TISSUE-SPECIFIC CELL-SURFACE ANTIGENS IN EMBRYONIC CELLS

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ABSTRACT

With the use of antisera prepared in rabbits against suspensions of live embryonic chick tissue cells, qualitative differences in cell surface antigens were demonstrated on cells from different embryonic chick tissues by immune agglutination and immunofluorescence. Unabsorbed antisera reacted with both homologous and nonhomologous cells; thorough absorption of the antisera with heterologous tissues removed cross-reacting antibodies, and the antisera acquired a high degree of tissue specificity. Thus, antiretina cell serum absorbed with nonretina cells or tissues, agglutinated only neural retina cells, and was shown by immunofluorescence tests to react specifically with the surface of retina cells, both in cell suspensions and in frozen tissue sections. Comparable results with antisera against cells from embryonic liver and other tissues demonstrated the existence of tissue-specific, phenotypic disparities in the antigenicities of embryonic cell surfaces, in addition to the presence of cell-surface antigens shared by certain classes of cells, and of antigens common to all cells in the embryo. The results are discussed in terms of the possible involvement of such phenotypic determinants in the specification of cell surfaces, in relation to cell recognition and developmental interactions.

It is generally assumed that the cell surface plays an important role in the control of embryonic morphogenesis, growth, and differentiation since it is closely involved in cell recognition, selective cell grouping, and various developmental cell interactions. Studies on reaggregation of embryonic cells in suspensions of mixed cell types have shown that cells can "recognize" like from unlike with respect to histotype and functional complementarity, and that they sort out and associate preferentially into tissue-specific groupings (1, 2). These studies have further indicated that cell recognition and histotypic cell association are mediated by specific macromolecules located in the cell surface and between cells which function as intercellular ligands. Preparations of tissue-specific cell ligants were obtained from several kinds of embryonic cells, including embryonic chick neural retina cells (1, 3, 4) and mouse brain cells (5, 6), as well as from different species of sponge cells (7); functional specificity was shown to be accompanied by antigenic specificity (4, 7). These results raised the important question whether surfaces of embryonic cells from different tissues possessed different antigenicities that would suggest the existence of tissue-specific cell-surface determinants. Preliminary observations (8) encouraged a detailed examination of this problem. The demonstration of such tissue-specific antigenic differences between cell surfaces would be of obvious interest and significance to further analysis of specification of cell surfaces and its role in developmental cell interactions and differentiation. In the experiments reported here,

the presence of tissue-specific cell-surface antigens on embryonic chick cells was demonstrated by techniques of immune agglutination and immunofluorescence.

MATERIALS AND METHODS

Chick embryos were from a randomly bred but essentially closed stock of White Leghorn chickens.

Preparation of Embryonic Cell Suspensions

Suspensions of single cells were prepared from the following tissues of the chick embryo: retina, liver, heart, thigh muscles, cerebrum. The embryonic ages of the tissues are stated below. The amounts of tissue and cells used were always adjusted to equivalence to compensate for differences in the size of the different organs.

Cell suspensions were prepared by trypsinization of the embryonic tissue according to the standard techniques of this laboratory (9–11). Briefly, freshly dissected embryonic tissue fragments were incubated at 38°C for 20–30 min in 0.5% solution of crystalline trypsin (Tryptar, Armour Pharmaceutical Co., Chicago, Ill.) in calcium- and magnesium-free saline (CMF), washed with chilled Tyrode's solution, transferred to the appropriate medium (4°C), and the cells were dispersed by pipetting.

Erythrocytes were obtained from defibrinated blood of adult White Leghorn chickens and used within 24 hr of collection after washing in Dulbecco's phosphate-buffered saline (PBS).

Aggregation of Cells

Dispersed cells were suspended in 3 ml culture medium in a 25 ml Erlenmeyer flask and rotated (70 rpm) at 38°C on a gyratory shaker for 3 hr to promote their reaggregation (for details of this procedure see reference 10). Cells to be used for immunization or for absorption of immune sera were aggregated in serum-free medium consisting of 99% Eagle's minimum essential medium (MEM), 1% L-glutamine, and 10 units/ml DNAse (11). For use in antibody assays, the cells were aggregated in 79% Eagle's MEM, 20% fetal bovine serum, and 1% L-glutamine. After 3 hr of aggregation, the clusters and cells were collected by centrifugation (3000 rpm for 3 min), washed twice in Tyrode's solution, transferred to the appropriate medium, and the clusters were disrupted into cells by pipetting. The resulting cell suspensions (referred to as "preaggregated cells") were used immediately. They consisted of approximately 90% single cells and 10% cell clusters (2–20 cells per cluster).

Antisera

Rabbits (male, New Zealand, albino) were injected with suspensions of preaggregated embryonic 10-day retina, liver, heart, or thigh muscle cells (10^8 cells in 1 ml Tyrode's solution). Each rabbit received only one kind of cell. Retina and liver cells were administered by combined intravenous, intramuscular, and intraperitoneal injections repeated at weekly intervals, and the rabbits were bled from the heart 7 days after the third injection. Heart and thigh muscle cells were injected only intravenously 2 wk apart and the rabbits were bled 7 days after the second injection. Sera from pre- and postimmunization bleedings were sterilized through an 0.45 μ Millipore membrane (Millipore Corporation, Bedford, Mass.), heat-inactivated (56°C; 30 min), and stored at 4°C.

Absorption of Antisera and Antibody Assays

Preaggregated embryonic cells were used to absorb the antisera and as indicator cells in antibody assays. The use of preaggregated cells was necessitated by the relative fragility of freshly trypsinized cells and the need for repair of their cell surface, and because residual trypsin in the suspensions of freshly trypsinized cells tended to degrade the antisera (see Results).

ABSORPTION OF ANTISERA

The serum was diluted 1:10 to 1:20 in phosphate-buffered saline (total volume 1 or 2 ml) and mixed with preaggregated cells or with freshly collected erythrocytes. After absorption overnight at 4°C on a gyratory shaker (70 rpm), the cells were removed by centrifugation, and the serum was sterilized through a Millipore membrane and stored at 4°C. Absorptions of a single serum with cells from two different organs were done consecutively.

Tissues from 10-day embryos were generally used for absorption to provide the rather large amounts of cells required to remove cross-reacting antibodies. Twice the amount of cells necessary to remove completely detectable antibodies to the absorbing tissues was used. In practice, we used cells from 20 to 40 retinas, livers, or cerebra; or 40 to 50 thigh muscles or hearts; or 1 ml packed erythrocytes, to remove reactive antibodies from the equivalent of 0.1 ml undiluted antiserum.

ASSAYS FOR CELL-SURFACE ANTIGENS

IMMUNE AGGLUTINATION: The agglutination reaction was performed in a microtiter system, using disposable V-well trays and 50-μl droppers and diluters (Cooke Engineering Co., Alexandria, Va.). The reaction mixture consisted of one part serum (serially diluted in twofold steps) and one part cell suspension, added in that order. The cell suspensions

were adjusted to approximately 40 × 10^6 cells/ml, so that 2 × 10^6 cells were added to each well. Dulbecco's PBS (pH 7.4) was the diluent throughout. Heat-inactivated preimmunization serum controls were included in each experiment.

After addition of the cells, the tray was sealed with transparent tape, incubated at 4°C for 20 min, then centrifuged (35 *g*, 10 min, 4°C), using a swinging-bucket carrier. The cells were resuspended by forcefully inverting the plate several times. Agglutination titers were confirmed by microscopic examination, and were rarely more than twofold higher than the titers assigned macroscopically. All these manipulations were done at 4°C, since at higher temperatures spontaneous aggregation of cells might have obscured the effects of immune agglutination.

IMMUNOFLUORESCENCE:

Cell Suspensions Indirect immunofluorescence was performed in the microtiter system using U-well trays. The reaction mixture was prepared as for the agglutination reaction. Approximately 10^6 cells were added to each well of serially diluted serum. After 20 min at 4°C, the cells were washed 3 times with PBS in the microtiter tray (225 *g*, 5 min, 4°C). To each well was added 0.025 ml of a 1:20 dilution of fluorescein-conjugated goat antirabbit IgG (Microbiological Associates, Inc., Bethesda, Md.); after 20 min at room temperature the cells were rewashed 3 times in PBS and placed on ice. More than 90% of the cells were viable as judged by exclusion of Trypan blue. Samples were withdrawn from each well and examined under glass cover slips with a Zeiss Ultraphot microscope (Carl Zeiss, Inc., New York), using a darkfield condenser, 2001 w mercury vapor arc lamp, BG 12 excitation filter, and No. 50 barrier filter.

Frozen Sections Eyes from chick embryos and young chickens were frozen in O.C.T. compound (Scientific Products, Inc., Detroit, Mich.) at −20°C; 4 μ sections were prepared in a cryostat, dried on alcohol-cleaned slides, and stored in a vacuum dessicator at 4°C. To test for immunofluorescence the sections were exposed to normal or hyperimmune rabbit serum (1:20 dilution) for 20 min at 4°C, washed 3 times in PBS, incubated for 20 min with fluorescein-conjugated goat antirabbit IgG (1:20 dilution), rewashed, and mounted in buffered glycerin.

RESULTS

Embryonic Neural Retina: Properties of Antiretina Serum

Immune Agglutination

Antiserum prepared in rabbits to suspensions of preaggregated 10-day embryonic retina cells was titrated against suspensions of 10-day embryonic retina and liver cells and against adult chicken erythrocytes. Results of a representative experiment are presented in Table I.

Both retina cells and erythrocytes were agglutinated by the antiretina serum; the liver cells were not agglutinated. However, after absorption of the antiserum with erythrocytes or embryonic liver cells, only the retina cells were agglutinated (Fig. 1 *a*, *b*, *c*). These absorptions removed all detectable cross-reacting antibodies to erythrocytes, and to liver cells as well, as demonstrated by immunofluorescence (see below). Absorption of the antiretina serum with retina cells abolished the ability of the serum to agglutinate both retina cells and erythrocytes.

Immunofluorescence

Indirect immunofluorescence tests showed that antiserum prepared against preaggregated 10-day embryonic retina cells contained antibodies to 10-day embryonic neural retina and also to other embryonic tissue cells that were tested (Table II). Absorption of the antiretina serum with preaggregated embryonic liver cells or with adult erythrocytes removed all detectable cross-reacting antibodies to embryonic liver, skeletal, and cardiac muscle cells, and to erythrocytes, but

TABLE I

Agglutinating Activity of Antiretina Cell Serum against Suspensions of Cells from Embryonic and Adult Chicken Tissues*

	Reciprocal agglutination titer‡		
Treatment of antiserum	Embryonic retina cells (10 day)	Embryonic liver cells (10 day)	Adult erythrocytes§
Unabsorbed	1280	<10	640
Absorbed with			
Adult erythrocytes§	640	<10	<10
10-day embryonic liver cells	1280	<10	<10
10-day embryonic retina cells	<10	<10	<10

* Prepared against preaggregated 10-day embryonic chick neural retina cells. (See Methods). Preaggregated embryonic cells were also used to absorb the antiserum and in the agglutination assay.

‡ Reciprocal agglutination titer of preimmunization serum was <10 against all cell types tested.

§ The erythrocytes were washed but not trypsinized.

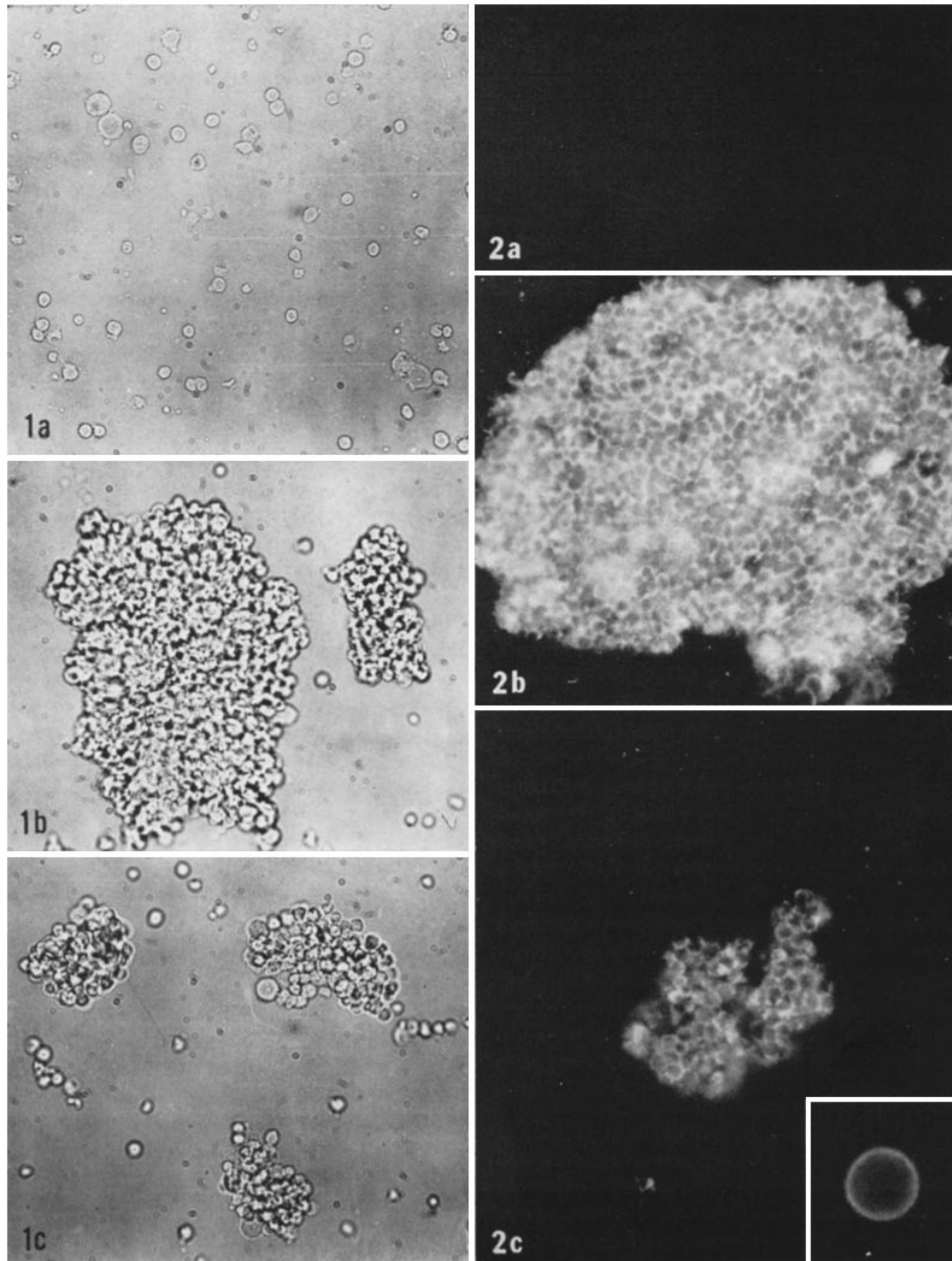


FIGURE 1 Suspension of neural retina cells from 10 day chick embryo exposed to rabbit serum and observed for aggutination. × 380. (*a*) Retina cells plus preimmunization rabbit serum diluted 1:10. No agglutination. Small, medium, and large cell types are normally present in the suspension. (*b*) Retina cells plus rabbit antiretina serum (absorbed with embryonic liver cells) diluted 1:10. Massive agglutination of the retina cells. (*c*) Retina cells plus rabbit antiretina serum (absorbed with embryonic liver cells) diluted 1:80. All retina cell types are represented in the agglutinates.

FIGURE 2 Suspension of neural retina cells from 10 day chick embryo exposed to rabbit serum, washed, and reacted with fluorescein-conjugated goat antirabbit IgG. × 380. (*a*) Retina cells plus preimmunization rabbit serum diluted 1:20. No fluorescence or agglutination. Exposure: 120 sec. (*b*) Retina cells plus rabbit antiretina serum (absorbed with embryonic liver and cerebrum cells) diluted 1:20. Marked agglutination and intense green fluorescence of all cells. Exposure: 30 sec. (*c*) Retina cells plus rabbit antiretina serum (absorbed with embryonic liver and cerebrum cells) diluted 1:80. Fluorescent-staining is restricted to surface of retina cells (see *insert*). Exposure: 45 sec.

TABLE II

Indirect Immunofluorescence of Antiretina Cell Serum Reacted with Cells from Embryonic and Adult Chicken Tissues

Treatment of antiserum	Reciprocal immunofluorescence titer						
	Embryonic retina cells (10 day)	Embryonic liver cells (10 day)	Embryonic cerebrum cells (10 day)	Embryonic skeletal muscle cells (10 day)	Embryonic heart cells (6 day)	Erythrocytes	
						Embryonic	Adult
Unabsorbed	2560	1280	2560	1280	2560	640	640
Absorbed with							
Adult erythrocytes	2560	< 20	1280	< 20	< 20	< 20	< 20
10-day embryonic liver cells	1280	< 20	640	< 20	< 20	< 20	< 20
10-day embryonic cerebrum cells	2560	< 20	< 20	< 20	< 20	< 20	< 20
10-day embryonic liver cells + 10-day embryonic cerebrum cells	1280	< 20	< 20	< 20	< 20	< 20	< 20
10-day embryonic retina cells	< 20	< 20	< 20	< 20	< 20	< 20	< 20

See legend for Table I and text.

left elevated titers of antibodies to retina and cerebrum cells. Absorption with cerebrum cells or cerebrum plus liver cells removed activity to all cell types tested, except retina; we will refer to this type of absorbed antiretina cell serum as *retina-specific antiserum.* Repeated absorption of this antiserum with nonretina tissue did not measurably reduce its antiretina activity; the implication from this concerning the possibility of qualitative differences in antigen makeup between cell surfaces from different embryonic tissues will be considered in the discussion.

All cell types in the retina cell suspension were stained by the retina-specific antiserum (Fig. 2 *a*, *b*). The fluorescing cells had the typical "ring" or "halo" appearance characteristic of antigen-antibody combination at the surface of intact cells (Fig. 2 *c*). There was no conspicuous cytoplasmic or nuclear staining. In the case of unabsorbed antiretina serum, cross-reacting antibodies coated the surfaces of all cell types found in the suspensions of liver, skeletal muscle, heart, and cerebrum cells and caused agglutination in all the cell suspensions, except liver.

ANTIBODIES TO SERUM PROTEINS

In determining the properties of antisera against chick cells it was first necessary to exclude the slight but theoretically conceivable possibility that antibodies formed against trace amounts of chicken serum proteins might have been involved in the immune reactions of the antiserum. This was tested by adding whole serum from a 4 wk old chicken to each well of serially diluted retina-specific antiserum (absorbed with liver and cerebrum cells), to determine if this addition would alter the reaction with cells. The reaction mixture consisted of 0.05 ml diluted antiserum, 0.05 ml undiluted chicken serum, and the standard number of preaggregated 10-day retina or liver cells. Results of agglutination and immunofluorescence tests showed no effect of the chicken serum, in that normal titers against retina cells were obtained, and in that there was no reaction with liver cells. Cross-reacting antibody titers of unabsorbed antiretina serum were similarly unaltered by the presence of chicken serum. Therefore, antibodies directed against chicken serum proteins are not responsible for either the tissue-specific or the cross-reactions of the antiretina serum.

ROLE OF TRYPSIN

Since trypsin was used to prepare the suspensions of retina cells that were injected into rabbits, it was essential to determine whether the antibodies that might have formed against residual trypsin in the immunizing inoculum were re-

sponsible for any of the activities of the antiserum. Furthermore, since proteolytic enzymes have been shown to unveil or activate antigens on cell surfaces (12–15), the question arose whether the specific antiserum was directed preferentially against trypsin-treated cell surfaces, or whether it would react with normal untrypsinized retina cells in the intact tissue. The following experiments show that the action of trypsin did not account for the observed immunological results.

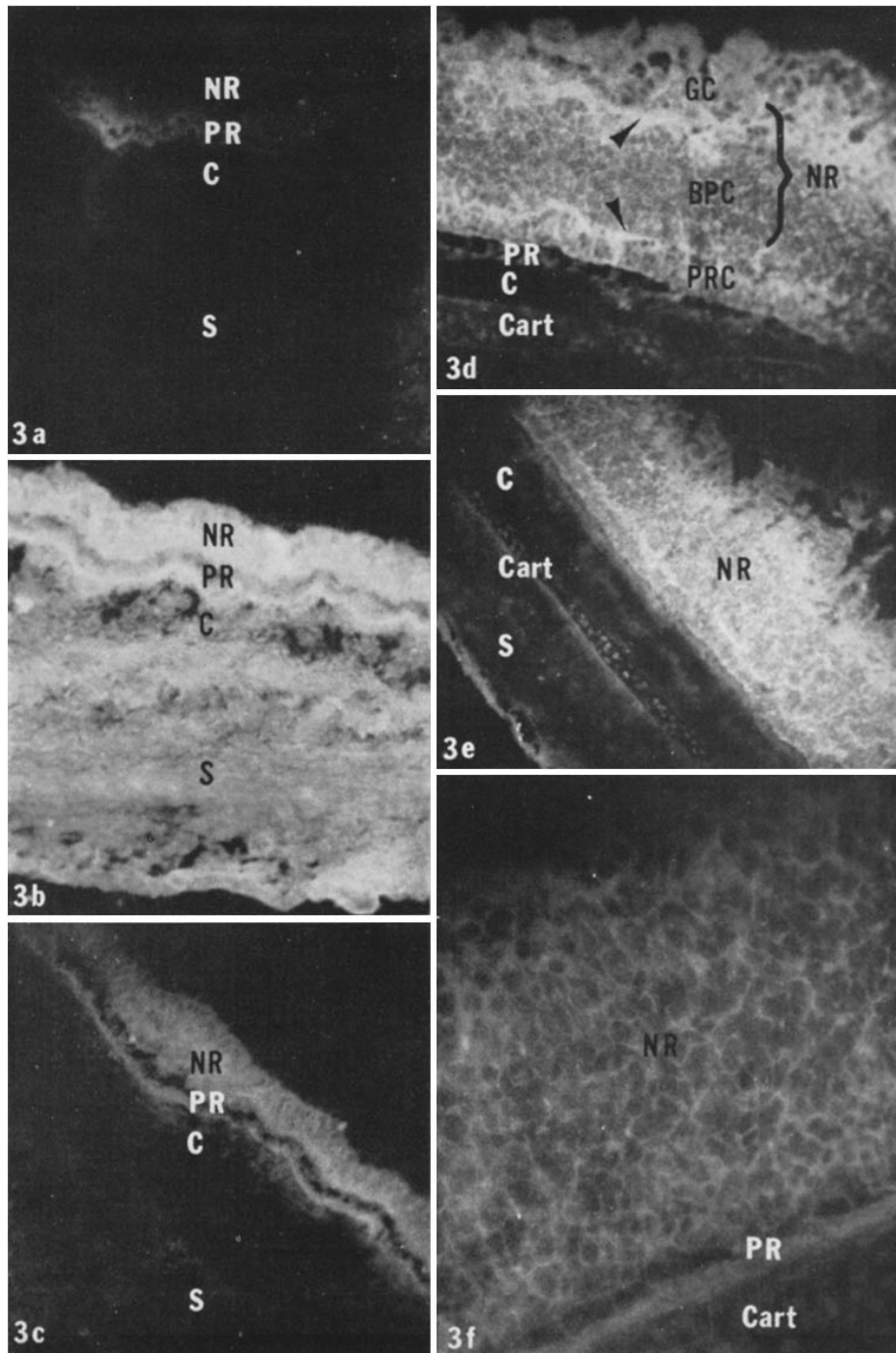
EFFECT OF TRYPSIN ON IMMUNE AGGLUTINATION: Preliminary tests using capillary precipitation and two-way gel diffusion techniques failed to demonstrate precipitating antibodies to trypsin in the antiretina serum. However, the addition of small amounts of trypsin (less than 100 units/ml) to diluted antiserum abolished its agglutinating activity on retina cells and other cell types. That this was not due to an immunological inhibition but to proteolysis was shown by the fact that this loss of agglutinating activity could be prevented by adding equimolar amounts of soybean trypsin inhibitor to the reaction mixture together with the enzyme. Similarly, the activity of retina-specific antiserum was not abolished by the addition of trypsin which had been previously inactivated with soybean trypsin inhibitor. Thus, the inactivation of the antiretina serum by active trypsin resulted from proteolytic effects on antibodies (16); therefore, its specificity was not due to antibodies against trypsin.

Since trypsinization can elicit the appearance on cell surfaces of antigens normally not accessible on these cells, it was next determined if the retina-specific antiserum would react with cells which had never been exposed to trypsin. This was tested by determining if the specific activity of this antiserum could be absorbed with intact retina, rather than with suspensions of retina cells, and by examining the staining of frozen tissue sections with the antiserum. The results showed that the antiserum reacted also with normal cell surfaces and excluded the involvement of trypsin treatment in the immunological specificity of the retina-specific antiserum.

ABSORPTION WITH INTACT RETINA TISSUE: Intact neural retina tissue, freshly removed from twelve 10-day embryos, was used to absorb 1 ml of a 1:10 dilution of antiretina serum. Serum thus absorbed was found to be completely nonreactive when tested by agglutination or by indirect immunofluorescence against suspensions of 10-day retina cells. On the other hand, absorption with fragments of embryonic liver or cerebrum did not abolish the reactivity of the antiretina serum for retina cells, attesting to the specificity of the absorption with the nontrypsinized retina tissue.

FROZEN SECTIONS: Frozen sections of 7- and 10-day embryonic chick eyes were stained by the indirect immunofluorescence procedure, using the unabsorbed antiretina serum and the absorbed retina-specific antiserum. As expected from the earlier results in this study, the unabsorbed antiretina serum stained all the layers of the eye (Fig. 3 *a*, *b*), not only the neural retina,

FIGURE 3 Frozen sections of unfixed 10-day chick embryonic eyes (sagittal sections) were exposed to rabbit serum (1:20 dilution), washed, and reacted with fluorescein-conjugated goat antirabbit IgG. 3 *a–c* are of an area of the retina near the origin of the ciliary muscle; Figs. 3 *d–f*, area near posterior region of the eye. Exposure: 60 sec. *NR*, neural retina; *PR*, pigmented retina; *C*, choroid; *S*, sclera; *Cart*, cartilage; *GC*, ganglion cell layer; *BPC*, bipolar cell layer; *PRC*, photoreceptor cell layer. (*a*) Eye section exposed to preimmunization rabbit serum. Only cytoplasmic autofluorescence (yellow-gray) of pigment epithelium is seen (the nuclei appear as dark "holes"). × 65. (*b*) Eye section exposed to antiretina serum (unabsorbed). All tissue layers fluoresce intensely. × 65. (*c*) Eye section exposed to retina-specific antiserum (absorbed with embryonic liver and cerebrum cells). Only neural retina cells are stained and show bright green fluorescence. Pigment epithelium cells show yellow-gray autofluorescence. No staining of choroid or scleral layers. × 65. (*d*, *e*) Eye section exposed to retina-specific antiserum (absorbed with embryonic liver and cerebrum cells). There is specific fluorescence of surfaces of photoreceptor (*PR*), bipolar (*BP*), and ganglion (*G*) cells; and intense fluorescence of axonal processes in inner and outer plexiform layers (arrows) of neural retina (*NR*). No nuclear staining is seen in any of the retinal layers. Slight cytoplasmic staining is present in the ganglion cell layer. Note nonspecific autofluorescence of pigment epithelium cytoplasm (*PR*) and slight autofluorescence in cartilage cell nuclei (*Cart*). (*d*) × 65; (*e*) × 40 (*f*) Higher magnification of area of retina (*NR*) adjacent to that seen in Figs. 3 *d*, *e*. Intersecting lines of fluorescence delineate surfaces of neural retina cells. Nuclei and cytoplasm appear as dark oval areas. Pigment cell cytoplasm (*PR*) and cartilage cell nuclei (*Cart*) fluoresce nonspecifically. × 400.


NR
PR
C
S
3a
NR
PR
C
S
3b
NR
PR
C
S
3c
GC
NR
BPC
PR
C
PRC
Cart
3d
C
Cart
NR
S
3e
NR
PR
Cart
3f

due to its content of cross-reactive antibodies to cells in connective tissue, endothelium, cartilage, and pigmented epithelium (cartilage and pigmented epithelium also show some autofluorescence). However, after absorption of the antiretina serum with embryonic liver and/or cerebrum cells, the resulting retina-specific antiserum stained only the neural retina cells (Fig. 3 *c–f*). Thus, cross-reactive antibodies to the various eye tissues, originally present in the antiretina serum, were removed by this absorption procedure. Taken together with the absorption of the antiretina activity with intact retina tissue (see Absorption with intact retina tissue, above), this selective reaction *in situ* of the retina-specific antiserum with retina cells confirms conclusively that the immunological specificity of this antiserum is directed against constituents normally present on the surface of neural retina cells.

It should be pointed out that the retina-specific antiserum reacted with all the cell layers of the neural retina, including the receptor, bipolar, and ganglion cells, but stained most intensely the cell processes in the inner and outer plexiform layers (Fig. 3 *d*, *e*). There was no nuclear staining. Although no attempt was made to remove antibodies to cytoplasmic constituents, the clearly visible fluorescent boundary lines between the retina cells indicated that the retina-specific antibodies were directed largely against cell-surface antigens (Fig. 3 *f*).

ABSORPTION WITH ERYTHROCYTES: The immunological properties of the antiretina serum were further examined in the following tests. 1 ml of a 1:20 dilution of antiretina serum was absorbed with 1 ml of packed adult chicken erythrocytes. The erythrocytes were washed 3 times in buffer before use but were not trypsinized. The reactivity of the absorbed serum with various embryonic cells was tested by indirect immunofluorescence (Table II). The results showed that cross-reactive antibodies to liver, skeletal muscle, and heart cells were removed by the erythrocytes; on the other hand, the titer of antibodies against 10-day retina cells was unaltered by this absorption, and that against cerebrum was only slightly reduced. These results with nontrypsinized erythrocytes exclude trypsin as a factor not only in the production of the retina-specific antibodies, but also in the formation of cross-reacting antibodies. Furthermore, they demonstrate the presence on adult erythrocytes of antigens shared by several types of embryonic tissue cells.

EMBRYONIC AGE AND PRESENCE OF RETINA-SPECIFIC CELL-SURFACE ANTIGENS

Preaggregated neural retina cells from 5- through 19-day chick embryos were reacted with retina-specific antiserum (absorbed with liver and cerebrum cells). The reaction was examined by indirect immunofluorescence. Reciprocal immunofluorescence titers of 640–2560 were obtained against retina cells of all ages tested. All the cell types present in the retina cell suspensions were stained, showing that the retina-specific cell-surface antigens are present at different developmental stages of neural retina cells in a form available for serological reaction; this does not necessarily imply that they are always functionally available to the same extent, or that their attributes other than their antigenicity do not change with differentiation.

These findings were corroborated by immunofluorescence tests on frozen sections from eyes of 5-, 10-, and 19-day chick embryos and a 28 day old chicken. In spite of the marked developmental differences, neural retinas at all these embryonic ages stained with the retina-specific antiserum. Although some cytoplasmic staining of retina cells could not be excluded, the pattern of staining was characteristic for reaction with cell-surface antigens.

Embryonic Liver: Properties of Antiliver Serum

Antiserum prepared in rabbits to suspensions of preaggregated embryonic liver cells from 10-day chick embryos (see Materials and Methods) weakly agglutinated liver cells, possibly because of the large size of these cells. However, indirect immunofluorescence tests showed that the antiliver serum contained antibodies specific for the surface of hepatocytes (liver parenchyma cells).

Results in Table III show that the unabsorbed antiliver serum, like the unabsorbed antiretina serum, cross-reacted strongly with cells from other tissues. Fluorescent antibody titers of 640–2560 were found against embryonic retina, cerebrum, skeletal muscle, and heart cells. When tested against liver cells, the unabsorbed antiserum reacted strongly against both parenchymal and mesenchymal elements. Parenchymal cells were readily identifiable under darkfield fluorescence microscopy by their large size, distinctive granularity, and yellow-brown appearance of the cytoplasm (Fig. 4 *a*). The other cells were predomi-

TABLE III

Indirect Immunofluorescence of Antiliver Cell Serum Reacted with Cells from Embryonic Chicken Tissues*

Treatment of antiserum	Reciprocal immunofluorescence titer					
	Liver cells (10 day)		Retina cells (10 day)	Cerebrum cells (10 day)	Skeletal muscle cells (10 day)	Heart cells (6 day)
	Parenchyma	Mesenchyma				
Unabsorbed	1280	2560	1280	640	1280	1280
Absorbed with						
10-day retina cells	1280	1280	< 20	< 20	640	640
10-day skeletal muscle cells	1280	< 20	< 20	< 20	< 20	< 20
12-day skeletal muscle cells	1280	< 20	< 20	< 20	< 20	< 20
10-day retina cells + 12-day skeletal muscle cells	1280	< 20	< 20	< 20	< 20	< 20
Adult erythrocytes	640	< 20	< 20	< 20	< 20	< 20
10-day liver cells	< 20	< 20	< 20	< 20	< 20	< 20

* Prepared against preaggregated 10-day embryonic chick liver cells; see legend in Table I and text.

nantly mesenchymal in nature (fibrovascular stroma and extramedullary hemopoietic cells).

Extensive absorption of the antiliver serum with 10-day embryonic retina cells removed all fluorescent staining activity for retina and cerebrum, but reduced only twofold or less the immunofluorescence titers against liver cells (parenchymal and mesenchymal cells), skeletal muscle, and heart cells. Absorption of the antiliver serum with 10- or 12-day embryonic skeletal muscle cells alone, or combined with embryonic retina cells, or its absorption only with adult chicken erythrocytes completely removed cross-reacting antibodies to retina, cerebrum, skeletal muscle, heart, and embryonic chick red blood cells (Table III). Furthermore, the absorbed antiserum no longer reacted with the mesenchymal elements in the liver cell suspensions; only liver parenchyma cells were stained (Fig. 4 *b*). Absorption of this *liver-specific antiserum* with liver cells removed all detectable antibodies to the liver cells.

These results demonstrate the presence in the liver-specific antiserum of antibodies directed preferentially against liver parenchyma cell-surface antigens; the unabsorbed antiliver serum contains also antibodies against cross-reactive antigens shared by liver with other tissues, such as mesenchymal and skeletal elements, neural cells, and adult erythrocytes.

Mesenchyme: Properties of Antisera against Cells of Mesenchymal Origins

Antisera prepared to suspensions of preaggregated embryonic skeletal muscle and heart cells (from 10-day embryos) were tested on different cell types by immunofluorescence without previous absorption, and after various absorptions (Tables IV and V). In addition to generally cross-reacting antibodies shared also with cells from other tissues, antibodies were detected which are shared primarily by skeletal muscle, heart, and other mesenchymal cells, but which could not be demonstrated by absorption to be exclusive for any one of these. Absorption of the antiheart serum with neural retina cells removed antibodies to retina and liver parenchymal cells but reduced only twofold the fluorescent antibody titers to heart, skeletal muscle, and liver mesenchymal cells (Table IV, Fig. 5 *a*, *b*, *c*). On the other hand, absorption with embryonic skeletal muscle cells, liver cells (including mesenchyme), or adult erythrocytes removed all detectable antibodies from the antiheart serum.

Similar tests with antiserum against embryonic skeletal muscle cells showed (Table V) that surface antigens present on embryonic skeletal muscle cells are also shared by heart, liver mesenchymal cells, and erythrocytes, but are not detectable on neural retina cells or liver parenchymal cells. Therefore, it appears that the antimuscle and antiheart sera (and probably also the antiliver serum described above) in addition to containing general cross-reactive antibodies, also contain antibodies directed more specifically against surfaces of cells of mesenchymal derivation. This suggests that different kinds of cells, all of which have a mesenchymal origin, share "mesenchymal" antigens which are not detectable by our procedures on cells with ectodermal or entodermal

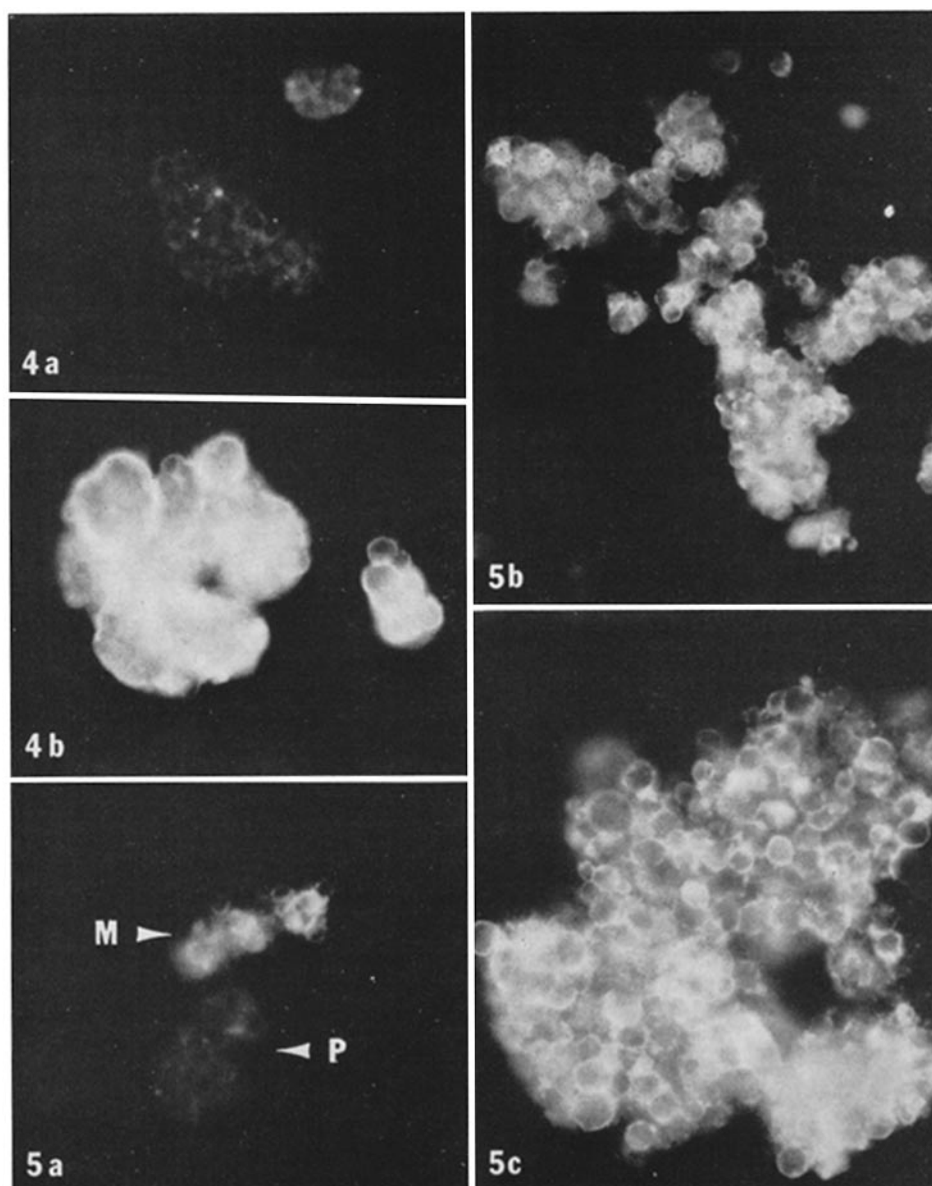


FIGURE 4 Suspension of viable cells from 10 day chick embryonic liver was exposed to rabbit serum (1:40 dilution), washed, and reacted with fluorescein-conjugated goat antirabbit IgG. × 380. (*a*) Liver cells plus preimmunization rabbit serum. Two clusters of liver parenchymal cells (hepatocytes) are shown. Unlike retina cells, hepatocytes clump during requisite centrifugations for the immunofluorescent procedure. Cytoplasm of hepatocytes has moderately bright yellow-brown autofluorescence. Nuclei appear dark. No specific fluorescence is present. Exposure: 120 sec. (*b*) Liver cells plus rabbit antiliver serum (absorbed with embryonic neural retina and skeletal muscle cells). Intense green fluorescence outlines hepatocytes. There is no nuclear or cytoplasmic staining. Liver mesenchymal cells do not fluoresce (not shown; see Fig. 5 *a*). Exposure: 20 sec.

FIGURE 5 Suspensions of viable cells obtained from various embryonic chick tissues were exposed to a 1:40 dilution of rabbit antiheart serum (absorbed with embryonic neural retina cells), washed, and reacted with goat antirabbit IgG. No fluorescence was observed using preimmunization rabbit serum. Exposure: 30 sec. (*a*) Suspensions of 10-day embryonic chick liver cells. Liver mesenchymal cells (*M*) show bright green fluorescence. Liver parenchymal cells (*P*) do not fluoresce specifically. × 380. (*b*) Agglutination and fluorescence of 6-day embryonic heart cells. × 400. (*c*) Agglutination and surface fluorescence of 10-day embryonic chick skeletal muscle cells. × 400.

TABLE IV

Indirect Immunofluorescence of Antiheart Cell Serum Reacted with Cells from Embryonic Chicken Tissues*

	Reciprocal immunofluorescence titer				
			Liver cells (10-day)		
Antiserum	Heart cells (6-day)	Skeletal muscle cells (10-day)	Parenchyma	Mesenchyma	Retina cells (10-day)
Unabsorbed	1280	2560	640	1280	1280
Absorbed with					
10-day retina cells	640	1280	< 20	640	< 20
10-day liver cells	< 20	< 20	< 20	< 20	< 20
10-day thigh muscle cells	< 20	< 20	< 20	< 20	< 20
Adult erythrocytes	< 20	< 20	< 20	< 20	< 20
10-day heart cells	< 20	< 20	< 20	< 20	< 20

* Prepared against preaggregated 10-day embryonic chick heart cells; see legend for Table I and text.

TABLE V

Indirect Immunofluorescence of Antimuscle Cell Serum Reacted with Cells from Embryonic Chicken Tissues*

	Reciprocal immunofluorescence titer				
			Liver cells (10-day)		
Antiserum	Skeletal muscle cells (10-day)	Heart cells (6-day)	Parenchyma	Mesenchyma	Retina cells (10-day)
Unabsorbed	2560	1280	640	1280	1280
Absorbed with					
10-day retina cells	1280	640	< 20	1280	< 20
10-day liver cells	< 20	< 20	< 20	< 20	< 20
10-day heart cells	< 20	< 20	< 20	< 20	< 20
Adult erythrocytes	< 20	< 20	< 20	< 20	< 20
10-day thigh muscle cells	< 20	< 20	< 20	< 20	< 20

* Prepared against preaggregated 10-day embryonic chick thigh muscle cells; see legend for Table I and text.

origins. Antibodies differentially specific for either embryonic heart or skeletal muscle cells could not be identified in the present experiments.

DISCUSSION

The present experiments have demonstrated the existence of tissue-specific antigens on the surface of embryonic chick cells. Using techniques of immune agglutination and indirect immunofluorescence, we have identified: (*1*) antigens which distinguish the surfaces of (*a*) embryonic neural retina from (*b*) embryonic liver epithelial cells; (*2*) surface antigens shared by embryonic retina and cerebrum cells; (*3*) antigens common to different cells of mesenchymal origin; and (*4*) antigens general to embryonic chick cells (these have not been examined further in the present study). The existence of tissue-specific antigens has been demonstrated previously in several embryonic systems, and antisera to such antigens have been shown to interfere with organogenesis when injected in vivo (17–19). However, in most instances these antigens have been identified as cytoplasmic constituents, or have not been characterized as to their precise subcellular location or origin.

Our results indicate that the retina-specific and liver-specific antigenic determinants are localized on the cell surface and are not detectable by our techniques on the surfaces of embryonic chick heart, skeletal muscle, connective tissue, and red blood cells. Similarly, antigens shared only by neural retina and cerebrum cell surfaces are not apparent on cell surfaces in other tissues;

and the surface antigenicity common to cells of mesenchymal origin is not detectable on the surfaces of retina or liver epithelial cells. We would like to emphasize that these differences, striking as they are, should not be interpreted as evidence of absolute absence of a given antigen from a cell surface on which it is not detectable immunologically; conceivably, this antigen could be "masked" somehow, or be present in undetectable amounts, or be grouped in clusters that may be difficult to detect; in this context, arguments concerning qualitative *versus* quantitative differences in antigenic make-up are not too rewarding. To us, the more meaningful issue is the existence of gross disparities in the comparative availability or prevalence of a given antigen on different cell types, since this might be a decisive factor in the specificity features of the cell surface and of cell interactions; in this sense, the striking differences demonstrated in the present study between cell surfaces from different tissue are of obvious interest and significance.

The tissue specificity of the retina cell-surface antigen was not only demonstrated on retina cells in suspensions, but was also confirmed on frozen sections of normal embryonic tissue. It was further corroborated by the absorption results: as there was upwards of 1000-fold difference in the efficiency with which homologous and heterologous tissues removed the tissue-specific antibodies from the antiretina serum, it is unlikely that minor quantitative differences in the distribution of these antigens could explain the striking tissue specificity. Obviously, trace amounts of antigen, if present on the heterologous cells, would have remained undetected.

It is not unexpected that cellular differentiation is reflected in the appearance of unique qualitative, quantitative, and structural patterns of molecules on the surfaces of cells (1, 2, 19–21). Recently, much interest has been directed towards identification of specific cell-surface antigens, in an attempt to explore the concept of "differentiation antigens" in such diverse processes as organogenesis, neoplasia, ontogeny of the lymphoid system, pathogenesis of autoallergic diseases, and formation of cell membranes. Various clinical and immunological observations on autoimmune diseases in humans and experimental animals strongly suggest that organ- and tissue-specific cell-surface antigens are present in adults (22–26). This has recently been confirmed by Sell et al. (27) in a study of organ-specific antigens in membranes from both normal and neoplastic human cells. In addition, cell-surface antigens peculiar to the human embryo have been described (28).

The conceivable roles of cell-surface antigens in the biological activities of embryonic cells are numerous. Of special interest to the present study are those concerned with cell recognition and morphogenetic interactions of cells. The existence and function of antigenically distinct cell-surface constituents have been demonstrated in the ligand-receptor systems involved in the specific aggregation and association of various sponge cells (7), in the tissue-specific aggregation of chick neural retina cells (3, 4), and in the neuraminidase-sensitive receptors involved in the homing mechanism of small lymphocytes to lymph nodes (29). In addition, specific cell-surface antigens have been shown to be involved in a variety of selective cell interactions; for example, the mating substances on bacteria (30) and yeasts (31); specific attachment sites for viruses (32–34) and mycoplasma (35) on bacteria and vertebrate cells; sperm-binding sites on the egg surface (36); and cell aggregation factors in slime molds (37).

A role for cell surface antigens in the determination of cell identity and cellular interactions is also suggested by studies on neoplastic cells. Neoplastic transformation, regardless of etiology, seems to be associated with loss of normal cell-surface antigens (12, 13, 38, 39) and appearance of new tumor-specific transplantation antigens (40). A causal relationship between these antigenic changes and the acquisition of neoplastic properties (e.g., loss of contact inhibition) (41) has been postulated (42); and neoplasms arising in adults were found to possess an antigen on their cell surfaces which is normally present only on embryonic cells (28, 43). Recent evidence indicates that normal embryonic tissue cells possess accessible receptors for lectins and that these become "masked" with differentiation and unmasked again in neoplastic transformation (14, 15). A common antigen has been described on the surfaces of tumor cells from diverse connective tissue elements (44). While it has not been established that this antigen is a normal component of human embryonic mesenchymal cells, this possibility arises in the light of our finding of a surface antigenicity common to various cells of mesenchymal origin.

The study of cell-surface antigens has been especially advanced in the analysis of the origins and functions of lymphocytes and other blood cells. In addition to histocompatibility ("transplantation") antigens (45) which are also present on nonhemopoietic cells, antigens have been found which are restricted to cells of the lymphoid system. Thus, a series of isoantigens characteristic of thymocytes and/or lymphocytes has been described (46). Antigens peculiar to plasma cells (47), macrophages (48), and platelets (49) have also been identified, and cell-surface antigens characteristic of functional subpopulations of lymphocytes have been found;[1] these specific cell-surface antigens seem intimately associated with the functional attributes of lymphoid cells of diverse origins. It, therefore, appears that differences in cell-surface antigenicities may be a characteristic, perhaps universal, attribute of cellular differentiation; in addition to cell-type or tissue-specific antigens, shared classes of antigens may typify the surfaces of cells with a common lineage, such as neural retina and brain cells; another class of surface antigens may be shared by cells of mesenchymal origin, such as various connective tissues, contractile and skeletal elements. Finally, antigens obviously exist that are common to all cell surfaces in the organism, or in a group of organisms; these would include transplantation antigens, blood group antigens, and various receptor substances which are not cell-type or tissue-type specific. The detailed analysis of these and other differences and similarities must await more precise methods for detecting and quantifying cell-surface antigens (50) and analyzing them biochemically (51). With respect to tissue-specific cell-surface antigens, it would be of interest to determine whether cells from homologous tissues, but from genetically different organisms, or from different species have closely similar cell-surface antigens (52); this question is particularly intriguing in view of the findings that cells from homologous tissues of different species can recognize their phenotypic similarities and can interact histogenetically (1, 2, 5, 53), and in light of the evidence that intercellular ligands isolated from mouse cerebrum cells can recognize and link also chick cerebrum cells (6). In this context it should be important to determine at what stage in embryogenesis do tissue-specific cell-surface antigens appear.

The nature and the function of the tissue-specific cell-surface antigens described by us in this report is not yet known. Many of the biologically active cell-surface antigens referred to in the above discussion are glycoproteins or glycolipids; others may be pure proteins; their function may involve structural (7) or enzymic (54) reactions. The tissue specificity of the embryonic chick tissue antigens demonstrated in this study weighs against their identity with classical histocompatibility antigens (52, 55), blood group antigens, or other cell-surface entities such as certain virus and lectin receptors (15) which are not tissue- or cell-type specific. Also, due to the use of cells from outbred strains of chickens, it is not possible to determine whether isoantigens are involved (56). What is presently clear is that tissue-specific cell-surface antigens exist in embryonic cells and represent a phenotypic expression of cell differentiation. These antigens could have a role in histospecific cell recognition and cell association during histogenesis and the organizational maintenance of tissue integrity. This possibility lends itself to examination.

[1] Goldschneider, I., and D. D. McGregor. Manuscript in preparation.

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