THE CYTOLOGICAL EFFECTS OF PESTICIDES ON HUMAN CHROMOSOMES

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ABSTRACT

THE CYTOLOGICAL EFFECTS
OF PESTICIDES ON HUMAN CHROMOSOMES

By

DAVID HENRY KRAUSE

Chromosomes of human peripheral leucocyte cells were analyzed for cytological damage attributable to pesticide usage. Widespread introduction of pesticides into the environment makes contact unavoidable and necessitates the development of an *in vivo* human monitoring system. Circulating leucocytes offer a convenient and sensitive indicator of potential genetic effects in humans.

Cells, exposed *in vivo*, were obtained from high-use sprayers of pesticides and cultured *in vitro*. Slides were prepared from these cultures and microscopically analyzed for chromosome aberrations. Selected pesticides were screened, using human amnion AV_3 cells, for effects on chromosome breakage and cell growth.

The incidence of chromosome breakage in the exposed group was slightly higher than in the control group. However, the data was inconclusive and further study was indicated. In the AV_3 tissue culture experiment, captan was shown to be of concern due to its increase of chromosome breakage and inhibition of cell growth.

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BY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

1.	Acaralate	Isopropyl 4,4'-diclorobenzilate
2.	Alar	Succinic acid 2,2-dimethylhydrazide
3.	Amitrole	3-Amino-1,2,4-triazole
4.	BHC	1,2,3,4,5,6-Hexaclorocyclohexane
5.	Captan	N-Trichloromethylthio-4-cyclohexene-1,2-dicarboximide
6.	Carbaryl	1-Naphthyl N-methylcarbamate
7.	Colcemide	N-Desacetyl-N-Methylcolchicine
8.	Cyprex	Dodecylguanidine acetate
9.	DDT	1,1,1-Trichloro-2,2-bis(p-chlorophenyl) ethane
10.	Ferbam	Ferric dimethyl dithiocarbamate
11.	Guthion	0,0-dimethyl-s-phosphorodithioate
12.	HEOD	1,2,3,4,10,10-Hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-exo-5,8-dimethanonaphthalene
13.	Imidan	N-(Mercaptomethyl)phthalimide S-(0,0-dimethyl phosphorodithioate)
14.	Karathane	2-(1-Methylheptyl)-4,6-dinitrophenyl crotonate
15.	Lead Arsenate	Dibasic lead arsenate
16.	Lime Sulfur	Calcium polysulfides and calcium thiosulfate
17.	Maneb	Manganous ethylene bisdithiocarbamate
18.	Napthaleneacetic acid	1-Napthaleneacetic acid
19.	Oil	
20.	Paraquat	1,1'-Dimethyl-4,4'-bipyridinium dichloride
21.	Parathion	0,0-Diethyl 0-p-nitrophenyl phosphorothioate
22.	Phaltan	N-(Trichloromethylthio)-phthalimide
23.	Phosphamidon	2-Chloro-2-diethylcarbamoyl-1-methylvinyl dimethyl phosphate
24.	Phygon	2,3-Dichloro-1,4-naphthoquinone
25.	Simazine	2-chloro-4,6-bis(ethylamino)-s-triazine
26.	Sulfur	Sulfur
27.	Streptomycin	2,4-Diguanidino-3,5,6-trihydroxycyclohenyl 5-deoxy-2-0-(2-deoxy-2-methylamino-α - glucopyranosyl)-3-formyl pentofuranoside

28.	Systox	2:1 0,0-diethyl-0-AND S-2-(ethylthio) ethyl phosphorothioate (thiono isomer) and 0,0-diethyl-S-2-(ethylthio)ethyl phosohorothioate(thiol isomer)
29.	TEPP	Tetraethyl pyrophosphate
30.	Thiodan	6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzo-dioxathiepin-3-oxide
31.	Zineb	Zinc ethylene bisdithiocarbamate

INTRODUCTION

During the past decade there has been increasing concern over the effects of the introduction of pesticides into our environment. Some pesticides are mutagenic and the normal tests for mammalian tolerance may be an insufficient clearance. A risk to mankind much greater than acute, subacute, or chronic toxicity may exist in subtle genetic effects of these chemicals. Pesticides as chemical mutagens are a hazard which has not been systematically investigated. The protection of public health depends upon determining the nature and severity of harm that may be caused by these agents. Chromosomes from human peripheral blood offer a convenient and sensitive *in vivo* monitoring system for estimating potential genetic damage in man.

The study of human chromosomes dates back almost a century, however it has only been in the last ten years that culture techniques have been made available to make analysis of large numbers of cells feasible. In fact, it was not until 1956 that the correct diploid number of somatic chromosomes in man was established. The first attempts to visualize and count the chromosomes of man were in 1882 by Flemming and in 1891 by Hansemann (Robinson, 1961). Due to poor techniques estimates varied greatly until 1923 when it was concluded by Painter, and generally agreed upon for the next 33 years by the majority of in-

vestigators, that the diploid number of chromosomes in a human somatic cell was 48. During this period an important discovery led to determination of the correct human diploid chromosomal number.

In 1952 Hsu and Pomerat and, simultaneously, Huges reported that a cell in metaphase swells when placed in a hypotonic citrate solution before fixing and staining. This excessive intake of water by the cells allows the chromosomes to separate making analysis of them easier. Using this new technique Tjio and Levan (1956) established a diploid somatic chromosome number of 46 for man, 44 autosomes and 2 sex chromosomes. This finding was confirmed by Ford and Hamerton (1956), Tjio and Puck (1958) and Chu and Giles (1959).

Three other techniques aided the rapid advance in human cytogenetics. Human chromosomes are analyzed at the metaphase stage of mitosis, however it is only by chance that one finds a cell in this stage. Ford et al. (1956) and Tjio and Levan (1956) made use of the action of colchicine as a mitotic blocking agent to hold cells at the metaphase stage of division by inhibiting spindle fiber formation. The addition of this chemical to culture media not only increased the number of cells suitable for analysis, but also somewhat shortened and dispersed the individual chromosomes.

Still, chromosome work on humans depended upon bone-marrow puncture or skin biopsy for material and upon classic squashing methods. These problems were alleviated, when in 1958 Rothfels and Siminovitch developed an air-drying method. The advantages of air drying of meta-

phase cells cultured on glass are that it provides well-spread chromosomes in one focal plane and there is a minimum of over-lay and scattering. In 1960 Nowell made an important discovery which permitted convenient chromosome analysis of a significant number of cells. He found that phytohemagglutinin would induce normal peripheral leucucytes from human blood to enter DNA systhesis and division. Since under normal conditions in the body only a very small percentage of the cells are undergoing mitosis, this finding was of great significance to chromosome analysis. The use of peripheral blood offered a material that was easily obtained with a minimum of discomfort.

A classic paper written by Moorhead et al. in 1960 combined the aforementioned techniques to provide a convenient method of culturing human leucocytes for critical analysis of chromosome morphology. Since the development of these tissue culture techniques and Moorhead's work, many slight modifications and simplifications have been made for the culturing of white cells from peripheral blood (Steinberger, 1964, Bishun, 1965, Uchida, 1966, Katz, 1970).

Even though the study of human chromosomes dates back to 1882, it has only been in the last decade that practical and convenient methods have been developed to allow a detailed visual study of the number and morphology of human chromosomes. Since 1959 when Lejeune (Hampton, 1964) demonstrated a chromosomal abnormality resulting in mongolism, there has been no doubt as to the clinical value of chromosome examination. In order to effectively use these methods in

the interpretation of chromosome aberration data, an understanding of chromosome morphology and cell division is necessary.

Using tissue culture techniques, the infrequently dividing cells of most normal body tissues undergo division in significant numbers. The cell cycle encompasses the time from the beginning of the growth phase to the end of mitosis. The period of non-division i.e. "no growth" is termed G_0 referring to the time after mitosis occurs but before the onset of growth processes. G_1 is a presynthetic period; a period in which net cell growth (protein, RNA, etc.) preceeds, as well as preparation for DNA synthesis. The second stage of the cell cycle is S or the DNA synthetic period. Last is the post-DNA synthetic period, G_2, followed by mitosis, M (Gilbert, 1964). Mitosis is divided further into four stages: prophase, when the chromatin begins to condense and spindle fibers begin to form; metaphase, when the nuclear membrane disappears and the chromosomes shorten and line up on the equatorial plate; anaphase, when the doubled chromosomes split and half of each migrates to each pole; and telophase, when nuclear membrane reforms, chromosomes elongate and the two daughter cells are formed. The cell then goes into interphase which includes G_0, G_1, S, and G_2.

Chromosomes are the carriers of the hereditary elements of cells. A normal human cell contains 46 chromosomes. They are paired, 22 pairs of autosomes and 1 pair of sex chromosomes. These chromosomes were classified into easily identifiable groups in 1960 at the University of Colorado in Denver (Book et al., 1960). By this classification there

are five groups: Group 1-3, large chromosomes with approximately median centromeres; Group 4-5, large chromosomes with submedian centromeres; Group 6-12, medium sized chromosomes with submedian centromeres which resemble the X chromosome; Group 13-15, medium sized chromosomes with nearly terminal centromeres; Group 16-18, short chromosomes with submedian centromeres (approximately median in 16); Group 19-20, short with approximately median centromeres; Group 21-22, very short acrocentric chromosomes (includes the Y chromosome). These groups are often referred to by letters as A through G respectively. This international nonmenclature system is referred to as the Denver system. It is possible to identify individual chromosomes within a cell by use of an idiogram. They may be identified only by comparison with each other within a cell due to the varying degrees of contractions of chromosomes existing between cells (Robinson, 1961). This is accomplished by arranging paper cutouts of the chromosomes into pairs and then rearranging by size and centromere location until their classifications become clear. This method is very time consuming and involves much uncertainty. Recently, investigators have turned to a more precise method by using scanning computers to measure and calculate the relative lengths of the arms (Environment, 1970).

This system of nonmenclature was further developed to describe variant chromosomes at a conference held in Chicago in 1966. They designated the short arm of a chromosome to be indicated by a p; the long arm by a q. Deletion from an arm of a chromosome by a minus sign,

and lengthening or an extra entire chromosome by a plus sign (German, 1970).

A gene, being a region of a DNA molecule residing in the chromosome, consists of purine & pyrimidine base pairs arranged in a specific sequence (code) which directs the manufacture of a particular enzyme or protein in the cell. The genetic code is altered by a spontaneous mutation rate at a level sufficient to permit rearrangments important to evolutionary development, but not so rapid as to exceed the ability of selective factors to eliminate deleterious mutations (Neel et al, 1969). Agents capable of increasing this rate should be of concern when they are commonly introduced into the environment, since their effects will most likely be deleterious. When this genetic code is altered it may manifest itself in a viable genetic defect, neoplasia or cell death. Damage to genetic material has been attributed to radiation, microorganisms and chemicals.

Chromosome aberrations may involve either a numerical change or a structural change. Microscopically the effects of these substances can be observed in metaphase as aneuploidy, polyploidy, mitotic index, gaps, breaks, fragments, inversions, deletions, multicentrics, rings and translocations.

Chromosome numerical changes include aneuploidy, polyploidy and mitotic index. Aneuploidy is any deviation in chromosome number from the normal diploid state. It may be seen as a result of chromosome stickiness due to the action of some agent inhibiting movement during

division which causes irregular distribution to daughter cells. In some cases it may be an inherited trait seen either in all cells or in clones of cells. Also, aneuploidy arising as artefacts in slide preparation is found in cells of normal individuals. To determine whether the aneuploidy is random or due to a particular chromosome a karyotype analysis must be used. Aneuploidy is characteristic of rapidly dividing cancer cells (Environment, 1970).

A polyploid cell is one which possesses two or more complete sets of chromosomes. It results from errors in division when the chromosomes double, but the cell doesn't divide. This effect can be produced by a prolonged exposure to colchicine.

Mitotic index is a comparision of dividing figures to non-dividing cells. It is included because it is a means of detecting effects of agents which may inhibit division.

Chromosome structural changes are of two kinds, deletion and exchange. The simple deletion is the result of a single break in the chromosome or chromatid. The exchange is a new rearrangment following the joining of breakage ends which result from separate breaks. Breakage is the disruption of chromatin material which occurs before an aberration forms. If breaks do not restitute, they may lead to deletion, duplication, inversion or translocation of a chromosome. If a break occurs only in one arm of an already duplicated chromosome, i.e. in a single chromatid, it is a chromatid-type aberration; if both chromatids are altered, it is a chromosome-type aberration. Chromosome-type

aberrations (rings, multicentrics, exchanges, acentric fragments) are seen as a defect in both arms and are produced only in G_0 or G_1 when the chromosome acts as if it were functionally single stranded (Brewen, 1967). That is to say that the DNA double helix is the smallest unit in which an aberration will be seen. Little is known of the effect of partial breaks, i.e. subchromatid-type aberrations. However, they would most likely result in an unstable state which would either undergo restitution or further aberration.

Of the possible chromosome-type aberrations only the translocation would not result in an acentric fragment. An acentric fragment consists of two pieces of chromosome, with no centromere. If the lesion occured in a single chromosome an acentric fragment consisting of two pieces from the same chromosome could result. This lesion could be the result of one event affecting both strands of the chromosome or two events affecting both strands separately. If the lesion is a result of two events, one or two acentric fragments could result, depending on whether the fragments rejoin, prior to replication. A polycentric chromosome results from exchange between two (dicentric) or more chromosomes. It appears in metaphase as a single chromosome with more than one centromere and an acentric fragment. A ring chromosome results from an intrachange within a single chromosome. As the name suggests it is seen in metaphase as a chromosome with its ends joined to form a ring and an acentric fragment. A trans-

location involves an exchange between chromosomes which, if equal and the position of the centromeres does not move, will not be seen. Usually, however, the exchange is unequal resulting in an unusually large chromosome. Generally a karyotype analysis is necessary to locate translocations.

An exchange is an intra-change if it occurs within a single chromosome or an inter-change if between chromosomes. The rearrangement is termed symmetrical (stable) if mitosis can occur without mechanical difficulty. An asymmetrical intra- or inter- change is one in which division is interfered with, as in the case of rings and multicentrics.

Chromatid-type aberrations are generally more stable than chromosome-type aberrations, in that they are still capable of segregating properly at anaphase. A chromatid gap is a non-staining area less than one chromatid width and no terminal dislocation. If the stained portion of the chromatid distal to the gap is displaced or the non-staining area is greater than one chromatid width the aberration is a chromatid break. Gaps are usually considered insignificant because often they are artefacts resulting from poor stain uptake. An inversion occurs when a portion of the gene sequence becomes rearranged as a result of breakage and reunion in reverse order. A pericentric inversion includes the centromere, whereas a paracentric does not. Inversions are very difficult to detect except when they involve the centromere and the breakage points are not equidistant from it. In

this instance, for example, an acrocentric chromosome could be converted to a metacentric and would be detected in karyotype analysis. A fragment when listed separately from breaks indicates that a piece of a chromosome was observed but was not attributable to any particular chromosome. Similar to this is a deletion, which indicates that a chromatid arm is shorter than usual, but there is no evidence of a fragment. A defect in both chromatids of a chromosome and at the same level is an isochromatid aberration, which results in an acentric fragment.

In human lymphocyte cultures both chromosome-type and chromatid-type aberrations could be found after the first *in vitro* division. Chromatid-type aberrations could result from damage in the lymphocyte mother cell or as a delayed effect of an agent affecting DNA synthesis during the *in vitro* division. Chromosome-type aberrations would be the result of an agent affecting the lymphocyte while it was in circulation since circulating human peripheral leucocytes are non-dividing. It should be noted that Bloom *et al.* in 1967 demonstrated clone formation in lymphocytes of atom bomb radiation exposed individuals. This would indicate that a small portion of the population may be actively dividing. The possibility exists that these cells are capable of dividing because of an aberration.

The circulatory human peripheral leucocytes are in G_0 *in vivo* and only enter G_1 when stimulated *in vitro* (Bond *et al.*, 1958; MacKinney *et al.*, 1962; Bender and Prescott, 1962). Sasaki and Norman (1966) have shown that the small lymphocyte finishes its transformation in 24

to 48 hours after stimulation and begins a period of cell proliferation with a generation time of 22 hours; G_1, S, G_2 and M are 6, 11, 3 and 2 hours, respectively. In a second paper, Sasaki and Norman (1967) showed that leucocytes may undergo up to three in vitro divisions in 72 hours. Furthermore, they indicated that 70% of the cells in a 72 hour leucocyte culture system were in their second in vitro division. This is contrary to the previously recognized paper of Bender and Prescott (1962) indicating that only one of seventy-five cells in a 72 hour leucocyte culture can be expected to be in second division. It is important to be sure that the cells only undergo one in vitro division because the results will more closely resemble the conditions in vivo. A single leucocyte could be represented by four metaphase cells in 72 hours possibly giving an unrealistic percentage of stable aberrations. Buckton and Pike (1964) stated that the frequency of leucocytes with unstable type aberrations (dicentrics, rings and acentric fragments) decreases with increasing culture time. Also they showed that an increase in polyploidy occurs in leucocytes cultured greater than 72 hours. Contrary to this, Honda, Kamada and Bloom (1968) found no evidence of a decrease in aberrations with increased culture time. They compared the normal 66 to 72 hour culture with a 46 to 50 hour culture method of Bloom and Iida (1967).

In 1969 Bender and Brewen offered an explanation which seemed to resolve the question. They found that a peak aberration rate occurred 54 hours after culture initiation which then dropped off with a second

peak, slightly smaller than the first, occurring at 78 hours. To account for this data they postulated that those leucocytes which divide in culture constitute at least two populations differing in the speed at which they reach mitosis. Furthermore they pointed out that not all leucocyte cultures reach second division at the same time. The rate varies with different donors, incubation temperature and culture techniques.

Since human circulatory leucocytes are essentially non-dividing (MacKinney et al., 1962; Bender and Brewen, 1969), it is necessary to know the life of a leucocyte within the body to determine the time span in which an effect could be seen. Aberrations seen in vitro are a conservative estimate of damage since severely damaged cells may die, there may be repair mechanisms operating to restitute damage or aberrations such as inversions and translocations may be undetected if the position of the centromere is not altered. The human leucocytes or white cells are of two basic types; those with granulated cytoplasm, granulocytes, and those without granules in the cytoplasm, agranulocytes. The agranulocytes include the small lymphocyte which merges through transition forms into the monocyte.

The lymphocyte originates from precursor cells in the lymphatic tissue of the lymph nodes (Bloom and Fawcett, 1962) from which they are released into the peripheral blood. The leucocyte cells which divide in culture are the small lymphocytes (MacKinney et al., 1962). This imposes another technical limitation on aberration data in that of

the cells surviving the effects of an agent, whether lethal or inhibiting division, only that portion of the lymphocytes which are antigen-responsive (phytohemagglutinin) will enter mitosis. Buckton and Pike (1964) estimated the mean survival time of this circulating lymphocyte population at several hundred days, a fraction of which may survive without division up to 10 years. Sasaki and Norman (1967) in their study of induced unstable aberrations reported a mean life of 530 days with some cells still existing without division at the end of the study, 2,698 days. Bloom and Hamilton (1969) showed that heavily irradiated atom bomb survivors had a number of unstable aberrations attributable to the exposure 20 years earlier. Brewen (1970) indicated it is now generally agreed that small lymphocytes have a mean life of between 4 and 5 years. This average value probably represents a series of lymphocyte populations with different survival times. Removal of unstable aberrations occurs in part when the lymphocytes enter division through antigenic stimuli and die as a result of the difficulties imposed by the aberration (Brown, 1967). As a result of the possible long life of lymphocytes, it becomes important to carefully check a subject's history for radiation therapy and other such mutagenic insults.

Although the effects of agents on chromosomes can be described morphologically, relatively little is understood of the mechanism of chromosome breakage and repair at the molecular level. The biochemical lesions that lead to chromosome aberrations probably vary with different breaking agents. A lesion involves an alteration of the

in DNA. The alteration may be either submicroscopic, a point mutation, involving a small number of nucleotides or a gross aberration, such as a break which generally inhibits further DNA duplication. There are a variety of modes by which chemical agents can cause strand breakage. A chemical may inhibit DNA replication by blocking one of the mononucleotide precursors to DNA. The base analog 5-fluorodeoxyuridine inhibits thymidylate synthetase, and, therefore, the conversion of deoxyuridylate to thymidylate is blocked (Taylor et al., 1962). Another base analog, 5-bromodeoxyuridine, causes aberrations by incorporation into the DNA strand (Hsu and Somers, 1961).

An agent may produce a chemical alteration of DNA. Ethylenimine is an alkylating agent and acts by facilitating depurination which has the effect of labilizing the sugar-phosphate backbone (Chang and Elequin, 1967). Interference with repair of DNA lesions is another possible action. This mode of action has been proposed for caffeine (Cleaver, 1968). In contrast to enzyme inhibition, DNA damage could result from the enhancement of an enzyme such as deoxyribonuclease (Paton, 1970). Colchicine is a chemical which does not act by altering DNA, but rather disrupts cell division by inhibiting spindle formation.

That aberrations could be induced artificially was demonstrated in 1928 by Muller with X-irradiation. Since then evidence of the health hazards of radiation has been extensive, including nuclear radiation, ultraviolet light and sunlight (Schmickel, 1967; Buckton et al., 1967; Bloom et al., 1970; Bloom and Hamilton, 1969; Bloom et al., 1966;

Bloom and Tijo 1964; Trosko et al., 1970). Recently, chromosome damage has even been attributed to viral infection (Nichols, 1966), specifically measles (Nichols et al.,1962) and chicken pox (Aula, 1964).

Chemicals comprise a larger group than radiation or microorganisms and have been widely introduced into the environment. The first demonstrated chemical mutagen, mustard gas, was reported in 1947 by Auerbach, Robson and Carr. Known chemicals capable of inducing aberrations consist of many commonly occurring agents such as cyclamate (Stoltz et al., 1970), caffeine (Ostertog, 1966), sodium nitrite (Malling, 1965), 3,4-benzpyrene (Epstein and Shafner, 1968), lysergic acid diethylamide (Egozwe et al., 1968), mitomycin C (Shaw and Cohen, 1965), and captan (Legator et al., 1967). These chemicals enter the body in such common forms as soft drinks, food, food additives, air and water pollutants, drugs, medications, and pesticides.

Pesticides are of particular concern because their widespread use, dispersion and persistance in the environment makes contact with minute amounts unavoidable. These dosages, although usually too small to produce an acute effect. still may posess the potential for chronic effects. The majority of pesticides have not been adequately tested for mutagenicity in humans. The research that has been accomplished is inconclusive and generally only involves a test with one organism (Mrak Commission, 1969). Evidence of mutagenic activity exists for malic hydrazide (McCarthy and Epstein, 1968), captan (Legator et al., 1967), tretamine, TEPA and apholate (Chang

and Klassen, 1968; Epstein and Shafner, 1968) in human cells. Wuu and Grant (1966; 1967) have reported a systematic study of pesticides using barley root tips as have others in various systems (Mrak Commission, 1969).

Although research in bioassay systems other than man is very informative, it cannot be said with certainty that the same cause and effect relationship will exist in man. Distribution of the chemicals may differ greatly in human organs and tissues or they may be detoxified by enzymes in human beings. On the other hand, compounds which are not mutagenic may be converted into mutagens in the human body. Because some pesticides are known to be mutagenic in lower organisms, a monitoring system must be employed to determine if man's exposure to these chemicals is detrimental enough to out-weigh the benefits. In humans the generation length makes it difficult to discover by direct observation that a chemical is producing mutations. A recessive mutation, if visible, would take at least two generations to be seen. Even a dominant mutation would not be seen until the next generation and by this time the effect, even if it were still possible to determine it, would have exposed large numbers. Therefore, it is important to detect a possibly carcinogenic, teratogenic or mutagenic agent early enough to rectify the cause. If the mutational effect were accompanied by chromosome breakage it could be detected by chromosome studies in human leucocytes.

MATERIALS AND METHODS

The population studied was selected from high-use sprayers of pesticides in the southwestern Michigan fruit-growing area. Dosage of the subjects was difficult to assess, even though accurate spray records were kept, and could be determined only to number of days of usage. Furthermore, precautionary measures could not be standardized. Control individuals were from the same area but had no direct contact with pesticides. The history of the subjects in the preceding twelve months was checked for diagnostic X-irradiation exclusive of chest X-rays. In 1968 the average age of the exposed group was 43.00 years and ranged from 24 years to 57 years. The control group had an average age of 46.74 years and ranged from 30 years to 79 years. In 1969 the average age of the exposed group was 44.74 years and ranged from 25 years to 58 years. The control group had an average age of 48.10 years and ranged from 32 years to 80 years. The study consisted of twenty control and twenty exposed male volunteers, examined each month during the spray season.

Three drops of whole blood, drawn by veinapuncture, were placed into a warm (37°C) culture tube of Grand Island Biological Company Chromosome Medium 1A and mixed well. The tubes were shipped

via special delivery mail in insulated containers to the laboratory. In 1969 the technique was altered slightly in that the blood was shipped in heparinized pipets and the cultures were initiated in the laboratory. The chromosome medium 1A contains culture fluid and fetal calf serum, heparin, antibotics, and phytohemagglutinin. The peripheral leucocyte cells, exposed in vivo, were cultured in vitro at 37°C for 72 hours. Three hours prior to harvest, 0.3 milliliter Colcemide (10 micrograms per milliliter) in isotonic saline was mixed into each culture. At 72 hours the culture was transfered to a 12 ml. centrifuge tube and spun down at approximately 500 RPM for 3 minutes. The supernatant was removed and three milliliters of one percent sodium citrate, warmed to 37°C, was used as a hypotonic solution to resuspend the cells. After ten minutes the cells were recentrifuged at approximately 500 RPM for 3 minutes and with a clinical centrifuge, all except a small portion of the supernatant was removed. The button of red and white cells was then mixed thoroughly. Two milliliters of freshly prepared cold Carnoy fixative (one part glacial acetic acid to three parts methanol) was added quickly and mixed thoroughly. Fresh Carnoy fixative was then added enough times to obtain a clear supernatant, resuspending the cells at each centrifugation (see above). Slides were either prepared at this time or the cells left in the fixative overnight. The cells were suspended in fixative a little more than the size of the button, prior to slide preparation. Clean slides were dipped in 70% methanol and two or three

drops of the suspended cells were allowed to fall on the slide from a height of 6 to 8 inches. Immediately the slide was passed through a flame to ignite the methanol. The water left on the slide after the flame burned out was drained off. The slide was then examined under the microscope to determine if the cell concentration had allowed maximum spreading. If the slide was not satisfactory, a second slide was made by either diluting or concentrating the cell suspension. The slides were either stained with aceto-orcein or Giemsa and made permanent with Permount®. Apparently normal metaphase plates (Figure 1) were selected for analysis under low-power (200X) magnification on the basis of their apparent intactness and on the quality of chromosome spreading. Once selected, the cell was included in the study. Aberrations scored were breaks (Figure 2), dicentrics, rings, translocations, fragments and deletions, as well as chromosome number. Gaps (Figure 3) were considered insignificant and not included in the data. Photomicrographs were taken using Kodak High Contrast Copy film and were printed on F-5 paper. All slides were number coded to reduce bias.

The in vitro tissue culture experiments used human peripheral leucocytes and human amnion AV_3 cells. Analytical standards of the pesticides were obtained from the Pesticide Repository, U.S.P.H.S., Perrine, Florida. The peripheral leucocytes were innoculated in the laboratory from a single donor and incubated as previously described. The human amnion AV_3 cells were plated into 60mm petri dishes in

Eagle's MEM medium (10% calf serum). The cells were exposed with pesticides during the final 12 hours of a 72 hour incubation period. The pesticide solutions were prepared in dimethyl sulfoxide not more than an hour prior to application. For the AV_3 growth experiment the pesticide was added at time zero approximately three hours after plating. The AV_3 cells were removed from the plates by means of a 5 minute contact with trypsin and then either fixed for chromosome analysis or counted in a hemacytometer.

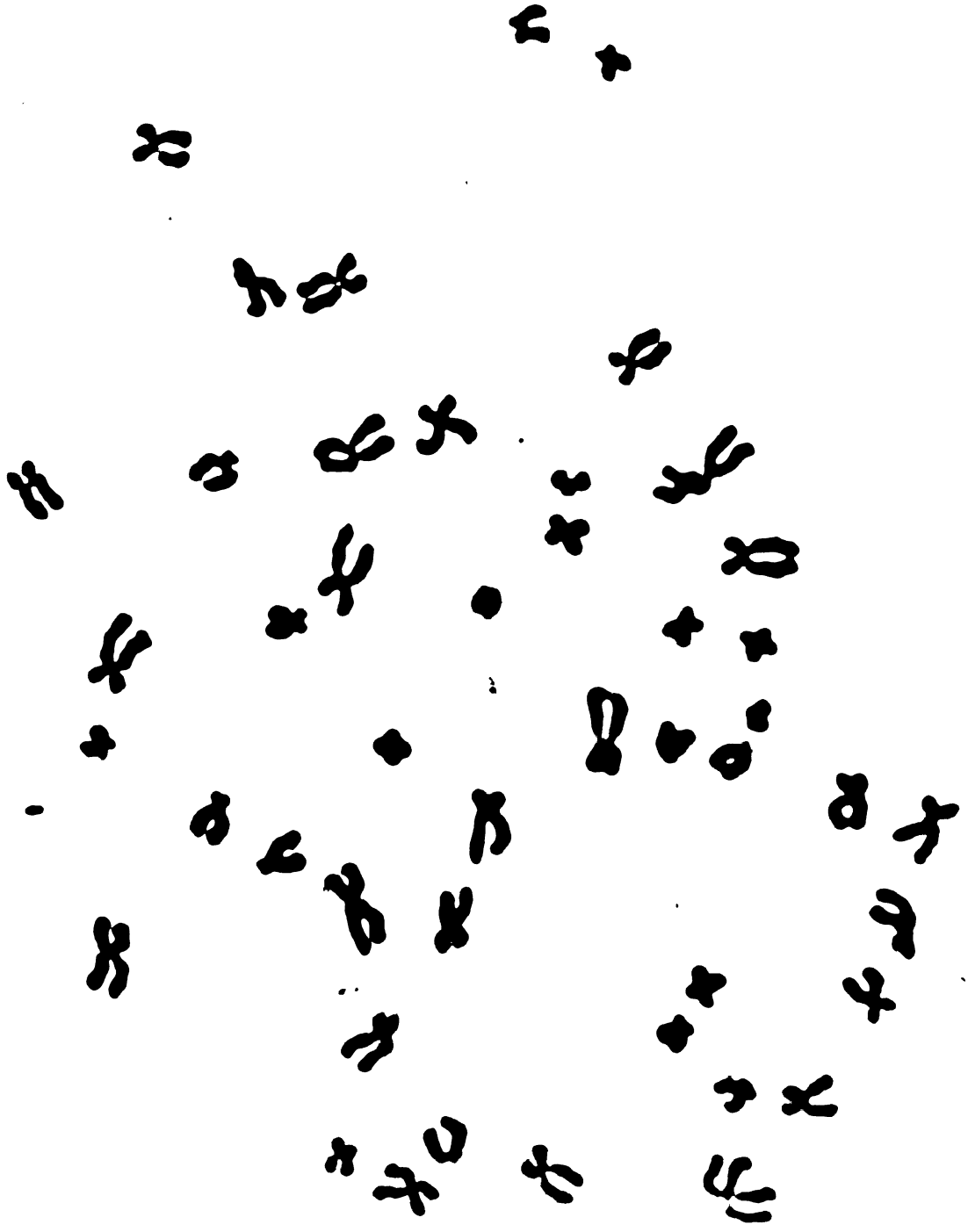

FIGURE 1. Normal human chromosome metaphase plate.

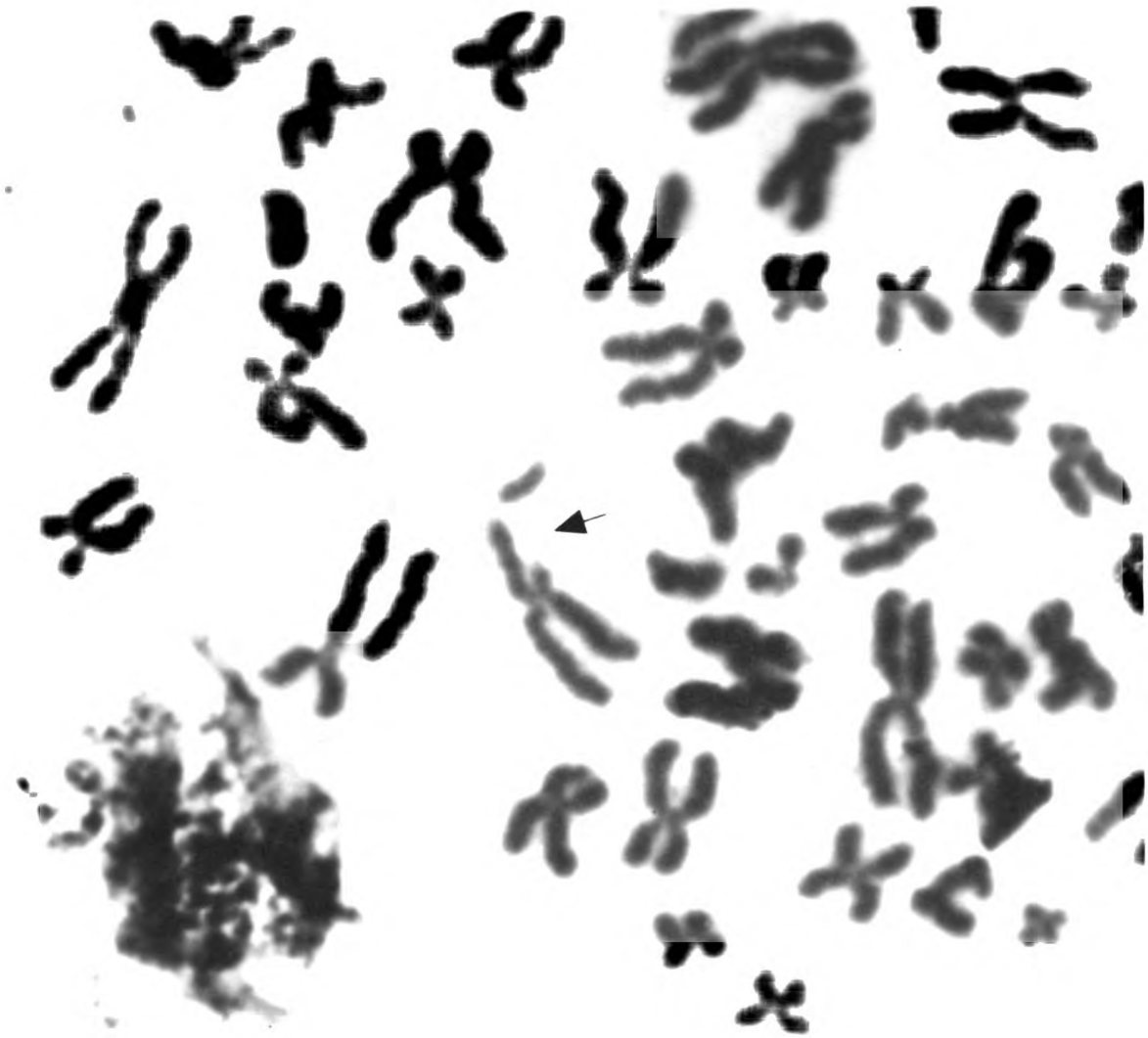

FIGURE 2. Break with associated fragment in a human chromosome.

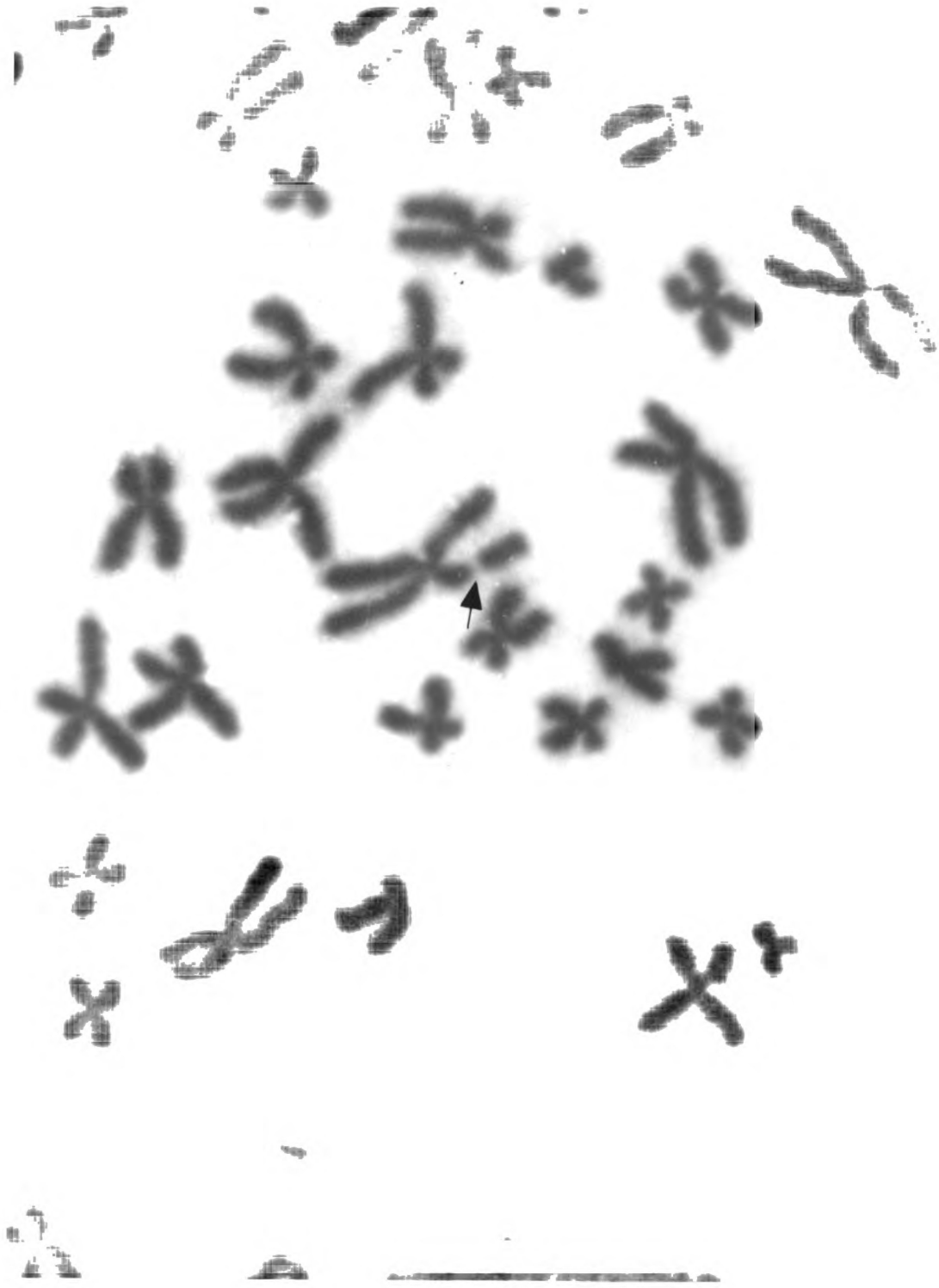

FIGURE 3. Gap in a human chromosome.

RESULTS

Rings, dicentrics and translocations were not observed during the two years of the study and only chromosome breaks with their associated fragments were recorded. In 1968 the total number of breaks in the user group was more than twice that of the control group (3.9 percent vs. 1.7 percent). This was not confirmed in 1969 where the user group was insignificantly higher than the control group (1.7 percent vs. 1.2 percent). In neither year did the amount of breaks prove to be statistically significant at the five percent level when tested on a fourfold contingency table. The number of breaks for the months of June and July in 1968 and July of 1969 were significant at the five percent level. In both years there appears to be a slight rise in chromosome aberrations during the peak of the spraying season. This was also noticed in the controls, but was more erratic and not as significant, (Table 1). The chromosome number distribution for the exposed group during both years was within the range for the controls (Table 2).

Table 3 indicates the wide variety of pesticides excountered by the user group during a typical spraying season. However, in Figure 4 it can be seen that only a small number of pesticides were encountered

by the group in significant amounts. The wide range of acute toxicities of the pesticides used is illustrated in Table 4. Those pesticides which were encountered in large amounts were tested in vitro on human amnion AV_3 cells and human peripheral leucocyte cells. A comparison was made of production of aberrations and interference with growth between these and other pesticides of varying toxicity. Tables 5 and 6 show that in the leucocyte cultures captan at a concentration of 1×10^{-4}M produced twelve percent breaks whereas only one other pesticide (carbaryl, two percent) produced any breaks at all. The Guthion culture produced the lowest number of diploid cells. Chromosome analysis of treated human amnion AV_3 cells showed a percentage of aberrations within the normal range at a concentration of 1×10^{-4}M. However, captan at a concentration ten times lower than the rest of the dosages (1×10^{-5}M) still had an aberration rate slightly above controls (Table 7). Growth studies indicated minimal reduction in cell growth after addition of pesticides at a concentration of 1×10^{-4}M, with the exception of the captan treated culture which did not survive (Table 8). The graph of the data (Figure 5) clearly demonstrates that captan at a concentration of 1×10^{-5}M inhibited cell growth for up to 48 hours.

TABLE 1. Effect of pesticides on chromosome aberration.

	No. of cells with aberrations/ No. of cells analyzed			
	Control breaks	%	Exposed breaks	%
1968				
May	2/212	.9	3/310	1.0
June	2/178	1.1	13/332	3.9
July	3/100	3.0	25/336	7.4
August	7/325	2.2	7/275	2.5
Total	14/815	1.7	48/1253	3.9
1969				
June	4/258	1.6	10/616	1.6
July	2/597	.3	11/600	1.8
August	7/225	3.1	8/330	2.4
September	1/294	.3	6/497	1.2
October	5/229	2.2	5/298	1.7
Total	19/1603	1.2	40/2341	1.7

TABLE 2. Effect of pesticides on chromosome number.

	Total No. Cells	Chromosome Number Distribution					
		<44	44	45	46	>46	Polyploid
1968							
Exposed	705	6	14	66	616	0	3
%		.9	2.0	9.3	87.4	0.0	.4
1969							
Control	560	3	7	26	517	2	5
%		.5	1.3	4.6	92.3	.4	.9
1969							
Exposed	720	2	13	37	654	6	9
%		.3	1.8	5.1	90.8	.8	1.2

TABLE 3. Distribution of exposed group to pesticides encounterd.[1]

	Number of days exposed during month.				
Pesticide	April	May	June	July	August
Captan	7	101	86	60	68
Carbaryl	0	4	16	40	32
Cyprex	66	80	94	33	17
DDT	6	32	46	10	8
Ferbam	0	25	35	21	3
Guthion	3	80	109	90	45
HEOD	5	45	31	7	0
Karathane	0	12	21	7	0
Lead Arsenate	0	22	29	21	2
Lime Sulfur	22	22	9	4	5
Oil	33	7	0	0	0
Parathion	7	44	51	41	20
Simazine	2	20	2	0	0
Sulfur	28	104	98	51	22
TEPP	0	0	4	8	23

[1] Other pesticides used less than twenty exposure days in any month: Acaralate, Alar, Amitrole, BHC, Imidan, Maneb, Napthaleneacetic acid, Paraquat, Phaltan, Phosphamidon, Phygon, Streptomycin, Systox, Thiodan, Zineb

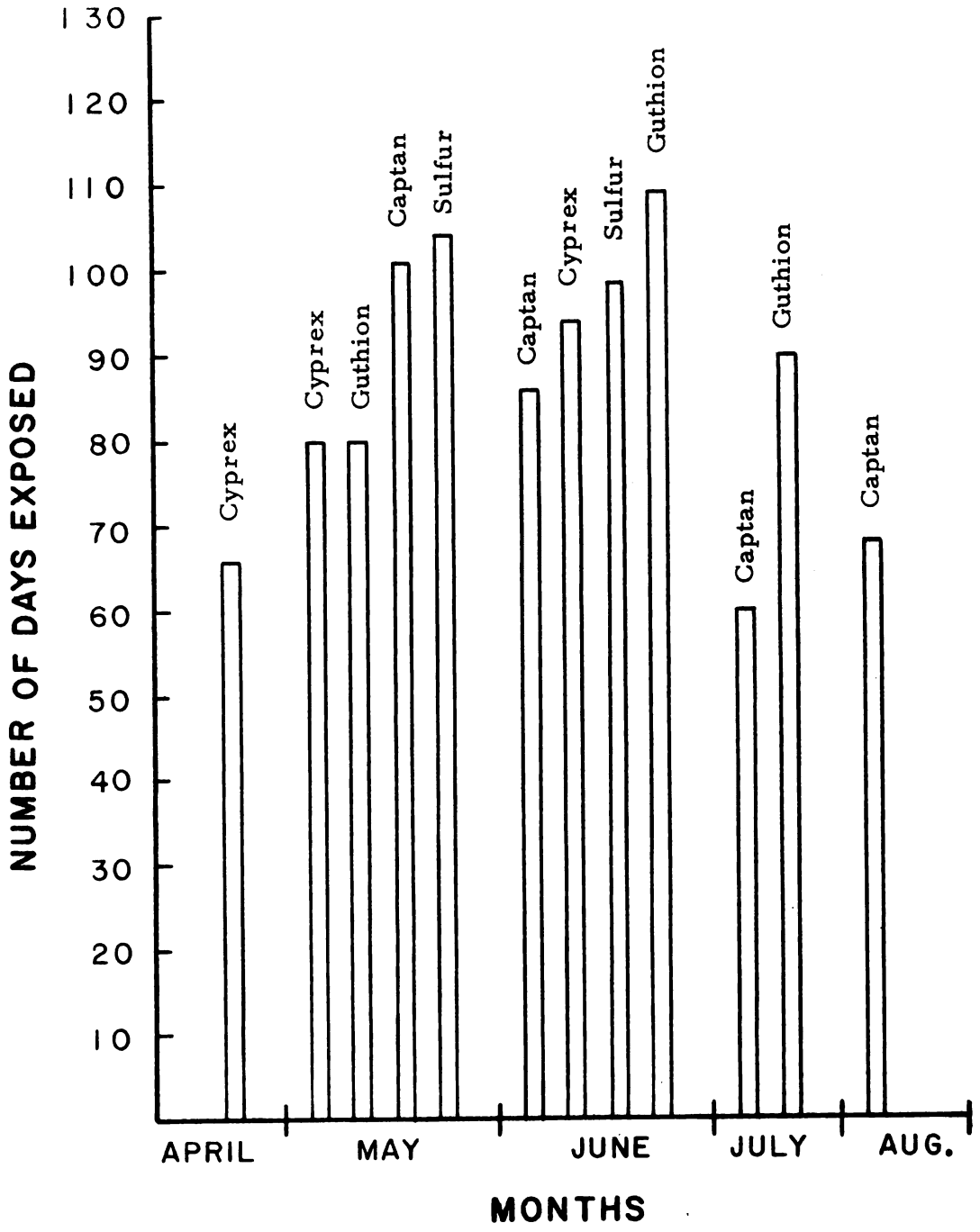

FIGURE 4. Comparative exposure of user group to frequently encountered pesticides.

TABLE 4. Acute oral toxicities of pesticides encountered by exposed groups.

Pesticide	Acute oral LD_{50}[2,3]
Captan	10,000.
Carbaryl	540.
Cyprex	600.-870
DDT	113.
Ferbam	17,000.
Guthion	10.-18.
HEOD	60.
Karathane	980.
Lead Arsenate	100.
Lime Sulfur	-
Oil	-
Parathion	6.-15.
Simazine	5,000.
Sulfur	-
TEPP	2.

[1] Pesticides used by exposed group twenty days or more during any month.

[2] The LD_{50} is based on rats. It is the number of milligrams of pesticide per 1000 grams of body weight of the test animal that is required to kill 50% of the test animals.

[3] Neumeyer, Gibbons, Trask, 1969.

TABLE 5. Effect of pesticides on chromosomes of human peripheral leucocyte cells in vitro.

Pesticide	Concentration	No. of Cells Analyzed	Rings Dicentrics Translocations	Breaks	Chromosome number distribution						
					Gaps	<44	44	45	46	>46	Poly-ploid
Captan	$1x10^{-4}$M	50	0	6	4	2	2	2	43	0	1
	$1x10^{-5}$M	50	0	0	2	1	2	5	40	1	1
Carbaryl	$1x10^{-4}$M	50	0	1	5	0	2	6	42	0	0
Cyprex	$1x10^{-4}$M	50	0	0	4	1	1	5	42	0	1
Guthion	$1x10^{-4}$M	50	0	0	2	2	2	4	39	2	1
HEOD	$1x10^{-4}$M	50	0	0	0	2	0	2	42	2	2
Parathion	$1x10^{-4}$M	50	0	0	3		1	5	43	1	0
Simazine	$1x10^{-4}$M	50	0	0	0	0	0	4	44	2	0
Sulfur	$1x10^{-4}$M	50	0	0	2	1	0	4	44	1	0
DMSO	$1.28x10^{-1}$M	50	0	0	0	0	2	2	46	0	0
Control		50	0	0	0	0	2	6	40	2	0

TABLE 6. Effect of pesticides on chromosomes of human peripheral leucocyte cells *in vitro*.

Pesticide	Concentration	No. of Cells Analyzed	PERCENT					
			Rings Dicentrics Translocations	Breaks	Gaps	Diploid	Aneuploid	Polyploid
Captan	$1x10^{-4}M$	50	0	12	8	86	12	0
	$1x10^{-5}M$	50	0	0	4	80	18	2
Carbaryl	$1x10^{-4}M$	50	0	2	10	84	16	0
Cypre x	$1x10^{-4}M$	50	0	0	8	84	14	2
Guthion	$1x10^{-4}M$	50	0	0	4	78	20	2
HEOD	$1x10^{-4}M$	50	0	0	0	84	12	4
Parathion	$1x10^{-4}M$	50	0	0	6	86	14	0
Simazine	$1x10^{-4}M$	50	0	0	0	88	12	0
Sulfur	$1x10^{-4}M$	50	0	0	4	88	12	0
DMSO	$1.28x10^{-1}M$	50	0	0	0	92	8	0
Control		50	0	0	0	80	20	0

TABLE 7. Effect of pesticides on chromosomes of AV3 cells.*

Pesticide	Concentration	No. of cells Analyzed	PERCENT		
			Rings Dicentrics Translocations	Gaps	Breaks
Captan	$1x\ 10^{-4}$ M	100	0	7	5
Captan	$1x10^{-5}$ M	100	0	5	4
Carbaryl	$1x10^{-4}$ M	100	0	4	2
Cyprex	$1x10^{-4}$ M	100	0	5	1
Guthion	$1x10^{-4}$ M	100	0	5	1
HEOD	$1x10^{-4}$ M	100	0	4	1
Parathion	$1x10^{-4}$ M	100	0	4	1
Simazine	$1x10^{-4}$ M	100	0	3	2
Sulfur	$1x10^{-4}$ M	100	0	4	1
DMSO	$1.28x10^{-1}$ M	100	0	3	1
Control		100	0	4	1

TABLE 8. Effect of pesticides on growth of AV3 cells.*

Pesticide	Concentration	No. of cells Plated	Exposure time in hours 0	24	48	72	96
Captan	$1x10^{-5}$ M	.460	.391	.377	.402	.704	1.017
Captan	$1x10^{-6}$ M	.460	.391	.579	.909	1.490	2.339
Carbaryl	$1x10^{-4}$ M	.460	.391	.452	.710	1.161	1.695
Cyprex	$1x10^{-4}$ M	.460	.391	.478	.755	1.224	1.608
Guthion	$1x10^{-4}$ M	.460	.391	.418	.665	1.110	1.476
HEOD	$1x10^{-4}$ M	.460	.391	.508	.709	0.971	1.272
Parathion	$1x10^{-4}$ M	.460	.391	.434	.685	1.074	1.462
Simazine	$1x10^{-4}$ M	.460	.391	.583	.796	1.365	2.114
Sulfur	$1x10^{-4}$ M	.460	.391	.612	.834	1.452	2.022
DMSO	$1.28x10^{-1}$ M	.460	.391	.598	.861	1.412	1.730
Control		.460	.391	.524	.854	1.409	2.293

* Figures are in millions of cells per plate

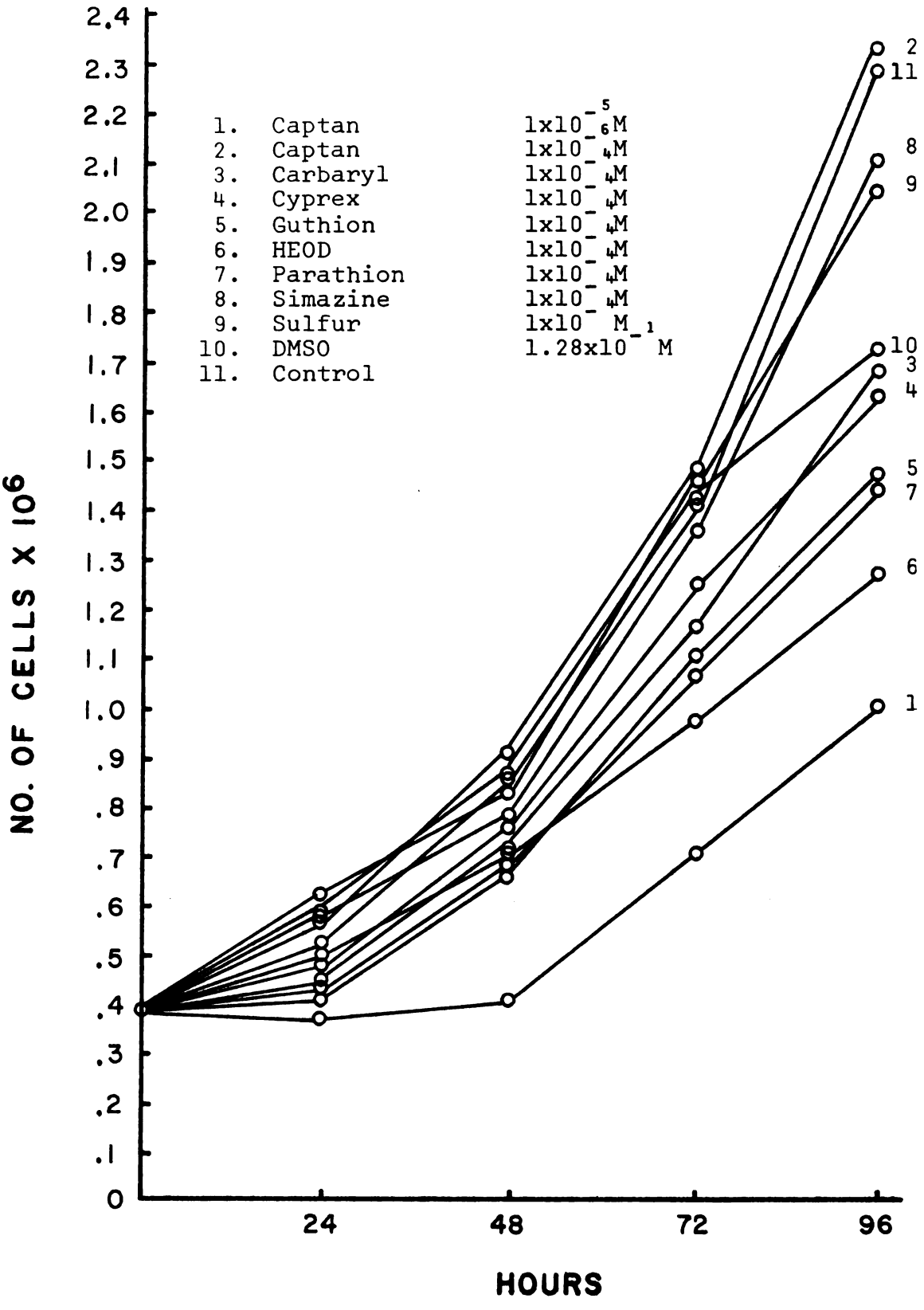

FIGURE 5. Effect of pesticides on growth of AV3 cells.

DISCUSSION

The problems inherrent in this type of research are two-fold. In order to show the significance of a small increase in aberration rate, large numbers must be employed. Secondly, and perhaps more important, was the difficulty of pinpointing an agent when such a wide variety of compounds was used. However, a study of this type can show, if usage increases, the chances of chromosome damage and whether further investigation is justified. The data obtained on chromosome breakage was inconclusive in itself, although further study seems to be implied when weighed in conjunction with the tissue culture data. There was an increase in aneuploidy but it was not statistically significant. Because of this slight increase in exposed individuals, all cells analyzed in future experiments should be counted. Aneuploidy should be of concern since cancer cells typically have more than the normal complement of chromosomes (Conen, 1967). Cancer cells have a higher metabolic rate and the ability to divide without restraint (Environment, 1970). Chromosomes (DNA) as the carriers of genes, control the activities of the cell. It is only when a cell has acquired the ability to outgrow other cells that a problem arises. An increase in the rate of chromosome abnormalities could increase the chances of forming a cancerous cell. This cell could possess a mutation which enhances its

proliferation, resists its death, or inhibits growth or surrounding cells.

The problem of multiple exposure will remain unless a group of formulators could be found who contact only one pesticide. However, this was not imperative in this experiment since the objective was to show whether pesticide exposed individuals would have a higher incidence of aberrations over controls. Only after this has been accomplished does it become necessary to pinpoint the particular pesticide, assuming that synergism does not play an important role. The improved techniques employed during the second year of the study did not completely solve the problem of inconsistent culture viability or standardize in all viable cultures the number of cells suitable for analysis. This is important since it can be seen that in some months very low numbers of cells suitable for analysis were obtained. New techniques recently available should provide additional data for improved statistical validity.

At the concentrations of pesticides used in tissue culture only captan appeared to have activity high enough to make it a potent and potentially dangerous environmental agent. Carbaryl also showed some activity and captan demonstrated an inhibiting action on cell growth. The tissue culture experiment serves only to provide supporting data and a comparison of frequently used pesticides. Virtually any chemical at a high concentration will cause damage to tissue culture cells. Also it can not be said that the effects in tissue culture will be the same as

those in the whole organism. This is because the biochemical processes of the body could activate or inactivate an agent, or inhibit its expression. The tissue culture data does present an interesting situation. Toxicity is the usual test used in determining the safety of a pesticide. Captan has a low toxicity when compared with other pesticides. However, of the pesticides tested, it had an inhibiting effect on cell growth and the highest aberration rate. It is interesting to note that the volunteer group consisted of individuals with a high exposure to captan. Usually low numbers of analysable cells were obtained, not because of a complete growth failure, but rather due to inadequate growth. It is possible therefore that the action of captan was a contributing factor.

Visable chromosome aberrations represent a gross means of evaluating genetic damage. Usually they would result in cell death. However, when chromosome aberrations are observed it must be assumed that point mutations could also be produced. Furthermore, when using human peripheral leucocytes as a monitoring system the assumption is made that the effects seen in somatic cells would also appear in germ cells. Chromosome aberrations in human leucocyte cells can only serve as a warning indicator of a potential genetic effect in humans.

SUMMARY

An attempt was made to use human peripheral leucocytes as an *in vivo* monitoring system of the subtle long term genetic effects of pesticides. The project was a pilot study to determine if a genetic risk existed. It demonstrated the feasibility of using human leucocytes, although the wide variety of pesticides used by the sprayers made etiological conclusions difficult. Alterations of the test system were suggested to increase the validity of the data. The incidence of chromosome breakage in the exposed group was only slightly higher than that in the control group and larger numbers would be necessary to demonstrate a significant increase. In general, assuming the adequacy of the test systems used, the pesticides tested *in vitro* did not seem to present a human health danger from the gross cytological viewpoint. However, captan was demonstrated to be of concern due to its increase of chromosome aberration rate and inhibition of cell growth.

Recommendations for future pesticide-chromosome research

1) Monitor larger numbers of subjects on a once a year basis due to the long mean life of lymphocytes.

2) Attempt to establish a formulator group, contacting only a specific pesticide, in addition to the sprayer group.

3) Screen all pesticides encountered in an in vitro tissue culture situation.

4) Investigate the use of an intraperitoneal diffusion chamber in experimental mice to have a controlled and more realistic screening system.

5) Count all cells analyzed

6) Collect and culture blood using the methods described by Katz et al., 1970.

7) Initiate two cultures per subject and harvest at 55 and 65 hours to assure significant mitotic plates.

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