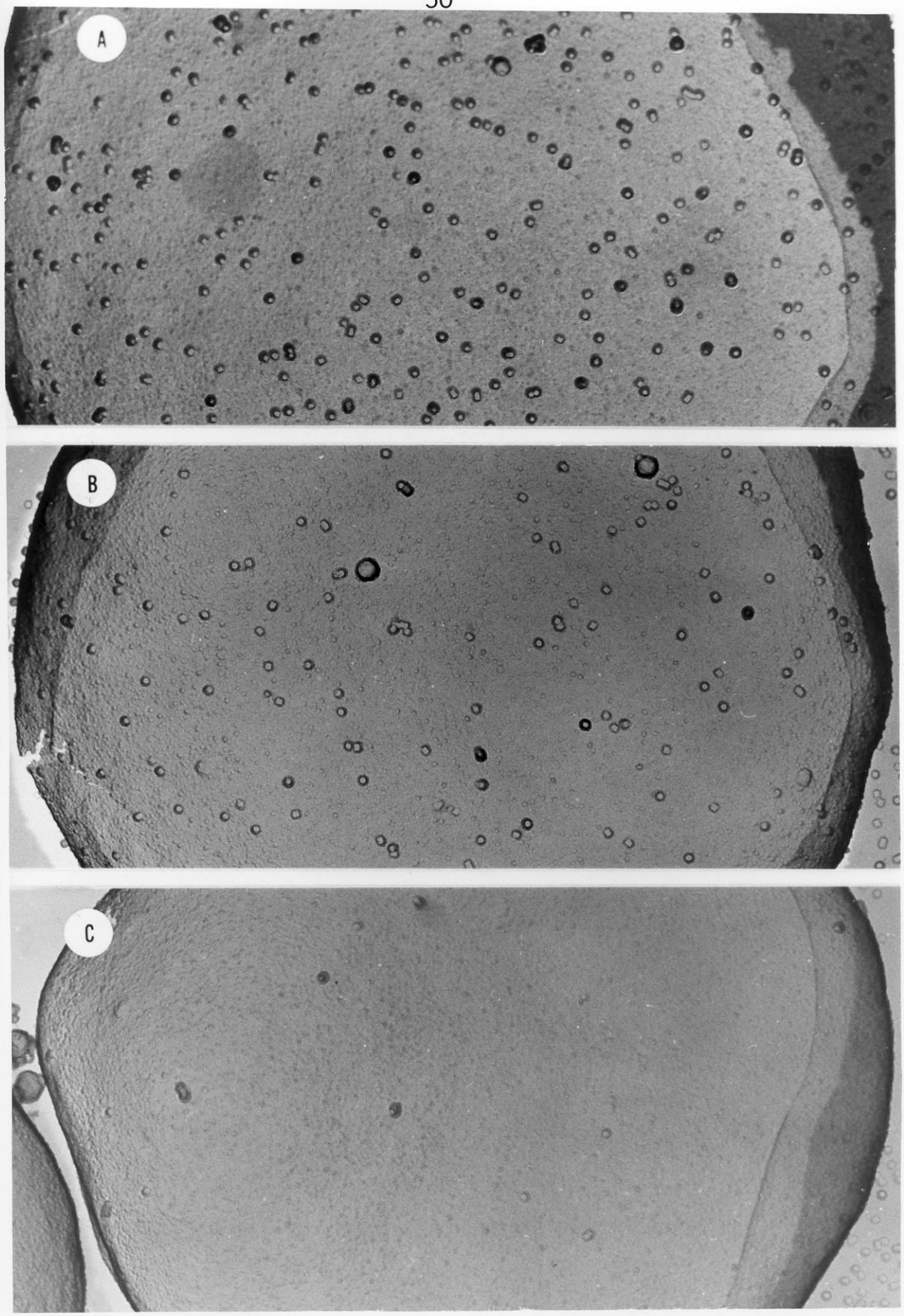

A
B
C

Table 1

Mean Labeling Values and J Titers for 8 Cell Populations

Target Cells	EM Labelling Value with anti-J (ST48) (Hcy/unit area) ± SE	J Titer^{-1}
1566	8 ± 1	-
1513	9 ± 1	-
1475	6 ± 1	-
1559	38 ± 2	64-128
1570	42 ± 1	-
1502	51 ± 4	128-256
1441	69 ± 4	1024
1428	102 ± 4	1024

except for #1570 cells which are genotypically negative for the J antigen. The cells with 69 or 102 hemocyanins have high J titers.

Setting aside for the moment the #1570 cells, the remaining 8 cell types display on their surfaces amounts of J antigen which are directly related to the antiserum dilution necessary to lyse those cells. That is, cells with fewer antigenic sites require a higher concentration of antibodies for complete lysis in unit time. Figure 3 shows electron-micrographs of labeled cells ranging in J titers from 0 to 1024.

Cells from cows #1428 and #1441 display the same lytic titer, but have different numbers of antigenic J sites by EM. Because of the different antigen binding capacities of IgG and IgM antibodies, the following experiments were performed to examine the labeling values for the two antibody classes on #1428 and #1441 cells. The anti-J ST 48 antiserum was fractionated into its IgG and IgM components and these immunoglobulin fractions were assayed serologically and by electron microscopy for their lytic and EM binding activities.

Bovine IgG and IgM from the anti-J ST 48 antiserum were purified as described in Materials and Methods. The resulting IgG and IgM fractions were assayed by immunoelectrophoresis (IEP) for purity. Figure 4 shows the IEP slides of the purified IgG and IgM, respectively. The anti-J IgG is contaminated with transferrin, and the IgM is contaminated with alpha-2 lipoprotein, but neither of these contaminations should affect the lytic activity of the immunoglobulin. The IgG preparation is not contaminated with IgM, and the IgM preparation is not contaminated with IgG.

Figure 3. Electronmicrographs of replicas of bovine red cells labeled with anti-J (ST48), RABI-Hcy.

(A) #1556 cell; J titer^{-1}: -

(B) #1502 cell; J titer^{-1}: 128-256

(C) #1441 cell; J titer^{-1}: 1024

(D) #1428 cell; J titer^{-1}: 1024

Magnification: 11,250×.

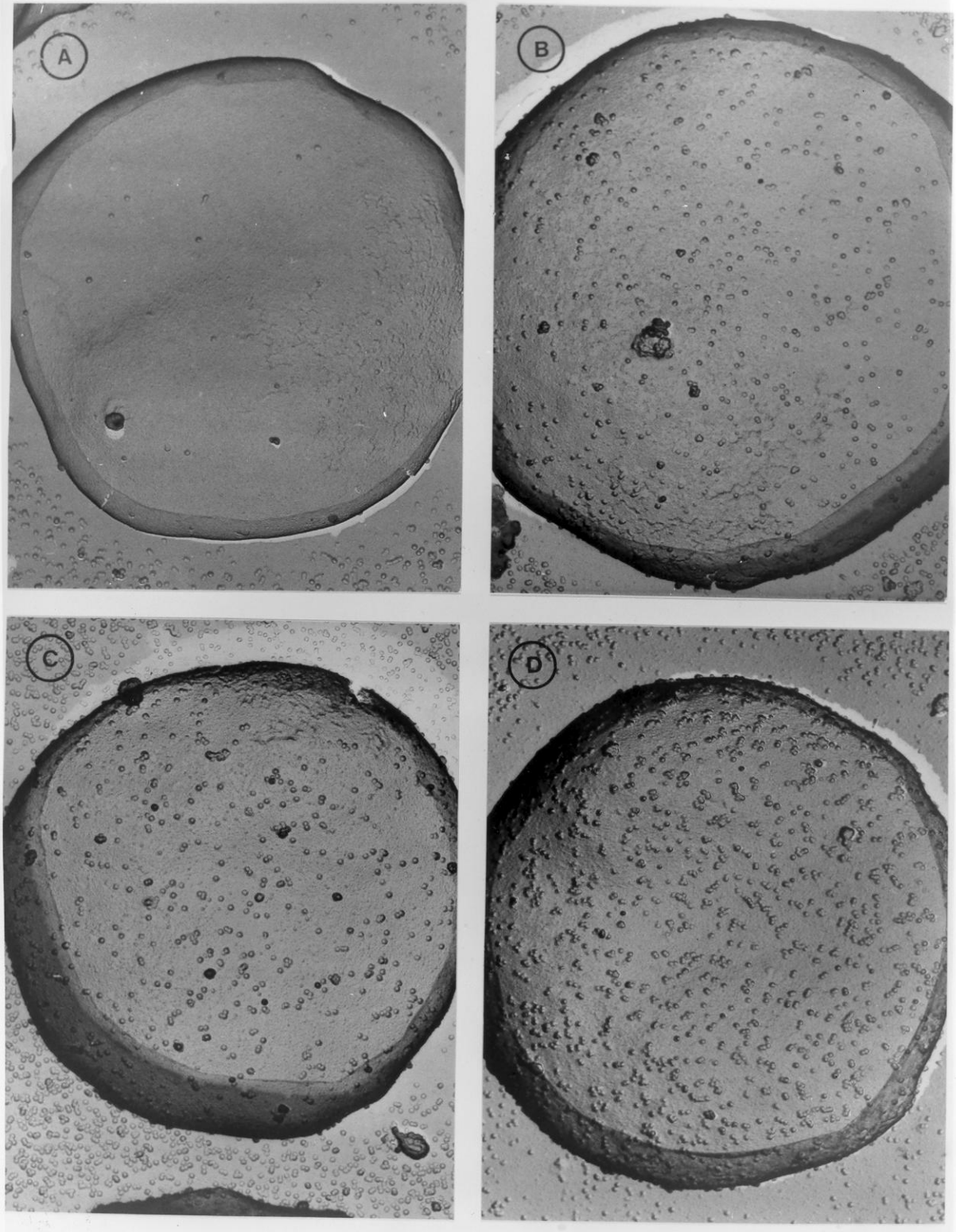
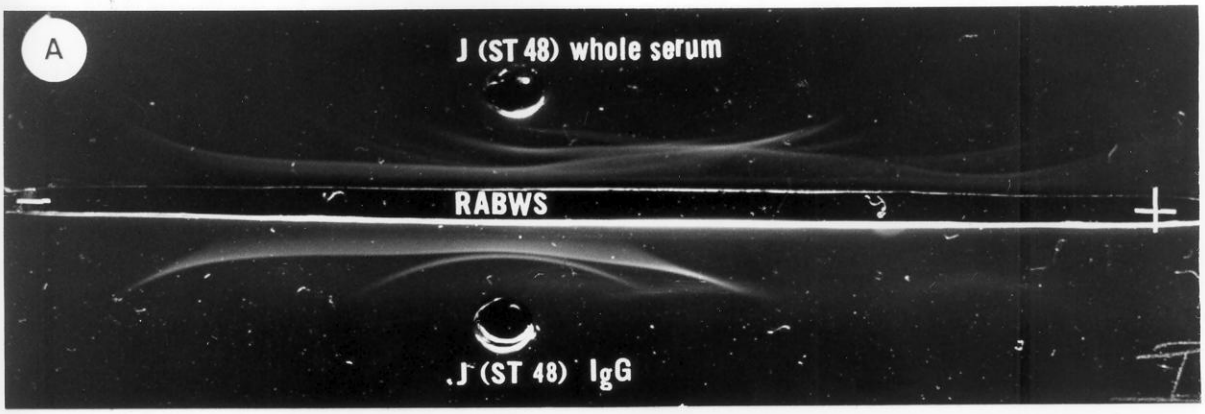
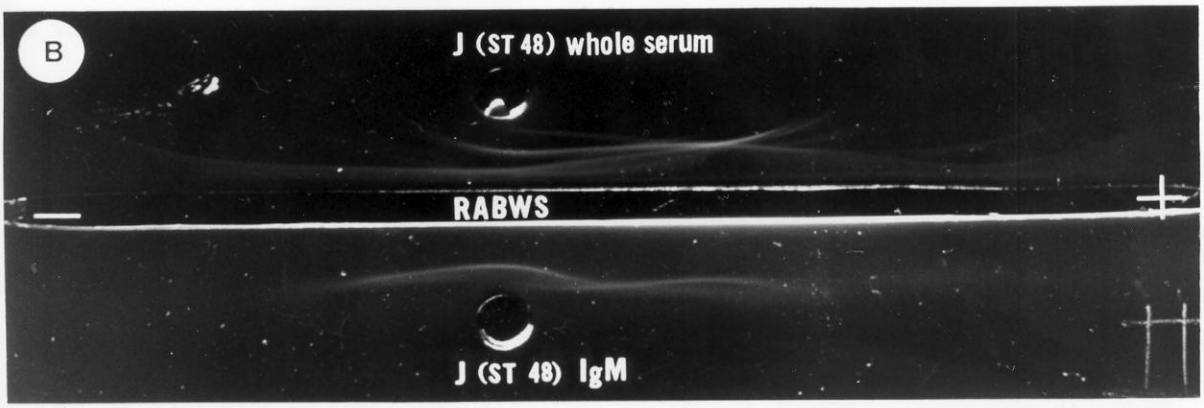

A
B
C
D

Figure 4. Immunoelectrophoresis of purified anti-J (ST48) IgG and anti-J (ST48) whole serum (A), and anti-J (ST48) IgM and anti-J (ST48) whole serum (B) diffused against RABWS.


A
J (ST 48) whole serum
RABWS
J (ST 48) IgG


B
J (ST 48) whole serum
RABWS
J (ST 48) IgM

The hemolytic assay of the purified anti-J IgG and IgM singly, pooled, and including whole anti-J ST 48 antiserum is shown in Table 2. Red cells from 6 different animals representing assorted J titers and EM binding of anti-J antibody were tested. The hemolytic activity seems to be divided between the IgG and IgM fractions, with more activity in the IgG fraction. Note that the pooled purified anti-J IgG and IgM do not approach the activity of the whole anti-J antiserum. This result is probably due to loss of antibody activity during the purification procedure. Cells #1441 and #1428 are lysed to the same extent by the purified anti-J IgG and IgM.

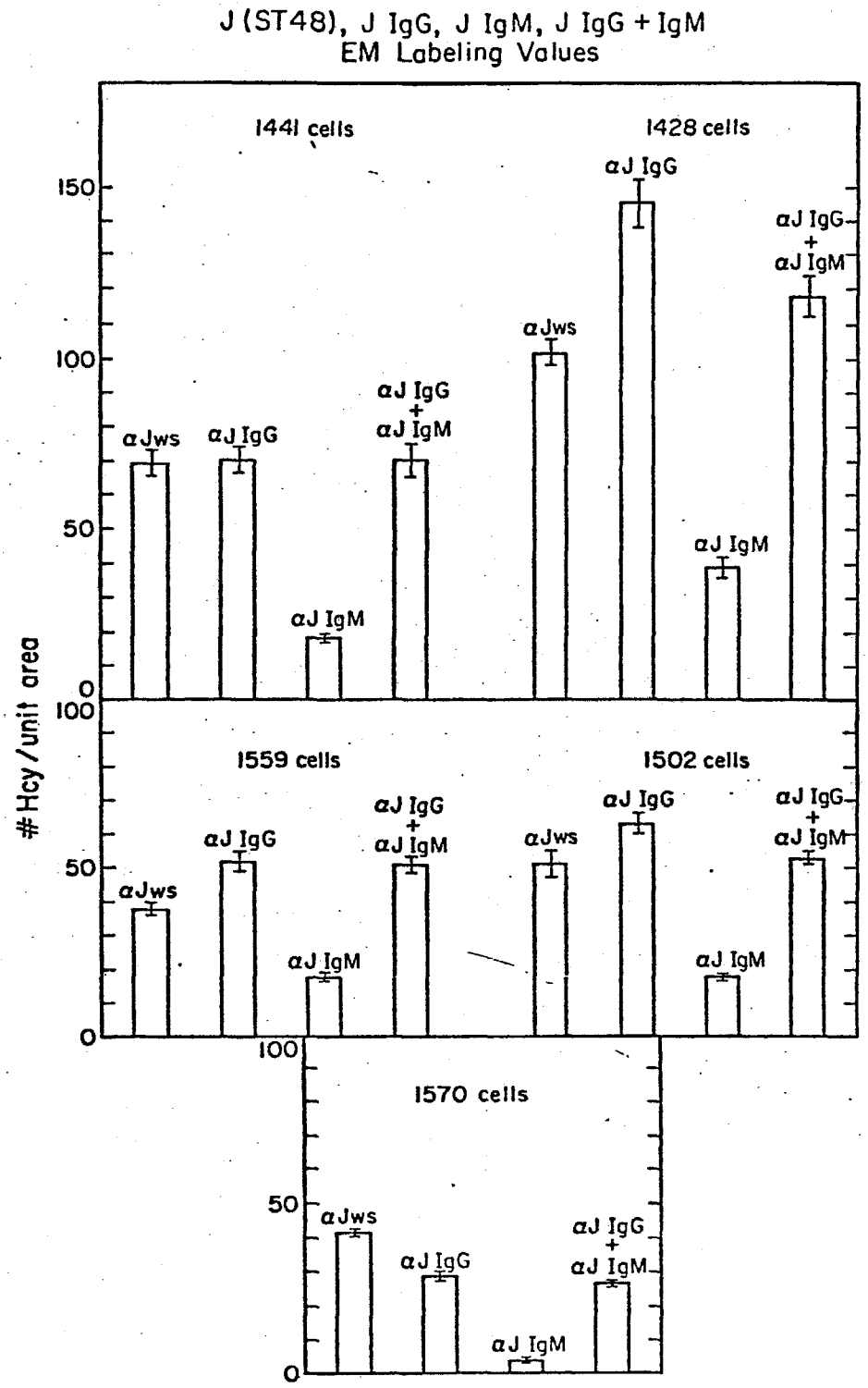
The purified immunoglobulin fractions were also used to label bovine red cells with the RABI-Hcy reagent and determine the number of antigenic J sites. The results of these EM experiments are shown in figure 5. As previously discussed, the quantity of J antigen on the surface of a given population of red cells is inversely related to the amount of antibody necessary to lyse that cell population. In all 5 cases examined, more anti-J IgG is taken up by the target cells than IgM; this quantitation is in the same direction as the lytic results. Also, #1502 and #1559 cells label approximately the same with the IgG and IgM fractions; the value for #1502 cells is slightly, but not significantly, higher. The anti-J whole serum labeling value is significantly higher for #1502 cells as compared to #1559 cells. #1428 cells again label more heavily than #1441 cells; the effect is very pronounced with the anti-J IgG fraction.

Table 2

Lytic Titers of Purified J (ST48) IgG, IgM, IgG + IgM, and J (ST48) Whole Serum for 6 Cell Populations

Cells	Anti-J (ST48) IgG $Titer^{-1}$	Anti-J (ST48) IgM $Titer^{-1}$	Anti-J (ST48) IgG + IgM $Titer^{-1}$	Anti-J (ST48) Whole Serum $Titer^{-1}$
1570	-	-	-	-
1566	-	-	-	-
1559	2	-	2	64-128
1502	8	-	4	128-256
1441	64	16	128	1024
1428	64	16	64	1024

Figure 5. EM labeling values for purified anti-J (ST48) IgG, IgM, IgG + IgM, and anti-J (ST48) whole serum.


J (ST48), J IgG, J IgM, J IgG + IgM
EM Labeling Values
#Hcy/unit area
1441 cells
1428 cells
1559 cells
1502 cells
1570 cells
αJws
αJ IgG
αJ IgM
αJ IgG + αJ IgM
0
50
100
150

In no case does the sum of the labeling values for the purified anti-J IgG and IgM applied separately equal the labeling value when the two reagents are applied to the target cells at the same time. Note in addition that the anti-J IgG labeling values for # 1428, #1559, and #1502 cells are higher than the anti-J whole serum labeling values.

One set of cells, #1570 cells, which are genotypically negative for the J antigen, binds hemocyanin well above background level, but labels less with the anti-J IgG fraction than with whole anti-J antiserum. These cells also show background levels of anti-J IgM binding while the other 4 cell types (which are J positive) display varied levels of anti-J IgM labeling all well above the background level.

Absorption of the anti-J whole serum with #1570 cells does not reduce the direct hemolytic titer of the J antiserum against J positive cells. Given the apparent serological absence of the J antigen on #1570 cells and the electronmicroscopic positive reaction of these cells with the anti-J antiserum, absorption experiments were conducted to fractionate the anti-J antiserum and separate these apparently different properties.

Anti-J antiserum was absorbed (see Materials and Methods) sequentially 4 times with #1570 cells. The resulting absorbed antiserum, J[1570], was tested by electron microscopic labeling and by direct hemagglutination assay for its activity on various target cells. All direct agglutination tests with the J[1570] reagent and anti-J whole

antiserum were negative as assayed against #1441, #1428, #1502, #1566, and #1570 cells.

The EM labeling results using the J[1570] reagent as compared to anti-J whole serum are shown in Table 3. The anti-J antiserum absorption with #1570 cells is complete because the background labeling value of #1570 cells with the J[1570] reagent is 10. The absorbed reagent and anti-J whole serum bind equally well to #1428 and #1566 cells. However, a quantitative difference is present in the binding of anti-J whole serum and J[1570] on #1570, #1441, #1559, and #1502 cells. The difference between the J whole serum and the J[1570] binding for these cells is significant ($p < 0.01$). These results indicate that #1570 cells possess an antigen which is reactive with antibodies in the whole anti-J antiserum and which are not directed against the J antigen. Cells #1441, #1559, and #1502 also appear to have this antigen, but #1428 and #1566 cells do not have the antigen.

Although #1570 cells were serologically negative by hemolysis and direct agglutination, an indirect or Coombs test demonstrated the presence of antibody on #1570 cells following incubation with the anti-J antiserum, ST 48. In this test the cells were preincubated with the ST 48 reagent and then reacted with RABI. If antibody had been absorbed to the cell surface the cells would be agglutinated by the RABI; however, if no antibody were bound to the target cells the RABI would not react with the red cells.

Table 3

Computed EM Labeling Values and Significance for Anti-J (ST 48) Labeling Value Minus Anti-J[1570] Labeling Value

Target Cells	Labeling Value with Anti-J Minus Labeling Value with Anti-J[1570]	Significant Difference Between J and J[1570] Labeling Values
1570	23	+
1441	23	+
1428	5	-
1559	15	+
1502	14	+
1566	1	-

Table 4 shows the results of the Coombs test on J positive, J negative, and #1570 cells. All J positive cells (#1502, #1559, #1441, and #1428) pretreated with J ST48 can be agglutinated by the indirect procedure. #1566 cells, which are J negative, are not agglutinated in the Coombs test. #1570 cells, which by direct methods are negative with the J ST 48 reagent, are positive in the indirect test.

According to our EM results, #1428 cells do not possess the new antigen. Therefore, absorption of the J ST48 reagent with #1428 cells should produce a reagent specific for the new antigen. Such a reagent, J[1428], was produced and the activity as tested by indirect hemagglutination is shown in Table 5. The absorption is complete as seen by the absence of J[1428] reactivity with the #1428 absorbing cells. The specific reagent indirectly agglutinated #1441, #1502, #1559, and #1570 cells, showing that these cells possess the new antigen, as previously indicated by the EM labeling studies using the J[1570] reagent.

Discussion

J Site Labeling

The mean labeling values of hemocyanin binding by EM and the serological J titers as determined by hemolytic assay suggest that for the J antigen the greater the quantity of antigen on the red cell surface, the smaller the amount of antibody necessary to lyse the cell.

Of a total of 9 cell populations studied by EM, two cell types do not at first glance fit the above pattern. #1441 and #1428 cells have the

Table 4

Indirect Agglutination Test of 6 Cell Populations

Pretreated with the Anti-J (ST 48) Reagent

Cells	J Phenotype	Cells Pretreated with	Serum	Agglutination
1566	-	Anti-J (ST 48)	RABI	-
1570	-	Anti-J (ST 48)	RABI	+
1502	J	Anti-J (ST 48)	RABI	+
1559	J	Anti-J (ST 48)	RABI	+
1441	J	Anti-J (ST 48)	RABI	+
1428	J	Anti-J (ST 48)	RABI	+
1566	-	Anti-J (ST 48)	RNS	-
1570	-	Anti-J (ST 48)	RNS	-
1502	J	Anti-J (ST 48)	RNS	-
1559	J	Anti-J (ST 48)	RNS	-
1441	J	Anti-J (ST 48)	RNS	-
1428	J	Anti-J (ST 48)	RNS	-
1566	-	--	RABI or RNS	-
1570	-	--	RABI or RNS	-
1502	J	--	RABI or RNS	-
1559	J	--	RABI or RNS	-
1441	J	--	RABI or RNS	-
1428	J	--	RABI or RNS	-

RABI: rabbit-anti-bovine-immunoglobulin.

RNS: rabbit normal serum.

Table 5

Indirect Agglutination Tests of 6 Cell Populations Pretreated with the Anti-J[1428] Reagent

Target Cells	J Phenotype	Cells Pretreated with	Serum	Agglutination
1566	-	Anti-J[1428]	RABI	-
1570	-	Anti-J[1428]	RABI	+
1502	J	Anti-J[1428]	RABI	+
1559	J	Anti-J[1428]	RABI	+
1441	J	Anti-J[1428]	RABI	+
1428	J	Anti-J[1428]	RABI	-
1566	-	Anti-J[1428]	RNS	-
1570	-	Anti-J[1428]	RNS	-
1502	J	Anti-J[1428]	RNS	-
1559	J	Anti-J[1428]	RNS	-
1441	J	Anti-J[1428]	RNS	-
1428	J	Anti-J[1428]	RNS	-
1566	-	--	RABI or RNS	-
1570	-	--	RABI or RNS	-
1502	J	--	RABI or RNS	-
1559	J	--	RABI or RNS	-
1441	J	--	RABI or RNS	-
1428	J	--	RABI or RNS	-

Anti-J[1428]: J (ST 48) absorbed with 1428 cells.
RABI: rabbit-anti-bovine-immunoglobulin.
RNS: rabbit normal serum.

same lytic titer but different EM binding values; and #1570 cells, which are genotypically negative for J, take up label by EM.

The IgG and IgM fractionation studies were performed to investigate the apparent discrepancy between EM binding and titer for #1441 as compared with #1428 cells. These studies do not explain the apparent discrepancy, but they do introduce two interesting points concerning the J antigenic sites on the red cell surface. On all of the cells studied, the combined single labeling values for purified anti-J IgG and IgM sum to less than the labeling value found when anti-J IgG and IgM are applied to the red cells at the same time. This observation suggests that anti-J IgG and IgM are binding to the same J antigenic sites; that is, the two immunoglobulin classes do not bind to unique sites on the red cell surface. The binding of specific antibody from whole serum therefore probably involves a competition between IgG binding and IgM binding. Because of the different antigen binding valencies of IgG and IgM, one may observe slightly aberrant EM binding values depending on which class of antibody is bound more quickly and tightly. The different binding values for #1441 and #1428 cells, despite their identical lytic titers, may be due to the greater binding of anti-J IgG to the #1428 cells than to the #1441 cells.

In 3 of the 4 cases of labeling J positive cells, the labeling value for the anti-J IgG fraction is higher than the anti-J whole serum labeling value. This observation also suggests that in labeling using whole anti-serum there is an interplay between IgG and IgM binding. Therefore, the anti-J IgG labeling value is probably a closer approximation

to the actual number of J antigenic sites than is the anti-J whole serum labeling value. Because of the different lytic properties of IgG and IgM antibodies, the lytic efficiency of an antiserum will depend not only on the quantity of the specific antibody, but also on the ratio of the classes of specific antibody.

The observation that #1570 cells by direct serological methods are negative for the J antigen but do label with the anti-J whole antiserum by EM implies that this antiserum contains antibodies directed against an antigen other than the soluble J antigen. The electron microscopic experiments using the absorbed anti-J reagent J[1570] suggest that this new antigen is not associated with the J antigen, because some J positive cells have the new antigen while others lack it, and vice versa. The indirect hemagglutination tests using the J[1428] reagent conclusively demonstrate the presence of an additional antigen on #1570 cells which is independent of the J antigen, and which is also found on #1441, #1559, and #1502 cells. These serological results are in agreement with the EM results using the J[1570] reagent. Therefore, the hemocyanin labeling of #1570 cells is detecting an antigen distinct from J. This new antigen does not display a lytic or an agglutinating reaction, and to our knowledge can only be detected by EM labeling or by indirect hemagglutination.

Correcting our values for this new antigen detected by the Coombs test and by EM, we have shown that the cell-bound value of J is indicative of the serological J titer of the cells. This result implies

that red cell lysis, a second-order effect of an antigen-antibody reaction, is directly proportional to antibody binding.

Z Site Labeling

Of a total of 11 experiments, 9 cell populations show mean labeling values which correspond to their genotypes. That is, cells homozygous for the Z specificity display approximately twice as much label as cells heterozygous for that specificity. Two cell populations do not display an amount of label in simple relationship to their dosage of the Z allele. Both of these populations show more label than one would expect according to their Z zygosity.

The anti-Z antiserum used in these experiments is a monospecific reagent according to lytic assays. The presence in this antiserum of non-lytic antibodies would not be picked up by the serological assay. It is probable that the additional labeling found on these two cell populations is due to the presence of an additional population of antibodies in the anti-Z antiserum which is of a non-lytic class, and which is directed against other molecules on the surface of the red cells. The situation may be similar to the anti-J ST 48 experience; that is, an additional antigenic red cell surface specificity is being detected by the anti-Z antiserum which is not detected lytically.

The results indicate that the allele dosage for an integrated cell surface antigen linearly corresponds to the amount of that antigen on the cell membrane. This observation implies that the Z gene codes for a gene product that is quantitatively expressed on the cell membrane

and is the molecule being tagged by our EM labeling reagents. The dosage studies for human red cell antigens mentioned in the introduction suggest that the quantity of antigen expressed in these systems is controlled by a single allele (Race and Sanger, 1962). Masouredis' study, however, suggests that a number of alleles, and even a number of genes, may be involved in Rh_0(D) antigen quantitative expression. In the enzyme systems, the enzyme levels seem to be controlled by single alleles. In our Z system study of bovine red cells the simple results also suggest that the quantitative expression of this antigen is under the control of a single allele, the Z allele.

CHAPTER II

CELL SURFACE TOPOLOGY AND EXPRESSION OF ANTIGENS AS FUNCTIONS OF INTRAGENIC, INTERGENIC, AND CIS-TRANS CONFIGURATIONS OF THEIR GENETIC CONTROLS

Introduction

A definition of the cell surface topology of genetically defined antigens may aid in understanding the genetic control and the membrane relationships of these antigens. One of the key questions in cell surface topology concerns the relative spatial arrangements of specificities. For example, do two specificities compete with each other for the same cell surface site, or are they independent of each other? The former would suggest that the antigens under study possess a common backbone structure, or that the specificities, though molecularly separate, are sufficiently close on the cell surface that labeling one specificity may sterically block labeling of the second specificity. The latter would imply that the chemical structures of the antigens are sufficiently separate on the cell surface that there is no steric blocking.

Numerous examples are found in the human Rh antigen system of differences in quantitative expression of Rh antigens. These phenomena have been explained in terms of cis-trans effects (Chown and Lewis, 1957; Race et al., 1959; Race and Sanger, 1962), but cross reactive antibody reagents account for the effects more clearly.

Similar studies have been conducted on mouse thymocyte surface antigens, and blocking is found with several pairs of antigens. Using a serological absorption procedure, Cresswell and Sanderson (1968) demonstrated that in the presence of bound antibody directed against certain murine H-2 specificities, the binding of additional antibody directed against a second H-2 specificity was inhibited. Kristofova

et al. (1971) and Lengerova and Peknicova (1973) noted that significant blocking was found with the specificities 4 (H-2 D end) and 11 (H-2 K end), if the specificities were coded for by genetic regions on the same haplotype (e.g., +4 +11/ -4 -11). No blocking was found if the specificities were coded by genetic regions on opposing haplotypes (e.g., +4 -11/ -4 +11). That is, specificities had to be coded by regions which were in a cis configuration to show the blocking effect.

Using similar serological techniques, Boyse and coworkers (Boyse et al., 1969) mapped the mouse thymocyte surface for the Tla, Ly A, Ly B, and Thy 1 antigens. They found that absorption of antibody directed against the gene product of one allele of any of the above antigens reciprocally blocked the absorption of antibody directed against the product of the allelic alternative. They concluded from this study that in the reciprocal blocking situation the allelic products occupied topographically close locations on the cell surface. Additional studies by Boyse and Old (1969) on the cell surface expression of the Tla antigens suggested that Tla and H-2, which are genetically rather closely linked, are also closely related on the cell surface. The Tla structural and regulatory genes probably lie immediately to the left of the D end of the H-2 region (Boyse and Old, 1971). In TL- thymocytes, or in thymocytes in which Tla has been suppressed by antigenic modulation, the expression of H-2 (D end) antigens is as much as two-fold higher than in TL+ cells. This quantitative difference in H-2

expression occurs only when the Tla structural genes are in a cis position relative to the Tla regulatory gene(s) (Boyse and Old, 1971).

The mouse studies also indicate that antigens which are controlled by genetic regions which are present on the same haplotype, that is in a cis position relative to each other, are either very close to each other on the cell surface or are antigenic moieties on single molecules. In the case of H-2 it has been chemically demonstrated that the specificities (e.g., 4 and 11) reside on separate macromolecules (Cullen et al., 1972). Therefore for H-2 the serological blocking experiments indicate that individual glycoprotein H-2 antigenic macromolecules are very close together on the cell surface.

The genetic organization of the genes coding for the bovine red blood cell specificities is not well understood (Stormont et al., 1945, 1951; Stormont, 1955, 1972). The chemical basis of these antigens has not been identified, and one does not know if the antigenic determinants detected by the antisera are each the product of a single gene, or if two or more determinants are on a single molecule which is the product of a single gene (Ferguson et al., 1942; Stormont et al., 1945, 1951; Stormont, 1955). Several kinds of experiments can be done to resolve the question. One can chemically characterize the antigenic specificities and observe if single macromolecules have multiple specificities. The complications and uncertainties encountered in solubilizing and characterizing cell surface antigens however make this option difficult (Wallach, 1972; S. Rosenberg, unpublished observations). Alternatively, one can observe how different antigens on genetically

defined cells behave with respect to each other. By choosing appropriate genetic combinations one can expect certain results for the "multiple specificities per single macromolecule" hypothesis and other results for the "single gene-single specificity" hypothesis.

Using our EM labeling system in which cell surface antigens are labeled indirectly with a marker molecule visible in the transmission EM, we can follow the expression of an antigen in the presence of bound antibody directed against another specificity. If bovine red cell specificities are determinants on common backbone macromolecules, then one would expect that labeling one specificity would probably sterically block the labeling of a second also on that macromolecule. This interpretation requires that the two specificities be close to each other, so that the labeling reagents sterically interfere with each other. If the red cell specificities represent various cross-reacting systems one would also expect interference. However, if the antigens are each coded for by individual genes, then the gene products might be independent on the cell surface. There would be no steric blocking problems if the two specificities being studied are sufficiently far apart.

The above considerations apply to specificities that are coded for on the same chromosomal homologue, that is, specificities which are coded for in the cis position. However, a structural situation analogous to the human ABO blood groups may exist. The human A and B alleles code for glycosyl transferases that attach terminal sugar residues to a backbone molecule shared by the A and B blood group substances

(Watkins, 1966). Therefore, the quantitative expression of the A and B antigens does not depend on the genetic organization of the A and B genes (cis or trans), but on the activity of the specific glycosyl transferases. Specificities whose genes are carried in the trans configuration should not display steric hindrance unless a structural situation analogous to the ABO system exists.

Given these hypotheses, we have examined the cell surface distribution of two specificities whose genes reside in the trans configuration in one and in the cis configuration in a second cell population. We have chosen antigens which belong to the C system of bovine blood groups and which map genetically in the same region (Stormont et al., 1951).

One can study the expression of an antigen in the presence of antibody plus label bound to a second related antigen using sequential double labeling techniques. We have determined the quantity and distribution of two antigens on singly labeled cells and compared this value to the quantity and distribution on doubly labeled cells. By using sequential antibody labeling one can detect the effect of antibody pretreatment on the expression of a second antigen.

We have also studied the cell surface distribution of two specificities whose genes reside in different genetic systems (C and Z systems) to examine possible cell surface interaction of the products of independent genes.

In the course of our cis-trans antigenic expression studies we have encountered two specificities which display a super-additive effect in antigenic expression at the cellular level. That is, pre-treatment of cells with labeled antibody directed against one specificity, followed by treatment with an antibody-label directed against a second antigen, yields a total labeling value which is greater than the sum of the two single label values. This observation is confirmed by serological experiments with pretreated cells, in which we find a corresponding increase in antibody uptake by the presensitized cells.

Results

Cis-trans Experiments

To study the cell surface relationship of antigens whose genes reside in cis conformation in the genome versus a trans configuration, two cell populations were chosen which display the R_2 and W specificities. #1428 cells exhibit the cis configuration, genotype C_1/R_2W, and #1502 cells display the trans configuration, genotype R_2/WX_2. The genotypes given for these cells are of the C blood group complex of bovine red cells.

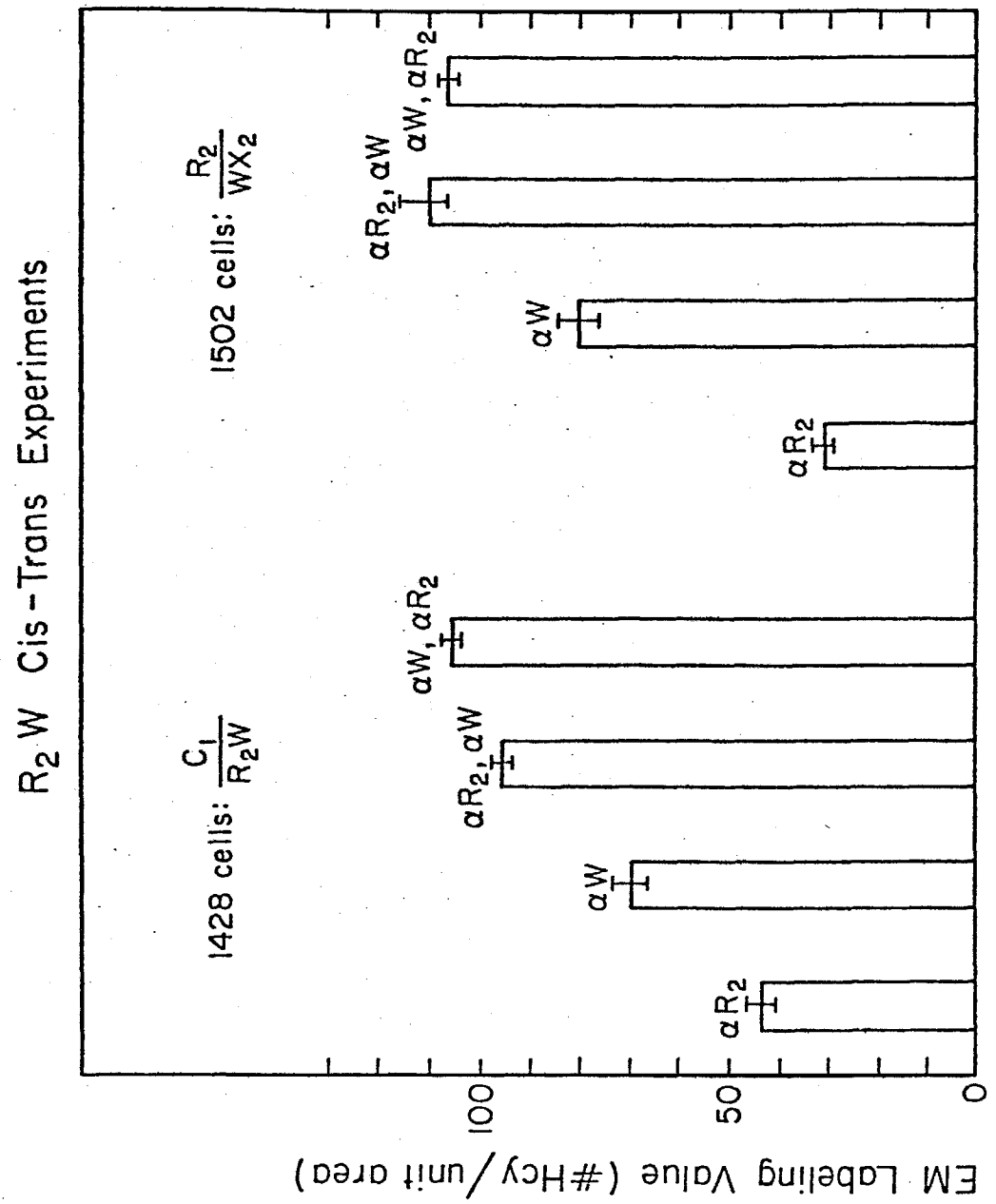
The surface antigens were reacted first with a monospecific bovine antiserum directed against the antigen under study. The bovine immunoglobulin was then reacted with RABI-Hcy, as described in the Materials and Methods section of this thesis. In the sequential double label experiments, labeling with the isoimmune antisera was performed

in both directions. That is, in one group of experiments cells were first treated with anti-R_2 antibody, then the labeling reagent, and then with anti-W antiserum followed by the labeling reagent (R_2, RABI-Hcy, W, RABI-Hcy). In a parallel set of experiments the sequence was reversed (W, RABI-Hcy, R_2, RABI-Hcy).

Figure 1 is a bar graph of the single label and sequential double label values of #1428 and #1502 cells. Looking first at #1502 cells, which display the trans configuration of the R_2 and W antigens (R_2/WX_2), the sum of the two single label values (R_2 plus W = 32 + 81) is 113, which is not significantly different ($p < 0.05$) from the labeling values found in the sequential double label experiments (111 and 107).

#1428 cells, which exhibit the cis configuration (C_1/R_2W), show single label values for the R_2 and W antigens of 44 and 70 hemocyanins per unit area respectively. In a sequential double label experiment in which R_2 is labeled first followed by W, the mean labeling value is 96. Labeling initially for W and secondly for R_2 gives a value of 106. The sum of the two single label values for R_2 and W (114) is significantly greater ($p < 0.01$) than the double label value in the R_2, W direction (96), but is not significantly different ($p > 0.05$) from the double label value in the W, R_2 direction (106). Pooling the two sequential double label experiments and comparing this mean labeling value to that of the sum of the two single label values yields a significant difference ($p < 0.01$) between these values.

Figure 1. EM labeling values for #1428 and #1502 cells labeled with anti-R_2(NORI) or anti-W(C301), followed by RABI-Hcy; sequential double labeling values for anti-R_2 + anti-W and anti-W + anti-R_2.


R_2 W Cis-Trans Experiments
EM Labeling Value (#Hcy/unit area)
0
50
100
1428 cells: $\frac{C_1}{R_2W}$
1502 cells: $\frac{R_2}{WX_2}$
αR2
αW
αR2, αW
αW, αR2
αR2
αW
αR2, αW
αW, αR2

Figures 2 and 3 show typical electron micrographs of #1428 and #1502 cells, respectively, in single label experiments for R_2 and W and sequential double label experiments in the R_2, W direction and in the W, R_2 direction.

R_2Z Position Experiments

Electronmicroscopic labeling studies were also used to examine the cell surface expression of two antigens whose genes are in independent genetic complexes. The C system and the Z system of bovine red cells were chosen for this study because the genes coding for the specificities in these systems are unlinked (Stormont , 1952). As previously noted, the Z system is a single factor system. The R_2 antigen was the marker followed in the C system because of familiarity with this antigen from the previous cis-trans studies. #1428 cells have the genotype C_1/R_2W and Z/-, and # 1502 cells have the genotype R_2/WX_2 and Z/-, for the C and Z systems, respectively.

Figure 4 shows the results of the R_2 Z position experiments. #1428 cells have mean labeling values of 44 (from the cis-trans experiments) and 74 for R_2 and Z, respectively. The sum of these two single label values (118) is not significantly different ($p > 0.5$) from the sequential double label values in the R_2, Z direction (118) or in the Z, R_2 direction (117). The single label values for #1502 cells are 32 (from the cis-trans experiments) and 60 for R_2 and Z, respectively. The sum of these two single label values (92) is not significantly different ($p > 0.05$) from the sequential double label value in the R_2, Z

Figure 2. Electronmicrographs of replicas of #1428 cells (C_1/R_2W) labeled with (A) anti-R_2(NORI), RABI-Hcy; (B) anti-W (C301), RABI-Hcy; (C) anti-R_2, RABI-Hcy, anti-W (C301), RABI-Hcy; and (D) anti-W, RABI-Hcy, anti-R_2, RABI-Hcy. Magnification: 11,250×.

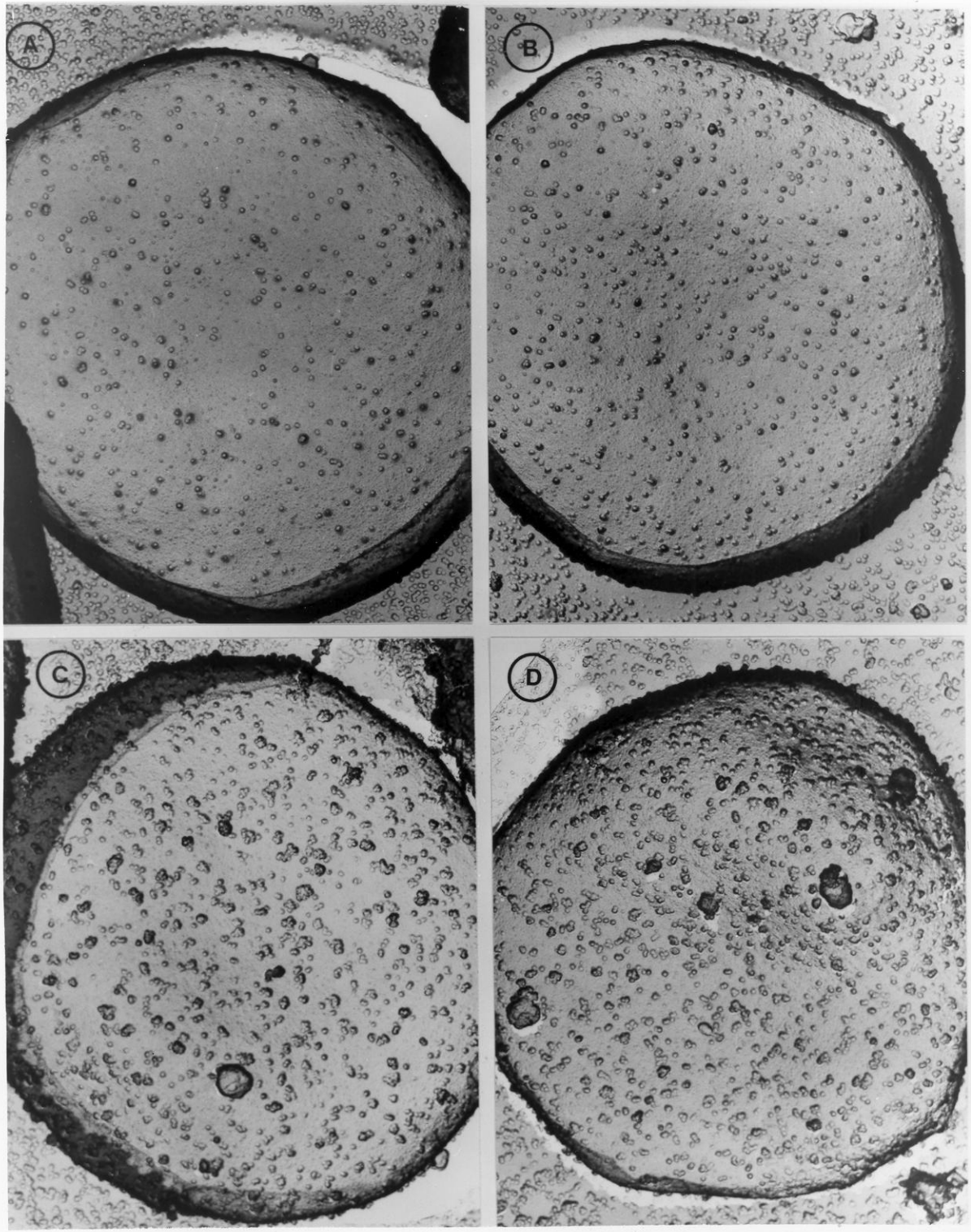

A
B
C
D

Figure 3. Electronmicrographs of replicas of #1502 cells (R_2/WX_2) labeled with (A) anti-R_2(NORI), RABI-Hcy; (B) anti-W (C301), RABI-Hcy; (C) anti-R_2, RABI-Hcy, anti-W, RABI-Hcy; and (D) anti-W, RABI-Hcy, anti-R_2, RABI-Hcy. Magnification: 11,250×.

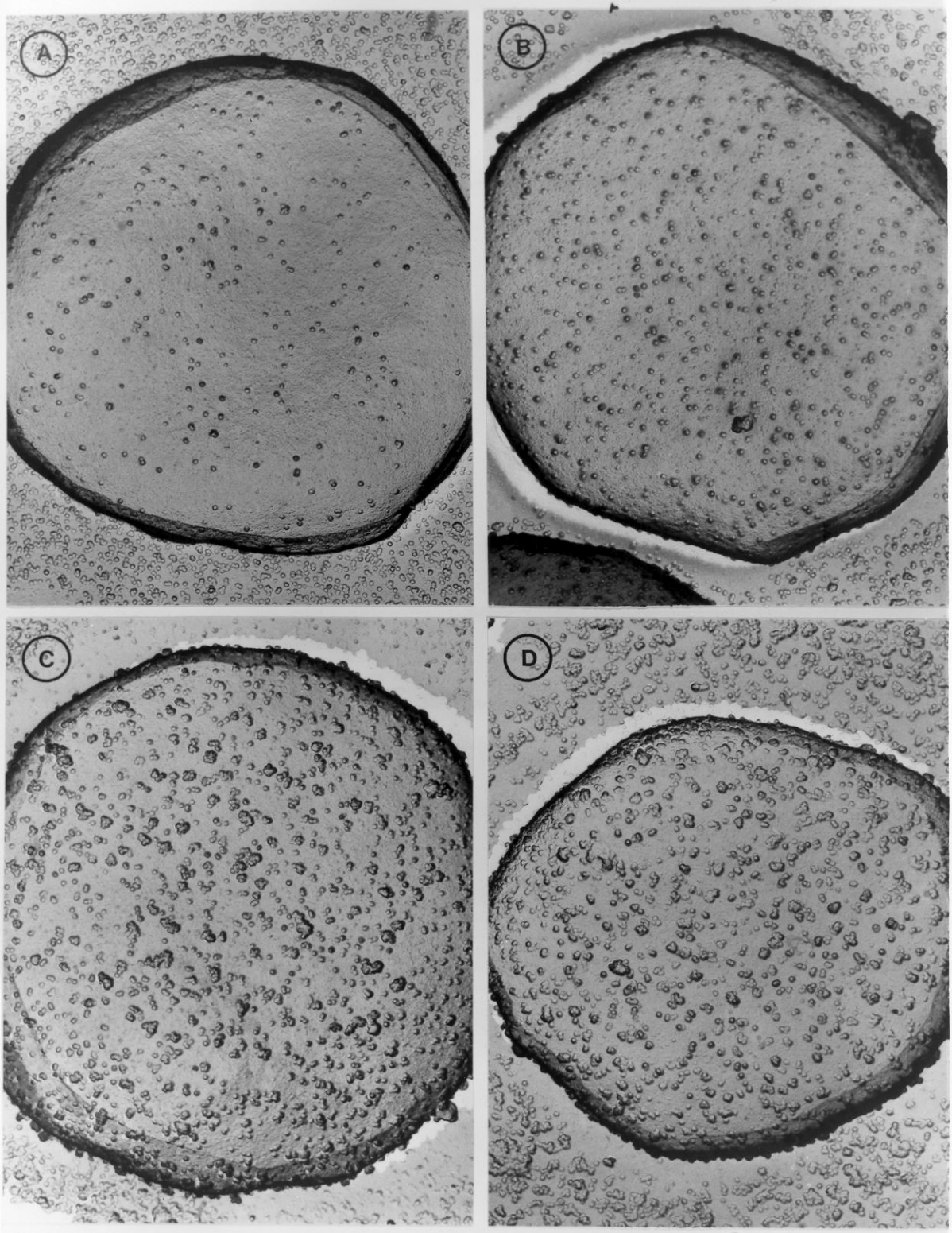
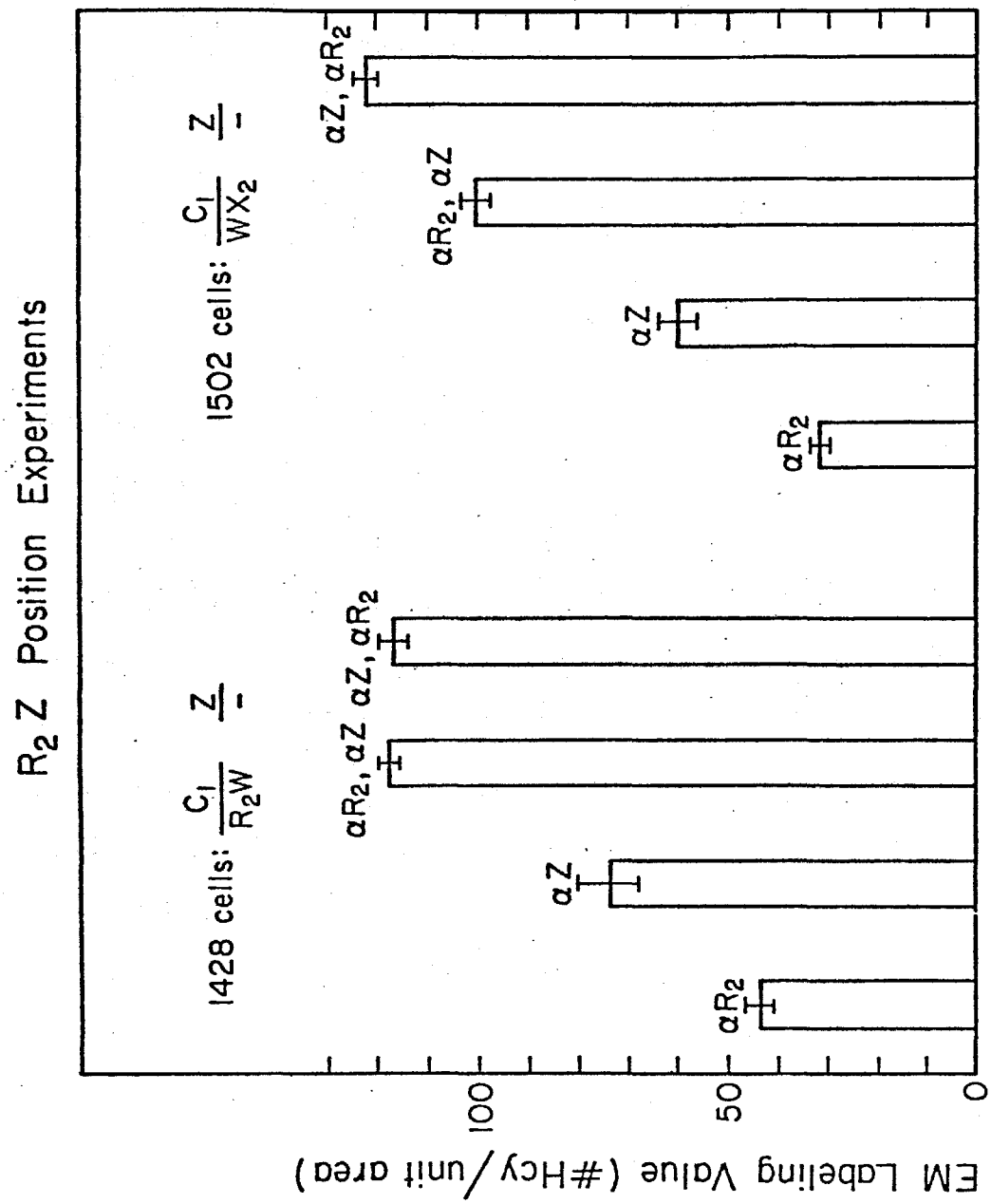

A
B
C
D

Figure 4. EM mean labeling values of #1428 cells and #1502 cells labeled with anti-R_2(NORI), RABI-Hcy, and anti-Z (ST 54) RABI-Hcy; double label values with anti-R_2 + anti-Z, and anti-Z + anti-R_2.


R2 Z Position Experiments
1428 cells: C1/R2W Z/-
1502 cells: C1/WX2 Z/-
EM Labeling Value (#Hcy/unit area)
0
50
100
αR2
αZ
αR2, αZ
αZ, αR2

direction (100), but is significantly less ($p < 0.01$) than the double label value in the Z, R_2 direction (122). The mean labeling value for the pooled results of the two double labeling experiments and the sum of the two single label values for #1502 cells are significantly different ($p < 0.01$). Note that for #1502 cells the sequential double labeling experiments give slightly higher values than the sum of the two single labeling experiments.

Figures 5 and 6 show typical electron micrographs of #1428 and #1502 cells respectively labeled for R_2, Z, R_2 + Z, and Z + R_2 antigens.

R_2X_2 EM Experiments

EM labeling experiments were also used to examine the cell surface relationship of antigens which are in the same blood group system but whose genes are not in any particular genetic configuration. Cells #1502 and #1559 both display the phenotype R_2X_2 in the C system. #1502 cells show the trans genotype R_2/X_2, but the exact genotype of #1559 cells is unknown and cannot be determined because family data are not available.

Single label experiments for the R_2 and X_2 antigens and sequential double labeling experiments were performed in the X_2, R_2 and R_2, X_2 directions. The results are plotted as bar graphs in figure 7A . When #1502 cells were labeled with an anti-R_2 antiserum in the usual single labeling procedure, a characteristic mean labeling value of approximately 26 was obtained. Labeling for the X_2 antigen gave a mean labeling value of 23. In a sequential double labeling experiment,

Figure 5. Electronmicrographs of replicas of #1428 cells (C_1/R_2W; Z/-) labeled with (A) anti-R_2(NORI) RABI-Hcy; (B) anti-Z (ST 54), RABI-Hcy; (C) anti-R_2, RABI-Hcy, anti-Z RABI-Hcy; and (D) anti-Z, RABI-Hcy, anti-R_2, RABI-Hcy. Magnification: 11,250×.

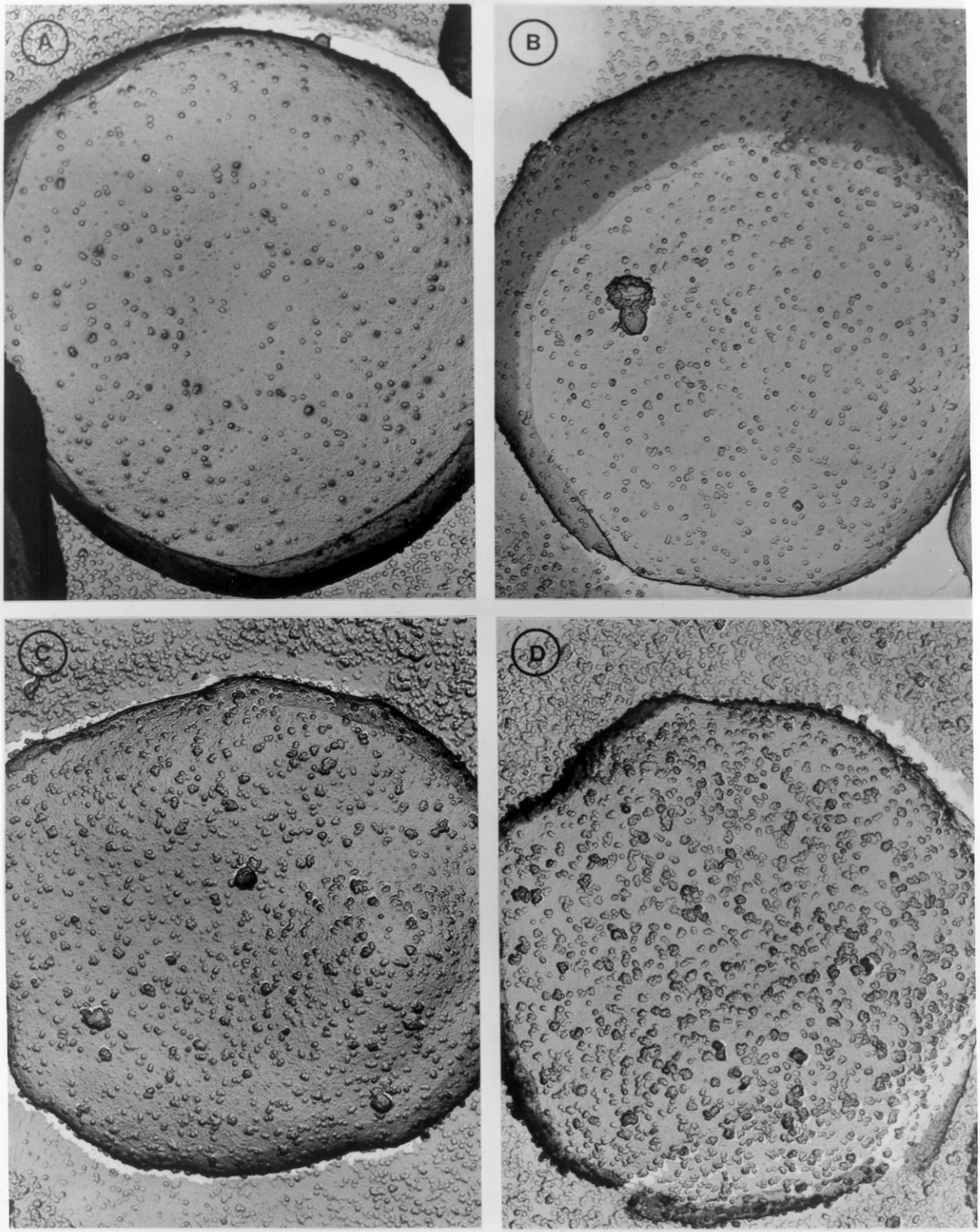

A
B
C
D

Figure 6. Electronmicrographs of replicas of #1502 cells (R_2/WX_2; Z/-) labeled with (A) anti-R_2 (NORI), RABI-Hcy; (B) anti-Z (ST 54), RABI-Hcy; (C) anti-R_2, RABI-Hcy, anti-Z, RABI-Hcy; and (D) anti-Z, RABI-Hcy, anti-R_2, RABI-Hcy. Magnification: 11,250×.

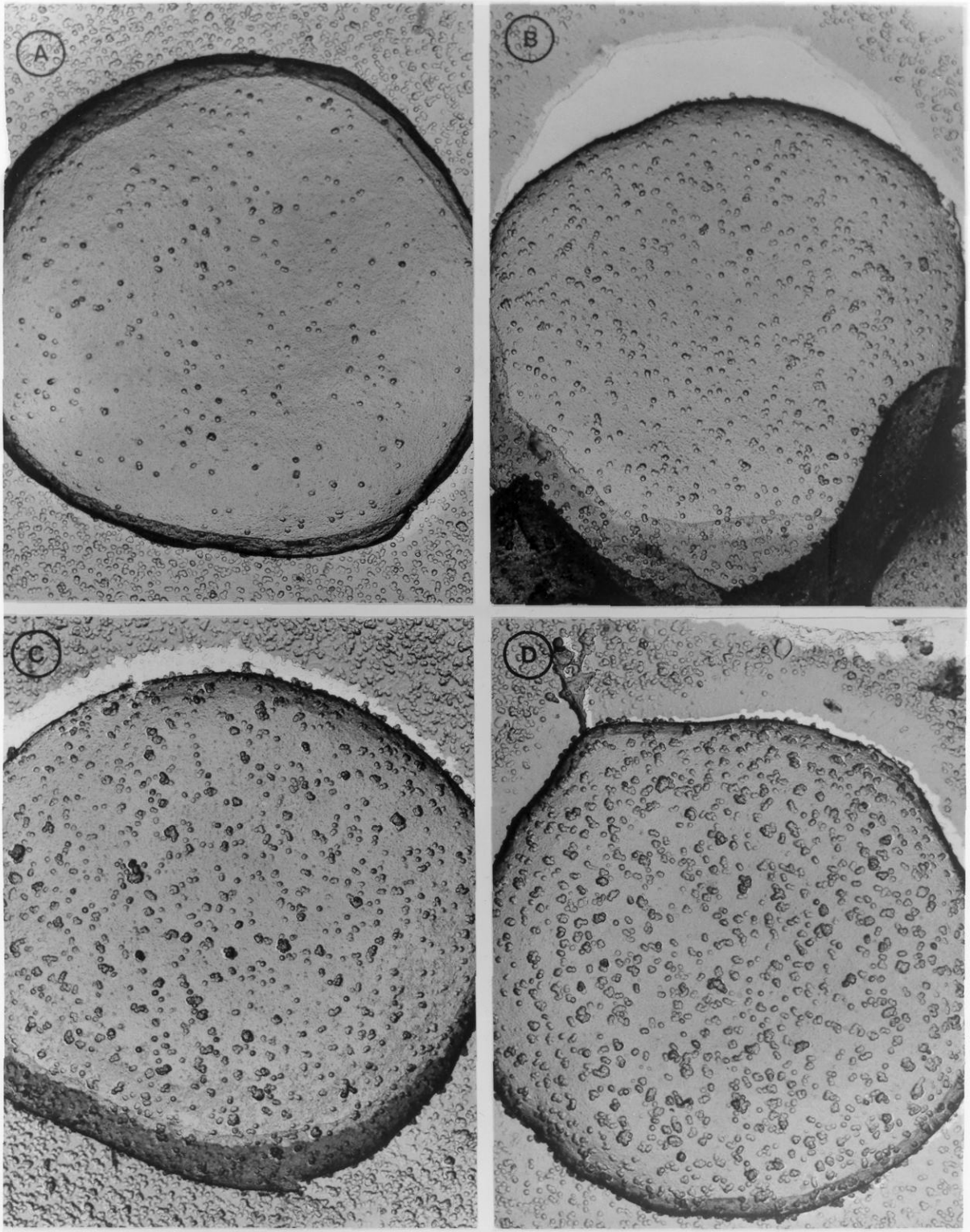
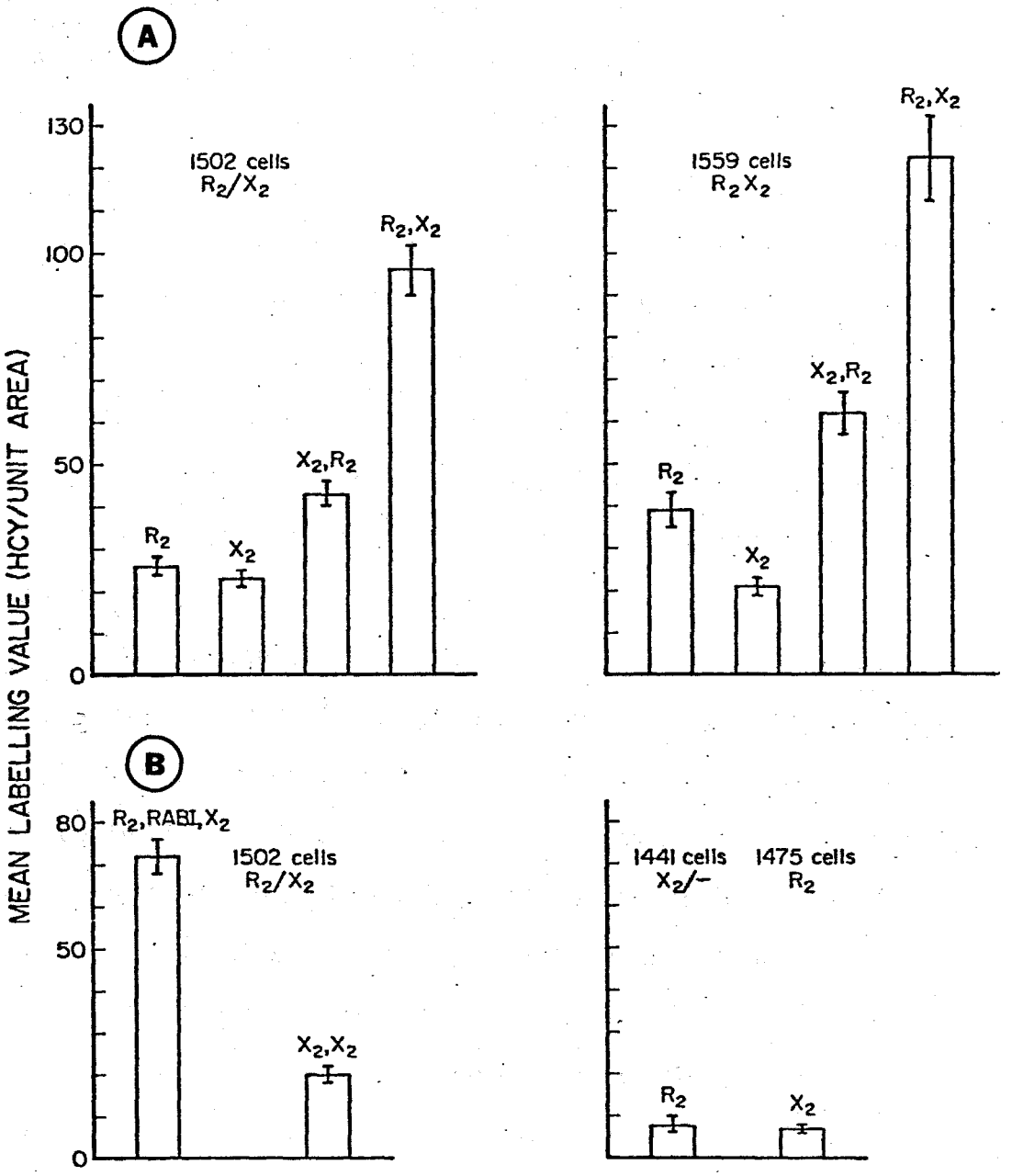

A
B
C
D

Figure 7

(A) EM mean labeling values for #1502 and #1559 cells labeled with anti-R_2 (NORI), RABI-Hcy; anti-X_2 (C228) RABI-Hcy; sequential double labeling values for X_2, R_2, and R_2, X_2.

(B) EM mean labeling value for #1502 cells labeled with anti-R_2, RABI, anti-X_2; sequential double labeling value for X_2. R_2 and X_2 labeling values on cells negative for these antigens.


A
130
100
50
0
1502 cells
R2/X2
R2
X2
X2,R2
R2,X2
1559 cells
R2 X2
R2
X2
X2,R2
R2,X2
MEAN LABELLING VALUE (HCY/UNIT AREA)
B
80
50
0
R2,RABI,X2
1502 cells
R2/X2
X2,X2
1441 cells
X2/-
1475 cells
R2
R2
X2

reacting first with anti-X_2 and secondly with anti-R_2, a mean labeling value of 43 was obtained, not significantly different from the sum of the two single label values with anti-X_2 and anti-R_2 (49). However, when the cells were first treated with the anti-R_2 reagent and secondly with the anti-X_2 reagent, then approximately twice the sum of the single labeling values was found (96 as compared to 49). We found the same phenomenon to be true for #1559 cells. In double label experiments, treating first with anti-X_2 and secondly with anti-R_2 gave a mean labeling value (62) approximately the sum of the two single label values (60). However, labeling first with anti-R_2 and then with anti-X_2 gave a labeling value (122) which is about twice the sum of the single label values (60).

To test whether the increased labeling was restricted to primary treatment with the anti-R_2 antiserum or if secondary application of anti-X_2 alone was responsible, we did the following control. #1502 cells were reacted first with anti-X_2 and RABI-Hcy and then a second time with anti-X_2 followed by RABI-Hcy. As shown in figure 7B, the mean labeling value for this experiment was 20, which is not significantly different from the single label value of 23 for #1502 cells reacted only once with anti-X_2. Therefore, pretreatment with anti-X_2 does not cause increased uptake of the same label.

We also wanted to know whether the additional sites we were labeling following R_2 sensitization of the cells were specifically binding anti-X_2 antibody. To answer this question we did a double label experiment in which we first treated #1502 cells with anti-R_2 and then

saturated these anti-R_2 antibodies with RABI not coupled to Hcy. The cells were then reacted with anti-X_2 antiserum and RABI-Hcy. The result is shown in figure 7B. The mean labeling value is 72, not significantly different from the value of 70 obtained by calculating the additional X_2 sites by subtracting the R_2 sites in the single label experiments from the R_2, X_2 sites (96 minus 26 = 70). We therefore conclude that the additional label we find on R_2X_2 positive cells following R_2 sensitization is specifically binding anti-X_2 antibody.

R_2X_2 Serological Experiments

According to the EM labeling results one should be able to measure a serological difference between R_2-sensitized #1502 cells and non-sensitized #1502 cells. If R_2-treated cells bind more anti-X_2 antibody than non R_2-treated cells, then the sensitized cells should be able to absorb a greater quantity of anti-X_2 antibody than do non-sensitized cells. The following absorbed antisera were therefore prepared.

Aliquots of #1502 cells were sensitized with anti-R_2 antiserum (1502-R_2) and used to absorb sequentially an anti-X_2 antiserum (X_2[1502-R_2]). As a control, non-sensitized #1502 cells were used to absorb another aliquot of the anti-X_2 serum (X_2[1502]). As a parallel to the EM experiments, anti-R_2 antiserum was also absorbed with either X_2-sensitized (R_2[1502-X_2]) or nonsensitized #1502 cells (R_2[1502]). (See the general Materials and Methods section of this thesis for the absorption procedure.)

The absorbed sera were tested in a lytic assay against cells which were X_2 positive R_2 negative (#1441 cells) and X_2 negative R_2 positive (#1499 cells). The serological results using the absorbed sera are shown in figure 8. On the ordinate is plotted antiserum titer and on the abscissa the number of absorptions beginning with the unabsorbed serum. Figure 8B shows that X_2-sensitized and non-sensitized cells have the same absorption capacity for anti-R_2 antibody, as we would expect from the EM studies. However, the R_2-sensitized cells shown in figure 8A have a greater absorptive capacity than the non-R_2 sensitized cells, as predicted by our EM studies. Testing the absorbed reagents against cells which lack the appropriate specificity shows no lytic activity, indicating that the absorbed sera are specific.

Discussion

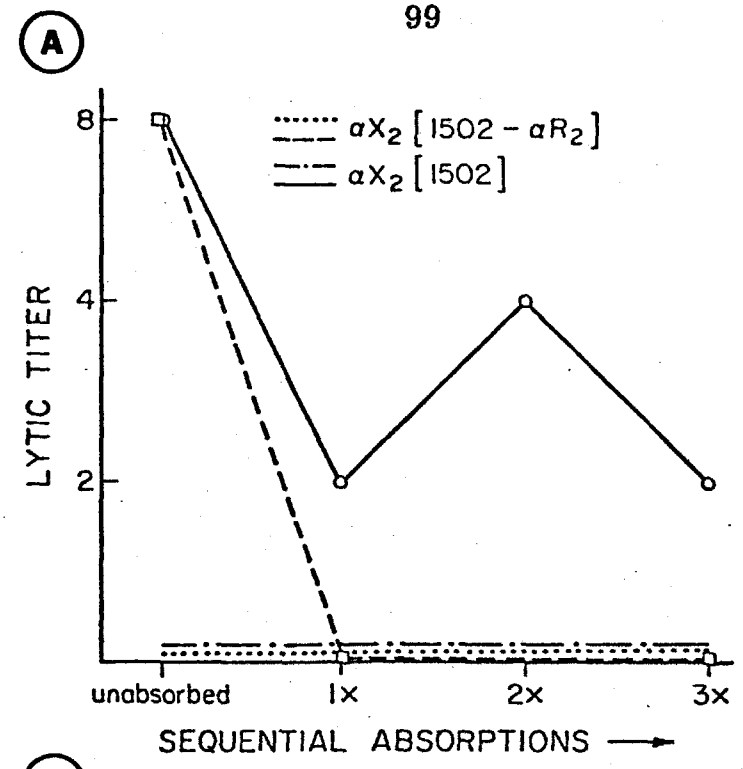
The sequential double labeling technique for visualizing cell surface antigens in the transmission electron microscope is an accurate method for assessing antigen topology and antibody blocking effects on pretreated cells. Clarity of label enables one to quantitate accurately the number of antibody binding sites. By reacting cells sequentially with two populations of antibody one can observe the effect of bound antibody on the subsequent labeling of a second antigen.

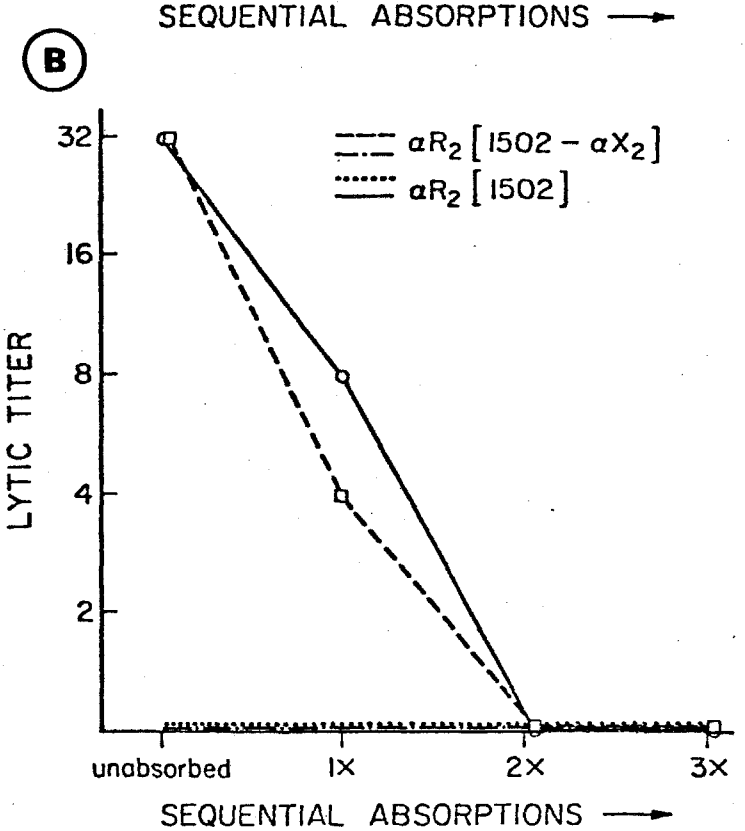
The cis-trans experiments, labeling the R_2 and W C system antigens, were designed to answer the question of whether the bovine red cell blood group antigens are each coded by separate genes and

Figure 8. Hemolytic assays of sequentially absorbed anti-X_2 (C228) and anti-R_2 (NORI) antisera.

(A) Anti-X_2 (C228) absorbed with 1502-R_2 (- - -) and 1502 (—·—) cells and tested against X_2 positive, R_2 negative target cells (———) and X_2 negative, R_2 positive target cells (— : —).

(B) Anti-R_2 (NORI) absorbed with 1502-X_2 (— : —) and 1502 (· · ·) cells and tested against R_2 positive, X_2 negative target cells (———) and R_2 negative, X_2 positive target cells (— : —).


A
8
4
2
LYTIC TITER
αX2 [1502 – αR2]
αX2 [1502]
unabsorbed
1x
2x
3x
SEQUENTIAL ABSORPTIONS


B
32
16
8
4
2
LYTIC TITER
αR2 [1502 – αX2]
αR2 [1502]
unabsorbed
1x
2x
3x
SEQUENTIAL ABSORPTIONS

hence have individual gene products, are multiple specificities residing on a common macromolecular backbone, or are cross-reactive substances sharing a common backbone. If blocking had been found, the multiple specificities per macromolecule or the cross-reactive substances hypotheses would have been supported. Our results, however, indicate that cells displaying the cis conformation (#1428 cells) show very little blocking; they are comparable to cells which exhibit the trans configuration (#1502 cells). We therefore conclude that the R_2 and W specificities are probably not side-groups of a common macromolecule or cross-reactive moieties sharing a common backbone. If the R_2 and W specificities are in either of these organizations then the antigenic determinants are sufficiently far apart that labeling them does not involve any steric hindrance. Our data support the possibility that the R_2 and W antigenic determinants are separate macromolecules on the surface and are coded for by distinct genes.

The labeling experiments for the R_2 and Z antigens, whose genes exist in the unlinked C and Z systems respectively, suggest that there is also no steric hindrance in labeling antigens whose genes reside in separate antigenic systems. The double label value for #1502 cells in the Z, R_2 direction is significantly greater than the sum of the two single label values. However, the double label value is only 20% higher than the corresponding double label value in the R_2, Z direction. This variation is small and does not approach the two-fold increase in sites

found in the R_2, X_2 experiments. Also, no increase is found in the corresponding experiment with #1428 cells. If we are seeing an enhancing effect in the R, Z systems (genetically independent antigens), it is marginal. The results imply that the independent gene products labeled are physically separate on the cell surface. They are far enough apart so that attaching an antibody-Hcy complex to one specificity does not interact in labeling a second specificity.

In contrast to the mouse thymocyte cell surface antigen studies (Boyse et al., 1968; Boyse and Old, 1969, 1971; Kristofova et al., 1971; Lengerova and Peknicova, 1973) discussed in the introductory section, we have found no blocking associated with the bovine red cell surface antigens examined. The authors working on the mouse thymocyte antigens concluded that the specificities they studied were closely associated and thus antibody directed against one specificity blocked antibody binding to a second topologically close specificity. Our R_2 and Z results in combination with the R_2 and W results strongly suggest that these bovine red cell blood group antigens are distinct macromolecules on the red cell surface, distant enough from each other so that antibody binding to one does not sterically interfere with antibody binding to a different antigen.

The R_2 and X_2 experiments were originally designed to study the interaction of two specificities in the same blood group system whose genes were not in any particular genetic configuration. Labeling in the X_2, R_2 direction gave as a labeling value the sum of the two single label

values for X_2 and R_2. Labeling in the R_2, X_2 direction, however, gave a labeling value twice the sum of the two single label values. We have shown that this super-additive result is characteristic of both of two cell populations, #1502 and #1559 cells, which display the R_2 and X_2 antigens. This revelation of additional sites which bind anti-X_2 antibody does not seem to be an artifact of the double-labeling procedure, because it is observed only in the R_2, X_2 labeling direction and not in the reverse labeling sequence. Furthermore, if the R_2 sites are treated with anti-R_2 and then saturated with RABI and labeled for X_2 sites, the number of X_2 sites is increased as found in the normal double labeling experiments. The site increase is not due to secondary labeling with anti-X_2, because double labeling using anti-X_2 both times gives the characteristic single label value for X_2.

The serological absorption experiments strongly support the EM labeling results and demonstrate again that #1502 or #1559 cells which have been sensitized with anti-R_2 have a heightened specific ability to take up anti-X_2 antibody.

The heightened specific uptake of anti-X_2 following anti-R_2 sensitization is consistent with any of several models. The most obvious explanation is that new X_2 sites are revealed on the red cell surface as a consequence of combination with anti-R_2 antibody. Alternatively, the antibody-R_2-antigen complex may be interacting somehow with the cell surface and mimicking the X_2 antigenic moiety. The EM and the serological experiments both indicate that the heightened

uptake is specific for anti-X_2 antibody, so any explanation of the phenomenon must involve a specific interaction.

In surveying the literature we can find no results analogous to this phenomenon for antibody treated cells. Examples of increased ligand uptake following protease treatment (Burger, 1969; Noonan and Burger, 1973) are known. The heightened quantitative expression of D end H-2 specificities in Tla modulated cells (Boyse and Old, 1969) is a possible example of increased expression of a second antigen in the presence of antibody bound to another antigen. Tla modulation is induced by binding anti-Tla antibody (Old et al., 1968), but the Tla H-2 phenomenon is encountered only when the Tla regulatory and structural genes are on the same haplotype as the genetic region coding for the H-2 specificities (Boyse and Old, 1971). Our R_2 X_2 phenomenon is found on cells which are trans for these specificities, so the situation is not completely analogous.

In addition to antibodies blocking the attachment of other antibodies, therefore, particular surface molecules may be altered by the attachment of antibodies to other macromolecules on the same membrane such that increased antigenic expression is found.

CHAPTER III

EFFECTS OF ENZYME TREATMENT ON THE EXPRESSION OF CELL SURFACE ANTIGENS AND CONCANAVALIN A BINDING SITES ON BOVINE RED CELLS: IMPLICATIONS FOR MEMBRANE STRUCTURE

Introduction

Concanavalin A (Con A) is a plant lectin isolated from the jack bean (Canavalia ensiformis) which specifically binds to terminal α-D-mannose-like residues (Inbar and Sachs, 1969). At physiological conditions the Con A molecule has four binding sites (Kalb and Lustig, 1968). Numerous studies show that normal nucleated cells are not agglutinated by Con A while leukemic, virally transformed, and trypsinized normal cells are agglutinated by Con A (Inbar and Sachs, 1969; Burger, 1969). Hakormori (1969) has shown that antisera directed against globosides and hematosides will agglutinate and hemolyze human fetal red cells to a greater extent than human adult red cells. Trypsinization of the adult red cells, however, makes them susceptible to lysis and agglutination to the same extent as the fetal red cells. Sanders (1962) has described an antigen on brown trout red cells that is present on red cells of young animals, but absent on red cells of adults. Papain treated adult red cells, however, also display the antigen. In these studies it is believed that the normal or adult cells have the particular molecules that the transformed or fetal cells have (Con A receptor or antigen), but that the molecule is in a cryptic form on the normal or adult cells and is exposed by proteolytic enzyme treatment or malignant transformation (Sanders, 1962; Burger, 1969; Inbar and Sachs, 1969; Hakormori, 1969). Recent evidence suggests that in addition to the exposure of the sites by trypsin treatment, a rearrangement of the sites on normal cells is also necessary for Con A agglutina-

tion to occur (Noonan and Burger, 1963; Rosenblith et al., 1973).

The increased agglutinability of red cells following trypsinization is found for many blood group antigens (Unger, 1951). It is believed that the trypsin treatment releases sialoclycopeptides from the cell surface revealing additional antigenic sites on the membrane (Winzler et al., 1967).

Freeze-cleavage and freeze-etch studies of the inner plane of the red cell membrane reveal 85Å particles lying on a smooth surface (Branton, 1966, 1969; Pinto da Silva and Branton, 1960; Tillack and Marchesi, 1970). These particles have frequently been interpreted as being globular proteins lying within the lipid membrane matrix (Marchesi et al., 1962; Pinto da Silva, 1973). The major glycoprotein of the red cell membrane isolated by the Marchesi group (Marchesi et al., 1972), called glycophorin, has been characterized as an amphipathic glycoprotein in which the N-terminal region has approximately 20 to 30 oligosaccharides branching from the polypeptide backbone, and the C terminal region lacks carbohydrate and is hydrophilic (Segrest, et al., 1972; Marchesi et al., 1972, 1973). Sequence data on 51 residues of this 50,000 molecular weight protein show that a cluster of 23 amino acids in the central section of the protein is hydrophobic in character (Segrest et al., 1972). Lactoperoxidase iodination coupled with cyanogen bromide and trypsin cleavage of glycophorin indicate that only the carbohydrate-rich N

terminus is exposed to the outer aqueous environment of the cell, while the hydrophilic C terminus is exposed to the internal cytoplasm (Segrest et al., 1972, 1973). The N terminus of glycophorin has been shown to carry the ABO and MN blood group antigens and the receptors for phytohemagglutinin (PHA), wheat germ agglutinin (WGA), and influenza virus (Marchesi et al., 1972). These antigens and receptors have been shown to overlie the 85Å intramembranous particles found on freeze-cleavage studies of red cells (Pinto da Silva et al., 1971; Marchesi et al., 1972; Tillack et al., 1972). Marchesi et al. (1972, 1973) have suggested that glycophorin is the major structural glycoprotein of the human red cell membrane and that it extends through the membrane with its N terminus carrying various antigenic sites and receptors, the C terminus passing into the cell cytoplasm, and the hydrophobic middle section interacting with the intramembranous particles. They and others (Pinto da Silva, 1971) point out that the number of intramembranous particles per erythrocyte is very close to the number of PHA receptors and A_1 antigenic sites per human erythrocyte, supporting the model that the intramembranous particles are physically attached to the cell surface antigens and receptor molecules.

Concanavalin A agglutinates some trypsinized bovine red cells, but does not agglutinate untrypsinized bovine red cells (Jaffe et al., 1972). Hines et al. (1972) have reported that those trypsinized bovine red cells that are agglutinated by Con A usually also have blood factor A.

These authors suggest that the bovine red cell Con A receptor and the A antigen are molecularly the same moiety.

In this section of this thesis, bovine red cells have been examined by electron microscopic and serological methods to study the possible relationship of the Con A cell surface receptor and the bovine red cell A antigen. The experiments indicate that the Con A receptor and bovine A antigen are molecularly independent entities. Freeze-cleavage experiments have also been performed to study the relationship between the bovine red cell A antigen and the inner structure of red blood cells. The evidence presented in this report supports the hypothesis that the bovine red cell surface antigenic determinants are physically associated with the intramembranous particles.

Materials and Methods

Bovine red cells were chosen for this study on the basis of their genetic constitution for the A antigen. #1441 cells are positive for the A_1 antigen, a subgroup within the bovine blood group A system, and #1502 cells genetically lack the A antigen. Hemolysis, agglutination, and EM labeling experiments with an anti-A antiserum, C304, and the RABI-Hcy reagent were done as described in the general Materials and Methods section of this thesis. Con A sites can be labeled with hemocyanin because of the affinity of Con A for hemocyanin (Smith and Revel, 1972). The sequential double labeling procedure for anti-A and Con A is similar to that described in the general Materials and Methods section for sequential double labeling with two antisera.

In these experiments, however, following application and incubation of anti-A, no hemocyanin was applied, but the Con A was then applied. In all hemocyanin labeling experiments the target cells were prefixed with glutaraldehyde prior to exposure to antisera as described in the general Materials and Methods section. In the freeze-fracture studies, the target cells were first incubated with antiserum and then glutaraldehyde fixed according to the procedure described in the general Materials and Methods section.

Results

Hemocyanin labeling studies were conducted to ascertain whether the A antigen and Con A binding sites are molecularly the same entity on bovine red blood cells. Table 1 shows the results of anti-A hemolysis, anti-A agglutination, Con A agglutination, and Con A combined with anti-EM labeling values on trypsinized and untrypsinized A positive and A negative target cells.

Anti-A Lysis, Agglutination, and EM Binding Results

In the hemolytic assay only the A positive (# 1441) cells are lysed by anti-A (titer: 256). Following trypsinization of the target cells the lytic titer of the A positive cells (# 1441-t) increases slightly (256 - 512), and the trypsinized A negative cells (# 1502-t) are still negative. Regardless of their A genotype, non-trypsinized cells (#1441 and #1502) are not agglutinated by anti-A. However, trypsinized A positive cells (#1441-T) are agglutinated by anti-A (titer: 8) and trypsinized A negative cells (#1502-t) are also slightly agglutinated by the anti-A reagent (titer: 2).

Table 1

Lytic Titers, Agglutination Titers, and EM Binding Values for A Positive (#1441) and A Negative (#1502) Cells Treated with Anti-A (C304) or Con A

(t = trypsinized cells)

Target Cells	A Phenotype	Reagent	Lytic Titer^{-1}	Agglutination Titer^{-1}	EM Labeling Value ± SE
1441	A	Anti-A (C304)	256	-	30 ± 3
1502	-	Anti-A (C304)	-	-	8 ± 1
1441-t	A	Anti-A (C304)	512	8	159 ± 7
1502-t	-	Anti-A (C304)	-	2	76 ± 4
1441	A	Con A		-	45 ± 2
1502	-	Con A		-	30 ± 3
1441-t	A	Con A		8	105 ± 4
1502-t	-	Con A		-	78 ± 2

The EM binding values for the anti-A antiserum, also shown in Table 1, correspond to the serological results. By EM labeling with the RABI-Hcy reagent, untrypsinized A positive cells (#1441) take up immunoglobulin from the anti-A reagent (presumably anti-A antibodies) (labeling value: 30), while the untrypsinized A negative cells (#1502) show only background labeling (8). Following trypsinization of the target cells, both A positive (#1441-t) and A negative (#1502-t) cells take up hemocyanin when incubated with the anti-A antiserum. However, the trypsinized A positive cells (#1441-t) take up approximately twice as much hemocyanin (158) as the trypsinized A negative cells (#1502-t; 76). Therefore, the EM binding values follow the agglutination titers even in the detail that the trypsinized A negative cells (#1502-t) do react slightly with the anti-A antiserum. Additional experiments to be described later demonstrate that the anti-A uptake by trypsinized A negative cells (#1502-t), as measured by agglutination and EM binding, is a non-specific uptake of immunoglobulin.

Con A Agglutination and EM Binding Results

As shown in Table 1, non-trypsinized cells are not agglutinated by Con A, although the trypsinized A positive cells (#1441-t) are agglutinated by Con A (titer: 8), and the trypsinized A negative cells (#1502-t) are not agglutinated. This observation is in agreement with the correlation reported by Hines et al. (1972) that A positive cells when trypsinized are agglutinated by Con A, while trypsinized A negative cells are not agglutinated by Con A.

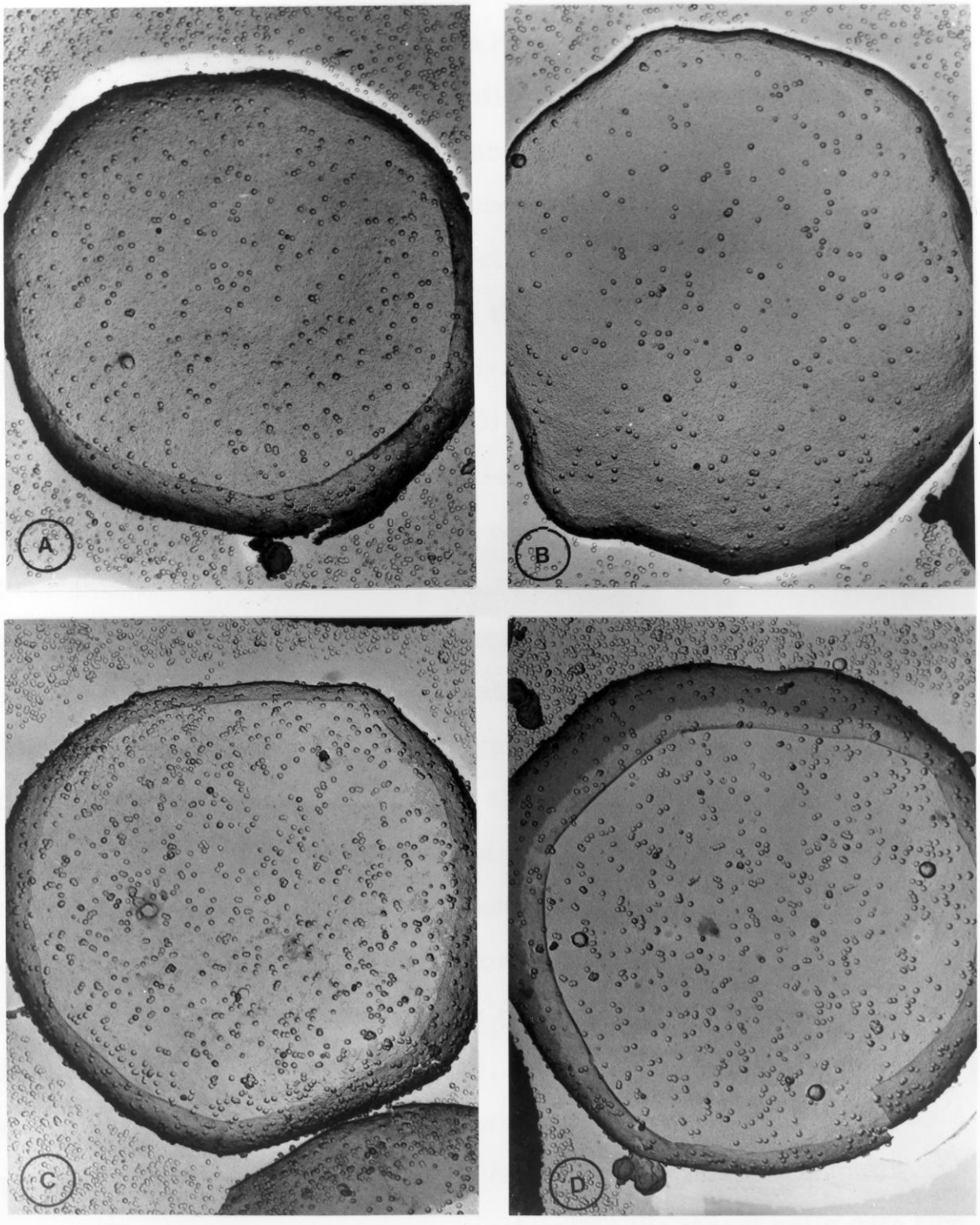
Figure 1 shows electronmicrographs of replicas of untrypsinized #1441 and #1502 cells labeled with Con A and hemocyanin, and trypsinized #1441 and #1502 cells labeled with Con A and hemocyanin, respectively. Note the random distribution of hemocyanin molecules on these cells.

Despite the inability of Con A to agglutinate untrypsinized cells, both the A positive (#1441) and A negative (#1502) cells bind Con A according to EM labeling (Table 1). The A positive cells (#1441) bind more hemocyanin (45) than the A negative cells (#1502: 30). Following trypsinization of the target cells both cell types show increased uptake of Con A. Trypsinized A positive cells (#1441-t) take up 105 hemocyanins per unit area, and trypsinized A negative cells (#1502-t) take up 78 hemocyanins per unit area. Therefore, the A positive cells (#1441) show a greater uptake of Con A than the A negative cells (#1502) for both trypsinized and non-trypsinized target cells.

Con A and Anti-A Binding on Pre-sensitized Target Cells

The Con A and anti-A experiments described show that for untrypsinized cells only those cells which genetically have the A antigen bind anti-A antibody, while both A positive and A negative cells bind Con A. For trypsinized target cells, however, both A positive and A negative cells take up anti-A antibodies and Con A, although there is a quantitative difference with the A positive cells reacting more than the A negative cells. To ask the question whether the A antigen sites and the Con A binding sites are molecularly identical, competition studies

Figure 1 Electronmicrographs of replicas of #1441 (A) and #1502 (B) cells labeled with Con A, hemocyanin; trypsinized #1441 (C) and trypsinized #1502 (D) cells labeled with Con A, hemocyanin.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were therefore necessary. In these experiments cells were treated or pre-sensitized with either anti-A antiserum (C304) or Con A as described in the general Materials and Methods section of this thesis. The serological and EM labeling values were then compared for the pre-treated cells versus the untreated cells.

Table 2 shows the results of these experiments. Untrypsinized A positive cells pretreated with anti-A (1441-anti-A) label to the same extent with Con A (labeling value: 45) as non-pretreated cells (45), indicating that the attachment of anti-A antibodies does not block the binding of Con A. The 1441-anti-A cells are not agglutinated by Con A. Pretreatment of trypsinized A positive cells with anti-A (1441-t-anti-A) and pretreatment of trypsinized A negative cells with anti-A (1052-t-anti-A) does not inhibit the agglutination of these cells by Con A (titers: 8, 2, respectively). The agglutination titers are identical for the pretreated and non-pretreated cells. Again it appears that the binding of anti-A to the target cells does not affect the agglutinating ability of Con A for these cells. The EM labeling values for these pretreated cells are slightly higher for the trypsinized A sensitized cells than for the trypsinized non-sensitized cells (#1441 cells: 123, 105, respectively; #1502 cells: 104, 78, respectively). This result indicates some enhancement of Con A binding following treatment of both genetically A positive and A negative trypsinized cells with the anti-A reagent.

The inverse experiments, pretreating the target cells with Con A and examining anti-A binding and activity, cannot be done by EM

Table 2

Lytic Titers, Agglutination Titers, and EM Binding Values for

A Positive (#1441) and A Negative (#1502) Cells Treated with Anti-A (C304) or Con A

Target Cells	A Phenotype	Reagent	Lytic Titer^{-1}	Agglutination Titer^{-1}	EM Labeling Value ± SE
1441-anti-A*	A	Con A		-	45 ± 2
1502-anti-A	-	Con A		-	
1441-t-anti-A*	A	Con A		8	123 ± 4
1502-t-anti-A	-	Con A		2	104 ± 3
1441	A	Anti-A (C304)	128		
1441-Con A*	A	Anti-A (C304)	64		
1502	-	Anti-A (C304)	-		
1502-Con A	-	Anti-A (C304)	-		

-anti-A: target cells pretreated with anti-A (C304).

- t-anti-A: target cells trypsinized and pretreated with anti-A (C304).

- Con A: target cells pretreated with Con A.

because the sensitizing Con A binds hemocyanin. However, such experiments can be done serologically. A positive (#1441) and A negative (#1502) cells were sensitized with Con A as described in the general Materials and Methods section, and examined for hemolysis with the anti-A antiserum. The results of these experiments are also shown in Table 2. In this particular experiment, untreated A positive cells (#1441) show a lytic titer of 128 with the anti-A antiserum; A negative cells (#1502) do not lyse. Con A pretreated A positive cells (#1441-Con A) show a titer of 64, one dilution lower in titer than the unsensitized cells. One dilution difference is not significant in such tests. The control experiments with the A negative cells (#1502) show negative lytic titers for sensitized (#1502-Con A) and non-sensitized (#1502) cells. Therefore, Con A pretreatment of the target cells does not affect the lytic activity of the anti-A antiserum.

Non-specific Labeling of Trypsinized A Negative Cells

In the above experiments, trypsinized A negative cells (#1502-t) were agglutinated by anti-A antiserum and took up label by EM when treated with the anti-A reagent. The following experiments were designed to test whether this uptake is specific or non-specific for anti-A antibody. That is, is there A antigen on the surface of these cells or are they only non-specifically "sticky?"

Trypsinized #1441 and #1502 cells were used to absorb the standard anti-A antiserum (A[1441-t], A[1502-t] reagents, respectively).

Exhaustive absorptions were performed as described in the Materials and Methods section of this thesis. The results of lytic assays and EM labeling experiments with these reagents are shown in Table 3. The A[1502-t] reagent gives a hemolysis titer of 64 when tested against A positive (#1441) cells and shows no activity against A negative (#1502) cells. The lytic titer for unabsorbed anti-A is 256. However, the lytic titer of the A[1441-t] reagent against A positive (#1441) cells is negative. Therefore, the #1502-t cells do absorb some anti-A antibodies, but do not approach the absorptive capacity of the #1441-t cells. The EM labeling results show that #1441 cells labeled with the A[1502-t] reagent have a reduced number of A sites (20) as compared to labeling with the unabsorbed A reagent (30). However, the absorption of the A antiserum with #1502-t cells is complete as shown by the background labeling value (13) for A[1502-t] on trypsinized #1502 cells. The EM labeling value for #1441-t cells with the A[1502-t] reagent (76) is also less than the labeling value for #1441-t cells with the unabsorbed anti-A reagent (158), indicating again that the #1502-t cells do absorb some antibody from the anti-A antiserum, but do not approach the absorptive capacity of #1441-t cells.

By EM labeling, the number of A sites for the #1502-t cells is 76 per unit area and the number of A sites on untrypsinized #1441 cells is 30. Therefore, if #1502-t cells were specifically absorbing anti-A antibodies, they should have an absorptive capacity at least as great as #1441 cells. However, from an original titer of 256, absorption with the #1441 cells reduces the anti-A antiserum titer to zero, while

Table 3

Lytic Titers and EM Binding Values for A Positive (#1441) and A Negative (#1502) Cells Treated with Anti-A (C304) Absorbed with #1441, #1502, 1502-t, or 1441-t Cells

Target Cells	Phenotype A	Reagent	Lytic Titer^{-1}	EM Labeling Value ± SE
1441	A	A[1502-t]	64	20 ± 1
1502	-	A[1502-t]	-	
1441	A	A[1441-t]	-	
1502	-	A[1441-t]	-	
1441-t	A	A[1502-t]		76 ± 2
1502-t	-	A[1502-t]		13 ± 1
1441	A	A[1441]	-	
1502	-	A[1502]	-	

A[1502-t]: Anti-A (C304) absorbed with 1502-t cells.

A[1441-t]: Anti-A (C304) absorbed with 1441-t cells.

A[1441]: Anti-A (C304) absorbed with 1441 cells.

A[1502]: Anti-A (C304) absorbed with 1502 cells.

t: trypsinized cells.

absorption with the #1502-t cells reduces the titer only to 64. Therefore, the #1502-t cells have a lower absorptive capacity than the #1441 cells. Presumably, continued absorption of the anti-A antiserum with the #1502-t cells would reduce the serum titer to zero.

Freeze-cleavage Results

Hemocyanin cell surface labeling techniques and freeze-cleavage procedures were used to study the relationship between the surface distribution of the A antigen and the distribution of the intramembranous particles exposed by freeze-cleavage on the inner membrane surface. Figure 2 shows electronmicrographs of A positive cells (#1441) treated with anti-A and RABI-Hcy (1441, anti-A, RABI-Hcy; figure 2A); treated with anti-A at 23 °C and freeze-cleaved (1441, anti-A, 23 °C; figure 2B); trypsinized and labeled with anti-A and RABI-Hcy (1441-t, anti-A, RABI-Hcy; figure 2C); and trypsinized and labeled with anti-A at 23 °C and freeze-cleaved (1441-t, anti-A, 23 °C; figure 2D). Figure 3 shows the corresponding experiments with A negative (#1502)cells. Note the random distribution of the hemocyanins and the intramembranous particles on the #1441 untrypsinized cells (figures 2A and 2B). The #1441-t cells, however, show large clusters of hemocyanin label (figure 2C) and also clustered intramembranous particles (figure 2D). The A negative (#1502) cells show a random distribution of the hemocyanins and intramembranous particles on untrypsinized cells (figures 3A and 3B, respectively). The trypsinized A negative cells (#1502-t) show some clustering of the hemocyanin label (figure 3C) although it is

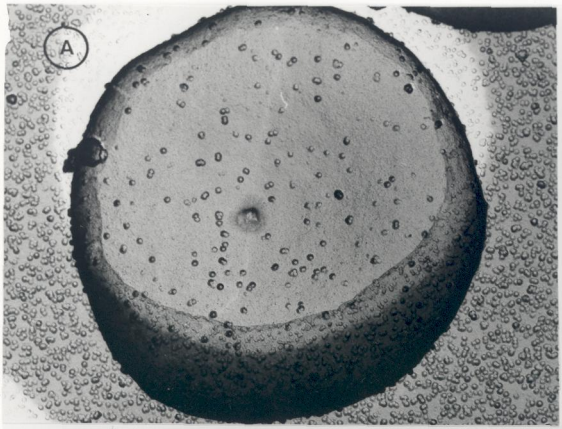
Figure 2. Electronmicrographs of replicas of A positive (#1441) untrypsinized and trypsinized cells labeled on their surfaces with anti-A antibodies; labeled with anti-A antibodies at 23°C and freeze-fractured.

(A) #1441 cells, anti-A (C304), RABI-Hcy. Magnification: 11,250×.

(B) #1441 cells, anti-A (C304; 23°C), freeze-fractured. Magnification: 136,400×.

(C) #1441 cells- trypsinized, anti-A (C304), RABI-Hcy. Magnification: 11,250×.

(D) #1441 cells- trypsinized, anti-A (C304; 23°C), freeze-fractured. Magnification : 136,400×.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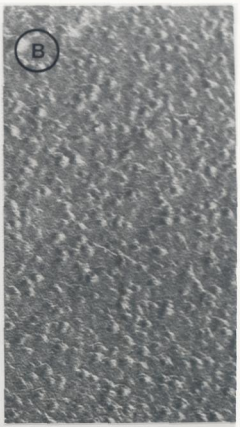
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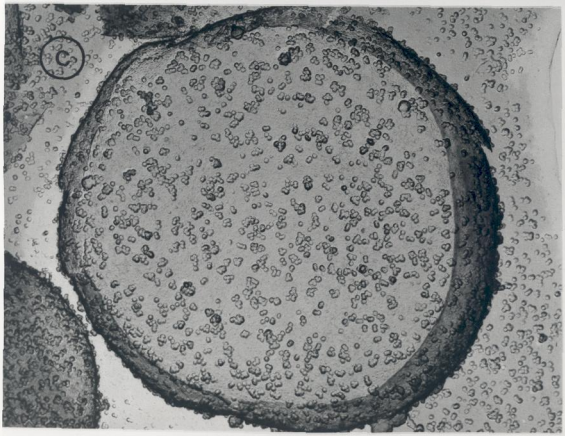

C


D

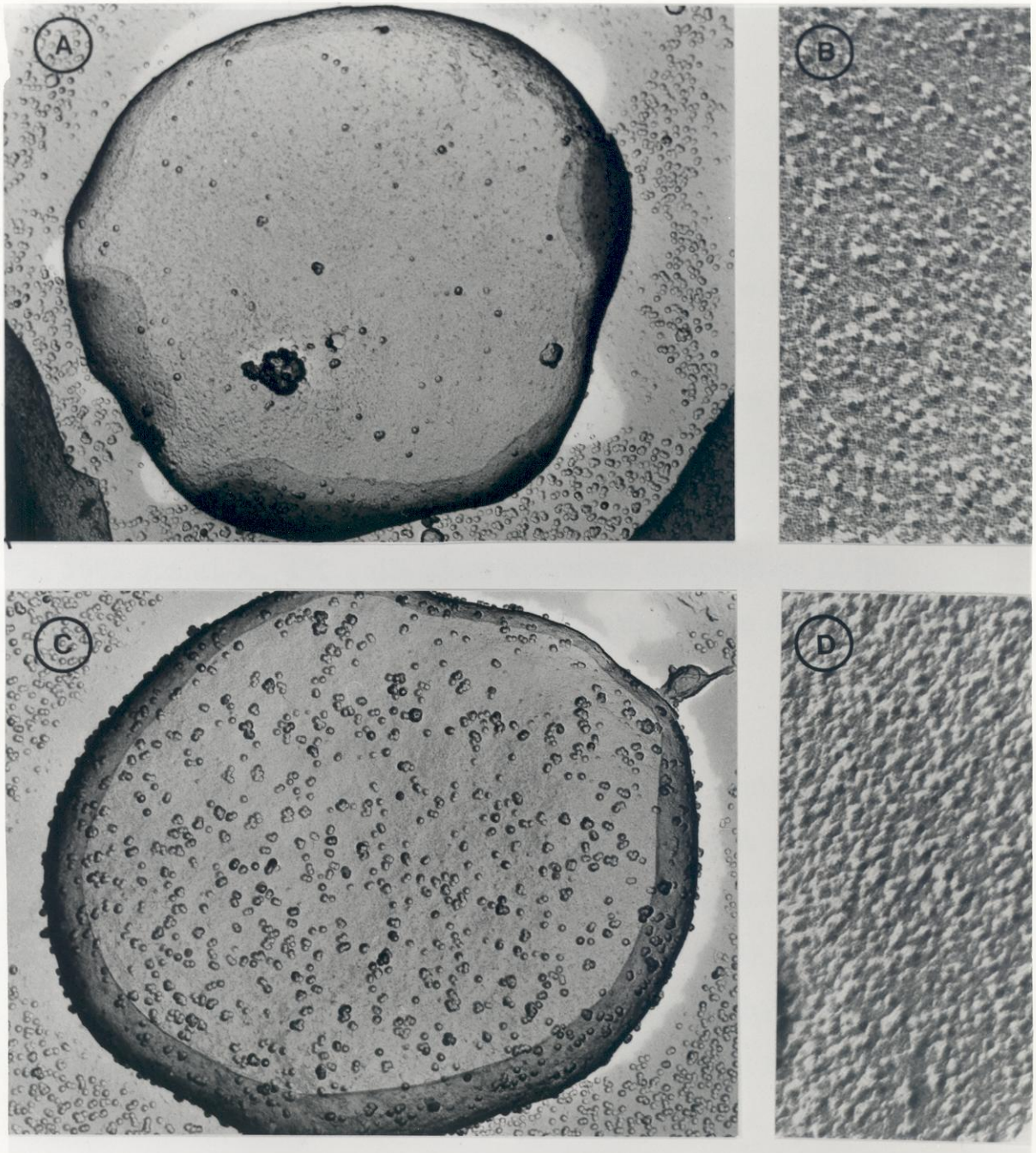
Figure 3. Electronmicrographs of replicas of A negative (#1502) untrypsinized and trypsinized cells labeled on their surfaces with anti-A antibodies; labeled with anti-A antibodies at 23°C and freeze-fractured.

(A) #1502 cells, anti-A (C304), RABI-Hcy. Magnification: 11,250×.

(B) #1502 cells, anti-A (C304; 23°C), freeze-fractured. Magnification: 136,400×.

(C) #1502 cells-trypsinized, anti-A (C304), RABI-Hcy. Magnification: 11,250×.

(D) #1502 cells-trypsinized, anti-A (C304; 23°C), freeze-fractured. Magnification: 136,400×.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not as extensive as on the #1441-t, anti-A cells (figure 2C). #1502-t cells treated with anti-A and freeze-cleaved show a random distribution of the intramembranous particles (figure 3D).

Additional freeze-fracture experiments were done to ascertain whether the A antigen clustering and the intramembranous particle clustering are temperature-dependent. Trypsinized A positive and A negative cells were incubated at 4°C for 15 minutes with anti-A antiserum (1441-t, anti-A; 1502-t anti-A, 4°C) and freeze-fractured as described in the general Material and Methods section of this thesis.

The 1441-t, anti-A, 4°C cells show clustering of the intramembranous particles as did the 1441-t, anti-A, 23°C cells (figure 2D). The 1502-t, anti-A, 4°C cells show a random distribution of the particles as did the 1502-t, anti-A, 23°C cells (figure 3D).

Discussion

Con A, Anti-A Sites

Hemolysis, agglutination, and EM binding studies have been performed to examine the relationship between Con A receptors and the bovine A antigen. Hines et al. (1972) have suggested that the Con A receptor and the bovine A antigen may be molecularly the same entity. The results presented in this report do not support that conclusion. These studies do confirm the observation by Hines et al. (1972) that trypsinized A positive cells are agglutinated by Con A while trypsinized A negative cells are not agglutinated. EM labeling studies demonstrate the presence of the A antigen on A positive bovine red cells and its

absence on A negative red cells, but both A negative and A positive red cells take up Con A regardless of trypsin treatment. A quantitative difference in Con A binding is found, with A positive cells labeling more than A negative cells. In the hemolytic assay, only the A positive cells are lysed by the anti-A antiserum, although some agglutination is found for trypsinized A negative cells. The EM labeling values parallel the agglutination results, showing some binding of anti-A to the trypsinized A negative cells.

The lack of identity of Con A receptors and the A antigen is shown by the experiments in which cells are pretreated with either anti-A antibodies or Con A (Table 2). Sensitization with either substance does not block the uptake of the other substance as measured by hemolysis, agglutination, or EM labeling. Some interference is found in Con A sensitized cells lysed with anti-A. However, the resultant reduction in anti-A titer is within the experimental error for the lytic technique. The EM binding value for anti-A sensitized cells reacted with Con A is in fact higher than the value for unsensitized cells.

The absorption experiments in which trypsinized A negative cells were used to absorb the anti-A antiserum indicate that these cells non-specifically take up antibody (and probably other substances) from the antiserum. The inability of these cells to completely absorb out the anti-A activity under conditions in which known A positive cells do absorb the specific antibody indicates that the A negative trypsinized cells are not specifically absorbing anti-A antibodies, but are merely

non-specifically sticky. The lytic assay of the absorbed antiserum supports this interpretation.

Con A Binding and Agglutination

The results described in this report indicate that despite binding of Con A to untrypsinized cells, these cells are not agglutinated by the lectin. Noonan and Burger (1973) have found a similar situation using ^{3}H-Con A to label normal and transformed cells. These authors suggest that in addition to increased Con A receptors following trypsinization, membrane constituents must be reorganized to permit agglutination. Rosenblith et al. (1973) report similar results with 3T3 mouse fibroblasts and virally transformed 3T3 cells. They also suggest that increased mobility of lectin receptors in the plane of the membrane, as induced by protease treatment, is a necessary prerequisite for cell agglutination by Con A.

The #1441-t and #1502-t cells treated with Con A and hemocyanin, however, show no clustering of hemocyanins. These cells have been prefixed, so surface molecule redistribution may have been prevented. Nevertheless, the #1441-t-Con A cells are agglutinated, demonstrating that in this particular case red cell agglutination by Con A is not accompanied by aggregation of the Con A cell surface receptors, in contrast to the suggestion by Rosenblith et al. (1973) and Noonan and Burger (1973).

Anti-A Freeze-Fracture and Hemocyanin Labeling Experiments

The aggregation of the intramembranous particles on only the A positive cells incubated with the anti-A antiserum strongly suggests that the intramembranous particles are physically associated with the cell surface bovine A antigen. This co-clustering supports the Marchesi model of the human red cell membrane (Marchesi et al., 1972, 1973; Tillack et al., 1972). Glycophorin is believed to be integrated in the membrane and attached to the intramembranous particles, while the N terminus of the glycophorin molecule protrudes to the outside of the membrane and carries various antigenic determinants and other receptors (Tillack et al., 1972).

The clustering of hemocyanin molecules on the surfaces of trypsinized red cells labeled with anti-A is at first puzzling. Because these cells are prefixed prior to the labeling procedure, one would expect that the bivalent antibody could not induce A antigen redistribution on these cells. Many investigators working with various systems have found that non-prefixed cells or cells incubated at 37°C with a multivalent reagent display clustering of the labeled sites, while prefixed cells or cells labeled at 4°C do not show clustering of label (Nicolson, 1971, 1973; Karnovsky et al., 1972; Rosenblith et al., 1973; De Petris et al., 1973).

Additional freeze-fracture experiments were performed to find out whether the prefixation with 0.1% glutaraldehyde is sufficient to prevent cell surface antigen redistribution on the hemocyanin labeled

cells. A higher concentration of glutaraldehyde was not used for prefixation because glutaraldehyde may not be effective in immobilizing the inner lipid matrix of the membrane. It was felt that a better approach would be to perform freeze-fracture experiments at 4°C, which is below the transition temperature of the membrane, and at which temperature the lipid membrane matrix should be immobilized (Edidin, 1974). The results of these experiments show that only the A positive cells show clustering of the intramembranous particles, while the A negative cells show a random distribution of these particles. Provided the membrane is in fact not fluid at 4°C, these experiments indicate that trypsinization induces the aggregation of the intramembranous particles only in those cells that possess the A antigen.

Returning to the clustering of the cell surface hemocyanin label, we can now conclude that this site aggregation could be caused by at least two mechanisms. Trypsinization could induce intramembranous particle aggregation in only those cells which possess the A antigen. Alternatively, trypsinization of A positive cells may reveal new A sites which are topographically in the area immediately surrounding those A sites which are normally displayed on untrypsinized A cells. Either of these mechanisms would account for our observations; other explanations are also possible.

If the aggregation of the intramembranous particles and the hemocyanins is an artifact of inadequate glutaraldehyde fixation or can occur below the transition temperature of the membrane, then our results

can be simply interpreted as reflecting cross-linking of the surface antigens by bivalent antibody, thus inducing the hemocyanin and particle clustering. It would be interesting to investigate this possibility further.

The uptake of anti-A antibodies by the trypsinized A negative cells is non-specific, reflecting an increased stickiness of these cells as shown by the inability of ordinary concentrations of these cells to completely absorb an anti-A antiserum as tested by EM binding and red cell hemolysis. Therefore, the clustering of hemocyanins on these cells is probably not representative of clustered A antigenic sites, but of other cell surface moieties. The absence of intramembranous particle aggregation on these cells in the freeze-fracture experiments indicates that the surface moieties being labeled are not physically connected with the intramembranous particles.

The experiments described in this report demonstrate that the bovine red cell membrane displays characteristics similar to other red cell membrane systems studied by other methods in the past (Marchesi et al., 1972). The surface A antigenic determinants as visualized by hemocyanin appear to be physically associated with the intramembranous particles. The antigenic determinants and the intramembranous particles of trypsinized cells redistribute in the presence of the specific bivalent antibody. Both trypsinized and non-trypsinized bovine red cells bind Con A, although trypsinized cells bind more Con A than the untreated cells, and only the trypsinized A positive cells are agglutinated by Con A. The EM and serological observations on the occurrence of the bovine A antigen and Con A receptors on the cell surface clearly demonstrate that

these two properties are molecularly independent entities, in contrast to the suggestion of Hines et al. (1972).

CHAPTER IV

BOVINE LEUKOCYTE ANTIGENS

Introduction

Bovine red blood cell surfaces display over 60 serological specificities grouped into 11 genetic systems (Stormont, 1962). These specificities and the phenogroups they form have been well characterized genetically and serologically. Believing that a study of bovine peripheral blood leukocytes for the presence of bovine red cell antigens is in order, we have surveyed 28 bovine red cell typing reagents for their leukocytotoxicity in a microcytotoxicity system. To our knowledge, only 3 other studies have been undertaken on bovine leukocyte antigens. Borovská and Demant (1967) reported that the H′ (S_2) and U_1 antigens of the S system are present on bovine lymph node leukocytes. In an abstract, Schmid and Cwik (1972) reported 28 lymphocyte and 37 granulocyte factors on bovine leukocytes. The latter study used reagents prepared by iso- and heteroimmunization with bovine leukocytes. Hruban and Simon (1973) reported the detection of the J antigen on bovine leukocytes using an immune serum. We have examined bovine red cell typing reagents to determine whether red cell polymorphism is reflected in peripheral blood leukocyte specificities.

Materials and Methods

Leukocyte typing was originally performed using the Amos one-step microcytotoxicity assay (Amos et al., 1969). Using this assay, 46 bovine red cell typing reagents, including 17 B complex reagents, 5 C complex reagents, 9 S complex reagents, 3 A complex reagents, F and V reagents, Z reagent, L_X reagent, 5 J reagents, M_2 reagent, and R′ and

S′ reagents were tested against subsets of cells from 56 donors. Subsequent testing was done using the Amos two-step microcytotoxicity test (Amos et al., 1969).

The preparation of cells for testing, and other technical details, are described in the general Materials and Methods section of this thesis.

Results

According to the one-step microcytotoxicity test, 30 of the antisera tested were selectively leukocytotoxic, one antiserum was cytotoxic for all leukocytes tested, and 15 antisera showed no cytotoxicity for the target leukocytes. In no case did a reagent cytotoxic for the target leukocytes give a plus and minus reactivity pattern which matched the red cell types of the leukocyte donors. Also, reagents that were not cytotoxic for leukocytes were nonreactive with leukocytes whose donors lacked the red cell antigen as well as with leukocytes whose donors possessed it.

During the course of subsequent typing it was found that if bovine leukocytes were typed using the Amos two-step assay (Amos et al., 1969), they reacted positively with more reagents than if they were tested in the one-step assay. In many cases those specificities which were weakly positive in the one-step assay were strong positives by the two-step assay. In no case was a specificity picked up by the one-step method and not detected by the two-step method.

Following the realization that the one-step and two-step assays gave different results, we repeated various typing tests using the two-

step assay and found the data to be repeatable. Twenty-eight red cell typing antisera were then tested for leukocyte cytotoxicity, using the two-step assay, against a cell panel consisting of leukocytes from 13 animals from the California State Polytechnic Institute at Pomona (Cal Poly Pomona) and the University of California at Davis (U.C. Davis) herds. The 28 red cell typing reagents were chosen for the subsequent leukocyte typing based on their reactivity in the one-step test.

The reagents tested in the two-step assay are listed by blood group system in Tables 1 and 2. Of the 28 reagents tested, 23 showed leukocytotoxicity (Table 1) and the remaining 5 were not cytotoxic for any of the leukocytes tested (Table 2). Table 1 shows the results of testing representative reagents from each of 10 genetic systems against leukocytes from the 13 donors. Except for the F (C338) reagent (no cells from F negative cows were included in the study), the patterns of leukocyte cytotoxicity do not coincide with the red cell types of the donors. The cell panel for the non-leukocytotoxic reagents shown in Table 2 consisted of cells from donors whose red cells were positive and from donors whose red cells were negative for the antigen under study.

One U_1 reagent (R414), one H′ reagent (ST61), and an H′ H′ reagent (R pool) were tested against a total of 34 cells. No relation was found between leukocytotoxicity and donor red cell antigens U_1, H′, or S (see Table 1), although H′ (ST61), H′ H′ (R pool) and S (C236) were

Table 1

Leukocytotoxic Activity of 23 Bovine Red Cell Typing Reagents

System	Reagent		No. of Cattle Tested	Leukocyte Positive[a] rbc positive	Leukocyte Positive[a] rbc negative	Leukocyte Negative rbc positive	Leukocyte Negative rbc negative	Weak[b] Positive Leukocyte Reaction
B	A'_x	C308	13	4	1	3	5	1
	O_1F'	C281	13	6	1	4	0	3
	E'_x	ST11	13	6	0	5	1	1
	B′	ST60	12	0	3	2	7	0
	T_1	C58	13	2	2	1	8	0
	B_1	C125	13	3	7	1	2	0
	K	C77	13	0	1	3	9	0
	sheep O	C50	13	4	0	7	1	1
C	W	C301	13	6	1	1	4	1
	R_2	NORI	13	5	7	1	0	0
	$X_2 + I$	ST39	13	6	4	2	1	0
F-V	F	C338	13	10	0	0	0	3

Table 1 (Continued)

System	Reagent		No. of Cattle Tested	Leukocyte Positive[a] rbc positive	Leukocyte Positive[a] rbc negative	Leukocyte Negative rbc positive	Leukocyte Negative rbc negative	Weak[b] Positive Leukocyte Reaction
Z	Z	ST54	13	7	2	0	1	3
	U_1	R414	0	0	1	4	11	1
S	H′	ST61	9	1	0	4	3	1
	S	C256	13	3	1	1	8	0
	H′ H′	R Pool	13	3	2	7	1	0
A	A	C304	9	3	0	4	1	1
L	L_X	C63	13	1	5	2	3	2
J	J	ST48	13	2	2	6	3	0
M	M_2	C320	13	1	6	0	4	2
R′ S′	R′	C285	13	7	3	0	1	2
	S′	C312	13	11	0	1	0	1
	anti-386		13	5	6	0	1	1

Table 1 (Continued)

[a] Greater than 35% killing is scored as a positive leukocyte reaction.

[b] Between background and 35% killing is scored as a weak positive leukocyte reaction.

Table 2

Non-Leukocytotoxic Red Cell Reagents

System	Reagent	
B	K′	C117
S	U′	C146
	U_2	C310
A	Z′	C175

selectively cytotoxic for certain target cells. This result is in contradiction to the report of Borovská and Demant, to the effect that bovine blood group antigens H' (S_2) and U_1 are present on bovine lymph node leukocytes.

Anti-386, a bovine isoimmune antiserum (see the general Materials and Methods section of this thesis), was also selectively cytotoxic for certain leukocytes, as shown in Table 1.

Discussion

The discrepancy between the one-step and the two-step assays originally came to our attention while we were conducting family studies. Four dam-offspring pairs had been leukocyte typed. At a later date, a blood sample from the sire #249, generously supplied by All West Breeders (Burlington, Washington), was typed with the one-step assay. It was found that all of the calves' apparent leukocyte antigens could not be accounted for by tests of their dams and sire. Therefore, the sire's leukocytes were retyped using the two-step assay. With the two-step procedure all of the calves leukocyte antigens could be attributed to either the dam or the sire. Unfortunately, the dams' and the calves' cells were no longer available for testing using the two-step assay. Additional studies were then conducted using the two-step assay exclusively. All of the typing data reported here were collected using the two-step assay.

The report of Borovská and Demant that S system red cell antigens are present on leukocytes (Borovská and Demant, 1967) has not been

supported by our studies. We have typed bovine peripheral leukocytes using a modified form of the microcytotoxicity assay used for detection of H-2 and HL-A antigens, while they used lymph node cells and a macrocytotoxic assay (Gorer and O'Gorman, 1956). Demant and Borovská found a statistically significant association between H′ (S_2) and U_1 in leukocytotoxic and hemolytic tests. We have tested one H′ reagent, one U_1 reagent, and an H′ H′ (H′ dosage reagent) and have found no such association. The results of Schmid and Cwik (1972) are consistent with ours in that they also found leukocyte specificities with iso- and heteroimmunization. We have prepared an anti-leukocyte reagent (anti-386) by specific isoimmunization and also note that this antiserum is selectively leukocytotoxic.

Hruban and Simon have reported that a J_2 reagent was cytotoxic for a panel of leukocytes and that the reaction pattern corresponded to the hemolytic reaction pattern. Our results with the J (ST48) reagent are not consistent with the results of Hruban and Simon. The pattern of leukocytotoxicity of the J (ST48) reagent does not correspond to the pattern of red cell hemolysis of the donor red cells. Hruban and Simon used the Terasaki microcytotoxicity procedure (Terasaki et al., 1965) while we have used the Amos two-step procedure (Amos et al., 1969). Our procedure introduces a wash step between antiserum and cell incubation, and the addition of complement. The two-step assay is necessary for certain sera which are partially denatured, anticomplementary, or which give weak actions (Amos et al., 1969). Initially,

we also used a one-step assay but found that we picked up more positive reactions using the two-step assay. The inconsistency of our results with the results of Hruban and Simon can be partially explained by different methods, but some reactions still remain at variance. The J_2 reagent of Hruban and Simon and our J (ST48) reagent could contain additional antibodies which are not detectable by the hemolytic assay, and which are directed against other specificities than the bovine red cell J antigen. Such a population of antibodies has been found in our J (ST48) reagent (Chapter I, this thesis). These additional antibodies could then give a leukocytotoxic pattern at variance with the J phenotype of the red cells of the leukocyte donor, as well as a variance between leukocytes tested with the J_2 and J (ST48) reagents.

We have tested representative reagents from each of the bovine red cell blood group systems (Tables 1 and 2). For the F(C338) reagent no cells from donors whose red cells were negative for F were included in the test panel and the F reagent was cytotoxic for all target leukocytes. In all other cases no reagent cytotoxic for the target leukocytes gave a plus and minus reactivity pattern which matched the red cell types of the leukocyte donors (Table 1).

The W (C301), S (C236), and S′ (C312) reagents show few reactions in the leukocyte positive - red cell negative and leukocyte negative - red cell positive classes (Table 1). These observations suggest a possible association of red cell and leukocyte antigens for these particular specificities. However, the reported test cell

leukocyte panel is small, and additional cell populations should be tested before any definitive conclusions can be drawn.

Reagents that were not cytotoxic for leukocytes (Table 2) were nonreactive with leukocytes from donors whose red cells possessed the antigen, as well as with leukocytes whose donors lacked the antigen. With the exception of the F antigen for which we have an incomplete test panel, the data indicate that bovine leukocyte antigens, as detected by the two-step microcytotoxicity test, are distinct from red cell antigens.

Because there is no apparent association of leukocyte reactivity with bovine red cell antigens, aside from the possibilities discussed, we are probably detecting different antigenic systems on leukocytes and red cells. In order to characterize the leukocyte antigens, absorptions should be done on our reagents (many of these antisera probably detect multiple specificities), and family studies should be undertaken.

The red cell typing reagents were prepared by iso- or hetero-immunization with either whole blood or washed cells. A red cell typing reagent prepared against red cells and leukocytes and rendered monospecific for a red cell antigen by absorption with red cells could still contain antibodies against various immunizing leukocyte antigens. The activity of our leukocytotoxic red cell typing reagents has probably been produced in this manner.

SUMMARY

The experiments described in this thesis show that electron microscopy can be productively used in conjunction with serological studies to examine questions in immunogenetics.

Using cells which are genetically and serologically defined for their zygosity with respect to the Z allele, it was found that homozygous (Z/Z) cells have approximately twice as much surface Z antigen as heterozygous (Z/-) cells. The Z antigen is an integrated cell membrane substance. In the case of the J substance, a soluble serum substance secondarily adsorbed to the red cell surface, cells display a quantity of J which is directly related to the J titer of the cells. In the J system, most of the antibody bound is of the IgG class, although in most cases some IgM is also bound. A new antigen has been described which is independent of the J antigen, and which is detectable by EM labeling and by indirect agglutination, but not by hemolysis.

Experiments which dealt with the cell surface expression of antigens in the presence of bound antibody directed against another antigen demonstrated the absence of steric blocking for three pairs of antigens labeled. This result indicates that the antigens within each pair were not so topographically close together on the cell surface that attaching an antibody and hemocyanin molecule to one of the antigens inhibited uptake of antibody and hemocyanin by the second antigen. It is suggested that these bovine red cell antigens are independent entities on the cell surface. The genetic organization that produces

antigens in such a topological configuration would have to allow for gene products which were sterically distant from each other on the cell surface by at least the diameter of one antibody molecule. This result does not eliminate the possibility that the bovine red cell antigens are coded for by series of multiple alleles, but it does imply that if the specificities are determinants on common backbone macromolecules then the antigenic determinants are topologically separated by distances at least as great as the diameter of an immunoglobulin molecule.

For one particular pair of antigens (R_2, X_2) sequentially double labeled cells take up twice as much label as the sum of the single labeled cells. This observation suggests that new antigenic sites are revealed in the presence of bound antibody directed against another specificity. The findings that the increased uptake of label occurs only on cells labeled first with one particular antibody, and that the phenomenon is not found for other pairs of antigens labeled or in the reverse sequence of labeling, suggest that this result is specific to the R_2 and X_2 antigenic determinants.

Increased quantitative expression of the A antigen and Con A receptors has been demonstrated for trypsinized as compared with non-trypsinized A positive target cells. Much of the increased labeling found on the trypsinized cells can be attributed to non-specific stickiness of the target cells, although additional specific A sites are revealed following enzymatic incubation of the A positive target cells. The clustering of surface A antigen found on the trypsinized A positive cells,

and the aggregation of the intramembranous particles seen on the freeze-fractured replicas, imply that the surface A antigen is physically attached to the intramembranous particles. The absence of clustering of intramembranous particles and specific surface antigen on cells negative for the antigen demonstrates the specificity of the reaction. The lack of agglutination, of surface antigen clustering, and of intramembranous particle aggregation on the untrypsinized cells, and the presence of surface antigen and intramembranous particle aggregation associated with red cell agglutination on the trypsinized cells, strongly suggest that membrane rearrangements are an integral part of the agglutination process.

Specific bovine leukocyte antigens have been demonstrated. The results presented here are preliminary. A comprehensive study should be conducted, including family studies, tests on normal serum, reagent absorptions to produce monospecific leukocyte typing antisera, and specific iso- and heteroimmunizations with bovine leukocytes.

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