*AN APPROACH TO THE MAPPING OF ANTIGENS ON THE CELL SURFACE**

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The antigenic individuality of the surfaces of cells from different members of a species (genotypic variation) has been an object of study from the time Landsteiner discovered the human ABO blood groups in 1900 to the present era of organ transplantation. Only recently, however, has attention been directed to the antigenic individuality of the surfaces of different cells from a *single* member of a species (phenotypic diversity), although it comes as no surprise to find that cellular differentiation associated with selective gene activation gives rise to serologically demonstrable differences in cell surface structure. Four of the five systems of alloantigens known to occur on thymocytes (Table 1) are limited to one or a few cell types. These four have special interest as models of quantitative phenotypic variation within one individual, and since they are cell surface components, they are presumably relevant to the organization of interdependent cell populations; it is hardly likely that such phenotypic variation is lacking in biological significance. Fortunately these antigens—θ, Ly-A, Ly-B, and TL—as well as H-2, exhibit both genotypic (allelic) variation and phenotypic variability (i.e., are alloantigens as well as "differentiation" or "cell-type-specific" antigens), and this makes their individual recognition possible. At least one additional thymocyte/lymphocyte-specific antigen, mouse-specific lymphocyte antigen (MSLA),[4] *lacks* allelic variability (being demonstrable only with heteroantisera) and is therefore subject to the restrictions on systems where segregation cannot be used. These differentiation antigens are of special interest because they constitute a physical basis for surface differences confronting different cells in the same individual, in contrast to genotypically determined differences confronting two cell populations in the contrived situation of homotransplantation.

We have answered, or can readily answer, the usual questions relating to any one of the antigens in Table 1, namely, "Is it present on a particular cell?" and "in what quantity?" But we know virtually nothing about the disposition of

Table 1. *Five antigenic systems demonstrable on mouse thymocytes by cytotoxic alloantisera.*

Alloantigenic system	Key reference	Cells on which antigen is represented
H-2	1	Majority
θ	2	Thymocytes, lymphocytes, and adult nervous tissue
Ly-A	3	Thymocytes and lymphocytes
Ly-B	3	Thymocytes and lymphocytes
TL (*Tla*)	4	Thymocytes

H-2 is poorly represented on thymocytes; the other four antigens are more strongly represented on thymocytes than on lymphocytes.

H-2 and *Tla* are linked; otherwise none of the five loci is linked to any other of the five.

any antigens on the cell surface nor whether they occur in patterns that can be elucidated and thus can serve as the basis of a systematic study of cell relationships. With this in mind, we have devised a method of plotting the position of antigens on the cell surface in relation to one another and have applied it to the five systems of antigens named in Table 1. The thymocyte was selected for study because it is the only cell on which all five are represented and because with the exception of H-2 all these antigens are more strongly represented on thymocytes than on other cells. The principle that emerges, and is used in plotting these antigens, is that when two cell antigens are sufficiently close, the absorption of antibody by one of them impedes the absorption of antibody by the other. This provides a measure of the proximity of any pair of antigens; we call it the "blocking" test.

Materials and Methods.—Antisera: See references 3, 5, and 6. H-2 is treated as two groups of antigens, specified by the "D" end and the "K" end of *H-2*, by use of the H-2^b/H-2^a crossover stocks H-2H and H-2I[1] and of H-2^a, which behaves as a crossover derivative of H-2^d/H-2^k,[1] in conjunction with the relevant antisera. In contrast to a reliance on tables of H-2 specificity, this approach precludes (for genetic reasons) interference by unrecognized H-2 antibodies in H-2 antisera of ostensibly more limited specificity. This fact is important in the blocking test (below) where an H-2 antibody that passes unrecognized because it happens to agglutinate poorly or to be poorly cytotoxic might block others and lead to an erroneous interpretation. The two groups of specificities are designated H-2(D) and H-2(K).

Cytotoxic test with mouse thymocytes: See reference 3.

Blocking test: Step 1: Viable thymocytes of selected phenotype are washed twice and resuspended (1.5×10^7/ml) in (*a*) an excess of selected antibody ("blocked" thymocytes) and (*b*) the same concentration of normal mouse serum ("standard" thymocytes). The two suspensions are incubated for 1 hr with repeated mixing at room temperature, except for TL antisera, when the cells are incubated on ice to prevent antigenic modulation.[5] All procedures thereafter are performed in the cold, except for the cytotoxic test. The thymocytes are then washed three times, the initial supernate of the "blocked" thymocyte preparation being tested for expected residual cytotoxicity, to verify that the thymocytes have in fact been exposed to excess antibody. Thymocyte counts are repeated and viability is checked with trypan blue (required viability >95%). *Step 2:* The absorption capacity of the "blocked" and "standard" thymocytes for antibody of the second selected specificity is determined quantitatively. The method for quantitating antigen on viable cells by their absorption of cytotoxic antibody is described fully elsewhere.[3] Briefly, aliquots of antiserum, diluted according to preliminary tests, are absorbed with a range of counted numbers of the thymocytes to be tested for 30 min in the cold with continuous shaking. *Step 3:* Each absorbed aliquot is tested for residual cytotoxicity against the appropriate test cell. *Calculation:* see Figure 1. These cytotoxic data (% test cells lysed by residual antibody, *ordinate*) are plotted against the numbers of thymocytes used for absorption (*abscissa*) and this provides an absorption curve for "blocked" and for "standard" thymocytes. The index of absorption is the number of thymocytes (*n*) that reduces the cytotoxicity of the aliquot to *50% test cells dead.* The per cent reduction in absorption capacity caused by the blocking antibody is given by

$$\frac{n(\text{blocked thymocytes}) - n(\text{standard thymocytes})}{n(\text{blocked thymocytes})} \times 100.$$

Results and Comments.—Relative positions of antigens specified by allelic genes: Tests with heterozygotes of all systems tested, H-2(D), H-2(K), *θ*, Ly-A, and Ly-B, gave similar results: the absorption of antibody related to one allele was blocked 34–44 per cent by previous absorption of antibody related to the

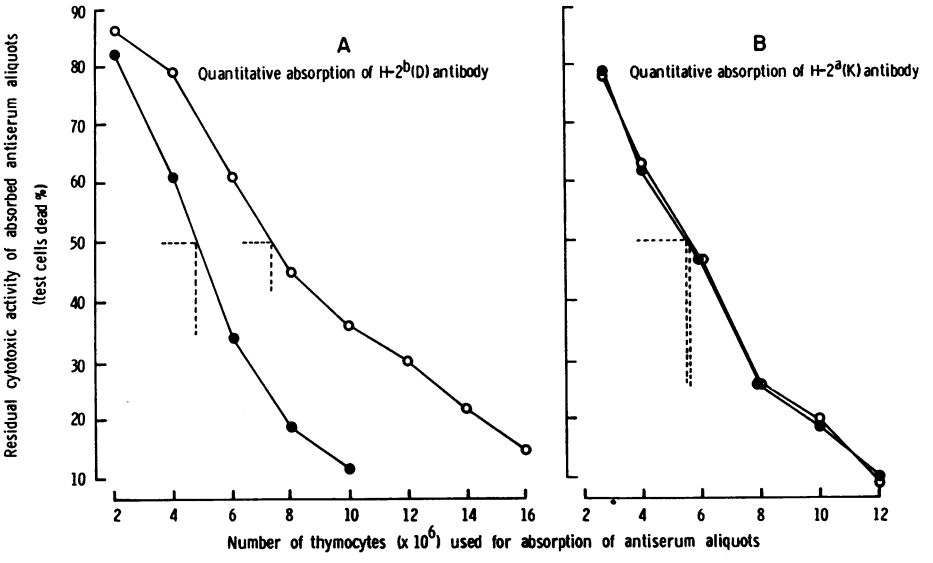

FIG. 1.—Example of the blocking method for determining whether two cell membrane antigens are closely adjacent to one another.

(*Step 1*) *Attachment of the blocking antibody:* H-2^b/H-2^a thymocytes were exposed to (*a*) excess *anti*-H-2^a(D) ("blocked" thymocytes, O——O——O), and (*b*) normal mouse serum ("standard" thymocytes, ●——●——●), and then washed three times.

(*Step 2*) *Quantitative absorption:* The two preparations of thymocytes were tested for their capacity to absorb (*a*) H-2^b(D) antibody and (*b*) H-2^a(K) antibody.

Abscissa: Numbers of "blocked" or "standard" thymocytes used to absorb aliquots of these antisera.

(*Step 3*) *Cytotoxic tests were performed with the absorbed H-2^b(D) and H-2^a(K) antiserum aliquots.*

Ordinate: Residual cytotoxic activity of antiserum aliquots.

Calculation of % reduction in absorption capacity:

$$(A)\ \frac{7.3 - 4.8}{7.3} \times 100 = 34 \text{ (first entry in Table 2)}$$

$$(B)\ \frac{5.6 - 5.4}{5.6} \times 100 = 3 \text{ (second entry in Table 2).}$$

Interpretation: Attached H-2^a(D) antibody partially blocks uptake of H-2^b(D) antibody but does not interfere with the uptake of H-2^a(K) antibody. Therefore H-2^a(D) antigen sites must be closely adjacent to H-2^b(D) sites, but H-2^a(K) must lie at some greater distance from H-2^a(D).

alternative allele (Table 2). (Heterozygotes of the necessary type do not occur in the TL system,[6] which therefore is not amenable to similar tests.) Thus the provisional rule is that antigens specified by pairs of alleles occupy closely adjacent positions. To illustrate how the figures denoting degree of blocking are derived, the absorption curves for the first two entries in Table 2 are calculated in the legend of Figure 1.

Relative positions of antigens specified by nonallelic genes: Three kinds of result were observed (Table 3).

(a) *No blocking:* This indicates that two antigens are relatively widely

spaced, although their distance apart may still be estimated by reference to a third antigen within blocking range of both of them (Fig. 2).

(b) *Reciprocal blocking:* As with *allelic* pairs (Table 2), either antibody of a pair blocks its partner to the same extent; this type of blocking indicates that two antigens are situated close to one another and that there are no considerable differences in their disposition (entries 1*b* and 2*a*, 3*d* and 4*c*, 4*e* and 5*d*, and 7*h* and 8*g* in Table 3).

(c) *Nonreciprocal blocking:* One member of a pair of antibodies blocks its partner but the other does not; such blocking implies that two antigens are close to one another but that there *is* a substantial difference in their disposition. The only example of this so far is θ, which is not affected by blocking with any antibody (column *f*, Table 3), although *anti-*θ blocks TL.2, TL.1, H-2(D), Ly-B, and Ly-A (line 6, Table 3). The least complicated assumption is that the quantity of θ antigen on the cell is greater than that of the antigens affected by θ blocking, which are thought to fringe an extensive θ area (Fig. 2). The fact that θ antibody blocks *several* systems, some of which are not within blocking range of one another, supports this interpretation.

Grouping of antigens specified by nonallelic genes: If we consider only the instances of reciprocal blocking, the antigens form two groups. In the first

TABLE 2. *Location of thymocyte antigens: proximity of alloantigens specified by allelic genes.*

Thymocyte	Specificity of blocking antibody	Specificity of serum	Quantitative Absorption: Reduction in absorption capacity (%)*
(I)† H-2^b/H-2^a	H-2^a(D)	H-2^b(D)	34 } Fig. 1
	H-2^a(D)	*H-2^a(K)*‡	3 } Fig. 1
	H-2^b	H-2^a(K)	37
	H-2^b	*H-2^b(K)*§	>80**
	H-2^a	H-2^b(K)	40
	H-2^a	*H-2^a(K)*§	>80**
(II)† Ly-A.2/Ly-A.1	Ly-A.2	Ly-A.1	40
(II)† Ly-B.2/Ly-B.1	Ly-B.2	Ly-B.1	36
(III)† θ-C3H/θ-AKR	θ-C3H	θ-AKR	44
	θ-C3H	*θ-C3H*§	>80**
	θ-AKR	θ-C3H	40
	θ-AKR	*θ-AKR*§	>80**

Interpretation: With heterozygous thymocytes of all systems tested, H-2(D), H-2(K), Ly-A, Ly-B, and θ, absorption of antibody corresponding to one allele is partially blocked by previous absorption of antibody corresponding to the other allele. In all these systems therefore, antigens specified by allelic genes must lie in close proximity to one another.

* In comparison with absorption capacity of thymocytes incubated in normal mouse serum (standard), as illustrated in Fig. 1.

† (I) = (C57BL/6 × A)F$_1$. (II) = (BALB/c × C3H/An)F$_1$. (III) = (C3H/An × AKR)F$_1$.

‡ Negative tests of this type illustrate that saturation with the first antibody produces no general inhibition of subsequent absorption of other antibodies, such as might be expected if agglutination were a significant factor.

§ Positive controls of this type were frequently included; absorption of the blocking antibody completely inhibits subsequent absorption of the *same* antibody, and this indicates complete saturation.

** No perceptible absorption by highest number of cells tested (5 × minimum number of standard thymocytes showing perceptible absorption).

TABLE 3. *Location of thymocyte antigens: proximity of alloantigens specified by nonallelic genes.*

SPECIFICITY OF BLOCKING ANTIBODY	SPECIFICITY OF SITE TESTED FOR BLOCKING (reduction in absorption capacity %)								
	TL.3	TL.2	TL.1	H-2(D)	Ly-B	θ	Ly-A	H-2(K)	
TL.3		41	0[b]	0	0	0	0	0	1[a]
TL.2	36		42[c]	0[d]	0	0	0	0	2
TL.1	—[e]	—[e]		31[f]	0	0	0	0	3
H-2(D)	0	0	32[c]		28	0	0	0	4
Ly-B	0	0	0	28		0	0	0	5
θ	0	40	31	36	40		24	0	6
Ly-A	0	0	0	0	0	0		30	7
H-2(K)	0	0	0	0[g]	0	0	34		8
	a[a]	b	c	d	e	f	g	h	

Each entry was determined as illustrated in Fig. 1. The use of thymocytes of several different phenotypes was dictated by serological requirements. The order in which the specificities are listed is that implied by the data.

In several instances, the serological details were varied, e.g., entry 4e. First test: absorption of H-2[b](D) antibody by H-2H cells reduced their *anti*-Ly-B.2 absorption capacity by 27.8%. Second test: absorption of H-2[a](D) antibody on A cells reduced their *anti*-Ly-B.2 absorption capacity by 28.0%.

The positive entries are symmetrical about the diagonal (i.e., blocking between pairs is reciprocal) with the exception of θ, which is blocked by none of the antibodies tested (column *f*), although θ antibody blocks several antigens (line 6).

A diagram based on these data appears in Fig. 2.

[a] The lines are numbered, and the columns lettered, to provide a reference system to entries in the table.

[b] Results in the range +5 to −5 are recorded as 0.

[c] Saturation of thymocytes with both *anti*-TL.2 *and anti*-H-2(D) raised the degree of blocking of the TL.1 site to 62%.

[d] Although *anti*-TL.2 did not block H-2(D) on TL.1, 2, 3 thymocytes, it did so on TL.2 thymocytes.

[e] Tests were not feasible because monospecific *anti*-TL.1 was not available.

[f] *Anti*-TL.1 was assumed to be responsible because *anti*-TL.3 did not block H-2(D), whereas *anti*-TL.1, 3 did.

[g] On lymphocytes also, no blocking was observed between H-2(D) and H-2(K), despite the much greater representation of H-2 on lymphocytes as compared with thymocytes, and the lesser representation of all the other antigens listed here.

group are TL.3, TL.2, TL.1, H-2(D), and Ly-B. Their cited order is related to position, for each member of this sequence is within blocking range of its neighbors but not of other members.

Antigens TL.1 and H-2(D) are closely adjacent, a conclusion already suggested by the greater representation of H-2(D) on TL− thymocytes as compared with TL+ thymocytes and by the increase in H-2(D) accompanying antigenic modulation.[5, 7] In this instance, close proximity of antigens (TL.1 and H-2(D))

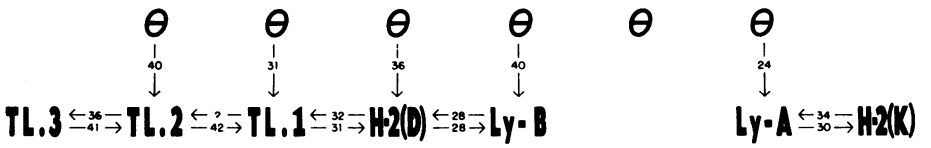

FIG. 2.—Relative positions of TL, H-2, Ly-A, Ly-B, and θ antigens on the thymocyte surface, according to a direct interpretation of Table 3. Indications as to linear order are strong, but at present the two-dimensional relations are obscure. Assumptions involved in plotting θ as shown are discussed in the text.

The figures show the % reduction in absorption capacity for one antibody (specificity indicated by arrowhead) produced by previous saturation with another antibody (specificity indicated by the tail of the arrow). (Data are taken from Table 3.)

parallels genetic linkage (*Tla* : *H-2(D)*) and implies that the genetic sequence is *Tla.3* : *Tla.2* : *Tla.1* : *H-2(D)* to conform with the sequence of antigens indicated by blocking (Fig. 2). But no principle applicable to other loci emerges, for H-2(D) and H-2(K) are not neighbors although specified by linked genes, and conversely, H-2(D) and Ly-B antigens *are* neighbors although specified by unlinked genes (Fig. 2).

The second group of antigens has two members, Ly-A and H-2(K). As *anti-θ* blocks Ly-A but not H-2(K), the sequence is apparently Ly-A, H-2(K) in relation to the first group (Fig. 2), if it is assumed that θ occupies a continuous and generally regular area within the unit of membrane envisaged for purpose of analysis, forming a bridge between the two groups. It is impossible to say how accurate this picture will prove to be, but if it is substantially correct, this leaves none of the test specificities isolated, and TL.3 and H-2(K) occupy peripheral or terminal positions.

Placing H-2(D) and H-2(K) in separate groups is contrary to a natural inclination in favor of a single exclusive space on the cell surface for products of the *H-2* locus, but it seems proper to propose only the simplest map that accords with the blocking data (Fig. 2). Moreover, wide separation of H-2 antigens specified by the two poles of H-2 accords with evidence that *H-2* produces or specifically modifies more than one macromolecule.[1, 8]

Does alternative occupancy occur at certain positions? Two of the loci studied, *Tla* and *H-2*, are compound, i.e., a single chromosome determines TL or H-2 antigens of more than one specificity. In both instances, the arrangement of antigens has a defined orientation: H-2(D) antigens do not compete for H-2(K) positions; TL.1 antigen does not compete for TL.3 positions; etc. Clearly, sites on the cell surface are not allotted at random for the product of any *Tla* structural gene or of any *H-2* gene, but specifically for the product of a segment of the locus, presumably of a *cistron*. Thus the location of TL and H-2 antigens is *gene*-determined rather than *locus*-determined. The alternative, random occupancy of a site assigned to a locus by any product of that locus, is excluded. (TL may be an exception, for TL+ thymocytes have less demonstrable H-2(D) antigen than TL− thymocytes,[7] and this suggests alternative occupancy of some sites by TL or H-2(D), although absence of TL may simply relieve a steric block to the attachment of H-2(D) antibody, which in absorption tests would simulate an actual increase in H-2(D) antigen.)

These data do not bear on whether a particular antigen is a direct (protein) gene product, or an indirect product such as the saccharide determinants of the human AB blood group specificities, whose intermediaries are enzymes specified by the A and B genes.[9] Nor do they indicate whether two or more determinants will be found on the same molecule in soluble preparations of antigens; for TL and H-2(D) are closely adjacent but have been found in different chromatographic fractions, whereas in the same experiments, H-2(D) and H-2(K), more distant from one another, appeared in the same chromatographic fraction.[10] The blocking methods show only the pattern of antigens on the intact membrane.

Although random occupancy of particular sites by *nonallelic* gene products, even of the same locus, is in general excluded, it is plausible that alternative occupancy occurs in the case of *allelic* products. Indeed it seems likely that a heterozygous cell is indifferent to occupancy of an allotted site by the product of one particular allele as opposed to the other, for this is *prima facie* implicit in the fact of allelic variation (cf. Watkin's view of the A-specifying and B-specifying enzymes of human AB heterozygotes as competitors for available carbohydrate substrate). Thus the degree of blocking observed with *allelic* products in heterozygotes may be influenced by alternative occupancy; this would give rise to sites of type *aa*, *aa'*, and *a'a'*, of which only the second would be subject to blocking by either *anti-a* or *anti-a'*.

Distribution of antigens on the cell as a whole: This is not the province of the blocking method but of visual techniques that employ fluorescein or ferritin attached to antibody to mark the cell surface. The former method indicated a "patchy" or "speckled" distribution of H-2 on thymocytes and a more nearly confluent pattern on lymphocytes,[11] which is in keeping with their higher concentration of H-2. If this is confirmed, a number of restrictions are placed on the cellular distribution of *other* isoantigens to conform with the groupings indicated by blocking; e.g., a patchy distribution of Ly-A and Ly-B also would be required, for the blocking data show the major part of these antigens to be not far removed from H-2.

The unit of examination inherent in the blocking method is not the cell but the least area of surface membrane enclosing a single pattern representative of all antigens. On the simplest interpretation of the data so far, alloantigens of the five systems examined comprise a single cluster (Fig. 2), which we take to form this basic unit and which presumably is a repetitive feature of the membrane. Whether it coincides with any "patch" identifiable by visual methods is unknown. Two major technical problems confront us here: first, the lack of well-tried markers other than ferritin that would allow separate visual identification of antibodies with different specificities, and second the difficulty of securing a two-dimensional view of the cell surface with the electron microscope, rather than the essentially one-dimensional view provided by conventional sections. Nothing less than a solution to these shortcomings may be adequate for satisfactory visual confirmation of conclusions reached by the blocking method.

Summary.—Where two cellular antigens of different specificities are situated in close proximity on the cell surface, the absorption of antibody by one of them impedes the subsequent absorption of antibody by the other. This reduction in

absorption capacity is measurable quantitatively and provides a method of mapping the antigenic features of the cell surface. It was applied to alloantigens of five systems represented on thymocytes, H-2, θ, Ly-A, Ly-B, and TL, and their relative positions on the thymocyte surface were thereby established.

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