The binding of monoclonal antibodies to cell-surface molecules

A quantitative analysis with immunoglobulin G against two alloantigenic determinants of the human transplantation antigen HLA-A2

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Monoclonal IgG₁ (immunoglobulin G₁) PA2.1 and MA2.1 antibodies recognize polymorphic sites of the human transplantation antigen HLA-A2. They are distinguishable because MA2.1 binds HLA-A2 and HLA-B17, whereas PA2.1 binds HLA-A2 and HLA-A28*. The affinities of PA2.1-Fab for HLA-A2, three HLA-A2 variants and HLA-A28* are similar and relatively low ($1.9 \times 10^7 \text{ M}^{-1}$). The affinities of MA2.1-Fab for HLA-A2, three HLA-A2 variants and HLA-B17 are similar and high ($1.2 \times 10^9 \text{ M}^{-1}$). The difference in affinity is due to the rates of dissociation, which give half-times of dissociation of 290 min for MA2.1-Fab and 4 min for PA2.1-Fab. For both Fab, equilibrium measurements and kinetic determinations gave consistent estimates for affinity. When PA2.1-F(ab)₂ or IgG is incubated with cells it reaches equilibrium within 3 h, with most molecules bound bivalently to the cell. Under similar conditions, MA2.1-F(ab)₂ does not reach equilibrium and a significant proportion of molecules bound with one and two sites are found. For the lower-affinity antibody (PA2.1), estimates of the binding constants for one- and two-site interactions could be made. By simple Scatchard analysis the avidity of F(ab)₂ or IgG is $1.3 \times 10^9 \text{ M}^{-1}$, giving an enhancement factor of 68 between bivalent and univalent binding. This is a measure of the equilibrium constant for the interchange between bivalent and univalent binding. Analysis of the results with more realistic models indicates that the actual value is larger (10^3 – 10^4 M^{-1}) than 68 M^{-1} . The avidities of F(ab)₂ and IgG for HLA-A2 are identical, showing the Fc does not interfere with bivalent binding to cells.

The human histocompatibility or HLA antigens are a polymorphic family of membrane glycoproteins that control the recognition of foreign antigens by thymus-derived lymphocytes (Matzinger & Zamoyska, 1982). The classical transplantation antigens are considered here and they consist of a polymorphic membrane-bound glycoprotein of M_r 43 000 associated with β_2 -micro-

globulin of M_r 12 000 [for review, see Dorf (1981) and Parham & Strominger (1982)]. In recent years, monoclonal antibodies have provided tools for analysing the genetic polymorphism, structure and function of HLA antigens. In many experiments one wishes to compare the effects of binding of panels of antibodies to different cell-surface HLA molecules. The interpretation of these experiments can be complicated if the antibodies are of different affinity and if the valency of their attachment varies. It is therefore important to have a working knowledge of how an antibody will bind to cellular or soluble antigen in experimental situations (Mason & Williams, 1980). In the present study the binding properties of the monoclonal antibodies PA2.1 and MA2.1 that define two highly specific alloantigenic determinants of the HLA-A2 $\uparrow$ molecule are described. In addition to A2, PA2.1 recognizes A28*,

Abbreviations used: IgG₁, immunoglobulin G₁; PA2.1 and MA2.1, monoclonal antibodies initially characterized by Parham & Bodmer (1978) and McMichael *et al.* (1980) respectively; HLA antigens, human histocompatibility antigens; F(ab)₂, bivalent antigen-binding fragment produced by *papain* digestion of IgG₁ [this is to distinguish it from F(ab')₂ which is commonly used for the bivalent fragment of IgG produced by *pepsin* digestion]; Fab, univalent fragment produced by mild reduction and alkylation of F(ab)₂; Fc, non-antigen-binding fragment produced by *papain* digestion of IgG; phosphate-buffered saline, Dulbecco's phosphate-buffered saline, pH 7.2 (Gibco, Grand Island, NY, U.S.A.).

$\uparrow$ For brevity HLA-A2 will be referred to as A2; likewise for other HLA molecules.

and MA2.1 recognizes B17. Despite these clear differences in serological specificity, MA2.1 and PA2.1 strongly compete for binding to soluble (Holmes & Parham, 1983) and cell-surface A2 (Ways & Parham, 1983). One aim of this study was to compare the binding of PA2.1 and MA2.1 to A2, A28*, B17 and to three A2 variants. A second aim was to further characterize the binding of monoclonal antibodies to cell-surface antigens in a system where high genetic polymorphism can be used to experimental advantage.

Materials and methods

Cells and media

Human B-cell lines and their HLA type are: MICH (A2,32; B27,15), IDF (A26,28*; B38,18), PREISS (A2,2; B15,15), WT49 (A2,2; B17,17) and DAUDI (HLA-A,B, and C-negative) from Dr. W. Bodmer; JY (A2,2; B7,7) and LB (A28,28; B40,40) from Dr. J. Strominger; M7 (A2,3; B35,53), DR1 (A2,11; B7,12), DK1 (A2,33; B44,40; CW3) and B17 (A1,2; B7,8) from Dr. W. Biddison (Biddison *et al.*, 1980a); A2M1 (A1, BW44,17) (Andreotti *et al.*, 1980) was from Dr. P. Cresswell; and LCL 721.53 (A2) from Dr. R. DeMars (Kavathas *et al.*, 1980). The HL-60 myeloid cell line that expresses HLA-B17 was from Dr. F. Brodsky (Collins *et al.*, 1977; Gallagher *et al.*, 1979). FMB (A1,w32; Bw17,Bw44) is from this laboratory. B-cell lines were maintained in RPMI 1640 medium supplemented with 10% (v/v) foetal-calf serum, penicillin (100 units/ml), streptomycin (100 units/ml), and L-glutamine (2 mM).

Monoclonal antibodies and their fragments

PA2.1 (IgG₁) and MA2.1 (IgG₁) were purified from the ascites fluids of BALB/c mice carrying the monoclonal-antibody-producing cell line as a tumour (Parham *et al.*, 1982). The cell lines may be obtained from Dr. Anne Hamburger at the American Type Culture Collection (A.T.C.C.) Cell Culture Department, 12301 Park Lawn Drive, Rockville, MD 20852, U.S.A.

F(ab)₂ and Fab fragments were prepared after digestion of IgG with papain as described previously (Parham *et al.*, 1982; Ways & Parham, 1983).

Radioactive labelling of antibodies

Radioiodination of IgG and F(ab)₂ was by the method of Hunter & Greenwood (1962) with modifications to permit quantification as described by Mason & Williams (1980).

¹²⁵I-Fab were made by mild reduction and alkylation of ¹²⁵I-F(ab)₂ (Parham *et al.*, 1982). ¹²⁵I-F(ab)₂ preparations were divided in two; one

half was reduced with 10 mM-cysteine at 37°C and alkylated with 50 mM-iodoacetamide in darkened tubes at 37°C, the other half was mock-treated with the addition of buffer alone at 37°C. Preparations of ¹²⁵I-Fab and ¹²⁵I-F(ab)₂ thus made were used in comparative studies.

Radioiodinated preparations were checked for activity and serological specificity by direct cell-binding assay on a panel of human B-cell lines of appropriate HLA type. The specific bindable radioactivity in the preparations ranged from 55 to 95%. In all cell-binding experiments the radioactivity bound to DAUDI cells that express no HLA-A,B,C was used as background and subtracted to give the specific binding (Parham *et al.*, 1982).

Cell-binding assays

Equilibrium binding assays were based on the methods of Trucco *et al.* (1980) as modified by Parham *et al.* (1982). Dilutions of ¹²⁵I-antibody were incubated with cells in phosphate-buffered saline/0.1% bovine serum albumin in 1.5 ml Microfuge tubes for 3 h at 4°C with continuous slow rotation. The tubes were centrifuged (1 min, Eppendorf Microfuge) and the supernatants quickly aspirated. Cell pellets were assayed for radioactivity bound. Background binding to DAUDI cells was 1–5% of the input. The results were analysed by the method of Scatchard (1949; Tinoco *et al.*, 1978). The quotient of bound to free radioactivity for duplicate samples was plotted against radioactivity bound and straight lines fitted by linear regression. The binding constant (*K*) and the number of antibody molecules bound per cell (*n*) were calculated from the equations:

$$K = \frac{-\text{grad } SV \times 10^{14}}{4.44 M}$$

and

$$n = \frac{\text{intercept } V \times 10^{21}}{1.66 NK}$$

where *S* = specific activity in c.p.m./μg, *V* = volume in ml, *N* = number of cells, grad = gradient, intercept = intercept on the ordinate of the graph, and *M* = *M_r* of antibody, where values of 150 000 for IgG, 100 000 for F(ab)₂ and 50 000 for Fab were used.

The rates of antibody association and dissociation were measured by the methods of Mason & Williams (1980). In association experiments the radioactivity bound was plotted against time (*t*) and the forward rate constant (*k*₊₁) calculated from the gradient at *t* = 0:

$$k_{+1} = \frac{\text{grad}}{[A^{\circ}]}$$

where grad = gradient of the plot and $[A^0]$ = the initial antigen concentration. In dissociation experiments the logarithm of the proportion of specific radioactivity remaining bound was plotted against time (in seconds). Straight lines were fitted by linear regression and the half-time dissociation ($t_{1/2}$) interpolated from the graph. k_{-1} was calculated from the equation:

$$k_{-1} = \frac{0.693}{t_{1/2}}$$

Indirect saturation binding assays were as described by Parham *et al.* (1982), based on the method of Morris & Williams (1975).

Results

PA2.1 antibody

1. *Equilibrium binding.* Equilibrium binding of ^{125}I -PA2.1-IgG, -F(ab) $_2$ and -Fab to cells of different HLA types was measured. In all experiments the antigen concentration was kept constant and the antibody concentration varied. Preliminary experiments established a range of concentrations that gave measurable bound and free antibody and the time required to reach a constant level of binding. Scatchard plots were linear, showing that a single affinity was being measured (Fig. 1a). The results were analysed by linear regression and gave correlation coefficients in the range 0.95–0.99. Values obtained for the affinity (K) and antigenic site number (n) are summarized in Table 1. Although the results obtained within a single experiment were consistent, some day-to-day variation in the values of K and n for similar experiments was observed. This variation was on the order of 2-fold. PA2.1-Fab bound to A2 or A28* with affinities $(1-3) \times 10^7 \text{ M}^{-1}$. The values for n were $5 \times 10^5 - 1 \times 10^6$ sites per cell. The affinities of PA2.1-Fab for different cells expressing A2, A28* and three variants of A2 were similar. The radioactivity bound to cells expressing other HLA-A,B specificities including wild-type A28 was indistinguishable from that bound to the DAUDI cell line that does not express HLA-A,B,C.

The binding of F(ab) $_2$ or IgG to cell-surface molecules is more complex than for Fab. They may bind with one or two combining sites and the effective strengths of these two modes of binding can be different. Following immunological convention, affinity refers to the binding constant of Fab or a single antibody combining site, and avidity refers

Fig. 1. *Equilibrium binding of PA2.1* The cell lines are: ●, MICH; △, DKI; ▲, DRI; ○, M7; ■, IDF. For Fab (a), incubation was in a volume of 0.125 ml with ^{125}I -Fab concentration in the range 1–30 $\mu\text{g}/\text{ml}$ at a specific radioactivity of $7.4 \times 10^5 \text{ c.p.m.}/\mu\text{g}$. For F(ab) $_2$ (b), incubation was 5 $\times 10^5$ cells in a volume of 1.0 ml with ^{125}I -F(ab) $_2$ concentration in the range 0.1–3 $\mu\text{g}/\text{ml}$ at a specific radioactivity of $6.2 \times 10^6 \text{ c.p.m.}/\mu\text{g}$.

to the apparent binding constants of the bivalent species F(ab) $_2$ and IgG.

Measurement of the binding of F(ab) $_2$ or IgG required lower concentrations of antigen and antibody than those used for Fab. Under the conditions used for Fab the reaction with F(ab) $_2$ or IgG was

‡ The term 'antibody' will be used collectively for IgG, F(ab) $_2$ and Fab.

Table 1. Binding constants (*K*) and site numbers (*n*) from equilibrium assays

Cell line	Antibody ...	PA2.1-IgG			PA2.1-F(ab) ₂			PA2.1-Fab			MA2.1-Fab		
		<i>D</i> [†]	10 ⁻⁷ × <i>K</i> (M ⁻¹)	10 ⁻⁵ × <i>n</i>	<i>D</i>	10 ⁻⁷ × <i>K</i> (M ⁻¹)	10 ⁻⁵ × <i>n</i>	<i>D</i>	10 ⁻⁷ × <i>K</i> (M ⁻¹)	10 ⁻⁵ × <i>n</i>	<i>D</i>	10 ⁻⁷ × <i>K</i> (M ⁻¹)	10 ⁻⁵ × <i>n</i>
MICH	Target	3	100	4.7	3	130	2.8	5	2.5	9.5	4	140	10
DR1	HLA antigen	2	110	2.0	2	110	3.3	3	1.6	7.2	3	120	10
DK1	Variant A2	2	58	6.4	2	120	4.5	3	1.9	10	3	96	10
M7	Variant A2	3	90	5.3	1	130	2.2	2	1.2	8.3	3	110	9
IDF	Variant A2	4	150	4.3	2	150	2.0	2	1.5	6.9	3	0	0
JY	A28*	2	100	1.9							4	110	15
PREISS	A2	4	140	11.0									
GFF	A2	2	140	8.6									
MFF	A2	2	160	5.6									
A2M1	B17												
HL-60	B17										4	92	11
WT-49	A2, B17										3	90	6
FMB	B17										3	160	25
Peripheral blood lymphocytes	A2	1	181	0.37							3	130	7
Mean for all B-cell lines			130	5.5		130	2.9		1.9	8.6		120	11

[†] *D* = number of determinations.

driven to completion. Conversely, under the conditions used for F(ab)₂ and IgG there was no significant binding of Fab. This indicated that the avidity of PA2.1 for cells was greater than the affinity. When the results for IgG and F(ab)₂ were submitted to Scatchard analysis, straight lines with high correlation coefficients were obtained, as shown in Fig. 1(b) for F(ab)₂. This suggested that a single mode of binding was being sampled, and, as univalent binding did not significantly occur under these conditions, it was therefore bivalent binding. The values of avidity for F(ab)₂ and IgG were similar and $(1-2) \times 10^9 \text{ M}^{-1}$, about 50-100-fold higher than for Fab (Table 1). The site numbers were between 30 and 50% of the Fab values, consistent with complete bivalent binding of F(ab)₂. The avidities of PA2.1-F(ab)₂ for cells expressing wild-type A2, variant A2s and A28* were not significantly different. The avidity of PA2.1-F(ab)₂ or -IgG for HLA-A,B,C antigens other than A2, A2 variants and A28* is at least 1000 times lower than for A2. PA2.1-IgG bound with similar avidity to peripheral-blood lymphocytes or B-cell lines, expressing HLA-A2, with similar avidity, though as previously observed the antigenic site number for peripheral-blood lymphocytes was about 30-fold less (McCune *et al.*, 1975; Parham *et al.*, 1979). The comparable avidity of IgG and F(ab)₂ shows that the Fc imposes little strain upon the relative motions of the Fab arms required to form the bivalent antigen-antibody complex at the cell surface.

2. *Kinetics of association and dissociation of PA2.1.* The rates of association and dissociation of PA2.1-Fab and -F(ab)₂ were measured on a single cell line (MICH) expressing wild-type A2, since the equilibrium assays had shown the affinity of PA2.1 for A2, A28* and A2 variants was similar. The results are summarized in Table 2. The initial association rate for F(ab)₂ was 4.4 times greater than for Fab. The half-time of dissociation of Fab in the absence of competing antibody was 4.2 min (Fig. 2). F(ab)₂ dissociated slowly under these conditions and a dissociation was measured in only four out of

nine experiments. They provide a lower limit for the half-time of dissociation of 50 min. With competing non-radioactive antibody the rate of dissociation of Fab only slightly increased, whereas F(ab)₂ dissociated much faster with a half-time of 17 min. Increasing the concentration of competing antibody beyond 50 µg/ml did not result in further increase in the rate of dissociation. This indicates that preferential rebinding of the combining sites of ¹²⁵I-F(ab)₂ occurs even when an excess of competing antibody is present. Values for the affinity of Fab and the avidity of F(ab)₂ calculated from the experimentally determined on and off rate constants

Fig. 2. *Dissociation of PA2.1* MICH or DAUDI cells (2×10^7) and 5×10^6 c.p.m. of ¹²⁵I-PA2.1-F(ab)₂ or ¹²⁵I-PA2.1-Fab were incubated at 4°C for 2 h in a total volume of 200 µl. Cells (10^6 , 10 µl) were then diluted in 1 ml of phosphate-buffered saline/0.1% bovine serum albumin with or without 50 µg of non-radioactive PA2.1-IgG/ml and allowed to dissociate at 4°C for various times. Δ , ¹²⁵I-PA2.1-F(ab)₂; $\blacktriangle$, ¹²⁵I-PA2.1-F(ab)₂ with 50 µg of PA2.1-IgG/ml; $\circ$, ¹²⁵I-PA2.1-Fab; $\bullet$, ¹²⁵I-PA2.1-Fab with 50 µg of PA2.1-IgG/ml.

Table 2. *Kinetics of binding of PA2.1*

	No. of determinations	F(ab) ₂	t _{1/2} (min)	No. of determinations	Fab	t _{1/2} (min)
(a) k ₊₁	3	$4.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$	NA*	3	$0.93 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$	NA
(b) k ₋₁ without competing antibody	4	$2.3 \times 10^{-4} \text{ s}^{-1}$	50	6	$2.7 \times 10^{-3} \text{ s}^{-1}$	4.3
(c) k ₋₁ with 50 µg of competing antibody/ml	5	$8.4 \times 10^{-4} \text{ s}^{-1}$	14	4	$3.1 \times 10^{-3} \text{ s}^{-1}$	3.7
(a)/(b) (k ₊₁ /k ₋₁) (K)		$1.8 \times 10^9 \text{ M}^{-1}$			$3.4 \times 10^7 \text{ M}^{-1}$	
Mean K from Table 1		$1.3 \times 10^9 \text{ M}^{-1}$			$1.9 \times 10^7 \text{ M}^{-1}$	

* NA, not applicable.

Fig. 3. *Equilibrium binding of MA2.1*
For Fab (a), 2×10^5 MICH (○), JY (△), A2M1 (□) or HL-60 (▽) cells were incubated in a volume of 1.0 ml with a concentration range of ^{125}I -Fab of 0.03–1 $\mu\text{g}/\text{ml}$ with specific radioactivity 4.2×10^6 c.p.m./ μg . For F(ab)₂ (b), 2×10^5 MICH (●), JY (▲), A2M1 (■) or HL-60 (▼) cells were incubated in a volume of 1.0 ml with a ^{125}I -F(ab)₂ concentration range of 0.03–1 $\mu\text{g}/\text{ml}$ with specific radioactivity of 4.2×10^6 c.p.m./ μg . The binding of ^{125}I -MA2.1-Fab to MICH cells (○) is included for comparison.

showed a good agreement with experimental values determined by equilibrium assays (Table 2).

MA2.1 Antibody

1. *Equilibrium binding.* MA2.1-Fab bound to A2, A2 variants or B17 with a similar affinity [$(0.9\text{--}1.7) \times 10^9 \text{M}^{-1}$] (Fig. 3a, Table 1). No binding to other HLA-A,B antigens including A28 and A28* was detected.

Scatchard plots for MA2.1-F(ab)₂ were heterogeneous, unlike that found with MA2.1-Fab or any of the PA2.1 species (Fig. 3b). At either end of the range were experiments that gave linear graphs with different slopes. For HL-60 and A2M1 the slope was low and approximates that of the Fab. MICH gave a steeper slope and JY an intermediate result that at high antibody concentrations approximated Fab binding to MICH and at low antibody concentrations F(ab)₂ binding to MICH (Fig. 3b). This suggested that, at the end point of the experiment, MA2.1-F(ab)₂ bound to JY in both univalent and bivalent forms. Lower antibody concentrations, higher cell numbers and cells with higher antigen density all favoured bivalent binding. As shown below, the rate of dissociation of MA2.1-Fab is low, and true equilibrium was probably not obtained in these assays, although the F(ab)₂ bound reached a plateau value. This would occur if the forward rate

Fig. 4. *Association of ^{125}I -MA2.1-F(ab)₂ (○) and ^{125}I -MA2.1-Fab (●) to MICH cells*

MICH or DAUDI cells (5×10^5) were added at various time points to 1 ml of buffer containing 10^5 c.p.m. of ^{125}I -MA2.1-F(ab)₂ or ^{125}I -MA2.1-Fab in 1.5 ml Microfuge tubes at 4°C. After 10 min, all tubes were centrifuged and the pellets counted for radioactivity bound. Each data point is the average of duplicates with the binding to the control cell line (DAUDI) subtracted.

of univalent binding to the cell significantly competes with the rate of change from univalent to bivalent attachment. The attainment of equilibrium then becomes controlled by the slow rate of dissociation. This will not occur with Fab provided that the experiment is designed to minimize localized high concentrations of cells or antibody.

2. *Saturation binding assays.* To test if MA2.1 binds in both univalent and bivalent modes, saturation binding assays with $F(ab)_2$ and Fab on different cell lines were performed. Such assays will favour univalent binding. $F(ab)_2$ bound bivalently to cells should bind the same amount of ^{125}I - $F(ab)_2$ rabbit anti-mouse IgG (^{125}I -RAM) as Fab. When bound univalently it should bind twice the radioactivity bound by Fab. Thus by comparing the radioactivity bound by Fab and $F(ab)_2$, one may calculate the proportion of $F(ab)_2$ bound in univalent and bivalent forms. There were considerable and reproducible differences between cells that were consistent with the results of equilibrium assays (Table

3). The range was 15–81% bivalent binding. The degree of bivalent binding is roughly correlated with antigen density.

3. *Kinetics of association and dissociation of MA2.1.* The rate of association of $F(ab)_2$ was slightly higher than for Fab, and in the same range seen with PA2.1 fragments (Fig. 4, Table 4).

Dissociation of MA2.1- $F(ab)_2$ or -Fab at 4°C in the absence of competing, non-radioactive, homologous IgG was not detected. At room temperature, dissociation of Fab ($t_{1/2} \approx 100$ min), but not $F(ab)_2$, occurred in the absence of competing IgG (Fig 5). When competing antibody at 100 µg/ml was included, dissociation of $F(ab)_2$ as well as Fab was seen. The rate was ≈ 9 times faster at room temperature than at 4°C for Fab and ≈ 8 times faster for $F(ab)_2$. At each temperature the dissociation rate for Fab was about twice that for $F(ab)_2$. The dissociation of Fab at 4°C in the presence of competing antibody gave a value for k_{-1} of 4.0×10^{-5} , corresponding to a dissociation time ($t_{1/2}$) of

Table 3. Indirect saturation binding assay of MA2.1- $F(ab)_2$ and MA2.1 Fab on cell lines

Cell line	Target antigen	¹²⁵ I-RAM bound (c.p.m.)				Percentage of F(ab) ₂ bound univalently	
		No antibody	[F(ab) ₂]	[Fab]	Mean* - DAUDI		$\left(\frac{[F(ab)_2] - [Fab]}{[F(ab)_2]} \times 100 \right)$
					F(ab) ₂	Fab	
DAUDI	None	1400	1600	1600			
MICH	A2	1600	16 200	11 000	14 600	9400	32
JY	A2.2	1903	41 400	35 100	39 800	33 500	19
LCL721.53	A2	4087	18 100	13 700	16 500	12 100	37
HL-60	B17	2228	13 700	8700	13 100	7100	85
A2M1	B17	1500	1200	8400	12 100	6800	78
LB	None	1000	1400	1300	0	0	
MST	None	1300	1300	1200	0	0	

* Mean values were calculated from determinations at three MA2.1 antibody concentrations (25, 12.5, 6.25 µg/ml) that were all at saturation. (The cell line DAUDI has no HLA and is used to give background values. 'Mean - DAUDI' is the mean radioactivity bound to the cell line under test minus the mean radioactivity bound to DAUDI.)

Table 4. Association and dissociation rate constants of MA2.1
Abbreviations used: NA, not applicable; RT, room temperature, 24°C.

	No. of determinations	$F(ab)_2$	$t_{1/2}$ (min)	No. of determinations	Fab	$t_{1/2}$ (min)
(a) k_{+1} (4°C)	2	$1.9 \times 10^5 M^{-1} \cdot s^{-1}$	NA	2	$1.4 \times 10^5 M^{-1} \cdot s^{-1}$	NA
(b) k_{-1} (4°C) without competing antibody	22	No dissociation		2	No dissociation	
(c) k_{-1} (4°C) with 100 µg of competing antibody/ml	4	$1.9 \times 10^{-5} s^{-1}$	610	4	$4.0 \times 10^{-5} s^{-1}$	290
(d) k_{-1} (RT) without competing antibody	3	No dissociation		3	1.2×10^{-4}	96
(e) k_{-1} (RT) with 50 µg of competing antibody/ml	2	1.9×10^{-4}	61	2	4.0×10^{-4}	29
(a)/(c) (k_{+1}/k_{-1}) (K)					$3.5 \times 10^9 M^{-1}$

Fig. 5. Dissociation of ^{125}I -MA2.1-F(ab) $_2$ and ^{125}I -Fab from M1CH cells

M1CH or DAUDI cells (2×10^7) and 5×10^6 c.p.m. of ^{125}I -MA2.1-F(ab) $_2$ or ^{125}I -MA2.1-Fab were incubated at 4°C for 2 h in a total volume of 200 μl . Cells (5×10^5 , 5 μl) were then diluted into 1.5 ml Microfuge tubes containing 1 ml of buffer at room temperature or at 4°C with 50 $\mu\text{g}/\text{ml}$ of non-radioactive MA2.1-IgG. Δ , ^{125}I -MA2.1-F(ab) $_2$, room temperature; $\blacktriangle$, ^{125}I -MA2.1-F(ab) $_2$, 4°C with 50 μg of MA2.1-IgG/ml; $\circ$, ^{125}I -MA2.1-Fab, room temperature; $\bullet$, ^{125}I -MA2.1-Fab, 4°C with 50 μg of MA2.1-IgG/ml.

≈ 5 h. The value of the equilibrium constant, K , derived from this value and the k_{+1} of 1.4×10^5 is $3.5 \times 10^9 \text{ M}^{-1}$ (Table 4), consistent with the value from equilibrium binding experiments. This suggests that the lack of dissociation measured in the absence of competing IgG is the result of dissociation and rebinding and not because the rate of dissociation is altered by the presence of the competing IgG.

Discussion

The specific HLA molecules recognized by PA2.1 or MA2.1 are bound with identical affinities. Affinities for other HLA-A,B molecules are not measurable. Thus A2 and A28* share the PA2.1 antigenic determinant, and A2 and B17 share the MA2.1 antigenic determinant. Biddison *et al.* (1980a, b) have characterized natural variants of HLA-A2. Cells expressing these variants type as HLA-A2 with alloantisera but are not killed by HLA-A2-restricted cytotoxic T-lymphocytes. MA2.1 and PA2.1 bind wild-type and variant A2 molecules with the same affinity. It is of great interest that these antigenic determinants are not affected by the structural changes that lead to loss of T-lymphocyte recognition.

PA2.1 has low affinity ($1.9 \times 10^7 \text{ M}^{-1}$) and MA2.1

has high affinity [$(1-2) \times 10^9 \text{ M}^{-1}$] for HLA-A2. This difference is a function of the dissociation constants and leads to qualitative differences in their cell-binding properties. MA2.1 forms a stable interaction with cell-surface antigen when bound by one or two combining sites. With MA2.1-IgG or F(ab) $_2$, equilibrium is not reached with incubation times (0-6 h) that are practical with live cells, and antibody is found bound with one and two sites over a wide range of conditions. Equilibrium assays gave reliable binding constants only for Fab.

By contrast, PA2.1 has a low affinity, and the single combining site dissociates rapidly from antigen. Stable interaction of PA2.1 with cells only occurs when it is bound in the bivalent form. The ratio between the avidity of PA2.1-F(ab) $_2$ and PA2.1-Fab gives a measure of the extra strength of binding contributed by the bivalency of the antibody. This has been called the 'enhancement factor' (Karush, 1976, 1978) and for PA2.1 is 68. This is related to the equilibrium constant for the reaction relating antibody bound with one and two combining sites.

In a theoretical treatment of the binding of bivalent antibodies to cell surfaces at equilibrium, Reynolds (1979) considered the reaction leading to bivalent binding in two steps, the step to univalent binding being in solution and that from univalent to bivalent binding being at the cell surface. This is shown below;

where A is antibody, B is HLA, and AB and BAB are the univalent and bivalent complexes. The asterisk refers to components that are at cell surfaces and where effective concentrations are calculated with respect to the surface volumes that they move in. Manipulation of these equations for conditions in which bivalent complexes predominate allows the determination of an expression for $1/v$, which is the ratio (mol/mol) of total antibody to HLA bound by antibody (Reynolds, 1979):

$$\frac{1}{v} = \frac{CV^*}{K_1 K_2 [L]^2} + 1$$

where C = number of cells/litre, and V^* = surface volume of one cell.

For PA2.1-F(ab) $_2$, but not MA2.1-F(ab) $_2$, this analysis is appropriate and the data from equilibrium binding experiments were used to calculate values for v and for $[L]$, which is the concentration of unbound HLA-A2. This required a value for the total HLA-A2 concentration, which was obtained from the Fab site number shown in Table 1. $1/[L]^2$ was calculated for different values of total HLA-A2 concentration within the range $2 \times 10^{-10} \text{ M}$ –

$6.6 \times 10^{-9} \text{ M}$, corresponding to 2.5×10^5 – 8×10^6 molecules per cell (Table 1). The graphs shown in Fig. 6 are of $1/v$ against $1/[L]^2$ and are those giving the best fit to a straight line. The best fit was with 2×10^6 HLA-A2 molecules per cell. K_2 was calculated from the gradient by using the affinity of Fab ($2 \times 10^7 \text{ M}^{-1}$) as K_1 and a value of V^* of 6.8×10^{-15} litre. V^* was obtained by assuming the cells were spheres of radius $6 \mu\text{m}$ and that cell-membrane-bound F(ab)_2 and HLA moved in a volume equal to the product of the cell-surface area and 15 nm ($150 \text{ \AA}$). The value of K_2 obtained was $1.3 \times 10^4 \text{ M}^{-1}$ for the best straight line and in the range $3 \times 10^3 \text{ M}^{-1}$ – $3 \times 10^4 \text{ M}^{-1}$ for the lines plotted in Fig. 6.

Crothers & Metzger (1972), after Greenbury *et al.* (1965), devised a method for predicting the ratio between the univalent and bivalent binding constants for IgG. They derived the relationship for bivalent antibody binding to a multivalent antigen:

$$K_2 = \frac{3VK_1}{\langle r \rangle Na}$$

where K_1 is the observed affinity for Fab, N is the number of cells in volume V in the standard state, a is the surface area of the cell and $\langle r \rangle$ is the average distance between the two combining sites of the antibody that they estimate to be 8.7 nm ($87 \text{ \AA}$).

Fig. 6. Analysis of ^{125}I -PA2.1-F(ab) $_2$ binding by the model of Reynolds (1979)

The results were obtained under similar conditions to those shown in Fig. 2. $1/v$ is plotted against $1/[L]^2$ where v is the ratio HLA-A2 bound (mol)/total F(ab) $_2$ (mol). $[L]$ is the concentration of free HLA-A2. Three values for the total concentration of HLA-A2 were used, based on 4×10^6 (□), 2.5×10^6 (Δ) and 2.0×10^6 (○) HLA-A2 molecules/cell. Straight lines were fitted by linear regression and their magnitudes used to calculate a value of K_2 .

Calculation of the ratio $3VK_1/\langle r \rangle Na$ for PA2.1 gives a value of $4.6 \times 10^3 \text{ M}^{-1}$ for K_2 . The cells were again assumed to be spheres of radius $6 \mu\text{m}$.

The avidities from Scatchard analysis probably underestimate the contribution of bivalent binding. More realistic models give values of K_2 for PA2.1 that are 3×10^3 – $3 \times 10^4 \text{ M}^{-1}$ which are significantly larger than 68 M^{-1} . These are all low compared with the theoretical maximum as given by one-quarter of the square of the single-site affinity (Reynolds, 1979). A contributing factor may be strain induced by complex-formation. The identical binding constant of PA2.1-IgG and F(ab) $_2$ eliminates involvement of the Fc region. Other variables that can decrease K_2 are due to the cell surface and are poorly understood. For example it has been assumed that the cells are perfect spheres with a uniform distribution of antigen. If true, then the mean separation of HLA-A2 molecules is about 50 nm ($500 \text{ \AA}$), a distance much greater than the separation of the Fab arms of an IgG molecule. Lateral diffusion of A2 in the lipid bilayer is therefore required for establishing bivalent complexes, and the diffusion rate will affect the value of K_2 . For HLA on B-cell lines the time taken for a movement of 50 nm is $\approx 0.1 \text{ s}$ (Smith *et al.*, 1982). This is slow compared with the motion of antibody from solution to the cell surface and can explain why high-affinity antibodies such as MA2.1 give significant amounts of univalent binding.

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