Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 608–612, January 1982
Immunology

Cell surface properties of HLA antigens on Epstein–Barr virus-transformed cell lines

(fluorescence/photobleaching/diffusion/monoclonal antibodies/patching)

LLOYD M. SMITH*, HOWARD R. PETTY*†, PETER PARHAM‡, AND HARDEN M. MCCONNELL*§

*Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, California 94305; and ‡Department of Structural Biology, Stanford University School of Medicine, Stanford, California 94305

Contributed by Harden M. McConnell, October 2, 1981

ABSTRACT **A number of monoclonal antibodies have been used to investigate the distributions and rates of lateral motion of the HLA-A,B, and -DR antigens on several Epstein–Barr virus-transformed B-cell lines. The lateral diffusion coefficients (*D*) of fluorescein conjugates of the monoclonal antibodies bound to the cell surface were determined by fluorescence recovery after pattern photobleaching. *D*s of HLA-A and -B were found to be comparable and of the order of 10^{-9} to 10^{-10} cm^2/sec for each of the seven monoclonal antibodies and four cell lines examined. The HLA antigens appear to be monomeric on the cell surface based on experiments using mixtures of arsanilic acid-conjugated and fluorescein-conjugated antibodies. Four monoclonal antibodies against DR antigens were examined. Two of these, Genox 3.53 and L243, labeled the cell surface uniformly and gave *D*s comparable to those obtained for the HLA-A and -B antigens. The other two, DA2 and 2.06, rapidly patched on the cell surface and were immobile. The DA2, L243, and Genox 3.53 antibodies bound outside of the caps formed with the arsanilic acid-conjugated 2.06 antibody and a second-step rhodamine-conjugated rabbit anti-arsanilate antibody. This is consistent with recent biochemical evidence that there are multiple distinct antigens coded for by the HLA-DR region.**

The major histocompatability complex codes for a series of polymorphic cell surface antigens important for the control of cellular interactions in immunity (1–6). Two different types of glycoprotein complexes provide the molecular basis for the HLA-A,B,C and HLA-DR antigens in humans. Their respective homologues in mice are the H-2D,K,L and Ia antigens. For convenience the major histocompatibility antigens from all species are collectively referred to as either class I or class II antigens (7).

Class I antigens consist of a polymorphic 42,000-dalton glycoprotein associated with β_2-microglobulin. The products of at least three loci are independently expressed on most murine and human cells. A primary structural relationship between both polypeptide components of class I antigens and immunoglobulin chains has been established (8–10). Solubilized HLA-A,B,C can be found as monomers, dimers, and higher oligomers, depending upon the conditions of isolation (11–14). However, it is not known whether class I antigens, like immunoglobulin, exist physiologically as a four-chain structure in the intact cell membrane.

Class II antigens, HLA-DR and Ia molecules, are a complex of two glycoproteins of about 34,000 and 29,000 daltons (15, 16). In the mouse it has been shown that two different sets of Ia molecules, I-A and I-E/C, are expressed (reviewed in ref. 17). In man, the situation is less clear. Partial NH_2-terminal sequences of HLA-DR antigens isolated from B-cell lines show homology only with the I-E/C polypeptides of the mouse and not with I-A products (18, 19). Similarly, serological analysis of the cross-reactions of mouse anti-Ia alloantisera on human cells showed a predominant correlation with I-E/C specificities (20, 21). The first concerted effort to define human B-cell alloantigens indicated that a single set of allelic HLA-DR molecules existed (22). However, certain broadly reactive antisera that were initially thought to define supertypic determinants are now considered as possible serological markers for additional sets of genetically distinct HLA-DR molecules (23). Several immunochemical studies support this idea (24–26). A number of monoclonal antibodies with specificity for human Ia-like antigens have been produced (27–35). Methods to differentiate biochemically and functionally between putative classes of human class II antigens by using such reagents would contribute to the clarification of this situation.

To investigate these questions, several monoclonal antibodies were used to analyze the distributions, interactions, and lateral diffusion of HLA-A,B,C,DR antigens on a number of Epstein–Barr virus (EBV)-transformed B-cell lines. Evidence is presented that the class I antigens are monomeric on the cell surface and also that there exist distinguishable sets of HLA-DR antigens.

MATERIALS AND METHODS

Antibodies. The monoclonal antibodies used in this work have been described. Specificities and references are as follows: PA2.1 (anti-HLA A2, ref. 36); BB7.2 (anti-HLA A2, refs. 37 and 38); BB7.1 (anti-HLA B7, ref. 37); MB40.2 (anti-HLA B7 or anti-HLA B40, ref. 39); W6/32 (anti-HLA-A,B,C monomorphic, refs. 40 and 41); BBM.1 (anti-β_2-microglobulin, ref. 37); MB40.1 (anti-HLA A28, A32, B7, B8, B40, ref. 42); DA2 (anti-DR monomorphic, ref. 43); Genox 3.53 (anti-DR 1,2,6, ref. 43); 2.06 (anti-DR monomorphic, refs. 31 and 44); and L243 (anti-DR monomorphic, ref. 29). Antibody 2.06 was the gift of H. M. McDevitt and L243 was the gift of Olivera Finn and Ron Levy. Rhodamine-conjugated F(ab')$_2$ rabbit anti-mouse antibody was obtained from Cappel Laboratories (Cochranville, PA). Rhodamine-conjugated rabbit anti-arsanilate antibody was from Becton Dickinson (Sunnyvale, CA). Fluorescein conjugates of the monoclonal antibodies were made by standard procedures using fluorescein isothiocyanate (45). Arsanilic acid-conjugated antibodies were prepared as described (46). The F(ab')$_2$ fragment of MB40.1 was obtained by pepsin digestion (47) followed by purification by Sephadex G-150 chromatography.

The cell lines JY (A2, B7, DR 4,6), Madura (A2, B40, DR 8), Priess (A2, B15, DR 4), LB(A28, B40, DR 6), and Daudi (-, -, DR 6) have been described (37). Cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 μg of penicillin and 50

Abbreviations: CB, cell buffer; *D*, lateral diffusion coefficient; PMNs, polymorphonuclear leukocytes; EBV, Epstein–Barr virus.

† Present address: Department of Biological Sciences, Wayne State University, Detroit, MI 48202.

§ To whom reprint requests should be addressed.

μg of streptomycin per ml, and 10 mM Hepes at pH 7.4, all from GIBCO. Cells were maintained at 2×10^5 to 2×10^6 cells per ml by splitting 1:2 to 1:3 every 2 or 3 days.

Labeling of Cells for Diffusion Measurements. Cells were taken from culture, washed two times in cell buffer [CB; 2.0 mM $CaCl_2$/1.5 mM $MgCl_2$/5.4 mM KCl/1 mM Na_2HPO_4/5.6 mM glucose/120 mM NaCl/0.2% bovine serum albumin (Calbiochem, fatty acid-poor), pH 7.4], resuspended to $1–2 \times 10^6$/ml in CB, and kept on ice until used (no longer than 4 hr). Typically, 10–15 μl of cells was incubated on ice with 5 μl of fluorescein-conjugated antibody (0.5–1 mg/ml in phosphate-buffered saline for 15 min. Cells were washed twice with 1 ml of cold CB, resuspended in 10 μl of CB, and deposited in a ring of vacuum grease on a glass slide. A coverslip was applied and diffusion measurements were made within 20 min of sample preparation, as described (48).

Double Labeling. In experiments using two fluorescent labels it was often necessary to view either label selectively. To observe fluorescein fluorescence (and block rhodamine fluorescence) with 488-nm excitation, we used a Zeiss FT 510 chromatic beam splitter and a Zeiss LP 528 barrier filter in conjunction with a 540-nm short-pass barrier filter (Ditric Optics, Marlboro, MA). Rhodamine fluorescence was observed by excitation with an HBO 50-W mercury lamp and a Zeiss 487714 filter set.

Capping experiments used established methods (49). Five microliters of arsanilic acid-conjugated antibody (≈0.1 mg/ml) was added to 15 μl of cells. After 15 min on ice, cells were washed in CB and resuspended at the original concentration. They were then incubated at 37°C with gentle agitation for 30 min with 5 μl or rhodamine-conjugated rabbit anti-arsanilate antibody. After a washing with CB, the cells were incubated on ice for 15 min with 5 μl of fluorescein-conjugated anti-HLA antibody. The cells were then washed in CB, resuspended in 10 μl of CB, and deposited in a ring of vacuum grease on a glass slide. A coverslip was applied and the sample was examined by fluorescence microscopy using a ×40 bright-field air objective (n.a. = 0.65) on a Zeiss Photomicroscope III epifluorescence microscope.

The extent of oligomerization of a cell surface antigen was studied by using double-label fluorescence methods (50). Ten microliters of cells was incubated for 15 min on ice with a mixture of the arsanilic acid and fluorescein conjugates of a monoclonal antibody. The cells were washed two times with CB and resuspended in 10 μl of CB, and a second step of rhodamine-conjugated rabbit anti-arsanilate antibody was added. The cells were incubated on ice for 15 min, washed, and examined by fluorescence microscopy as described above. When the antigen is oligomeric, and the arsanilic acid conjugate is present in excess of the fluorescein conjugate in the first step incubation, the green (fluorescein) fluorescence patches with the red (rhodamine) fluorescence. If the antigen is monomeric, the green does not patch with the red.

Fluorescence photomicrographs were taken with the aid of an image intensifier (Ni-Tec, Skokie, IL) and Kodak 2475 recording film at ASA 3200.

RESULTS

The measured diffusion coefficients (*D*s) of monoclonal anti-HLA antibodies are given in Table 1. The relatively large standard errors of these values are due to both cell-to-cell variability on a given day and significant day-to-day variability. A Fab fragment of the W6/32 antibody exhibited lateral distribution and diffusion similar to those of the other antibodies. Thus, the valency of the antibody did not significantly affect the lateral motion of the antigen.

In previous work (48) it was shown that W6/32 IgG, but not Fab, rapidly patched on peripheral blood monocytes and polymorphonuclear leukocytes (PMNs) but not on peripheral blood lymphocytes. The possibility existed that this patching was due to interaction of the Fc stem of the antibody with the cell surface Fc receptors. Therefore, patching was studied with an $F(ab')_2$ fragment of the MB40.1 antibody. The distribution of this antibody fragment on the peripheral blood cells was identical to that obtained previously, indicating both that the Fc stem was not involved in the patching phenomenon and that the particular site to which the antibody binds is not of importance in the patching.

The following experiment provides evidence that the HLA antigens are monomeric on the surfaces of EBV-transformed B-cell lines. JY cells were incubated with a mixture of arsanilate-conjugated and fluorescein-conjugated monoclonal antibodies. The arsanilate conjugate was patched or capped by using a rhodamine-conjugated rabbit anti-arsanilate antibody, and the degree of copatching of the green (fluorescein) fluorescence was determined by visual observation. Three monoclonal antibodies were used in separate experiments: PA2.1, BBM.1, and BB7.1. The green fluorescence always remained uniformly distributed in spite of the extensive patching of red fluorescence; 40 or more cells were examined in every case. Appropriate control experiments were performed to demonstrate competitive binding of the two conjugates to the cell surface and to show absence of detectable nonspecific binding of any of the antibody reagents. In some experiments the relative amounts of arsanilate and fluorescein conjugates were varied over a wide range, to give both weak red fluorescence with bright green fluroescence and weak green fluorescence with bright red fluorescence. The same results were obtained when cells were starved for glucose in the

Table 1. Diffusion coefficients of monoclonal antibodies to HLA antigens determined on four human cell lines

	D, $cm^2 \times 10^{10}$/sec			
Antibody*	JY	Madura	Priess	LB
PA2.1 (A2)	5.9 ± 2.0 (5)	11.9 ± 7.6 (5)	7.2 ± 3.8 (4)	X
BB7.2 (A2)	9.0 ± 6.2 (3)	ND ND	6.8 ± 2.2 (7)	X
BB7.1 (B7)	10.9 ± 5.9 (4)	X	X	X
MB40.2 (B7,B40)	4.0 ± 1.1 (6)	6.4 ± 3.3 (4)	X	7.9 ± 4.3 (4)
W6/32 (A,B,C)	5.3 ± 2.4 (6)	ND	5.7 ± 3.5 (6)	5.8 ± 3.6 (5)
Fab W6/32	3.5 ± 1.3 (4)	ND	ND	ND
BBM.1 (β_2M)	5.4 ± 3.1 (8)	8.3 ± 3.8 (2)	4.7 ± 3.4 (7)	1.9 ± 0.6 (5)
MB40.1 (A28,32 B7,8,40)	4.3 ± 1.3 (6)	ND	X	10.1 ± 1.3 (2)
DA2 (DR monomorphic)	P	ND	ND	P
Genox 3.53 (DR 1,2,6)	9.9 ± 3.0 (9)	X	X	9.5 ± 4.1 (6)
CA2.206 (DR monomorphic)	P	ND	ND	ND
L243 (DR monomorphic)	5.1 ± 2.4 (7)	ND	ND	6.0 (1)

Measured with fluorescein conjugated monoclonal antibodies against HLA antigens. Numbers are mean ± SD; in parentheses are the number of measurements made for the indicated cell/antibody combination. X, inappropriate antibody/cell combination; ND, not determined; P, patchy and immobile (see text). Cell HLA haplotypes are given in text.

* Antibody specificity is shown in parentheses.

presence of 5 mM 2-deoxyglucose to increase intracellular levels of oxidized glutathione. A control experiment in which a rhodamine-conjugated rabbit anti-mouse $F(ab')_2$ was used as a second-step reagent did give complete co-capping of the red and green fluorescence.

The properties of four monoclonal antibodies against DR antigens were investigated. L243 and Genox 3.53 gave uniform fluorescence on the surfaces of EBV-transformed cells and also smooth exponential recovery of fluorescence after pattern photobleaching. The diffusion coefficients were comparable to those obtained with anti-HLA-A,B,C antibodies (Table 1). In contrast, 2.06 and DA2 behaved quite differently. The distribution of fluorescence immediately after labeling on ice was often relatively uniform. However, this became rapidly nonuniform as a function of time at room temperature: in about 10 min the distribution became highly uneven. This patching was qualitatively different from that seen previously with W6/32 on human peripheral blood cells in that (*i*) it occurred more slowly, and (*ii*) there were fewer patches but they were larger. In some experiments the cells were preincubated with 20 mM ethylamine and 5 mM lidocaine. No significant effect on the patching was observed. DA2 and Genox 3.53 are both IgG_1 (37), indicating that the observed difference is not a function of antibody type. DA2 was fluoresceinated at a lower fluorescein-to-protein ratio than the other antibodies, indicating that over-fluoresceination of the antibody was not responsible for the differences.

Fluorescence recovery curves obtained from these cells were highly nonexponential and often exhibited sharp increases or decreases in fluorescence intensity, possibly due to the movement of patches on the cell surface. The patching induced by DA2 was more extensive than that due to 2.06. The DA2 also often caused agglutination of the cells. Fluorescent DA2 was concentrated at the interfaces between cells. This agglutination was not observed with the other antibodies. Representative fluorescence photomicrographs of the four antibodies bound to JY cells are shown in Fig. 1.

Co-capping experiments were carried out with arsanilate-conjugated 2.06 which was capped by using a rhodamine-conjugated rabbit anti-arsanilate antibody. A fluorescein-conjugated monoclonal anti-DR was then added, and the cells were examined for the presence of green fluorescence outside of red caps. The data are shown in Table 2. The control of adding fluorescein-conjugated 2.06 gave green fluorescence outside of red caps in only 10% of the cells, indicating that the antigen to which it binds was removed from the cell surface. However, each of the other three antibodies showed binding outside of the red caps on 85–100% of the cells. L243 and Genox 3.53 gave a uniform green fluorescence, and DA2 gave the usual patchy and nonuniform fluorescence.

DISCUSSION

All monoclonal antibodies bound to HLA-A,B,C antigens show similar behavior on the EBV-transformed cell surface. The mea-

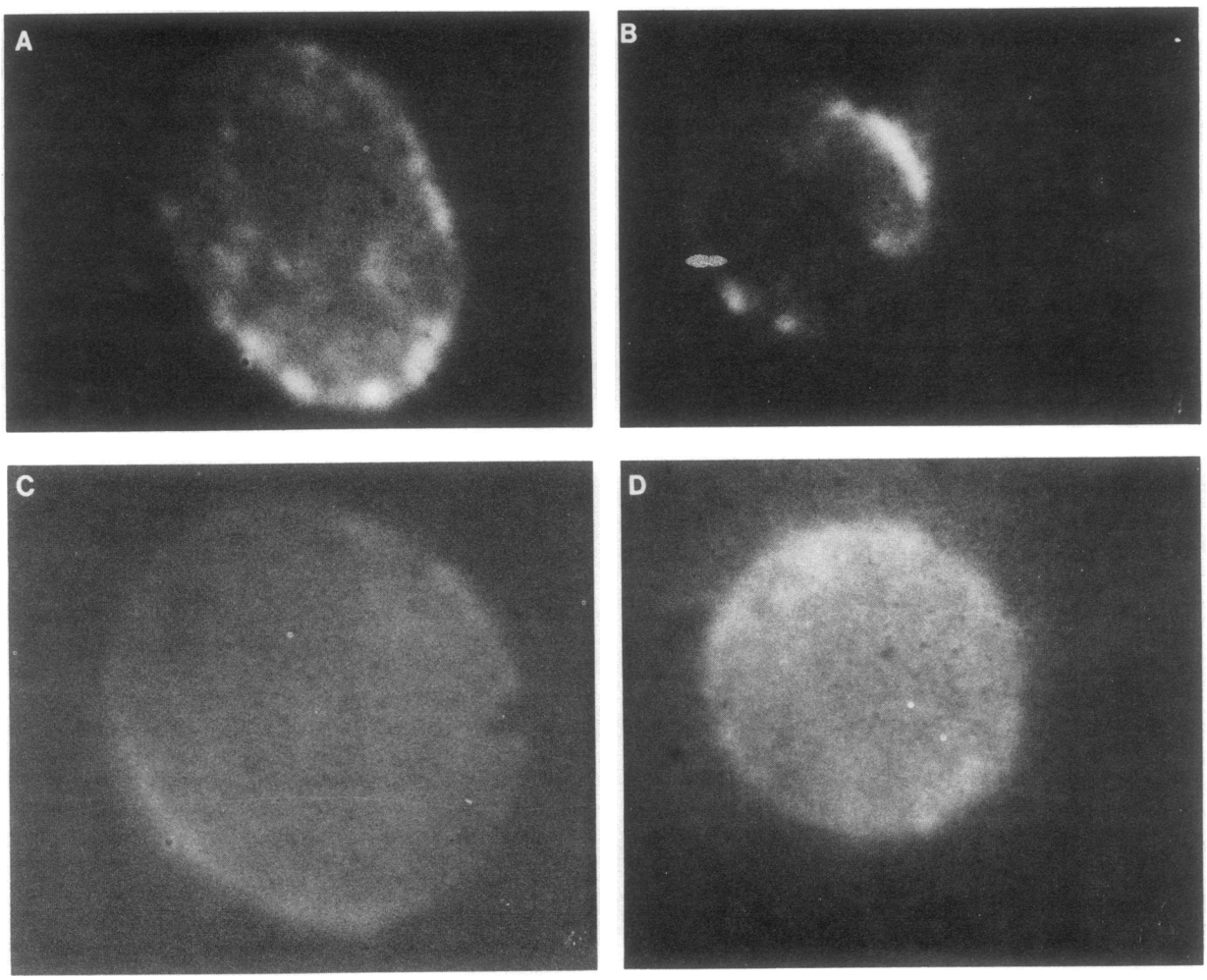

FIG. 1. Fluorescence photomicrographs of fluorescein-conjugated monoclonal anti-DR antibodies bound to JY cells. (*A*) DA2 antibody; (*B*) 2.06 antibody; (*C*) L243 antibody; (*D*) Genox 3.53 antibody. (×3000.)

Table 2. Co-capping of DRw antigens

Fluorescein-conjugated third-step antibody	Green fluorescence outside red caps	
	Present %	Absent, %
2.06	10	90
DA2	100	0
L243	85	15
Genox 3.53	86	14

Arsanilate-conjugated 2.06 antibody was capped by using a rhodamine-conjugated rabbit anti-arsanilate antibody. A third-step fluorescein-conjugated monoclonal antibody was added, and the cells were examined for green (fluorescein) fluorescence outside of red (rhodamine) fluorescence in caps. In each experiment, 20–40 cells were counted. The experiment was carried out on two separate occasions with similar results.

sured values of D are in the range 10^{-9} to 10^{-10} cm^2/sec, consistent with previous results in the literature for HLA antigens (51) and with values commonly reported for mobile membrane proteins (52). It is likely that the 10^{-9} cm^2/sec value for D is that for a protein unhindered by cytoskeletal components because this is comparable to the values obtained for proteins in cholesterol-containing model membranes (53, 54). The smaller values probably reflect some hindrance of the motion of the protein. The measurements of lateral motion showed a high degree of variability, and on several occasions no motion was measurable. In contrast, measurements of lipid motion on such cells gave highly reproducible results (data not shown). Edidin and Wei (51) also observed considerable variability in the diffusion of HLA and H2 antigens on heterokaryons, suggesting that this may be a general property of these molecules. Diffusion rates were not affected by a number of variables, including cell density, temperature, oxygen in the buffer, and treatment with lidocaine (data not shown). The variability may reflect the interaction of the HLA antigens with the cytoskeleton, either in a direct covalent manner or via a cytoskeletal matrix impeding diffusion as proposed by Koppel *et al.* (55). The restriction of motion by intracellular components is now well documented (56, 57), although the precise mechanisms remain unclear.

HLA-A,B,C antigens appear to be monomeric on the cell surface of EBV-transformed cells as indicated by the co-capping experiments. They showed that HLA antigens are not present as homodimers (e.g., HLA A2 HLA-A2). We have also used monoclonal antibodies to show that certain heterodimers (e.g., HLA A2 HLA B7) are not present (data not shown). The latter conclusion is in accord with previous co-capping experiments using alloantisera against H-2 antigens (58–61). Several artifacts might flaw the valency measurement. Examples are monovalent binding of the antibody, a rapid association/dissociation modification, presence of a large fraction of unconjugated antibody in the antibody conjugates, or presence of inactive light chains. None of these effects appear to play any substantial role in the work reported here.

In earlier studies of H-2 and HLA antigens it was found that, under nonreducing conditions of isolation, disulfide-linked dimers were formed (14). This suggested that HLA dimers might exist on cell surfaces. In addition, the existence of cytoplasmic residues that are sometimes phosphorylated (62) or that may serve as substrates for transglutaminase (63) suggests the possibility of linkage of HLA to the cytoskeleton. This could make HLA antigens functionally multivalent. The lateral diffusion (Table 1) and valency measurements are relevant to this question. The results of co-capping experiments clearly show that, for the cells and conditions used, the HLA-A,B,C antigens are functionally monomeric. The D values for HLA-A,B,C, antigens (Table 1) suggest they have dynamic and variable interaction with other cellular components which impedes their motion.

Biochemical evidence has been reported indicating the existence of antigens encoded by multiple loci in the DR region (24, 25, 29). The co-capping experiments reported here strongly support this contention. Three other anti-DR antibodies were bound outside caps made with the 2.06 antibody. The 2.06 antibody itself, however, did not bind outside its own cap, demonstrating that the antigen to which it binds had been completely capped. The ability of a given antibody to patch on the cell surface did not appear to correlate with its specificity for a given antigen: both DA2 and 2.06 caused patching without a second-step antibody, but the antigen to which DA2 binds did not co-cap with 2.06. The simplest interpretation of these data is that there are three different DR antigens on the cell surface: L243 and Genox 3.53 bind to one, DA2 binds to another, and 2.06 binds to a third. Thus, L243 and Genox 3.53 do not patch in the absence of a second-step antibody, and they diffuse uniformly. 2.06 does patch, and DA2 also patches but does not co-cap with 2.06. Biochemical studies have also indicated the existence of at least three distinct DR antigens (24).

In unpublished experiments carried out in collaboration with J. McNicholas and P. Jones (Biology Sciences Department, Stanford University) cells from the murine B-cell line CH1 were specifically labeled with seven different monoclonal antibodies against Ia antigens. Some of the antibodies were specific for *I-A* region products, and some for *I-E/C* region products. In no case was any patching observed. This suggests that either (*i*) the patching phenomenon is characteristic of the human system or, more likely, (*ii*) this patching is specific for certain cell types. Support for the latter possibility follows from the observed patching of HLA class I antigens on only certain types of peripheral blood leukocytes (48). The intracellular "invariant chain" known to be associated with Ia and DR antigens (31) may also play a role in the patching of DR antigens. The degree of expression of inactive light chains by the "monoclonal" antibody-secreting cell line in principle could also affect complex formation on the cell surface, although we consider this to be unlikely.

In earlier work (48) it was shown that W6/32 patched rapidly and extensively at 4°C on the cell surface of polymorphonuclear leukocytes and monocytes, but not on lymphocytes. In the present work (*i*) it is shown that the Fc domain of the antibody is not required for this patching, and (*ii*) a qualitatively different patching is observed with two monoclonal anti-DR antibodies on human B-cell lines. Such patching may play a role in cell–cell interactions involving these antigens. At least two effects may give rise to patching. One, the antigen is oligomeric on the cell surface, such that the monoclonal antibody causes crosslinking and patch formation. Two, the binding of monoclonal antibody to the antigen somehow "triggers" the cell such that it redistributes the antigen. The redistribution of the HLA antigens on peripheral blood leukocytes occurs rapidly at 4°C, and the binding of W6/32 to PMNs does not trigger a respiratory burst or Ca^{2+} uptake (unpublished data). Thus, it is likely that this patching phenomenon is related to an oligomeric antigen rather than to cell triggering. Similar conclusions may apply to the DR antigens.

The main conclusions of the present paper regarding the motion, distribution, and oligomerization of cell surface antigens require that different monoclonal antibodies against the same antigen show similar behavior. This is clearly the case for all of our experiments involving the HLA-A,B,C antigens, and we have implicitly assumed this to be true for the DR antigens.

This work was supported by National Science Foundation Grants PCM 80-21993 (to H.M.Mc.C.) and 80-17834 (to P.P.).

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