SYMPOSIUM: *Etiology of Chromosomal Abnormalities*

The Role of Viruses in the Etiology of Chromosomal Abnormalities

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THE ROLE OF viruses in the etiology of chromosomal abnormalities is receiving considerable attention and stimulating many investigations and a growing literature. In this review, I will not attempt an exhaustive survey of all of the literature and all of the current work but rather will present a review of the work we have been doing and cite some pertinent references of others in an effort to illustrate the general problems. All of the work that will be presented has been done in conjunction with several collaborators, including Professor Albert Levan of the University of Lund.

A search for the etiology of cancer was the origin of our interest in chromosomal abnormalities associated with viruses. It is well known that around the turn of the century two theories of carcinogenesis were put forth, the somatic mutation theory that is attributed to Theodore Boveri and the viral theory that arose primarily from the work of Peyton Rous when he found that a chicken cancer was produced by a virus. Since that time, adherents of these two hypotheses have usually been in opposing camps. We thought it pertinent to look for common ground between the two hypotheses and decided to see what effects viruses had on chromosomes.

EFFECTS OF VIRUSES ON CHROMOSOMES

Rous Sarcoma Virus

For this we utilized initially two systems. One of these was a strain of the Rous sarcoma and the other was measles. First, some of the observations that have been made with the Schmidt-Ruppin strain of the Rous sarcoma will be presented. The original and usual strain of the Rous sarcoma described by Dr. Rous produced sarcomas in chickens and various other fowls but not in other animals. The Schmidt-Ruppin strain, found by Dr. Schmidt-Ruppin (1959) and studied extensively by one of our colleagues, C. G. Ahlström (Ahlström and Jonsson, 1962; Ahlström, Jonsson, and Forsby, 1962), is a strain that, in addition to producing tumors in chickens and fowl, produces tumors in a large variety of mammals.

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Tumor material induced by this virus, when taken from a chicken and injected into mice, rats, hamsters, guinea pigs, and rabbits, gives rise to tumors that can be passed serially from one generation of the mammal to the next or in a "zig-zag" fashion from the mammal back into the chicken. A similar strain also produces tumors in monkeys (Munroe and Windle, 1963), and to my knowledge any animal that has been injected in the newborn period has responded with the production of tumors.

Our initial mammalian chromosome studies with this virus were in the rat. The rat was selected because of the relative ease with which good chromosome preparations were obtained and because some of the rat chromosomes have distinctive morphology. Rat tumors were studied *in vivo* both in the zero passages and after serial transplantation and *in vitro* by planting a zero passage tumor in tissue culture and passing this serially. Diploid rat embryo cells in tissue culture to which the virus was added were also studied (Nichols, 1963).

In vivo tumors were induced in the rats by injecting chicken material, and when a tumor arose it was removed and chromosome preparations made directly. In the zero and early passages done in this way, it was frequently observed that the cells comprising a tumor had a normal karyotype. However, in some of the tumors, there were changes in number of chromosomes around the diploid level but no new chromosome types were identified (Nichols, 1963). When the tumors were passed for several generations in the rats and were examined again, new chromosome types were found as well as changes in number. In order to have these changes in structural type of chromosome, chromosome breakage and reunion must have taken place earlier.

The next phase of the study of the rat material was to grow one of the zero passage tumors in tissue culture. The cells grew quite slowly, and it took one to two weeks before the cells formed a confluent sheet and could be passed. During this time, the chromosomes were studied and were found to have a normal diploid rat karyotype. At passage 4, the cells underwent changes characteristic of a cell transformation, including a change in cell morphology and and a rapid increase in the growth rate of the cells. When chromosome preparations were made from passage 4 material, a significant proportion of the cells had chromosome breaks, acentric fragments, ring chromosomes, large metacentrics, and probable dicentrics. After this, the cells continued to grow rapidly and took on the characteristics of an established tissue culture cell line. These cells continued to produce tumors in all injected rats, and, although the cells were no longer diploid, they did not show the striking chromosome abnormalities that were seen during the cell transformation. Most cells of the transformed line now have a normal chromosome number of 42 but exhibit subtle morphologic changes. These cells have been tested for the presence of virus, and, by the ordinary plaquing techniques on chick embryo fibroblasts or chorioallantoic membranes of embryonated eggs, we have not been able to detect any virus. However, if the cells are injected into chickens, tumors develop which have chicken chromosomes, indicating that a new tumor induction has been accomplished rather than a heterotransplant. Thus,

TABLE 1. CHROMOSOME BREAKAGE AND MITOTIC INHIBITION IN HUMAN LEUKOCYTE CULTURES AFTER ADDITION OF SCHMIDT-RUPPIN STRAIN OF ROUS SARCOMA VIRUS AT VARIOUS TIMES AFTER ESTABLISHING CULTURE

Chromosome Breakage

Exp.	Stage	Rous Cells	Rous Breaks	Rous (%)	Control Cells	Control Breaks	Control (%)
1.	metaphase	50	12	24	50	1	2
2.	metaphase	50	8	16	100	8	8
3.	metaphase	50	13	26	50	3	6
		Cells	Abnormal Anaphase	(%)	Cells	Abnormal Anaphase	(%)
4.	anaphase	50	10	20	50	1	2

Mitotic Index

Exp.	Rous at Planting Cells	Mitosis	(%)	Rous at 36 Hrs. Cells	Mitosis	(%)	Control Cells	Mitosis	(%)
1.	1000	3	0.3	1000	19	1.9	1000	31	3.1
2.	1000	2	0.2	1000	25	2.5	1000	23	2.3
3.	1000	3	0.3	1000	12	1.2	1000	14	1.4

there is either a low dosage of virus present in these cells or there is a virus in a latent or incomplete form that finds the necessary component to complete itself in the chicken.

The next approach in these studies on the rat consisted of establishing diploid rat embryo tissues in culture and treating these with the Schmidt-Ruppin virus. We made observations on the first and second divisions after the addition of virus and found frequent chromosome breaks.

In summary, chromosome breakage was associated with virus effects on rat cells *in vivo* and *in vitro* in three systems studied.

Because this virus causes tumors in a large number of animal species, studies of the effects of the virus on human leukocytes were initiated (Nichols, Levan, Coriell, Goldner, and Ahlström, 1964). Table 1 summarizes some of the early work which showed inhibition of mitosis when the virus was added at the time the cultures were established and the production of chromosome breaks when the virus was added at 36 hours, after DNA synthesis had already started. This has now been repeated in 11 more experiments (Nichols, Levan, Heneen, and Peluse, 1965). The chromosome breakage varied from 19 to 30% (Table 2).

Figure 1 is an example of the breaks produced in human leukocytes. These cells were studied for virus multiplication at the same time, and no evidence was found that the virus was multiplying or shedding virus particles into the media. These observations of chromosome changes induced by the Schmidt-Ruppin virus in human leukocytes agree with the work of Jensen *et al.* (1964), who demonstrated morphologic changes and focus formation in human skin cells inoculated with Schmidt-Ruppin virus.

Measles Virus

At this point, we were convinced of the chromosome breaking capacity of

TABLE 2. ELEVEN ADDITIONAL EXPERIMENTS SHOWING FREQUENCY OF CHROMOSOME BREAKS IN HUMAN LEUKOCYTE CULTURES AFTER THE ADDITION OF SCHMIDT-RUPPIN ROUS SARCOMA VIRUS

Experiment No.	Number of Cells	% Cells with Breaks	Number of Chromosomes with Breaks
1	115	26.1	49
2	251	24.3	77
3	76	19.7	18
4	61	19.7	17
5	113	25.7	39
6	50	26.0	18
7	194	32.0	91
8	201	29.4	91
9	226	29.6	102
10	282	25.9	127
11	50	32.0	29
Total	1619	27.0	658

the Schmidt-Ruppin virus in various cell systems, and we wondered whether this effect was restricted to cancer viruses or whether other viruses also had this potential. In order partially to answer this question, we started the study of the second system, measles (Nichols *et al.*, 1962; Nichols, 1963). The two main reasons for selecting measles virus were the ease of clinical diagnosis of measles and the occurrence of a measles epidemic at the time the studies were undertaken. Initially, a group of patients with clinical measles were studied by collecting blood on alternate days for three or four bleedings. Chromosome preparations were made, and we observed a high number of chromosome breaks for a very short time interval in these patients. The number of cells with chromosome breaks in this series of five patients varied from 32 to 70% usually on the third to fifth day after the onset of rash. This work has been confirmed by Aula (1965), Gripenberg (1963), and others but was not confirmed by Harnden (1964) or Tanzer *et al.* (1963). The reasons for these differences in observations lead to interesting speculations. Different strains of viruses may occur in different epidemics, or selection of viral mutants may occur at different times in any given epidemic. It may also be that other viruses either interfere with or enhance the measles virus effect. Neither in our studies nor those of others have virus agents coincident with the measles been sought.

In the next part of the study, a group of patients who were treated with live attenuated measles vaccine were investigated (Nichols, 1963). These were divided into four groups. The first group of patients was immune to measles by virtue of having previously had measles. The second group were susceptible patients who received Edmondston Type B vaccine; the third, susceptible patients who received a more attenuated vaccine; the fourth group, susceptible patients who received gamma globulin plus the more attenuated vaccine.

In these studies, the third group (those who were treated with the more at-

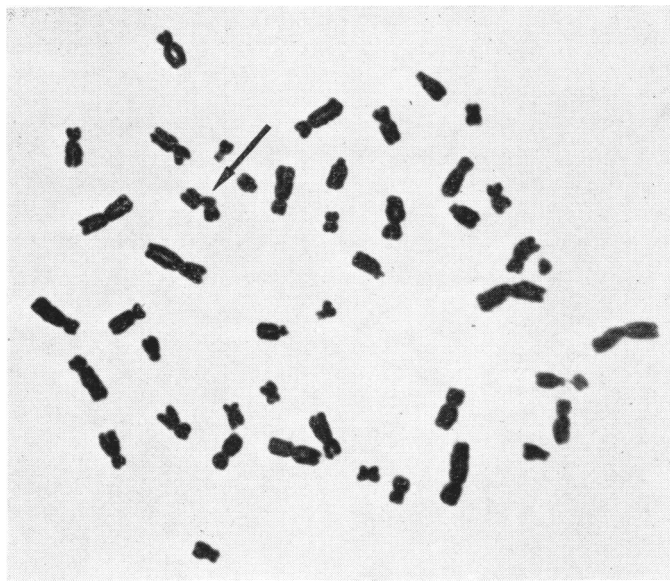

FIG. 1. Human leukocyte showing chromosome break (arrow) after addition of Schmidt-Ruppin Rous virus *in vitro*.

tenuated vaccine alone) could not be analyzed because of the quality of the slides; however, from the other preparations some generalizations could be made. First, breaks were seen with the attenuated vaccine but never in as high a percentage of cells as with clinical disease. Second, the patients who were immune by virtue of having had measles or those who received gamma globulin with a more attenuated vaccine did not tend to have breaks over the control levels. The breakage here was of the same type as seen with the clinical disease, and the type of breakage that was seen throughout the measles study fits the description by Östergren and Wakonig (1954) of the delayed isolocus break. In this type of break, one sees a range of abnormality from a secondary constriction in only one chromatid—or, as in the typical example, a secondary constriction in one chromatid with a corresponding break in the other—to a complete break in both chromatids.

After these studies *in vivo, in vitro* systems for the study of the virus were set up. In these, we utilized four tissue culture systems (Nichols, Levan, Aula, and Norrby, 1964, 1965). The first was an established heteroploid tissue culture cell line, Lu 106, that originated from human embryo lung. Human leukocytes, human diploid kidney cells, and human diploid lung cells were also used. In the studies of Lu 106, human leukocytes and human diploid kidney cells, breakage different from the delayed isolocus type was observed. This we termed "chromosome pulverization" because of the severe fragmentation of the chromosomes. It is interesting to note that in the human diploid lung cells single breaks were seen. This was also noted by Aula (1965). The Boués, studying rubella virus in different tissue culture systems, also found a difference in reaction depending on the types of cells used, but in their case

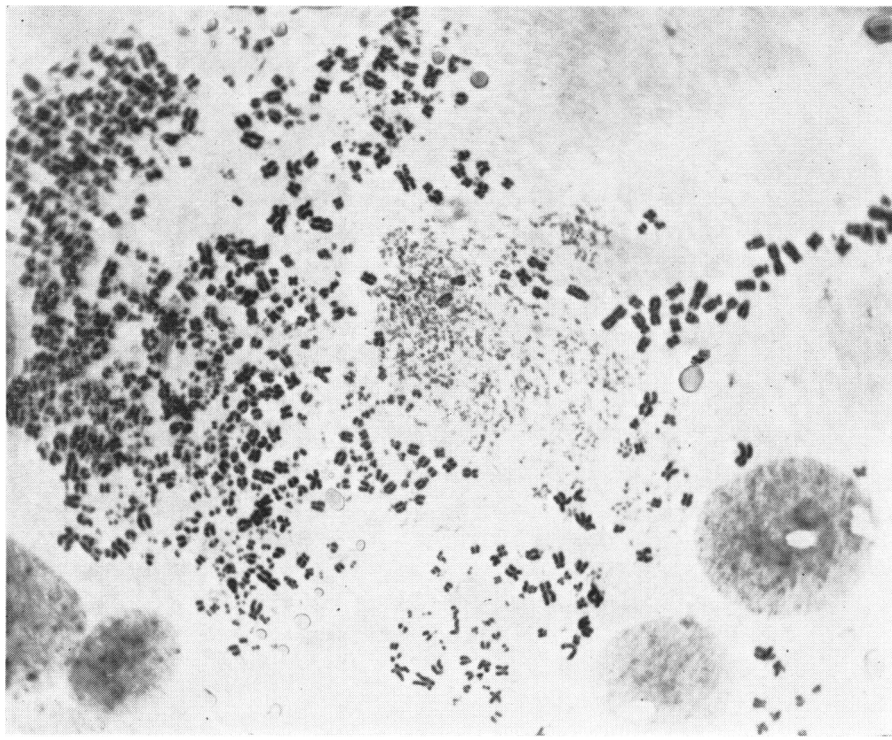

FIG. 2. Mitosis in a syncytium of Lu 106 cells after exposure to measles virus. The various nuclear chromosome groups demonstrate different stages of fragmentation and contraction (from Nichols, Levan, Aula, and Norrby, 1964).

some cells exhibited breaks while others did not, depending on the cell types used (Boué, Plotkin, and Boué, 1965).

The pulverization phenomenon occurs in cells that have fused into syncytia. These may consist of a very few or very many nuclei, and, although there is usually some degree of mitotic synchronization in the syncytia, the effects on the various groups of chromosomes frequently differ markedly. In Fig. 2, a syncytium exhibiting pulverization, some chromosomes are completely fragmented, while others seem to be relatively intact, and still others are in intermediate stages.

Harnden (1964) has reported a similar phenomenon *in vivo* in patients who have been immunized with yellow fever vaccine. Also, Stich, Hsu, and Rapp (1964) have depicted a similar process in human lung cells treated *in vitro* with herpes simplex virus. Pulverization has also been seen in bone marrow cells after *in vivo* treatment with deoxyriboside analogs by Block *et al.* (1965). During the past summer, further studies have been made in conjunction with Dr. Erling Norrby on the induction of this phenomenon by the measles virus (Norrby, Levan, and Nichols, 1965). In addition to a pool of normal infectious virus, Dr. Norrby prepared fractions of the virus that possessed either hemolytic activity or hemagglutinating activity, with little or no infectivity.

It was observed that the pulverization phenomenon took place when the

hemolytic component of the virus was added but not when the hemagglutinating component of the virus was added. Thus it would appear that the chromosome pulverization phenomenon is induced by a virus component or product, but we cannot be sure that viable virus is not necessary, since the methods used for elimination of infectivity do not completely abolish infective virus. These pulverization effects can be seen within an hour after the addition of virus or the hemolytic fraction, and this rapidity of appearance of such severe chromosomal effects and syncytial formation, coupled with the activity of the hemolytic fraction of the virus, lead us to believe that this is probably a different effect than the single breaks previously described.

Another chromosomal effect seen in this work was the result of the cell fusion and syncytial formation previously mentioned. This has been studied by Dr. Waheeb Heneen at our laboratory. These syncytia can consist of a few to a great many nuclei, and when these nuclei divide they seem to be partially synchronized. This leads to many abnormal spindle formations, especially the sharing of common centrosomes. Probably most of these cells would die and not go through further divisions and cleavages, but if some cells do survive it would result in changes in ploidy as well as the possibility of genetic recombinations resulting from the abnormal spindle mechanisms. An instance of centrosome sharing by more than one metaphase is seen in Fig. 3.

It can be said then that viruses are capable of producing at least three types of change involving the chromosomes. The first of these is the single break, the second is chromosome pulverization, and the third is cell fusion and spindle abnormalities. Although all of these are interesting phenomena, the one that in our opinion is most likely to have mutagenic significance is the single break. While most cells exhibiting any of the phenomena, including loss of significant chromosome material from single breaks, would probably die, it is possible that these breaks are an indicator for point mutations that occur in less severely affected cells. This would then be similar to X radiation, in which the number of visible breaks reflects incidence of mutation. This has led to attempts to study the mechanism of these single breaks.

MECHANISM OF SINGLE BREAKS

The first approach has been the use of radioactively labeled virus. However, at the present time, our data are inconclusive. Rapp and Hsu (1965) have utilized tritiated thymidine in diploid hamster cells that were not in synthesis period so that any thymidine incorporated was incorporated by virus particles. In these studies they found that there was no radioactivity localized to chromosomal regions. While these are interesting and elegant data, they do not completely answer the question as to whether chromosomes are involved in viral nucleic acid replication, since it may be necessary to study the chromosomes during their synthesis period to detect interchange or interaction between these two sources of nucleic acids.

Effects of Base Analogs

The second approach was based on the dissimilarity between X-ray induced

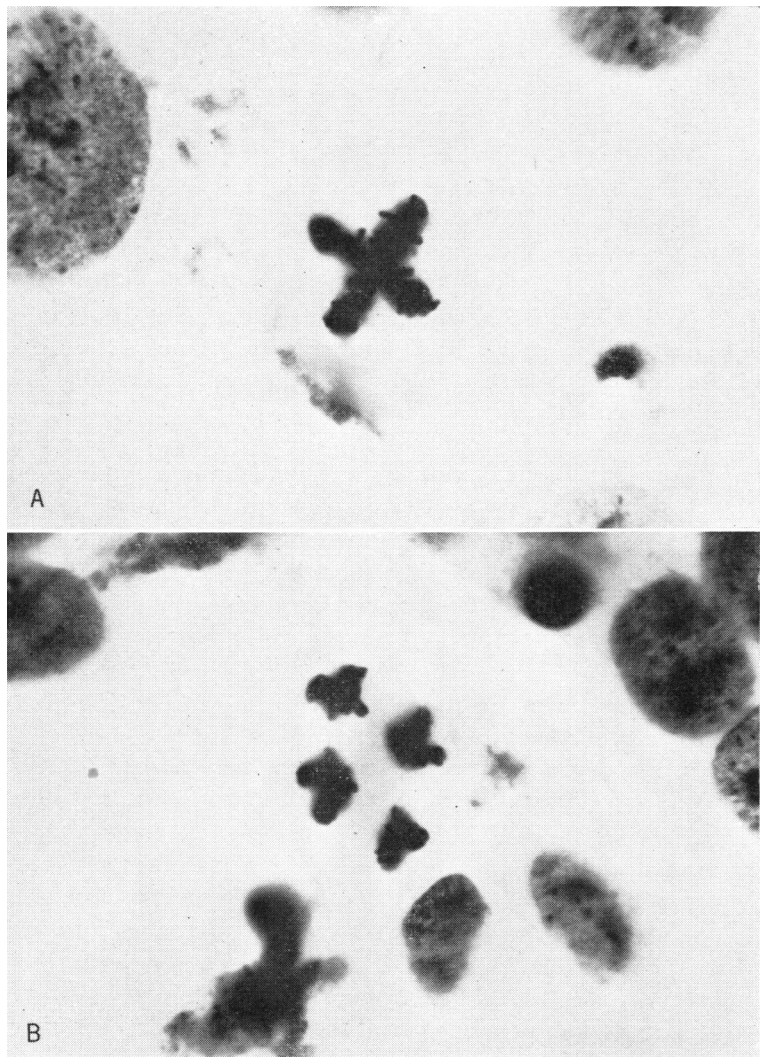

FIG. 3. Abnormalities of mitosis due to cell fusion after exposure to measles virus. a. Four metaphase plates arranged radially and sharing common centrosomes. b. Anaphase in a plate similar to the one seen in (a). Because of shared centrosomes the chromatids from adjacent nuclear groups have come together, approximately doubling the chromosome number of each nuclear group.

breaks, in which there are large numbers of reunions and recombinations, and breaks associated with viruses, in which reunions and recombinations are quite uncommon. At the time the original measles work was in progress, we were aware of the work by Taylor, Haut, and Tung (1962) with the deoxyriboside analog fluorodeoxyuridine in plant materials and also the work of Kihlman (1963) with deoxyadenosine, also in plant material, which produced breaks of a type similar to those seen with the viruses.

In collaboration with Kihlman (Kihlman, Nichols, and Levan, 1963), we studied deoxyadenosine and arabinosylcytosine in human leukocytes and

found that they produced breaks morphologically indistinguishable from those produced by viruses. We have also observed similar effects with the analog arabinosyladenine. These compounds are very similar to the four normally-occurring deoxyribosides that are found in DNA. For instance, arabinosylcytosine differs from cytidine only in the stereochemical relationship of one hydroxyl group at the 2-carbon position of the sugar moiety. Deoxycytidine differs from these in having a hydrogen at the 2-carbon position. These seemingly minor variations in structure are associated with major differences in the production of chromosomal abnormalities. The analogs produce inhibition of DNA synthesis, and it is thought that the chromosome breaks are also due to this DNA inhibition. This hypothesis was put forward by Taylor (Taylor, Haut, and Tung, 1962) and others and is supported by the fact that there can be a reversal or inhibition of the breaking effects of these analogs by adding an excess of the normal deoxyriboside.

This theory is now disputed by Bell and Wolff (1964) and by Brewen (1965), who think that these effects are separate and that, while the analogs do inhibit DNA synthesis, this inhibition is not the basis of the chromosome breaks. Their data are based on analog studies combined with autoradiographic studies. I do not wish to pursue this discussion at the present time but will note that the similarity of the virus and analog breaks and the hypothesis that the analog breaks might be due to the inhibition of DNA synthesis lead to the interesting possibility that the virus breaks are also due to the inhibition of DNA synthesis.

In comparative studies of viral and analog systems, we have found that there are several similarities between the viruses and the ribosides: Both produce mitotic inhibition; breaks caused by the two agents are morphologically similar; and both produce their effects in a rather short time after addition (Nichols and Heneen, 1964).

On the basis of these similarities, attempts have been made to duplicate viral effects using analogs. Koprowski *et al.* (1962) and Moorhead and Saksela (1965) have shown that SV40 virus can induce cell transformations. These transformations involve changes in cell morphology, changes in the rate of growth of the cells, and chromosomal abnormalities when the viruses were added to human diploid cell strains. We have undertaken a study of human diploid cell strains to which arabinosylcytosine has been added (Nichols and Heneen, 1964), reasoning that if the mechanisms are similar, then the analogs might be capable of the same type of cell transformation. Immediately after the analogs were added, a high incidence of open breaks were found similar to those discussed in the leukocyte preparations. After a period of recovery it was observed that there were some changes in cell morphology, and when the chromosomes were studied at later passages there were changes in the chromosomes similar to those seen in the viral transformed cells. These consisted of dicentrics and other rearrangements. An increase in the growth rate has not been seen as yet and, if anything, the growth rate of these cells seems decreased.

Another comparative approach is based on the fact that the analogs can be inhibited or reversed by excess of the normal deoxyribosides. Here attempts

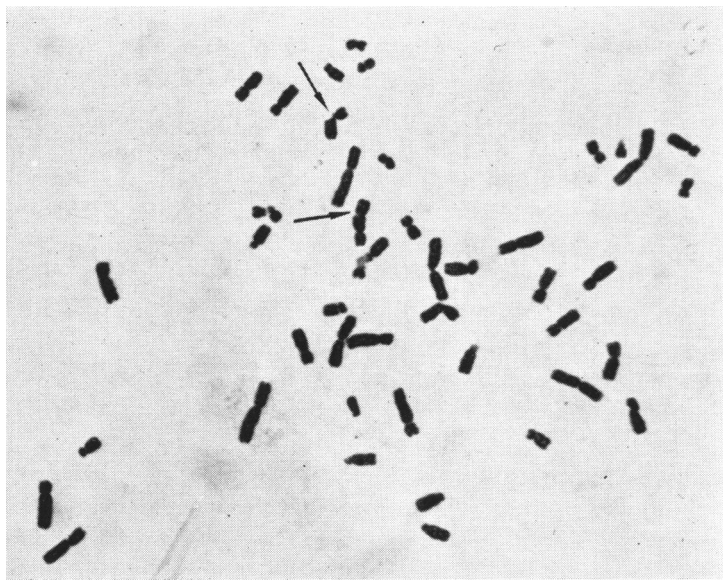

FIG. 4. Human leukocyte showing two chromosome breaks (arrows) after addition of cytidine triphosphate.

to reverse the viral effects by the ribosides or deoxyribosides are in progress. The initial phases of this work, the effect of the ribosides in their triphosphate form alone, in combination with each other, and in combination with the Schmidt-Ruppin strain of Rous sarcoma virus, have been completed (Nichols, Levan, Heneen, and Peluse, 1965). These results are not quite as we had expected. Rather than inhibition of the Schmidt-Ruppin virus by the ribosides, we found that there was no effect by three of the ribosides and that cytidine triphosphate actually was synergistic with the Rous virus. Cytidine triphosphate by itself also had a powerful chromosome breaking effect (Fig. 4). This synergistic activity between CTP and Schmidt-Ruppin virus is another similarity that can be added to the previous list.

SUMMARY

In summary, viruses can produce at least three changes involving chromosomes. The first is single breaks, the second, pulverization of chromosomes, and the third, cell fusions and spindle abnormalities. We feel that the greatest mutagenic potential is in the single break effect and have noted the similarity of these breaks to those induced by various ribosides and deoxyriboside analogs. Attempts to study the mechanisms of these breaks by comparing them with deoxyribosides and also using autoradiographic virus tracers in the cells are in progress.

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