Survival *in Vitro* of Nitrogen Mustard-Treated Peripheral Lymphocytes (35801)

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(Introduced by A. R. Kemmerer)

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The present investigation explores the *in vitro* effects of nitrogen mustard (HN_2) on peripheral lymphocytes. This drug and an analog, cyclophosphamide, have been used in therapy of rheumatoid arthritis (1–3). Rheumatoid (RA) and nonrheumatoid (N-RA) peripheral white blood cells were studied with two objectives in mind:

1. To determine the viability of lymphocytes incubated with nitrogen mustard *in vitro* for periods up to 7 days;

2. To explore, in part, the capabilities of surviving cells.

Materials and Methods. Lymphocytes were obtained from 50 ml of heparinized blood from adult subjects and separated by differential centrifugation (4, 5). The cell-rich plasma was aspirated to within 2 mm of the red blood cell surface. An average of 87% purity was achieved.

Culture medium was Eagle's minimum essential medium enriched with 20% pooled human cord serum[1] plus 50 units of penicillin–streptomycin and 2.9 mg of glutamine/ml. From 1–2 $\times$ 10^6 lymphocytes were cultured with HN_2[2] in 1 ml of medium. Drug dosages ranged between 0.1 and 100 μg/ml. The HN_2 combines with reactive compounds of cells within a few minutes (6). To provide adequate nutrient, 4 ml of medium were added/tube after 15-min incubation at room temperature. Cultures were then incubated vertically at 37° until time of sacrifice. Preparations of sedimented cells were made on cover slips, fixed with acetic acid–methanol 1:3 for 20 min, or Spraycyte,[3] and stained with acridine orange (7) or Wright.

Procedures. Viability. The viability of lymphocytes from 9 subjects was determined: 3 N-RA on days 1, 3, 5, and 7, after treatment with HN_2 ranging from 0.1 to 10 μg; and additional studies on 3 RA and 3 N-RA on days 3 and 7 at drug levels of 10 and 100 μg. Presence of viable cells was determined by exclusion of trypan blue (0.04% in isotonic saline). Results of hemacytometer counts by two individuals were averaged.

Reactivity to phytohemagglutinin (PHA)[4]. Lymphocytes from 3 RA and 3 N-RA subjects were treated with 2, 10, and 100 μg HN_2 and cultured for 3 days. The supernatant was decanted and cells were cultured for an additional 3 days in the presence of 0.1 ml of PHA-P (1:20 in calcium and magnesium-free saline) in 5 ml of medium. Positive and negative control preparations were included. At 6 days, slides were prepared from sedimented cells, stained with Wright, and assayed by two investigators. A positive response was judged by the presence of large mononuclear cells with deeply basophilic cytoplasm often containing vacuoles. The nuclei were large with fine, homogeneously stained chromatin.

Longevity. Small populations of lymphocytes alone are short-lived in culture (8).

[1] 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, Tucson, Arizona.

[2] Nitrogen mustard, mechlorethamine hydrochloride, Mustargen, Merck, Sharp & Dohme, West Point, Pa. 19486.

[3] Spraycyte Fixative, Clay-Adams, Inc., New York.

[4] Phytohemagglutinin (PHA-P), Difco Laboratories, Detroit, Mich. 48201.

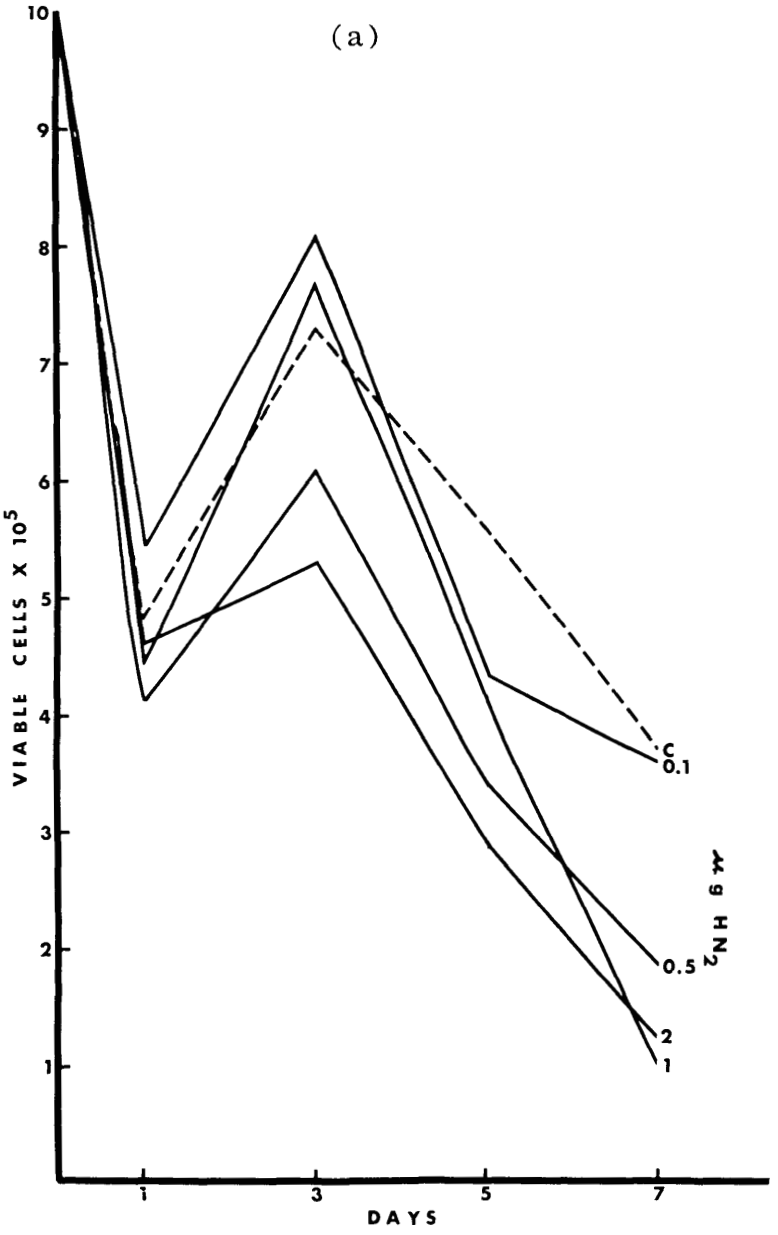
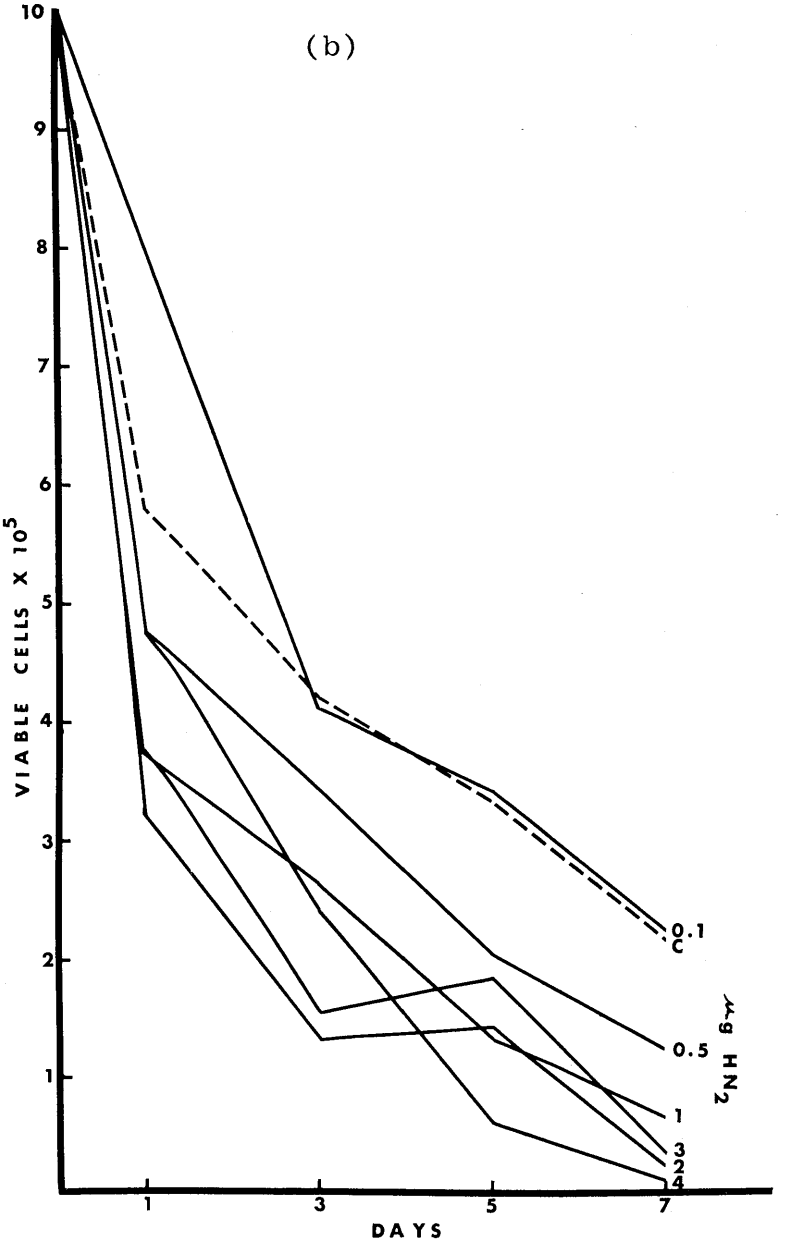


FIG. 1a, b, c. Viability of peripheral blood lymphocytes from 3 N-RA subjects treated *in vitro* with varying dosages of HN_2: trypan blue dye exclusion.

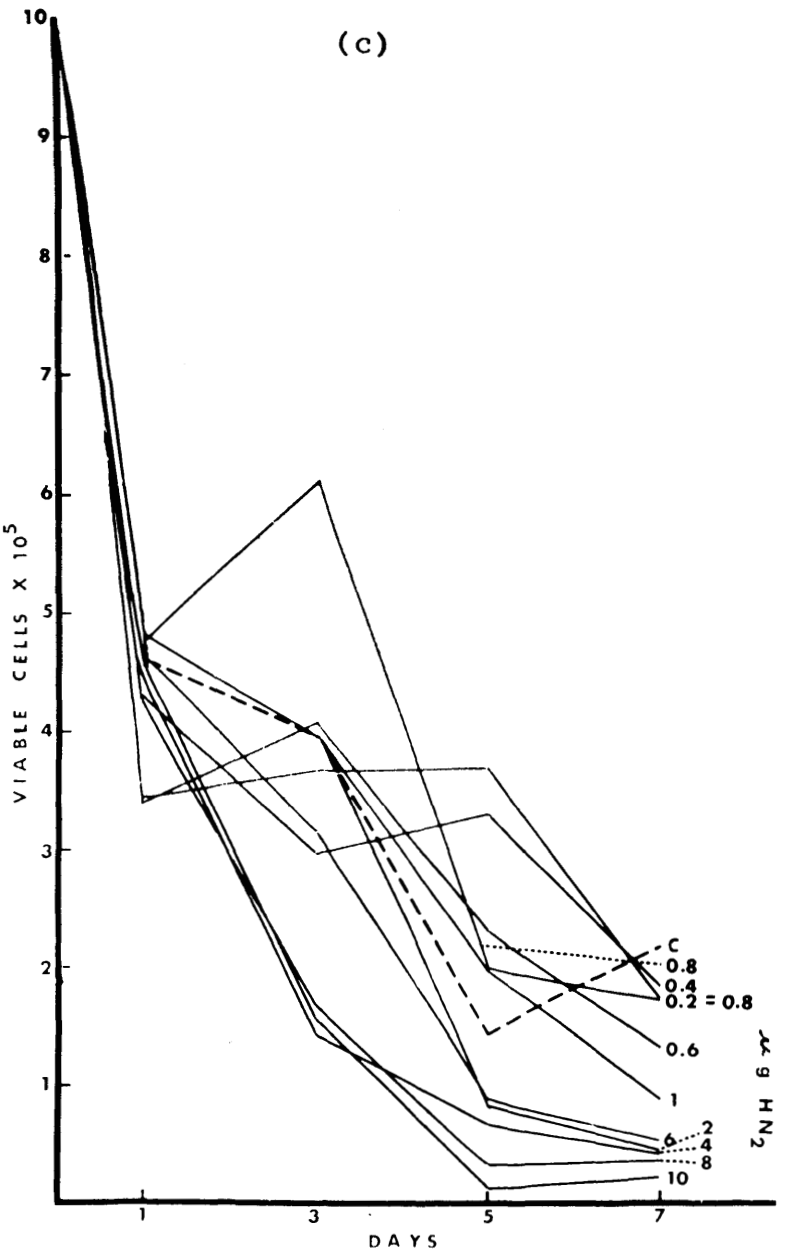
The feeder layer technic of Stanfield *et al.* (9) provided a means for continuing study of the treated lymphocytes. Briefly, it was as follows: monolayer subcultures of human synovialis were trypsinized 48 hr prior to use, washed in medium, and 5×10^4 cells grown in Leighton tubes on 10×50-mm cover slips. These cultures consisted only of cells capable of adhering to glass. The tube slip was transferred to the bottom cover slip of a Rose chamber (10). Lymphocytes which had been treated with 1 μg HN_2 for 3 days were washed with BSS and layered by pipet on the feeder layer. The cells were covered with dialysis membrane to provide support and to maintain cells in plane of focus for



phase microscopy and cinemicrography.

Results. Viability. Under conditions of the viability study, at no time were 100% of the HN_2-treated lymphocytes destroyed. A rapid fall in viable lymphocytes occurred during the first day. At 7 days, in 10- and 100-μg preparations, as determined by trypan blue dye exclusion, 2.5–5% of the initial population were still viable. In controls, the range was 22–48%. The number of viable untreated lymphocytes varied considerably from test to test.

An unexpected effect was observed: to the fifth day of culture, some levels of dosage actually proved stimulatory, compared to the controls (Figs. 1a, b, c, and 2).



Presence of viable cells in 10 μg HN_2 preparations on day 3 was verified by examination of Wright and acridine orange stains (Figs. 3 and 4).

Reactivity to PHA. Positive lymphocyte response to PHA occurred in all cultures which had first survived a 2-μg dosage of HN_2. Positive results were also observed at 10 μg in all 3 RA and in one N-RA determination. In two of the 3 N-RA at 10 μg, and in all preparations at 100 μg there was no response to PHA. Instead, the picture was one of massive destruction with only an occasional intact small lymphocyte (Figs. 5 and 6).

Longevity. Control lymphocytes after

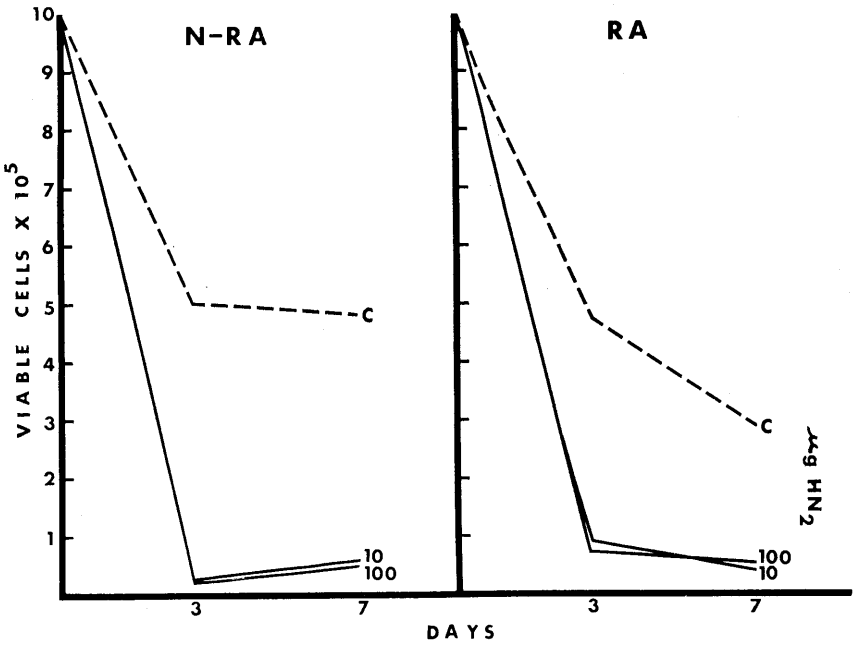


FIG. 2. Averaged viability determinations on peripheral blood lymphocytes from 3 N-RA and 3 RA subjects treated *in vitro* with HN_2: trypan blue dye exclusion.

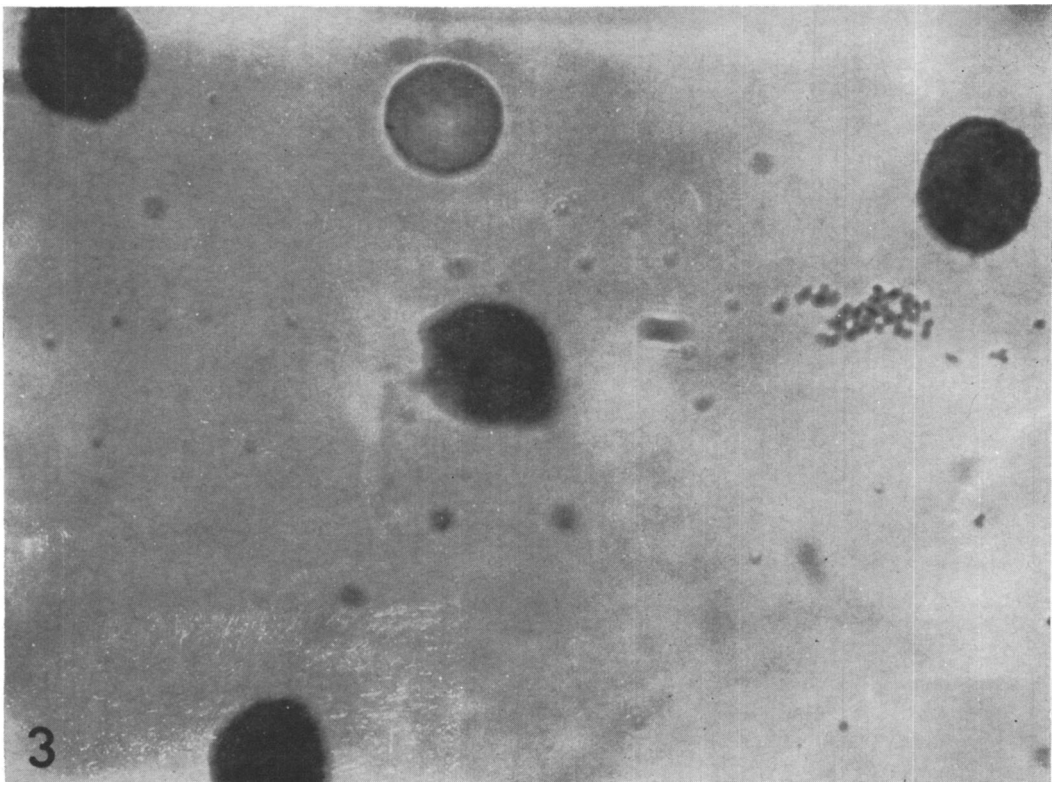


FIG. 3. Wright stain, control lymphocytes at 7 days; 100× obj.

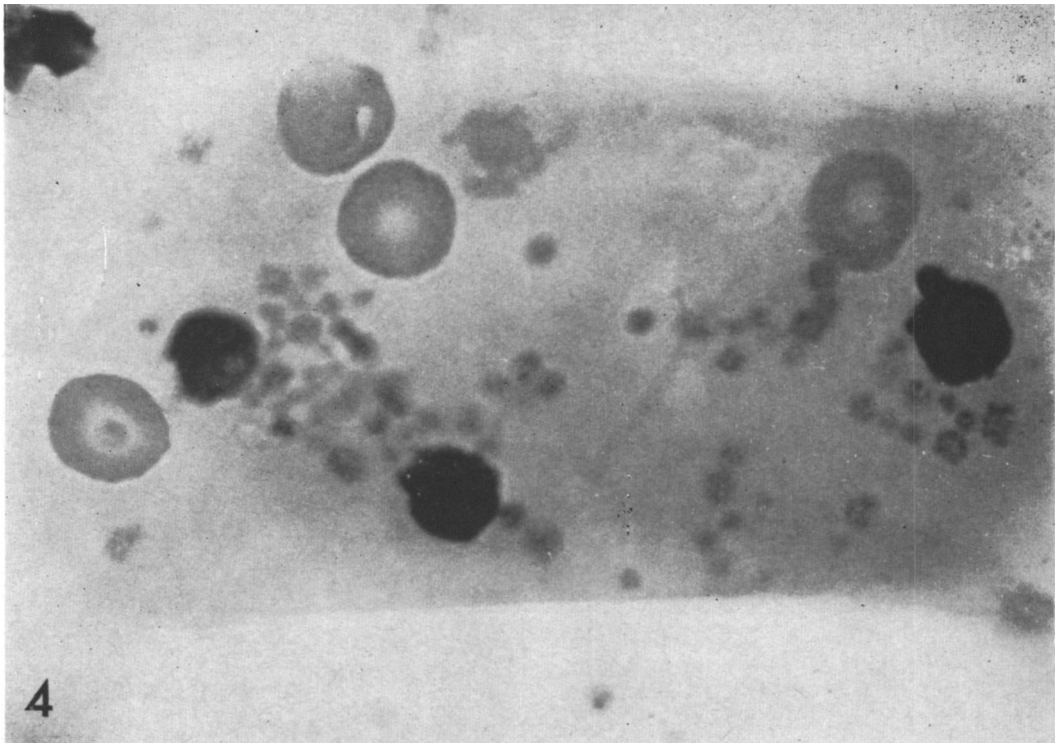


FIG. 4. Wright stain, surviving lymphocytes, 100 μg of HN_2 for 7 days. Note shrunken appearance; 100× obj.

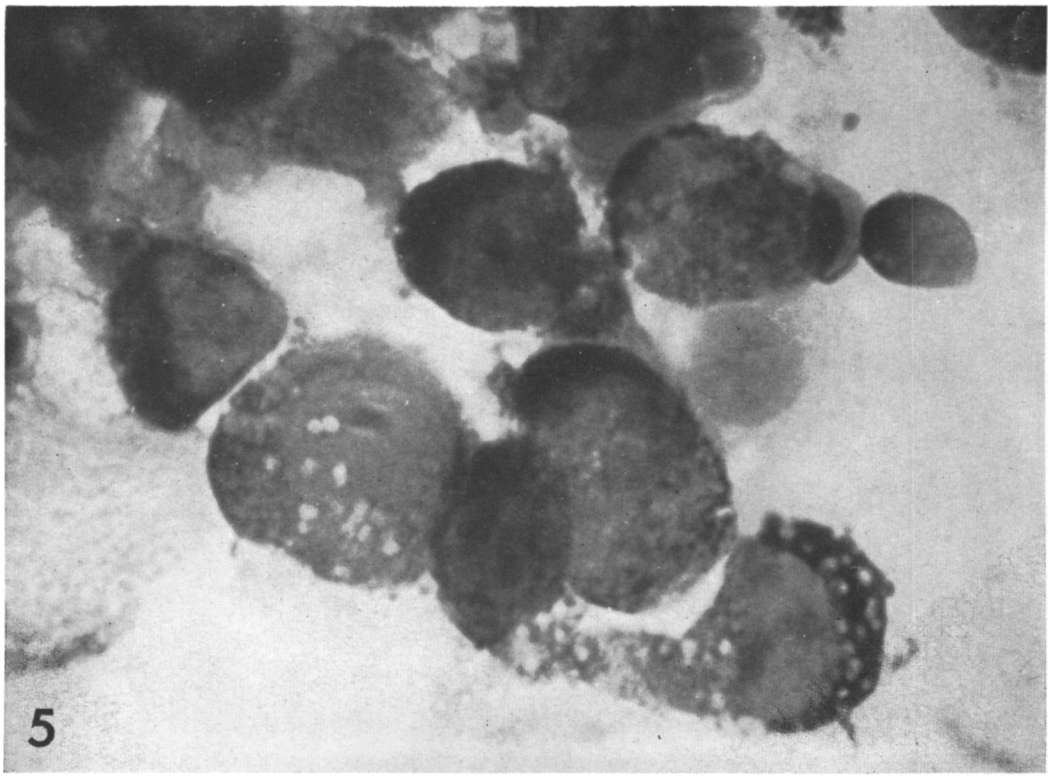


FIG. 5. Wright stain, control lymphocytes at 6 days; PHA administered on day 3; 100× obj.

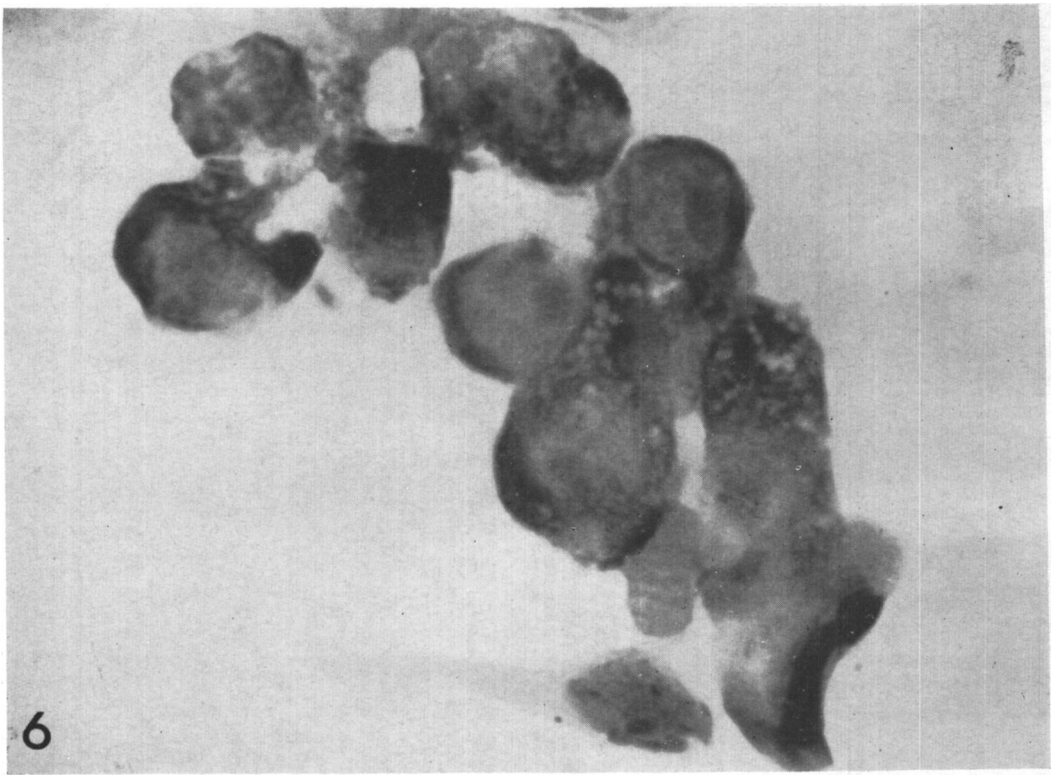


FIG. 6. Wright stain, lymphocytes surviving 10 μg of HN_2 at 6 days; PHA administered on day 3; 100× obj.

3-days incubation rarely lived longer than 24 hr when placed on feeder layer. However, one culture surviving 1 μg of HN_2 for 3 days was subsequently maintained in viable condition on feeder layer for more than one year. Fresh nutrient was supplied to the culture at weekly intervals. Lymphocytes exhibited the typical motility of untreated cells and their viability was documented by cinemicrography time-lapse at both 8 and 10 months (Fig. 7). A lymphocyte in mitosis was observed at 1 month (Fig. 8).

Discussion. The effect of HN_2 on lymphocytes from human peripheral blood was observed over a period of 7 days. Under conditions of this study, dosages equivalent to 50 times the therapeutic level of HN_2 did not destroy all of the lymphocytes in either the RA or N-RA preparations. These findings differ from those of Schrek and Stefani (11) who reported that no lymphocytes survived 5 μg of HN_2 *in vitro* at 6 days. On the other hand, *in vivo* work with rats revealed a small population of lymphocytes were able to withstand large dosages of HN_2 (12). The *in vitro* work presented here suggests that a similar population of lymphocytes exists in both RA and N-RA individuals.

The stimulatory effect of HN_2 in the viability studies provoked considerable interest. At the highest level of viability above the controls, some cells showed a blastoid response when examined by acridine orange fluorescence (7). Other mammalian cells during and following treatment with HN_2 *in vitro* have been found to contain greatly increased quantities of RNA and protein and up to a twofold increase of DNA (13–15).

The blastogenesis observed in lymphocytes stimulated with PHA after surviving HN_2 establishes that the cells were not only viable but, in addition, were capable of a positive response.

Nitrogen mustard inhibits mitosis (16). However, a cell in mitosis was observed 1 month after surviving HN_2. Characteristic

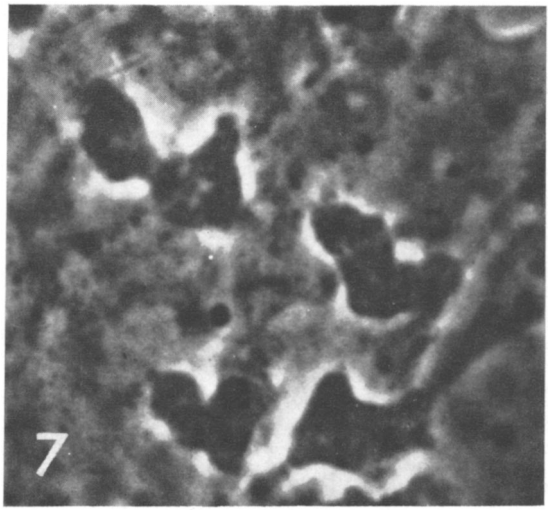


FIG. 7. Lymphocytes surviving 1 μg of HN_2, photographed on fibroblast feeder layer at 8 months; Cine abstract.

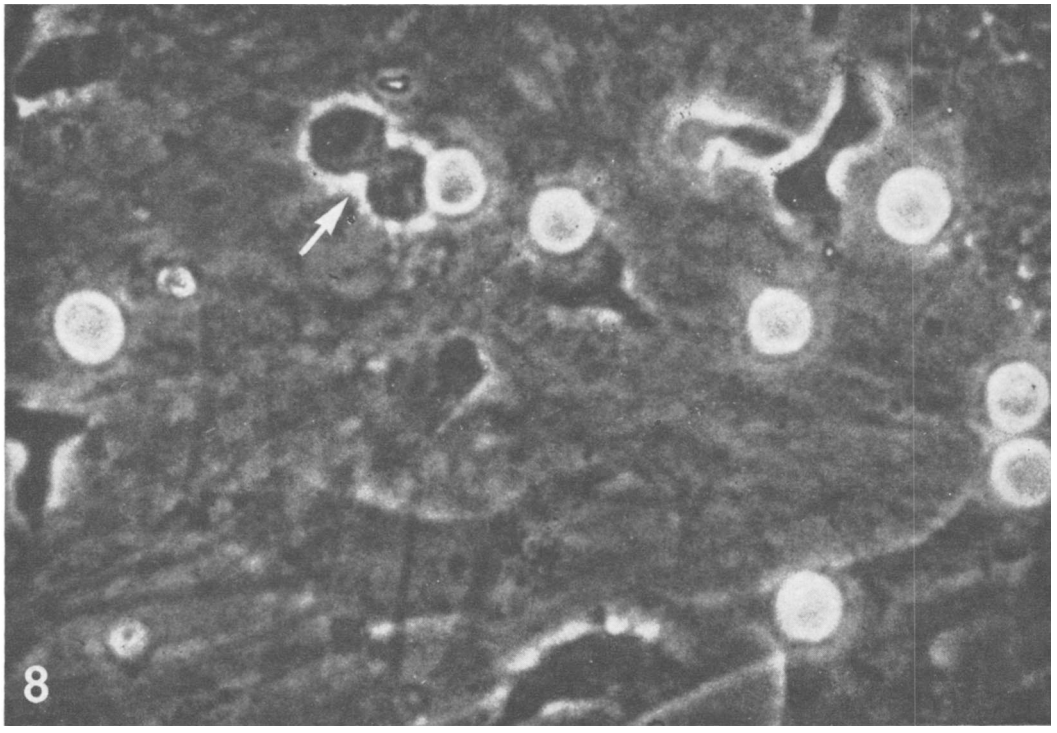


FIG. 8. Lymphocytes surviving 1 μg of HN_2 photographed on fibroblast feeder layer at 1 month; note mitosis; 40× obj.

motility following mitosis identified the cell as a lymphocyte.

In the present investigation, RA lymphocytes behaved similarly to N-RA lymphocytes.

Summary. A small population of peripheral blood lymphocytes survived *in vitro* dosages of nitrogen mustard up to those equivalent to 50 times the therapeutic level.

Some surviving cells were capable of a

positive response to phytohemagglutinin.

The data suggest the possibility of a nitrogen mustard-resistant or adaptive population of lymphocytes.

The authors gratefully acknowledge the editorial assistance of Miss Margaret L. Doorly in preparation of this manuscript.

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Received Apr. 1, 1971. P.S.E.B.M., 1971, Vol. 137.