

Human Bone Marrow Lymphocytes

CYTOTOXIC EFFECTOR CELLS IN THE BONE MARROW OF NORMAL INDIVIDUALS

ANTHONY S. FAUCI, JAMES E. BALOW, and KAREN R. PRATT

From the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT This study was undertaken to determine the capability of lymphocytes in the bone marrow of normal individuals to mediate nonspecific killer cell functions in assays of phytohemagglutinin (PHA)-induced cellular cytotoxicity, and antibody-dependent cellular cytotoxicity (ADCC) against ^{51}Cr -labeled chicken erythrocyte target cells. Relatively pure mononuclear cell suspensions were obtained from bone marrow aspirates in 30 normal volunteers by sucrose gradient centrifugations and from the peripheral blood of the same individuals by Hypaque-Ficoll density centrifugations. At an effector:target ratio of 10:1, the PHA-induced cellular cytotoxicity of peripheral blood was $78.8 \pm 1.3\%$, while that of bone marrow was not significantly less at $66 \pm 9\%$ ($P > 0.1$). At low effector:target ratios, the ADCC of bone marrow was negligible, while at higher effector:target ratios (20:1) bone marrow ADCC was $69 \pm 3.7\%$, which was comparable to that of peripheral blood. The lymphocytes themselves in the mononuclear cell suspensions of both peripheral blood and bone marrow were capable of cytotoxic activity since depletion of monocytes from the suspensions by adherence to rayon wool and G-10 Sephadex columns did not remove the cytotoxic activity. Blocking of the Fc receptor on the effector cells by the addition of aggregated gamma globulin to the cultures suppressed the ADCC but not the PHA-induced cellular cytotoxicity of both peripheral blood and bone marrow, indicating that ADCC is dependent on an Fc receptor on the effector cell in both compartments. These studies demonstrate that the bone marrow of normal humans contains populations of lymphoid cells which have highly efficient killer cell capacities. It is uncertain what portion

of these cells arise in the bone marrow and what portion enter the bone marrow parenchyma as part of the circulating lymphocyte pool. These findings have relevance in the clearer understanding of the killer cell potential of grafted human marrow, as well as the bone marrow sequestration of functionally capable lymphocyte subpopulations in disease states and during chemotherapy.

INTRODUCTION

Various lymphocyte populations in both animals and man have been demonstrated to function as effector cells in a variety of cytotoxic reactions including specific target cell killing by sensitized effector cells, nonspecific mitogen-induced cellular cytotoxicity against a neutral target such as a chicken red blood cell (CRBC),¹ and cytotoxicity by a nonimmune effector cell against an antibody-coated target cell (1, 2). Specific target cell killing by sensitized effector cells is generally felt to be a function of thymus-derived (T) lymphocytes (3-5). On the other hand, cytotoxicity by nonimmune effector cells against antibody-coated target cells has been shown to be independent of T cells (6, 7) and dependent on the presence of a receptor for immunoglobulin (an Fc receptor) on the surface of the effector cells (8, 9). This Fc receptor is found predominantly on certain non-T lymphocytes (10, 11) as well as on a small fraction of T lymphocytes

¹ *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; B, bone marrow-derived; BM, bone marrow; Con A, concanavalin A; CRBC, chicken red blood cell; E:T ratio, effector to target cell ratio; FCS, fetal calf serum; Ig, gamma globulin; MEM-S, Eagle's minimal essential media; PB, peripheral blood; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PMN, polymorphonuclear leukocyte; PWM, pokeweed mitogen; T, thymus-derived.

Received for publication 2 July 1975 and in revised form 8 December 1975.

(12), in addition to monocyte-macrophages (13) and polymorphonuclear leukocytes (PMN) (14). Mitogen-induced cellular cytotoxicity against erythrocyte targets can be mediated by a variety of lymphocyte and non-lymphocyte effector cell populations (1, 15). Although both mitogen-induced cellular cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) against chicken erythrocyte targets are nonspecific functions and not manifestations of killer cell activity resulting from specific sensitization of the host, they do reflect the cytotoxic potential of the effector cell (1). In human studies, particularly with normal subjects in whom specific sensitization cannot be a consideration, these assays, although not direct reflections of naturally occurring *in vivo* phenomena, do serve as simple and highly reproducible measures of cytotoxic effector cell capabilities of various cell populations.

Lymphocyte-mediated cytotoxic activity has been demonstrated in a variety of lymphoid organs in animals (1, 2). In humans, however, most studies have been done on peripheral blood (PB) in which both T lymphocyte and non-T lymphocyte-mediated cytotoxic capacities have been demonstrated (1). There is very little information on the functional capacities of lymphocytes in human bone marrow (BM). In a recent study (16) employing cell separation techniques yielding relatively purified populations of mononuclear cells from BM aspirates in normal volunteers, it was demonstrated that human BM contains several distinct subpopulations of lymphocytes. These purified BM mononuclear cell suspensions responded well to stimulation with the mitogens phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM). These findings could not be explained by contamination of BM aspirates with PB. In the present studies, using these same techniques (16) to obtain purified BM mononuclear cell suspensions, PHA-induced cellular cytotoxicity and ADCC of unfractionated as well as adherent-cell depleted BM mononuclear cells were compared with that of similarly fractionated PB mononuclear cells in a large number of normal volunteers in order to further delineate the functional capacities of human BM lymphocytes.

METHODS

Subjects. BM aspirates were performed on 30 normal adult volunteers, 14 men and 16 women aged 19-27 yr. Details of the procedure were explained individually to each volunteer, and informed consent was obtained. The subjects were all in excellent health, and were taking no medications at the time of the study.

BM aspirates. BM aspirates were performed as previously described (16). Briefly, aspirates were obtained from the posterior superior iliac crest after preparation of the area with iodine, 75% alcohol, and sterile draping. Xylocaine (1%) was used to anesthetize the overlying skin and periosteum. 1.0-2.0 ml of aspirate was withdrawn via a Rosenthal needle into a syringe containing acid citrate anti-

coagulant. A smear of the aspirate was then made. Only those aspirates which contained macroscopically visible bone spicules and fat globules were used. A 35-ml sample of heparinized PB was also drawn from each individual. Total nucleated cell counts were done on the BM aspirate, as well as on a sample of PB with a Coulter counter (model F_n, Coulter Electronics, Fine Particle Group, Hialeah, Fla.). Differential counts of 400 cells were done by the same observer throughout the study on Wright-Giemsa-stained smears of both PB and BM.

Preparation of cell suspensions. Mononuclear cell-rich suspensions were obtained from BM aspirates as previously described (16). Briefly, BM cells were dispersed from clumps and spicules by repeated aspirations through a 1-ml pipette, and erythrocytes were removed by hypotonic lysis. Sucrose gradients of 15-35% (increments of 5%) in Hanks' balanced salt solution and 20% fetal calf serum (FCS) (Industrial Biological Labs., Inc., Rockville, Md.) were prepared. Gradients of 35% sucrose at the bottom through 15% at the top (2 ml of each 5% solution) were layered in a 1.5 × 9-cm plastic tube to give a total of 10 ml. BM cells (4×10^7 - 2×10^8) suspended in 2 ml of Eagle's minimum essential media (MEM-S) (Grand Island Biological Co., Grand Island, N. Y.) were layered over the top of the gradient and centrifuged at 100 *g* for 7 min at 20°C. The upper half (4.5 cm) of the gradient containing the mononuclear cell-rich suspension was collected, washed three times in MEM-S, and a cell count was performed in a Coulter counter. Cyto-centrifuge smears of the suspensions were made, stained with Wright-Giemsa stain, and 400 cell differential counts were performed by the same observer throughout the study. These mononuclear cell-rich suspensions from the upper portion of the sucrose gradient were used for all BM studies except where indicated.

We have previously demonstrated (16) that after fractionation of BM aspirates on sucrose gradients, the resulting mononuclear cell suspensions were comprised almost exclusively of cells from the BM parenchyma with minimal contribution of mononuclear cells from the inevitable contamination by PB. This was shown to be the case by simultaneously drawing blood and performing BM aspirates on subjects who had been reinfused the previous day with radioactive chromium-labeled autologous erythrocytes. The radioactivity in equivalent volumes of BM and PB samples were compared to determine the percent contamination by volume of the BM samples with PB. It was found that even with maximal contamination by volume of BM aspirates with PB, the number of actual PB mononuclear cells contained in the total mononuclear cells from the BM aspirate was relatively minor.

In certain experiments, in which the cytotoxic capacities of nonlymphoid BM cells were assayed, the bottom layer of the gradient which contained predominantly bands, mature neutrophils, and myeloid and erythroid precursors was collected and used.

Mononuclear cells (lymphocytes and monocytes) were obtained from the 35-ml sample of heparinized PB (drawn at the time of the BM aspirate) by the Hypaque-Ficoll method (17). Throughout this paper, cell suspensions will be referred to as "mononuclear cells" when they are unfractionated and contain both monocytes and lymphocytes. The term "lymphocytes" will be used to describe mononuclear cell suspensions depleted of adherent monocytes.

Cytotoxicity assays. PB and BM mononuclear cells were assayed for two separate types of lymphocyte-mediated cytotoxicity: PHA-induced cellular cytotoxicity and ADCC against radioactive (⁵¹Cr-labeled) CRBC targets.

PHA-induced cellular cytotoxicity assays were performed by a slight modification (18) of a previously described method (19), and ADCC was performed as previously described (20). Briefly, labeled targets were prepared as follows: Sterile chicken blood mixed with an equal volume of Alsever's solution (Flow Laboratories, Inc., Rockville, Md.) was stored at 4°C and used within 7 days of being drawn. Immediately before labeling, cells were washed three times with phosphate-buffered saline (PBS), pH 7.4, and brought to a concentration of 10^8 /ml in MEM-S with 10% FCS. 0.1 ml of this suspension was put in a 9.5 × 1.5-cm plastic tube and 0.1 ml of sodium chromate (Amersham/Searle Corp., Arlington Heights, Ill.; 1 mCi/ml) containing 100 μ Ci of ^{51}Cr was added. The mixture was incubated at 37°C for 30 min with gentle agitation every 10 min. The cells were then washed three times at 4°C in MEM-S containing 10% FCS and reconstituted with 1 ml of MEM-S and 10% FCS.

Rabbit and guinea pig anti-CRBC antisera for use in the ADCC assay were prepared as follows: CRBC were washed three times in PBS. 0.3 ml of packed CRBC was mixed in an emulsion with 2 ml of complete Freund's adjuvant and injected in several locations subcutaneously into New Zealand White rabbits and strain 13 guinea pigs. This was repeated two times at 2-wk intervals. 2 wk after the last injection, animals were bled and serum was prepared, heat inactivated at 56°C for 60 min, and stored in 2-ml aliquots at -20°C. Dilutions of antibody were prepared in MEM-S and stored at 4°C. Some ADCC experiments were done with rabbit anti-CRBC antiserum and some were done with guinea pig anti-CRBC antiserum as will be indicated.

For the PHA-induced cellular cytotoxicity, PHA, MR 68, lot K4402 (Wellcome Reagents, Ltd., Beckenham, England) was added to cultures to obtain final concentrations of 1.0, 10, and 100 $\mu\text{g}/\text{ml}$. For the ADCC, 10-fold dilutions of anti-CRBC antiserum from 10^{-1} through 10^{-5} were used. The effector to target cell (E:T) ratios used were 1:10, 1:1, and 10:1 for PHA-induced cellular cytotoxicity, and 1:10, 1:1, 5:1, and 20:1 for ADCC. Cultures done at a 1:1 E:T ratio had 1×10^6 effector lymphocytes and 1×10^6 labeled CRBC targets for a total of 2×10^6 cells in culture. As the E:T ratio was varied greater and less than 1:1, the total number of cells in culture was kept at 2×10^6 in order to preserve consistent culture conditions. At certain E:T ratios, this required the addition of small numbers of unlabeled CRBC. Cultures with and without PHA or anti-CRBC antiserum were performed in triplicate in 1 × 7.5-cm plastic tubes. Into each culture tube was added 1 ml of lymphocytes at the appropriate concentration in MEM-S with 10% FCS, varying concentrations of PHA or antiserum in 0.1 ml of MEM-S, or 0.1 ml of MEM-S alone as control, and 0.1 ml of varying concentrations of ^{51}Cr -labeled CRBC depending on the E:T ratio. The cultures were incubated at 37°C in 5% CO_2 in air and 100% humidity. PHA-induced cellular cytotoxicity was optimal at 40 h and ADCC at 24 h, i.e., specific cytolysis in the presence of PHA or anti-CRBC antiserum minus cytolysis in cultures without PHA or antiserum peaked at these time intervals. The respective data will be reported for these durations of culture. The culture tubes containing a total volume of 1.2 ml were then spun at 1,000 *g* for 10 min at 4°C and 0.6 ml of the supernate was pipetted into a separate tube. The 0.6-ml supernatant tube and the remaining 0.6-ml pellet were counted separately in an automatic gamma counter (series 1185, Searle Analytic, Inc., Des Plaines, Ill.). The percent of ^{51}Cr released by the CRBC targets into the supernate was determined by the following formula (18): per-

cent ^{51}Cr release = $100 \times (\text{supernatant cpm} \times 2) / (\text{supernatant cpm} + \text{pellet cpm})$. The degree of cytotoxicity is expressed as the percent ^{51}Cr release in the presence of PHA or anti-CRBC antiserum minus the percent ^{51}Cr release in the absence of PHA or anti-CRBC antiserum. The percent ^{51}Cr release in the presence of PHA or anti-CRBC antiserum and in the absence of mononuclear cells was consistently less than 10% at the termination of the incubation periods.

Removal of adherent cells. Adherent cells were depleted from PB mononuclear cells in three subjects by passage of cells over rayon wool columns (21), and from PB and BM mononuclear cells in one subject by passing mononuclear cells over a G-10 Sephadex column (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) (22, 23). This was done in order to determine the effect of depletion of monocytes from the mononuclear cell suspensions (lymphocytes and monocytes) on PHA-induced cellular cytotoxicity and ADCC. $4-8 \times 10^7$ PB or BM mononuclear cells in 2 ml of MEM-S and 10% FCS were layered over the top of the column which consisted of either 2 g of washed (0.2 M HCl and distilled water) rayon wool packed in a 20-ml plastic syringe, or a 20-ml bed volume of washed (PBS, pH 7.4) G-10 Sephadex poured into a 35-ml plastic syringe. Both columns were primed with several 20-ml washes of MEM-S and 10% FCS. After layering of cells on the top of the columns, cells were washed through at room temperature without incubation on the column with 60 ml of MEM-S and 10% FCS at an elution rate of 2-3 ml per min. The percent of depletion of PB monocytes after passage through the rayon wool column was $72 \pm 5\%$, and the yield in the eluate was $60 \pm 1\%$ of the total cells put on the column. After passage through the G-10 Sephadex column, the percent depletion of PB monocytes was 95% with a 59% yield of the total cells put over the column, and the percent depletion of BM monocytes was 60% with a 50% yield of the total cells put over the column. There was no significant change in the relative proportions of T lymphocytes as measured by the spontaneous sheep erythrocyte rosette assay and BM-derived (B) lymphocytes as measured by the complement receptor assay. These surface marker assays were performed as previously described (16).

Cytotoxicity of PMN-enriched suspensions. In order to determine the relative cytotoxic capacities of PMNs in the PB and BM, PMN-enriched suspensions were obtained as follows: The erythrocyte-PMN button obtained after Hypaque-Ficoll centrifugation of PB was resuspended in an equal volume of autologous plasma and mixed with an equal volume of 6% dextran in normal saline (Abbott Laboratories, North Chicago, Ill.). The suspension was placed in a glass cylinder and let stand at room temperature for 40 min. The PMN-rich supernate was collected, residual erythrocytes were lysed by hypotonic lysis, and the cells were washed three times in MEM-S. This separation procedure yielded cell suspensions containing greater than 95% PMNs. PMNs were obtained from BM suspensions by collecting the lower half of the sucrose gradient described above for obtaining purified mononuclear cells. This layer contained approximately 50% bands and mature PMNs, and the remainder predominantly early myeloid and erythroid forms. These PMN-enriched suspensions of PB and BM cells were assayed for PHA-induced cellular cytotoxicity and ADCC in the same manner as the mononuclear cells.

Inhibition of Fc receptors by aggregated gamma globulin (Ig). The suppression of ADCC by either removal of cells expressing Fc receptors or blocking of Fc receptors

by a variety of means including reaction with aggregated Ig is well known (9). The effect of inhibition of Fc receptors by aggregated Ig on ADCC as well as PHA-induced cellular cytotoxicity was determined as previously described (24, 25). Aggregated Ig was obtained by heating fraction II, human Ig (Pentex Biochemical, Kankakee, Ill.), 25 mg/ml, at 63°C for 30 min. A control preparation was ultracentrifuged at 45,000 rpm for 120 min to remove spontaneous aggregates. Aggregated Ig was added to the culture tubes in a final concentration ranging from 0.05 through 5 mg/ml, and cytotoxicity was compared with that of cultures in which an equal amount of aggregate-free Ig had been added. The E:T ratios were varied from 1:1 through 20:1 and the antibody dilutions from 10^{-3} through 10^{-6} .

RESULTS

Mononuclear cell-enriched BM suspensions prepared from sucrose gradients. The marked enrichment of BM aspirates for mononuclear cells (mature and immature lymphocytes and monocytes) by the sucrose gradient technique has been described in detail in a previous study (16). The same degree of purification and yields were attained in the present study. Unfractionated BM contained a mean \pm SEM of $15.4 \pm 0.9\%$ mononuclear cells. After sucrose gradient purification, cell suspensions from the upper layer of the gradient contained $76.1 \pm 2.1\%$ mononuclear cells with a yield of $81 \pm 3.7\%$ of the total mononuclear cells originally put on the gradient. The remainder of the cells from the upper layer of the gradient were predominantly nucleated erythrocytes and myeloid and erythroid precursors. The cells from the lower half of the gradient were comprised of less than 5% mononuclear cells and contained predominantly cells of the myeloid series in all stages of maturation (mature PMNs, band forms, metamyelocytes, and myelocytes). The viability of cells after separation on sucrose gradients was always greater than 90%.

Mononuclear cell suspensions of PB obtained by Hypaque-Ficoll separation contained approximately 70–85% lymphocytes, 15–30% monocytes, and less than 1% PMNs.

Cytotoxicity assays. The PHA-induced cellular cytotoxicity against ^{51}Cr -labeled CRBC of simultaneously assayed mononuclear cell suspensions from the PB and BM of five subjects is shown in Fig. 1. At the lowest E:T ratio of 1:10 there was no detectable cytotoxicity in BM, while PB showed slight, but clear-cut cytotoxic activity at a PHA concentration of 1 $\mu\text{g}/\text{ml}$. As the E:T ratio was increased to 1:1, there was a marked increase in the PB cytotoxicity while the BM showed a substantial degree of cytotoxicity. The greater degree of PHA-induced cellular cytotoxicity of PB compared to BM at this low E:T ratio was clearly seen in a larger group of subjects ($n = 22$) not shown in Fig. 1, in which simultaneous cytotoxicities of PB and BM were assayed over a range of PHA concentrations of 1.0, 10, and 100 $\mu\text{g}/\text{ml}$ at a fixed E:T ratio of 1:1. When the maximal

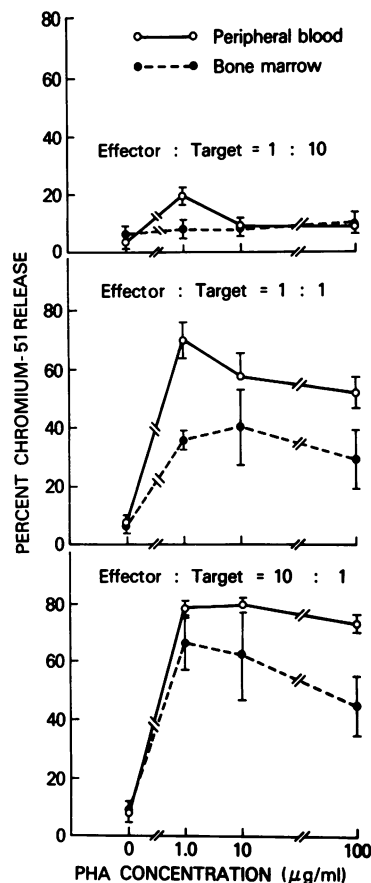


FIGURE 1 PHA-induced cellular cytotoxicity of mononuclear cell suspensions from PB and BM against ^{51}Cr -labeled CRBC. Simultaneous assays were done on the PB and BM of five subjects. The E:T ratios were varied from 1:10 through 10:1. Data are given as the mean \pm SEM percent isotope release in the five subjects at each E:T ratio, at varying concentrations of PHA.

cytotoxicities of PB and BM were compared at this E:T ratio of 1:1, PB showed a significantly greater cytotoxicity of $62.8 \pm 3.3\%$ compared to BM with $28.7 \pm 2.6\%$ ($P < 0.001$, Student's *t* test). However, as seen in Fig. 1, which shows the values in five other individuals at various E:T ratios and at each concentration of PHA, as the E:T ratio was increased from 1:1 to 10:1, the cytotoxicity of PB increased only slightly with a flattening of the PHA concentration curve such that a concentration of 10 $\mu\text{g}/\text{ml}$ of PHA was no longer supramaximal and suppressive, but was essentially maximal with $78.8 \pm 1.3\%$ cytotoxicity. Of note is the fact that at this E:T ratio of 10:1 the BM cytotoxicity increased to a peak of $66 \pm 9\%$, which is not significantly different from the peak cytotoxicity of PB at this E:T ratio ($P > 0.1$).

For the assay of ADCC, two separate antibody preparations were employed: guinea pig anti-CRBC antibody and rabbit anti-CRBC antibody. In the series of experi-

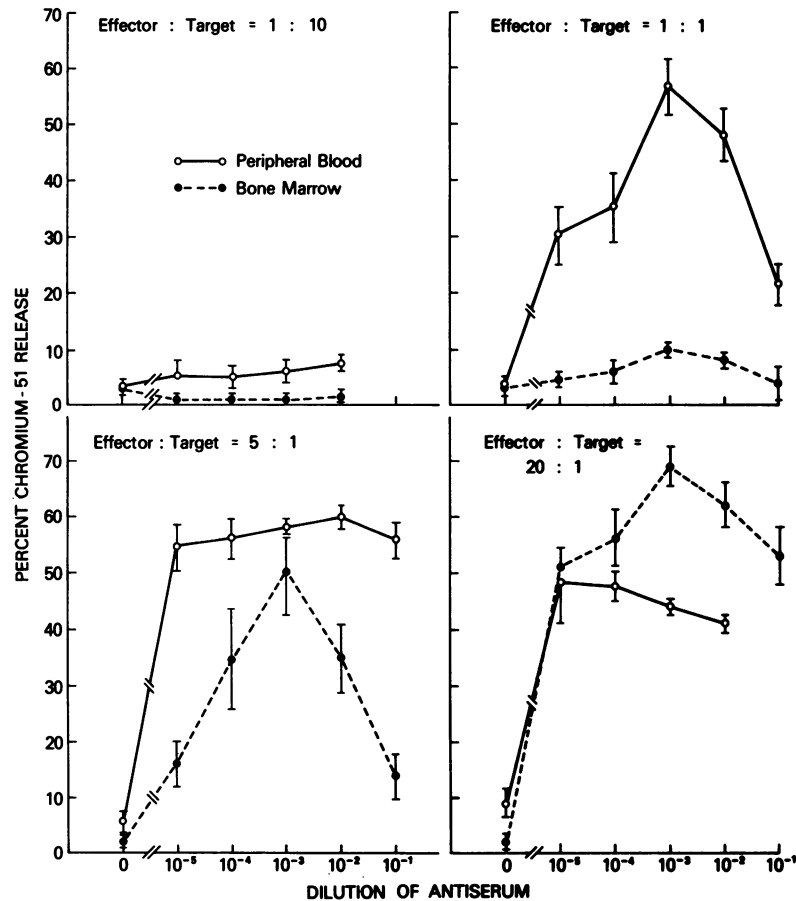


FIGURE 2 ADCC of mononuclear cell suspensions from PB and BM. Targets were ^{51}Cr -labeled CRBC in the presence of varying dilutions of rabbit anti-CRBC antiserum. The E:T ratios were varied from 1:10 through 20:1. Data are given as the mean \pm SEM percent isotope release in five subjects at each E:T ratio, at varying dilutions of rabbit anti-CRBC antiserum.

ments done early in the study using the guinea pig anti-CRBC antiserum with final concentrations ranging from 10^{-1} through 10^{-5} in 10-fold dilutions, 10^{-3} proved the optimal dilution. All experiments were carried out at an E:T ratio of 20:1. 22 subjects underwent simultaneous PB and BM assays for ADCC using the guinea pig antiserum. The peak cytotoxicity for PB was $37.9 \pm 2.2\%$, which was slightly, but significantly greater than that of BM at $30.5 \pm 1.7\%$ ($P < 0.02$).

The rabbit anti-CRBC antiserum proved more efficient in the ADCC assay than the guinea pig antiserum and was used in the subsequent kinetic studies. ADCC assays using the rabbit antiserum were done simultaneously on the PB and BM of five subjects. The results of these studies are shown in Fig. 2. At an E:T ratio of 1:10, there was no detectable cytotoxicity in the BM, while the PB showed only minimal cytotoxicity. As the E:T ratio was increased to 1:1, there was a marked increase in the cytotoxicity of PB to a peak of $56.4 \pm 5.2\%$ at an antibody dilution of 10^{-3} . This value is not significantly different

from the maximal attainable ADCC of PB at any antibody dilution or E:T ratio. At this E:T ratio, the BM cytotoxicity was still comparatively low. As the E:T ratio was further increased to 5:1, there was very little increase in the peak cytotoxicity of PB, while a flattening of the antiserum dilution curve was seen. However, at this E:T ratio, there was a marked increase in ADCC of BM with a sharp peak at an antibody dilution of 10^{-3} . This peak BM cytotoxicity of $48.8 \pm 7.6\%$ at an E:T ratio of 5:1 is not significantly different from the peak PB cytotoxicity of $59.8 \pm 2.1\%$ at the same E:T ratio ($P > 0.2$). Of note is the fact that as the E:T ratio was further increased to 20:1, the PB cytotoxicity was suppressed somewhat as compared to the values at an E:T ratio of 5:1. On the other hand, at this high E:T ratio, the BM cytotoxicity continued to increase to a maximum of $69 \pm 3.7\%$, which is significantly greater than the PB maximal cytotoxicity of $48 \pm 7.3\%$ at this particular E:T ratio ($P < 0.05$). Thus, at low E:T ratios, the ADCC of PB was significantly greater than that of BM. How-

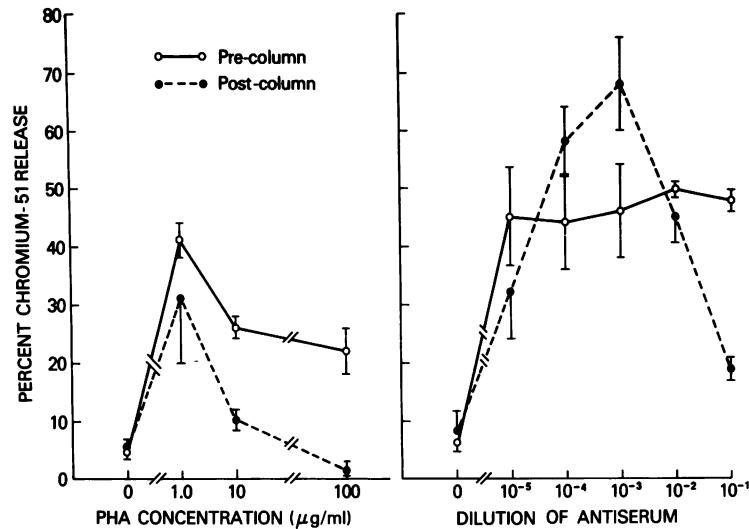


FIGURE 3 Effect of removal of adherent cells on the PHA-induced cellular cytotoxicity and ADCC of PB mononuclear cell suspensions. Cytotoxicity assays were performed on cell suspensions before and after passage through a rayon wool column to remove adherent cells. E:T ratios were 1:1 and 20:1 for PHA-induced cellular cytotoxicity and ADCC, respectively. Data given are the mean \pm SEM percent isotope release in three subjects at each concentration of PHA and antiserum.

ever, as the E:T ratio was increased, the ADCC of BM was comparable and even exceeded that of PB.

Removal of adherent cells. As shown in Fig. 3, depletion of adherent cells from PB mononuclear cell suspensions of three subjects by passage over a rayon wool column resulted in no significant change ($P > 0.2$) in the peak cytotoxicity of the PHA-induced cellular cytotoxicity. However, at supramaximal concentrations of PHA (10 and 100 $\mu\text{g/ml}$), there were significant decreases in cytotoxicity after removal of adherent cells ($P < 0.001$ for 10 $\mu\text{g/ml}$ and $P < 0.01$ for 100 $\mu\text{g/ml}$ of PHA). Thus, while the peak PHA-induced cytotoxicity of PB mononuclear cells was not decreased by removal of adherent cells, the configuration of the PHA concentration curve was altered. Removal of adherent cells from PB mononuclear cell suspensions also altered the configuration of the antiserum dilution curve in the ADCC assay (Fig. 3). Although this post-column peak value of $68 \pm 8\%$ at 10^{-3} antiserum dilution was somewhat higher than the pre-column value of $46 \pm 8\%$, the difference was not significant ($P > 0.1$).

In order to determine the effect of removal of adherent cells from BM mononuclear cell suspensions on PHA-induced cellular cytotoxicity and ADCC, a G-10 Sephadex column was used since there was a paucity of BM mononuclear cells available after sucrose gradient purification, and preliminary studies showed the G-10 Sephadex column to be more efficient than rayon wool in removal of adherent cells. The effect of removal of adherent cells from PB and BM mononuclear cell suspensions of

a single subject is shown in Fig. 4. There was little if any change in the PHA-induced cellular cytotoxicity and ADCC of PB or BM cells after removal of adherent cells. As with the rayon wool columns used for PB (Fig. 3), there was very little difference in the peak cytotoxicities, and any suppression of cytotoxicity by removal of adherent cells was seen only at the supramaximal concentrations of PHA and antiserum in the PHA-induced cytotoxicity and ADCC assays, respectively. Hence, these data show that removal of adherent cells caused some alteration in the shapes of the concentration curves for PHA and antiserum but did not significantly change the peak cytotoxicities of PB and BM mononuclear cells in either the PHA-induced cellular cytotoxicity or the ADCC assays.

Cytotoxicity of PMN-enriched PB and BM cell suspensions. The relative cytotoxic capacities of mononuclear cells as compared to PMN-enriched suspensions in PB and BM of four subjects are shown in Table I. Of note is the fact that the background cytotoxicity (i.e., in the absence of PHA or anti-CRBC antiserum) was quite low for both the mononuclear cell and PMN-enriched suspensions of PB and BM. Hence, mature PMNs as present in the PB, or immature forms (predominantly band forms and myeloid precursors) as present in the BM suspensions, exhibited very little nonspecific (background) killing. This is quite similar to the mononuclear cell suspensions from these compartments. In addition, PMNs from PB were equally as efficient in killing in the PHA-induced cellular cytotoxicity assay as were

TABLE I
Relative Cytotoxic Capacities of Mononuclear Cell and PMN-Enriched Suspensions from PB and BM*

Cell source	Cell type	Percent ⁵¹ Cr release			
		PHA-induced cellular cytotoxicity		ADCC	
		No PHA	PHA (1 μg/ml)	No antibody	Antibody (10 ⁻⁵)
PB	Mononuclear	8±1	79±2	3±1	52±3
PB	PMN‡	7±1	84±1	3±1	76±2
BM	Mononuclear	6±1	50±1	3±0.3	69±1
BM	PMN§	4±0.3	44±1	3±1	69±2

* Simultaneous assays done on the different cell suspensions from PB and BM of four individuals. Cultures were carried out for 24 h for ADCC and 40 h for PHA-induced cellular cytotoxicity.

‡ Greater than 95% mature PMNs.

§ Mature PMNs, 10–17%; bands, 35–45%; myelocytes and metamyelocytes, 30–40%; remainder, predominantly mononuclear cells and erythroid precursors.

mononuclear cells, and even more efficient than mononuclear cells in killing in the ADCC assay. Furthermore, cell suspensions from BM which were enriched in immature granulocytic forms (predominantly bands) were equally as efficient as BM mononuclear cells in both PHA-induced cellular cytotoxicity and ADCC.

Inhibition of Fc receptors by aggregated Ig. Aggregated Ig had no effect on the PHA-induced cellular cytotoxicity of either PB or BM mononuclear cells at any concentration of Ig. In particular, in three subjects at an E:T ratio of 1:1 with an Ig concentration of 5 mg/ml, PHA-induced cellular cytotoxicity of PB was 69±2% in the absence of and 71±8% in the presence of aggregated Ig. For BM, PHA-induced cellular cytotoxicity was 44±3% in the absence of and 42±3% in the presence of aggregated Ig. The effect of aggregated Ig on ADCC was variable depending on the E:T ratio, the aggregated Ig significantly ($P < 0.01$) inhibited the ratio of 5:1 and a low antiserum concentration of 10⁻⁵, concentration of antiserum, and the concentration of Ig. At a high E:T ratio of 20:1, there was little or no effect at any antiserum concentration, while at an E:T ADCC of PB and BM as shown in Table II.

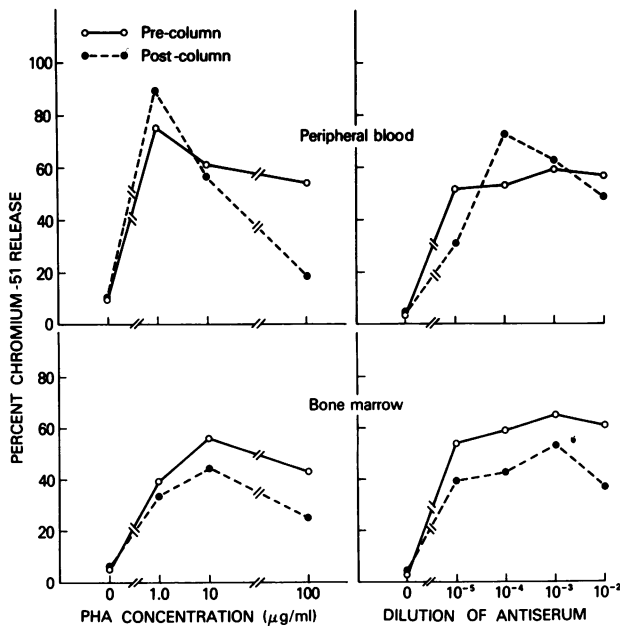


FIGURE 4 Effect of removal of adherent cells on the PHA-induced cellular cytotoxicity and ADCC of PB and BM mononuclear cell suspensions. Cytotoxicity assays were performed simultaneously on PB and BM cell suspensions of a subject before and after passage through a G-10 Sephadex column to remove adherent cells. E:T ratios were 1:1 and 20:1 for PHA-induced cellular cytotoxicity and ADCC, respectively. Data are given as the percent isotope release at each concentration of PHA and antiserum.

DISCUSSION

Previous studies have demonstrated that the BM of normal humans contains identifiable subpopulations of lymphoid cells with T-lymphocyte surface markers, B-lymphocyte surface markers, and a relatively large proportion (approximately 65% of the mononuclear cells present) without readily detectable surface markers (16). It was shown that these cells could not be accounted for by contamination of BM aspirates with PB, and indeed were contained within the BM parenchyma itself. In addition, these BM cell suspensions responded to the mitogens PHA, Con A, and PWM. These studies showed that the human BM was an organ whose immunological capabilities exceeded that of supplying lymphoid stem cells whose mature functions would be exhibited in peripheral organs. They suggested that the BM possessed a degree of immunocompetence in and of itself. This has

been substantiated clinically by studies of human BM transplantation which have shown that donor BM contains a relatively mature population of cells capable of mounting a severe, early graft versus host reaction in graft recipients (26). The present study further demonstrates that the human BM contains populations of lymphoid cells with cytotoxic effector capabilities similar to those of PB. Specifically, BM cell suspensions markedly enriched for mononuclear cells (precursor as well as mature lymphocytes and monocytes) were highly efficient in two separate killer cell assays. In the PHA-induced cellular cytotoxicity assay, PB mononuclear cells manifested near maximal killing at a relatively low E:T ratio of 1:1 (Fig. 1). At this low E:T ratio, the cytotoxicity manifested by the same total number of BM mononuclear cells was significantly less than that of PB ($P < 0.001$). However, as the E:T ratio was increased to 10:1, the BM showed slightly, but not significantly less PHA-induced cellular cytotoxicity than PB. This occurred because the PB was already functioning at near maximal killing capacity at an E:T ratio of 1:1. Relatively little was gained in killing efficiency by an increase in E:T ratio to 10:1. On the other hand, the BM nearly doubled its killing efficiency to a level comparable to that of peak PB cytotoxicity by an increase in E:T ratio to 10:1. This is true most probably because of the relatively high proportion and/or efficiency of cells with effector capacities compared to the total mononuclear cell population in PB. On the other hand, the BM cells, with a relatively low proportion and/or efficiency of effectors, increased their killing substantially by an increase in E:T ratio. A low efficiency of mononuclear effector cells in the BM parenchyma may well reflect the lack of functional maturity of this population of cells.

It has recently been demonstrated that mitogen-induced cellular cytotoxicity can be mediated by several different effector cell types including both T and B lymphocytes (15). Hence, one must be cautious in attributing the differences in PHA-induced cellular cytotoxicity between PB and BM at various E:T ratios to the relative proportions of recognizable T and B lymphocyte populations in these two compartments (16). In addition, it is possible that the PHA-induced cellular cytotoxicity of BM mononuclear cell suspensions is due at least in part to other populations of cells in these suspensions including the relatively large proportion of mononuclear cells without detectable surface markers.

The ADCC of BM mononuclear cells is even more impressive than the PHA-induced cellular cytotoxicity of BM. It is noteworthy that the comparative kinetics of the PB and BM ADCC assays are similar to those seen in the PB and BM PHA-induced cellular cytotoxicity studies (Figs. 1 and 2). Again, this phenomenon may be indicative of the presence in the BM mononuclear cell

TABLE II
Effect of Aggregated Ig on ADCC of PB and BM Mononuclear Cells*

	Aggregated Ig added to culture	% ^{51}Cr release
	<i>mg/ml</i>	
PB ($n = 3$)†	0	50±2.9§
	0.05	31±3.4
	0.5	17±5
	5.0	12±3.5
BM ($n = 3$)	0	46±3.8
	0.05	26±4.3
	0.5	17±4.3
	5.0	5±1.9

* E:T ratio of 5:1; antiserum dilution of 10^{-5} .

† n , number of subjects studied.

§ Mean±SEM percent isotope release.

suspensions of a population of effector cells similar to or identical with those found in the PB, only in a lesser proportion, such that only at higher E:T ratios do they manifest a killing capacity comparable to that of PB mononuclear cells. The possibility also exists that different populations of cells which are dissimilar either in true identity or in stage of development are mediating the ADCC observed in these two compartments.

Of note is the fact that the addition of aggregated human Ig to the cultures of both PB and BM mononuclear cells resulted in suppression of ADCC. This finding indicates that the mechanism of the observed ADCC of both PB and BM mononuclear cells in the present study is consistent with that described in previous studies, i.e., it is mediated through the Fc receptor of the effector cell (8, 9).

It has been clearly demonstrated in several previous studies that PMN from human PB possess Fc receptors and are highly efficient in mediating ADCC (14, 24). In the present study it was shown that suspensions of BM cells enriched in cells of the myeloid series (predominantly bands and metamyelocytes) were equally as effective as purified BM mononuclear cells in mediating both ADCC and PHA-induced cellular cytotoxicity (Table I). Since the proportion of mature PMNs in these suspensions was so small, and the ADCC cytotoxicity of the fraction was comparable in magnitude to the purified mature PMNs of the PB (Table I), it is likely that the immature forms, probably bands, contributed at least somewhat to the cytotoxicity. Thus, apart from the well recognized cytotoxic capacities of PB mononuclear cells, human PB as well as BM contains other populations of cells, particularly PMNs which are highly efficient

effector cells in both the PHA-induced cellular cytotoxicity and the ADCC assays.

The mononuclear cell suspensions of both PB and BM in the present study contained variable amounts of monocytes. Monocytes also possess Fc receptors (13), and can function as effectors in ADCC (9). In addition, PHA-induced cellular cytotoxicity can also be mediated by monocytes (1). Hence, in order to determine the cytotoxic capacities of purified PB and BM lymphocytes depleted of monocytes, cytotoxic assays were performed on the mononuclear cell suspensions before and after removal of monocytes by column adherence (Figs. 3 and 4). The results clearly demonstrate two main points. First, BM as well as PB lymphocytes depleted of monocytes are highly effective in mediating PHA-induced cellular cytotoxicity and ADCC. Second, monocytes in both compartments also possess a degree of cytotoxic capability since their removal, though not significantly affecting the peak cytotoxicity, does alter the concentration curves of PHA and antiserum in the respective assays.

Several mature lymphocyte functions have been demonstrated in the BM in animal studies. These have included response to various mitogens (27, 28), to various antigens (29, 30), the ability to mediate contact sensitivity (31) as well as the graft versus host reaction (32, 33), in addition to mitogen-induced cellular cytotoxicity against erythrocyte targets (34). There is very little previous information concerning the functional capacities of human BM lymphocytes. In an earlier paper, different subpopulations of lymphocytes were demonstrated in the BM of normal humans (16). In the present study, it has been further shown that the BM contains populations of lymphocytes which are very efficient mediators of PHA-induced cellular cytotoxicity, as well as ADCC.

It is uncertain whether the lymphocytes which are mediating these functions arise *de novo* within the BM or arrive there as part of the recirculating lymphocyte pool (35) which can gain free access into and out of the BM parenchyma (36, 37). In this regard, in addition to the BM serving as the direct source of B lymphocytes (38), it has been clearly shown that mature peripheral lymphocytes, particularly T lymphocytes can and do sequester in the BM in certain disease states (39), as well as during corticosteroid administration (40-42).

The present finding of cytotoxic effector cell activity in normal human BM lymphocytes demonstrates that these cells have the potential to function as killer cells. The relationship of these findings to the graft versus host reaction frequently associated with BM transplantation (26) remains to be clarified. Further studies in this area will hopefully elucidate the phenomenon of BM sequestration of functionally capable lymphocyte subpopulations, as well as the specific sensitivity of these subpopulations to various chemotherapeutic regimens.

ACKNOWLEDGMENTS

The authors wish to thank Mrs. Rhoda Hubert for expert technical assistance.

REFERENCES

1. Perlmann, P., and G. Holm. 1969. Cytotoxic effects of lymphoid cells in vitro. *Adv. Immunol.* 11: 117-193.
2. Cerottini, J. C., and K. T. Brunner. 1974. Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. *Adv. Immunol.* 18: 67-132.
3. Cerottini, J. C., A. A. Nordin, and K. T. Brunner. 1970. In vitro cytotoxic activity of thymus cells sensitized to alloantigens. *Nature (Lond.)* 227: 72-73.
4. Cerottini, J. C., A. A. Nordin, and K. T. Brunner. 1970. Specific in vitro cytotoxic activity of thymus derived lymphocytes sensitized to alloantigens. *Nature (Lond.)* 228: 1308-1309.
5. Henney, C. S. 1973. On the mechanism of T-cell mediated cytolysis. *Transplant. Rev.* 17: 37-70.
6. Van Boxel, J. A., J. D. Stobo, W. E. Paul, and I. Green. 1972. Antibody dependent lymphoid cell-mediated cytotoxicity: no requirement for thymus-derived lymphocytes. *Science (Wash. D. C.)* 175: 194-196.
7. Zigelboim, J., R. P. Gale, A. Chiu, B. Bonavida, R. C. Ossorio, and J. L. Fahey. 1974. Antibody dependent cellular cytotoxicity: cytotoxicity mediated by non T-lymphocytes. *Clin. Immunol. Immunopathol.* 3: 193-200.
8. MacLennan, I. C. M. 1972. Antibody in the induction and inhibition of lymphocyte cytotoxicity. *Transplant. Rev.* 13: 67-90.
9. Perlmann, P., H. Perlmann, and H. Wigzell. 1972. Lymphocyte mediated cytotoxicity in vitro. Induction and inhibition by humoral antibody and nature of effector cells. *Transplant. Rev.* 13: 91-114.
10. Basten, A., J. F. A. P. Miller, J. Sprent, and J. Pye. 1972. A receptor for antibody on B lymphocytes. I. Method of detection and functional significance. *J. Exp. Med.* 135: 610-626.
11. Jondal, M., H. Wigzell, and F. Aiuti. 1973. Human lymphocyte subpopulations: classification according to surface markers and/or functional characteristics. *Transplant. Rev.* 16: 163-195.
12. Stout, R. D., and L. A. Herzenberg. 1975. The Fc receptor on thymus-derived lymphocytes. I. Detection of a subpopulation of murine T lymphocytes bearing the Fc receptor. *J. Exp. Med.* 142: 611-621.
13. Huber, H., M. J. Polley, W. D. Linscott, H. H. Fudenberg, and H. J. Müller-Eberhard. 1968. Human monocytes: distinct receptor sites for the third component of complement and for immunoglobulin G. *Science (Wash. D. C.)* 162: 1281-1283.
14. Henson, P. M. 1969. The adherence of leukocytes and platelets induced by fixed IgG antibody and complement. *Immunology.* 16: 107-121.
15. Muchmore, A. V., D. L. Nelson, H. Kirchner, and R. M. Blaese. 1975. A reappraisal of the effector cells mediating mitogen induced cellular cytotoxicity. *Cell. Immunol.* 19: 78-90.
16. Fauci, A. S. 1975. Human bone marrow lymphocytes. I. Distribution of lymphocyte subpopulations in the bone marrow of normal individuals. *J. Clin. Invest.* 56: 98-110.
17. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21: 77-89.

18. Sherwood, G., and R. M. Blaese. 1973. Phytohaemagglutinin-induced cytotoxic effector lymphocyte function in patients with the Wiskott-Aldrich syndrome (WAS). *Clin. Exp. Immunol.* **13**: 515-520.
19. Perlmann, P., H. Perlmann, and G. Holm. 1968. Cytotoxic action of stimulated lymphocytes on allogenic and autologous erythrocytes. *Science (Wash. D. C.)*. **160**: 306-309.
20. Perlmann, P., and H. Perlmann. 1970. Contactual lysis of antibody-coated chicken erythrocytes by purified lymphocytes. *Cell. Immunol.* **1**: 300-315.
21. Rosenstreich, D. L., J. T. Blake, and A. S. Rosenthal. 1971. The peritoneal exudate lymphocyte. I. Differences in antigen responsiveness between peritoneal exudate and lymph node lymphocytes from immunized guinea pigs. *J. Exp. Med.* **134**: 1170-1186.
22. Ly, I., and R. I. Mishell. 1974. Separation of mouse spleen cells by passage through columns of Sephadex G-10. *J. Immunol. Methods.* **5**: 239-247.
23. Schwartz, R. H., A. R. Bianco, B. S. Handwerger, and C. R. Kahn. 1975. Demonstration that monocytes rather than lymphocytes are the insulin-binding cells in preparations of human peripheral blood mononuclear leukocytes: implications for studies of insulin-resistant states in man. *Proc. Natl. Acad. Sci. U. S. A.* **72**: 474-478.
24. Gale, R. P., and J. Zigelboim. 1975. Polymorphonuclear leukocytes in antibody-dependent cellular cytotoxicity. *J. Immunol.* **114**: 1047-1051.
25. Calder, E. A., S. J. Urbaniak, W. J. Penhale, and W. J. Irvine. 1974. Characterization of human lymphoid cell-mediated antibody-dependent cytotoxicity (LDAC). *Clin. Exp. Immunol.* **18**: 579-593.
26. van Bekkum, D. W. 1972. Use and abuse of hemopoietic cell grafts in immune deficiency diseases. *Transplant. Rev.* **9**: 3-53.
27. Osmond, D. G. 1968. Culture of lymphocyte-rich fractions of bone marrow with phytohemagglutinin and pokeweed mitogen. *Anat. Rec.* **160**: 402.
28. Claman, H. N. 1974. Bone marrow T cells. I. Response to the T cell mitogens, phytohemagglutinin and concanavalin A. *J. Immunol.* **112**: 960-964.
29. Singal, S. K., and M. Richter. 1968. Cells involved in the immune response. IV. The response of normal and immune rabbit bone marrow and lymphoid tissue lymphocytes to antigens in vitro. *J. Exp. Med.* **128**: 1099-1125.
30. Yoshida, Y., and D. G. Osmond. 1971. Blastogenic response of lymphocytes separated from bone marrow to allogeneic lymphoid cells in vitro. *Immunology.* **21**: 767-779.
31. Asherson, G. L., M. Zembala, and B. Mayhew. 1974. Passive transfer of contact sensitivity by bone marrow cells and evidence for their origin from immunized lymph nodes. *Int. Arch. Allergy Appl. Immunol.* **46**: 256-260.
32. Cohen, J. J., and H. N. Claman. 1971. Hydrocortisone resistance of activated initiator cells in graft versus host reactions. *Nature (Lond.)*. **229**: 274-275.
33. Chen, M. G., G. B. Price, and T. Makinodan. 1972. Incidence of delayed mortality (secondary disease) in allogeneic radiation chimeras receiving bone marrow from aged mice. *J. Immunol.* **108**: 1370-1378.
34. Kirchner, H., and R. M. Blaese. 1973. Pokeweed mitogen-, concanavalin A-, and phytohemagglutinin-induced development of cytotoxic effector lymphocytes. On evaluation of the mechanisms of T cell-mediated cytotoxicity. *J. Exp. Med.* **138**: 812-824.
35. Ford, W. L., and J. L. Gowans. 1969. The traffic of lymphocytes. *Semin. Hematol.* **6**: 67-83.
36. Rosse, C. 1972. Migration of long-lived lymphocytes to the bone marrow and to other lymphomyeloid tissues in normal parabiotic guinea pigs. *Blood.* **40**: 90-97.
37. Röpke, C., and N. B. Everett. 1974. Migration of small lymphocytes in adult mice demonstrated by parabiosis. *Cell Tissue Kinet.* **7**: 137-150.
38. Abdou, N. I., and M. Richter. 1970. The role of bone marrow in the immune response. *Adv. Immunol.* **12**: 201-270.
39. Borella, L., and A. A. Green. 1972. Sequestration of PHA-responsive cells (T-lymphocytes) in the bone marrow of leukemic children undergoing long-term immunosuppressive therapy. *J. Immunol.* **109**: 927-932.
40. Cohen, J. J. 1972. Thymus-derived lymphocytes sequestered in the bone marrow of hydrocortisone-treated mice. *J. Immunol.* **108**: 841-844.
41. Fauci, A. S. 1975. Mechanisms of corticosteroid action on lymphocyte subpopulations. I. Redistribution of circulating T and B lymphocytes to the bone marrow. *Immunology.* **28**: 669-680.
42. Fauci, A. S. 1975. Corticosteroids and circulating lymphocytes. *Transplant. Proc.* **7**: 37-40.