

DIFFERENCES IN GENE COPY NUMBER CARRIED BY
DIFFERENT MHC ANCESTRAL HAPLOTYPES

Quantitation after Physical Separation of Haplotypes
by Pulsed Field Gel Electrophoresis

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The MHC is bounded by the telomeric class I HLA genes and the centromeric class II HLA genes. The central genes include the C4, C2, Bf, 21 hydroxylase (Cyp21), TNF, BAT, and other genes. Most, if not all, of these genes are duplicated and polymorphic. In the case of C4 and Cyp 21, it is known that the gene copy number can vary from one to three per haplotype (1-3).

We have used the term "ancestral haplotype" (AH)¹ to describe conserved haplotypes that appear to be identical between subjects not known to be related (4-6). These AHs have a specific content of alleles at all MHC loci and have a particular genomic length as determined by pulsed field gel electrophoresis (PFGE) (6). In local caucasoids, some 30 AHs, and recombinants between these, account for almost 90% of the haplotypes found after family studies. These 30 AHs account for almost all of the known linkage disequilibria and almost all but the lowest frequency alleles so far identified (4). It has already been shown that certain AHs have a particular gene copy number at the C4 and Cyp21 loci (3).

The quantitation of gene copy number can be rather difficult. The simpler approaches, such as counting the number of fragments after RFLP analysis (7), are subject to degrees of uncertainty so that progress has been relatively slow. Densitometric comparison of the intensity of hybridizing fragments can be helpful in determining gene copy number. However, this approach is only really useful when fragments are compared within one lane so as to eliminate differences due to variations in DNA concentration and degree of restriction enzyme digestion.

PFGE allows the analysis of fragments of more than one megabase, and in heterozygous subjects, the physical separation of AHs of different lengths (4, 6). In this study we show that densitometric comparison of different AHs after PFGE separa-

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¹ *Abbreviations used in this paper:* AH, ancestral haplotype.

tion gives the expected results when the gene copy number is already known. Accordingly, we have used this approach to estimate the gene copy number in other situations and we have shown that different AHs carry specific numbers of several MHC genes.

Materials and Methods

Cell Lines. EBV-transformed cell lines were obtained from fully genotyped individuals with selected AHs (Table I). For the present study, heterozygous cell lines were of particular value. Disease associations with these AHs have been reported previously (8-11).

DNA Preparation, Restriction Enzyme Digestion, and PFGE. The methods have been described previously (6, 12, 13). Briefly, genomic DNA was prepared from EBV-transformed cells and embedded in low melting temperature agarose plugs. DNA plugs containing 6×10^5 cells were treated with Proteinase K, washed in Tris-EDTA buffer (pH 7.5), equilibrated with the appropriate restriction buffer and digested with restriction enzymes as described (6, 12). Electrophoresis was performed as described (6).

Southern Hybridization. After PFGE, the gels were stained in ethidium bromide and appropriate measurements were recorded for the lambda and yeast markers. The gels were then treated with HCl and NaOH, to depurinate and denature the DNA, followed by transfer to nylon membranes by alkaline transfer (14). The membranes were hybridized with probes labeled by the random priming method (15), washed, and exposed to x-ray film according to standard methods. Probes were stripped off the membranes by treatment with NaOH. Stripping was carefully monitored by overnight exposure to x-ray films and the membranes were then used for reprobing (6).

Human cDNA probes were used to quantitate gene copy numbers. The probes for Bf (16), C4 (17), and DRB (18) were provided for study in the 10th International Histocompatibility Workshop (19). All have been fully characterized (2, 20-23). The 1.6- + 0.66-kb Cla I + Bam HI probe for Bf appears to be specific for this gene. The 0.5-kb Bam HI + Kpn I C4 probe (pATA) hybridizes with the 5' ends of C4A and B. The 0.786-kb Hind III + Sal I DRB probe including most of the coding sequence may cross-hybridize with other class II genes under low stringency conditions. The 2.1-kb Bam HI probe for Cyp21 (pC21/3c) was kindly provided by Dr. P. White (24) and has been shown to react with Cyp21A and B (2, 3, 21, 25). The 0.8-kb Eco RI TNF- α probe, which contains the entire coding region of mature TNF, was kindly provided by Dr. H. M. Shepard (26; Genentech Inc., South San Francisco, CA) and has been fully characterized (27, 28). There is no detectable hybridization of this probe with TNF beta contained within genomic clones of 8.1 or 57.1 (Du et al., manuscript in preparation).

Evaluation of Gene Copy Number by Densitometry. Quantitative determination of gene copy number by densitometry was carried out with a video densitometer (Bio-Rad Laboratories, Richmond, CA; Model 620, settings: filter frequency, 4.0 lines/mm; enhancement, 0.1/mm; boost factor, 1). Integrated areas under the curve corresponding to specific DNA bands were used for the calculation of optical density (OD) ratios. The comparative estimation of gene copy numbers carried by different haplotypes was only performed when two distinct haplotype-specific DNA fragments were obtained from a single heterozygous individual and it was possible to compare these two haplotypes by signal differences as determined by densitometry (Fig. 1). The haplotype-specific DNA fragments were defined by PFGE analysis of a number of homozygous cell lines (6). It should be emphasized that the present approach depends upon the physical separation of each haplotype, complete stripping before reprobing, and quantitative densitometry.

Results

In the first instance we selected subjects known to be heterozygous for the AHs of interest and established EBV-transformed cell lines. DNA from selected cell lines

TABLE I
Ancestral Haplotypes Used in This Study

AH	No.	HLA A	Cw	B	Bf	C4A	C4B	DR	DQ	Gene copy no.		
										C4	Cyp21	DRB*
7.1	7	3	7	7	S	3	1	2	6	2	2	3
8.1	10	1	7	8	S	Q0	1	3	2	1	1	3
18.2	1	33	5	18	F1	3	Q0	3	2	1	1	3
44.1	1	2	5	44	S	3	Q0	4	7	3-4	2	4
44.2	1	29	4	44	F	3	1	7	2	2	2	4
57.1	3	1	6	57	S	6	1	7	9	2	2	3-4
60.3	1	2	3	60	S	Q0	2	13	?	3-4	3	3-4
62.1	5	2	3	62	S	3	3	4	8	2	2	4
62.2	4	?	3	62	S	4	2	4	3	?	?	4
65.1	1	?	8	65	S	2	1+2	1	5	3	3	2
27.1	1	?	2	27	S	3	1	4	?	2	2	4

Gene copy numbers were assigned to each AH at C4, Cyp21, and DRB loci by using the observed O.D. ratios and the data provided in Fig. 5.
 ?, Not defined; *, provisional assignment.

was used in the PFGE studies. After Southern transfer, the blots were probed sequentially paying careful attention to stripping after each hybridization.

As shown in Fig. 1, AHs could be separated and their densities compared. It was immediately obvious that the ratios between the shorter and longer haplotypes depended upon the probe used.

Densitometric scanning allowed objective determination of densitometric ratios. For example, it was clear that there are approximately twice as many C4 and Cyp21 genes on the 57.1 AH as compared with the 8.1 AH.

To confirm that these densitometric ratios segregated with haplotypes, family studies were undertaken. As shown in Figs. 2 and 3, consistent results were obtained, confirming that it was possible to separate AHs and to use objective densitometry. At the same time we were able to compare ratios in siblings so that an estimate of scatter was possible.

Since there are several variables determining the density of fragments after Southern blotting and hybridization, we were concerned about the reproducibility of the assay. The effective exposure time was studied by probing with C4 after digestion with Mlu I or Not I. For each fragment the absolute OD area increased between 1 and 8 d as expected. Importantly, the ratio between fragments remained constant over this period.

Reproducibility was examined by comparing samples within and between runs. After digestion with Not I and probing for C4, three different heterozygous cell lines gave ratios of ~0.6, 1.0, and 1.4 (see Fig. 4, second panel). When the three cell lines were triplicated on the same run or repeated on three separate occasions, ranges of 0.4-0.6 (mean \pm SD = 0.53 \pm 0.07), 0.9-1.2 (mean \pm SD = 1.04 \pm 0.08), and 1.3-1.6 (mean \pm SD = 1.43 \pm 0.12), respectively, were obtained; there was no overlap even when tested on completely different runs.

Having demonstrated the potential value of our approach we examined several

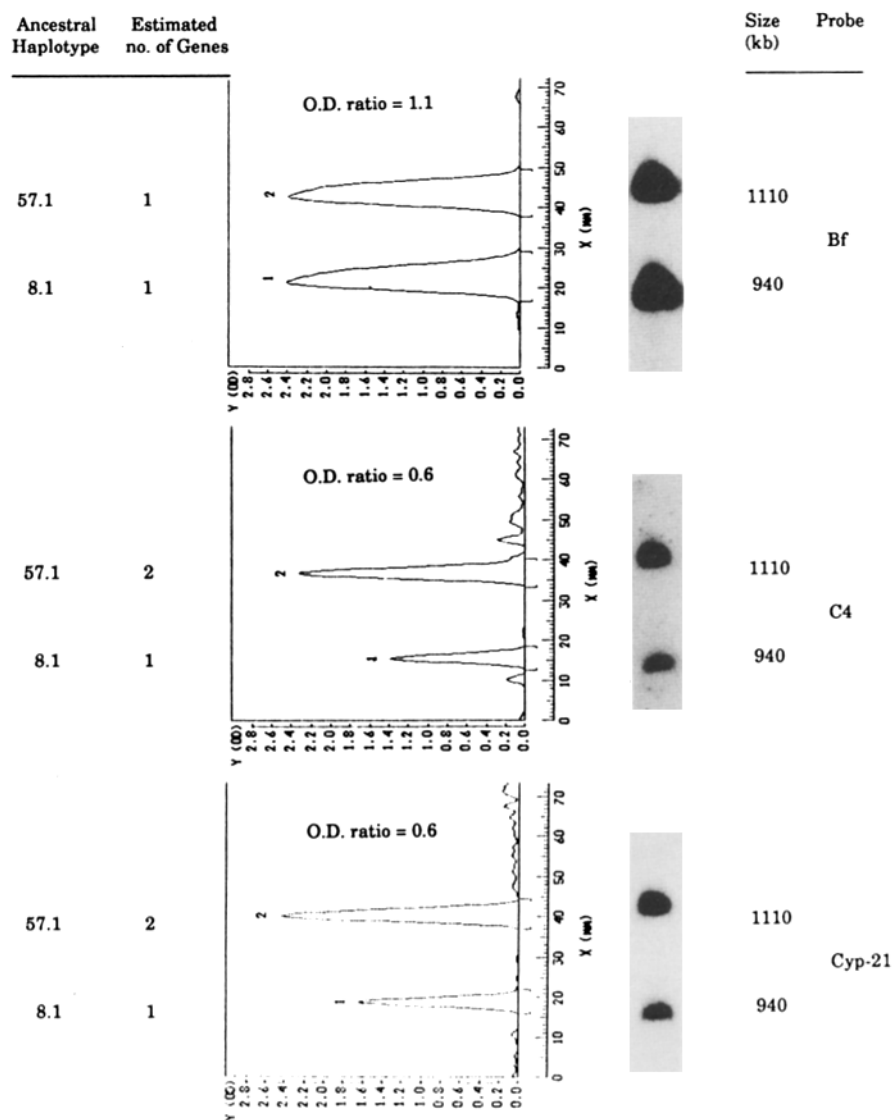


FIGURE 1. Single C4 and Cyp21 genes on the 8.1 ancestral haplotype demonstrated by densitometry on PFGE separated DNA fragments. The genotype of the cell line is HLA A1, B8, BfS, C4AQQ, C4B1, DR3, DQw2 (8.1); A32, B57, BfS, C4A6, C4B1, DR7, DQw9 (57.1). After digestion with Not I the two AHs were separated by PFGE. After Southern transfer, the membrane was hybridized sequentially with each of the three probes. Since it is known that the 8.1 and 57.1 AHs are carried by the 940- and 1,110-kb fragments, respectively (6), it was possible to compare the densities of the two haplotype-specific fragments. As shown quantitative scanning gave ratios of 1.1 for Bf and 0.6 for C4 and Cyp21A+B respectively. All AHs including 8.1 and 57.1 carry only one copy of Bf so that a heterozygous subject would be expected to give an optical density ratio of 1. Numerous previous studies have shown that the 8.1 AH carries only one copy of C4 because of a deletion resulting in C4AQQ. The observed OD ratio of 0.6 is close to that expected of 0.5. Similarly the 8.1 AH lacks Cyp21A and again the observed OD ratio is close to that expected. It should be emphasized that the present approach depends upon the physical separation of each haplotype, complete stripping before reprobing and quantitative objective densitometry.

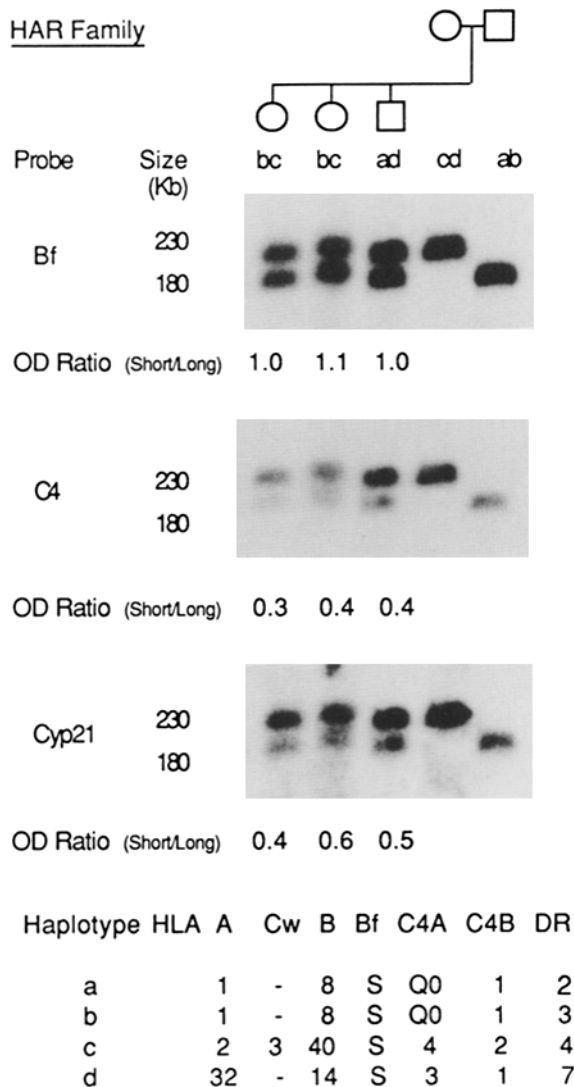


FIGURE 2. Haplotype-specific fragments segregate within families and can be used to quantitate gene copy number. It can be seen that after Mlu I digestion the father (ab) is homozygous for fragments of 180 kb, whereas the mother (cd) is homozygous for fragments of 230 kb. Comparison of optical densities in the children revealed ratios close to the expected 1.0 for Bf. Since both of the 180-kb fragments carry the BfS, C4AQ0, C4B1 genotype derived from the 8.1 AH, d carries BfS, C4A3, C4B1. With the C4 probe the a/d and b/c ratios were 0.4 and 0.3-0.4 respectively. It is concluded that haplotypes c and d contain either two or three copies of C4. Similar results were obtained after reprobng with Cyp21A + B. Thus the ratios for a/d and b/c were 0.5 and 0.4-0.6, respectively, suggesting that both the c and d haplotypes contain two copies of Cyp21A or B. If equal numbers of C4 and 21 hydroxylase are assumed, it appears that haplotypes a and b have one of each and c and d have two of each. It is likely that the expected ratios of 0.5 may actually yield ratios of 0.3-0.6.

heterozygotes carrying AHs with known gene copy numbers (Fig. 4). As shown in Fig. 5, similar ratios were obtained when haplotypes with equal gene copy numbers were compared, irrespective of whether there were one or two genes on each haplotype. No overlap was observed when haplotypes with 1:2 or 2:4 genes were compared with those with 1:1 or 2:2 genes. On the other hand, we have not established that the technique would be able to measure reproducible differences between 2:3 or 3:4 genes.

From the results shown in Figs. 4 and 5, it is often possible to define the likely gene copy number given a particular OD ratio. The ranges so obtained were essentially the same as those expected from the comparison of siblings (see Figs. 2 and 3).

Having demonstrated that the approach provides predictable results when gene

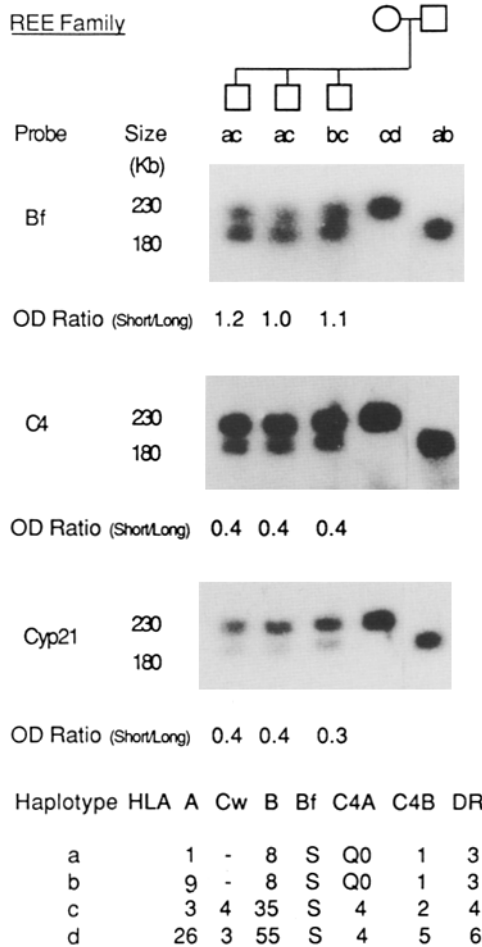


FIGURE 3. Family segregation analysis demonstrates correct estimation of gene copy number by densitometric comparison. A situation similar to that described in Fig. 2 was observed in family REE. With Bf probing after Mlu I digestion haplotypes a, b, and c showed similar density in three children as indicated by ratios close to 1.0 (*top*). With C4 (*middle*) and Cyp21A + B (*bottom*) probes the OD ratios for b/c and a/c were 0.3 and 0.4. Since haplotypes a and b contain the BfS, C4AQ0, C4B1 complotype derived from the 8.1 AH, it is estimated that these haplotypes carry one copy of C4 and 21 hydroxylase genes, whereas haplotype c carries two copies of each.

copy number is known, we then studied other AHs (Table I) in heterozygous individuals. Analysis of heterozygotes containing one characterized and one uncharacterized AH enabled us to estimate gene copy numbers at C4, Cyp21, and DRB loci. As shown in Table I, there are differences in the numbers of genes carried by different AHs. The number is always the same for those AHs where multiple examples have been tested. In the case of DRB, the probe may cross-hybridize with other class II genes under certain conditions, but it appears that the number of genes may relate to DR specificity determined by serology. There may be exceptions as in the case of 57.1, but further studies are required.

The blots used to estimate gene copy number for C4, Cyp21, and DRB were re-stripped and re-probed for TNF- α . Once again, haplotype-specific differences were obtained (Fig. 6). Depending upon the ancestral haplotypes, the ratios varied over the range found for C4, Cyp21, and DRB; the shorter 57.1 gave more than twice the density of the longer 8.1 AH. By contrast, the shorter 7.1 yielded only half the signal of the longer 62.1. Using cell line TRE carrying 57.1 and 8.1, it was possible

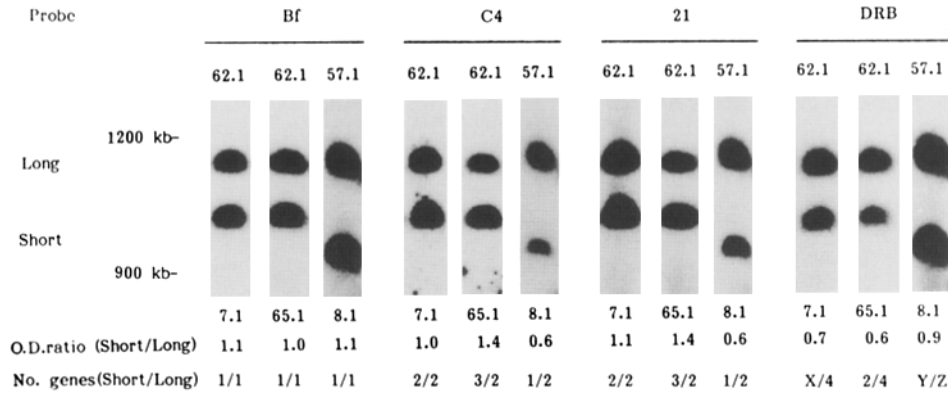


FIGURE 4. Quantitative determination of gene copy numbers carried by different AHs after physical separation of genomic DNA by PFGE. PFGE Southern blots of samples digested with Not I were probed with Bf, C4, Cyp21A + B, and DRB probes ensuring complete stripping after each hybridization. The gene copy numbers carried by each fragment or haplotype were estimated from the OD ratio ranges as shown in Fig. 5. It is important to note that with the Bf probe all the three cells showed ratios of ~1.0 (also seen in Figs. 2 and 3). However, various ratios were seen after probing for C4, Cyp21, and DRB and therefore different numbers of genes were assigned to different haplotypes as shown. Four DRB genes were assigned to 62.1 based on references 43 and 44. Accordingly, the 7.1 may have 3 DRB genes (X), while the 8.1 and 57.1 AHs may carry Y and Z genes, respectively (Y and Z represent 3-4 genes).

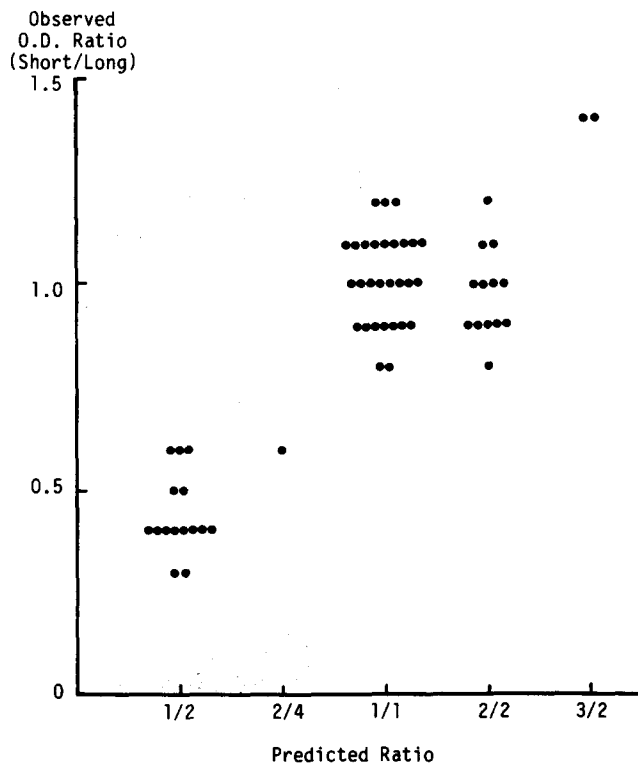


FIGURE 5. Gene copy number is reflected by OD ratios with ranges derived from individuals with known gene copy number. A number of heterozygous cell lines with two distinct haplotype-specific DNA fragments ("short" and "long") have been physically separated by PFGE. Densitometric comparison showed that the observed OD ratios fit those predicted from fully genotyped individuals with known gene copy numbers for Bf (1:1, mean \pm 2 SD = 1.0 ± 0.22), C4 and Cyp21 (1:2, mean \pm 2 SD = 0.43 ± 0.20 ; 2:2, mean \pm 2 SD = 0.96 ± 0.26 ; and 3:2), and DRB (2:4).

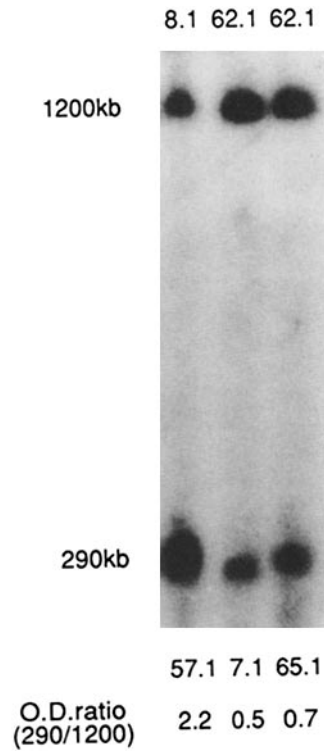


FIGURE 6. Differences in fragment intensities with a probe for TNF after physical separation of ancestral haplotypes by PFGE. Three heterozygous cell lines were digested with Not I and the component AHs were separated by PFGE. The sizes of the Not I/TNF fragments are as described previously (6). As shown, there were major differences in fragment intensities when two AHs were compared and therefore different densitometric ratios were obtained. On the left, it can be seen that 8.1 and 57.1 differ by a factor of ~2. A reciprocal difference is seen when 62.1 is compared with 7.1. In the third lane 65.1 is somewhat less intense than 62.1.

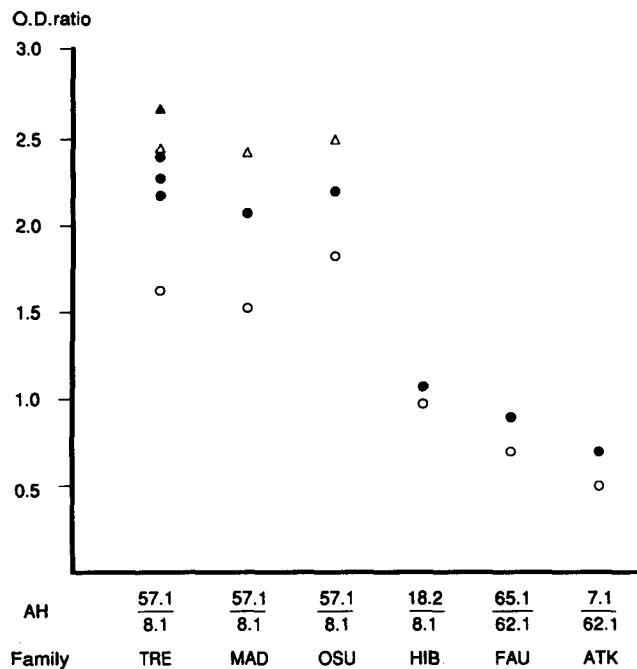


FIGURE 7. After PFGE separation of AHs, probing for TNF reveals differences in densitometric ratios. Six heterozygous cell lines were subjected to PFGE after Not I digestion. As shown in Fig. 6, two DNA fragments could be demonstrated after probing for TNF. AHs 57.1, 18.2, 65.1, and 7.1 are carried by shorter fragments (~290 kb), while AHs 8.1 and 62.1 are represented by longer fragments (~1,200 kb). As shown, OD ratios are similar for the cell lines TRE, MAD, and OSU with the same two AHs. Different OD ratios were obtained with the cell lines HIB, FAU and ATK. Results were similar when a cell line was repeated within (same symbols) and between runs (different symbols).

to compare results within and between runs (Fig. 7). Results were similar on all occasions. Three unrelated subjects with the same AHs were compared; the results in all three were very similar, especially within the same run. When a cell with 8.1 and 18.2 was tested on two separate runs, ratios of ~ 1.0 were obtained, suggesting that there is at least a twofold difference between 18.2 and 57.1.

Discussion

Until recently, the MHC has been seen as a cluster of HLA genes principally concerned with presentation and other antigen-specific phenomena. Other genes within the complex have been regarded as accidental or irrelevant (29, 30).

Following the pioneering work of Alper et al. (31) and Dupont et al. (32), it became quite clear that alleles at the C2, Bf, C4, and Cyp21 loci are associated with particular alleles at the HLA class I and class II clusters and that some HLA-associated diseases are actually due to abnormalities of the central genes (e.g., C2 deficiency, C4 deficiency, and 21 hydroxylase deficiency). During the same period our studies of autoimmune diseases had revealed that certain diseases were associated with particular supratypes (or combinations of alleles at various MHC loci). This led us to suggest that the association might reflect quantitative changes in the expression of MHC genes other than the HLA genes known at the time (8, 33). Subsequently, we began to characterize supratypes associated with disease and showed, in a variety of situations, that it was the supertype rather than any particular allele which provided the best marker of disease susceptibility (9–11, 34, 35).

By studying the genomic structure of particular supratypes, it became obvious that we were actually identifying ancestral haplotypes viz. highly conserved structures with a particular content of alleles, deletions, and duplications throughout the MHC (3, 4, 6).

These conclusions were confirmed in many respects through collaborative studies undertaken during the 10th International Histocompatibility Workshop.

Following family studies of local Caucasoids we have identified 30 AHs that (with recombinants between 2 or 3 of these) account for almost all alleles at all MHC loci and that explain the observed linkage disequilibria between alleles at two or more loci. Thus, the MHC can be seen in the context of a rather limited number of AHs that have remained essentially unique throughout human evolution. Each has a particular length and a particular content of alleles, deletions, and duplications. Elsewhere we have argued that many of these AHs can be identified, in large measure, in nonhuman primates, although some appear to be of more recent origin (36). Those AHs that contain large deletions are associated with autoimmune diseases.

These concepts suggest that the MHC has been conserved en bloc and that central non-HLA genes may be of critical importance. As but one example, it is now known that C2, Bf, C4, and TNF are all potential regulators of B cell differentiation and function, and we favor the hypothesis that a particular cascade of different polymorphic forms of these gene products may have greater or lesser consequences on global antibody production. Thus, the central part of the MHC may be important in terms of antibody-mediated defense and autoantibody-mediated autoimmune disease.

To test some of these hypotheses we require methods for quantitating gene copy

numbers within the MHC. Ideally the method should be appropriate for all genes, and perhaps even for those that have not been characterized fully at present.

In approaching this aim we were favorably influenced by our experience in quantitating the protein products of the C4 genes. This approach, pioneered by Christiansen et al. (37), has been extended following the introduction of improved techniques in protein allotyping (38, 39) and now allows the accurate detection of from 0 to 3 functional C4 genes per haplotype (40, 41).

The present report describes our experience with objective densitometric assessment of gene copy number after PFGE and Southern blotting. We have taken advantage of the fact that PFGE allows physical separation of many AHs so that two AHs can be compared within the one lane. Further, by taking account of the fact that the gene copy number of C4, Cyp21, and DRB can be deduced for some AHs (1-3, 42-44) we have been able to evaluate the system and develop appropriate techniques for the quantitation of other MHC genes.

Gene copy number is a function of the particular AH, at least in the case of C4 and Cyp21. The same appears to be true of DRB, although further studies using locus-specific probes are required. To date we have shown that the DRB-SIV subprobe (44) gives similar results to those presented above (Zhang et al., manuscript in preparation). AHs with the same serological specificity have the same or similar numbers of DRB genes. Thus, immunological reactions apparently reflecting the DRB serological specificities could actually relate to the gene copy number as well as the protein sequence.

It should be emphasized that the approach described does not distinguish between functional genes and pseudogenes. For example, in the case of Cyp21, most AHs contain one pseudogene (21A) and one functional gene (21B) but we include both. Similarly in the case of DRB, pseudogenes will be included (44). By contrast most, if not all, C4 genes are functional.

With respect to TNF, our results reveal at least twofold differences between AHs. While variations in gene copy number may be responsible, interpretation is more complicated than in the case of C4, Cyp21, and DRB. The TNF probe should be specific for TNF- α under the present conditions but it is not yet known how much different AHs vary with respect to TNF sequence and organization. Recently we have shown that the same TNF- α probe can distinguish between AHs with or without a Nco I site within TNF- β (27) and there may be far more sequence polymorphism of the TNF gene family than previously appreciated (Du et al., manuscript in preparation). Furthermore, some AHs contain hybrid C4 and DRB genes and it is possible that a similar phenomenon occurs elsewhere within the MHC, possibly including hybrids between different TNF genes. These possibilities are under investigation at present.

Since Not I separation results in fragments of very different length, quantitation of TNF will be less precise than in the case of C4, Cyp21, and DRB. However, since the OD ratios varied from ~ 0.5 -2.5 (see Fig. 7), and since the differences were haplotype specific, we conclude that there can be important qualitative and/or quantitative differences between at least some haplotypes. It may be relevant that TNF appears to be an important mediator involved in the regulation of B cell function (45, 46) and differences in regulation or gene copy number could influence the amount of product produced. A recent study has shown that production of TNF is related

to H-2 haplotypes in the mouse (47), and a similar possibility has been suggested in man; individuals with HLA DR2 have low production of TNF by monocytes activated with LPS (48-50). Moreover, HLA DR3 subjects may also be low responders when compared with subjects with HLA DR7, DR4, and DR1 (48, 49). These observations may relate to the present results since HLA DR2 and DR3 are carried by AHs 7.1, 8.1, and 18.2, respectively, and these three AHs have less TNF signal than 57.1 and 62.1 carrying HLA DR7 and DR4, respectively.

The 8.1 AH is particularly interesting because it contains a C4 null (8, 33), a polymorphism of TNF at the RFLP level (27), and as shown here, reduced gene copy numbers of C4, Cyp21, DRB, and possibly TNF. Any or all of these characteristics could be relevant to its association with autoimmune diseases such as SLE. Recently, Jacob and McDevitt (1988) have shown that TNF delays the development of a lupus-like disease in a murine model (51). Others have extended these observations (52). We therefore propose that predisposition to autoimmune diseases may be related to relative deficiencies of the products of central MHC genes.

Summary

We have examined the hypothesis that MHC ancestral haplotypes have a specific content of genes regulating the extent of autoimmune reactions. Gene copy number was quantitated by objective densitometry after PFGE was used to separate heterozygous AHs of different lengths. Initially we analyzed examples of known gene copy number at the C4 and 21 hydroxylase loci and showed that the approach provides predictable results.

We then studied heterozygotes containing one characterized and one uncharacterized AH with particular attention to the gene copy number at the C4, Cyp21, and DRB loci. Each AH studied has a characteristic gene copy number at each locus studied. The same may be true of TNF, but other possibilities must be considered.

AHs are markers for extensive chromosomal segments including particular numbers of several functional genes. Since AHs mark susceptibility to autoimmune disease, differences in gene copy number may be implicated.

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