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Chemical and Immunological Characterization of HL-A-linked B-lymphocyte Alloantigens

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HL-A-linked B-lymphocyte alloantigens are a highly polymorphic system of alloantigens. They are expressed on the surface of B lymphocytes and are encoded by genetic loci in the major histocompatibility complex (MHC) of humans, HL-A (Winchester et al. 1975; Mann et al. 1975a,b; Legrand and Dausset 1975; Barnstable et al., this volume; van Rood et al., this volume). One of these loci is either identical to or in very strong linkage disequilibrium with the HL-A *D* locus, the strongest genetic locus causing mixed lymphocyte culture (MLC) stimulation in man (van Rood et al., this volume). The HL-A-linked B-cell alloantigens are distinct serologically (and chemically, as will be shown below) from the earlier-discovered HL-A A,B antigens. The HL-A A,B antigens are also distinguished by their much wider tissue distribution and by their presence on T as well as B lymphocytes.

The purification of papain-solubilized HL-A-linked B-cell alloantigens has been reported recently (Humphreys et al. 1976). They contain polypeptides of 23,000 (p23) and 30,000 (p30) m.w. The papain-solubilized products have been characterized immunologically but not chemically. Antisera to these products are potent inhibitors of the MLC reaction and precipitate from detergent-solubilized extracts polypeptides of 27,000 and 35,000 m.w. (Cresswell and Geier 1975; Humphreys et al. 1976).

This paper describes the isolation of HL-A-linked B-cell alloantigens in intact form, their subunit structure and immunologic properties, and the chemistry of their subunits. The implications of the homologous subunit structure of these antigens and their similarities to murine and guinea pig immune-associated (Ia) antigens are discussed. Portions of this work will be described in more detail elsewhere (T. A. Springer, J. F. Kaufman, C. Terhorst and J. L. Strominger, in prep.; T. A. Springer, J. F. Kaufman, L. A. Siddoway, D. L. Mann and J. L. Strominger, in prep.).

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METHODS

Gel electrophoresis. One-dimensional sodium dodecyl sulfate (SDS) slab gel electrophoresis (Studier 1973) and two-dimensional SDS gel electrophoresis of cross-linked samples (Wang and Richards 1974) were as described. Two-dimensional electrophoresis, with isoelectric focusing in the first dimension and SDS gel electrophoresis in the second dimension, was by a method previously described (O'Farrell 1975), except that in some cases 10% sucrose was substituted for 8 M urea. Proteins were stained with Coomassie blue (Vesterberg 1971).

Antiserum preparation. Antisera were prepared against SDS gel-purified proteins (Lazarides and Weber 1974), and the modified method will be described in detail elsewhere. Briefly, the lectin-column eluate (see Results) was purified by SDS slab gel electrophoresis; gel slices containing the appropriate proteins were minced, homogenized with Freund's adjuvant, and injected into rabbits, which were bled 6 weeks later; and antisera and preimmune sera were titered against RPMI 4265 cells to determine whether antibodies had been formed.

Cytotoxicity assays. Antisera were tested against B and T lymphocytes purified from peripheral blood (Mann et al. 1975b) and against paired T and B cultured cell lines derived from three individuals (Mann 1975a). Detergent-soluble antigens were preincubated with bovine serum albumin (BSA) to prevent detergent-mediated lysis of target cells (Springer et al. 1974) and then tested for their ability to inhibit in the cytotoxicity assay either rabbit xenoantisera or platelet-absorbed human HL-A-linked B-cell alloantisera (van Rood et al. 1975).

Preparation of peptides for chemical characterization by SDS gel electrophoresis. Material from pools A and B (see below) was combined, alkylated with 3 mM iodoacetic acid, precipitated with five volumes acetone, dissolved in SDS gel buffer, and

heated at 100°C for 5 minutes; proteins were purified by preparative electrophoresis in 0.8 × 22 cm, 10% polyacrylamide pre-eluted tube gels (Weiner et al. 1972). After elution, and in some cases after being completely reduced with 5 mM dithiothrietol (DTT) in SDS and alkylated with an excess of iodoacetic acid, the proteins were chromatographed twice on a 0.9 × 40-cm Sephadex G-50 column equilibrated with 20 mM NH_4HCO_3 to remove SDS and substances which interfered in the Edman degradation, and were lyophilized for amino acid analysis, NH_2-terminal sequencing, and peptide mapping.

Chemical analysis. Amino acid compositions were determined by hydrolyzing samples in 5.7 N HCl, 0.1% 2-mercaptoethanol (ME) for 24, 48, and 72 hours and applying samples at two concentrations to a Beckman 121M analyzer equipped with a microbore single-column system. Sialic acid content was determined as described by Warren (1959). The tryptophan content of proteins denatured in 0.01% SDS was determined using a modified version of the method of Edelhoch (1967). The spectrum of *N*-acetyl tryptophanamide was measured in 0.01% SDS and found to be the same as in 6 M guanidine·HCl, except that it was blue-shifted 1 nm. NH_2-terminal sequencing of 10–30 nmoles of peptides was performed using [^{35}S]-phenylisothiocyanate (New England Nuclear) in an updated Beckman 890B sequencer as described by Terhorst et al. (1976). Phenylthiohydantoins were identified after thin-layer chromatography on polyamide sheets by visual inspection under ultraviolet (UV) light and by autoradiography. Isoleucine and leucine were identified on the amino acid analyzer after back-hydrolysis. Peptides (5 nmoles) were also sequenced using the SDS-Edman-Dansyl technique (Weiner et al. 1972).

RESULTS

Purification

Membranes were prepared from RPMI 4265 lymphoblastoid cells and solubilized with the nonionic detergent Brij 99:Brij 97 (2:1). After high-speed centrifugation, the detergent-soluble supernatant was adsorbed to and eluted from a *Lens culinaris* lectin affinity column (Springer et al. 1974; Springer et al. 1977a). The eluate was filtered on a Bio-Gel A-5m column (Fig. 1), and fractions from the area of the column containing protein were analyzed by SDS gel electrophoresis (Fig. 1, inset) and placed in pools A, B, and C. The material applied to the Bio-Gel A-5m column consisted predominantly of six polypeptides of molecular weights 105,000 (p105), 44,000 (p44), 34,000 (p34), 29,000 (p29), 16,000 (p16), and 12,000 (p12). p105 and p16 were in sharp peaks at 900,000 m.w. (pool A), and p44 and p12, the subunits of HL-A A,B antigens, were in a sharp peak at 400,000 m.w. (pool C), with some aggregated p44, 12 at higher molecular weight. p29 and p34, which are the subunits of the HL-A-linked B-cell alloantigen (see below), chromatographed at an intermediate molecular weight in a broad peak indicative of partial aggregation (pools A and B) and were largely separated from p44, 12. The fact that the ratio of p29 to p34 was constant in all fractions of the column was consistent with noncovalent association between p29 and p34. Preparations were conducted in the presence of 1 mM DTT, as above, and also in the absence of DTT (Strominger et al. 1976a). The latter preparations were somewhat less pure, but p29 and p34 showed a similar elution pattern during Bio-Gel A-5m filtration.

Subunit Structure

After preparation in the absence of DTT, the fractions from Bio-Gel A-5m filtration enriched in p29 and p34 were electrophoresed on SDS gels after incubation with 0.6% SDS at various temperatures, in the presence of iodoacetamide (IAA) or ME (Fig. 2A, B). Comparison of the patterns at different temperatures showed that the HL-A subunits, p44 and p12, were dissociated and electrophoresed identically after incubation at all temperatures. However, p29 and p34 were associated in a complex (p29,34) at an apparent molecular weight of 55,000 in SDS at 21°C or 38°C but were dissociated at 100°C. Interestingly, when IgG was similarly treated with ME and SDS at 21°C, IgG L chains were dissociated, but H chains remained associated noncovalently (at the ME concentration employed [Olins and Edelman 1964]) in an H_2 dimer which calibrated at 90,000 m.w. (Fig. 2D). Since dissociation into p29 and p34 occurred at 100°C in the presence of 10 mM iodoacetamide (Fig. 2A) to prevent disulfide interchange, p29 and p34 were not linked by disulfide bonds. Two-dimensional SDS gels of the same preparation using electrophoresis without reduction in the first dimension, followed by reduction and electrophoresis in the second dimension, also showed that although p44 disulfide-linked dimers were present in these preparations, p29 and p34 were not disulfide-linked (Strominger et al. 1976a; Springer et al. 1977b). The mobilities in reduced gels (Fig. 2B) were different than in unreduced gels (Fig. 2A), particularly in the case of p29. The identity of reduced and unreduced bands was confirmed by cutting out bands from unreduced gels, reducing, and reelectrophoresing them.

To determine the number of p29 and p34 subunits which were noncovalently associated, pool B was cross-linked with a reagent containing a cleavable S-S bond, dimethyldithiobis-(propionimidate), and was electrophoresed on two-dimen-

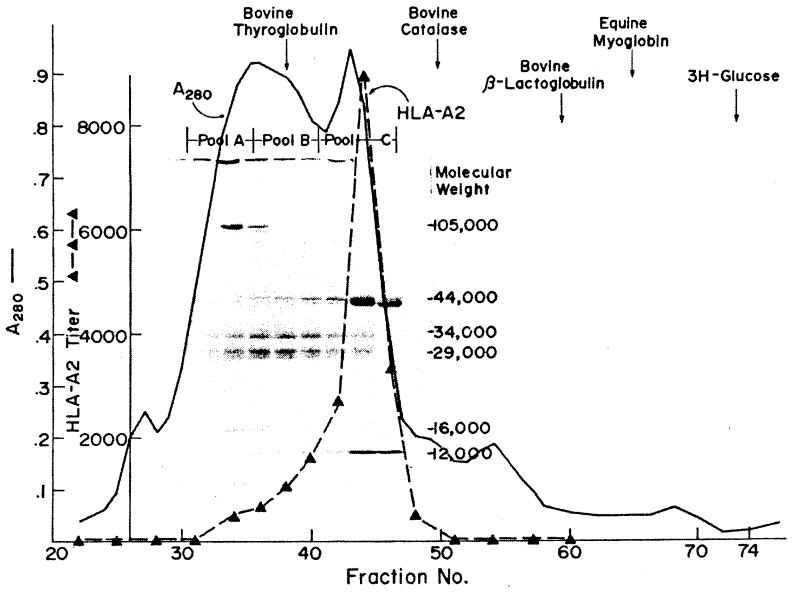

Figure 1. Bio-Gel A-5m filtration and SDS gel electrophoresis profile of p29,34. Bio-Gel A-5m filtration of the lectin column eluate will be described in detail elsewhere (Springer et al. 1977a). Equal volumes of the even-numbered fractions 30–48 were precipitated with 5 volumes of acetone and electrophoresed on 11% polyacrylamide-SDS slab gels, after boiling in 5% ME sample buffer. They are shown aligned with their respective Bio-Gel fractions.

sional SDS gels, unreduced in the first dimension and reduced in the second dimension (Fig. 3) (Wang and Richards 1974). Spots of p29 and p34 were observed vertically aligned with each other below the diagonal of non-cross-linked proteins and calibrated at 64,000 m.w. in the first dimension, showing that they are noncovalently associated in a $(p29)_1(p34)_1$ structure. $(p29)_2$ and $(p34)_2$ dimer molecules were not observed. Since p29 and p34 were better separated from one another in the first, unreduced dimension than in the second, reduced dimension, and separation on SDS gels was linear with log molecular weight in this range, $(p29)_2$ and $(p34)_2$ molecules would have yielded diagonally related spots in the two-dimensional gel. Cross-linking with this reagent was inefficient, but a higher yield of cross-linked p29 and p34 (~ 70%) could be obtained with Lomant's reagent (Lomant and Fairbanks 1976), which was similar to the yield obtained when p44, 12 was cross-linked (T. A. Springer et al. 1977b). The smear of p29 and p34 in the gel towards higher molecular weight in dimension one indicated that some p29,34 was aggregated. This aggregation was overcome in SDS at 21°C (Fig. 2), indicating that aggregates are not held together as tightly as the $(p29)_1(p34)_1$ structure and are probably composed of multiple units of the latter structure. The molecular weight of 55,000 observed for p29,34 in SDS at 21°C was lower than the molecular weight of 64,000 observed for cross-linked p29,34, which had been heated in SDS at 100°C. p29,34 in SDS at 21°C retained antigenic activity (see below) and therefore possessed a folded conformation with electrophoretic properties which would be expected to differ from those of cross-linked, heated, unfolded p29,34.

An association between p29 and p34 was also demonstrated in experiments utilizing isoelectric focusing in dimension one and SDS gel electrophoresis in dimension two (Fig. 4). In Figure 4A and B, focusing was conducted in the absence of urea. When the equilibriated focusing gel was not heated before SDS gel electrophoresis in the sec-

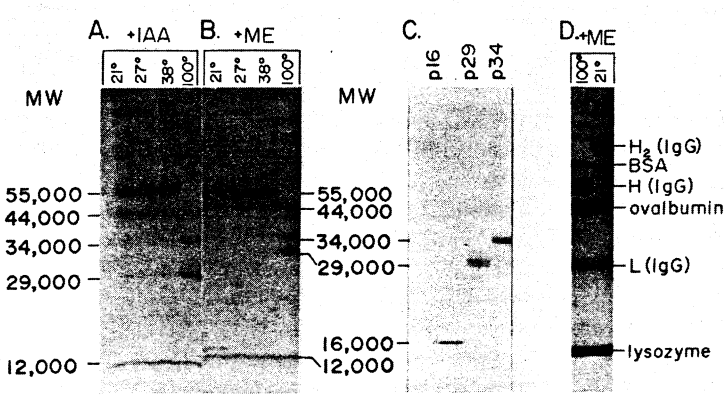

Figure 2. SDS gels of p29,34 preparations after various treatments. *(A)* SDS gel electrophoresis in 10% polyacrylamide slab gels of material purified, in the absence of DTT, by lectin affinity chromatography and gel filtration (Strominger et al. 1976a) after incubation with 0.6% SDS and 10 mM IAA for 30 min at 20°C, 27°C, and 38°C or for 5 min at 100°C. *(B)* Same as above, except incubation was in the presence of 5% ME instead of IAA. *(C)* Two μg of each polypeptide eluted from gels was electrophoresed in an SDS-7–15% acrylamide gradient gel. *(D)* A mixture of standards (IgG, bovine serum albumin [BSA], ovalbumin, and lysozyme) were electrophoresed after treatment as in *B*.

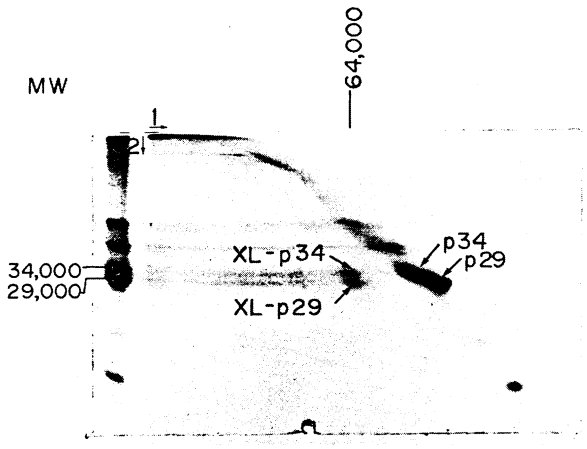

Figure 3. Two-dimensional electrophoresis in SDS gels of cross-linked p29,34. Pool B was dialyzed vs. 50 mM triethanolamine, pH 8.4, cross-linked with 1 mg/ml dimethyl dithiobis-(propionimidate) (Pierce) and electrophoresed in a 5% first-dimensional gel and a 10% second-dimensional gel. A portion of the cross-linked material was also applied to a sample slot in the second dimension to the left of the tube gel. Cross-linked components are designated XL.

ond dimension, p29,34 migrated in a complex calibrating at 55,000 m.w. (Fig. 4A) but, when heated, migrated as dissociated p29 and p34 (Fig. 4B). Although p29 and p34 were somewhat smeared in the focusing dimension, perhaps due partly to aggregation, their positions in the focusing dimension were exactly aligned with one another, and the ratio of stain in p29 and p34 was constant in all positions. p29 and p34 dissociated after focusing were found in the same region in focusing gels as the p29, 34 complex at 55,000 m.w., further demonstrating that the 55,000-m.w. complex is composed of p29 and p34. When samples were focused in denatured form in 8 M urea in the first dimension (Fig. 4C), p29 focused at pI = 6.1 and p34 focused at pI = 5.2. Since p29 and p34 have such different pI's under denaturing conditions, it is unlikely that their association in the same region of the focusing gel under nondenaturing conditions is fortuitous.

Antiserum to p29,34

Since the subunits of p29,34 remain noncovalently associated in the presence of SDS, it was hypothesized that p29,34 in SDS would retain a folded conformation and be active as an antigen. Therefore, the p29,34 complex that had been incubated in SDS at 21°C, as well as dissociated p29 and p34 that had been heated at 100°C in SDS, were purified by preparative SDS gel electrophoresis as described in Methods. Dissociated p29 and p34 did not elicit antibodies detectable by precipitation in gel or by their ability to lyse lymphocytes in any of the three rabbits into which they were injected. However, three rabbits injected with associated p29,34 produced an antiserum lytic for cultured B cells, and the antiserum with the highest titer was studied, unadsorbed, in detail.

This anti-p29,34 serum was tested against three pairs of cell lines with B- or T-lymphocyte character, each pair having been derived from the same individual (Table 1). The antiserum was high-titered (1:500) and had a much greater titer against the B cell line than against the T cell line of each pair. The antiserum was also tested diluted 1:5 against a panel of peripheral B and T cells purified

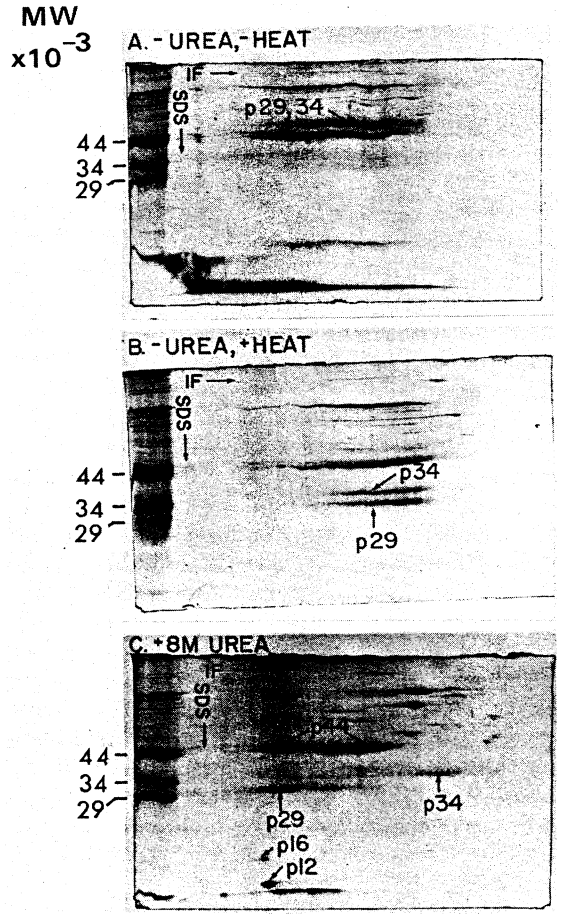

Figure 4. Two-dimensional gels, using isoelectric focusing in dimension one and SDS gel electrophoresis in dimension two, demonstrating association of p29 and p34. The lectin column eluate was separated by isoelectric focusing in dimension one; gels were equilibrated with SDS sample buffer for 30 min, laid atop an SDS slab gel; and SDS gel electrophoresis was conducted in a 10% polyacrylamide gel in dimension two, with the following modifications: *(A)* focusing without urea, plus 10% sucrose; *(B)* same as in *A* except that after equilibration in SDS sample buffer, the tube gel was heated at 80°C for 5 min; *(C)* focusing in 8 M urea. A sample, which had been boiled, was applied to each second-dimension gel in a slot to the left of the isoelectric focusing gel. The alkaline end of the isoelectric focusing gel is to the left.

Table 1. Lysis of B and T Cultured Lymphoblastoid Cells by Anti-p29,34 Serum

Cell line	B- or T-cell character	Titer[a]
8392	B	250
8402	T	10
PA3	B	100
CEM	T	10
SB	B	500
HSB	T	50
4265	B	500

[a] Dilution giving 50% lysis.

Table 2. Lysis of Anti-p29,34 Serum of Panels of Purified, Peripheral B and T Lymphocytes

Target cell	Lysed/total
B	46/48
T	1/48

from 48 individuals (Table 2). It lysed B cells from almost all individuals and thus does not show allospecificity. The antiserum lysed T cells from only one individual, and this reaction may have been due to experimental error. The antiserum is thus entirely B-cell-specific at high dilutions and, at low dilutions, is reactive with T lymphoblastoid cells but not with normal peripheral T lymphocytes.

Immunoreactivity of p29,34

To determine whether subunit association was correlated with antigenic activity, samples of pool B were treated with or without SDS at several temperatures, and aliquots were electrophoresed on SDS gels (Fig. 5) and tested for inhibition of the rabbit anti-p29,34 serum (Fig. 6). During treatment with SDS, a higher concentration of SDS and a lower concentration of the nonionic detergent Brij 99:97 were used, compared with the experiment in Figure 2, in order to provide harsher denaturing conditions. Although material treated with SDS at 21°C retained 50% inhibitory activity and little subunit dissociation occurred, at 80°C inhibitory activity was completely lost and the subunits were completely dissociated. At 57°C, intermediate amounts of subunit dissociation and loss of inhibitory activity occurred. p29,34 was more stable in the absence of SDS and retained full antigenic activity and subunit association after 30 minutes at 57°C. Thus the p29,34 complex retains a conformation in SDS similar to its native state, since antibodies made against it react with the native form of p29,34 on the surface of B lym-

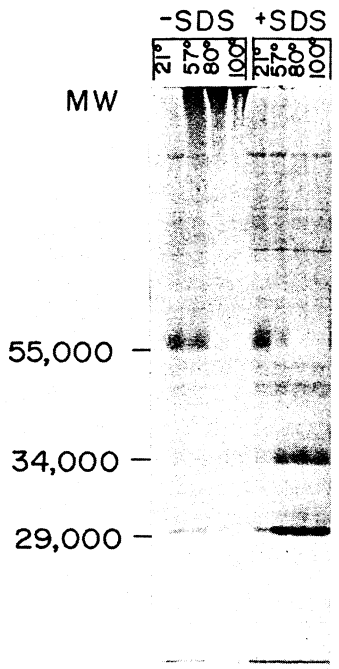

Figure 5. Heat stability of the p29,34 complex, measured by SDS gel electrophoresis. Pool B, which contained 2% Brij 99:97, was concentrated and removed from excess detergent by ion-exchange chromatography as described by Springer and Strominger (1976). Samples were 1.2 mg protein/ml and contained 0.2% Brij 99:97. They were heated at 20°C, 57°C, or 80°C for 30 min, or at 100°C for 5 min, with no additions, or in the presence of 2% SDS or 2% SDS and 2 mM DTT (not shown). Aliquots were withdrawn for assay with anti-p29,34 serum, and, to the remainder, SDS was added to samples not already containing it; to samples not containing DTT, iodoacetamide was added to a final concentration of 50 mM. SDS gel electrophoresis was in 9–13% polyacrylamide slab gels.

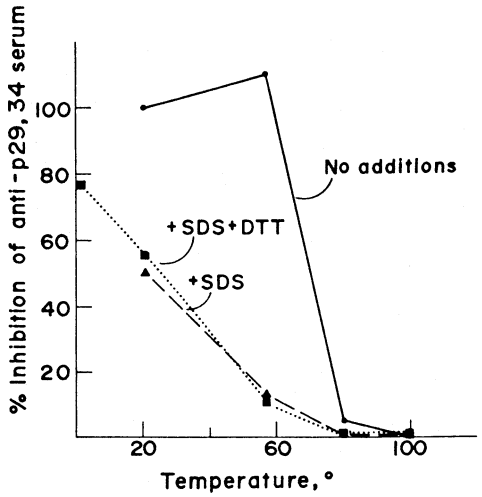

Figure 6. Heat stability of p29,34 measured by inhibition of rabbit anti-p29,34 serum. Samples were heated in the presence of no additions, 2% SDS, or 2% SDS and 2 mM DTT as described in the legend to Fig. 5, and aliquots were withdrawn for the inhibition of cytotoxicity assay using anti-p29,34 serum.

Table 3. Amino-terminal sequences

	Residue 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
p29	Gly	Asp	Thr	Pro			Phe	Leu	Glu	Gln					
p34	Ile	Lys	Glu	Glu		Val	Ile		Gln	Ala	Glu	Phe	Tyr	Leu	Asn
p44[a]	Gly	Ser		Ser	Met	Arg Val	Tyr	Phe	Phe Tyr	Thr	Ala Ser	Val	Ser	Arg	Pro
p12[b]	Ile	Gln	Arg	Thr	Pro	Lys	Ile	Gln	Val	Tyr	Ser	Arg	His	Pro	Ala

[a] HL-A A2 and B7, 12 sequences from Terhorst et al. (1976) and Springer and Strominger (1976).
[b] From Smithies and Poulik (1972).

phocytes, and p29,34 which has been treated with SDS can inhibit this reaction. Upon dissociation in the presence of SDS, the p29,34 subunits lose antigenic activity and presumably undergo drastic conformational changes.

Alloreactivity of p29,34

To demonstrate that p29,34 is indeed the HL-A-linked B-cell alloantigen, pool B from gel filtration, which contained the majority of alloreactive material, was further purified by preparative SDS gel electrophoresis. The band at an apparent molecular weight of 55,000, the p29,34 complex, was eluted and tested for its ability to inhibit alloantisera in the cytotoxicity assay. An HL-A-linked B-cell alloantiserum, Po (van Rood et al. 1975), which is lytic for the cells from which antigen is derived, RPMI 4265, was completely inhibited. The inhibition was specific, since an HL-A-linked B-cell alloantiserum which does not lyse RPMI cells but does lyse IM-1 cells was not inhibited in its ability to lyse IM-1. In addition, an HL-A A2 serum, Davis, which is lytic for RPMI 4265, was not inhibited. Therefore, SDS gel-purified p29,34 contains the Po antigenic determinant (and presumably products allelic to Po) and does not appear contaminated by HL-A A (and presumably HL-A B) antigens.

Chemical Characterization

The subunits of HL-A-linked B-cell alloantigens, p29 and p34, were chemically characterized after dissociation at 100°C in SDS and a final purification by preparative SDS gel electrophoresis. Analytical electrophoresis of the eluted polypeptides (Fig. 2C) showed them to be homogeneous. The peptides were also shown to be homogeneous by NH_2-terminal analysis using the SDS-dansyl technique and by automatic NH_2-terminal sequencing (Table 3). The NH_2-terminal sequences of p29 and p34 were quite different from each other. The sequences of the p44 and p12 subunits of HL-A A, B antigens are shown in Table 3 for comparison. If an insertion was placed between residues 1 and 2 of p29, it would have identical residues with p44 at positions 1 and 8, and many of the others would not be chemically unsimilar. p12 (β_2-microglobulin) and p34 both have isoleucine residues at positions 1 and 7. Therefore, the possibility has not been ruled out that a low degree of homology at the NH_2 terminals might exist between p29,34 and p44, 12, and a decisive answer must await further sequencing.

The amino acid compositions of p29 and p34 were determined (Table 4), and the composition of p44 from detergent-soluble HL-A A,B antigens is included for comparison. The polarities of p29 and p34 are not unusually low and are comparable to that of p44. The mole percent compositions of p29 and p34 were very similar to one another and were also similar, but to a lesser extent, to that of p44. The compositions were compared by the S△Q method (Marchalonis and Weltman 1971) to estimate the degree to which these proteins might

Table 4. Amino Acid and Sialic Acid Composition

	Moles/100 moles			Residues/molecule	
Amino acid	p29	p34	p44[a]	p29	p34
CM-Cys	1.50	1.21	1.31	3.1 (3)	3.0 (3)
Asp	7.87	8.93	8.73	16.2 (16)	22.3 (22)
Thr[b]	6.70	6.65	6.87	13.8 (14)	16.6 (17)
Ser[b]	6.87	5.32	6.77	14.2 (14)	13.2 (13)
Glu	12.62	11.61	12.65	26.0 (26)	29.0 (29)
Pro	4.82	6.26	5.06	9.9 (10)	15.6 (16)
Gly	7.93	6.89	7.67	16.4 (16)	17.2 (17)
Ala	4.85	5.27	8.32	10.0 (10)	13.1 (13)
Val[c]	8.14	8.48	6.19	16.8 (17)	21.1 (21)
Met	1.52	1.46	1.51	3.1 (3)	3.6 (4)
Ile[c]	3.33	4.89	3.45	6.9 (7)	12.2 (12)
Leu	8.56	9.17	6.94	17.7 (18)	22.9 (23)
Tyr	3.76	2.73	4.32	7.8 (8)	6.8 (7)
Phe	5.31	5.98	3.06	10.9 (11)	14.9 (15)
His	2.83	2.70	2.91	5.8 (6)	6.7 (7)
Lys	3.88	4.45	3.87	8.0 (8)	11.1 (11)
Arg	7.01	5.56	7.19	14.5 (14)	13.8 (14)
Trp	2.51	2.44	3.2	5.2 (5)	6.1 (6)
Polarity[d]	47.8	45.3	49.0		
Sialic acid				2.2	2.6

Values in parentheses are rounded to the nearest integer.
[a] From Springer and Strominger (1976).
[b] Extrapolated to zero time values.
[c] 72-hr values.
[d] Calculated according to the method of Capaldi and Vanderkooi (1972).

Table 5. SΔQ Values

	p12	p29	p34	p44
p29	103			
p34	94	16		
p44	225	27	43	
Erythrocyte[a] membrane	159	41	28	44

[a] Lipid-extracted human erythrocyte membrane protein (Rosenberg and Guidotti 1968).

be evolutionarily related (Table 5). SΔQ values lower than 50 have been taken to be indicative of sequence homology. p29 and p34 were highly related by this method, with an SΔQ = 16, and were as similar as HL-A A2 is to HL-A B7, which also have an SΔQ = 16 (Strominger et al. 1976b). p44 was also indicated to be related to p29 and p34, with SΔQ values of 27 and 43, respectively. However, we do not consider the latter values of 27 and 43 to be significant, since similar values were obtained in comparisons to human erythrocyte whole membrane protein (Rosenberg and Guidotti 1968). p12 was not related to the other proteins by this method.

The sialic acid content (Table 4) was 2.2 and 2.6 moles/mole for p29 and p34, respectively, and thus they are both sialoglycoproteins with amounts of sialic acid similar to that of HL-A A,B antigen heavy chains (Terhorst et al. 1976). Staining of SDS gels with periodic acid-Schiff reagent (PAS) also showed that p29 and p34 were glycoproteins. Since glycan moieties which have affinity for the *L. culinaris* lectin are generally linked by a glycosylamine bond to asparagine and are branched oligosaccharides with galactose-*N*-acetylglucosamine sequences in the outer chains and mannose residues in the core (Kornfeld et al. 1971), the carbohydrate compositions of p29 and p34 are suggested to be similar to that of p44 (Terhorst et al. 1976).

Since p29 and p34 are glycoproteins, their peptide molecular weights were probably lower than estimated by SDS gel electrophoresis. They were assumed to be approximately 24,000 and 29,000, respectively, or 5000 lower than estimated by SDS gel electrophoresis, as is also the case for the heavy chain of HL-A A, B antigens (Terhorst et al. 1976). With these molecular weights as a starting point, the peptide molecular weights of p29, p34, and p16 were calculated from the amino acid compositions (Boyer et al. 1973) and were found to be 23,600, 28,400, and 15,600, respectively. This is in good agreement with the above SDS gel electrophoresis estimates, and the latter molecular weights were used for calculating amino acid composition in residues per molecule (Table 4). Although p29 is of lower molecular weight than p34, it contained one more residue than p34 of two amino acids, serine and tyrosine. Three cysteines were found in p29 and p34. p29 and p34 that had not been totally reduced and alkylated, but which had been mildly reduced and alkylated before addition of SDS, were also analyzed and were found to contain 0.8 and 1.0 residue of CM-cysteine per molecule, respectively. These may be free sulfhydryls, or, alternatively, a portion of the molecules may have been denatured, leading to easy reduction of their disulfides. The amino acid analysis results establish that both p29 and p34 contain at least two intrachain half-cysteines or two sulfhydryls which are deeply buried and resistant to alkylation. Since the mobility of p29 in SDS gels (Fig. 2) is faster when unreduced (it calibrates at an apparent molecular weight of 25,000), it must contain a disulfide bridge rather than resistant sulfhydryls.

Although limited NH_2-terminal sequencing did not reveal homology between p29, p34, and p44, there was no reason to suspect that homologies, if they exist between these polypeptides of dissimilar size, would necessarily begin at the NH_2 terminus. Furthermore, the amino acid compositions suggested homologies, and therefore these proteins were compared by peptide mapping. Carboxymethylated p29 and p34, as well as p44, all from cell line RPMI 4265, were labeled with ^{125}I after heating at 100°C in the presence of SDS and, after removal of SDS, were digested with trypsin. As a control for artifactual spots due to free iodine, another protein from the lectin column eluate, p16, was also analyzed by peptide mapping. Comparison of the ^{125}I-tyrosyl tryptic peptides in one dimension (chromatography) (Fig. 7A) showed that p29 and p34 have an almost identical peptide pattern. Spots due to free iodine, present in all samples, migrated close to the solvent front in one-dimensional maps and moved out of the area shown on the two-dimensional maps. Two-dimensional maps (Fig. 7B) showed that p29 and p34 share six major tyrosyl tryptic peptides. p29 and p34 also contain unique peptides. The number of peptides observed agreed well with the amino acid analysis results showing that p29 and p34 contain eight and seven tyrosines, respectively. The high proportion of shared tyrosyl tryptic peptides shows that p29 and p34 have highly homologous structures. If the tyrosyl tryptic peptides are representative of the remaining three-quarters of the tryptic peptides, p29 and p34 would be identical throughout most of their sequences. p44 contains many unique peptides. It also contains one peptide in the same position as a peptide of both p29 and p34. These three peptides were also confirmed to have identical mobilities during electrophoresis in one dimension (data not shown). The comigration of a p44 peptide with peptides from p29 and p34 suggests a low degree of homology between p44 and the latter proteins. However, a firm conclusion must await further characterization of this peptide. p29 and p34 could not be proteolytic products of p44 since four peptides not present in p44 were found in p29 and p34.

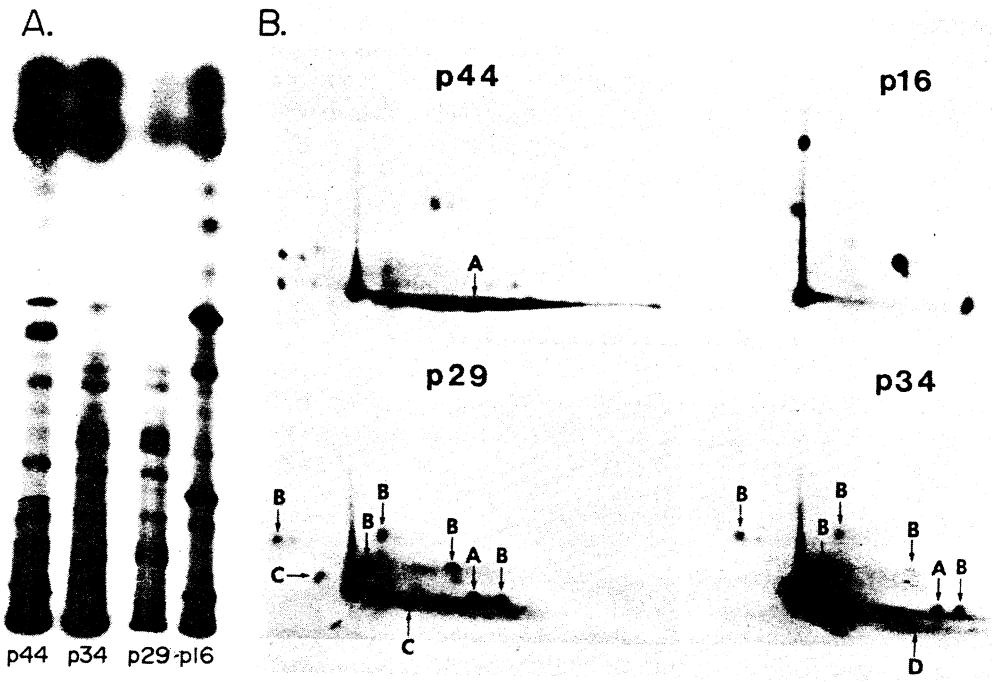

Figure 7. [^{125}I]tyrosine-labeled tryptic peptide maps of p16, p29, p34, and p44. Peptides were labeled with ^{125}I in the presence of SDS, digested with trypsin, and separated on silica gel G (Yen et al. 1976). HL-A antigens were purified (Springer et al. 1977a), and p44 was separated from p12 by preparative SDS gel electrophoresis. *(A)* Chromatography dimension only. *(B)* Chromatography (upward) followed by electrophoresis (anode to right). The origin is the intensely radioactive spot near the lower left corner. Labeled spots: A represents peptides common to p29, p34, and p44; B represents peptides common to p29 and p34; C represents peptides unique to p29; and D represents peptide unique to p34.

Several pieces of evidence showed that p29 was not a proteolytic breakdown product of p34. p29 contained one more serine and tyrosine than p34 and two unique tyrosine tryptic peptides. In addition, p34 contained a tyrosine residue at position 13 (Table 1), and therefore proteolytic cleavage from the NH_2 terminal would have produced a product containing *two* fewer tyrosines than p29. Proteolysis of p29 and p34 during preparation or storage was never noted, and the ratio of the two polypeptides always remained constant, as determined by SDS gel electrophoresis. Equimolar amounts of p29 and p34 were recovered after preparative SDS gel electrophoresis. Proteolysis would have been expected to result in a mixture of molecules of $(p34)_2$, $(p29)_2$, and $(p29)_1$ $(p34)_1$ structures, but only molecules of the last structure were found in cross-linking and in two-dimensional isoelectric focusing and SDS gel electrophoresis experiments. p29 and p34 also seemed unique, as determined by papain digestion (see below), since one polypeptide was converted to p23 and the other to p30. Therefore, p29 and p34 appear to be the products of duplicated genes.

DISCUSSION

The properties of detergent-solubilized HL-A-linked B-cell alloantigens may be compared to those of the papain-solubilized form of these molecules. The latter contain polypeptides of 23,000 m.w. (p23) and 30,000 m.w. (p30), inhibit HL-A-linked B-cell alloantisera, and elicit a B-cell-specific antiserum in rabbits that can inhibit MLC reactions (Humphreys et al. 1976). Preliminary experiments have shown that p29,34 is essentially totally immunologically cross-reactive with p23,30 (J. Kaufman, unpubl.) and that p29, 34 is degraded by papain to p23,30 (A. DeFranco, unpubl.). Cresswell and Geier (1975) used papain-solubilized material to prepare a B-cell-specific antiserum which was a potent inhibitor of the MLC reaction. When tested with detergent-solubilized material, this antiserum precipitated polypeptides of molecular weights very similar to those of p29, 34. The same polypeptides have also been identified by Snary et al. (this volume) as the components of HL-A-linked B-cell alloantigens. As yet, little is known about the chemistry of the papain-solubilized products. It will be of interest to determine whether both p29 and p34 are anchored in the membrane, and whether their membrane orientation and integration are similar to that of the p44 subunit of HL-A A,B antigens (Springer and Strominger 1976).

The subunit association between p29 and p34 and the alloantigenic determinant of p29,34 were unexpectedly stable to SDS at room temperature. Most proteins would be denatured under these conditions, including HL-A A,B antigens. A few

proteins, including the influenza virus hemagglutinin (Skehel and Waterfield 1975), are stable to SDS treatment. This stability allowed preparative SDS gel electrophoresis to be used as the last step in p29,34 purifications. This antigen elicited in rabbits a high-titered, B-cell-specific antiserum (anti-p29, 34), which exhibited a pattern of reactivity toward human leukemias (L. Chess, pers. comm.) identical to that observed for anti-p23, 30 (Schlossman et al. 1976). A finding of major clinical importance was that two cytologically indistinguishable forms of acute lymphoblastic leukemia can be distinguished with B-cell alloantigen serological reagents such as anti-p23, 30 (Fu et al. 1975; Schlossman et al. 1976). p29,34 can be readily obtained in highly pure form and is thus well suited for preparation of antisera. These antisera should be of great use both in identifying cell-surface markers and in functional studies.

Chemical characterization has led to an overall view of the structure of HL-A-linked B-cell alloantigens. They are composed of two, tightly noncovalently associated sialoglycoprotein subunits of 29,000 and 34,000 m.w. p29 and p34 have different NH_2 terminals but in other regions have highly homologous sequences. It will be interesting to determine whether the strong noncovalent interactions between p29 and p34 occur between their homologous regions.

The large extent of homology between p29 and p34, which cannot be explained by proteolysis, appears to be the result of a gene duplication event. A gene duplication event in the MHC has also given rise to the HL-A A and HL-A B antigens (Terhorst et al. 1976). Although the initial event generating separate p29 and p34 genes may have occurred in the MHC, one of these genes might have been subsequently translocated to a different chromosome. Homologous protein subunits are often found to be specified by unlinked genes, as is the case for immunoglobulin L and H chains (Gally and Edelman 1972) and hemoglobin α and non-α chains (Kabat and Koler 1975). On the other hand, more than one gene for B-cell alloantigens is contained in HL-A (Mann et al. 1976; van Rood et al., this volume). It therefore is important to establish which of the p29, 34 subunits bears the Po alloantigenic determinant and whether the other subunit also bears a determinant specified in HL-A.

The structure of HL-A-linked B-cell alloantigens appears similar to those of mouse and guinea pig Ia antigens (Cullen et al. 1974; Silver et al., this volume; Schwartz et al., this volume). Moreover, the sequence presented by Schwartz et al. (this volume) for the smaller chain of guinea pig Ia antigens is identical to that of human p29 in three out of the seven positions for which information is available, suggesting that these polypeptides are homologous. Such homologies have been demonstrated among the classical, serologically defined histocompatibility antigens of mouse, guinea pig, and human (see table compiled by Silver, this volume).

In addition to their biochemical similarities, murine Ia antigens and human HL-A-linked B-cell alloantigens share the following properties: (1) Their genes are in the MHC and are in intimate association with MLC genes. (2) Antisera to these products are potent inhibitors of the MLC reaction. (3) They have similar tissue distributions, being strongly expressed on B lymphocytes, weakly expressed on T lymphocytes, and absent from most if not all nonlymphoid tissues (Shreffler and David 1975; Cresswell and Geier 1975; Schlossman et al. 1976; Humphreys et al. 1976).

Little is known about the biological function of HL-A-linked B-cell alloantigens. However, it is increasingly evident that many products of the MHC function in the immune system. The finding that HL-A-linked B-cell alloantigens are composed of homologous subunits might be a key to understanding their function. In other proteins with homologous subunit structure (immunoglobulins, hemoglobins, and isozymes), this type of structure has functional implications. These proteins contain interchangeable subunits specified by sets of duplicated and subsequently mutated genes, and their functional specificity can be modulated by controlling which genes of each set of duplicated genes are expressed.

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