Proc. Nat. Acad. Sci. USA
Vol. 71, No. 10, pp. 3998–4001, October 1974

Heterogeneity of HL-A Antigen Preparations Is Due to Variable Sialic Acid Content

(histocompatibility/neuraminidase/isoelectric focusing/carbohydrate/papain)

PETER PARHAM, ROBERT E. HUMPHREYS, MERVYN J. TURNER, AND JACK L. STROMINGER

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Contributed by Jack L. Strominger, July 1, 1974

ABSTRACT **Purified, papain-solubilized preparations of HL-A antigen from cultured human lymphoblastoid cells (HL-A2 and HL-A7,12 from RPMI 4265 cells and a mixture of HL-A3, W-25, HL-A12, and HL-A27 from IM-1 cells) show substantial charge heterogeneity in isoelectric focusing gels. This heterogeneity can be ascribed largely to variable numbers of sialic acid residues on each molecule. Neuraminidase (EC 3.2.1.18) treatment of the HL-A antigens as a function of time altered the band patterns in a manner demonstrating that up to three sialic acid residues are present on both first locus ("LA") and second locus ("Four") antigens. Neuraminidase treatment did not alter the specificity or specific activity of the purified antigens.**

The major serologically defined histocompatibility antigens, called HL-A and H-2 in man and in the mouse, respectively, are cell surface products of genes in a chromosomal region governing several modes of immunologic function. This region is bounded by the two genes that determine the serologically defined antigens. They are conveniently called the first and second loci, but are often referred to as the "LA" and "Four" loci in the HL-A system of man or "K" and "D" loci in the H-2 system of the mouse. Because the products of these genes are expressed codominantly, four different antigenic specificities may be found in an individual (1). More than 16 alleles have been defined serologically at each locus in man, and greater than 40 alleles at each locus in the mouse. This high degree of polymorphism is probably intrinsic to the function of these antigens. Their function in relation to graft rejection or immune surveillance is not yet clear.

HL-A antigens released from cell membranes with papain have been purified to homogeneity (as defined by sodium dodecyl sulfate or alkaline buffer gel electrophoresis) from human spleen cells (2) and from cultured human lymphoblastoid cells (3, *). These antigens have been shown to contain two polypeptide chains, one of 34,000 daltons and the other of 12,000 daltons (3, *). The smaller, carbohydrate-free polypeptide has been shown to be β_2-microglobulin, which has amino-acid sequence homology to the C_H3 domain of IgG (refs. 4–7 and earlier references cited therein). The sequence of the larger glycopeptide has not been determined because preparative quantities of immunologically pure antigens have not been separated from the mixtures of papain-derived antigens. This separation of immunologic specificities by biochemical techniques has been impeded by charge heterogeneity of the 34,000-dalton glycopeptide. This heterogeneity has been demonstrated by isoelectric focusing and isotachophoresis, and by unusually broad peaks of antigen on DEAE-cellulose chromatography (*, 8, 9). Charge heterogeneity could result from several kinds of molecular variations: (*1*) microheterogeneity of amino-acid sequence as a result of fusion of a constant region containing an allotypic HL-A determinant with a variable region, as occurs in the immunoglobulins, (*2*) microheterogeneity of amino-acid sequence due to gene duplication with relatively minor evolutionary diversion, (*3*) variable amidation of side chain carboxyl groups, (*4*) "frayed ends" and variably excised peptides caused by papain or autolytic enzymes during growth of the cells and isolation of the HL-A antigens, and (*5*) differences in sialic acid content of the side chain carbohydrate.

This paper reports that the electrophoretic heterogeneity of the glycopeptide of papain-solubilized HL-A is due largely to its variable sialic acid content.

MATERIALS AND METHODS

HL-A antigens were prepared as described (3, *, †). The three preparations used in this study were HL-A2 and a mixture of HL-A7 and HL-A12 from the cell line RPMI 4265, and a mixture of four specificities, HL-A3, W-25, HL-A12, and HL-A27, from the IM-1 cell line. Antigenic activity was assayed by inhibition of cytotoxicity, using the ^{51}Cr release method (10, 11) adapted to a microtiter plate (†). The following antisera were used: BC (HL-A3), DAL(HL-A27), and Davis 67 (HL-A2) were a gift from Dr. Bernard Amos. BEL(HL-A27), SLA (HL-A12), FS (HL-A12), EVA (W-25), and MS (HL-A10) were a gift from Dr. Tom Fuller. SAN (HL-A7) was the gift of Dr. Arnold Sanderson. Jackson (HL-A7) was provided by the Transplantation Immunology Branch of the National Institute of Allergy and Infectious Diseases.

Treatment of HL-A Antigens with Neuraminidase. Neuraminidase (EC 3.2.1.18; acylneuraminyl hydrolase) was purchased from Behring Diagnostics and shown to be protease free, as determined by the release of trichloroacetic acid-soluble counts from ^{125}I-labeled casein. In time course experiments, 5 aliquots of HL-A antigen (10–20 μg each) in 25 μl of 50 mM acetate buffer (pH 5.5), containing 20 mg/ml

* Turner, M. J., Cresswell, P., Parham, P., Mann, D. L., Sanderson, A. R. & Strominger, J. L. (1974) *J. Biol. Chem.*, submitted.

† Humphreys, R. E., Gonzalez-Porque, P., Mann, D. L. & Strominger, J. L. (1974) *Transplantation*, submitted.

TABLE 1. *Specific activities of various HL-A preparations, before and after treatment with neuraminidase*

HL-A preparation	Neuraminidase treatment	HL-A specificity					
		HL-A2	HL-A3	W-25	HL-A12	HL-A27	HL-A7
HL-A2	Before	120*	0				
	After	90	0				
HL-A7, 12	Before		1	0	2	100	100
	After		1	0	2	150	150
HL-A3, W-25; A-12, A-27	Before		100	6	2	400	0
	After		150	9	3	600	1

* HL-A activity in units/mg $\times 10^{-3}$. None of the changes shown is significant.

of NaCl and 1 mg/ml of $CaCl_2$, were incubated at 37° in silanized tubes. To 1 aliquot was added 5 μl of buffer and to each of the rest, 5 μl (2.5 international units) of neuraminidase solution. Reactions were stopped by addition of 9 M urea to bring the total urea concentration in the sample to 6 M. As each successive tube was removed from the incubation, a further 5 μl of neuraminidase was added to each of the remaining tubes. Incubation times were 0.5, 1, 2, and 4 hr, and the control tube was removed with the final digestion tube. Bromophenol Blue (1 μl of 0.05%) was added to each sample, and they were run on pH 5–7 isoelectric focusing gels containing 6 M urea (see below). The 4-hr incubation, with a final ratio of 1 unit of enzyme to 1 μg of HL-A antigen, was found to give the maximal change in the banding pattern of HL-A antigen. In experiments to measure the cytotoxic inhibitory activity after treatment with neuraminidase, a 4-hr digestion was used with 1 unit of neuraminidase per μg of HL-A antigen being added initially and an equal quantity being added after 2 hr.

Isoelectric Focusing. Analytical isoelectric focusing was performed in 7.5% polyacrylamide gels, 10 cm in length, according to the method of Wrigley (12). Ampholines (LKB Produkter), pH ranges 5–7 and 4–6, were used either in the presence or absence of 6 M urea. Gels were run at 400 v for 16–20 hr at 4° and then fixed with two changes of 10% trichloroacetic acid–7.5% sulfosalicylic acid for 1 hr at 55°, stained in 0.1% Coomassie blue in 25% methanol–7.5% acetic acid. Gels assayed for HL-A antigenic activity were cut into 3-mm slices and eluted into 50 μl of 20 mM Tris buffer (pH 8.1). The pH gradients were determined by elution of 5-mm slices of a blank gel into 1 ml of water.

RESULTS

Activity of Neuraminidase-Treated HL-A Antigens. HL-A antigens from both cell line RPMI 4265 (preparations of HL-A2 and a mixture of HL-A7,12) and cell line IM-1 (a mixture of HL-A3, HL-A12, W-25, and HL-A27) were treated with neuraminidase as a function of time and amount of neuraminidase. No change in specific activity from untreated antigen was observed (Table 1). Low inhibition by the specificity controls (HL-A3 for antigens from 4265 cells and HL-A7 for IM-1 antigens) also remained constant. Thus, removal of sialic acid groups did not alter the specificity or specific activity of the HL-A antigen. Increased levels of lysis of cells at low dilutions of neuraminidase-treated HL-A antigens was demonstrated to be due to increased nonspecific lysis of cell by neuraminidase, as reported by Grothaus *et al.* (13).

Patterns of Antigen in Urea Isoelectric Focusing Gels on Treatment with Neuraminidase. Treatment of purified HL-A2 with neuraminidase as a function of time and increasing amounts of enzyme are shown in Fig. 1. The untreated antigen (gel A) shows, in addition to the β_2-microglobulin band at pI 6.4, three other bands in the pI range 6.1–6.3. On treatment with neuraminidase (gels B–E), the β_2-microglobulin band remained constant but a shifting of stained material was seen among the other bands. Stainable material moved from lower to higher pI values, as would be expected if successive sialic residues were being removed; after 4 hr most of the stainable material was present in a new band at pI 6.35. The series of 4 distinct bands probably corresponds to HL-A molecules having 0, 1, 2, or 3 terminal sialic acid residues. The original HL-A2 antigen preparation had an analytical value of 2.1 sialic acid residues per mole. Thus, the major band in gel A probably contains two sialic acid residues, and the two minor bands, one and three residues.

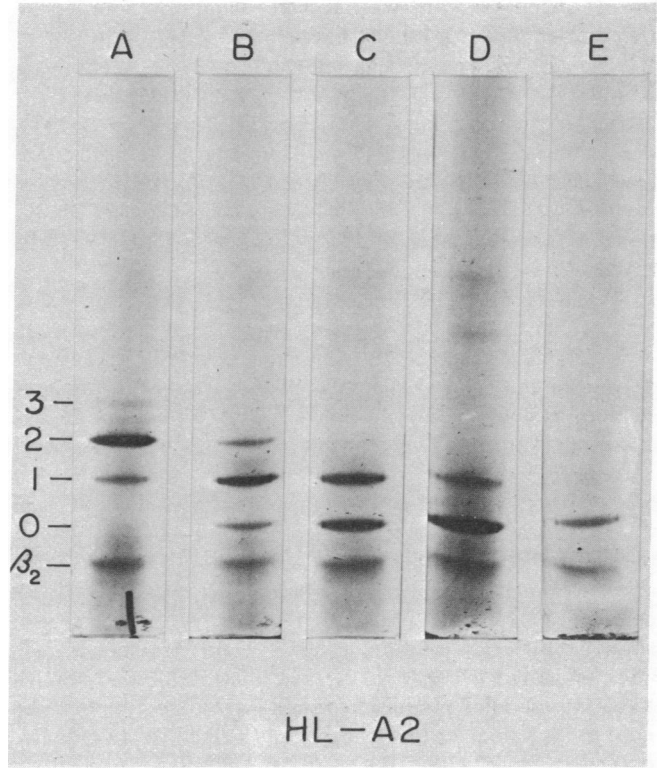

FIG. 1. Isoelectric focusing gels in 6 M urea of preparations of HL-A2 antigen treated with neuraminidase (see *text* for details).

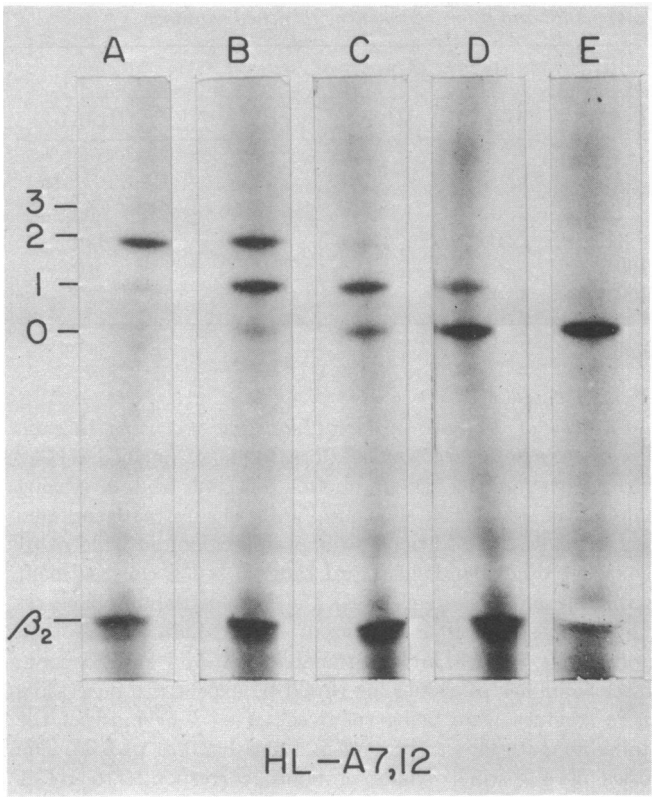

FIG. 2. Isoelectric focusing gels in 6 M urea of preparations of HL-A7,12 antigen treated with neuraminidase.

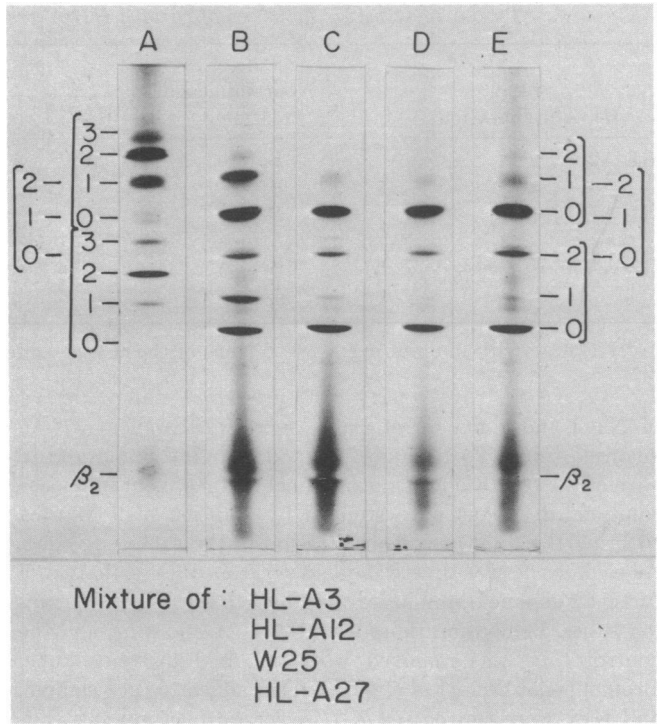

FIG. 3. Isoelectric focusing gels in 6 M urea of preparations of HL-A3, W-25, HL-A12, and HL-A27 antigens treated with neuraminidase.

A parallel experiment with the preparation of HL-A7,12 is shown in Fig. 2. The pattern is analogous to that seen with HL-A2, except that the original three bands (in addition to β_2-microglobulin) are all more acidic (pI 5.1–5.3). A single band at pI 5.35 remained after exhaustive treatment with neuraminidase (gel E). This result suggests that the HL-A antigen molecules having specificities 7 and 12 either have identical isoelectric points or are present in such unequal concentrations that only one of them was seen on the gel. If the former, more likely explanation is correct, then it explains the considerable difficulty experienced in efforts to separate these two specificities.

Treatment of the HL-A antigen preparation from IM-1 cells (which contain four specificities) with neuraminidase is shown in Fig. 3. Here the situation was more complex, although the same patterns of behavior occurred. The β_2-microglobulin band (pI 6.4) remained constant in position and intensity. The limit product of neuraminidase treatment (gel E) revealed only three additional bands at pI 5.1, 5.35, and 5.7. It seems likely that the intermediate band at pI 5.35 was HL-A12, since it corresponded in position to the band seen in the limit product of treatment of the HL-A7,12 preparation from RPMI 4265 cells (Fig. 2). The band pattern in gels A–E can be interpreted as representing three series of stainable materials containing 3, 2, 1, and 0 sialic acid residues (as suggested in Fig. 3). The most intensely staining series, whose limit product has the lowest pI, 5.1, and which appears as a doublet in some gels, most likely represents two of the three specificities HL-A3, W-25, and HL-A27.

When IM-1 antigens were eluted from isoelectric focusing gels run in the presence of 6 M urea, less than 1% of the HL-A activity could be recovered. Recombination of the eluates from the β_2-microglobulin band and other bands under several conditions failed to reconstitute HL-A activity. HL-A antigenic activity was judged not to exist in the isolated 34,000-dalton component obtained by this method.

Patterns of Untreated and Neuraminidase-Treated Antigen in Isoelectric Focusing Gels Without Urea. To see if different bands of the IM-1 antigen, as seen on isoelectric focusing, corresponded to different HL-A specificities, focusing gels *without urea* were run of the normal and neuraminidase-treated antigen. Gels were stained; parallel gels were sliced, eluted, and assayed for antigenic activity. Only two of the four specificities of IM-1 cells were followed in these experiments, HL-A3 and HL-A27. The banding pattern, pH profile, and activity profiles of the untreated antigen are shown in Fig. 4, and the profiles for the neuraminidase-treated antigen are shown in Fig. 5. The specificity of antigenic activity was maintained after isoelectric focusing, as judged by the separation of peaks of HL-A3 and HL-A27 activities and by the low level of inhibition with an HL-A7 antiserum (SAN-71) in both Figs. 4 and 5. The level of HL-A7 inhibitory activity was less than 250 units per slice and was, therefore, not diagrammed.

Four peaks of HL-A3 and three peaks of HL-A27 activities were seen in the untreated antigen, largely overlapping (Fig. 4). This pattern was reduced to two bands after neuraminidase treatment (Fig. 5). SDS gels revealed that both of these immunologically active bands contained both the small subunit (β_2-microglobulin) and large subunit found in HL-A antigen preparations. A sharp band at pI 6.4 was dissociated β_2-microglobulin.

The activity profile of neuraminidase-treated antigen showed that HL-A3 has a higher pI and was separable from

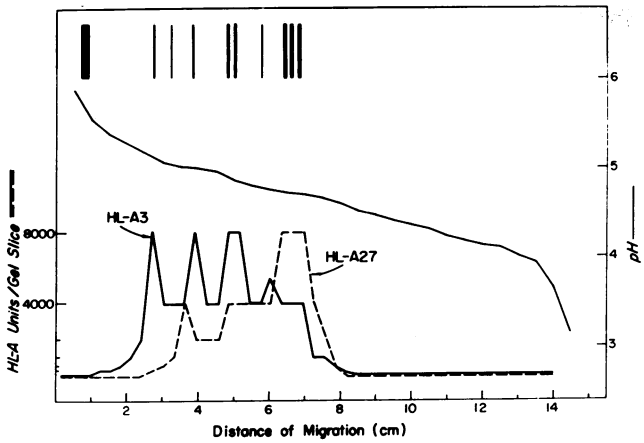

FIG. 4. HL-A3 and HL-A27 activities eluted from slices of an isoelectric focusing polyacrylamide gel run with IM-1 antigens. A diagram of a gel stained with Coomassie blue and run in parallel and the pH of the gradient are also shown. HL-A7 activity (specificity control) was less than 250 units per slice (not diagrammed). The dark band of the highest pI is β_2-microglobulin.

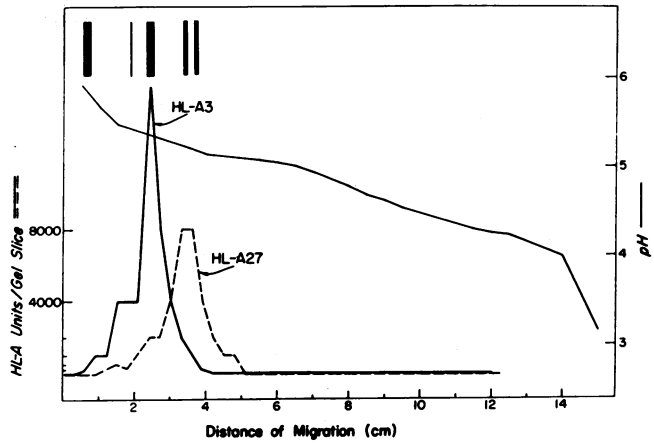

FIG. 5. Neuraminidase-treated IM-1 antigens run in parallel with the gel of Fig. 4.

HL-A27 (Fig. 5). The band with the highest pI, 5.7, in the urea gel (Fig. 3) may, therefore, represent HL-A3 and, thus, HL-A27 would be included in the material at lowest pI, 5.1 (presumably together with W-25).

DISCUSSION

These experiments establish that much of the electrophoretic heterogeneity of the 34,000-dalton glycopeptide of HL-A antigens is caused by variation in the number of sialic acid residues. Each glycopeptide contains 1, 2, or 3 sialic acid residues, with the species containing two residues predominating in the papain-solubilized product. Removal of the sialic acid residues allows the separation of some antigens by electrophoretic means. Neuraminidase treatment may therefore offer a step in the large-scale preparation of serologically specific HL-A antigens. Since immunological activity is retained after removal of sialic acid residues, they are clearly not involved in immunological specificity.

It is worth noting that the resolution of apparent heterogeneity by neuraminidase treatment leaves one with a few bands to which separate HL-A activities can be ascribed. This lack of electrophoretic heterogeneity after neuraminidase treatment implies the lack of amino-acid sequence microheterogeneity (as does occur in immunoglobulins). This observation allows one to approach determination of the amino-acid sequences of HL-A antigens with confidence. In addition, one cannot impute to HL-A molecules receptor function with such diverse demands as those placed on immunoglobulins.

Although amino-acid sequence homology has been shown between β_2-microglobulin and the C_H3 region of IgG, homology of the 34,000-dalton glycopeptide with immunoglobulin domains remains to be demonstrated. Lessening of the electrophoretic heterogeneity by removal of sialic acids will assist the quantitative preparation of HL-A antigens and hasten the elucidation of the amino-acid sequence of the glycopeptide.

This study was supported by a research grant from the U.S. Public Health Service (AI-09576). P.P. was supported by a fellowship from the Kennedy Memorial Trust, London. R.H. was supported by a fellowship from The Anna Fuller Fund.

1. Cullen, S. E., Schwartz, B. D., Nathenson, S. G. & Cherry, M. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1394–1397.
2. Sanderson, A. R. & Batchelor, J. R. (1968) *Nature* **219**, 184–187.
3. Cresswell, P., Turner, M. J. & Strominger, J. L. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1603–1607.
4. Nakamuro, K., Tanigaki, N. & Pressman, D. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2863–2865.
5. Grey, H. M., Kubo, R. T., Colon, S. M., Poulik, M. D., Cresswell, P., Springer, T., Turner, M. J. & Strominger, J. L. (1973) *J. Exp. Med.* **138**, 1608–1612.
6. Peterson, P. A., Rask, L. & Lindblom, J. B. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 35–39.
7. Cunningham, B. A., Wang, J. L., Berggard, I. & Peterson, P. (1973) *Biochemistry* **12**, 4811–4821.
8. Hess, M. & Smith, W. (1974) *Eur. J. Biochem.* **43**, 471–477.
9. Hess, M. & Davies, D. A. L. (1974) *Eur. J. Biochem.* **41**, 1–13.
10. Sanderson, A. R. (1964) *Brit. J. Exp. Pathol.* **45**, 398–408.
11. Wigzell, M. (1965) *Transplantation* **3**, 423–431.
12. Wrigley, C. W. (1968) *J. Chromatogr.* **36**, 362–365.
13. Grothaus, E. A., Flye, M. W., Yunis, E. & Amos, D. B. (1971) *Science* **173**, 542–544.