

IN VITRO EFFECTS OF NITROGEN MUSTARD ON
RHEUMATOID AND NON-RHEUMATOID CELLS

by

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STATEMENT BY AUTHOR

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ABSTRACT

Leading investigators subscribe to the theory that rheumatoid arthritis is an auto-immune disease. Immuno-suppressive agents have been of therapeutic value in treatment of the disease. Effects of one of the immuno-suppressive agents, nitrogen mustard, on rheumatoid and non-rheumatoid synovialis fibroblasts and peripheral blood lymphocytes were measured in vitro. Results were assayed by means of acridine orange fluorescent stain, Wright stain, and viability studies.

Fibroblasts from both groups showed similar response to the drug. Within a narrow range of concentrations there was an increase in nucleolar and cytoplasmic RNA.

Lymphocytes also responded with an increase in nucleolar and cytoplasmic RNA but within an even narrower range of drug concentration. Rheumatoid lymphocytes showed a greater percentage increase of RNA than did non-rheumatoid cells. No mitotic figures were observed.

A selective population of lymphocytes survived in vitro treatment with nitrogen mustard. The survivors regained their mitotic activity and responded to phytohemagglutinin. The behavior of the small population of lymphocytes which persisted in vitro despite high dosage of nitrogen mustard is provocative and warrants further investigation of its potential to respond to immunological stimuli.

INTRODUCTION

Immuno-suppressive agents, such as nitrogen mustard, cyclophosphamide, azathioprine, and 6-mercaptopurine have been shown to be effective in the treatment of rheumatoid arthritis (1-3). The present investigation was undertaken to determine the in vitro effect of one of these drugs on fibroblasts and lymphocytes from rheumatoid and non-rheumatoid individuals. The fluorochrome dye acridine orange was used in assay and the cells positively identified with Wright stain.

Arthritis is one of the oldest diseases known to mankind having been found in man 2,000,000 years ago (4). Rheumatoid arthritis (RA) is a constitutional disease which manifests itself as a profound disturbance to various tissues of the body, particularly the joints, and leads to chronic, progressive crippling (5). There is still no known cause or cure for rheumatoid arthritis. Among the theories considered in the etiology are viral or bacterial infection, a faulty immune mechanism, heredity, endocrine defects, and metabolic abnormalities (6, 7). Two widely considered theories are that RA is an auto-immune or a cross-immune disease (6-9). Osgood defines them as follows: Auto-immunity--"production of antibody in response to the presence of an undenatured antigen normally present;" Cross-immunity--"ability of antibodies to react with chemical groups closely related in structure to that of the antigen which led to their production (9)." The fact that immuno-suppressive agents have been of therapeutic value in the disease lends support to the auto-immune or cross-immune theories.

There are two currently proposed ideas concerning the mechanism of these reactions: A) Burnet's "forbidden clone"--lymphocytes which proliferate from a cell which produced antigen to an autologous component (8); and B) Hollander's theory--interaction of lymphocytes, plasmacytes and polymorphonuclear leukocytes where the lymphocyte produces an antigen to which the plasmacyte provides an antibody. A complex of the two proteins is formed, ingested by the polymorphonuclear cell, which in turn liberates lysozymes which cause inflammation (10). The second theory could be an extension of the first; the two are not mutually exclusive.

Nitrogen mustard (HN_2) is among the most effective immunosuppressive agents (11) and cytotoxic agents. It inhibits antigen uptake, inhibits mitosis, inhibits synthesis of DNA (12, 13), RNA, and protein; alters nucleic acid bases, and ultimately causes cell destruction (11). Each of these actions specifically may be effective in combating the theorized causes of auto-immune diseases. In addition, as an alkylating agent, HN_2 combines with the prosthetic groups of enzymes (14).

Cytotoxic agents are more effective in blocking the inductive phase of antibody formation than the secondary immune response (15). Schwartz and Dameshek (16) suggest the constant stimulus to the antibody-forming system in auto-immune disease, due to the continual presence of antigen, results in a chronic immune response analogous to the primary response. Inhibition of continuing antigen release and antibody production is a possible explanation for the response of RA to HN_2 .

The severe side effects of HN_2 , such as vertigo, nausea, jaundice, thrombosis, diminished hearing, alopecia, depression of peripheral blood cells, limit its use in the clinical treatment of RA (17). An analog of HN_2 , cyclophosphamide (CP), has less severe side effects, but these are still so disturbing that the drug is used with extreme caution and then only in patients refractory to other treatment. In one long-term study, 75% of patients treated with CP responded favorably over a period of five years (1).

As the clinical work with this drug progressed, the question arose as to its in vitro effect on RA and Non-RA cells. Unfortunately, CP is not effective in vitro. It is activated in vivo by the phosphoamidases to form HN_2 (18). Although these enzymes are present in all cells to some degree, they do not occur in sufficient quantity in vitro to activate CP. Therefore, HN_2 , the active component itself, must be used for all in vitro work.

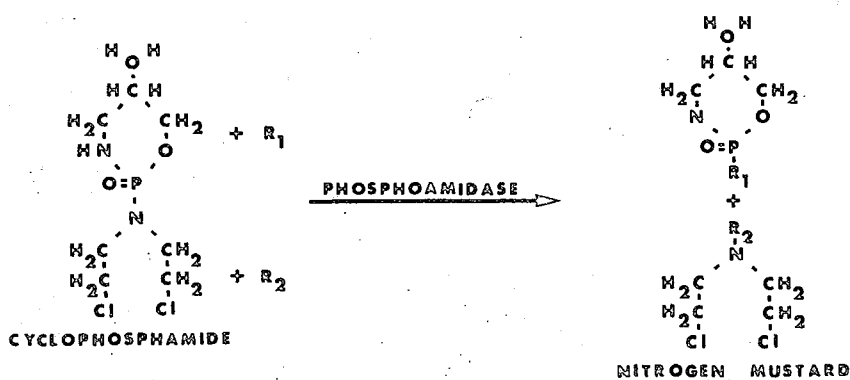


Figure 1. Cyclophosphamide Cleavage to Form Nitrogen Mustard in Vitro

R's are organic radicals. R_2 could be the prosthetic group of an enzyme, nucleic acid base, or reactive groups of amino acids: amino, sulfhydryl, carboxyl, and phenolic hydroxyl.

HN_2 is a nitrogen analog of sulfur mustard which was first synthesized in 1854, its vesicant properties described by Meyer in 1887 (19). During World War I, medical attention was first focused on the blistering action of mustard gas on the skin, eyes, and respiratory tract. In 1919, Krumbhaar and Krumbhaar (20) noted that mustard gas poisoning was characterized by leucopenia, aplasia of the bone marrow, dissolution of the lymphoid tissue, and ulceration of the gastrointestinal tract. It was demonstrated that nitrogen mustard caused extensive regression of established tumors in mice (21). This research led to use of similar chemotherapy in human cancer. Much more recent is its use in the treatment of diseases of the auto-immune type (1-3, 22).

Interruption of lymphocytic activity is an important mechanism in immuno-suppression. Immuno-suppression has been obtained in humans through thoracic duct drainage of lymphocytes (23). The therapeutic value of cyclophosphamide in rheumatoid arthritis may thus be due merely to the fact that the drug selectively affects leukocytes (including lymphocytes) prior to damaging other hematopoietic components: granulocytes, thrombocytes (platelets), and erythrocytes and prior to affecting other cells of the body (24). Thus, by regulating dosage, HN_2 can be used for immuno-suppression without seriously disturbing the other cells in the body.

As an alkylating agent, in addition to its effect on enzymes mentioned above, HN_2 combines with nucleic acids and with the organic radicals of the amino acids: amino, sulfhydryl, carboxyl, and phenolic

hydroxyl (15). The multiplicity of the possible reactions of alkylating agents with biological materials makes difficult the designation of a single reaction as the cause of the cytotoxicity of HN_2 .

The most important pharmacological actions of the nitrogen mustards are those which disturb fundamental mechanisms concerned with cell growth, mitotic activity, cell differentiation, and function. Proliferating cells manifest altered chromosome structure. Even if the changes are not so extensive as to be lethal, they may nevertheless be reproduced indefinitely as inherited mutations (24).

In the present in vitro study, fibroblasts and lymphocytes were investigated. RA is a disease of the connective tissue (25), and fibroblasts comprise the major formed elements (26). Many rheumatologists believe that the disease is first manifested in the synovial lining of the joints (9, 26, 27). Therefore, synovialis procured at open surgery was used as the source for fibroblast culture. The lymphocyte was chosen because it is believed to play an important role in RA (8, 9). This small, ubiquitous cell has been the subject of intensive research. It is directly involved in auto-immune diseases and tissue-homograft rejection (8). Furthermore, the lymphocyte, as one of the leukocytes, falls in the category of cells first destroyed by HN_2 in vivo (11, 18, 20, 24) as noted above.

Miller and Cole (28) reported that a small population of rat lymphocytes survive large dosages of HN_2 in vivo. It seemed of importance to investigate whether lymphocytes from humans could also survive treatment with HN_2 in vitro. Since small populations of lymphocytes

alone are short-lived in culture (29, 30), the feeder layer technic (29) provided a means for continuing study of the treated lymphocytes.

Acridine orange is a sensitive means for morphological differentiation of cells (31-33). It forms chemical bonds with RNA and DNA which fluoresce orange-red and apple green, respectively. The specificity of this reaction is evidenced by the absence of fluorescence when cells are treated with RNase and DNase. The intensity of RNA fluorescence is directly proportional to RNA content. Variations of cytoplasmic RNA during maturation is significant. The immature blast forms contain large amounts of RNA with concomitant fluorescent intensity and as the cell matures, the RNA content and intensity decrease (32).

The two-fold object of this study was first, to determine the effects of HN_2 on both RA and Non-RA synovialis fibroblasts and peripheral blood lymphocytes; and secondly, to determine if human lymphocytes could survive a massive dosage of HN_2 in vitro.

MATERIALS AND METHODS

Fibroblasts

Specimens of human synovialis were obtained surgically from two patients;*

RA - 42 year old male, rheumatoid spondylitis, arthroplasty of the left hip.

Non-RA - 39 year old male, torn medial meniscus, menisectomy of left knee.

At the time of surgery, tissue was placed in a sterile container in Eagle's minimum essential medium (MEM) (34) with penicillin-streptomycin 50 units each/ml. In the laboratory, the tissue was dissected with Bard-Parker blades into explants, each approximately 1 mm. square. Following established technics (30, 35) approximately 20 fragments were placed in a 2-oz. prescription bottle and covered with perforated dialysis membrane to support the tissue in early stages of growth. Each container received 5 ml. MEM, enriched with 20% bovine calf serum, or pooled human cord serum,** with penicillin-streptomycin 50 units each/ml. and glutamine 2.9 mg./ml. The two enriched media were designated BCS and PHCS respectively. All cultures were stoppered and incubated at 37°C.

*Specimens obtained at open surgery through the interest and kind assistance of the orthopaedic surgeons of Tucson.

**Cord bloods obtained through the generous cooperation of the Staffs in Maternity Services at Davis-Monthan Air Force Base Hospital, St. Joseph's Hospital, and Tucson Medical Center.

The dialysis membrane was removed after a monolayer of cells had formed approximately three weeks later. The cells were removed from the glass by light trypsinization, as follows: culture medium was decanted, 2 ml. of 0.2% trypsin was introduced, and the culture incubated for approximately 5 minutes. The action of trypsin was stopped by addition of 2 ml. of PHCS. The mixture was centrifuged at 800 r.p.m. for 10 minutes and supernatant fluid removed by aspiration. Cells were resuspended in PHCS, and cultured again in 2 oz. prescription bottles. Forty-eight hours prior to challenge, cells were trypsinized, washed and hemacytometer count made. Cells (2×10^5) were suspended in 1 ml. PHCS, and planted in short Leighton tubes* containing tube slips (10 x 50 mm.).

Treatment with Nitrogen Mustard - (HN_2)**

HN_2 was diluted in Earle's balanced salt solution (BSS) (36) in 13 concentrations ranging from 0.1 - 200 mcg./0.1 ml. Each dilution of the drug was incorporated in 1 ml. PHCS and added to the Leighton tube cultures 48 hours following transfer. After 24 hours incubation, the medium was decanted and the tube slips washed three times with BSS, fixed with methyl alcohol, and stained with acridine orange.***

*Flat-sided culture tubes, 3-3/8" long, Bellco, Vineland, N.J.

**Nitrogen mustard, mechlorethamine hydrochloride, MUSTARGEN (R); Merck Sharp & Dohme, West Point, Pa.

***Acridine orange, Hartman-Leddon Co., Philadelphia, Pa.

Viability

At 24 hours, duplicate Leighton tube preparations which had received 0.1, 10, and 200 mcg. HN_2 , plus two Control tubes were trypsinized, centrifuged, and washed with BSS. Cells were dispersed in PHCS and inoculated into assembled Rose perfusion chambers (37), incubated for 24 hours, and observed by phase microscopy.

Lymphocytes

Peripheral blood lymphocytes were obtained from 14 RA and 11 Non-RA individuals. From these, 284 separate determinations were made and 1,000 cells counted on each.

Method of obtaining lymphocytes from peripheral blood by differential centrifugation was a modification of the Jago technic (38). Briefly, it is as follows: approximately 50 ml. blood are obtained by venipuncture into a siliconized syringe and transferred to two 40 ml. siliconized conical tubes containing approximately 0.1 ml. heparin/ml. blood, to prevent clotting. The tubes are carefully inverted at least 12 times. The stoppers are removed, inside rim of the tubes cleansed with sterile gauze, and fresh stoppers inserted. Tubes are centrifuged at 500 r.p.m. for 10 minutes. Differential centrifugation produces three layers: red blood cells; buffy coat of white blood cells; and plasma with platelets. First, platelet-rich layer at the surface of the plasma is removed by aspiration with a heparinized Pasteur pipette. The buffy coat is then aspirated with a pipette inserted to within 2 mm. of the red blood cells. Care is taken to aspirate only the "cloud-like" layer. The heavier, clumped, stringy layer--primarily composed

of polymorphonuclear cells--is avoided. The cell suspension is centrifuged at 1200 r.p.m. for 10 minutes, supernatant decanted and pellet resuspended in PHCS. Total cells are counted in a hemacytometer and mononuclear cells roughly approximated. Mononuclear cells ($1-2 \times 10^6$) are dispensed in 1 ml. PHCS/tube (13 x 100 mm.).

Treatment with Nitrogen Mustard (HN_2)

Dilutions of HN_2 in BSS were made with a range varying from 0.1 - 4 mcg./0.1 ml. The drug was added to the lymphocytes and, 15 minutes later, an additional 4 ml. PHCS were added to each tube.

Cultures were incubated 72 hours in vertical position. Slide preparations of sedimented cells were made on 1 x 3" slides, or 22 x 22 mm. coverlips. Coverlip preparations were preferred since they caused less mechanical destruction of the fragile lymphocytes. A drop of cells was placed on one slip, covered with another, and the two carefully pulled apart and allowed to air dry. Both coverlips were used in assaying. Preparations were fixed with acetic acid-methanol 1:3 for 20 minutes, or Spraycyte,* and stained with acridine orange and/or Wright.

Viability

Approximately one million lymphocytes were dispensed/tube and incubated with varying concentrations of HN_2 . Hemacytometer counts were made on Days 1, 3, 5, and 7. At time of sacrifice, supernatant was decanted, 0.5 ml. of 0.04% Trypan Blue in saline was introduced/tube,

*Spraycyte (R) Fixative, Clay-Adams, Inc., New York, N.Y.

and counts made within 30 minutes. Non-viable cells took up the stain. Viable cells appeared iridescent and faintly yellow, compared to the non-viable purple cells.

Treatment of Surviving Lymphocytes

1. Repeated Challenge with HN₂: Lymphocytes treated, as above, for 72 hours were washed and challenged with a second dosage of 2 mcg. HN₂ for an additional 72 hours. Slide preparations were assayed with Wright stain.
2. Phytohemagglutinin - (PHA)*: Lymphocytes were treated with 2 mcg. HN₂ for 72 hours. The cells were washed with BSS and cultured for an additional 72 hours with PHA, a plant mitogen isolated from the kidney bean, Phaseolus vulgaris (39, 40). A 1:20 dilution of PHA was made in calcium- and magnesium-free saline (41). Each tube received 0.1 ml. of this dilution in 5 ml. PHCS. Slides were prepared from sedimented cells and stained with Wright.
3. Long-Term Study on Feeder Layers: Trypsinized fibroblasts (2×10^5) were cultured for 48 hours on tube slips in Leighton tubes. The tube slip was transferred to the bottom cover slip of a Rose chamber. Lymphocytes which had been treated with 1 mcg. HN₂ for 72 hours were washed and layered by pipette on the feeder layer of fibroblasts. The cells were covered with dialysis membrane and the chamber assembled in routine fashion. The membrane provided support and maintained the cells in plane of focus for later phase microscopy and Cinémicrography (30, 42).

*Phytohemagglutinin (PHA-P), Difco, Detroit, MI 48201.

Fluorescence Microscopy

Acridine orange: Tube slip preparations of fibroblasts and cover slip preparations of lymphocytes were prepared and fixed, as previously described. Fresh stain was made before each use since a precipitate forms after 24 hours. Diluent for each solution was isotonic saline prepared with triple-distilled water. Slips were agitated continuously during the staining period.

Method of staining was a variation of the technic of Schiffer, (32). The pH was regulated to produce maximum brilliance from each cell type: optimum pH for fibroblasts was 5.0; for lymphocytes, 3.8.

Solutions and timing schedules were as follows:

1. 10-20% acridine orange in McIlvaine's buffer, pH 5.0* (fibroblasts) or 2% acetic acid, pH 3.8 (lymphocytes)--5 seconds.
2. 2% ethyl alcohol--15 seconds.
3. Isotonic saline--5 seconds.

The wet tube slip was inverted on a 1 x 3" slide and the edges sealed with Synthetic Resin.** Best results were obtained when the slides were read at once. However, they could be stored in an airtight box at 4°C. for as long as a week without fading.

A Zeiss microscope equipped with a cardioid darkfield condenser and a Wratten 2B barrier filter was used. The illuminator was an American Optical Fluorolume unit with an Ozram HB 200 mercury lamp and UG 5 filter. These filters transmit blue, and long ultraviolet light to the slide but protect the eyes of the observer.

*102.6 ml. 0.2 M. Na_2HPO_4 + 97.4 ml. 0.1 M. citric acid.

**Harleco Synthetic Resin (R)--Hartman-Leddon Co., Philadelphia, Pa.

Cell Enumeration

Acridine Orange

Fibroblasts: Intensity of RNA was scored 0 through 4+, with intensity of cytoplasm of control cells as reference point.

Lymphocytes: One thousand cells were counted on each slide, care being taken not to duplicate the same fields. Cells were categorized as normal, intermediate, or blastoid on the basis of size, intensity of cytoplasmic and nucleolar RNA, nuclear characteristics, and cytoplasmic/nuclear ratio.

Identification of Cells

To identify lymphocytes accurately, photomicrographs were taken of identical cells stained first with acridine orange, de-stained, and re-stained with Wright. Unsealed slide preparations were used to facilitate the procedure. Photomicrographs were made with Ektachrome X (EX-135) film at 12 seconds exposure, or Kodachrome II Daylight (K-135) film at 30 seconds exposure. Photomicrographs of acridine orange stained cells were taken with a 40X objective and 10X eyepiece. A drawing of the cells and their immediate surroundings was made and the location recorded by means of a microscopic Kazeeff cell finder. Slide was submerged in isotonic saline and the coverlip gently removed. The slide was de-stained with methyl alcohol and Wright stain was applied as usual. Photomicrographs of the identical Wright stained cells were taken under tungsten illumination. Kodachrome II (KPA-135) film was used with 2 seconds exposure, using the 100X oil immersion objective and 10X eyepiece.

RESULTS

Fibroblasts

Cytochemical

HN ₂ Concentrations	Description
Controls	Cytoplasm stained brick-red; nucleoli, orange; nuclei, apple green. Nuclear wall defined and without evidence of rigidity. Note Figure 2. Color reproduction is lost in printing.
0.5 - 5 mcg.	RA intensity greater than Non-RA at lower concentration - (0.5 mcg.). Both RA and Non-RA cells showed marked increase of cytoplasmic RNA. Non-RA nucleoli showed marked increase in intensity at 1 - 5 mcg. RA nucleoli showed an increase in RNA in only one preparation - 2 mcg. Nuclear wall shows pronounced rigidity. Note Figure 3.
10 - 30 mcg.	Intensity of cytoplasmic RNA leveled off but was slightly greater than in Controls in both RA and Non-RA.
50 mcg.	Few cells remained.
200 mcg.	100% destruction.

After the cytoplasm had disappeared altogether (noted as minus on Table 1) the nuclear wall was still intact. This phenomenon occurred through the 50 mcg. level in both RA and Non-RA, and at 100 mcg. in one preparation of rheumatoid cells.

Table 1. RNA of Fibroblasts Treated with Nitrogen Mustard in vitro
Fluorescent Intensity Assayed by Acridine Orange

HN ₂ mcg.	RNA FLUORESCENCE											
	NUCLEOLAR						CYTOPLASMIC					
	RA			NON-RA			RA			NON-RA		
	1	2	3	1	2	3	1	2	3	1	2	3
0	++	++	++	++	+++	++	+	+	+	+	+	+
0	++	+	+	+	+++	+	+	+	+	+	+	+
0	++	+	+	+	+++	+	+	+	+	+	+	+
0.1	++	+	++	+	++	+++ +++	++	+	++	++	+	++
0.2	+++ -	+	++	+	++	++	-	++	++	-	+	++
0.5	++	+	+	+	++	+++	++	++++	++++	+	+++	++
1.0	+	+	+	== ++++	++> ++++	++	++	++++	+	++++	+	++
2.0	+++ +++	+	++	+++	++	+++	++	++++	++	++++	++	+++
5.0	++	++	++	+++	++	+++ ++++	++	++	+++	+	+	++++
10	++	+	++	+	++	+++ +++	++	+	++	-	++	+++
20	++	+	++	+	-> ++	++	++	+	+	++	+	++
30	+	+	+	+	-	+++ -	++	+	-	-	++	++
40	+++ -	NC	NC	-	-	-	NC	NC	++	-	NC	-
50	-	NC	-	-	++	-	NC	NC	-	-	-	-
100	-	NC	-	NC	-	NC	++	NC	-	NC	-	NC
200	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC

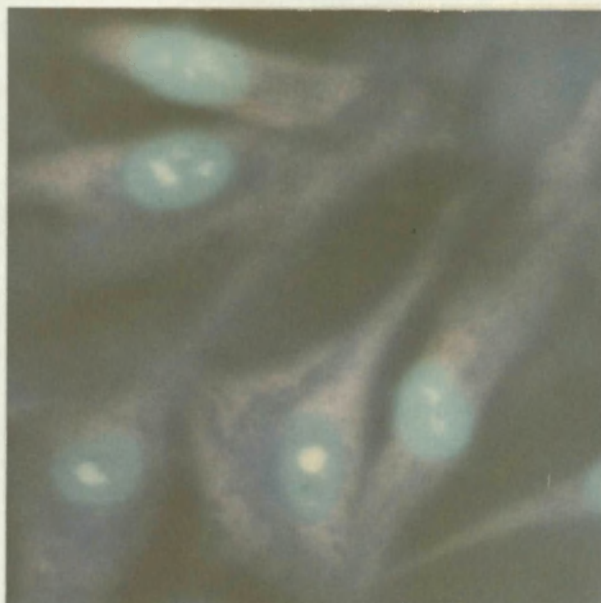
Numerals indicate separate determinations on transferred fibroblasts.

+: RNA in cytoplasm of Control

++ to ++++: RNA increase above Control

-: Absence of RNA

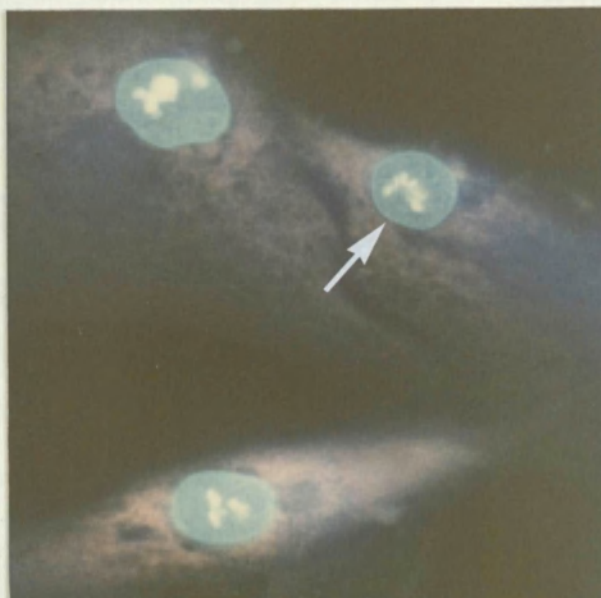
NC: No cells



Acridine Orange Stain 40 X Obj.

Figure 2. Control Fibroblasts in vitro 72 hours.

Brick-red cytoplasmic RNA, orange nucleolar RNA, apple-green DNA. (Color reproduction lost in printing).
Kodachrome II Daylight Film, Exp. 30 Sec.



Acridine Orange Stain 40 X Obj.

Figure 3. Fibroblasts with 10 mcg. HN_2 in vitro 72 hours.

Note increased intensity of fluorescence, pronounced rigidity of nuclear wall.
Kodachrome II Daylight Film, Exp. 30 Sec.

Viability--Phase Microscopy

Controls	Healthy, well spread-out spindle cells. Distinct nuclear detail. Well-defined nuclear and cell walls.
0.1 mcg. HN ₂	No observable difference from Control.
10 mcg. HN ₂	Healthy, viable cells. No aggregations.
200 Mcg. HN ₂	All cells destroyed.

Lymphocytes

Cytochemical

Assayed by acridine orange, lymphocytes challenged in vitro with varying dilutions of HN₂ sometimes resulted in a "blastoid" lymphocyte, containing an increased quantity of RNA. The response occurred over a narrower range of concentrations of HN₂ than of the fibroblasts. However, this phenomenon did not always occur. Lymphocytes in mitosis were never observed. See Table 2.

Figure 4 shows the RA cells responded to HN₂ above the Control, ranging to 40.8%. The highest response in Non-RA cells was 7.9%. Both showed greatest response at the 1 mcg. and 2 mcg. levels.

As illustrated in Figure 5, averages of 44 determinations (21 RA and 23 Non-RA) showed the greatest difference in percent transformation occurred at the 1 mcg. level, an average of approximately 5%.

Table 2. Percent Lymphocyte Transformation Following Treatment with Nitrogen Mustard in vitro

Subject	Sex	Age	Treatment	HN ₂ (mcg.)					
				0.0	0.2	0.5	1.0	2.0	
RHEUMATOID	1	m	22	CP 16 wks.	1.2	1.1	3.1	2.9	1.8
				24 wks.	9.2			50.0	
				28 wks.	39.1		39.4	50.0	26.1
	2	m	38	156 wks.	6.4		8.5		
	3	f	43	35 wks.	11.2				14.7
	4	f	51	78 wks.	2.9		3.2	3.0	2.6
	5	f	55	0 wks.	0.6	0.6	3.5	1.4	2.1
				0 wks.	0.4	0.2	0.2	0.3	1.0
				12 wks.	1.8	1.7	2.1	1.9	8.5
	6	m	59	None	0.4	0.1	0.1	0.3	0.8
	7	m	60	Gold	0.4		0.1	0.1	0.1
	8	m	63	CP 5 wks.	0.6	2.2	1.0	4.0	0.8
				16 wks.	1.1		3.4	1.7	1.3
	9	m	65	40 wks.	6.9		3.4	16.7	14.3
			69 wks.	2.6		9.7	1.8	23.1	
10	m	68	Gold	0.3	0.3	0.2	0.9	2.1	
11	f	68	Gold	0.5	0.2	0.8	0.7	1.7	
12	f	68	Gold	0.0	0.8	0.6	1.4	1.4	
				1.4			4.3		
13	f	69	None	1.5	1.0	0.8	0.8	3.0	
14	f	71	Gold	3.0		4.5	4.6	18.0	
NON-RHEUMATOID	1	f	24		0.5	0.0	0.3	1.6	1.1
					0.2	1.4	1.0	4.0	4.7
	2	m	29		0.5	1.3	1.0	1.0	1.5
	3	f	31		1.3	0.3	8.0	1.8	1.9
					1.6	8.1	1.3	6.0	9.4
					1.6	6.5	3.2	2.0	7.6
					0.0	2.1	3.3	0.5	1.9
					0.0	0.1	0.0	0.0	0.6
	4	f	35		0.2	0.4	1.1	0.3	0.0
	5	m	41		0.0	0.1	0.8	0.4	1.3
					0.8	1.0	0.1	0.2	0.6
	6	m	45		1.6	2.4	1.0	3.5	6.1
	7	m	46		1.1	0.9	1.1	1.1	
					0.9	1.0	0.6	1.2	2.7
					1.3	0.7	1.4	1.7	0.7
					1.4	0.6	1.6	1.5	
					0.4	1.9	1.8	6.5	6.4
8	m	46		0.1	0.4	1.0		1.6	
9	f	48		3.0	3.1	4.2	10.9	3.8	
				3.7	2.0	6.7	3.0	2.8	
		49		0.6	0.2	0.8	4.2	3.9	
10	m	52		1.9	0.3	0.8			
11	f	61		1.2	1.6	0.3	2.4	1.0	

HN₂: Nitrogen Mustard
 CP : Cyclophosphamide

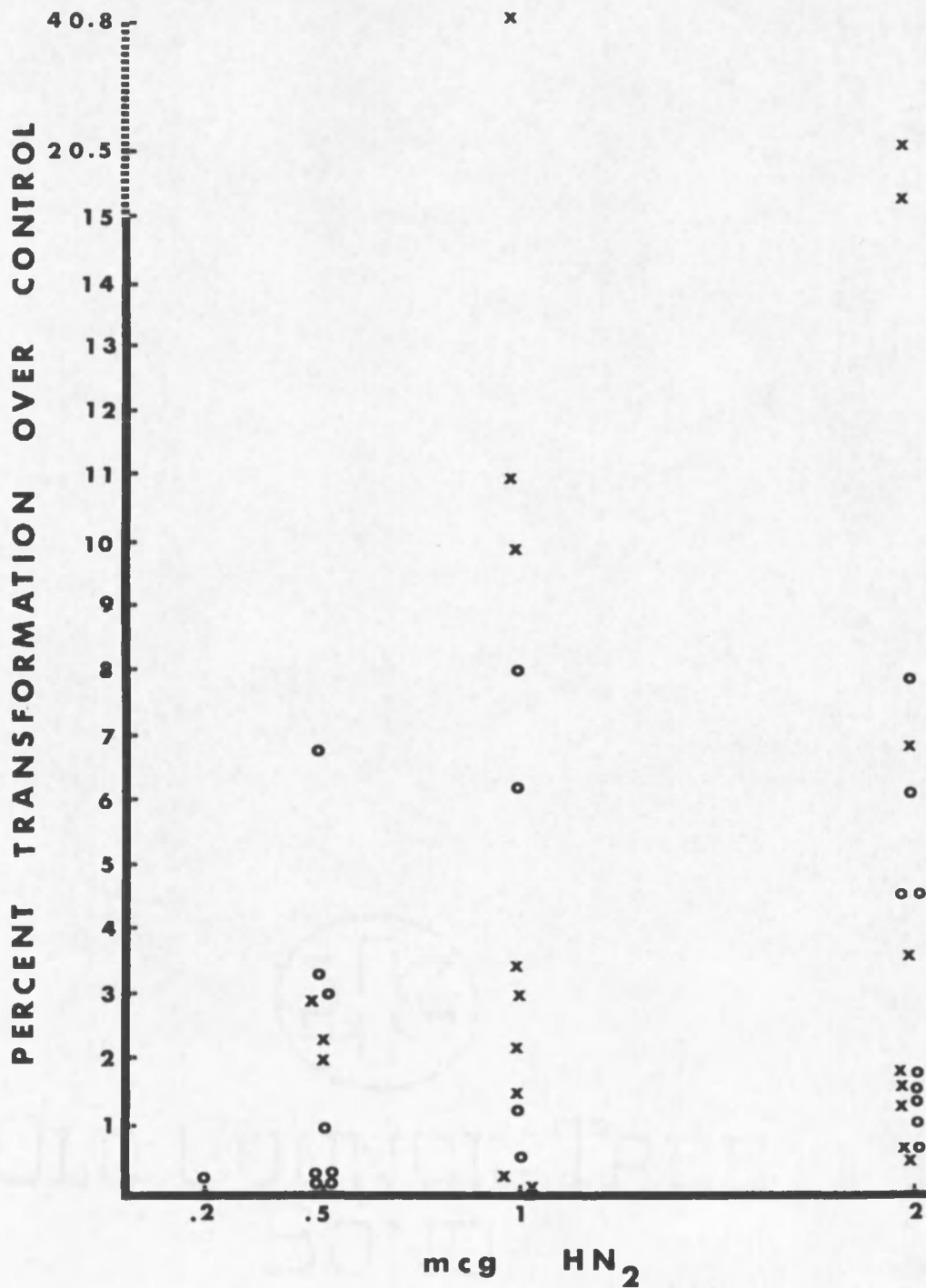


Figure 4. Percent Lymphocyte Transformation Over Control Values Nitrogen Mustard, in vitro, 72 Hours

X = RA

O = Non-RA

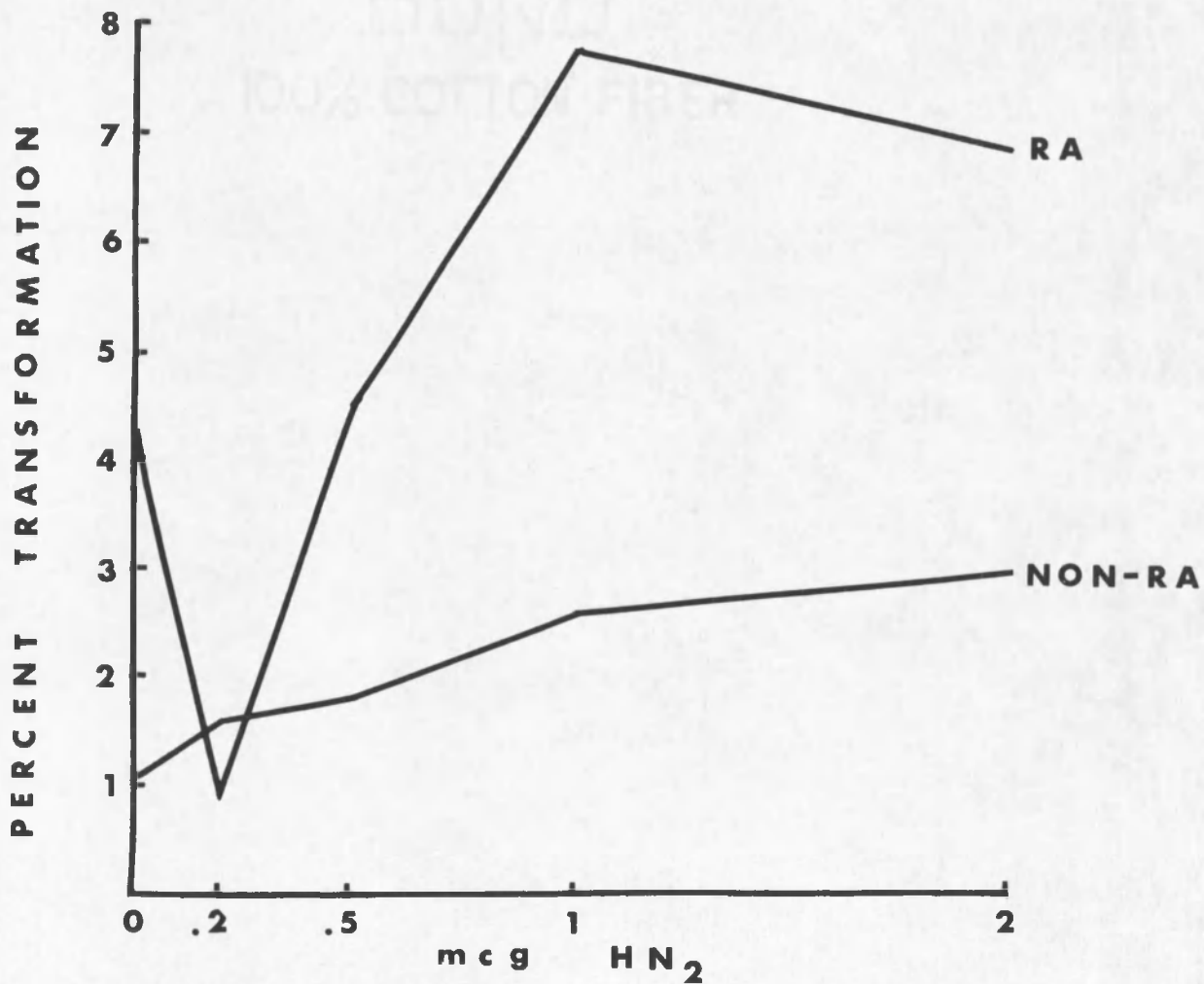


Figure 5. Averages of 21 RA and 23 Non-RA Lymphocyte Transformation Determinations with Nitrogen Mustard, in vitro, 72 Hours

Lymphocytes exhibited the following characteristics:

Acridine orange

Normal: Small, oval cells with small amount of orange-red cytoplasm. Nucleus, apple green with a few orange nucleoli.

See Figure 6A.

Intermediate: Increase in cell size, intensity of cytoplasmic and nucleolar RNA over Controls.

See Figure 6B.

Blastoid: Marked increase in intensity of RNA, prominently stained nucleoli, reticulated nucleus, increased cytoplasmic/nuclear ratio and greatly enlarged cells.

See Figure 6C.

Wright

Normal: Round or oval cells, purple staining nuclei, relatively coarse chromatin structure and small rim of clear blue cytoplasm, often containing indistinct nucleoli.

See Figure 6D.

Intermediate: No observable difference over Control except slight increase in size.

See Figure 6E.

Blastoid: Greatly enlarged cell with nucleus of fine chromatin structure, deeply basophilic cytoplasm, increased cytoplasmic/nuclear ratio, and distinctly stained nucleoli.

See Figure 6F.

Figure 6. Lymphocytes Cultured with Nitrogen Mustard 72 Hours:
Identical Cells Stained with Acridine Orange and Wright

ACRIDINE ORANGE

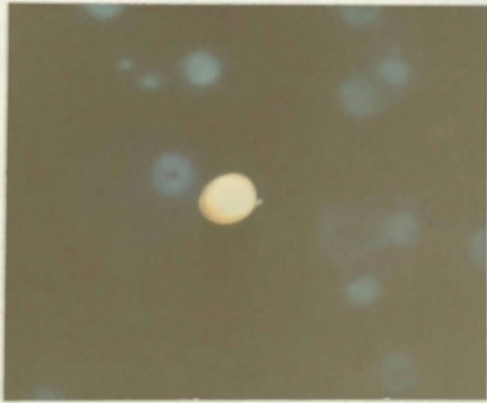
- A. Control.
Control lymphocyte exhibits apple green nuclear DNA with small rim of orange-red cytoplasmic RNA.
- B. Intermediate.
Note increase in cell size, intensity of cytoplasmic and nucleolar RNA over Control.
- C. Blastoid.
Marked increase in intensity of RNA, reticulated nucleus, increased cytoplasmic/nuclear ratio, enlarged size.

Acridine orange photomicrographs taken at 40X objective. Kodachrome II Daylight at 30 sec. or Ektachrome X, 12 sec. exposure.

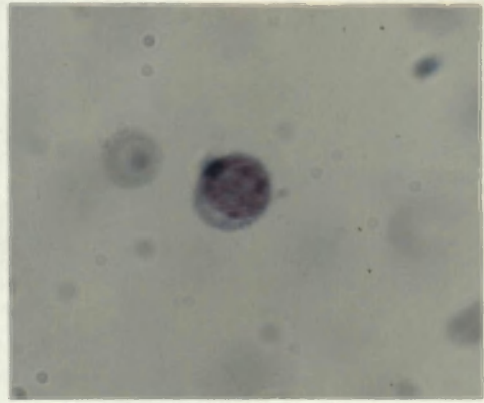
WRIGHT

- D. Control.
Control lymphocyte has purple nucleus with coarse chromatin and a small rim of clear blue cytoplasm.
- E. Intermediate.
Slight increase in size over Control. No other observable difference.
- F. Blastoid.
Enlarged cell with deeply basophilic cytoplasm, increased cytoplasmic/nuclear ratio.

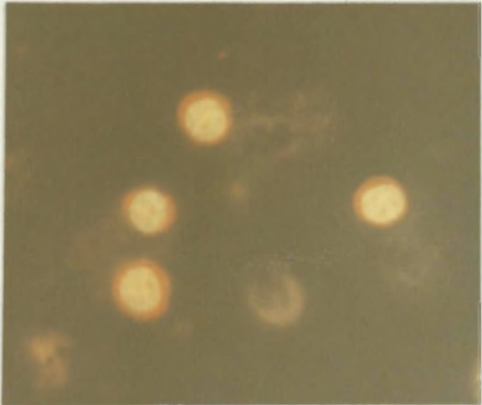
Wright photomicrographs at 100X objective. Kodachrome II A, 2 sec. exposure.



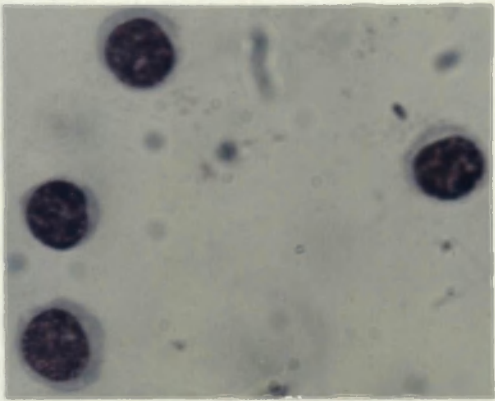
A



D



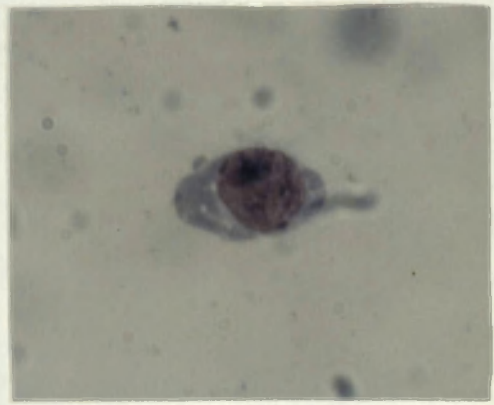
B



E



C



F

Acridine Orange

40 X Obj.

Wright

100 X Obj.

Figure 6. Lymphocytes Cultured with Nitrogen Mustard 72 Hours:
Identical Cells Stained with Acridine Orange and Wright

Viability--Trypan Blue Exclusion

At no time were 100% of the lymphocytes destroyed. Even at a concentration of 4 mcg. HN_2 for seven days, approximately 1.5% of the initial inoculum survived. See Figure 7.

Surviving Lymphocytes

1. A small population of lymphocytes which survived a 2 mcg. dosage of HN_2 for 72 hours, survived an additional 2 mcg. for a second 72 hours. Massive destruction of cells left too few survivors for an accurate differential count. Healthy appearing small lymphocytes were recorded on photomicrographs. See Figures 8 and 9.
2. PHA response was positive in lymphocytes from three RA and four Non-RA determinations which had survived a 2 mcg. dosage of HN_2 for 72 hours. Massive destruction of cells occurred during the total incubation of six days, leaving too few cells for an accurate differential count. See Figure 10.
3. Lymphocytes which survived a 1 mcg. dosage of HN_2 were subsequently cultured on feeder layer. The culture was maintained in viable condition without transfer for more than one year in the original Rose chamber preparation. Fresh nutrient was supplied at weekly intervals. The cells exhibited the typical motility of untreated lymphocytes and their viability was documented by Ciné time-lapse at 8 months. See Figure 11.

An occasional mitotic figure was observed, showing that in vitro lymphocytes were able to recover from the mitotic-arresting effect of HN_2 . See Figure 12.

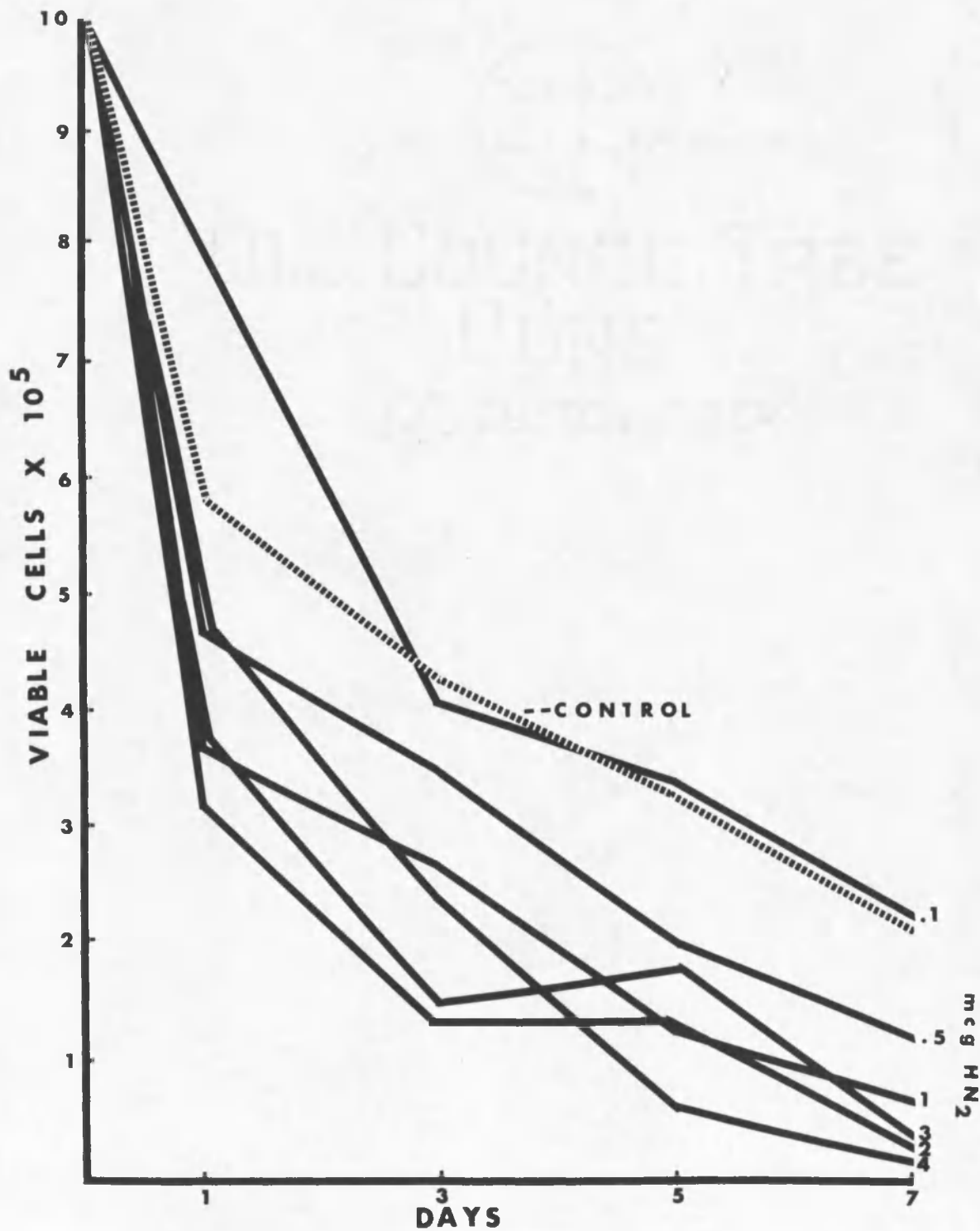
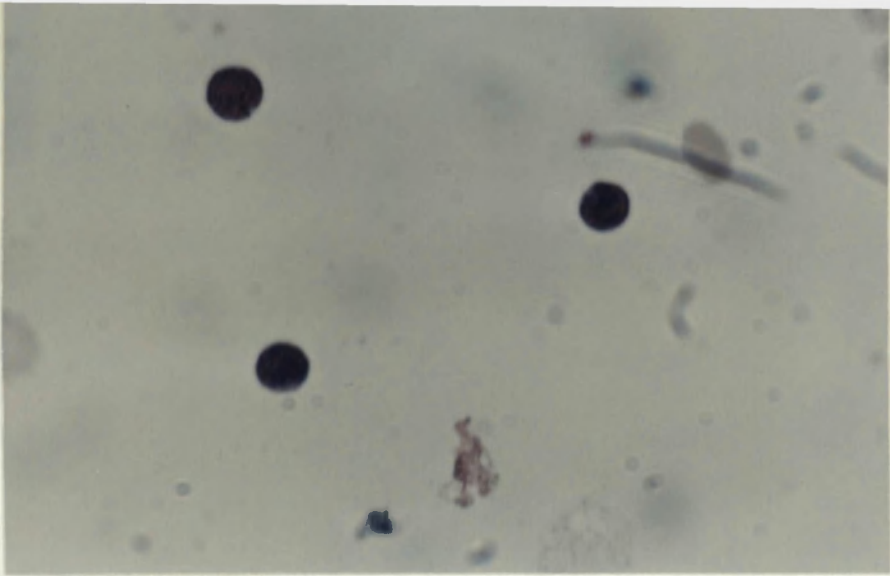


Figure 7. Lymphocyte Viability after 7 Days in vitro with Nitrogen Mustard: Trypan Blue Dye Exclusion

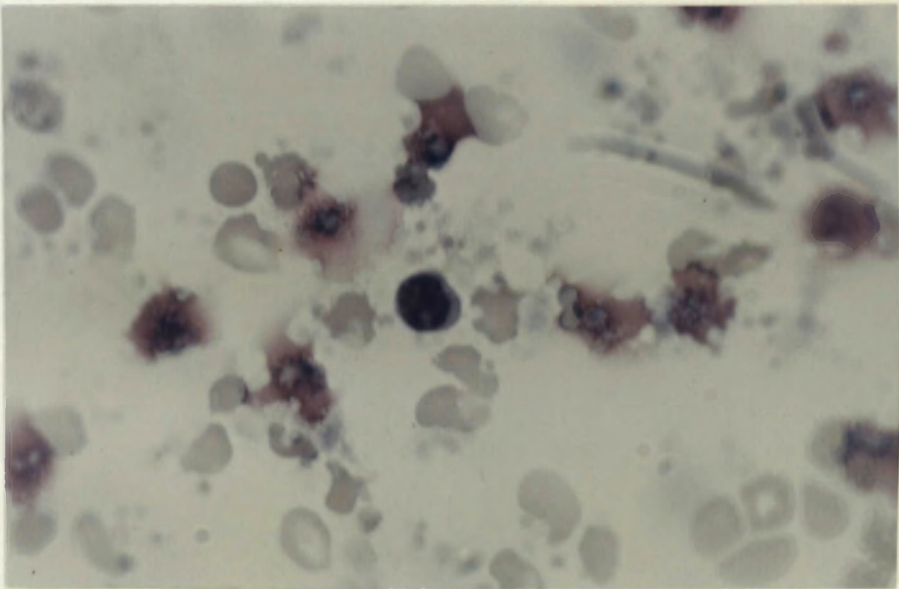


Wright Stain 100 X Obj.

Figure 8. Control Lymphocytes 6 Days in vitro.

Note characteristic dark purple nuclei with small rim of clear blue cytoplasm.

Kodachrome II Daylight 2 Sec. Exp.

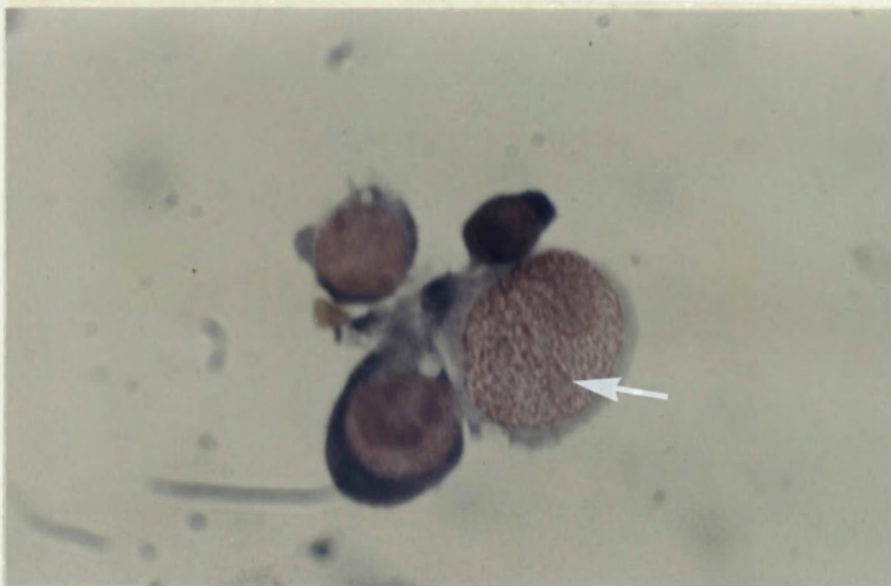


Wright Stain 100 X Obj.

Figure 9. Surviving Lymphocytes: 2 mcg. HN_2 for 72 Hours Followed by an Additional 2 mcg. for a Second 72 Hours in vitro

Note healthy condition of cell and the surrounding massive cellular destruction.

Kodachrome II Daylight 2 Sec. Exp.

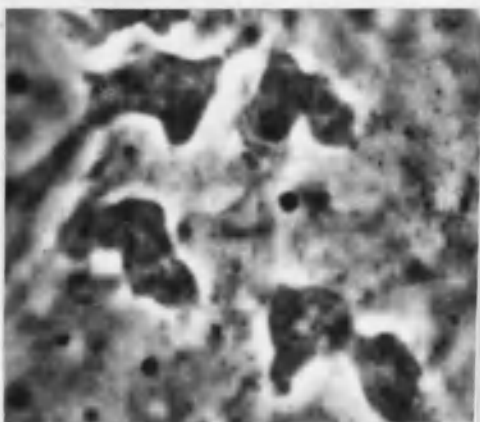


Wright Stain

100 X Obj.

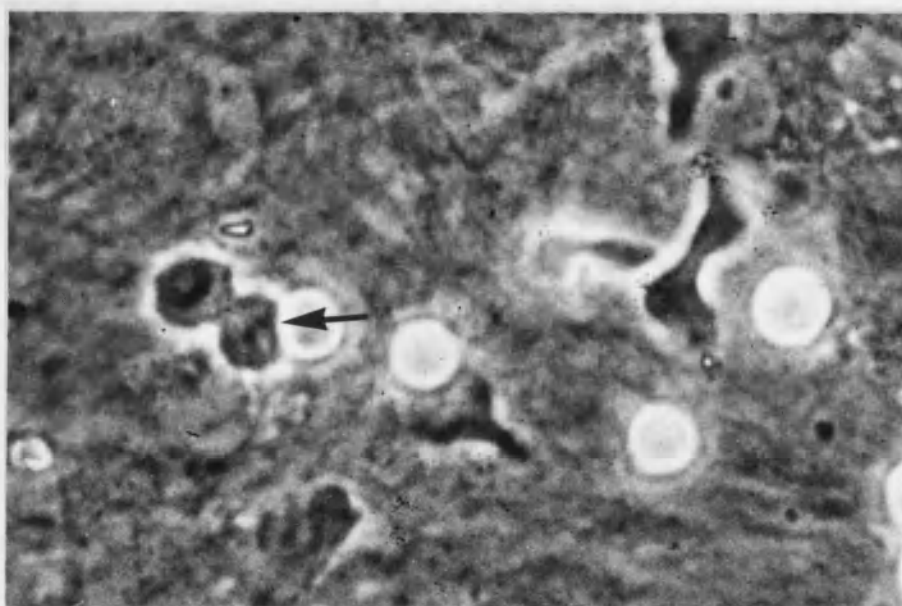
Figure 10. Lymphocytes Incubated with HN_2 for 72 Hours, Followed by PHA for an Additional 72 Hours.

Lymphocytes are in different stages of reaction: small, intermediate and blastoid with a concomitant increase in size. Note nucleoli and fine chromatin structure of nucleus in one blastoid cell as indicated by arrow.



Abstract from Ciné. 40 X Obj.

Figure 11. Nitrogen Mustard-Surviving Lymphocytes on Fibroblast Feeder Layer 8 Months in vitro



Print from Photomicrograph

40 X Obj.

Figure 12. Mitosis in Nitrogen Mustard-Surviving Lymphocyte on Fibroblast Feeder Layer 1 Month in vitro

DISCUSSION

Results of the present investigation demonstrated that there was little difference between reactions of rheumatoid (RA) and non-rheumatoid (Non-RA) fibroblasts treated with nitrogen mustard (HN_2) in vitro. Both showed a marked increase of RNA over Controls but only within a narrow range of dosage.

RA lymphocytes, however, showed a greater response to HN_2 above the Control than Non-RA lymphocytes. This was manifested by increased intensity in RNA in the cytoplasm and nucleoli. It is not surprising that one should find a difference here since HN_2 selectively affects the leukocytes (11, 18, 24, 28) and cyclophosphamide (CP), its analog, is also effective in treating RA (1-3).

Mitosis was never observed in lymphocytes treated with HN_2 in vitro. The increase of RNA following HN_2 in vitro demonstrated in both cell types confirms other reports that during and following HN_2 in vitro, mammalian cells contain greatly increased quantities of RNA and protein (43-45), and also contain up to a two-fold increase in DNA. These cells were enlarged as were the lymphocytes in the present study. The investigators reported that there were no mitotic figures. Similar effects were also produced by X-irradiation (43). For this reason, HN_2 is referred to as a radio-mimetic drug. The results of the present investigation and those referred to above contradict Shohat, et al., who state that HN_2 failed to produce visible morphological changes in

human lymphocytes in vitro (46). They were comparing its action to the blistering effect of Elatericin A, and may have disregarded less obvious changes. It is also possible they did not test within the narrow range of concentrations in which the morphological changes reported here were observed.

Although HN_2 selectively affects leukocytes first, the results of the present investigation have shown it does not kill the entire population of human lymphocytes in vitro. This result parallels a report (28) that in vivo long-lived lymphocytes and plasma cells in rats were resistant to cyclophosphamide (CP) whose chemically active component is HN_2 (18).

The selective population of lymphocytes that survived HN_2 in this study was found to be capable of responding to PHA and, when studied by time-lapse cinémicrography, exhibited the characteristic motility of untreated lymphocytes on feeder layers. The surviving lymphocytes were observed in mitosis, which demonstrated that the inhibition of mitosis by HN_2 is reversible when the cells are no longer in contact with the drug. The same phenomenon is observed in vivo when cessation of HN_2 treatment results in regeneration of lymphocytes (24).

With the total effect of the HN_2 inhibition of RNA, and ultimate cell death, it must be considered the increase in RNA in cells treated with HN_2 in vitro may be a pre-agonal response of the cell. In this connection, the small population of survivors cannot be overlooked.

Of paramount interest is the fact that a selective population of lymphocytes did survive massive HN_2 dosage, responded to phytohemagglutinin (PHA), and survived on feeder layers for extended observation. This aspect of the present investigation warrants further study.

Additional viability studies are indicated. Stimulation of nitrogen mustard surviving lymphocytes with specific antigens could prove to be of value.

SUMMARY

The present investigation was undertaken to determine the effects of nitrogen mustard (HN_2) on cultured cells of rheumatoid (RA) and non-rheumatoid (Non-RA) subjects: fibroblasts from synovialis and peripheral blood lymphocytes; and to determine whether human lymphocytes could survive massive dosage of HN_2 in vitro.

The following results were observed:

RA and Non-RA fibroblasts respond more or less equally to HN_2 in vitro, as assayed by acridine orange.

RA lymphocytes showed a greater response to HN_2 than Non-RA lymphocytes, as evidenced by increased RNA content in their cytoplasm and nucleoli.

The selective population of lymphocytes which survived treatment with HN_2 in vitro, showed the capacity to regain mitotic activity and to respond to phytohemagglutinin (PHA).

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