Sequential Changes in Spleen Cell Chromosomes during Friend Virus Leukemia

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SUMMARY

Infection of BALB/c mice with varying doses of Friend virus resulted in the appearance of abnormal chromosome numbers in spleen cells. Polyploid cells were found following infection with all doses of virus used. Statistically, however, polyploidy showed no correlation with virus dose, time after infection, or their combined interaction. A total of 0.9% of all infected metaphase figures examined showed the hyperdiploid number of 41 chromosomes. This hyperdiploid number was not clustered at any stage of the disease and was not higher than that of the control mice. The number of abnormal cell configurations in infected animals did differ significantly from noninfected animals throughout the disease process, but no 1 normal diploid number predominated. A significant correlation was found between the number of secondary chromosomal constrictions and the progression of the disease. As the spleen weight increased, the number of secondary constrictions per metaphase figure increased. There was an inverse relationship between increased spleen weight and the number of normal 2N cells with no secondary constrictions. Since an increased number of these secondary constrictions appeared early in the disease process even with a low dose of Friend virus, this change is considered to have significant mutational importance.

INTRODUCTION

The rapid and extremely proficient transformation of normal spleen cells to malignant cells during infection of susceptible mice with FV² (3, 5) makes this system ideal for studying virus-induced chromosomal aberrations. This system provides a transition from the normal to the neoplastic state under controlled conditions with a reproducible interval between inoculation and the appearance of histologically recognizable tumors. Previous reports (19, 20) have documented the presence of chromosomal abnormalities in FV disease but have not included critical information concerning either the relationship of these changes to virus dose or the rate of these changes through time. Since these previous reports on FV-induced chromosome abnormalities, FV has been further purified and its virulence increased by using selected strains of mice, to the point where a large dose of virus results in the death of almost all animals within 6 weeks following infection. The present study was limited to 5 weeks and not extended for the longer time period used in earlier reports (19, 20). In these latter studies, animals had a longer latent period before the viral leukemia resulted in animal death.

This paper reports the quantitation of sequential changes in spleen cell karyotype following the inoculation of 76 BALB/c mice with serial dilutions of FV. Secondary constrictions have been observed as a feature of normal mouse spleen cells (10). The present paper indicates a significant difference between the frequency of secondary constrictions in normal cells and those from infected mice. With the well-established parameters of the numbers and type of chromosome abnormalities in FV infection with known virus doses, the relative role of these abnormalities in the leukemic process can now be determined by inhibiting the disease with a variety of antineoplastic agents. On the basis of these findings, it is hoped to establish a quantitative relationship between the frequency of secondary constrictions through the disease process and the frequency of these same aberrations during drug-induced remissions of leukemia.

MATERIALS AND METHODS

Mice. Female BALB/c mice were obtained from Cumberland View Farms, Clinton, Tenn. The BALB/c strain was chosen because of its demonstrated susceptibility to FV, 96 to 98% of infected mice showing evidence of FV disease (3). The mice were kept in groups of 6 to 8 during the experimental period and given food and water ad libitum.

Virus. The origin and preparation of the stock FV pool used in this investigation has been previously reported (1, 2). The virus pool of titer 10⁴.⁰ i.e., that dose which infects 50% of the animals per ml (2, 12), was maintained at −65° as a 20% suspension of infected BALB/c mouse spleens in sucrose stabilizer.

Spleen Cell Chromosome Preparations. A modification of the method of Fox and Zeiss (4) was used in preparing spleen cells for chromosome analysis. Ninety min before animals were sacrificed they were given i.p. injections of 0.75 ml of 0.2% Colcemid (Ciba Corporation, Summit, N. J.). A total of 6 to 10 slides was made from each suspension of isolated, fixed spleen cells. Cells were affixed to the slides by a freeze-flame dry technique.

Cells were stained with synthetic acetoorcein, dehydrated, and mounted in Euparol Vert. After initial scanning at X 200, a total of 30 metaphase figures for each animal was critically examined under oil immersion at X 1250. Cells which appeared under low magnification to be complete, with nonoverlapping, well-spread chromosomes, were selected for
counting. For several mice, 30 countable cells could not be found.

The chromosome data included counting the number of chromosomes, assessment of polyploidy, and counting the number of abnormalities found in each metaphase cell. The primary abnormality was achromatic zones or secondary constrictions (18) noted near the centromere. The change in the number of these secondary constrictions was noted for virus dose and elapsed time. No other chromosome abnormalities observed appeared with any appreciable number or in any consistent pattern in any of the mice tested.

Questionable figures were examined independently by 2 people. Cells, once chosen, were not rejected because of chromosome number, size, shape, or degree of staining. Selected cells were photographed with Kodak high contrast copy film.

Experimental Design. Groups of 30 mice received i.p. 0.2 ml of either a 10\(^{-1}\), 10\(^{-3}\), or 10\(^{-5}\) dilution of the stock virus prepared in sucrose stabilizer. Uninfected mice with and without Colcemid treatment served as controls, and 14 of these control animals were sacrificed after the same time interval as experimental mice. A total of 6 Colcemid-treated, virus-infected mice from each dilution was sacrificed weekly for 5 weeks.

During the experiment, control mice did not demonstrate any deviation from the normal spleen weight of an adult mouse. In contrast, the infected mice developed splenomegaly through the course of the experiment, giving a reliable indicator of the degree of infection.

Statistical Analysis. Standard methods for computerized statistical analysis were used for analysis of variance (Anova) in the different data categories. Where needed, various transformations of the data were carried out to fulfill the assumptions required for analysis of variance techniques used. Other bivariate analysis correlations were examined by computer routines found in the book by Sokal and Rohlf (16).

### Results

**Spleen Weights.** All mice infected with the Friend leukemia virus exhibited hyperplasia of the erythroid elements of the spleen with a resultant splenomegaly that increased progressively during the 5-week course of study. The average spleen weights with their standard error for all control and infected animals are shown in Table 1. The spleen weight increases proportionately both with time and with virus dose when compared with the control values. The analysis of variance for the transformed spleen weights showed significant interaction at the 99% confidence level for dose, weeks, and dose X weeks.

**Chromosome Numbers.** Among the 2149 metaphase cells examined from infected animals, 21 or 0.9% exhibited 41 chromosomes. This is contrasted to 3 of 131 cells (2.3%) among the control animals showing this abnormality. Only 3 cells from the infected animals and none from control animals fell between counts of 41 chromosomes and the cells which were interpreted to be polyploid. In many instances, a chromosome number of less than 40 was noted (Table 1).

A significant difference was shown between the chromosome numbers in the various virus doses and for the different time intervals. This difference was established through analysis of variance (Anova) of cells from infected animals that did not have a count of 40 chromosomes. The raw data were prepared for this analysis by adding a constant (0.01) to remove zero values and then transforming the data to 

\[
\theta = \arcsine \sqrt{p + 0.01}, \quad \text{where} \quad p \text{ is the proportion of cells not having a count of } 40 \text{ chromosomes.}
\]

The arcsine and square root transformations were utilized to satisfy the underlying assumptions required for analysis of variance (16). Significance was shown with respect to dose, time following infection, and the interaction between dose and time.

Similar analysis was made on the proportion of diploid spleen cells from infected animals with normal mitotic

<table>
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<th>Table 1</th>
<th>Metaphase chromosome numbers and average spleen weights per control animal and those infected with various FV dilutions</th>
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<td><strong>Dilution</strong></td>
<td><strong>Time following infection (wk)</strong></td>
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\(^a\) Mean ± S.E.  
\(^b\) No. in parentheses, average number per animal.
patterns. Only at Week 2 did the proportion of normal cells differ significantly between the 3 virus dilutions used and then only between the highest and lowest doses of virus. The proportion of cell configurations showing abnormal numbers differed significantly from the controls after the 1st week. Throughout the experiment, the infected animals always exhibited a greater proportion of abnormal cells with respect to diploid chromosome number.

Polyploid cells were observed during the course of infection with all 3 doses of virus, but none were observed in any of the control cells. In the infected animals the frequency of polyploidy, as compared with the diploid number, showed no dependency of the observed polyploid state upon dose, week, or their combined interaction. However, in consideration of the dependency of the observed polyploid state upon dose, week, and in these cells an average number of secondary chromosome constrictions was obtained for each animal where data permitted. Those cells with abnormal chromosome counts were included within the analysis; however, those that contained no constrictions were ignored. Table 2 shows the average number of constrictions per cell with the standard error among those cells demonstrating constrictions. This table also includes the proportion of such cells for each successive week of the experiment. An increase was noted for each of the virus dilutions through time with respect to the proportion of cells showing 1 or more secondary constrictions. The percentage of cells with constrictions and the average number of constrictions per cell were greater in virus-infected animals, with the sole exception of Week 1, 10⁻³ virus dose. As the spleen weight increased so did the number of spleen cells with these secondary chromosome constrictions and the average number of secondary constrictions found in each cell.

A correlation of spleen weights and the average number of constrictions per metaphase figure in those cells demonstrating constrictions is shown in Chart 1. The 2 variables Y₁, the spleen weights transformed to \( \sqrt{x + 0.5} \), and Y₂, average number of constrictions per figure, correlate in a population ellipse at the 95% confidence level. This ellipse demonstrates that, as the spleen weight increases along the F-E axis, the average number of chromosome constrictions in each cell increases proportionately to the limits designated by the boundaries of the ellipse.

A similar correlation was carried out with the use of the variables Y₁, the spleen weight transformed to \( \sqrt{x + 0.5} \), and Y₂, the arcsine \( \sqrt{p + 0.01} \), where p is the proportion of normal diploid cells with no constrictions among those examined. Chart 2 exhibits the plotted points of the 95% confidence ellipse demonstrating the strong and significant correlation coefficient of these 2 variants. This analysis indicates that, as the spleen weight increases, the probability of finding metaphase figures with no secondary constrictions decreases.

DISCUSSION

Statistically valid alterations from the normal diploid chromosome number in FV-infected BALB/c mice were found.
Chromosome Changes in FV Leukemia

Coordinates A to H show the 95% confidence ellipse limits.

Chart 1. Correlation of spleen weight and average number of chromosomal constrictions in metaphase figures of spleen cells containing chromosomal constrictions during FV leukemia. Coordinates A to H show the 95% confidence ellipse limits.

Chart 2. Correlation of spleen weights to proportion of normal diploid cells among mitotic figures examined during FV leukemia. Coordinates A to H show the 95% confidence ellipse limits.

in this study. However, it does not seem possible to attach any absolute significance to these differences as no consistent abnormal number or pattern was obtained. Only a small number of infected mice, 0.9%, exhibited the hyperdiploid number of 41 chromosomes, and this abnormality was neither clustered in any virus dose nor at any specific time. This is in marked contrast to the increase in the number of cells with 41 chromosomes during the Friend disease reported by other authors (19, 20). Tsuchida and Rich (19), using 3 ICR/Ha Swiss mice, reported that 9% of 147 cells examined from these animals late in the disease period showed the 41-chromosome number. Wakonig-Vaartaja (20) reported that 1 of 3 mice infected with FV had cells with a high incidence of 41 chromosomes. In this mouse, 42% of the 50 cells were analyzed to be carrying this higher number of chromosomes. The strain of mice used in this latter experiment was not reported. The difference between these 2 studies and the present study might have its basis in the strain differences between mice or between virus stocks. Each experiment reporting the high incidence of 41 chromosomes observed this result in very few animals. This observation may have been due to a chance fluctuation not observed in the present experiment. Tsuchida and Rich observed their finding in the “late disease group” comprised of animals infected for 50 to 65 days. The present study comprised a 35-day period.

Polyploid cells were not found in increased numbers except in the 10⁻¹ virus dose and then only after the 1st 2 weeks of infection. The sporadic finding of this alteration, as with the occurrence of cells with 41 chromosomes, would be consistent with the hypothesis that these abnormalities result from, rather than initiate, the leukemic process. The increase in the proportion of cells with these abnormal numbers would be a secondary feature of these spleen cells that previously had been transformed by FV.

The finding of an increased number of secondary constrictions early in the disease process and with all 3 doses of FV is of interest. There was a positive increase in secondary constrictions as the spleen weight increased. This correlation is in agreement with the results of Tsuchida and Rich (19). They showed that infection with FV resulted in a 2- to 3-fold increase in the number of secondary constrictions late in the disease process (50 to 65 days after inoculation). Although we were never able to find this large an increase in the number of secondary constrictions, the proportion of cells that showed 1 or more such constrictions increased with time during the experiment. Along with an increase over control values in the average number of constrictions per cell, the total infected cells that showed this abnormality increased to include almost all of the cells studied by the end of the experiment.

Recent reports have demonstrated the presence of or an increase in secondary constrictions in a wide variety of oncogenic processes. Miles and O'Neill (11) have reported secondary constrictions in tumor cells that showed no other cytological abnormality. A marked increase in secondary constrictions was found by Ito et al. (7) in 2 human cell lines derived from embryonic cultures exposed to human leukemic fluid in vitro. These cell lines showed not only this marked increase of secondary constriction but also the presence of a herpes-type virus particle. This herpes-type particle is morphologically similar to the agent known to exist in Burkitt’s lymphoma cell lines, and a similar increase in secondary constrictions has been reported in these lymphoma cells (6, 8).

Secondary constrictions have been reported to be complete breaks in DNA (14), but these breaks apparently do not separate from the chromosome because of the presence of the chromosome matrix. This type of chromosome aberration was observed early in the disease in our studies, even with a low dose of FV, and the aberrations progressively increased through the course of the study. If secondary constrictions are interpreted to be breaks and since they appear early in the spleen cell transformation process, they would likely be considered of great mutational importance (13). We interpret the secondary constriction to be a facet of FV disease that produces DNA breaks. Once the initial lesion is produced, other cellular factors may influence the relative observed recovery of these constrictions. Since secondary constrictions...
are observed in control animals, some aspect of cell differentiation or heterochromatization may serve to fix these aberrations in FV-infected cells. Alternatively, selection of cells bearing secondary constrictions may occur during the course of the leukemia. The observed quantitative relationship between time-dose-aberration appears substantial regardless of the specific interpretation applied to explain the process of increasing the aberration through the course of the disease.

The frequency of chromosomal abnormalities found in various human leukemias has a tendency to return closer to normal values during periods of spontaneous or drug-induced remissions (15). To determine whether the increased secondary constrictions that are induced by FV infection would revert to near normal numbers, we retarded the leukemic process with 7,12-dimethylbenz(a)anthracene. Previous reports have shown this strongly carcinogenic hydrocarbon to be an effective inhibitor of all parameters of FV leukemia when used in small weekly doses (1, 2). Although the 7,12-dimethylbenz(a)anthracene in itself can induce a range of chromosomal abnormalities (9, 17), our initial studies show a significant retardation in the appearance of secondary constrictions in spleen cells in FV-infected animals treated with this carcinogen.

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REFERENCES

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