silization. The thermolytic degradation products of flavonoids under various conditions of *p*H may provide a valuable thermometric tool in geochemical studies of relatively recent sediments.

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4. Bentonite clay was extracted in a Soxhlet apparatus with a mixture of benzene and methanol (3 : 1 by volume). Extracts after 50 hours were gas chromatographed to determine the presence of contaminants.
5. A total of 91 permutations of temperature (from 60° to 180°C in 10° increments) and *p*H (from 5.5 to 8.5, at increments of 0.5 unit) were performed with each standard compound. Preliminary experiments indicated that ranges of 80° to 130°C and *p*H = 6.5 to 8.5 were sufficient to determine the rate and nature of thermolytic alterations.
6. High-resolution mass spectra were obtained on a Perkin-Elmer GEC-AEI MS 904 mass spectrometer. Mixtures of nonvolatile components derived from simulation experiments were separated on Elmer-Perkin preparative (model F-3D) and analytical (model 154) gas chromatographs (the latter incorporates a 1.5 percent SE-30 column).
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17 February 1977

HLA Variants of Cultured Human Lymphoid Cells: Evidence for Mutational Origin and Estimation of Mutation Rate

Abstract. *Variants of a diploid lymphoid cell line that show a loss of HLA-B27 antigen occur randomly in time and independently of exposure to the alloantiserum used for their isolation. From these and previous findings of variant stability, inducibility by mutagens, and the absence of linked variation, we conclude that most HLA variants arise by mutation. The mutation rate for HLA-B27 loss is* 8×10^{-7} *per cell per generation.*

A variety of methods has been proposed for assessing the carcinogenic and mutagenic potentials of environmental agents (*1*), but few methods have been described for measuring mutations directly in human somatic cells. We have developed a selective system to isolate variants of HLA, the major human histocompatibility complex (MHC), in cultured lymphoid cells (*2*). The MHC is a genetic region of higher organisms about two map units in length containing several loci coding for cell surface alloantigens with functions in self-recognition and defense (*3*). These alloantigens are potentially powerful markers for studying mutagenesis in human cells and for defining the mechanisms which give rise to somatic cell variants. Somatic genetic approaches in turn can be used to study the genetic organization of the MHC and the structure and function of its gene products. We have now isolated and partially characterized over 100 such variants at the HLA A and B loci in two lymphoid lines. Previous evidence excludes certain possible mechanisms of HLA variant formation, and suggests that the variants arise from genetic changes (*2*, *4*–*6*), but the evidence for genetic changes has not been definitive, limiting the usefulness of HLA variants in immunogenetic studies.

An important feature of genetic variation is that it occurs randomly in time and independently of exposure to the agents used for selective isolation of the variants. Determining whether antigenic variants are genetic in origin is important because exposure of cells to antiserum directed against cell surface components, such as that used for selection of HLA variants, can under certain circumstances induce epigenetic changes in antigenic expression (*7*). We now report evidence from fluctuation analysis and reconstruction experiments that HLA variants occur randomly in time and independently of exposure to alloantiserum. From the fluctuation data we have also obtained an estimate of the mutation rate at an HLA locus. These and other quantitative studies of alloantigenic variant formation (*6*) have been facilitated by development of a system in which variants can be isolated by a single exposure to selective conditions (*2*).

Cell line T5-1, an established human diploid line of B lymphoid cell origin (*8*), was used for these studies. It is heterozygous for the linked loci HLA A and B and phosphoglucomutase 3 (PGM3); its HLA haplotypes are Al-B8 and A2-B27 (*6*). Wild-type clones for determining the rate of spontaneous mutation were isolated by plating 3000 T5-1 cells in nutrient agarose; 10 to 14 days later, colonies were picked with a Pasteur pipette, transferred to liquid medium, and grown to 5×10^8 to 2×10^9 cells. Just prior to selection, the modal cell volume and volume distribution of each clone were de-

Table 1. The number of resistant cells in different wild-type clones.

Mean number of cells per clone	Number of cells per sample	Number of samples	Resistant cells per sample*	Mean	Variance†	Ratio of variance to mean	χ^2	*P*	Mean number of resistant cells per clone‡	Mutation rate§
			Experiment 1							
1×10^9	4×10^6	10	9 0 28 0 0 0 2 0 0 0	3.9	76.1	19.5	183.	<.0001	9,750	1×10^{-6}
			Experiment 2							
2×10^9	4×10^6	8	0 2 6 0 4 2 0 8	2.7	6.3	2.3	23.1	<.005	13,500	7.5×10^{-7}
			Experiment 3							
5×10^8	4×10^6	5	0 0 0 1 8	1.8	10.4	5.7	27.1	<.001	2,250	6×10^{-7}

*Each number is for the resistant cells (measured as colonies) from a sample of 4×10^6 cells from a wild-type clone. †Corrected for sampling. ‡Calculated from the average number of resistant cells per sample times the (1/sampling fraction) × (1/cloning efficiency). The average cloning efficiency was 0.10. §Number of mutations per cell per generation [calculated from equation 8 in (*18*)].

Table 2. The number of resistant cells in different samples from the same culture.

Number of samples	Number of cells per sample	Number of resistant cells added*	Mean number of resistant cells per sample	Variance	Ratio of variance to mean	χ^2	P
			Experiment 1				
7	1×10^6	0	1.14	0.81	0.71	4.26	.6
			Experiment 2				
15	1×10^6	63	5.93	3.78	0.64	8.92	.5
			Experiment 3				
16	1×10^6	83	7.75	7.27	0.94	14.06	.5

*In experiments 2 and 3, small numbers of cells from the resistant subline 5.11.5 were added to the T5-1 culture prior to sampling to ensure that there would be sufficient resistant cells for counting; indicated are the number of 5.11.5 cells added per 1×10^6 T5-1 cells.

termined by means of a Coulter counter and size plotter and compared to a known diploid stock of T5-1 to ensure that the clones were diploid. (In some cases, the cellular DNA content of the clones was also compared to a diploid T5-1 stock culture by single cell microfluorometry. Tetraploids could readily be distinguished from diploids by either method.) Selection was performed as described (*2, 9*) with an absorbed monospecific HLA-B27 alloantiserum (Associated Biomedic Systems 5200) and complement on four replicates of 1×10^6 cells from each wild-type clone, after which the cells were plated in nutrient agarose. After 14 days of incubation, clones which had survived selection were counted, picked, grown in liquid medium, and tested on two or more occasions for resistance to cytotoxicity by the selecting antiserum and complement. A clone was considered resistant if its cytotoxic titer (the reciprocal of the dilution of antiserum which killed 50 percent of the cells) was consistently one-fourth or less that of wild-type cells. A resistant clone was considered equivalent to a resistant cell in the selected population.

The results of three fluctuation experiments are shown in Table 1. In each experiment the number of resistant cells varied widely from wild-type clone to clone. If this were due solely to sampling error, the number of resistant cells per wild-type clone should have a Poisson distribution, and the variance in the number of resistant cells per wild-type clone should equal the mean. In fact, the variance significantly exceeded the mean in each of the three experiments. In three control experiments (Table 2), in which replicate samples were drawn from the same uncloned stock T5-1 culture, on the other hand, the average numbers of resistant cells per sample and their variances were approximately equal, indicating that no unusual sources of experimental error were associated with selection and plating. The large differences in the number of resistant cells in the fluctuation tests must therefore reflect real differences in the number of resistant cells per wild-type clone, indicating that the variants arose randomly in time and were not induced by exposure to antiserum and complement during selection. If resistant clones arise from resistant cells present in the population at the time of selection, the number of resistant clones recovered after selection should be proportional to the number of resistant cells present in the selected population. The results shown in Table 2, in which numbers of resistant cells were added to T5-1 cells in the three experiments, suggest such a proportionality. An experiment in which different numbers of resistant cells were added to T5-1 cells in a single experiment is shown in Table 3; again, the number of resistant colonies is proportional to the number of resistant cells in the selected population.

These findings are consistent with a genetic mechanism of origin of HLA variants. They are inconsistent with certain epigenetic mechanisms such as antigenic modulation, "capping," and suppression which (i) are induced by antiserum, (ii) occur in most or all cells of an exposed population, and (iii) revert spontaneously at various times after removal of the antiserum (*7*). The formation of HLA variants is distinguishable from these epigenetic phenomena not only by the fact that it is not induced by antiserum, but also by the fact that HLA variants grown under nonselective conditions after isolation retain the variant phenotype indefinitely (*2, 6*). Hyman and Stallings have used a sib selection method to show that Thy-1 cell surface alloantigenic variants of cultured mouse tumors also arise independently of exposure to antiserum (*10*).

Table 3. The number of resistant colonies resulting from selection against cell mixtures containing small numbers of antiserum-resistant cells and large numbers of antiserum-sensitive cells. Selection was carried out with B27 antiserum against 1×10^6 T5-1 cells to which were added cells of the B27-resistant subline 5.11.5. Cloning efficiencies of cells exposed to medium in place of antiserum were 0.16 and 0.15 for T5-1 and 5.11.5, respectively.

Number of cells in mixture		Number of resistant colonies*
T5-1	5.11.5	
1×10^6	0	1
1×10^6	54	8
1×10^6	162	19
1×10^6	430	91

*Average from two replicates.

Concerning the nature of the genetic changes in HLA variants, we have found that known mutagens at appropriate concentrations increase HLA variant frequency in T5-1 cells by greater than two orders of magnitude (*6*). Of some 30 variants induced by the alkylating agent ethyl methanesulfonate and the acridine derivative ICR-191, none have genetic lesions which extend as far as 0.8 map unit, the distance between HLA A and B. These mutagen-induced variants are probably single gene mutants. The spontaneously occurring variants examined thus far have similarly restricted genetic lesions (*11*), arise at random (this report), and most probably are also single gene mutants. In addition, some but not all variants, both spontaneous and mutagen-induced, have residual reactivity for antiserums of the selecting specificity (*6*) or new antigenic determinants detectable with absorbed heteroantiserums (*5*). We believe it likely that these changes result from missense mutations in HLA structural genes, but chemical characterization of the altered gene product will be necessary to confirm this in any given variant. It also cannot be excluded that some variants might arise from mutations at other than the structural genes or by mechanisms which do not alter the nucleotide sequence.

The close correlation recently found between the mutagenic and carcinogenic properties of environmental agents (*12*) demonstrates the need for systems capable of measuring mutagenesis and analyzing mechanisms of variation in human somatic cells. Other than in the studies reported here, however, the mutation rate has been measured for only one locus, the X-linked structural locus for hypoxanthine-guanine phosphoribosyltransferase (HGPRT), in human diploid cells (*13*), and for about ten loci in all mammalian cell lines (*14*). In a number of the latter, the cell lines used were hyperdiploid, and chromosome segregation and other nonmutational mechanisms

were not excluded as causes of variation. Chromosome segregation can occur at high rates in hyperdiploid cells (*15*), and conclusions based on studies of variant formation in such cells may not be applicable to diploid cells. Heterozygous markers linked to the selected marker are helpful in distinguishing between chromosomal segregation and intrachromosomal mechanisms of variation and are essential for detailed characterization of intrachromosomal mechanisms of variation. HLA markers and selective systems are particularly useful in this regard because there are four closely linked HLA alloantigenic loci which in turn are linked to the structural loci for the polymorphic enzymes PGM3 and glyoxalase I.

The mutation rate we have obtained for HLA B-27, approximately 1×10^{-6} per cell per generation is close to that found for HGPRT in diploid human fibroblasts (*13*). The gametic mutation rate for an H-2 gene in mice (H-2 is the mouse homolog of HLA) obtained by a grafting procedure was 5×10^{-1} per gene per generation (*16*). For the mutation rates in gametes and somatic cells to be compared, the rate for gametes must be divided by the number of doublings preceding formation of mature gametes, which DeMars has estimated to be 10^2 to 10^3 (*17*). Application of this correction factor results in good agreement between the mutation rate in vivo for H-2 and the rate in vitro for HLA. This agreement may be fortuitous, however; it could reflect offsetting differences related to the different methods of mutant detection, errors in the assumptions made in calculating the correction factor, and possible intrinsic differences in the mutability of germinal as opposed to somatic cells or of particular genes. These questions should be resolvable by further studies of somatic and germinal mutation rates. Systems such as the HLA system which allow for evaluation of mutagenesis and for quantitation and characterization of mechanisms of variation in diploid somatic cells should be valuable in this regard as well as in assessing the risks of environmental agents to humans.

Note added in proof: Since submission of this manuscript we have isolated two variants of T5-1 with B8 antiserum in which both B8 and A1 are affected. It thus appears that not all HLA variants of T5-1 are single gene mutants.

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19 AUGUST 1977

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18 February 1977; revised 5 April 1977

Polarity Reversal in Nerve-Free Hydra

Abstract. *Hydra experimentally depleted of all nerve cells and nerve stem cells (interstitial cells) demonstrate normal polarity reversal. Thus hydra epithelial cells are capable of controlling complex developmental patterning phenomena.*

The control over developmental patterning in hydra is frequently ascribed to neurosecretory cells (*1*). Hydra's developmental gradients, for example, have been postulated to be due to gradients in nerve cell density along the column (*2*) as well as to the diffusion gradients of the neurosecretory materials that act as developmental hormones (*3*). Regeneration, cell determination, budding, cell cycle duration, and cell differentiation are all considered to be influenced by neurosecretory cells (*4*). However, we are finding that experimentally denervated hydra grow, bud, and develop rather normally (*5, 6*). To determine the developmental capabilities of denervated hydra we have analyzed the kinetics of polarity reversal in these nerve-free animals. Polarity reversal is one of the most complex developmental traits analyzed in hydra and therefore represents the most severe test of developmental capability in nerve-free hydra.

An isolated segment of hydra's column regenerates with strict polarity. A hydranth (hypostome and tentacle whorl) regenerates from the end that was

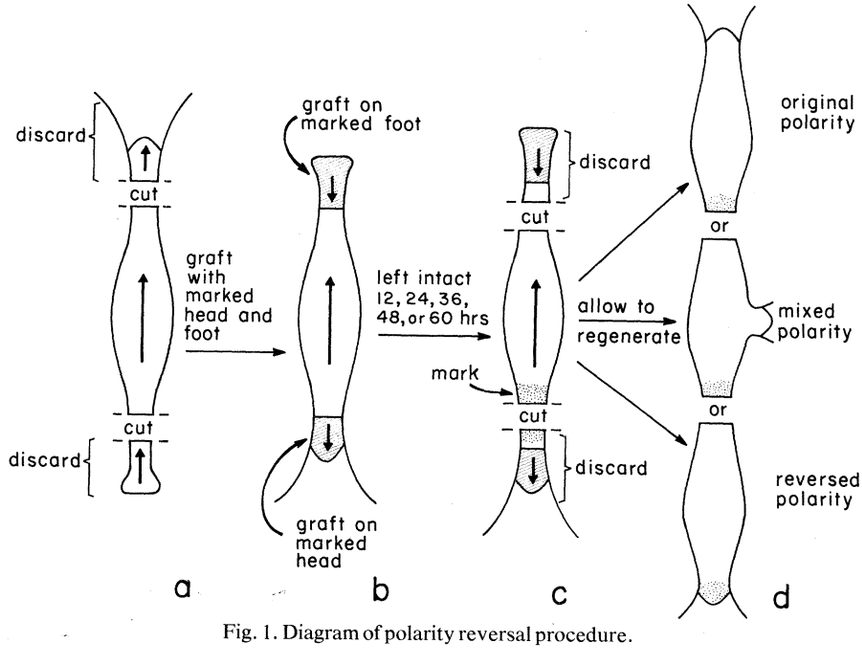

Fig. 1. Diagram of polarity reversal procedure.