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IMMUNOCHEMISTRY OF RAT LUNG TUMORIGENESIS

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals, "Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

Michael G. MacNaughton

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Changes in cell mediated immunity have been identified in relation to tumorigenesis. Most chemical carcinogens were found to be immunosuppressive. Cytogenetic changes and impaired DNA repair-synthesis were also found to be associated in chemical carcinogenesis. Measurement of temporal variability of immunologic, cytogenetic and DNA repair changes in relation to exposure to

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chemical carcinogens and throughout initiation and progression of lung cancer in an animal model is the subject of this research project. The first phase of this investigation includes the in vitro measurement of cellular immune response as well as sister chromatid exchange and DNA replication and repair synthesis in spleen, thymus and blood lymphocytes in control rats. This project was initiated in November of 1980; therefore, this annual report reflects only 8 months of work. It is projected that Phase I be concluded by the end of 1981. This will be followed by Phase II which will include the measurement of cellular immune parameters, sister chromatid exchange and DNA replication and repair synthesis in spleen, thymus and blood lymphocytes throughout tumorigenesis in rats intratracheally exposed to 3-methylcholanthrene (MCA).

Study Design

PHASE 1

TIME PERIOD	WORK	LOCATION
November, 1980-November, 1981	A. Quantitate in control rats 1. Lymphocyte subpopulations 2. Lymphocyte mitogen stimulation 3. Sister chromatid exchange 4. DNA replication and repair synthesis	UCI
	B. Test Feasibility of shipping tissue from THRU to UCI	UCI & THRU
March, 1981-November, 1981	Induction of rat lung tumors by intratracheal inoculation of MCA, dosimetry and pathology	THRU

PHASE II

December, 1981-December, 1982	Biological testing of rats exposed intratracheally to MCA	UCI & THRU

PREFACE

This is the first Annual Report for the Project on Immunochemistry of Rat Lung Tumorigenesis, a subprogram of the Toxic Hazards Research program performed by the Department of Community and Environmental Medicine of the University of California, Irvine (UCI) on behalf of the Air Force under Contract Number AF F33615-80-C-0512. This report describes the research activities at UCI from November, 1980 through May, 1981. During this period, H.A. Guirgis, Ph.D. was Principal Investigator for the research project, Paula Sweet served as Senior Medical Technologist beginning in January 1981 and Jeff Elliott served on a part-time basis as a research assistant.

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IN VITRO TESTING OF CELL MEDIATED IMMUNE RESPONSE

INTRODUCTION

Most chemical carcinogens are immunosuppressive. Whether or not immunosuppression is a prerequisite of tumorigenesis is still under investigation. It has been shown that small doses of 3-methylcholanthrene (3MC) are cumulatively immunosuppressive and that both humoral and cell-mediated immune responses are affected (Stjernsward, 1969, 1972 and Prehn, 1963).

The relationship of locally growing tumors to their host is variable. These host-tumor relationships reflect the interactions of many factors (Vaage, 1974; Mikulska, 1966): the need for continuous stimulation of the host by tumor antigens for the maintenance of immunity; immunosuppressive effects of large tumors mediated possibly by release of excessive amounts of circulating soluble tumor antigens that preempt potentially cytotoxic lymphocytes; development of suppressor lymphoid cell populations; and blocking serum factors (Riggins, 1964; Stjernsward, 1968; Lausch, 1969; Bard, 1969; Chandradasa, 1973; Basombrio, 1972; Baldwin, 1974; Yoshida, 1963; Rosenau, 1966). However, spleen, lymph node or peritoneal cells of tumor bearing animals are often capable of expressing in vitro and transferring in vivo anti-tumor immunity (Deckers, 1971; Wepsic, 1970; Milas, 1974). The magnitude of this activity depends on the rate of tumor growth and size of the tumor.

Transfer of immune spleen and lymph node cells with resulting inhibition of chemically induced tumor has been accomplished in a syngeneic immune system (Old, 1962). Since sensitized lymphoid cells exert a strong antitumor effect in vitro, it is also plausible to postulate a direct action of lymphoid cells (cytotoxic lymphocytes) on tumor cells in vivo or a distant action of the same cells but conveyed by soluble mediators (Mackler, 1971). In experiments in which donor lymphocytes were labeled with ^{3}H-uridine, they settled in the spleen of the recipient rather than in the tumor, and the antitumor effects of donor lymphocytes were abrogated by spleenectomy. When sensitized sheep lymphoid cells were used to treat a chemically induced sarcoma in the rat, lymphoid cells in the lymph nodes 90-120 hours after immunization were found to be the most effective. The sheep cells settled in the spleen of the rat and were eliminated within a few days. However, spleen cells of the host were shown to synthesize sheep Υ globulins (Alexander, 1968). The appearance of sheep-type globulin with rat lymphoid cells coincided with significant but temporary regression of tumor. The

interpretation offered is that messenger cells instruct by means of RNA transfer, lymphoid cells or the host to react against the tumor, either by antibody production or by cell-mediated reaction (Alexander, 1967). Thus, in this class of lymphoid cells, the messenger cell exerts its action by instructing the host's lymphoreticular system to undertake a specific immune reaction.

The suppressor function of immune responsiveness has been attributed to a special class of T lymphocytes. The suppressor cells, which normally maintain homeostasis by preventing autoimmune reactions, do so either by suppressing helper and amplifying T cells or by suppressing specifically the activity of non-T cells; antibody production by B cells is not commonly the subject of suppressor T cell activity. Suppressor T cells exert their effect on the tumor-bearing host by molecular mediators with a molecular weight lower than that of the serum albumin (Fujimoto, 1975). The targets of regulatory activity of suppressor T cells are both B and T lymphoid cells. Interactions between T cells are thought to be well balanced; i.e., for lung T cell-dependent augmentation (helper and amplifier function) there is an equal but opposite T cell mediated suppression (Gershon, 1974). Not only antigen-antibody complexes but also competing antigens and certain mitogens such as concanavalin-A induce suppressor cells (Glasgow, 1974; Rich, 1975). Suppressor T cells may be determined using colony inhibition assay or microplate cytotoxicity assays. In mice with tumors exceeding a certain size, rapid decrease and disappearance of all cytotoxic activity followed. Three or four weeks after surgical removal of tumor, the cytotoxicity was fully restored. This fluctuation of lymphocytic cytotoxicity depended on tumor size (Youn, 1973; LaFrancois, 1974).

In 1959 it was reported that phytohemagglutinin (PHA), an extract of the red kidney bean (Phaeolus Vulgaris), could stimulate the transformation of human small lymphocytes in culture (Hungerford, 1959). The in vitro lymphocyte response to PHA was found to be of value in the classification of the primary immune-deficiency diseases (Seligmann, 1968). Work in experimental animals suggests that PHA induces the transformation of T lymphocytes (Rodey, 1969; Greaves, 1968; Doenhoff, 1970; Owen, 1971; Jones, 1972; Lischner, 1967). Recent findings indicate that at least a portion of those lymphocyte responding to PHA are also B lymphocytes (Philips, 1973). PHA was the first non-specific stimulant of lymphocytes transformation discovered. It is classed as nonspecific but this merely signifies that the mode of action is not clearly known (Oppenheim, 1968). A number of other nonspecific stimulants of in vitro lymphocyte transformation have also been

found. These included Pokeweed mitogen (PWM), an extract of Phytolacca Americana, and Concanavalin A (Con A). PWM appears to stimulate mainly B lymphocytes (Knudsen, 1974; Weber, 1973; Weksler, 1974; Cisco, 1974; Stockman, 1971) while Con A stimulates T lymphocytes (Jondal, 1975). These may represent T lymphocytes at different stages of maturation than those stimulated by PHA (Chess, 1974). The number of T lymphocytes in peripheral blood has a direct positive correlation with the in vitro lymphocytic response to PHA and a direct negative correlatión with the in vitro lymphocytic response to PWM (Sengar, 1975).

ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC)

Cytotoxic tests essentially consist of testing the permeability of cells after their incubation with antibody and complement. If the cytotoxic antibody combines with the target cells, the complement is fixed and cell permeability increases. It is usual to assess cell permeability by adding a solution of trypan blue or eosin which penetrates dead cells.

Functionally, this extracellular cytotoxic mechanism would be expected to be of significance where the target is too large for ingestion by phagocytosis, e.g. solid tumors. It could also act as a backup for T-cell killing when antibody production might otherwise lead to protection of the target from attacks by T cells. Through blocking of the surface antigens, the evolution of ADCC mechanisms would ensure that the antibody-coated target was still vulnerable.

Methods and Procedures

Preparation of Lymphocyte-cell Suspensions

1. Spleen Cells

The spleen is obtained from a sacrificed rat and, using sterile technique, it is transferred to a petri dish.

The spleen is cut into small pieces and squashed with a U-shaped plastic "Tissue Squasher." The resulting cell suspension is pipetted into a large glass test tube and 10 ml (Minimal Essential Medium) MEM containing 100 µg/ml Gentamycin is used to rinse the petri dish and is added to the tube. The cell suspension is centrifuged at 1000 rpm for 10 min. at 4°C. The supernatant is aspirated and discarded. The lymphocytes are washed 3 times before cell-counting and determination of viability.

2. Blood-lymphocytes

Whole blood is diluted in MEM and this suspension is layered on top of ficoll-hypaque gradient. The tubes are centrifuged for 30 min. at 1500 rpm at 4°C. The top layer is aspirated and discarded. The lymphocyte layer on top of the ficoll-hypaque is removed and pooled into a large tube containing 10 ml MEM. An aliquot (0.2 ml) is removed for a cell-count and viability determination using the dye exclusion procedure.

3. Thymus Cells

Thymocytes are prepared by essentially the same method used for the spleen except that the thymus is placed in a glass test tube rather than a petri dish and the tissue is homogenized with a pestle-like tissue grinder. From this point on the method is the same for thymus as for the spleen.

Procedure for Enumeration of Suppressor T-cells

Lymphocytes are separated from rat whole blood and spleen using the lymphocyte separation medium (Bionetic Laboratories Products, Kensington, ME), resulting in minimum red blood cell contamination. Cells (2×10^5/well) and antisera (50 µl each) are added to wells of microculture plates and incubated at 4°C for one hour followed by the addition of complement (50 µl). The plates are incubated at 37°C for 1 hour and kept refrigerated until the addition of the stain (eosin) and microscopic examination. Experiments were designed to establish the appropriate antibody titer, the dose of the complement, and the cell concentration. The antiserum dilutions ranged from 1:103 to $1:10 \times 10^4$. The complement dilutions ranged from 1:5 to 1:40. A new vial of complement is reconstituted for each experiment to ensure high activity. Eosin (0.2%) in saline is used in place of trypan for dye exclusion since a greater contrast between live and killed cells can be seen microscopically, and percent viability is calculated.

Results

The results of the most recent experiment using rat, spleen and thymus lymphocytes are presented in Table 1. At the 1:5 complement dilution there is some whole spleen lymphocyte cytotoxicity present between 1:5,000 and 1:10,000 dilutions of antibody. When using thymus lymphocytes at 1:10 dilution of complement, cytotoxicity is observed with antisera diluted 1:5000 times.

TABLE 1. PERCENT VIABILITY OF SPLEEN AND THYMUS LYMPHOCYTES AT DIFFERENT DILUTIONS OF COMPLEMENT AND ANTISERUM

	ANTISERUM DILUTION					
	1:	$5x10^3$	$10x10^3$	$20x10^3$	$50x10^3$	$10x10^4$
	COMPLEMENT DILUTIONS					
	*CONTROL	99	100	99	99	100
	UNDILUTED	93	86	89	90	91
SPLEEN	1:5	73/75	80	80	81	65
	1:10	76	70	78	77	79
	1:20	78	69	80	77	80
	1:40	74	76	59	70	69
		$5x10^3$	$10x10^3$	$20x10^3$	$50x10^3$	$10x10^4$
	*CONTROL	98	99	98	100	100
	UNDILUTED	100	100	98	97	100
	1:5	81	83	86	87	65
THYMUS	1:10	57	67	73	86	88

*heat-inactivated guinea pig complement

IN VITRO MITOGEN STIMULATION IN SPLEEN AND THYMUS LYMPHOCYTES

In vitro response of lymphocytes to specific antigens and plant mitogens has been used to demonstrate the level of cellular immune response. Impairment in that response upon in vitro testing has been found to be associated with the presence of malignant disease in both animal models and in human experience. Furthermore, the magnitude of the impairment in lymphocyte response to mitogens has been correlated with tumor burden.

To test lymphocyte mitogen activation, it is necessary to quantitate the proliferation of sensitized cells on contact with specific mitogens in vitro. Quantitation may be accomplished by microscopic examination and enumeration of the change in cell morphology to large blast-like cells with paler staining nuclei and basophilic cytoplasm. Quantitation may also be done by measuring the incorporation of ^{3}H-thymidine following incubation with cells during transformation. Similar changes can be induced in lymphocytes using certain plant mitogens such as phytohemagglutinin (PHA), Concanavalin A (Con A) and Leucoagglutinin (LA). These are termed polyclonal activators because they react with the cell surface non-specifically and produce the same series of cellular events.

Methods and Procedures

Lymphocytes were separated and counted and viability was tested (using trypan blue exclusion microscopic examination). The cells were cultured in microculture plates, $1x10^5$ cells per well. Roswell Park Memorial Institute (RPMI) 1640 medium was used throughout this experiment. Two mitogens, PHA (2.0%) and leucoagglutinin (LA) (20 µg/ml), were used and control wells with no mitogen were also prepared. At 72 and 96 hours after incubation at 37°C in a 5% CO_2 humid incubator, ^{3}H-thymidine (0.5 µ/well) was added to all wells and the plates were further incubated for 6 hours. The cells were harvested using the cell harvester and radioactivity was counted using a liquid scintillation spectrometer.

Results

The data on mitogen stimulation in spleen and thymus are presented in Figures 2, 3 and 4. In Figure 1, data are presented on rat spleen cells using Concanavalin A (Con A), Leukoagglutinin (LA). Phytohemogglutinin (PHA), and pokeweed mitogen (PWM) in different concentrations. The highest stimulation of the spleen cells was found when 2% PHA was used followed by LA at 1-2%.

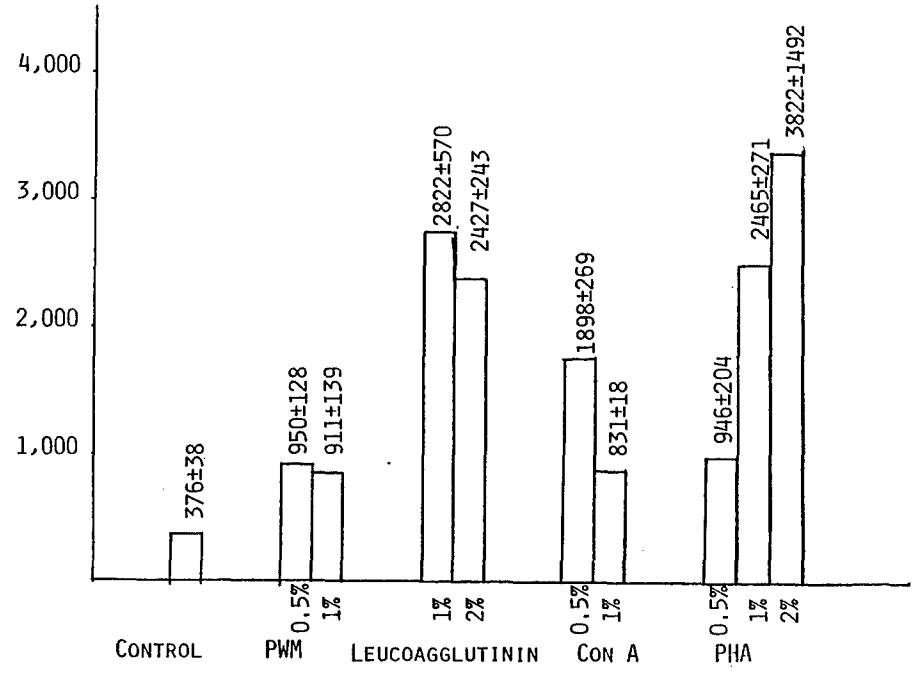

FIGURE 1. Rat Spleen Lymphocyte Stimulation Using Different Mitogens

The effect of different serum supplement is shown in Figure 2. It can be seen that using fetal calf serum (FCS) mitogen activation in rat spleen lymphocytes was higher than newborn calf serum (NCS) when PHA and Leucoagglutinin were used. Seventy-two and 96 hour culture times were tested using spleen lymphocytes (Figure 3). As can be seen, using both PHA (2%) and LA (20 µg/ml) mitogen activation was higher at 72 hours than 96 hours.

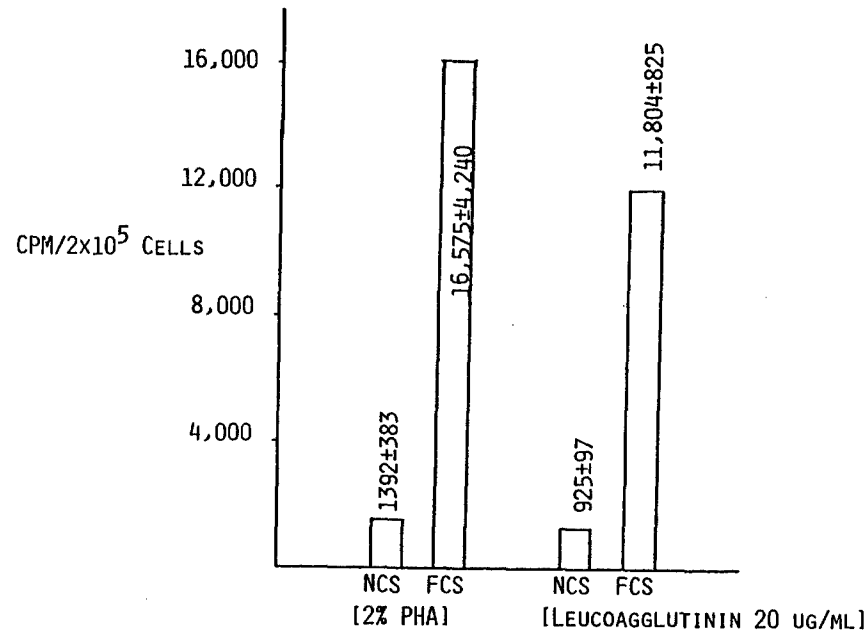

FIGURE 2. Effect of Different Supplement on Mitogen Activation in Rat Spleen Lymphocytes

DNA REPLICATION AND REPAIR SYNTHESIS IN RAT LYMPHOCYTES

INTRODUCTION

DNA repair synthesis has been studied in vitro in humans and in vivo and in vitro in animal models after treatment with carginogens as well as chemotherapeutic agents. This is usually done by measuring the incorporation of ^{3}H-thymidine into non-replicating DNA (Stich and Keiser, 1974; Smith and Hanawalt, 1976a,b; Cleaver, 1973; Craddock et al., 1976; and Lieberman et al., 1971). The increased incorporation of ^{3}H-thymidine in non-replicative cells is termed "repair" or "unscheduled DNA synthesis." This is a subject of controversy (Melzer,1979). However, it seems that this phenomenon,

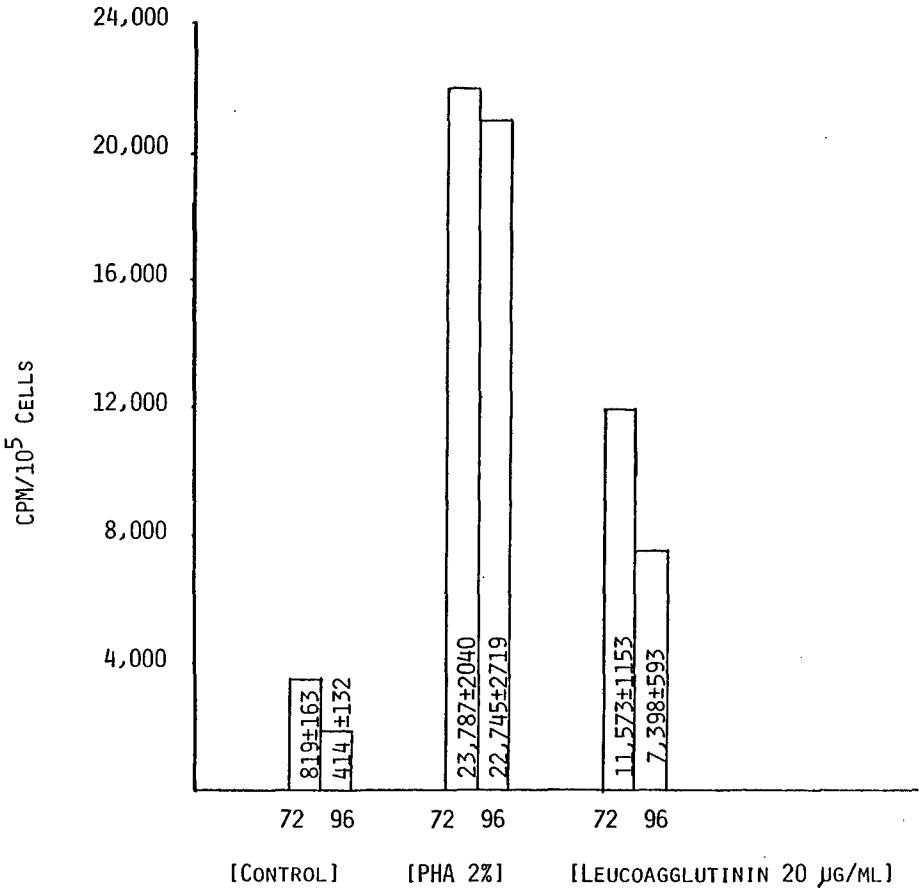

Figure 3. EFFECT OF CULTURE TIME ON MITOGEN ACTIVATION IN RAT SPLEEN LYMPHOCYTES

if not indicative of DNA repair, suggests a direct carcinogen-DNA interaction or carcinogen-induced replication DNA synthesis.

METHOD AND PROCEDURES

a) Lymphocytes are separated using the procedure already described.

b) Cells are suspended in phosphate buffered saline in petri dishes (10×10^6 cells in 10 ml/dish) and exposed to UV light at height of 7½".

c) Control cells are prepared in the same manner with no UV exposure.

d) Cell suspensions are centrifuged and resuspended in media, cell counts and viabilities are determined, and cell concentrations are adjusted to 2×10^6 cells/ml.

e) Cells are plated in microculture plates with and without hydroxyurea (HU) and ^{3}H-thymidine.

f) Microculture plates are covered and mixed, then incubated in a humidified CO_2 incubator at 37°C for 2 and 4 hours.

g) Cells are harvested and washed at the end of incubation periods on glass fiber discs.

h) The discs are counted using a Tracor liquid scintillation counter and the counts are expressed as DPM.

RESULTS

DNA replication and repair synthesis in pooled spleen lymphocytes were measured to compare effects of exposure to different media, to 4-nitroquinoline oxide (4NQO) and to UV light. Table 2 shows the results on spleen lymphocytes using different concentrations of 4NQO and exposure to UV. As can be seen, more repair was found using the lower concentration of 4NQO (5×10^{-6}M) although replication was not significantly different using the two concentrations. It can also be seen that the 10^{-5} concentration of 4NQO was toxic and no DNA repair activity was observed at the three hour incubation period.

UV exposure was also used to induce unscheduled DNA repair synthesis. The UV source (shortwave UV lamp, Minerallight, UVS-11 from Ultra-violet Products, Inc., 115 volt, 60 cycles. 0.12 amps, 250 nm wavelength) is placed in position at a height allowing 1 joule/m^2/sec as determined by use of a Jagger type ultraviolet meter which has been calibrated against a Yellow Springs UV meter. An uncovered petri dish containing 10 ml cell-suspension at 2×10^6 lymphocytes per ml in phosphate buffered saline is centered below the UV source during the duration of treatment. After treatment the cells are centrifuged, resuspended in RPMI 1640 medium, counted and viability determined before use. The results show that thymus lymphocytes demonstrate no repair using 4NQO and minimum repair with UV treatment. Using rat spleen lymphocytes, DNA repair synthesis was found after exposure to 4NQO, while UV treatment at most

TABLE 2

DNA REPLICATION AND REPAIR SYNTHESIS IN RAT SPLEEN CELLS
EFFECTS OF INCUBATION TIME AND EXPOSURE TO 4NQO AND UV EXPOSURE

Treatment	Control Replication	HU	Treated Cells	Treated + HU	DNA Repair
Incubation Time: 2 Hours		DPM ± S.D./2 x 10^5 cells			
4NQO					
1 x 10^{-5}M	1331 ± 266	140 ± 53	900 ± 118	293 ± 18	153 ± 40
5 x 10^{-6}M			1027 ± 27	269 ± 43	129 ± 48
*UV 10 sec.	1250 ± 41	136 ± 70	1545 ± 83	427 ± 73	291 ± 72
15 sec.			1672 ± 62	474 ± 63	338 ± 67
20 sec.			1636 ± 56	424 ± 14	288 ± 50
Incubation Time: 3 Hours					
4NQO					
1 x 10^{5}M	2173 ± 112	106 ± 33	660 ± 68	165 ± 14	59 ± 25
5 x 10^{6}M			1122 ± 124	353 ± 75	247 ± 58
UV 10 sec	1951 ± 128	137 ± 22	1839 ± 145	425 ± 16	288 ± 19
15 sec			2099 ± 75	529 ± 56	392 ± 43
20 sec			1951 ± 52	685 ±103	548 ± 74

UV is delivered at 1.38 x 10^8 ergs/sec.

exposure times induced higher repair. UV treatment for 20 seconds appears to induce maximum DNA repair synthesis. A summary of the results is presented in Figure 4 where it is shown that replication was greater at a 3 hour incubation period than at a 2 hour incubation and that DNA repair was higher when cells were exposed to UV for 20 seconds.

SISTER CHROMATID EXCHANGES IN RAT LYMPHOCYTES

INTRODUCTION

Sister chromatid exchange (SCE) has been studied in animal models, both in vivo and in vitro, as well as in human cells such as skin fibroblasts and lymphocytes. Data suggest that SCE might be useful in testing carcinogenicity, mutagenicity and predisposition to certain diseases.

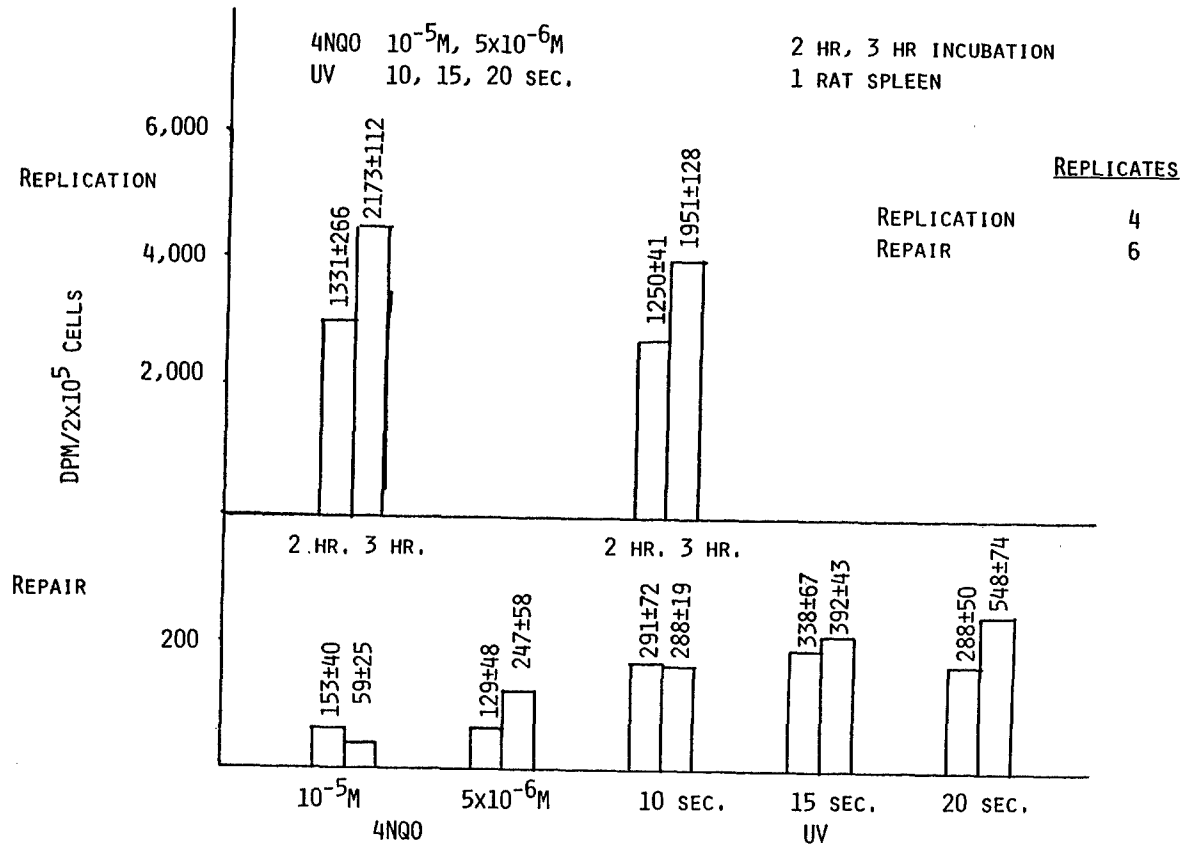

Figure 4. DNA REPAIR IN CONTROL RATS

SCE has been described in cells from different species. Average frequencies of 5.1 to 27.3 SCEs per cell have been reported in human peripheral lymphocytes (Kato, 1977). Within a species there may be wide intra- and inter-individual variation in SCE frequency. The variation between individual humans does not seem to depend on age or sex (Schneider, 1977; Lambert, 1978). Smokers show a higher SCE rate than non-smokers (Lambert, 1978). It has been suggested that differences in genotype may explain differing SCE frequencies between individuals of a given species (Galloway and Evans, 1975).

In general, the average number of SCEs in each chromosome is proportional to chromosome length (Morgan and Crossen, 1977; Latt, 1974a). Human peripheral lymphocytes, however, contain more SCEs in the B group chromosomes (numbers 4 and 5) and fewer in the E, F and G group chromosomes (numbers 16-22) than would be expected based on length (Morgan and Crossen, 1977). There is evidence for a higher

SCE frequency in the mid-arm regions of the chromosomes than in the end regions or areas near the centromere (Latt and Jurgens, 1977). This discrepancy may be due to the relative difficulty of ascertaining SCEs near the ends and centromeres of chromosomes rather than real difference. By staining sequentially for sister chromatid differentiation and G-banding, Latt (1974a) found a non-random distribution of SCEs within individual chromosomes with the majority occurring in interband regions (stained light in G-banded chromosomes). Cells cultured for simultaneous G-banding and SCE identification have shown SCEs in G-bands (stained dark in G-banded chromosomes) (Hoo and Parslow, 1979).

METHODS AND PROCEDURE

Lymphocytes are cultured at a concentration of 1.0×10^6 cells/ml in a total volume of 5 ml/culture using the following medium: Roswell Park Memorial Institute 1640 (RPMI 1640) (Grand Island Biological Co.), 1 mM/ml Glutamine (Grand Island Biological Co.), 100 µg/ml Gentamicin (Upjohn), 20% fetal calf serum (Microbiological Associates), 2% Phytohemagglutinin (PHA) (Grand Island Biological Col), 2% Leucoagglutinin (Pharmacia) and 0.5% Concanavalin A (ConA) (Pharmacia). The cultures are incubated for 72 hours at 37°C in a 5% CO_2 humidified incubator. At 72 hours Colcemid (Grand Island Biological Co.) is added to the cultures to give a final concentration of 0.05 µg colcemid per ml of culture medium. The cultures are returned to a CO_2 incubator for a period of 1 hour after which they are treated with a solution of 0.075 M KCl, fixed with a 3:1 mixture of methanol and acetic acid, and chromosome spreads are prepared on microscope slides. The slides are air-dried. They are stained with Hoechst 33258 at a concentration of 50 µg/ml in Sorensen's buffer for 10 minutes and rinsed in distilled H_2O. Slides are placed in a shallow pan, flooded with Sorensen's buffer, covered with saran wrap and exposed to intense illumination from cool-white fluorescent lamps for 3 hours. The slides are heated in a solution of 0.3 M NaCl and 0.03 M Na Citrate at 60°C for 30 minutes after which they are stained with a solution of 3% Gurr's R-66 Giemsa (Searle) in Na phosphate buffer for 15 minutes, rinsed in Na phosphate buffer (pH 6.8), and left to air dry overnight before cover slipping. This method is still undergoing modification, leading to establishment of a finally satisfactory procedure.

RESULTS

Several different conditions were examined in an attempt to develop a reproducible method for sister chromatid exchange using normal rat lymphocytes. These conditions are as follows:

1. Six different culture media were used to test for maximum growth conditions (Table 3).

TABLE 3. SIX FORMULAE FOR TISSUE CULTURE MEDIA

A		B	
Minimum Essential Medium (MEM)	78%	MEM	78%
Fetal Calf Serum (FCS)	20%	Newborn Calf Serum (NCS)	20%
Leukoagglutinin (LA)	2%	LA	2%
C		**D**	
MEM	78%	MEM	78%
FCS	20%	NCS	20%
Phytohemagglutinin (PHA)	2%	PHA	2%
E		**F**	
RPMI 1640 (commercially available)	78%	RPMI 1640 (commercially available)	78%
FCS	20%	FCS	20%
PHA	2%	LA	2%

2. Duration of culture incubation with bromodeoxyuridine (BrDU) was varied (72, 96 hours) to achieve two cell divisions and hence to insure the incorporation of the BrDU in DNA and eventually differential staining and possible scoring of sister chromatid exchanges.

3. Variability in exposure to hypotonic solution of potassium chloride before fixation and staining of cultured lymphocytes. (The purpose is to maximize the number of scorable metaphases where chromosome spreads are adequate for microscopic examination.)

Duplicate slides of each treatment were examined to quantitate overall stain quality (0, no staining through 3+, good staining) and the numbers of chromosome spreads present (0, 0-5 spreads; 1+, 5-15 spreads; 2+, 15-25 spreads; 3+, > 25 spreads). When spreads were found, the number of cell divisions were determined from the staining characteristics of the paired chromosomes.

Figures 5 and 6 show metaphases of spleen lymphocytes SCE and Table 4 shows the marker of first and second division cells and the

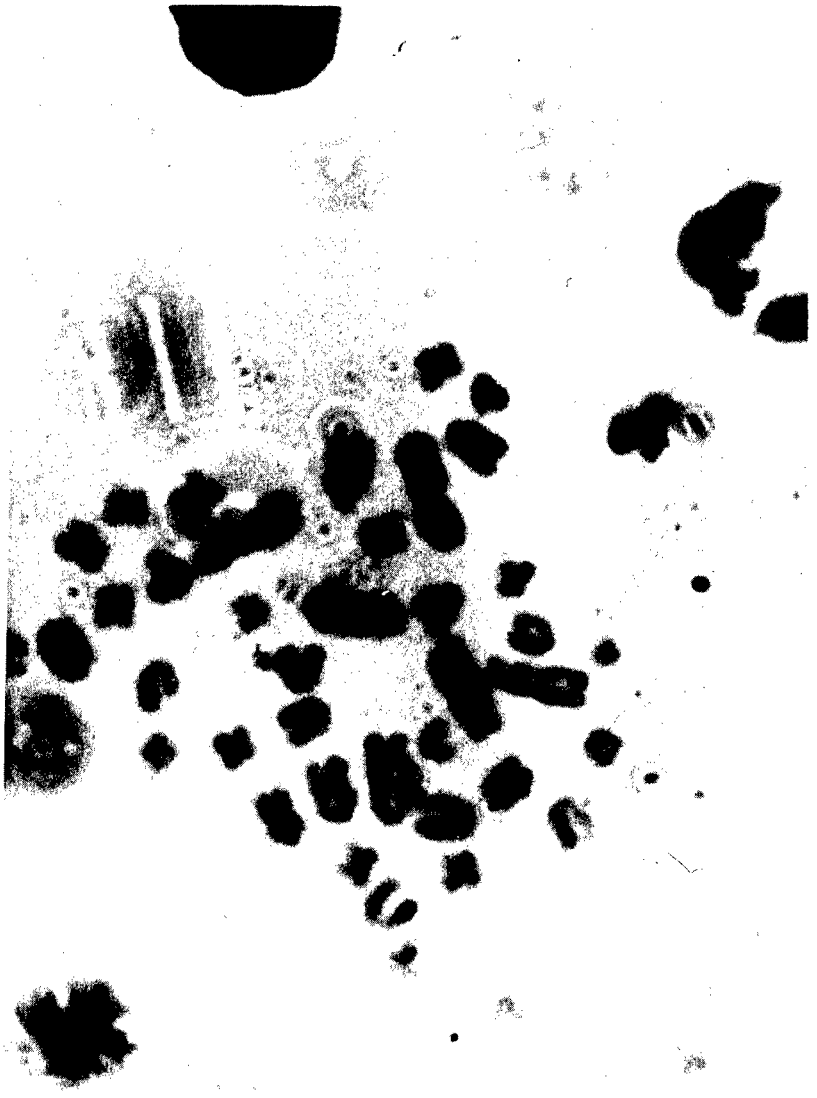

FIGURE 5. An example of a second division spread of R47C#2 at 18.5 x 90.4. Nikon camera and microscope settings were: automatic, ASA 100, hv 10, mag 100 x, upper and lower filters - blue.

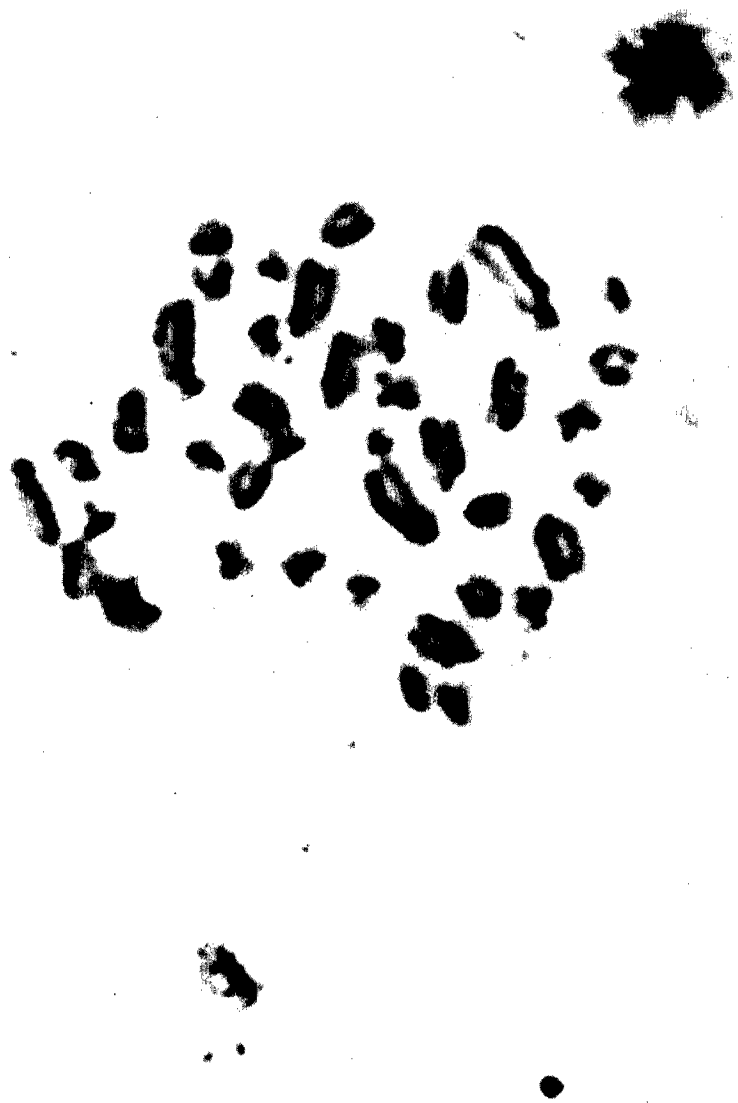

FIGURE 6. Print made of second division spread of R47C#2 at 9.8 x 90.4. Nikon camera and microscope settings were: ASA 100, hg 10, mag 100x, exposure using manual 1 second setting and blue upper and lower filters.

TABLE 4. FREQUENCY OF FIRST AND SECOND METAPHASES IN CULTURED RAT SPLEEN LYMPHOCYTES

Total Metaphases	Range From	To	Mean
Percent metaphases in first division	92	436	236
Percent metaphases in second division	31	54	40

number of scorable metaphases per microscope slide where clear differential staining and complete chromosome spreads are identifiable. Using the method described above, it is clear that we are able to quantitate SCE's in spleen lymphocytes from control rats.

3-METHYLCHOLANTHRENE STUDY

INTRODUCTION

In Phase II of this research project, tissue from rats intratracheally exposed to 3-methylcholanthrene (3MC) was sent from THRU to our laboratories at UCI. The objective of this experiment was to find out whether it is necessary to separate rat lymphocytes from blood, thymus and spleen and suspend them in tissue culture media before shipment from Dayton to Irvine. For this reason, Mr. Scott Bowers of the Toxic Hazards Research Unit was trained in this procedure during one week at UC Irvine; upon his return to the following experiments were conducted.

METHODS AND PROCEDURES

Rats were anesthetized, and whole blood was collected from the vena cava using a heparinized syringe containing RPMI 1640 tissue culture medium. One aliquot of the blood was immediately layered on lymphocyte separation medium and after centrifugation the separated cells were washed and counted. The separated blood lymphocytes were placed into test tubes, wrapped and placed on ice in a closed container. The other aliquot of blood was wrapped and placed similarly. The spleen and thymus were removed from two anesthethized animals and placed in sterile test tubes containing RPMI tissue culture medium. The lymphocytes from one spleen and one thymus were suspended in media, washed and counted. The remaining tubes containing whole tissue and the tubes containing cell

suspensions were wrapped and placed on ice in a closed container. Blood and tissue cell suspensions from the above procedures were shipped from Dayton to UCI. They were then removed from containers and cell suspensions were counted and stored again for another 24 hours. Another sample of whole blood, spleen and thymus was removed, cell suspensions were prepared and cells were counted and viabilities were determined. This procedure was followed until cell counts and/or viabilities were below the range normally accepted for subsequent assays (< 50%).

RESULTS

The results of two separate experiments are presented in Tables 5 and 6. When comparisons were made, it was clear that if whole tissue was kept 24 hours after shipping followed by lymphocyte separation, the recoverability and viability of lymphocytes was similar to that of the fresh preparations. Both cell recovery and viability were reduced at 3 or 4 days.

TABLE 5. CELL COUNTS AND VIABILITIES OF LYMPHOCYTES FROM THYMUS AND BLOOD AFTER SHIPMENT AND STORAGE

	Thymus					
	Day 1		Day 2		Day 3	
	Total Cells	Viability %	Total Cells	Viability %	Total Cells	Viability %
Whole	44	96	16	99	56	86
	32	98				
			27	99		
Cell	18	91	39	98	30	85
Suspension	45	71	27	99		
			15	53		
			32	24		
			Blood			
Whole	5.6	100	4.3	100		
Whole Lymphocyte Separated	1.54	100	4.5	100	3.0	100
			3.8	100		
			6.6	99		

These experiments have continued and in addition to cell counts and viabilities, some of the immunological tests using these cells have been performed to determine any changes in lymphocyte function.

TABLE 6. CELL COUNTS AND VIABILITIES OF LYMPHOCYTES FROM SPLEEN AFTER SHIPMENT AND STORAGE

	Total Cells	Viability %	Total Cells	Viability %	Total Cells	Viability %
Whole	332	96	345	96	496	96
Tissue	205	99	261	92	217	57
	343	96	370	99		
Cell	256	98	175	96	151	95
Suspension	205	99	159	99		
	323	97	242	94		
			193	94		
			298	96		
			324	98		

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