[CANCER RESEARCH 50, 4584–4587, August 1, 1990]

Molecular Nature of *in Vivo* Mutations in Human Cells at the Autosomal HLA-A Locus[1]

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ABSTRACT

The mutations present *in vivo* in normal human cells were studied at the HLA-A locus by isolating mutant lymphocytes using antibody-complement immunoselection and cloning at limiting dilution. The molecular basis for mutation in 127 mutant lymphocytes from 10 individuals was determined by studying a variety of polymorphic gene loci on both arms of chromosome 6. No change was detected in 78 mutants (61.4%), gene deletion was detected in 11 (8.7%), and mitotic recombination was detected in 38 (29.9%). Neither gene conversion nor chromosome loss was detected. These observations document the mechanisms responsible for gene loss in normal human cells *in vivo*, emphasize the importance of mitotic recombination, and indicate the similarity between mutational mechanisms in normal cells and in cancer cells.

INTRODUCTION

Recent studies of a number of childhood and adult forms of cancer have emphasized the importance of "tumor suppressor genes" and have suggested that loss of function of both copies of these genes may be of key importance in the initiation or progression of cancer (1–3). The best studied such gene is the retinoblastoma gene situated on chromosome 13. Evidence suggests that an initial inherited or acquired mutation, either a point mutation or a deletion, is then followed by a second acquired event, either an unrelated mutation or a chromosomal event which results in loss of the remaining wild-type gene and homozygosity (usually) or hemizygosity for the initial mutation. The mechanism for homozygosity appears in many cases to be mitotic recombination followed by segregation. The situation with other tumors is as yet less well defined but is likely to be similar, as loss of heterozygosity for markers localized to specific chromosomes has been recognized in a number of types of tumor.

The recognition of the importance of mutational and chromosomal mechanisms in the initiation and/or progression of many types of cancer has emphasized the importance of delineating similar mechanisms in normal mammalian cells *in vivo*. A number of studies have addressed this problem by using the HPRT[2] locus (4, 5) and have quantitated the normal frequency of HPRT mutants in human lymphocytes. These studies have shown that a proportion, approximately 15%, are associated with gene deletion (6–8) and preliminary unpublished evidence suggests that most of the remainder are due to point mutations or microdeletions. However, the HPRT locus is situated on the X chromosome, and therefore cannot detect interchromosomal events responsible for gene loss, as there is only one functional gene present. For this reason, and in order to obtain quantitative information from a second gene locus, we developed an assay for *in vivo* mutant lymphocytes using the HLA-A locus (9, 10). Mutant lymphocytes were isolated by antibody-complement immunoselection and were found to be present in a frequency of approximately $2–3 \times 10^{-5}$ in young adults. Quantitation of gene dosage suggested that a substantial proportion of gene loss mutants were associated with allelic duplication, *i.e.*, homozygosity, for the nonmutated HLA-A gene (11). We have now studied in detail the mutational spectrum at the HLA-A locus and have mapped polymorphic loci in both arms of chromosome 6 in order to more precisely delineate the genetic mechanisms responsible for gene loss at this locus.

MATERIALS AND METHODS

Gene Loci and Individuals Studied. The polymorphic loci studied on chromosome 6 were, proceeding from telomere to telomere, factor XIIIA, HLA-A, HLA-B, PCH6, and glutathione *S*-transferase (GST-II), which are on the short arm, and *myb* and TCP1 which are on the long arm. We studied a number of healthy adults who were known phenotypically to be heterozygous for either HLA-A2 or HLA-3 and were known genotypically to have convenient restriction fragment-length polymorphisms at the HLA-A locus. They were screened for heterozygosity at the above loci on chromosome 6, and 10 individuals, who were heterozygous at most loci, were selected for study (Table 1). Their ages ranged from 25 to 59 and their mean age was 36.

Isolation of Mutant Clones. Lymphocytes mutated at HLA-A were isolated by immunoselection and enumerated by culture at limiting dilution (9). Selection was performed after holding separated lymphocytes in culture medium at 37°C overnight, and involved exposure to antibody (BB7.2 for HLA-A2 and GAP or XI23 for HLA-A3) for 45 min followed by complement (Van Haeringen Laboratorium, Wageningen, The Netherlands) for 60 min. Selected cells were grown at 10^4 cells/well and unselected cells at 2 cells/well in microplates for 16–18 days. Wells showing growth were tested by a microtoxicity assay to exclude those in which growth originated from wild-type cells which had escaped selection. Mutant frequency was calculated as the ratio of the cloning efficiency for selected cells to the cloning efficiency for nonselected cells.

Expansion and Study of Clones. Presumptively mutant clones were again immunoselected to ensure elimination of any contaminating wild-type cells and then expanded further in microplates until $>20 \times 10^6$ cells were available. From each individual 15–20 mutant clones were initially expanded; owing to failure of some clones to maintain expansion a total of 5–17 clones from each individual was available for study. For each clone HLA typing for Class I antigens was performed by microcytotoxicity on an aliquot of cells and DNA was extracted from the remainder using standard methods.

Appropriately restricted DNA was electrophoresed, Southern transferred to a nylon membrane, and hybridized to ^{32}P-labeled probes for the various gene loci on chromosome 6, for the immunoglobulin heavy chain (IgH) gene, and for the T-cell receptor β chain gene. Details of the loci and probes used are shown in Table 2. Gene dosage for HLA-A was determined by double probing for IgH and HLA-A, measurements of the autoradiographic signal by laser densitometry, and calculation of the ratio of the signal for each HLA-A band to that for the IgH band. The immunoglobulin heavy chain gene is situated on chromosome 14 and it acted as an internal control for quantitation of total DNA.

Criteria for Classification of Mutations. Mutations were classified

Received 1/11/90; revised 4/13/90.

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[1] This study was supported by the National Health and Medical Research Council of Australia and the Anti-Cancer Foundation of the Universities of South Australia.

[2] The abbreviations used are: HPRT, hypoxanthine-guanine phosphoribosyltransferase; GST, glutathione *S*-transferase.

Table 1 *Informative polymorphisms in individuals studied*

Individual	XIII	HLA-B	pCH6	GST-II	*myb*	TCP-1
C. C.	+[a]	+	+	−[b]	−	−
C. M.	+	+	+	−	−	+
J. M.	+	+	+	+	+	−
J. R.	+	+	+	−	+	+
M. H.	+	+	+	+	+	−
M. K.	−	+	+	+	+	−
M. M.	+	+	−	−	−	+
M. Mc.	+	+	−	+	+	−
R. D.	+	+	+	−	+	+
T. B.	+	+	+	−	−	−

[a] +, polymorphism present.
[b] −, polymorphism absent.

Table 2 *Loci studied and probes used*

Locus	Map position	Probes	Source
Factor XIIIA	6p24-6pter	F13A	Dr. P. Board
HLA-A	6p21.3	pHLA2.1	Dr. H. Orr
HLA-B	6p21.3	pHLA1.1	Dr. H. Orr
pCH6	6p21.3	pCH6	American Type Culture Collection
Glutathione *S*-transferase	6p12	pGST-II-*Pva*II	Dr. P. Board
myb	6q21-23.1	pHM2.6	American Type Culture Collection
TCP1	6q25.1-6qter	pB1.4	American Type Culture Collection
T-cell receptor β chain	7q	T-cell breakpoint	Dr. T. Mak
IgH	14q32.3	HuμC	Dr. P. Leder

into those which did or did not show a gene change on Southern blotting. Those which did were classified according to the nature of the gene change as deduced from study of all of the loci on chromosome 6 which were informative for that individual. The criterion for a mutation due to a simple deletion was loss of the selected HLA-A2 or HLA-A3 band, single gene dosage for the other HLA-A allele, and no loss of heterozygosity at any other gene locus. The criterion for gene conversion was loss of the selected HLA-A allele, homozygosity (double gene dosage) for the unselected allele and no evident loss of heterozygosity for other alleles. The criterion for mitotic recombination was loss or alteration of the selected allele, homozygosity for the unselected allele, and loss of heterozygosity at factor XIIIA (for HLA-B if factor XIII was not informative) with or without loss of heterozygosity at other loci on the short arm. The criterion for chromosomal hemizygosity was loss of heterozygosity for loci on both arms of chromosome 6 together with single gene dosage of the unselected HLA-A allele; and the criterion for chromosomal homozygosity was the same except for double gene dosage of the unselected allele. The distinction between hemizygosity and homozygosity at HLA-A was made by considering the ratio of the autoradiographic signals for the unselected HLA-A gene and the immunoglobulin heavy (IgH) chain gene. Ratios were normalized as previously described (11) and double gene dosage, *i.e.*, homozygosity, was regarded as being present when the ratio was greater than 2 standard deviations above the mean for wild-type clones and for mutant clones not showing gene loss. Two mutants which did not meet this criterion were interpreted as showing homozygosity on the basis of a ratio of between 1.5 and 2.0 S.D. above the mean on repeated probing, loss of heterozygosity at factor XIIIA, and homozygosity of the remaining factor XIIIA gene.

RESULTS

Mutant Frequency. The mutant frequencies in the 10 subjects are shown in Table 5; the geometric mean of 2.84×10^{-5} and the 95% range of $1.6–5.1 \times 10^{-5}$ are in good agreement with previous data (9, 10).

Molecular Analysis. In total, 132 clones were expanded after selection; at final phenotyping five were found to express the selected HLA-A allele and were discarded as being wild-type clones. The results of genotyping in the final 127 mutant clones are shown in Table 3. No change at the HLA-A locus was observed in 78 clones (61.4%; 95% confidence limits, 51.8–70.7%); none of these clones showed gene changes at any other locus when studied (XIIIA in 53, HLA-B in 17, pCH6 in 12, GST-II in four, *myb* in three, TCP-1 in 32). Neither gene conversion nor whole chromosome loss was seen. From these negative data the 95% confidence limits for the true percentage of chromosome loss are 0–3.7%. Deletion was seen in 11 clones (8.7%; 95% confidence limits, 4.2–15.7%). In nine clones the HLA-A band was completely lost whereas in two clones a new band of altered mobility was observed. In these two clones it is conceivable that mutation resulted from a translocation or inversion rather than a deletion. In one clone the deletion was quite large as it involved loss of the HLA-B gene which is approximately 1000 kilobases centromeric to HLA-A. Mitotic recombination was detected in 38 clones (29.9%; 95% confidence limits, 21.6–39.5%) and the crossing-over point could be regionalized from the loci at which heterozygosity had been lost or retained (Table 4; Fig. 1). For any particular polymorphism in an individual, all clones characterized by loss of heterozygosity had always lost the same DNA band(s) which suggests that the band(s) represented the allele syntenic to the selected HLA-A allele.

After correcting for the different number of clones studied in the 10 individuals the mean percentage of mutants due to mitotic recombination was 27.8% but the results indicated a significant difference between individuals ($\chi^2 = 47.3$; d.f. = 9). The highest frequency of mitotic recombination was found in T. B. and J. M. Previous studies of other mutant clones from these individuals had shown a high frequency of homozygosity at HLA-A, which provided further evidence that the frequency of mitotic recombination was truly high in these individuals. The absolute frequencies of total mutant lymphocytes and the various types of mutants are shown in Table 5. The total mutant frequency for an individual shown in Table 5 is the mean of all estimations performed in the laboratory on that individual. The

Table 3 *Molecular changes responsible for mutation*

	No. of clones			
Individual studied	No evident change	Deletion	Recombination	
C. C.	5	2	0	
C. M.	10	1	2	
J. M.	5	1	10	
J. R.	10	0	5	
M. H.	9	1	1	
M. K.	15	1	1	
M. M.	4	0	1	
M. Mc.	2	2	5	
R. D.	14	3	0	
T. B.	4	0	13	
Total	78	11	38	127
	61.4%	8.7%	29.9%	100%

Table 4 *Loss of heterozygosity in the 38 clones mutated owing to recombination*

Locus	Informative clones	Loss of heterozygosity		95% confidence limits (%)
		No.	Percentage	
Short arm				
XIIIA	37	37	100	91–100
HLA-B	38	33	87	72–96
pCH6	31	27	87	70–96
GST-II	17	6	35	14–62
Long arm				
myb	14	0	0	
TCP-1	8	0	0	

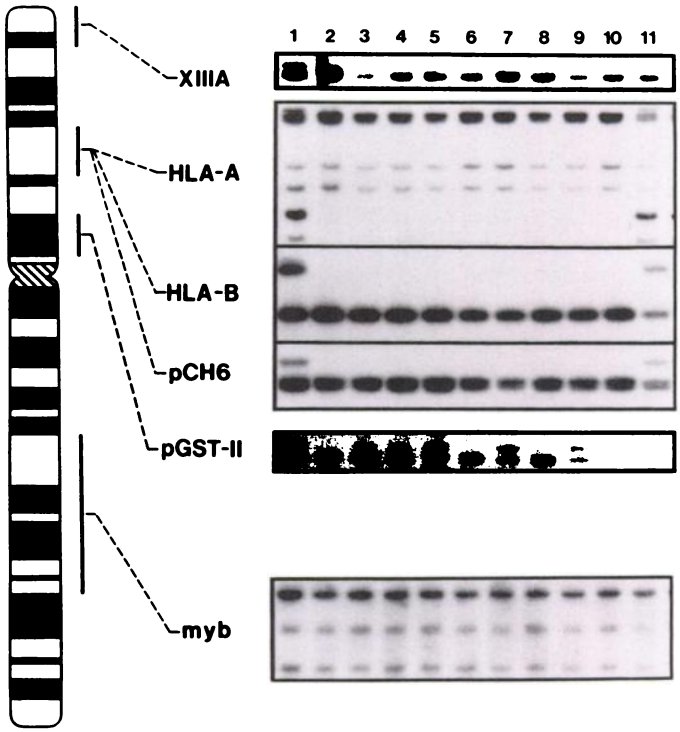

Fig. 1. Molecular analysis in 11 mutant clones from J. M. Clones 2–10 have resulted from mitotic recombination. In *clones 3, 4, 5, 7, 9,* and *10* heterozygosity at GST-II is retained indicating that the crossing-over point is between GST-II and pCH6. In *clones 2, 6,* and *8* heterogeneity at GST-II is lost indicating that the crossing-over point is centromeric to GST-II.

Table 5 *Absolute frequencies of various types of mutant lymphocytes*

Individual studied	Mutant frequency × 10^{-6} Total	No detectable change	Deletion	Recombination
C. C.	24	17.1	6.9	0
C. M.	23	17.7	1.8	3.5
J. M.	30	9.4	1.9	18.8
J. R.	40	26.7	0	13.3
M. H.	28	22.9	2.5	2.5
M. K.	30	26.4	1.8	1.8
M. M.	29	23.2	0	5.8
M. Mc.	37	8.2	8.2	20.6
R. D.	15	12.4	2.6	0
T. B.	38	8.9	0	29.1
Mean		17.3	2.6	9.5
95% confidence limits for the mean		12.7–21.9	0.8–4.4	3.0–16.0

mean number of estimations performed on an individual was 4.3.

HLA-B Phenotyping. All clones showing phenotypic loss of one HLA-B allele also showed loss of the selected HLA-A allele on genotyping; in any one individual the HLA-B allele lost was always the same and was presumably the allele syntenic to the selected HLA-A allele. For individuals having an informative polymorphism at HLA-B, there was an absolute association between loss of one HLA-B allele on phenotyping and loss of one HLA-B allele on genotyping. In 34 clones showing loss of HLA-B, the loss was due to mitotic recombination in 33 and to a simple deletion involving HLA-A and HLA-B in one. Family studies were performed for two individuals and confirmed that the HLA-B phenotype lost in mutant clones from those individuals was always syntenic to the selected HLA-A allele.

***In Vivo* Clonality.** The possibility that a number of the mutant lymphocytes identified in an individual could be clonal, *i.e.*, descendants of a single mutated precursor, was tested by gene probing to determine the arrangement of the gene for the T-lymphocyte antigen receptor β chain. A total of 119 clones from the 10 individuals was studied. The same rearrangement was seen in three pairs of clones. In one pair the same rearrangement was seen despite digestion with four restriction enzymes; in two pairs the same rearrangement was seen after digestion with two enzymes but, for one of the two pairs, one member of the pair showed gene loss of the selected HLA-A allele whereas the other member did not. Assuming that the mutations occurred in postthymic lymphocytes, the data provided a population best-estimate of 3.4% (2 × 2/119) for the proportion of HLA-mutant lymphocytes which are "siblings."

DISCUSSION

Study of the number and nature of mutant cells in a population of cells has the aim of making inferences concerning the number and nature of initial mutations affecting that population. There are several potential sources of bias in such inferences although their importance in practice is often unclear. Mutations may not produce any phenotypic change; such mutations will not be detected by any system based on phenotypic selection but they are unlikely to be of biological importance. Mutations, particularly point mutations, may produce a phenotypic change but one which is not detectable by the selection system being used. A proportion of HPRT mutations may be missed for this reason. Mutant cells may not be recovered in the *in vitro* selection system owing to the nature of the mutation which has occurred. Large deletions or recombinations, or chromosomal hemizygosity or homozygosity may lead to such extensive genetic change that the mutated cells may die or proliferate poorly. However, such extensively altered cells are also likely to die or proliferate poorly *in vivo* and are therefore probably unlikely to be of biological importance. Finally, the phenotypic change resulting from the mutation may not be neutral but may lead to an *in vivo* selective disadvantage to the cell. This may occur for HPRT-mutant lymphocytes but it is not known whether or not it occurs for HLA-mutant lymphocytes.

Kinetic and statistical factors may also influence the number of mutant cells recovered. Lymphocytes proliferate *in vivo* and a proportion of HPRT-mutant lymphocytes have been identified as clonal descendants of a single ancestral lymphocyte, usually postthymic, in which a mutation had occurred (8). Mutant frequency is conventionally expressed as a proportion and both the numerator and denominator lymphocytes of the statistic are subject to clonal expansion. Consequently the calculated value for mutant frequency should not be biased by the existence of clonal expansion, and should be the best estimate for mutation frequency, at least at the population level. However, clonal expansion does lead to skewing in the distribution of mutant frequencies and very high values can occur in occasional individuals and lead to difficulty in interpretation. The present data on clonality, which was detected at a population frequency of only 3.4%, suggest that it is less important for the HLA system than for the HPRT system. This may well reflect the greater pool of HLA mutants present *in vivo* which would tend to lessen the dominating effect of any expansion of a single clone *in vivo*. It remains possible that clonality of HLA mutants may be a significant phenomenon in a few individuals, but to determine this will require much more data.

The data for the numbers and types of mutations at the HLA locus obtained in the present study can be compared with previous data for the HPRT locus (6, 7, 12, 13). For the HPRT

gene the measured mutant frequency at age 36 is approximately 6×10^{-6}. Emerging data suggest that approximately 15% of mutations are associated with gene deletion and the majority of the remainder are due to point mutation or microdeletion. Based on these data the absolute frequency of HPRT mutations due to deletion is approximately 1×10^{-6} and that of mutants not showing any abnormality on Southern blotting is approximately 5×10^{-6}. The values for HPRT mutants of these types are similar although slightly lower than those for HLA-A mutants as shown in Table 5. These differences may be real, reflecting different mutability at the two loci, but they may well be explained by secondary factors such as different stringencies of the two mutation assay or *in vivo* selection against HPRT mutants, which have been discussed above and previously (10). Even if there is a true difference in mutability between the two loci, it must be small and be considerably less than 1 order of magnitude.

An important phenomenon observed in the present study was mitotic recombination which resulted in HLA-A gene loss in 29.9% of mutants. Although not strictly a mutation in the narrow sense, mitotic recombination followed by segregation will lead to heritable alteration of the genetic material of a cell, and it can thus be regarded as a mutation in the broad sense. Mitotic recombination has previously been difficult to document in the normal cells of higher organisms. Although there have been a number of suggestions that it does occur (14–19), at least in tumor cell lines, the present data clearly show that it is a common phenomenon in normal human T-lymphocytes. Until other cell types are studied, there will remain some reservation as to the generality of the phenomenon, as it is conceivable that general gene recombination is unusually frequent in lymphocytes owing to the activity of the recombinational mechanisms responsible for immunoglobulin and T-receptor gene rearrangement. The observation of a significant difference between individuals in the frequency of mitotic recombination is intriguing and is presently being analyzed by family studies.

Although only a limited number of polymorphic loci were studied, the pattern of loss of heterozygosity as shown in Table 4 suggests that the crossing-over point for recombination occurred fairly randomly along the short arm of chromosome 6. However, the observation that approximately 13% of the crossing-over points were located between PCH6 and HLA-A could indicate some element of nonrandomness, either in crossing-over or in mutant recovery, and there are two mechanisms which theoretically could lead to this. Firstly, the extensive homologies around the HLA genes could predispose to recombination in this region. Secondly, mutants resulting from crossing-over close to the centromere would develop homozygosity for many genes and would therefore be more likely to be eliminated as the result of expression of deleterious recessive genes.

These results are important for an understanding of the molecular genetics of normal cells as they provide information on the number and type of mutations which occur in humans *in vivo*. They are also important for an understanding of the molecular genetics of carcinogenesis, as they provide a link between the mutational changes in normal cells and mutational changes in cancer cells. Mitotic recombination is clearly important as an etiological mechanism in retinoblastoma and, by inference, in other tumors characterized by loss of heterozygosity. In a study of 33 cases of familial retinoblastoma Nordenskjold and Cavenee (3) found that loss of the remaining wild-type retinoblastoma gene was due to chromosomal hemizygosity in one case, to mitotic recombination in four cases, and to either chromosomal homozygosity or mitotic recombination in 19 cases. Our results suggest that chromosomal hemizygosity or homozygosity is uncommon in normal cells, at least for chromosome 6, perhaps because the resultant extensive gene hemizygosity or homozygosity is likely to lead to cell death owing to expression of deleterious recessive mutations. However, the high frequency of mitotic recombination we have observed at the HLA-A locus suggests the hypothesis that mitotic recombination is common at many gene loci and becomes important for carcinogenesis as a result of affecting loci important in cell growth and differentiation. Study in normal cells of the mechanism of mitotic recombination and the factors which affect its frequency will thus provide information very relevant to carcinogenesis.

ACKNOWLEDGMENTS

We thank Drs. P. Board, H. Orr, P. Leder, and T. Mak for the supply of DNA probes.

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