tion proves conclusively that the hybrid was formed from three different parental cells.

A simplistic view of somatic cell hybridization indicates that each hybrid is the result of a fusion between two cells—one human cell and one mouse cell. Although generally multiplicities of one mouse cell and one human cell do occur, other multiplicities are possible and do occur. Nabholz *et al.* (*1*) and Matsuya *et al.* (*1*) have reported multiplicities of greater than 1 : 1 in hybrids of human and mouse cells as well as in hybrids of mouse cells. Similar observations in our own laboratory show that cells from a fusion between RAG and WI-38 occur in multiplicities of 1 : 1 as well as 2 : 1.

Three possible explanations for the formation of hybrids with the greater multiplicities have been previously stated. In the case of the triple hybrid reported here, we have shown that three different cells are capable of fusing and forming a viable, long-lived hybrid. In one other instance, a bona fide triple hybrid has been reported (*12*). Knowles *et al.* reported the rescue of infectious SV40 after fusion between three different cells transformed by SV40. In this instance, the fusion between the three cell types was carried out sequentially. Suspensions of the two transformed cell types were exposed to Sendai virus; then the third cell type was added to the cell mixture. Also, it is not established that their triple hybrid would proliferate indefinitely. It is unclear how many generations this triple hybrid underwent after rescue of the virus.

Additional questions remaining to be answered are: Do higher multiplicities arise also by fusion of tetraploid and diploid cells or by fusion of single human and mouse cells with subsequent asynchronous replication of the mouse cell; in the case of triple hybrids, do the cells hybridize simultaneously, or as a series of events separated in time; and at what frequency do viable hybrids of higher multiplicities arise?

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References and Notes

1. M. Nabholz, V. Miggiano, W. Bodmer, *Nature* **223**, 358 (1969); Y. Matsuya, H. Green, C. Basilico, *ibid.* **220**, 1199 (1968).
2. R. Klebe, T. R. Chen, F. H. Ruddle, *J. Cell Biol.* **45**, 74 (1970).
3. J. W. Littlefield, *Science* **145**, 709 (1964).
4. L. Hayflick and P. S. Moorehead, *Exp. Cell Res.* **25**, 585 (1961).
5. T. B. Shows, F. H. Ruddle, T. Roderick, *Biochem. Gen.* **3**, 25 (1969).
6. R. DeLorenzo and F. H. Ruddle, *ibid.*, p. 151.
7. F. H. Ruddle, T. B. Shows, T. Roderick, *Genetics* **58**, 599 (1968).
8. W. Szybalski and E. H. Szybalska, in *Approaches to the Genetic Analysis of Mammalian Cells* (Univ. of Michigan Press, Ann Arbor, 1962), pp. 11–27.
9. T. T. Puck, P. Marcus, S. J. Cieciura, *J. Exp. Med.* **103**, 273 (1956).
10. C. M. Boone and F. H. Ruddle, *Biochem. Gen.* **3**, 119 (1969).
11. M. Weiss and H. Green, *Proc. Nat. Acad. Sci. U.S.* **58**, 1104 (1967).
12. B. B. Knowles, F. C. Jensen, Z. Steplewski, H. Koprowski, *ibid.* **61**, 42 (1968).
13. Supported by PHS grant GM-09966.

23 October 1970; revised 10 December 1970 ■

Cell Surface Antigens: Serial Sectioning of Single Cells as an Approach to Topographical Analysis

Abstract. *The topographical distribution of H-2 antigens on the surfaces of C57BL/6 mouse thymocytes and lymph-node lymphocytes was investigated by a new technique. Single cells were reacted with visually labeled antibody directed against H-2 antigen. Each cell was processed for electron microscopy individually and was serially sectioned. Models constructed from serial electron micrographs provided detailed views of the entire cell surface and showed that H-2 antigen occurs on thymocytes in small isolated regions and on lymphocytes in large interconnected regions.*

Electron microscopy with visually labeled antibody has shown that H-2 and other mouse alloantigens, as well as virus-specified antigens, occur on discrete areas of the cell surface rather than being distributed uniformly over the entire cell (*1, 2*). These antigens provide valuable markers for studying the topographical organization of cell surface constituents. However, conventional use of the visual labeling method restricts the investigator to defining the distribution of antigen on the perimeter of single sections, and permits only limited inferences as to its representation on the cell as a whole.

In order to determine the precise distribution of surface antigens, we have serially sectioned single lymphocytes and thymocytes exposed to antibody labeled with visual markers, enabling us to construct models of the complete surfaces of these cells. We chose to work with single cells maintained in droplets to avoid the repeated cycles of centrifugation and resuspension required for the preparation of labeled cell suspensions, which could cause cellular damage and loss of labeled antibody.

For this initial study, H-2 antigens on the cell surface were labeled by the indirect method of Hämmerling *et al.* (*3*); southern bean mosaic virus (SBMV) (*4*) was used as the visual marker. Cells were reacted first with the appropriate H-2 antibody (γG), then with the hybrid antibody (rabbit anti-mouse γG/rabbit anti-SBMV), and finally with SBMV.

To observe the distribution of H-2 antigen on mouse lymphoid cells, we prepared models of the surfaces of four thymocytes and four lymph-node lymphocytes from C57BL/6 mice. More than 20 antigens are specified by the *H-2* locus, and mouse strains express characteristic constellations of antigens (designated H-2^a, H-2^b, and so forth) (*5*). The C57BL/6 strain is H-2^b. The cytotoxic antiserum used was C57BL/6/H-2^k anti-C57BL/6 (H-2^b) prepared in an H-2 congenic mouse strain to ensure limitation of serological reactions to H-2 (the cytotoxic titer against H-2^b lymph node cells was 1/640). This was diluted 1 : 10; the hybrid antibody and SBMV were diluted to 0.5 mg of protein per milliliter. The following negative controls were used: (i) an H-2^b antiserum to H-2^a, which should not react with C57BL/6 cells, was substituted for positive antiserum; (ii) normal mouse serum was substituted for antiserum; (iii) all mouse serum was omitted; (iv) the C57BL/6 test cells were replaced by cells from the strain in which the H-2 antiserum was prepared, C57BL/6/H-2^k. For each of these four controls, one thymocyte and one lymphocyte were serially sectioned.

During the reactions, each cell was processed separately in droplets (volume, 0.3 μl) under a layer of silicone oil (dimethylpolysiloxane, Dow Corning 200 fluid, viscosity 100 cs) in the bottom of a plastic petri dish (60 by 15 mm). Medium 199 was used for suspending cells and for diluting reagents. After each step of the reaction sequence the cells were washed with medium 199 containing 2 percent γG-free fetal calf serum (Grand Island Biological); this protein supplement

was necessary for maintaining cell viability. Silicone oil was equilibrated with medium 199 at 37°C for several days before use to avoid dehydration of the droplets. Micropipettes, hand-drawn from soft-glass Pasteur pipettes to a terminal bore diameter of about 50 μm, were used to dispense droplets and to isolate and transfer cells. Operation of the micropipettes was by oral suction through a connecting tube, fine control being afforded by a column of silicone oil at the terminal end which eliminates capillarity and moderates applied pressure. All procedures were performed with the aid of a Zeiss IV stereomicroscope, equipped with substage transilluminator and zoom optical system, under darkfield illumination, and at magnifications of $\times$ 20 to $\times$ 100. A simple cooling device, through which ice-water flows, was constructed from plastic petri dishes to accommodate the dish in which cells were manipulated, and enabled us to process all cells in the cold.

Viable suspensions of thymus or lymph node cells were prepared at a concentration of 1 to 2 $\times$ 10^6 cells per milliliter. A few cells were introduced into a droplet of medium 199 under silicone oil, and from this source individual cells were transferred to separate droplets; cells which settled to the bottom of the droplets were selected because invariably these were healthy, and also because most of the droplet fluid can then be removed and replaced repeatedly by micropipette, without disturbing the cell, which remains at the bottom but will not attach to the surface of the dish. In this manner, cells were incubated for 30 minutes in each reagent (mouse alloantibody, hybrid antibody, SBMV) and after each incubation were washed several times for 15 minutes, all at 10° to 12°C. We prefer this to the alternative of transferring cells between droplets for the following reasons. (i) Possible damage to cells resulting from repeated handling by micropipette is avoided; (ii) since the cells remain at the bottom of the droplets, continuous observation is unnecessary, and therefore more than one cell can be processed at the same time; and (iii) loss of the cell, a frequent consequence of transfer from droplet to droplet, is substantially reduced.

Each cell was fixed with 1 percent glutaraldehyde in 0.1*M* cacodylate buffer for 15 minutes, briefly rinsed with buffer, and transferred to a special chamber (*6*) filled with buffer. By exerting gentle fluid pressure through the micropipette, cells were positioned and attached to the Epon-coated glass surface forming the bottom of the chambers. Postfixation with 1 percent OsO_4 buffered with cacodylate, staining with 0.5 percent uranyl acetate, dehydration, and embedding in Epon were achieved by successive replacement of the chamber fluids by micropipette. To facilitate handling, chambers containing embedded cells were inverted and mounted on Epon blocks prepared in BEEM (*6*) embedding capsules, by application of fresh Epon; cylindrical portions of BEEM capsules slipped over the Epon

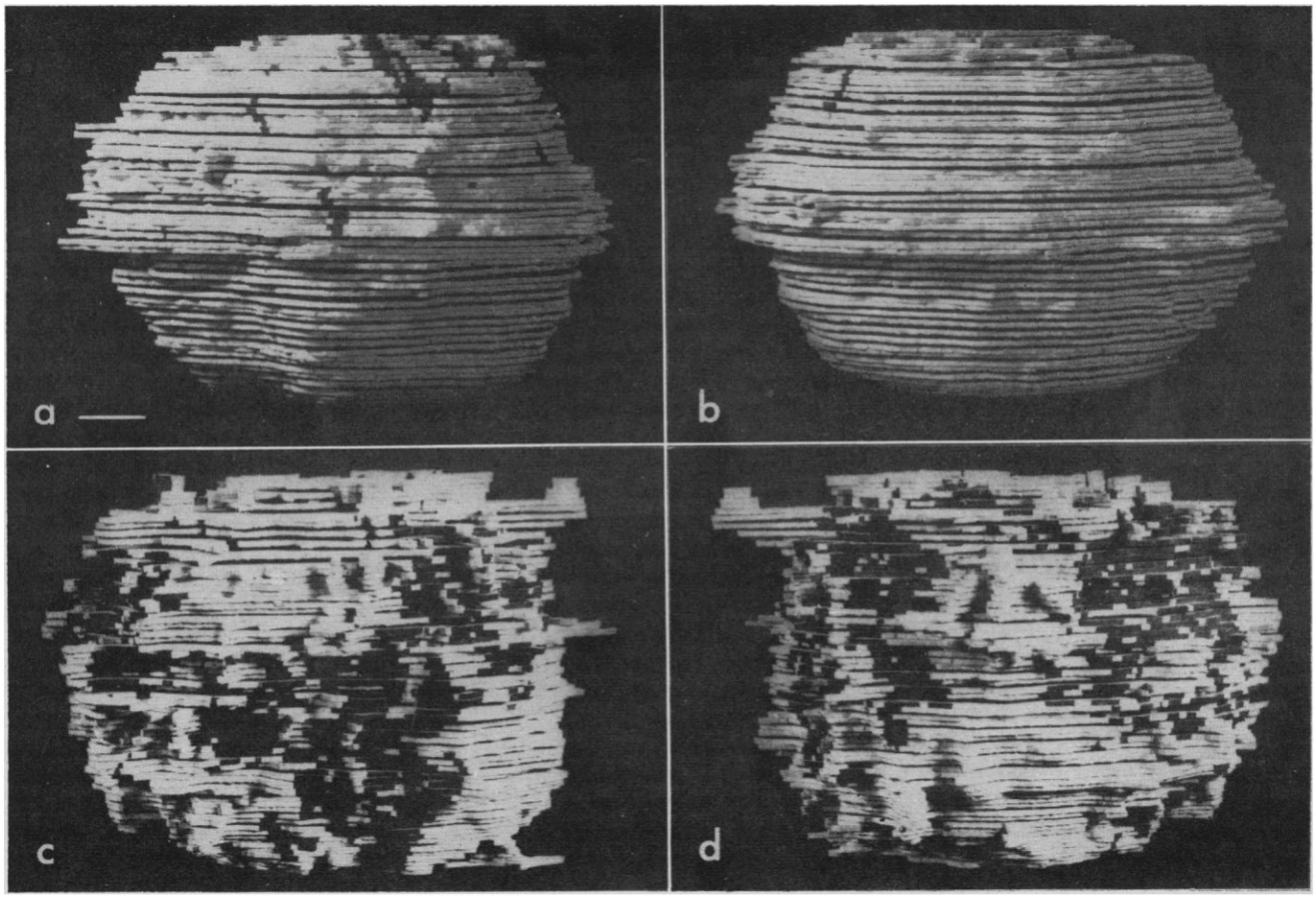

Fig. 1. Distribution of H-2 antigens (dark areas) on the surfaces of a thymocyte and a lymphocyte. (a and b) Approximately opposite sides of a thymocyte (No. 2) showing small H-2^+ areas of various sizes. (c and d) Opposite sides of a lymphocyte (No. 11), showing numerous protrusions, most of which are H-2^-. Both models were constructed to represent a magnification of $\times$ 28,000. Scale represents 1 μm.

Table 1. Summary of data for serially sectioned thymocytes and lymphocytes, including only cells from which 90 percent or more of the sections were recovered. Cells 1 to 4 and 9 to 12 were sectioned at an average thickness of about 900 Å, other cells at about 1200 Å.

Cell		Antiserum	Sections recovered	Sections lost	Surface area occupied by SBMV (% of total cell surface)*
Antigen specificity	No.				
		Thymocytes			
$H\text{-}2^b$	1	$H\text{-}2^k$ vs $H\text{-}2^b$	48	3	2.6
$H\text{-}2^b$	2	$H\text{-}2^k$ vs $H\text{-}2^b$	54	2	1.6
$H\text{-}2^b$	3	$H\text{-}2^k$ vs $H\text{-}2^b$	45	4	1.5
$H\text{-}2^b$	4	$H\text{-}2^k$ vs $H\text{-}2^b$	50	4	0.6
		Thymocyte controls			
$H\text{-}2^b$	5	$H\text{-}2^b$ vs $H\text{-}2^a$	38	0	0
$H\text{-}2^b$	6	Normal mouse serum	36	3	0
$H\text{-}2^b$	7	No antiserum	38	2	0
$H\text{-}2^k$	8	$H\text{-}2^k$ vs $H\text{-}2^b$	41	2	0
		Lymphocytes			
$H\text{-}2^b$	9	$H\text{-}2^k$ vs $H\text{-}2^b$	56	3	35
$H\text{-}2^b$	10	$H\text{-}2^k$ vs $H\text{-}2^b$	65	4	22
$H\text{-}2^b$	11	$H\text{-}2^k$ vs $H\text{-}2^b$	63	0	26
$H\text{-}2^b$	12	$H\text{-}2^k$ vs $H\text{-}2^b$	53	5	21
		Lymphocyte controls			
$H\text{-}2^b$	13	$H\text{-}2^b$ vs $H\text{-}2^a$	48	2	0
$H\text{-}2^b$	14	Normal mouse serum	47	4	0
$H\text{-}2^b$	15	No antiserum	42	4	0
$H\text{-}2^k$	16	$H\text{-}2^k$ vs $H\text{-}2^b$	42	0	0

* Total length of SBMV-positive sectors (all sections) divided by total length of perimeter (all sections) ×100.

blocks supported the chambers perpendicularly during mounting. The chambers were subsequently removed, the positions of cells identified as thymocytes or small lymphocytes on the basis of size (small cells, 5 to 8 μm in diameter, were selected preferentially) were determined by examination with a light microscope, and excess Epon was trimmed away from around the cells to form blocks 75 by 25 μm).

A Sorvall MT-2 microtome was used for serial sectioning; groups of five sections were mounted over the 600-μm diameter holes of Formvar-covered and carbon-reinforced single-hole copper grids, by a method similar to that of Galey and Nilsson (*7*): sections were picked up freehand, using naked grids as fine loops, and these grids were transferred with entrapped water to grids with support films; evaporation of the water resulted in smooth adhesion of the sections to the coated grids, after which the transfer grids were readily removed. Sections were doubly stained with uranyl acetate and lead citrate and examined with a Siemens 1A electron microscope; serial micrographs were made at a magnification of × 6000.

Models of the cell surface were constructed from the serial electron micrographs by tracing the outlines of projected images of each cell section (from the original negatives) onto artists' mounting board (1.5 mm thick); the cardboard sections were cut out, sectors labeled with SBMV were marked along the edges, and the sections were pasted together. To ensure that sections of different thicknesses were represented proportionally, a single layer of board was used for the thinnest sections, while "spacers" were added to thicker sections (or were used as substitutes for lost sections).

The models show, for the first time, the topographical distribution of H-2 antigen on the cell surface. The substantially greater representation of H-2 on lymphocytes than on thymocytes (Table 1 and Fig. 1) was anticipated from known results of electron microscopy (*1*) and quantitative absorption (*8*). The restriction of H-2 to discrete regions of the cell surface was also anticipated from previous immunoelectron microscopy (*1*). Inspection of individual models and comparison of different models does not reveal any readily discernible pattern of H-2 representation.

There are many more protrusions on the lymphocytes than on the thymocytes, and these protrusions are most commonly $H\text{-}2^-$. The $H\text{-}2^+$ areas are frequently studded with small $H\text{-}2^-$ regions which often are the sites of surface protrusions. Phagocytic vesicles containing SBMV are occasionally present in the cytoplasm of thymocytes and lymphocytes, immediately below the cell surface; that these engulfed markers are attached to antigen originally at the cell surface is indicated by the lack of SBMV in similar vesicles of control cells.

The main purpose of this report is to draw attention to the feasibility of building models of this type, and we should be reluctant to draw any firm conclusions from the limited data so far available. We perceive the next crucial technical advance to be the application of two visually distinct markers for two different antigens, which will greatly extend the information to be gained from cell models. At that point we may hope to answer hitherto unapproachable questions regarding the antigenic topography of cells in various states of differentiation, both normal and malignant.

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References and Notes

1. T. Aoki, U. Hämmerling, E. deHarven, E. A. Boyse, L. J. Old, *J. Exp. Med.* **130**, 979 (1969); confirming earlier indications from immunofluorescence by J. C. Cerottini and K. T. Brunner, *Immunology* **13**, 395 (1967).
2. T. Aoki, E. A. Boyse, L. J. Old, E. deHarven, U. Hämmerling, H. A. Wood, *Proc. Nat. Acad. Sci. U.S.* **65**, 569 (1970).
3. U. Hämmerling, T. Aoki, E. deHarven, E. A. Boyse, L. J. Old, *J. Exp. Med.* **128**, 1461 (1968).
4. U. Hämmerling, T. Aoki, H. A. Wood, L. J. Old, E. A. Boyse, E. deHarven, *Nature* **223**, 1158 (1969).
5. G. D. Snell and J. H. Stimpfling, in *Biology of the Laboratory Mouse*, E. L. Green, Ed. (McGraw-Hill, New York, ed. 2, 1966), p. 457.
6. Each chamber consists of the truncated portion of a polyethylene BEEM embedding capsule (Better Equipment for Electron Microscopy), with the tip (trimmed down to expose a 1-mm-square opening) cemented onto a 5-mm square of carbon- and Epon-coated glass. The glass squares were cut from cover glasses which were carbon-coated by evaporation and then covered with a 2- to 3-μm layer of Epon (proper thickness indicated by red and green interference colors), spread with a glass rod and allowed to polymerize. The carbon permits removal of the glass after the cell is embedded, and becomes the block face (the level at which serial sectioning is initiated). Positioning the cell slightly below the carbon in this manner permits adjustments in sectioning within the first few sections before the cell is encountered.
7. F. R. Galey and S. E. G. Nilsson, *J. Ultrastruct. Res.* **14**, 405 (1966).
8. E. A. Boyse, E. Stockert, L. J. Old, *Proc. Nat. Acad. Sci. U.S.* **58**, 594 (1967).
9. Supported by National Cancer Institute grant CA 08748 and grants from the John A. Hartford Foundation, Inc., and Health Research Council of the City of New York (contract I-325). We thank E. G. Diacumakos for advice and assistance in manipulating single cells; O. Kloesman and H. Boudakian of Rockefeller University for photographing the models; W. Matz and J. Marchese for other photography; and J. Goldstein for assisting in model construction.

19 November 1970