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AN APPROACH TO THE CHARACTERIZATION OF STEM CELLS IN MOUSE BONE MARROW

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The competence of bone marrow to promote recovery of hemopoietic function in irradiated recipient mice has been observed to decrease on serial transplantation (Barnes et al., 1959; van Bekkum and Weyzen, 1961; and Barnes et al., 1962). There is evidence, however, that the protective ability of the serially transplanted marrow can be reconstituted by addition of lymphoid cells harvested from normal lymph nodes (Barnes et al., 1962). It was the purpose of this study to investigate the nature and cellular basis of variations in the proliferative capacity of a given marrow cell population in the course of syngeneic (isologous) transplantation. A quantitative measure of this capacity of the marrow was obtained by determining the ability of the marrow to form colonies in the spleens of irradiated recipient mice (Till and McCulloch, 1961) and by measuring incorporation of I^{131}-labeled iododeoxyuridine (I^{131}UdR) -- a specific deoxyribonucleic acid (DNA) precursor -- in such spleens. The proliferative capacity, as evaluated by these techniques, was related to the cellular constitution of the marrow, as determined by differential cytologic analysis of marrow smears, in an attempt to identify the responsible stem cells.

Experimental Methods

Animals. Twelve-week-old (BALB/c X A/He)F_1 hybrid mice of both sexes were used. The mice were obtained from Cumberland View Farms, Clinton, Tennessee, at the age of 5 weeks and housed thereafter 10 to a cage in temperature-humidity controlled quarters. They were given Purina Chow and chlorinated drinking water ad libitum.

Irradiation. A General Electric Maxitron 300 X-ray machine was used, with the following factors: 300 kvp, 20 mA, 4.75 mm Be inherent filtration and 3 mm Al added filtration, 93.5 cm target-to-mouse distance, hvl of 0.5 mm of Cu. The dose rate in air was 80 r/min. During exposure, the mice were placed in a partitioned, revolving Lucite cage.

Bone marrow transplantation. Donor mice of the same sex as the recipients were killed by exsanguination and bone marrow cells were harvested from femurs and tibias. The cells were suspended in cold Tyrode's solution. Total nucleated cell counts and counts of eosin-stained cells were made by means of a hemacytometer. The concentration of eosin-unstained cells was adjusted immediately thereafter to the desired level and cells were infused by tail vein injection into recipient mice, generally within 2 hours after the donor was killed.

Estimation of colony-forming units. The method used is fully described elsewhere by Till and McCulloch (1961). A total of 20,000 and 40,000 donor cells was injected into recipient mice. Ten days later their spleens were removed, fixed in Bouin's fluid and examined with the naked eye to count visible nodular colonies. The number of nodules per 10,000 donor cells was then determined, and standard errors were calculated on these values.

Estimation of I^{131}UdR uptake. The use of I^{131}UdR for studies of cell turnover was proposed by Gitlin et al. (1961). Graded doses of bone marrow cells were infused into groups of recipient mice. Generally, each cell dose was given to at least five mice, and five cell doses were employed to obtain dose-response curves. Five days after injection, each recipient mouse received intraperitoneally 0.5 μC of I^{131}UdR (synthesized by W. L. Hughes; see Gitlin et al., 1961). No stable iodine was given beforehand. Spleens were not removed until 6 hours later to allow excretion of non-incorporated I^{131} radioactivity. Each whole spleen was placed in a glass test tube and its radioactivity was measured in a 2 X 2 inch well-type scintillation counter (Packard Auto Gamma Spectrometer, Series 410A). With each experiment, the spleen I^{131}UdR uptake of irradiated -- not cell-injected -- mice (radiation controls) was also determined to enable correction of uptake values for host, as opposed to donor, contributions. The I^{131}UdR uptake of the spleen was expressed in percentage of the total radioactivity injected. The method will be fully described elsewhere (Cudkowicz et al., in preparation).

Statistical procedures. Tests of significance were made using logarithms of the I^{131}UdR percentage uptake values to stabilize the variance.

When regression analysis was indicated, straight lines were fit by the method of least squares. Aberrant values were tested by the largest F-ratio method (Nair, 1948).

Cytological analysis of bone marrow. Bone marrow cells were expressed from tibias or femurs, smeared on glass slides, air-dried and stained with Wright-Giemsa. In some instances, marrow donors were given a single intraperitoneal injection of 0.5 μC of tritiated thymidine (H^3TdR) per gram of body weight one-half hour before preparation of marrow smears. The H^3TdR

specific activity was 2.9 C/mM. Smears were fixed in methyl alcohol, exposed for 10 days in Kodak NTB2 liquid emulsion, developed, and stained with Giemsa through the emulsion.

Experimental design (FIGURE 1). A single dose of 10^7 syngeneic bone marrow cells was injected into lethally irradiated (850 r) primary recipient mice unless otherwise stated. Such mice were killed at intervals up to 40 days after transplantation, and their bone marrow was retransplanted into X-irradiated (700 r) syngeneic secondary recipients to evaluate its proliferative capacity. Samples of the marrow to be retransplanted were smeared for cytological analysis. At the same time, an additional primary marrow recipient was injected with tritiated thymidine to obtain autoradiograms of marrow smears. Cells injected into the secondary recipients were allowed to colonize for 5 or 10 days, depending on whether their growth in the recipient's spleen was measured by I^{131}UdR uptake or colony formation, respectively. Secondary recipients were exposed to only 700 r of total-body irradiation, a dose not lethal to 100 per cent of the mice, since optimal proliferation of donor cells, as measured by the I^{131}UdR method, was obtained whenever the X-ray dose exceeded 600 r (TABLE 1). That irradiation of recipient mice represents a maximal stimulus for proliferation and differentiation of injected donor hemopoietic cells is also borne out by the lack of effect of added erythropoiesis-stimulating factor in such mice (Hodgson, 1962). Under these circumstances, therefore, the number of colonies and the I^{131}UdR uptake in the recipient's spleen depend only on the number of competent, colony-forming cells injected with the donor marrow.

Results

Colony-forming ability of retransplanted marrow cells. When bone marrow was retransplanted within 10 days after infusion into primary recipient mice, its ability to colonize the spleen was drastically reduced (FIGURE 2). The larger dose (4×10^4 nucleated cells per mouse) used at the 10-day interval was not sufficient to induce formation of even one colony in 20 spleens, so that a precise evaluation of the number of colony-forming units, i.e., of stem cells, could not be obtained at this time interval. Later, the colony-forming capacity of the marrow gradually recovered, overshot at 30 days, and appeared to have returned to normal values at 40 days after primary transplantation. The results are consistent with the findings of van Bekkum and Weyzen (1961) that marrow at 7-14 days after primary transplantation is not efficient in promoting survival of lethally irradiated, syngeneic recipients. That colony formation and protection of lethally irradiated animals are two interrelated properties of mouse bone marrow has also been indicated by Till and McCulloch (1962) during this conference.

(F-2)

I^{131}UdR uptake by retransplanted marrow cells. The incorporation of I^{131}UdR in the spleen of irradiated, cell-injected mice resulted almost entirely from incorporation of the compound into DNA of donor cells in S phase at the time of I^{131}UdR injection. Small amounts of I^{131}UdR were detected in spleens of radiation control animals, generally not exceeding 0.05 per cent of the injected I^{131}UdR. Correction has been made for this fraction of the total I^{131}UdR uptake in colonized spleens. The relation between the mean I^{131}UdR uptake in spleens of a group of mice and number of nucleated normal bone marrow cells injected into the animals 5 days prior to I^{131}UdR labeling is given in FIGURE 3 and TABLE 1. In FIGURE 3, the logarithm base 10 of percentage

(F-3)

of incorporation has been plotted against the logarithm base 2 of cell doses, and a least-square straight line has been fitted to the points. The data do not deviate significantly from linearity throughout a range of cell doses from 0.05 to 1.60 X 10^6 donor cells per recipient mouse. In TABLE 1, results are listed from several independent experiments using normal bone marrow cells and various radiation doses to recipient mice. In these experiments the uptake values were adjusted to an arbitrarily chosen unit number of 10^6 donor marrow cells. The uptake of each group does not deviate significantly from that of the other groups, and a common weighted average uptake could be calculated, therefore, for normal (BALB/c X A/He)F_1 marrow cells.

Cell dose-response curves of I^{131}UdR uptake were obtained also for marrow cells retransplanted at various time intervals after primary transplantation. For convenience, only three of these lines have been plotted in FIGURE 3: in these instances as well as at all other time intervals straight lines were obtained. The equality of their slopes was tested, with the conclusion that all the lines could be considered as deviating randomly and not significantly from parallel lines. Thus, lines were fit using a common slope, and the distance on the ordinate between them gives the difference in I^{131}UdR uptake between given numbers of donor marrow cells. The displacement to the right of the lines obtained at 30, 10, and 5 days indicates that also with this technique of evaluation of proliferative capacity the transplanted marrow appears to be less efficient than normal marrow in colonizing irradiated secondary recipients. That experimental conditions remained comparable throughout is indicated by the equality of the slopes of all dose-response lines.

Results of two separate retransplantation experiments are presented in FIGURE 4. In one case 10^7 nucleated marrow cells were given to the primary

(F-4)

recipients (left section of FIGURE 4). In the other experiment, 2 X 10^7 marrow cells were given (right section of FIGURE 4). The I^{131}UdR uptake per unit number of marrow cells decreased markedly 5 days following primary transplantation and returned later to higher values in both experiments. However, the ability of the transplanted marrow to give origin to I^{131}UdR-incorporating cells recovered at different rates in the two experiments, recovery being faster when the initial marrow inoculum was larger.

Cytological analysis of transplanted marrow. Marrow smears from donor mice employed in the experiments presented in FIGURE 4 were studied. Smears from two mice infused initially with 10^7 cells were available at each time interval; smears from five mice infused initially with 2 X 10^7 cells were also available at each time interval at which I^{131}UdR uptake was determined. One hundred intact marrow cells were counted in each case, and the various morphologic subtypes were pooled into several major categories to simplify presentation of the results (FIGURES 4 and 5); i.e., (1) myeloblasts and promyelocytes, (2) myelocytes and early metamyelocytes, (3) erythropoietic elements, and (4) lymphocytes. Not shown are values for late metamyelocytes, granulocytes, megakaryocytes, monocytes, histiocytes, reticulum cells, hemohistioblast, and other unidentified forms.

(F-5)

Conspicuous during the first 5-10 days after transplantation was the marked "shift to the left" in the marrow, with a preponderance of immature myeloid and erythroid precursors and a relative paucity of mature granulocytes and small lymphocytes. At later intervals, progressive maturation was noted, the differential returning essentially to normal by the 40th day. The large relative numbers of myeloblasts and erythroblasts in the marrow on the fifth day contrast with the depressed proliferative

capacity of the marrow at the same time. It is clear, then, that these cells do not give rise to splenic nodules, despite the hematopoietic character of such nodules (Till and McCulloch, 1961). Presumably, a less differentiated precursor of such cells is the colony-forming stem cell in question.

The close correlation between the lymphocyte content (FIGURE 4) of the marrow and its proliferative capacity (FIGURES 2 and 4) suggests that the lymphocyte might be the stem cell responsible for splenic repopulation. To test this hypothesis, autoradiograms of marrow smears were examined to determine whether marrow lymphocytes incorporated tritiated thymidine more actively than normal soon after transplantation into a lethally irradiated recipient. Preliminary results of such examinations suggest that this is indeed true; i.e., cells having the morphology of the small lymphocyte were seldom labeled in a normal marrow (FIGURE 6), but shortly after transplantation into a lethally irradiated recipient such cells (FIGURE 7) were seen to be labeled in greater numbers. They were also seen to be labeled relatively intensely; whether this is because of their small nuclear size or because of greater uptake of tritiated thymidine remains to be determined.

F-6

F-7

Discussion

The competence of mouse bone marrow to produce hemopoietic elements may be assayed by infusing the marrow into syngeneic recipients whose hemopoietic sites require repopulation because of previous lethal irradiation. In response to the maximal stimulation for hemopoiesis induced thereby, one would expect that immediate changes of the marrow's cellular composition would occur. One can speculate that: (1) the transplanted marrow cell population, during hyperactive hemopoietic stimulation, would contain a larger number of stem cells than normal marrow. On the other hand (2), it is known that serial retransplantation exhausts or unbalances the marrow stem cell pool (Barnes et al., 1959; van Bekkum and Weyzen, 1961; Barnes et al., 1962), suggesting that stem cells may produce differentiated elements to the extent that maintenance of the self-perpetuating cell compartment is compromised. A sufficiently severe disruption of this compartment, such as might be produced by repeated transplantation, might preclude a return to a normal hemopoietic marrow.

The results of these experiments show that a biphasic variation in proliferative capacity (i.e., in stem cell content) occurred after transplantation of marrow cells; a sharp decrease during the first 10 days after transplantation, followed by recovery within 40 days, when the initial cell inoculum amounted to 10^7 nucleated cells. Our present results favor the hypothesis that the stem cell pool is depleted during the first days after injection of marrow into an irradiated recipient.

The ability of adult transplanted mouse bone marrow to proliferate seems from the results to correlate better with the presence of lymphocytes than of erythroblasts, myeloblasts, and other cells, since changes in marrow

proliferative capacity on retransplantation, significant also in survival studies (van Bekkum and Weyzen, 1961), were invariably accompanied by parallel changes in the relative number of lymphocytes in the marrow. Furthermore, short-term labeling studies with H^3TdR indicate that lymphocytes in the normal marrow are capable of division, and that even more of them become capable of division after transplantation. Although these findings do not prove that erythroid and myeloid elements are derived from the small lymphocyte, they strongly suggest a link between this cell and hemopoiesis. On the other hand, the possible role of other cells (reticulum cell, hemohistioblast) cannot be excluded without further study. The frequency of these cells in our material was too low, however, to be determined accurately.

The involvement of the lymphocyte in hemopoietic cell production has been postulated and challenged on the basis of different types of evidence (see review articles in Ciba Foundation Conferences, 1960, 1961). Conflicting ideas have arisen, probably mainly because of the functional heterogeneity (Gowans, 1962; Porter and Cooper, 1962; and Caffrey et al., 1962) of this cell population contrasted with its morphological homogeneity. The lymphocyte we observed in our mouse bone marrow (FIGURE 6) has the same morphology as the lymphocyte described by Yoffey (1960) in guinea pig marrow and by Gowans (1962) and Porter and Cooper (1962) in rat lymph. Yet, the cells observed in the lymph apparently differ from those in the marrow, at least in not labeling immediately after a short single exposure to H^3TdR (Gowans, 1962; Porter and Cooper, 1962; Caffrey et al., 1962). Other differences and similarities remain to be established.

Summary

The competence of bone marrow cells to form hemopoietic colonies and take up I^{131}-labeled iododeoxyuridine in the spleen of X-irradiated mice was studied in relation to time after infusion of such cells into primary, lethally irradiated recipients. The proliferative capacity, as evaluated by these methods, was related to the cellular constitution of the transplanted marrow, as determined by differential cytological analysis.

When bone marrow was assayed within 10 days after primary transplantation, its ability to colonize secondary recipient spleens was drastically reduced, although myeloblasts and erythroblasts were relatively abundant in the donor marrows. The normal proliferative capacity returned only after 30 or more days following transplantation, depending on the size of the primary marrow inoculum, and was correlated with the reappearance in the marrow of normal numbers of lymphocyte-like cells. These cells were, furthermore, capable of incorporating tritiated thymidine in response to "flash" labeling. The results indicate that after infusion into recipients to be repopulated, the marrow cell population undergoes changes affecting the stem cell pool size, and, furthermore, that the proliferative capacity of such marrow is correlated less well with its content of erythroblasts and myeloblasts than with its content of lymphocytes. The results also suggest that the small lymphocyte may be a pluripotent hemopoietic stem cell.

Acknowledgement

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TABLE 1

I^{131}UdR UPTAKE/10^6 NORMAL BONE MARROW CELLS 5 DAYS AFTER INFUSION INTO SYNGENEIC RECIPIENTS

Recipients		Mean uptake in spleen (per cent)	95 per cent Confidence limits
No.	X-ray dose (r)		
6	600	0.456	0.347 - 0.600
6	700	0.409	0.311 - 0.538
25	700	0.395	0.340 - 0.458
22	700	0.400	0.334 - 0.479
23	700	0.446	0.374 - 0.532
15	700	0.378	0.306 - 0.466
7	800	0.438	0.340 - 0.565
6	900	0.527	0.401 - 0.693
110		Weighted average 0.420	0.384 - 0.459

Legends for Figures

FIGURE 1. Schematic outline of experimental procedure.

FIGURE 2. Colony-forming ability of retransplanted marrow in relation to time after initial transplantation.

FIGURE 3. Splenic uptake of I^{131}UdR in relation to number of bone marrow cells injected and time of retransplantation.

FIGURE 4. Ability of marrow to promote uptake of I^{131}UdR in relation to time of retransplantation and lymphocyte content.

o———o I^{131}UdR uptake o- - - -o lymphocyte content

FIGURE 5. Marrow granulocyte and erythrocyte precursors in relation to time after transplantation.

FIGURE 6. Autoradiogram of normal bone marrow smear. One labeled (L) and several unlabeled (U) lymphocytes. Giemsa X 600.

FIGURE 7. Autoradiogram of transplanted (5 days) marrow smear showing heavily labeled lymphocytes (L) and labeled blast cells (B). Giemsa X 1200.

11,585

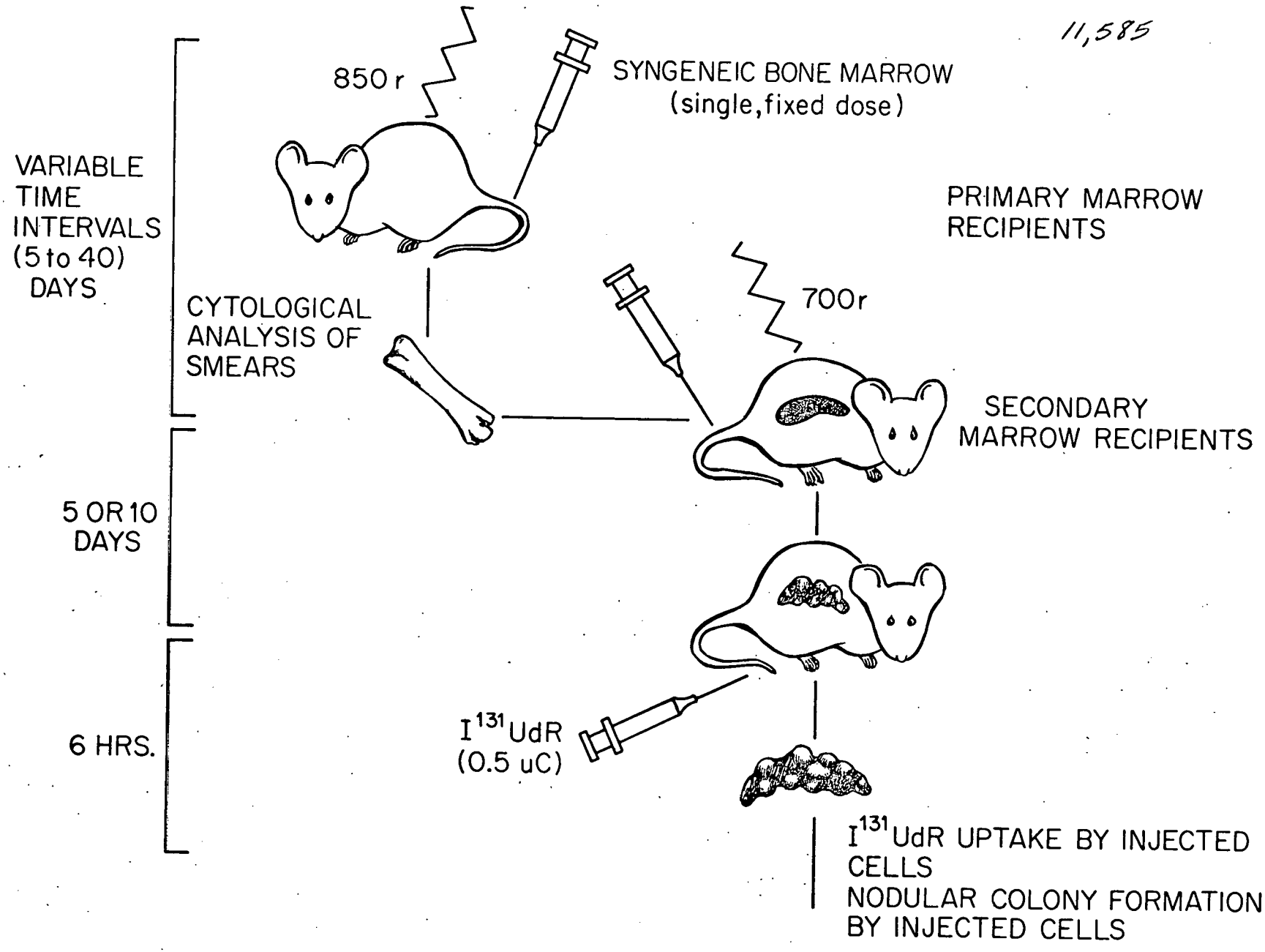
850 r
SYNGENEIC BONE MARROW
(single, fixed dose)
PRIMARY MARROW
RECIPIENTS
VARIABLE
TIME
INTERVALS
(5 to 40)
DAYS
CYTOLOGICAL
ANALYSIS OF
SMEARS
700r
SECONDARY
MARROW RECIPIENTS
5 OR 10
DAYS
6 HRS.
I131UdR
(0.5 uC)
I131UdR UPTAKE BY INJECTED
CELLS
NODULAR COLONY FORMATION
BY INJECTED CELLS

FIG. 1

11,586

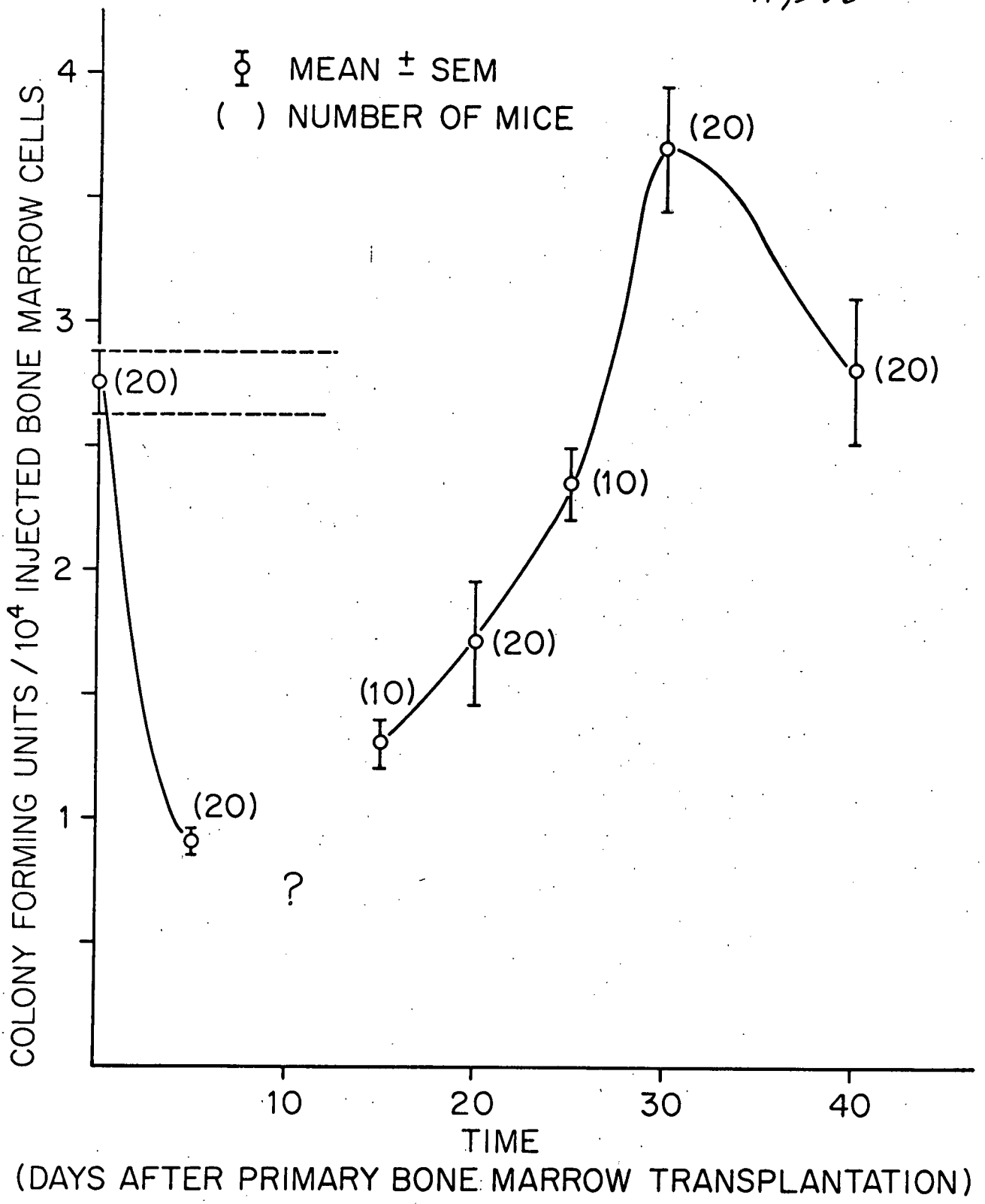
MEAN ± SEM
( ) NUMBER OF MICE
COLONY FORMING UNITS / 10^4 INJECTED BONE MARROW CELLS
4
3
2
1
(20)
(20)
(10)
(20)
(10)
(20)
(20)
?
10
20
30
40
TIME
(DAYS AFTER PRIMARY BONE MARROW TRANSPLANTATION)

FIG 2

11,587

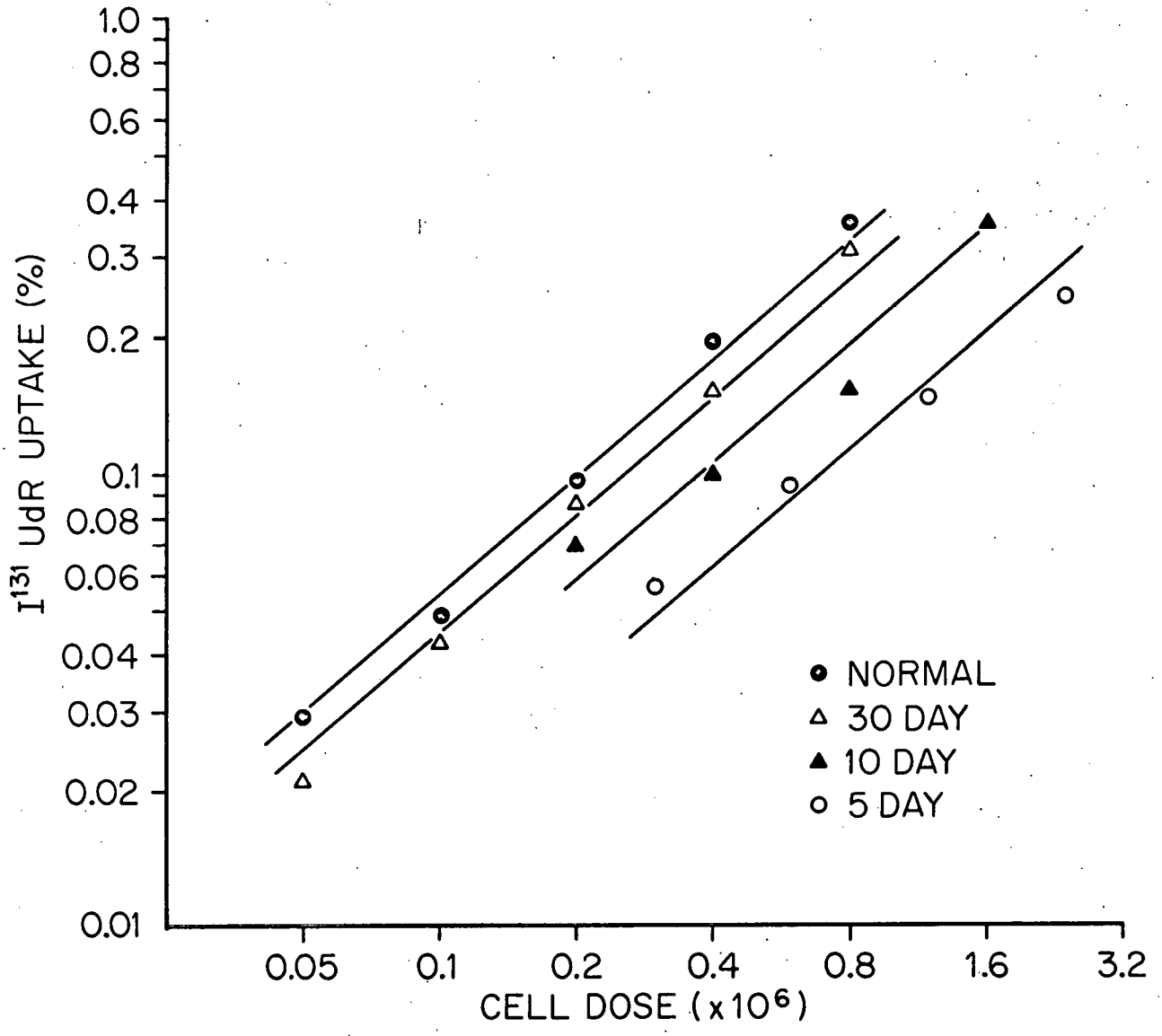
1.0
0.8
0.6
0.4
0.3
0.2
0.1
0.08
0.06
0.04
0.03
0.02
0.01
I131 UdR UPTAKE (%)
0.05
0.1
0.2
0.4
0.8
1.6
3.2
CELL DOSE (x10⁶)
NORMAL
30 DAY
10 DAY
5 DAY

FIG 3

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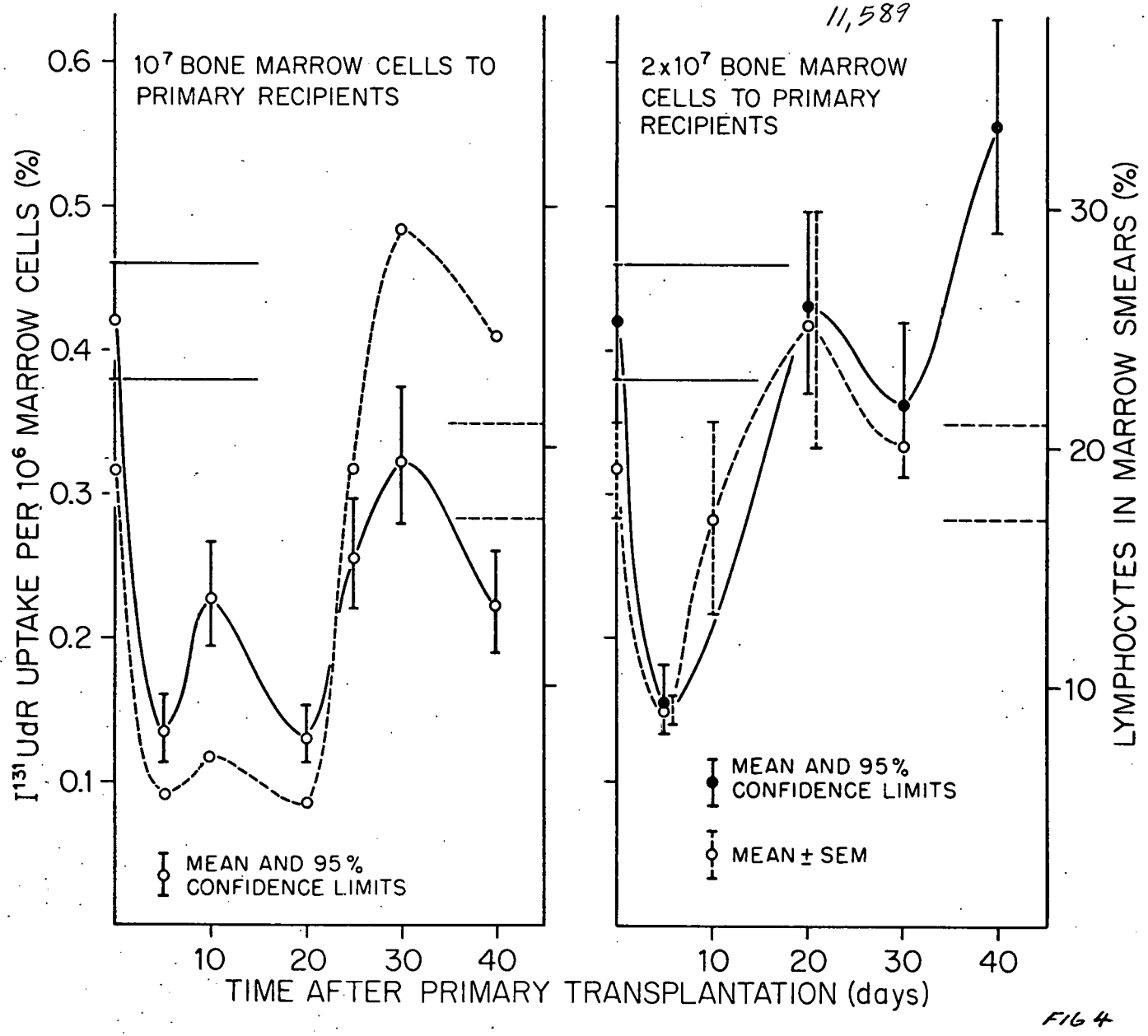
10⁷ BONE MARROW CELLS TO PRIMARY RECIPIENTS
2x10⁷ BONE MARROW CELLS TO PRIMARY RECIPIENTS
I¹³¹UdR UPTAKE PER 10⁶ MARROW CELLS (%)
LYMPHOCYTES IN MARROW SMEARS (%)
0.6
0.5
0.4
0.3
0.2
0.1
30
20
10
MEAN AND 95% CONFIDENCE LIMITS
MEAN ± SEM
10
20
30
40
TIME AFTER PRIMARY TRANSPLANTATION (days)

FIG 4

11,588

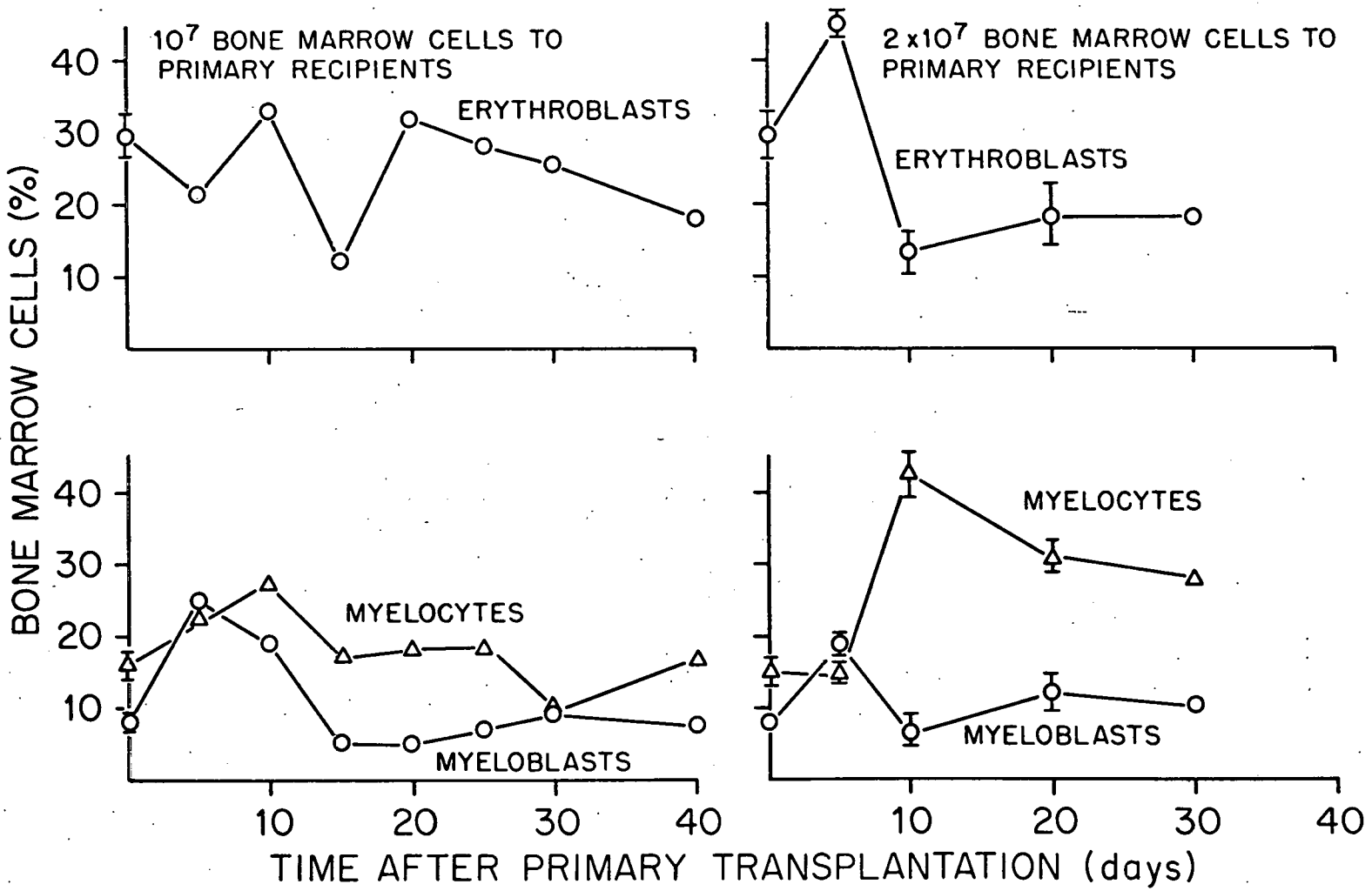
10^7 BONE MARROW CELLS TO PRIMARY RECIPIENTS
ERYTHROBLASTS
2×10^7 BONE MARROW CELLS TO PRIMARY RECIPIENTS
ERYTHROBLASTS
MYELOCYTES
MYELOBLASTS
MYELOCYTES
MYELOBLASTS
BONE MARROW CELLS (%)
10
20
30
40
TIME AFTER PRIMARY TRANSPLANTATION (days)

FIG 5

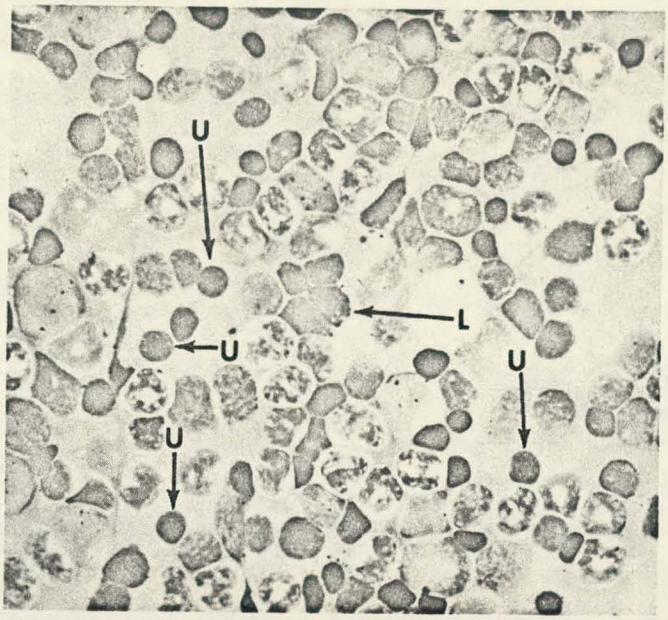

Fig. 6

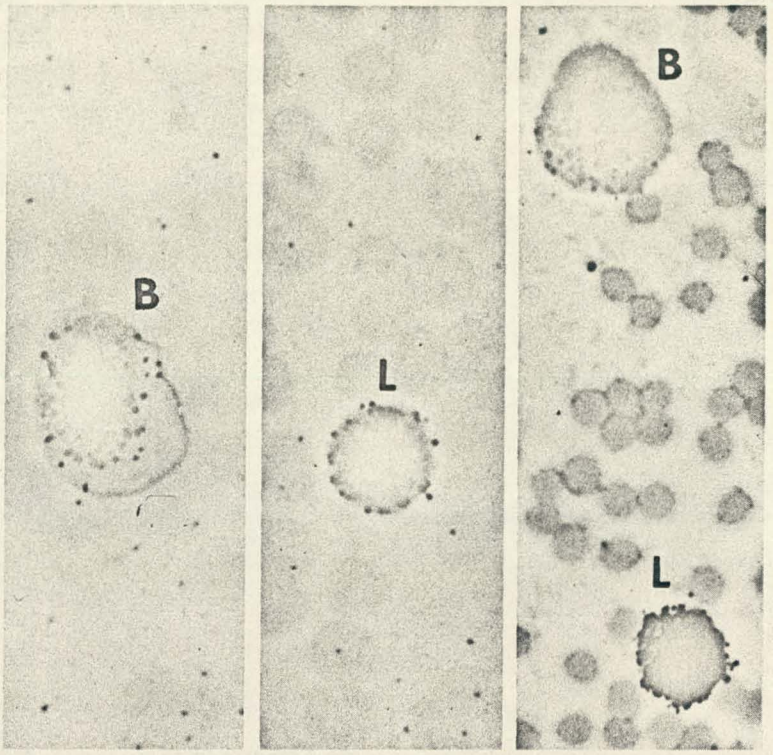

Fig. 7