Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 1351–1355, March 1987
Immunology

Insulin binding to human B lymphoblasts is a function of HLA haplotype

(major histocompatibility complex/cell surface receptors/HLA-A2/HLA-B5)

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Communicated by Christian B. Anfinsen, October 23, 1986

ABSTRACT **A variety of genetic and biochemical evidence points to an association between major histocompatibility complex (MHC) haplotype and several types of cell surface receptors including epidermal growth factor and insulin receptors. We report evidence for such associations between human class I MHC antigens, HLA antigens, and specific insulin binding sites on human B lymphoblasts. We have measured insulin binding to cells of an HLA-heterozygous, Epstein–Barr virus-transformed B-cell line, LCL 721, and to derivative mutants from which all or part of the HLA complex had been deleted. The affinity, K_a, of insulin binding sites is $\approx 10^8\ M^{-1}$ in mutants expressing antigen HLA-B5 together with other HLA antigens and in mutants expressing only HLA-C. HLA-A1; HLA-A1,B8; HLA-A2,C; and HLA null mutants (not expressing any HLA antigens) bind insulin to sites with an affinity of $\approx 10^9\ M^{-1}$.**

Products of the major histocompatibility complex (MHC) have been characterized largely in terms of their antigenicity and their role in immune responses (1, 2). However, the MHC may have other functions than antigen presentation. MHC haplotype affects cell physiology in a number of ways that are apparently unrelated to immune response (3, 4). Although many of these effects may be assigned to known MHC genes, a number of effects cannot readily be explained and imply that the identified MHC antigens function as, or affect the functioning of, cell surface receptors (4–6)—particularly receptors for peptide hormones such as epidermal growth factor and insulin. Recently, immunoprecipitation experiments by several groups have shown that insulin receptors are specifically coprecipitated with MHC class I antigens (6–8), providing good evidence that MHC antigens interact directly with peptide hormone receptors. These studies do not show such interactions in intact cells and were not designed to show either the effects on hormone binding of these interactions or the association of particular MHC antigens with hormone receptors.

A wide range of MHC antigen-loss mutants of the Epstein–Barr virus-transformed human B-cell clone B-LCL 721 has been selected (e.g., 9, 10). These mutants fail to express one or more of the human MHC antigens, HLA antigens. If, as the biochemical results imply, HLA antigens associate with insulin receptors, then we would expect to find differences in HLA binding between different HLA-loss mutants. We have studied the binding of insulin to the parent B-LCL 721 and to 17 mutant cell lines. The K_a values for insulin binding vary 20-fold between parent and mutant cells. Some mutant cells show only one type of low-affinity binding site, whereas others show both high- and low-affinity sites. These insulin binding patterns are associated with the expression of particular HLA class I antigens and are similar for all cell lines expressing the same HLA class I antigens.

MATERIALS AND METHODS

Reagents. Porcine insulin and bovine serum albumin, radioimmunoassay grade, were purchased from Sigma. ^{125}I-labeled insulin at a specific activity of 2200 Ci/mmol (1 Ci = 37 GBq), was purchased from DuPont Biomedical Products (Boston). Phthalate oils for separation of free from cell-bound radioactivity were purchased from Aldrich.

Cell line B-LCL 721 was established by transforming B cells from a normal female donor with Epstein–Barr virus. All mutants used were isolated after γ-ray mutagenesis of cells followed by selection for antigen-loss mutants with complement and appropriate cytotoxic antibody. The origin of B-LCL 721 and the basic procedures for irradiating the cells and isolating mutants have been described (9, 10). Publications describing the origins of the mutants used are cited in Table 1. In the text, mutants derived from B-LCL 721 will be identified by decimal numbers—e.g., .45 for 721.45. Mutants that do not express one or more MHC class I antigens—e.g., A-null, B-null, etc.—were isolated by a second irradiation of cells previously selected for loss of one MHC haplotype and further appropriate immunoselection for loss of part of the remaining haplotype.

The *HLA-C* alleles of B-LCL 721 cells are unknown, because repeated complement-dependent cytoxicity tests with panels of standard typing alloantisera have yielded inconsistent results. Nevertheless, there is evidence that these cells express HLA-C antigens. An HLA-C locus-specific probe (H. T. Orr, personal communication) made from some of the 3′-untranslated region of a cloned gene encoding HLA-Cw3 (16) reacts with mRNA from B-LCL 721 and mutant .45 cells. The probe also reacts with mRNA from the A-null B5$^+$ mutant .144 and the A2$^+$ B-null mutant .53, both of which lack mRNA that reacts with HLA-A locus-specific and HLA-B locus-specific probes (17). Mutant .184, which has homozygous deletions of both the HLA-A and HLA-B loci (11), binds the anti-class I monoclonal antibody W6/32 and contains mRNA that reacts with the HLA-C locus-specific probe (Y.S., B. Koller, D. Nordquist, D. Geraghty, H. T. Orr, and R.D.M., unpublished results).

The HLA-B-null phenotype of mutants .134 and .174 results from a defect in a post-transcriptional step in class I antigen expression (11). In all other mutants, Southern and RNA blotting analyses show that the lack of expression of antigen is due to the absence of either the gene or mRNA encoding the antigen. Mutant .221 is apparently completely deficient in class I mRNA. The expression of β_2 microglobulin genes is not impaired in any of the class I-null mutants

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Abbreviation: MHC, major histocompatibility complex.

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Table 1. Insulin binding by B-LCL 721 B lymphoblasts and by derived mutants

Cell line	HLA phenotype A	B	C	High-affinity insulin binding K_a ($\times 10^{-8}$ M^{-1})	Sites per cell	Reference
				Group I*		
721.181	–	–	C	1.1 ± 0.7	4,900 ± 2500	†
721.184	–	–	C	0.9 ± 0.3	6,000 ± 2000	11
				Group II*		
721.144	–	B5	C	3.3 ± 0.7	1,800 ± 400	12
				Group III*		
721.1	A2	B5	C	1.5 ± 0.4	40,000 ± 1200	9
721.19	A2	B5	C	1.4 ± 0.5	6,000 ± 2000	9, 12
721.38	A2	B5	C	0.7 ± 0.1	48,000 ± 4800	9
721.45.1	A2	B5	C	2.1 ± 0.2	12,000 ± 1200	9, 10, 12
				Group IV		
721	A1	B8	C‡	3.4 ± 1‡§	1,000 ± 250	9
	A2	B5	C			
				Group V		
721.13	A1	B8	C	6.2 ± 2.8	6,000 ± 3600	9
721.114	A1	B8	C	7.4 ± 8.4	2,400 ± 4200	10
721.124	A1	B8	C	19.0 ± 26	600 ± 60	10
721.127	A1	B8	C	4.7 ± 1.2	2,000 ± 500	10, 12
				Group VI		
721.53	A2	–	C	14 ± 9	1,000 ± 700	12, 13
721.134	A2	–	C	8.7 ± 12	700 ± 1000	10, 11
721.174	A2	–	C	24 ± 32	1,600 ± 2000	11, 15, 26
				Group VII		
721.221	–	–	–	10 ± 3.6	1,200 ± 400	†

–, Minus.
*Data are best fit by a one-site model.
†Y. S., B. Koller, D. Nordquist, D. Geraghty, H. T. Orr, and R.D.M., unpublished results.
‡See *Materials and Methods* for discussion of HLA-C in these cells.
§SD.

(ref. 11; and Y.S., B. Koller, D. Nordquist, D. Geraghty, H. T. Orr, and R.D.M., unpublished results).

Cell Culture. Cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 15% (in some cases, 10%) heat-inactivated fetal calf serum (Reheis, Phoenix, AZ). Some cells were cultured in the same medium supplemented with unmodified fetal calf serum.

Insulin Binding Assay. Cells, grown to a density of 3–4 × 10^5 cells per ml, were pooled and washed with Dulbecco's phosphate-buffered saline containing 0.1% bovine serum albumin. They were then incubated in suspension for 1 hr at 37°C in a 5% CO_2 atmosphere in RPMI 1640 medium containing 1% bovine serum albumin, an incubation that dissociates bound insulin from the cells. After incubation cells were suspended to give 1 × 10^6 cells per 100 μl, the highest concentration of cells that could be used without significant degradation of the added tracer insulin. Dilutions of labeled insulin in unlabeled insulin were added to replicate (usually triplicate) samples in a volume of 100 μl. Insulin concentration ranged from 80 pM to 0.1 μM, with one additional sample at 10 μM. The mixtures, in 11 × 75 mm plastic tubes, were incubated at a room temperature of 22°C with intermittent shaking for 90 min. These time and temperature conditions gave maximum insulin binding. After incubation, 160-μl aliquots of the sample were layered over dibutyl phthalate/dioctyl phthalate (1.1:1) in chilled Microfuge tubes. The samples were centrifuged in a Beckman Microfuge model 152 for 3 min to pellet the cells through the oil barrier. Tips of the tubes containing the cell pellets were clipped, and then radioactivity was counted in a γ radiation counter.

Data Analysis. The binding data were analyzed on Scatchard coordinates to estimate affinity and number of insulin receptors. We analyzed all data using the LIGAND program (18). LIGAND fits raw binding data—not requiring subtraction of nonspecific binding that is usually determined in terms of labeled ligand bound in 100-fold molar excess of unlabeled ligand. In this program all data are analyzed and the best polynomial equation is fit to that data.

Data for all cell types were initially fit to a one-site model. Parameters from such fits were used for a fit to a two-site (high- and low-affinity receptors) model. In some instances, even with sufficient data, we could not fit a two-site model to our data. In those cases where the data were fit by both one- and two-site models, two-site models were chosen when they significantly reduced the variance of the fit from that given by a one-site model. Logarithmic transformations of the calculated affinities were statistically compared following the suggestion for transformation and calculation of confidence intervals given by ref. 19. We compared affinities for insulin receptors on different B-LCL 721 mutants using the Duncan new multiple-range test as given by Li (20), an approach we had previously used to compare levels of cyclic AMP in livers of different mouse strains (21).

RESULTS

It has been reported that B-lymphoblasts, but not resting B cells, bear insulin receptors (22). We found that all 18 B-LCL 721-derived lymphoblasts examined did indeed bind insulin specifically, although to various extents. The quality of the insulin receptors also varied from cell to cell. LIGAND fit some cell line binding data best to a two-site model (Fig. 1*A*); other data sets were best fit by a one-site model (Fig. 1*B*). The low-affinity sites found for two-site models had affinities or K_a values in the range from 2.5 to 11 × $10^7 \cdot M^{-1}$. A Duncan new multiple-range test indicated no significant differences between the low-affinity sites of different mutants.

High-affinity insulin binding by groups of B-LCL 721-derived cell lines expressing the same HLA class I antigens is summarized in Fig. 2. Details of binding for each cell line are given in Tables 1 and 2. Only a single class of insulin binding sites could be measured on cells of the parent line, B-LCL 721, and on all mutant lines expressing HLA-B5 (A2 B5 C and – B5 C). Cells of two mutant lines expressing only HLA-C also carry a single type of insulin binding site. K_a values for this site range from 0.7 to 3.4 × $10^8 \cdot M^{-1}$. Two types of insulin binding sites were detected on cells of four A1 B8 C mutant lines (all lacking antigens A2 and B5) as well as on cells of the $A2^+$ $B5^-$ mutants .53, .134, and .174. Mutant .221, which does not even express HLA-C, also bound insulin with high affinity.

Each of the cell lines is an independently derived clone selected for loss of an HLA antigen. Hence, the finding that all cell lines expressing the same HLA antigens bound insulin in the same manner suggests that our results are not due to clonal variation in insulin receptors or independent of HLA. That is, we predict all B-LCL 721 clones expressing the same HLA antigens (no matter how derived) will bind insulin with approximately the same affinity. We further tested this prediction by measuring the insulin binding to drug-resistant clones of cell lines 721.184 and 721.53 and cell lines 721.184TgR4 and 721.53TgR4, which were derived from the original mutants by selection for thioguanine resistance (13). We found that clone 721.184TgR4 has a single class of low-affinity insulin binding sites, as does the parent clone 721.184, and that clone 721.53TgR4 carries both high- and low-affinity binding sites (Table 3). Thus, clones selected for another mutation, such as drug resistance, but expressing the same HLA antigens as their parent cells continue to express the same insulin binding phenotype as those parent cells.

We compared the binding of insulin by different B-LCL 721 cell clones using Duncan's new multiple-range test (20), a test

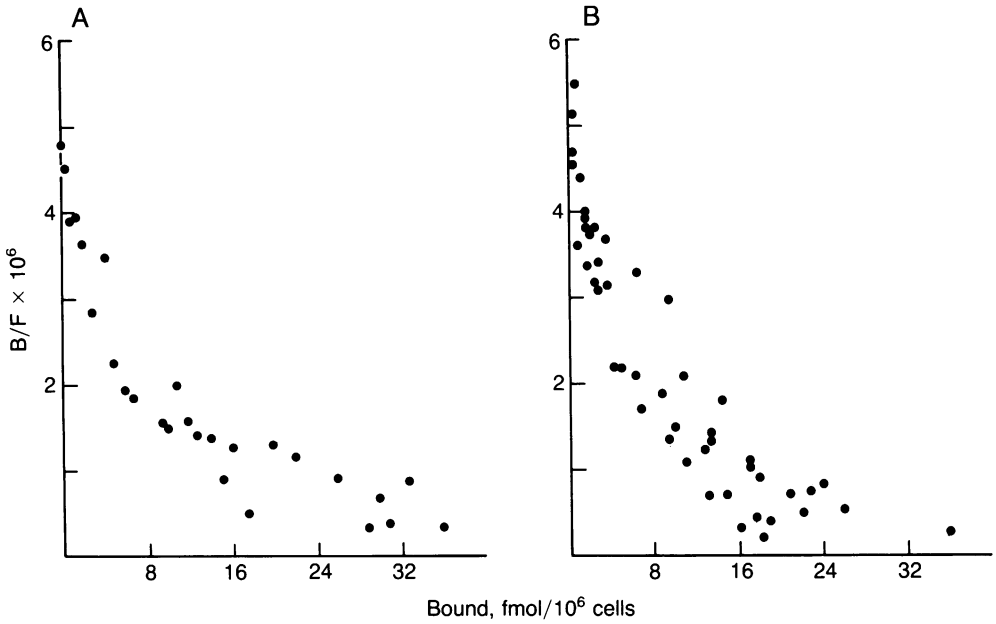

FIG. 1. Scatchard analyses of insulin binding to B-LCL 721-derived lymphoblasts. Nonspecific binding is not subtracted. (*A*) Mutant .174. The data are pooled from three experiments. The high values of bound/free (B/F) insulin extrapolate to a value of ≈6 fmol of insulin bound per 10^6 cells, or ≈3000 molecules bound per cell. LIGAND found the data best fit by a two-site binding model. (*B*) Mutant .45.1. The data are pooled from three experiments. The high values of B/F extrapolate to approximately 0.3 fmol per 10^6 cells, or ≈150 molecules per cell. LIGAND found the data best fit by a one-site binding model.

that allows the comparison of many mean values, each associated with some SEM. Fig. 3 shows K_a values for high-affinity insulin binding grouped by the Duncan new multiple-range test. Each B-LCL 721 mutant listed on the ordinate is represented by a horizontal line. The line length indicates the other B-LCL 721 mutants (listed on the abcissa) with similar K_a values ($P > 0.01$). Two groups of values do not overlap. One of these groups contains all the $A2^+$, $B5^+$ mutants—.45.1, .19, .1, and .38, together with the A-null, B-null, and C^+ lines .181 and .184; the second group contains all other lines.

The number of high-affinity insulin binding sites *R* varied greatly from cell line to cell line. Results of a Duncan new multiple-range analysis are given in Table 2. Values of *R* fall into two major groups differing significantly from each other. One group contains the parent B-LCL 721 and eight cell lines that are *neither* $A2^+$, $B5^+$ *nor* A-null, B-null and are C^+. The second major group contains one $A2^+$, $B5^+$ line (.19), and both A-null, B-null, C^+ lines (mutants .181 and .184). Besides the two main groups, the test defines three more groups, each with one member, all of which carry haplotypes A2 with B5.

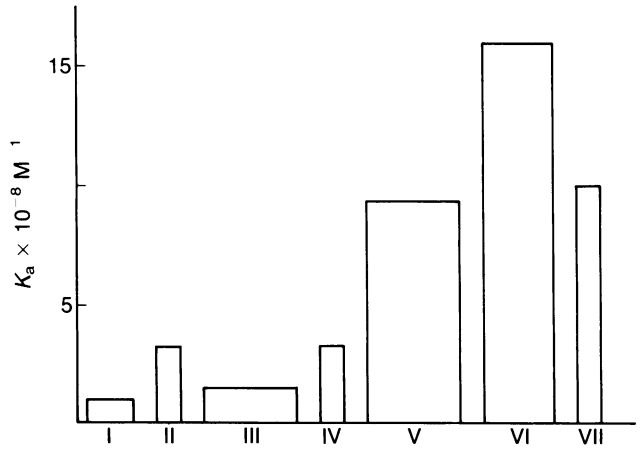

FIG. 2. Insulin binding to B-LCL 721 mutants as a function of class I HLA antigen expression by the mutants. Height of each bar indicates the average affinity, K_a, of insulin binding sites in a group of cell lines with the same HLA phenotype. Bar width indicates the number (1–4) of cell lines in the group. Haplotype constitution of groups are as follows: I, – – C; II, – B5 C; III, A2 B5 C; IV, A2 B5 C and A1 B8 C; V, A1 B8 C; VI, A2 – C; VII, – – –.

These groupings are quite similar to those based on K_a. However, *R* and K_a are not independently determined; hence, this similarity is expected. The values apparently do reflect the actual numbers of high-affinity insulin binding sites, because measurements of the binding of a specific anti-insulin receptor antibody (23) agreed reasonably well with calculated values when binding of the antibody was measured for mutant .45.1 (6000 molecules bound per cell vs. 12,000 sites calculated) and for mutant .53 (1200 molecules bound per cell vs. 1100 sites calculated).

The K_a for insulin binding correlates well with the presence or absence of HLA-B5 on cells. The difference between the A-null, B-null, C^+ cells of mutants .181 and .184 and those that are A-null, B-null, C-null as in mutant .221 suggests that HLA-C in the absence of HLA-A2 is associated with reduced affinity for insulin and with a single type of binding site.

DISCUSSION

Previous studies have shown that B lymphoblasts, but not resting B cells, bear insulin binding sites with affinities of the order of 10^9 M^{-1} (22). Consonant with these observations, we

Table 2. Ranking of cell line HLA haplotypes for number of high-affinity insulin receptors per cell

Cell line	HLA haplotype A	B	C	*R* (receptors per cell)
721.124	A1	B8	C	600 ± 60
721.134	A2	–	C	700 ± 1000
721	A1	B8	C	1,000 ± 250
	A2	B5	C	
721.53	A2	–	C	1,100 ± 700
721.221	–	–	–	1,200 ± 400
721.174	A2	–	C	1,600 ± 2000
721.144	–	B5	C	1,800 ± 400
721.127	A1	B8	C	2,000 ± 500
721.114	A1	B8	C	2,400 ± 4200
721.181	–	–	C	4,900 ± 2500
721.19	A2	B5	C	6,000 ± 2000
721.184	–	–	C	6,000 ± 2000
721.45.1	A2	B5	C	12,000 ± 1200
721.1	A2	B5	C	40,000 ± 1200
721.38	A2	B5	C	48,000 ± 4800

Table 3. Insulin binding by selected B-LCL 721 clones and their drug-resistant mutants

Cell line	HLA phenotype A	B	C	Thioguanine sensitivity	High-affinity insulin binding ($\times 10^8 \cdot M^{-1}$)	Sites per cell
721.53	A2	–	C	Sensitive	14 ± 9	1,100 ± 700
721.53 TgR4	A2	–	C	Resistant	10 ± 4	1,600 ± 300
721.184	–	–	C	Sensitive	0.9 ± 0.3	6,000 ± 1500
721.184 TgR4	–	–	C	Resistant	2 ± 0.3	17,000 ± 1500

find that cells of the Epstein–Barr virus-transformed lymphoblast line B-LCL 721 and HLA mutants derived from these cells bind insulin at sites varying more than 10-fold in affinity. The number of sites per cell ranges from $\approx 10^3$ to $<10^5$. Cells from 9 of the 18 cell lines examined appear to bear a single type of binding site, whereas cells of 9 other cell lines appear to carry both high-affinity and low-affinity insulin binding sites.

The affinity of insulin binding by cell lines derived from B-LCL 721 cells differs as a function of HLA haplotype and antigen expression. The insulin binding affinity does not appear to vary between clones of a single haplotype, even when the time of derivation of these clones differed by several years, or, in the case of cell lines B-LCL 721.184 TgR4 and B-LCL 721.53 TgR4, when clones had been further selected for other mutations.

Different patterns of variation can be derived from the data, but three variational themes seem evident. First, Fig. 3 shows that the cell lines formed two nonoverlapping groups in which the affinity of insulin binding depended strongly on the haplotype that was expressed. Mutants from which only the A1, B8-containing haplotype had been eliminated expressed the A2, B5-containing haplotype and displayed relatively low-affinity insulin binding. The second group contained those mutants from which only the A2, B5-containing haplotype had been eliminated, and these cells displayed relatively high-affinity insulin binding.

Second, the variation in binding of insulin depended more on variations in class I phenotype than on varied expression of class II (i.e., DP, DQ, DR) antigens. For example, within the high-affinity binding group, mutants .174 and .134 have similar class I phenotypes but differ dramatically in class II phenotype: mutant .134 expresses DP2, DQ1, and DR1 class II antigens, whereas mutant .174 lacks expression of any defined class II antigens, because the genes that encode them have been deleted (11, 15, 26).

Third, the high-affinity insulin binding group, which includes those cell lines that are $B5^-$ and express only the A1, B8 haplotype, also includes four B-null mutants—.53, .134, .174, and .221—that were derived by γ-ray mutagenesis and immunoselection from cells that originally contained only the A2, B5 haplotype. Elimination of B5 expression from cells that originally displayed low-affinity binding often was associated with a pronounced increase in the affinity of insulin binding. However, this association was not completely consistent, because mutants .181 and .184 are also HLA-B-null but display low-affinity insulin binding.

In summary, all of the lines that express HLA-B5, either alone or together with other class I antigens, bind insulin with moderate to low affinity, $K_a = 0.7–3.3 \times 10^8\ M^{-1}$, and appear to carry only a single type of binding site. On the other hand, all lines that lack HLA-B5 and are positive for other HLA-A or HLA-B antigens bind insulin with affinities in the range of $4.7–20 \times 10^8\ M^{-1}$ and appear to carry two types of insulin binding sites.

No HLA class I antigens could be detected in cells of line 721.221, but these cells do bind insulin with high affinity, clearly indicating that class I antigens, while affecting the quality and affinity of insulin binding sites, are not [as has been suggested (5, 6)] obligate parts of the insulin receptor. The result with cell line 721.221 also suggests that HLA-C antigens do affect the binding of insulin to its receptor.

The number of high-affinity insulin receptors varies greatly from cell line to cell line. Generally, mutants with lower-affinity receptors appear to have more of these receptors. The calculated numbers of binding sites roughly correlate with the number of receptors measured with a specific anti-insulin receptor antibody (23). Thus, the calculated values are unlikely to be artifacts of the curve-fitting procedure. The reciprocal variation of affinity and receptor number probably is due to the interdependence of values for these two variables. However, some increased receptor levels may reflect selection during cloning for efficient utilization of insulin as a growth factor.

Low-affinity sites were not reported in earlier work on the insulin binding sites of lymphoblasts (22), perhaps due to the limited range of insulin concentrations used to determine receptors. The low-affinity insulin binding sites reported here have values that fall outside the usual range of plasma insulin concentrations, *ca.* $10^7\ M^{-1}$. However, receptors of this

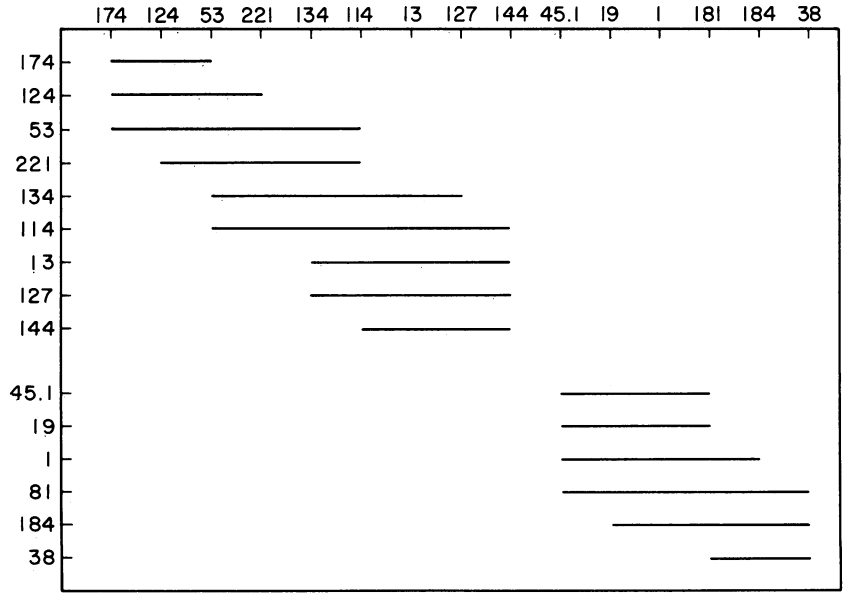

FIG. 3. Duncan new multiple-range analysis of high-affinity insulin binding to B-LCL 721-derived lymphoblasts. The horizontal lines indicate the extent to which the K_a for a given strain, listed on the ordinate, differs from K_a values for the strains listed on the abscissa ($P > 0.01$). K_a of parent B-LCL 721 overlapped both of the analyzed groups.

affinity have been reported to be functional in liver cells treated to eliminate higher-affinity sites (24); thus, they may be functional in B lymphoblasts. The low-affinity sites may also be, in fact, specific for insulin-like growth factors.

Previous work on haplotype influence on hormone binding to mouse liver indicated that H-2 type, and in particular *H-2D* and *H-2K* alleles, affected the affinity of liver membranes for insulin and glucagon (25). In all but one haplotype, glucagon binding to membranes was described by a two-site model. However, one H-2 haplotype, H-2K was associated with single-site binding of lower affinity than seen for all of the other haplotypes. These results are strongly echoed in the present work on insulin binding to lymphoblasts.

The results described here relate to recent demonstrations that some fraction of insulin receptors is specifically coprecipitated with class I and class II MHC antigens (6–8). The coprecipitation experiments show that the receptors and MHC antigens do interact. Our experiments give further evidence for this insulin receptor–antigen interaction and indicate some haplotype constraints at a physiologically important hormone binding site.

We thank Mrs. Taiyin Wei and Mr. Samuel Liu for expert technical assistance. Y.S. is a predoctoral trainee supported by Public Health Service Training Grant GM07133. This work was supported by Grants AI 18914 (M.E.) and AI 15486 (R.D.M.) from the National Institutes of Health. This is paper 2887 from the Laboratory of Genetics of the University of Wisconsin and contribution 1340 from the Department of Biology, The Johns Hopkins University.

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