Antigenic Analysis of Cells and Tissues: Common Antigens on Human Erythrocytes and in Cultured Cells.* (31607)

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The human erythrocyte membrane is immunologically characterized not only by blood group-specific antigens but also by species-specific components. Blood serologists recognize the latter mainly as a nuisance in that the preparation of certain test sera necessitates removal of species-specific antibodies by means of absorption. However, at least one example can be cited in which species-specific red cell antigens have been put to practical use, that is, in various methods for species determination of established mammalian cell culture strains(1,2,3). In the species-specific hemagglutination test, antisera are produced in guinea pigs against cell strains to be examined. The sera are then titrated for hemagglutinins against erythrocytes of various species being under consideration as origin of the unknown cell strain. There is no interference in this test from blood group-specific reactivity since established cell strains have lost the main blood group antigens of the original donor(4,5). Methods based on this principle are employed for characterization of reference cell strains, for exclusion of mix-up or accidental contamination between cell strains of different species, to verify purity of cell strains used for virus vaccine production, etc.(6). The antigens involved have been found to be proteins(7). They were demonstrated in all parts of the cultured cell including the nucleus(8).

In recent years a comprehensive analysis of the antigenic composition of human cells and tissues has been undertaken in this laboratory. So far we have been able to distinguish and to define 24 antigenic components by means of immunodiffusion techniques(9,10, 11). The water phase of mechanically disrupted and ether-extracted cells or tissues served as antigen. Mono- or oligospecific antisera were prepared in various animal species, particularly guinea pigs(12,13). It was of interest to correlate these findings with our earlier work on species-specific hemagglutinogens in cultivated cells and to analyze the species-specific antigenicity of the human erythrocyte membrane more precisely. The methodological approach obviously had to be changed as the antigen extraction procedure used for tissues and cultured cells was not applicable for red cell membranes. Instead, oligospecific reference sera were absorbed with red blood cells. Then, the absorbed sera were subjected to immunodiffusion tests with defined cell culture extracts as test antigens. Disappearance or significant weakening of specific precipitation lines would indicate successful absorption of the antibody in question and, hence, would prove the presence of the corresponding antigen on the erythrocyte membrane. As described in this paper, it was possible to demonstrate on the erythrocyte membrane 8 distinct antigenic components of the type characterized above although, for technical reasons, only 15 out of 24 specific cell antigens were studied so far.

Materials and methods. Preparation of cell and tissue antigens for animal immunization and for agar immunodiffusion tests was carried out as described previously(10). Human cell strains were cultured as monolayers according to standard procedures in Eagle's medium supplemented with 17% newborn calf serum. No antibiotics were added to assure early detection of microbial contaminants. Cultures were periodically checked for sterility and especially for the absence of mycoplasma. The growth medium was replaced 24 hours before harvest by Eagle's medium without serum supplement in order to exhaust intracellular serum components. Monolayers were washed with balanced salt solution and scraped off the glass. A 50% (v/v) cell suspension was mechanically disrupted by means of a teflon pestle (Tri-R) at high speed (2000 rpm) in the presence of half the volume alundum powder (60 mesh). After settling of alundum and teflon pieces the

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homogenate was used directly for immunization. For obtaining test antigens, the homogenate was extracted with ether for 4 hours at 4°C to remove lipids which tend to impair the clarity of immunodiffusion reactions.

Reference antisera against cellular homogenates were prepared in groups of guinea pigs or other animal species as previously described (10,13). Extended immunization schedules were advantageous with most animal species. At least in guinea pigs no significant differences regarding the potency of immune sera were observed when time schedule and dosage varied. For any of the tests and experiments the sera were inactivated at 56°C for 30 minutes.

Agar double immunodiffusion tests were carried out according to the micromethod of Crowle(14) with minor modifications.

Erythrocytes for serum absorption and species-specific hemagglutination were obtained from donors with different blood groups. The washed erythrocytes were pooled so that all major blood group components were included.

The *species-specific hemagglutination test* was carried out in disposable plastic trays with round bottoms as previously described (5). The serum titrations were carried out in 2-fold dilution steps ranging from 1:6 to 1:3840. The test volumes were 0.1 ml of serum dilution plus 0.025 ml of a 2% erythrocyte suspension. After carefully mixing, the covered trays were incubated at 37°C for 60 minutes and placed on an X-ray film viewer for reading the agglutination end points.

Experiments and results. At the time of these investigations 15 antigenic components had been defined in human cells and tissues. Reference sera were selected which contained the specific antibodies for each of these antigenic components. The sera were individually absorbed with human erythrocytes as follows. After repeated washing a 20% suspension of pooled human erythrocytes was distributed in equal amounts of 1.0 ml into screw cap tubes (16 × 75 mm in size). Just before each serum absorption an adequate number of tubes was centrifuged for 10 minutes at 1800 rpm with a rotor diameter of 45 cm bottom to bottom. The supernatant fluid was carefully removed with a capillary pipette and the rest of the fluid drained out with strips of filter paper. In this way each tube contained exactly equal amounts (approximately 0.2 ml) of packed red cells. For absorption 0.2 ml amounts of undiluted sera were filled into the tubes and gently mixed with the red cells. The tubes were incubated for 30 minutes at room temperature during which period they were gently stirred every 5 to 10 minutes. This was followed by centrifugation for 5 minutes at 1800 rpm and another 5 minutes with the speed increased to 2500 rpm. The supernatant sera were recovered carefully and subjected to 2 or 5 more courses of absorption. With each absorption there was a loss of about 0.01 ml of serum. At the same time a slight dilution factor was introduced from residual diluent caught in the intercellular space of the packed erythrocyte sediments. An adjustment was made accordingly on the nonabsorbed serum aliquots. It was based on the calculation that under the conditions of this experiment each absorption added a dilution factor of approximately 1:1.04.

Absorbed and nonabsorbed sera were comparatively examined in immunodiffusion tests with a HeLa or Chang conjuctiva cell extract as indicator antigen; simultaneously they were titrated in a species-specific hemagglutination test against human erythrocytes, the same pool as used for absorptions. In the immunodiffusion experiments standard conditions were maintained and arrangement patterns were employed by which the specific precipitation lines under study could be identified without ambiguity.

It is known that guinea pig sera give H-type precipitation reactions(10,12), *i.e.*, formation of precipitation lines is inhibited with either antibodies or antigen being in excess of the optimum proportional amount. Therefore, any shift in the quantity of a specific antibody as a result of absorption readily weakened the corresponding precipitation line or made it altogether disappear. Any such observation was confirmed as a specific absorption effect by examining the sera in different dilutions. The amount of antibody absorbed was in this way estimated and quantitatively related to corresponding shifts in species-specific hemagglutination titers.

TABLE I. Effect of Absorption with Human Erythrocytes on Capacity of Anti-Human Cell Reference Sera to Precipitate Defined Specific Antigen Components in Human Cells and to Agglutinate Human Erythrocytes.

					Species-specific hemagglutination		
			Precipitation line if serum was		Reciprocal titer if serum was		
Reference serum No.	Times absorbed with human RBC	Cellular antigenic component* reacting	Non-absorbed	RBC absorbed	Non-absorbed	RBC absorbed	% Hemagglutinin absorbed
15/5	6	# 1 (A_1)	+	+	>3840	<6	>99.8
		# 2 (B_1)	+	0			
		#10 (C_{12})	+	0			
33/4	6	#15 (B_3)	+	0	3840	6	99.8
		# 8 (C_4)	+	+			
22/2	6	# 1 (A_1)	+	+/0	>3840	<6	>99.8
		# 2 (B_1)	+	(+)			
		# 4 (B_8)	+	+			
		#14 (G^*)	+	0			
21/2	6	# 1 (A_1)	+	+/0	3840	6	99.8
		# 2 (B_1)	+	(+)			
		#12 (G_1)	+	+			
3/2	6	— (Z)	+	0	960	120	87
		# 1 (A_1)	+	0			
		#13 (G_2)	+	0			
28/2	3	# 1 (A_1)	+	+/0	960	240	75
		# 4 (B_8)	+	+			
19/5	3	# 1 (A_1)	+	(+)	480	240	50
		#11 (D)	+	+			
17/5 (Fig. 1)	3	# 1 (A_1)	+	+/0	480	240	50
		# 6 (B*9)	+	+			
		# 9 (C*11)	0	+			
7/7	3	# 1 (A_1)	+	+	<6	<6	—
		# 5 (B'_8)	+	+			
		#16 (D_2)	+	+			

* Designations according to those used in earlier publications(10,11).

+ = line present; (+) = line fading; 0 = line has disappeared; +/0 = line is split into 2 or more components, one of which has disappeared (Fig. 1).

The results of these experiments are compiled in Table I.

Closer evaluation of the data reveals some interesting details. The behavior of the antigen #1 (A_1) points to its heterogenicity. One of its components is present on the erythrocyte membrane, the other is not. Correspondingly, two different specificities of A_1-antibodies can be distinguished. Those in serum #3/2 react only with the A_1-component that is present on the erythrocyte membrane. The antibodies in sera #15/5 and #7/7 react only with the component not present on the erythrocyte membrane. Sera #22/2, #21/2, #28/2, and #17/5 contain both types of A_1-antibodies. As a consequence, the composite A_1-precipitation line splits toward the well containing erythrocyte-absorbed serum (Fig. 1).

Hemagglutination titers vary considerably among the sera chosen for this experiment. It cannot be overlooked that sera with the highest hemagglutination titers (#15/5, #33/4, #22/2, and #21/2) contain the immunologically closely related B_1 or B_3-antibodies and that these are absent in the low-titered sera. However, several other data make a causal relationship questionable. The effect of erythrocyte absorption was not always congruent in both the hemagglutination and the B_1 or B_3-precipitation reactions. With sera #22/2 and #21/2 complete removal of hemagglutination activity was achieved, yet the B_1-precipitation line remained faintly visible. A 99.8% loss of hemagglutination activity corresponds to 10 serial two-fold dilutions of serum. In control experiments the B_1-line had completely disappeared already

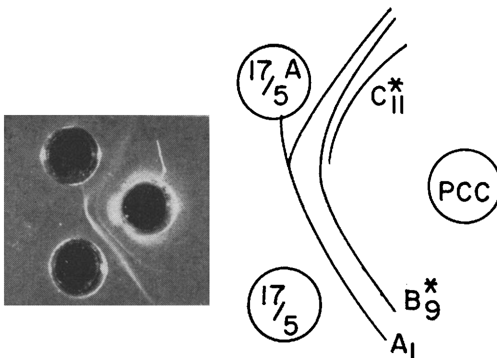

FIG. 1. Agar double immunodiffusion test of non-absorbed and erythrocyte-absorbed reference serum #17/5 against a whole cell antigen extract of the Chang conjunctiva strain. Direct close-up photography with indirect illumination. See also Table I (serum #17/5). Abbreviations used in the drawing: 17/5 = the well containing nonabsorbed serum #17/5; 17/5A = the well containing serum #17/5 absorbed 3 times with pooled human erythrocytes; PCC = the well containing Chang conjunctiva cell antigen extract; A_1, B*9, C*11 = specific reaction lines as listed in Tables I and II.

after 3 to 4 dilution steps. Therefore, antibodies other than B_1 and B_3 must be assumed to contribute heavily to the hemagglutinating potency of those sera. It even appears likely, *e.g.*, from the results obtained with serum #33/4, that the antibody specificities listed are not the only ones to account for excessively high hemagglutination titers.

A peculiar absorption effect was observed on the #9 (C*11)-reaction of serum #17/5. In the non-absorbed serum C*11-antibodies were present in excess preventing the formation of the precipitation line. Absorption decreased the amount of C*11-antibodies down to the level of optimum proportion. Only then the line became visible (Fig. 1), in this way proving the presence of the C*11-antigen on the erythrocyte membrane.

In Table II, the cellular antigen components are listed and their relationship to the antigenic composition of the human erythrocyte membrane is indicated.

Discussion. When this experiment was undertaken we had little reason to expect a positive result. Therefore, the demonstration of 8 antigenic components on the erythrocyte membrane out of the 15 cellular antigens studied came almost as a surprise. It must be realized that the cell and tissue antigens which we had investigated and defined by means of immunodiffusion techniques actually represented soluble and diffusible entities. In contrast, the antigens which we set out to explore by erythrocyte absorption represented structural elements of the erythrocyte membrane. We have to conclude that the 8 antigens common in HeLa cell extracts and on the human erythrocyte membrane either exist in structural as well as soluble form in the human body or they are readily freed and solubilized from their structural bounds. It should be mentioned in this connection that none of the antigenic components described have been found to be constituents of human serum(9).

The number of cellular antigens demonstrated and defined to date has already reached 24 and there is good indication that more will be added to the map. Accordingly it can be assumed that the list of common erythrocyte antigens is not yet complete. Quantitative calculations concerning the absorption effect on precipitating *vs.* hemagglutinating antibodies must also be interpreted in this sense.

It appears that many or most of these antigens are species-specific. However, observations have been made which force us to question the validity of this definition at both ends. Some antigens cross species boundaries, *e.g.*, the A_1-antigen, a component

TABLE II. Human Cellular Antigens* and Their Relationship to Human Erythrocyte Membrane.

Antigenic components* of human cells and tissues	Present on human erythrocyte membrane
# 1 (A_1)	+/0
# 2 (B_1)	+
# 4 (B_8)	0
# 5 (B'_8)	0
# 6 (B*9)	0
# 8 (C_4)	0
# 9 (C*11)	+
#10 (C_{12})	+
#11 (D)	0
#12 (G_1)	0
#13 (G_2)	+
#14 (G*)	+
#15 (B_3)	+
#16 (D_2)	0
#— (Z)	+

* Designations according to those used in earlier publications(10,11).

\+ = antigen present; 0 = antigen not present; +/0 = only part of a complex antigen present.

of which has also been demonstrated in the mouse L-cell strain and in tissues of certain inbred mouse strains(12). Others might well possess characteristics of isoantigens, *e.g.*, those which have not uniformly been found in every human cell strain(10,11). The latter possibility would have to be verified by comparatively analyzing these antigens in many individuals. Once the isoantigenic nature of some of these antigens is established, the door would be open for genetic studies and possibly even practical applications. On the other hand, connections to transplantation and transfusion problems might have to be taken into consideration.

Summary. Antisera were available which had been prepared in guinea pigs and other animal species against established human cell strains such as HeLa, etc. By means of agar-immunodiffusion tests numerous cellular antigens had been defined. Individual sera had been analyzed regarding the antibody specificities they contained. In the present study, selected reference sera were absorbed with pooled human erythrocytes and then subjected to immunodiffusion tests against cell antigen extracts of known composition. Disappearance or significant weakening of specific precipitation lines indicated successful absorption of a specific antibody and, hence, the presence of the corresponding antigen on the erythrocyte membrane. This was established for 8 out of 15 specific cellular antigens investigated. An attempt to correlate precipitating and species-specifically hemagglutinating antibodies was in part satisfactory. It was emphasized in the discussion that precipitable cellular antigens exist in soluble form whereas the corresponding antigens on the erythrocyte membrane are structurally bound.

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Differences in Serological Properties of Macroglobulins from Cancer And Normal Sera.* (31608)

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Previous investigations have revealed that sera from patients with cancer flocculate unsensitized particles of bentonite, while sera from normal humans give negative or low titered reactions. Both the cancer and normal sera had to be heated to 50°C for 30 minutes in order to establish this difference in reactivity. In addition, electrophoretic studies showed that when bentonite particles were added to cancer sera, alpha-2 globulins were reduced markedly, which was not the case when the particles were added to normal human sera(1).

Using Sephadex particles as molecular sieves, serum globulins were separated from

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