Mutations in human lymphocytes commonly involve gene duplication and resemble those seen in cancer cells

(gene deletion/somatic recombination/chromosome reduplication/HLA)

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ABSTRACT Mutations in human lymphocytes are commonly due to gene deletion. To investigate the mechanism of deletion for autosomal genes, we immunoselected lymphocytes mutated at the HLA-A locus and cloned them for molecular analysis. Of 36 mutant clones that showed deletion of the selected HLA-A allele, 8 had resulted from a simple gene deletion, whereas 28 had resulted from a more complex mutational event involving reduplication of the nonselected HLA-A allele as indicated by hybridization intensity on Southern blots. In 3 of the 28 clones, retention of heterozygosity at the HLA-B locus indicated that the reduplication was due to recombination between the two chromosomes; but in the remaining 25 clones, distinction could not be made between recombination and chromosome reduplication. The results indicate that mutations in normal somatic cells frequently result in hemizygosity or homozygosity at gene loci and, thereby, resemble the mutations thought to be important in the etiology of various forms of cancer.

Functional loss of both wild-type alleles of a critical gene is now recognized as being an important mechanism in the pathogenesis of several human cancers (1–11). Some of these cancers have a familial form in which loss of one allele is inherited and a second mutational event produces an acquired loss of the allele on the homologous chromosome. In the sporadic form of such cancers, both mutational events are presumptively acquired in the tumor stem cell. Several genetic mechanisms for the acquired loss of alleles in human tumors have been observed and include simple gene deletion, whole chromosome loss, whole chromosome loss with reduplication, and recombination. However, the importance of these genetic mechanisms in normal mammalian cells is not known.

Studying the genotypic basis of mutations in human somatic cells has required the development of techniques for selecting T lymphocytes mutated at particular loci and growing them clonally to appreciable numbers. The best-studied locus has been that for the X chromosome-linked gene encoding hypoxanthine phosphoribosyltransferase (HPRT) (12–14). At this HPRT locus, a substantial proportion of spontaneous and induced somatic mutations have been associated with gene deletion (15–17). However, the HPRT locus, being X chromosome-linked and therefore functionally hemizygous, has a number of disadvantages for study of mutation and, in particular, will not detect events that involve interaction between homologous chromosomes. For this reason and to study mutations at an autosomal locus, we have used immunoselection with antibody and complement, as previously used for lymphoblastoid cell lines (18, 19), to isolate and study human T lymphocytes mutated at the HLA-A locus, which is on chromosome 6. We found that approximately 40% of spontaneous HLA mutants and 78% of mutants produced by x-radiation or mitomycin showed deletion of the gene for the selected HLA-A allele (20). In this report we show that gene deletion is commonly associated with reduplication of the other HLA-A allele, which indicates that somatic recombination or chromosome loss with reduplication is commonly responsible for gene-loss mutation in normal somatic cells.

MATERIALS AND METHODS

Clones Studied. Clones were isolated from three individuals who had the HLA phenotype A2, A1 or phenotype A2, A11. There were 7 wild-type clones and 63 mutant clones, of which 22 were spontaneous mutants present in vivo, 22 had been produced by in vitro mutagenesis of G4 lymphocytes with x-radiation, and 19 had been produced by in vitro mutagenesis (20) of G4 lymphocytes with mitomycin.

Isolation of Mutant Clones. The isolation procedure used has been described in detail (20, 21). Spontaneously mutant clones present in vivo were immunoselected from freshly isolated lymphocytes separated from peripheral blood by Ficoll/Hypaque centrifugation; mutant clones resulting from exposure to x-rays or mitomycin were immunoselected from lymphocytes cultured for 7–10 days after mutagenesis to allow mutations to be expressed. Immunoselection of HLA-A2-loss mutants was performed by incubating 10⁶ lymphocytes for 30 min at 4°C in 100 μl of the anti-HLA-A2 antibody BB7.2 (obtained from the American Type Culture Collection). After centrifugation the cells were resuspended in 100 μl of rabbit complement (Commonwealth Serum Laboratories, Parkville, Victoria, Australia) for 1 hr at 37°C and then cultured in microwells (2 x 10⁶ cells per well) with added phytohemagglutinin, irradiated lymphocytes, and a source of interleukin 2. Nonselected cells were cultured similarly but at two cells per well.

Plates were scored after 16–20 days, and wells showing growth were tested for HLA-A2 positivity by microcytotoxicity with antibody and complement. Negative wells were regarded as being mutant clones and were expanded to approximately 50 x 10⁶ cells for full HLA-A and HLA-B phenotyping and for DNA extraction.

Southern Analysis. DNA was digested with HindIII, electrophoresed, and transferred onto nylon membranes, which were probed with an HLA-A-specific probe from the 3' untranslated region of the gene (22). At very high stringency, this probe is specific for the HLA-A locus, but with the conditions used in the present experiments, it also hybridizes to three anonymous genomic regions. The two HLA-A alleles can be separately identified because of the presence of a restriction fragment length polymorphism (23). Laser densitometry was used to measure the signal intensity of the various HLA-A bands, with the areas under the hybridizing
bands being objectively determined by the turning points on the densitometer integrator. Calculations were made of the ratios of the signal intensities for each HLA-A band and the signal intensity for a probe for the immunoglobulin heavy chain constant region (24) which had been hybridized to the filter at the same time as the HLA-A probe. Each clone was studied at least twice by electrophoresis and Southern transfer, and one to three probings were performed on each filter, resulting in a total of two to five, usually four, comparative readings per clone. Calculations of a ratio between an HLA-A band and the immunoglobulin band therefore corrected for different amounts of DNA loaded per electrophoresis track and for different film development conditions but did not correct for differences in the ratio of specific activities for the two probes in different probings. For this reason, in pooling all hybridization data, the ratio for each clone for a particular hybridization was weighted according to the mean ratio for undeleted clones from the same hybridization. Ratios were regarded as abnormal if they fell outside the range of 2 SD from the mean for wild-type and non-deletion-mutant clones.

RESULTS

Of the 63 mutant clones studied, 27 did not show any change at the HLA-A locus on Southern analysis and 36 showed deletion involving the HLA-A2 gene. An example of an autoradiograph with wild-type clones and non-deletion and deletion-mutant clones is shown in Fig. 1; the autoradiograph shows the HLA-A2 signal (band 1), the signal for the other HLA-A allele (band 5), the three cross-hybridizing sequences (band 1–3), and the immunoglobulin band. Visual inspection of autoradiographs suggested that many deletion mutants showed an increase in intensity of the band derived from the nonselected HLA-A allele, and this was confirmed by quantitation using densitometry. The densitometric HLA-A immunoglobulin gene ratios for each band for individual clones are shown in Fig. 2, and the geometric means of the HLA/immunoglobulin gene ratios for each band are shown in Table 1. Deletion clones fell into two groups—a minority of 8, in which signal intensity of the nonselected allele was the same as that for wild-type clones and for non-deletion-mutant clones, and a majority of 28, in which the signal intensity was abnormally increased. The 8 mutants having a normal signal intensity had all been produced by x-radiation, and they all retained phenotypic heterozygosity at the HLA-B locus. In these clones the signal intensities for the three cross-hybridizing bands were usually low, being approximately half those observed for wild-type and deletion clones (Table 1). The intensity of band 3 was reduced in 5 of the 8 clones, that of band 2 was reduced in these 5 clones and 1 additional clone, and that of band 1 was reduced in these 6 clones and 1 additional clone.

We interpret these findings to indicate that the three cross-hybridizing sequences are situated close to the HLA-A gene in the order of bands 1, 2, and 3, with band 1 closest to the HLA-A locus, and that all 8 mutants were simple deletion mutants in which the genomic loss was sufficiently large to frequently involve one, two, or three of the cross-hybridizing sequences.

In 28 mutants showing deletion at the HLA-A2 locus, the mean signal intensity for the nonselected allele was exactly twice that observed in wild-type and non-deletion-mutant clones, as shown by the HLA-A/immunoglobulin gene ratio being 1.48 as opposed to 0.74 for wild-type clones and non-deletion-mutant clones. This is a strong indication that the nonselected allele had been reduplicated. Gene reduplication occurred in all of the 8 spontaneous deletion mutants, in 9 of the 17 deletion mutants produced by x-radiation, and in all of the 11 deletion mutants produced by mitomycin.

In 25 of these 28 mutants showing reduplication, heterozygosity at the HLA-B locus was lost, but in 3 of the 28 clones, heterozygosity at HLA-B was retained.

DISCUSSION

In our previous study of HLA-A mutants (20), gene deletion occurred in 40% of spontaneous mutants and 78% of mutants induced by x-radiation or mitomycin. In that study, 70 mutants showing genotypic deletion of the HLA-A gene, phenotypic loss of one HLA-B allele, presumably that belonging to the same haplotype as the selected HLA-A gene, was observed in 35, whereas of 49 mutants not showing deletion, phenotypic loss of an HLA-B allele was observed in only 2. Loss of HLA-B in the HLA-A deletion mutants could have been due to whole-chromosome loss, to the presence of large deletions (the map distance between the HLA-A and HLA-B loci is about 0.8 centimorgans), or to a variety of recombinational events involving the homologous chromosome.

The present study has analyzed the molecular events responsible for gene deletion. In 8 mutant clones showing deletion, the mutational event was a simple deletion that was of variable size but in no instance was large enough to involve the HLA-B locus. All of these mutants had been produced by x-radiation. In 28 mutant clones showing deletion, the mutational event was more complex as indicated by concomitant reduplication of the nonselected HLA-A allele. Our results cannot rule out the possibility that duplication of the nonselected allele had occurred syntetically, but it is difficult to conceive of a genetic mechanism that could produce gene loss on one chromosome and exact gene doubling on the homologue. We suggest that gene duplication resulted from whole-chromosome loss and reduplication.

Fig. 1. Southern analysis of wild-type (tracks 1, 2, 3, 11, and 12) and HLA-A mutant clones. Bands: 1–3, cross-hybridizing sequences; 4, the selected HLA-A allele; and 5, the nonselected HLA-A allele. Tracks 8, 9, and 13 are deletion mutants, and track 10 is a marker track. Genomic DNA isolated by standard techniques was digested with restriction enzyme HindIII.
or from one of several recombinational events. Such recombinational events could include (i) recombination with gene conversion involving either homologous chromosomes prior to DNA synthesis or non-sister chromatids after DNA synthesis, or alternatively, (ii) mitotic recombination occurring after DNA synthesis involving one chromatid from each of the homologous chromosomes and followed by segregation. In 3 of the clones, each of which was spontaneous, heterozygosity at the HLA-B locus was retained, thus excluding chromosome reduplication and directly implicating recombination as the mutational mechanism. In the other 25 clones, heterozygosity at the HLA-B locus was lost, and it was not possible to distinguish between chromosome reduplication and recombination occurring centromeric to HLA-B.

Several other groups have suggested that mutations in nonneoplastic mammalian cells may be associated with recombination or chromosome reduplication (25–30). In particular, Potter et al. (30) demonstrated that a doubling of gene dosage was associated with recombination in a mutant subclone of a murine virus-transformed cell line; and Langlois et al. (29) observed the occurrence of rare variant erythrocytes having the MM or NN phenotype in normal individuals of the MN blood group phenotype. Of these studies, only that of Langlois et al. involved normal somatic cells present in vivo, but analysis was only possible at the phenotypic level because erythrocytes, which are nonnucleated, were studied.

Functional hemizygosity or homozygosity of critical gene loci was initially reported in retinoblastoma (2, 3) and Wilms tumor (4–7) but is now being reported in an increasing variety of cancers (8–11). Cavenee et al. (31) studied chromosome 13 in 33 cases of retinoblastoma and found that loss of heterozygosity in 19 cases was due to loss of chromosome 13 and reduplication of the homolog and in 4 cases was due to recombination between the two chromosomes 13. Fung et al. (32) studied the retinoblastoma gene in 17 cases of retinoblastoma and found evidence for gene deletion, recombination, and chromosome reduplication in individual cases. Our studies of HLA-A mutations in normal cells confirm that gene deletion or recombination is the mechanism responsible for a proportion of mutations. The data do not permit distinction between recombination and chromo-

![Graph](image.png)

**Fig. 2.** Densitometric ratios for the autoradiographic bands identified in Fig. 1. Band 4 is the band assigned to HLA-A2. Results are expressed as the densitometric ratio of signal for a given band to the signal for the gene for immunoglobulin (lg) heavy chain, weighted as described in the text. The ratios are shown bounded by 2 SD from the mean of wild-type clones (WT) and clones showing no deletion (ND) at the HLA-A2 locus. Each datum point represents the geometric mean of two to five comparative readings per clone (see text). Clones showing deletions (D) of band 4 fall into two groups, those showing an increase in densitometric ratio in band 5 (○) and those showing no such increase (□).

<table>
<thead>
<tr>
<th>Bands</th>
<th>Wild-type clones</th>
<th>Nondeletion mutants</th>
<th>Deletion mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Replicated</td>
</tr>
<tr>
<td>1</td>
<td>0.26 (0.29–0.24)</td>
<td>0.25 (0.27–0.24)</td>
<td>0.26 (0.27–0.24)</td>
</tr>
<tr>
<td>2</td>
<td>0.57 (0.59–0.54)</td>
<td>0.47 (0.50–0.45)</td>
<td>0.44 (0.46–0.42)</td>
</tr>
<tr>
<td>3</td>
<td>0.73 (0.75–0.72)</td>
<td>0.68 (0.71–0.66)</td>
<td>0.50 (0.60–0.55)</td>
</tr>
<tr>
<td>4</td>
<td>0.76 (0.79–0.73)</td>
<td>0.67 (0.69–0.64)</td>
<td>0.50 (0.60–0.55)</td>
</tr>
<tr>
<td>5</td>
<td>0.74 (0.80–0.69)</td>
<td>0.74 (0.77–0.72)</td>
<td>1.48 (1.54–1.43)</td>
</tr>
</tbody>
</table>

Intensities are expressed as the ratio of the HLA-A band signal to the immunoglobulin gene signal. Bands 1–3 represent cross-hybridizing sequences, band 4 is the selected HLA-A allele (A2), and band 5 is the nonselected HLA-A allele (A1 or A11). The results are expressed as the geometric mean, with the range (in parentheses) being the mean ± 1 SEM. The HLA-A probe was an HLA-A 3' untranslated region genomic probe (23).
some duplication as the mechanism in the remainder. We are presently characterizing such mutants further by karyotyping and mapping chromosome 6 with restriction fragment length polymorphisms, and this should unambiguously define the mechanisms involved.

In any event it is clear that the types of mutational events that occur in normal nonneoplastic somatic cells, both in vivo and in vitro, resemble the types of mutational events that occur in cancer cells. The mutations responsible for carcinogenesis are probably typical mutations that assume importance because they involve one or more critical sites.

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