Distribution of T, B and 'null' lymphocytes in maintenance hemodialysis patients

Groups	Total lymphocytes per mm³ (mean ± SE)	T lymphocytes (mean ± SE) %	per mm³ [a]	B lymphocytes (mean ± SE) %	per mm³	'Null' lymphocytes (mean ± SE) %	per mm³
Control ($n = 24$)	2577 ± 124	38.4 ± 1.9	974 ± 51	24.1 ± 2.4	611 ± 71	37.5 ± 3.4	992 ± 115
Nephritis ($n = 23$)	1691 ± 361	26.2 ± 4.0	664 ± 216	18.3 ± 2.5	329 ± 86	55.5 ± 4.6	697 ± 120
P-value [b]	< 0.0125	< 0.005	< 0.10	< 0.05	< 0.01	< 0.0025	< 0.05

[a] The mean was calculated from each individual's value. [b] According to Student's t-test.

sera of patients with SLE[7], lipoid nephrosis, and glomerulonephritis[8]. LIES et al.[9] demonstrated that these lymphocytotoxic antibodies were directly formed against T cells.

It has been postulated that infection by a latent virus may alter the T cells in such a way as to trigger the formation of T cell specific cytotoxins in SLE[10]. These toxins can destroy the T cells. This destruction resulted a decrease in the T cells as observed in our patients. On the other hand, the decrease in T cells may be associated with a deficiency in suppressor T cells[11]. This deficiency allows the B cells to produce a variety of autoantibodies. These autoantibodies, such as antinuclear antibody, could participate in the production of immune complex disease such as glomerulonephritis[12]. This interpretation is supported by a recent report of HUSTED et al.[13] who observed an increased incidence of antinuclear antibodies in chronic dialysis patients.

In conclusion, we observed a decrease in the circulating T and B lymphocytes in a group of patients with chronic renal failure who were being maintained on hemodialysis. Accompanied by the decrease in the T and B cells, there was an increase in the percentage of the 'null' cells. The cause of the renal disease in these patients was the result of various forms of immunologic glomerulonephritis. Therefore, additional studies are needed to determine whether similar changes in T, B, and 'null' cells also occur in renal failure due to non-immunologic causes.

Summary. The T and B lymphocytes in peripheral blood were reduced in patients with glomerulonephritis treated with hemodialysis as compared to normals. Although the absolute number of 'null' cells was also decreased, the percentage of 'null' cells was increased in these patients.

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[7] K. K. MITTAL, R. D. ROSSEN, J. T. SHARP, M. D. LIDSKY and W. T. BUTLER, Nature, Lond. *225*, 1255 (1970).
[8] B. S. OOI, A. R. ORLINA and L. MASAITIS, Lancet *2*, 1348 (1974).
[9] R. B. LIES, R. P. MESSNER and R. C. WILLIAMS JR., Arth. Rheum. *16*, 369 (1973).
[10] R. P. MESSNER, F. D. LINDSTROM and R. C. WILLIAMS, JR., J. clin. Invest. *52*, 3046 (1973).
[11] A. C. ALLISON, A. M. DENMAN and R. D. BARNES, Lancet *2*, 135 (1971).
[12] R. P. MESSNER, Arth. Rheum. *17*, 339 (1974).
[13] F. C. HUSTED, K. D. NOLPH and G. C. SHARP, abstracts 7th Ann. Meeting Am. Soc. Nephrology (1974), p. 40.
[14] This work was supported in part by a Grant, No. CA 14876, from the National Cancer Institute. Ms. RITA GIULIANO gave valuable technical assistance.

Fetal Calf Serum Alters Cyclic Adenosine 3′,5′-Monophosphate's Effect on Heme Synthesis in vitro[1]

Reports are often contradictory about the effect of cyclic adenosine 3′,5′-monophosphate (cAMP) on heme synthesis in vitro. GORSHEIN and GARDNER[2], for example, showed that cAMP stimulates radioiron incorporation into heme of human marrow cells. DUKES[3] illustrated that dibutyryl cAMP potentiates the effect of erythropoietin (EPO) on heme synthesis of rat marrow cells. BYRON[4] showed that cAMP increases the sensitivity of mouse marrow cells to tritiated thymidine suicide. Among those finding no effect are GRABER et al.[5] and BOTTOMLEY et al.[6].

This paper presents new data on the role of cAMP in erythropoiesis in vitro. It also highlights the importance of GOLDWASSER's[7] advice about independently evaluating each lot of fetal calf serum used in erythropoietic studies, perhaps explaining or reconciling such inconsistencies in the literature.

Materials and methods. Rat marrow cells were cultured by a variation of the technique described by GOLDWASSER and GROSS[8]. Details of the procedure are presented by OLANDER[9]. Standard medium comprised 65% National Collection Type Culture 109 (Micro Biological Associates) containing 100 units penicillin and 100 µg streptomycin per ml, 30% fetal calf serum (Lot R2230U or Lot C2220L, Grand Island Biological) and 5% isologous rat serum.

[1] This work was performed at the Laboratory of Experimental Hematology, Department of Zoology, the University of Nebraska-Lincoln, Lincoln, Nebraska 68508, USA.
[2] D. GORSHEIN and F. H. GARDNER, Blood *36*, 847 (1970).
[3] P. P. DUKES, Blood *38*, 822 (1971).
[4] J. W. BYRON, Nature, Lond. *234*, 39 (1971).
[5] S. E. GRABER, M. CARRILLO and S. B. KRANTZ, Proc. Soc. exp. Biol. Med. *141*, 206 (1972).
[6] S. S. BOTTOMLEY, W. H. WHITCOMB, G. A. SMITHEE and M. Z. MOORE, J. Lab. clin. Med. *77*, 793 (1971).
[7] E. GOLDWASSER, in *Peptide Hormones* (Eds. S. A. BERSON and R. S. YALOW; North-Holland Publishing Company, Amsterdam 1973), p. 1091.
[8] E. GOLDWASSER and M. GROSS, in *Hemic Cells in Vitro* (Ed. P. FARNES; Williams and Wilkins, Baltimore 1969), p. 36.
[9] C. P. OLANDER, Effects of Cyclic Adenosine 3′,5′-Monophosphate on Erythropoiesis in Vitro (Ph. D. dissertation, University of Nebraska-Lincoln, 1974), p. 14.

Rat serum was preincubated for 30 min at 37 °C in the presence of ferric nitrate to make a final ferric ion concentration of 0.2 μg per ml of complete medium. In some experiments the standard medium was modified by substituting amorphous zinc-free insulin for fetal calf serum in a final concentration of 0.5 μg per ml.

Male Long-Evans rats between 180 and 220 g were sacrificed by a sharp blow to the base of the skull. Marrow cells were flushed from aseptically removed tibiofibulae or femora by injecting medium into the end with a 23-gauge needle. Passing the marrow suspension through two 100-mesh stainless steel screens removed bone chips, cell clumps and debris.

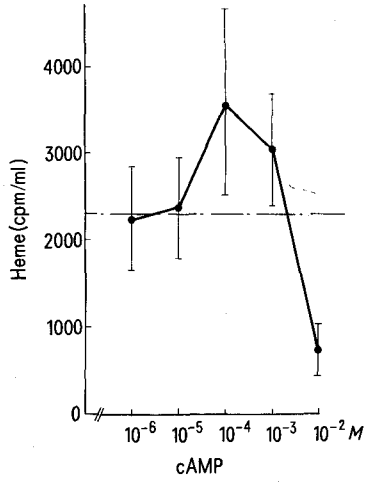

Fig. 1. Effect of cAMP on the 4-hour rate of heme synthesis of rat bone marrow cells. Cells were cultured for 24 h in medium containing fetal calf serum; ^{59}Fe was added 4 h before termination. Counts per min per ml of cyclohexanone are plotted against cAMP concentration. Initial concentration was 30.0×10^6 nucleate cells per ml. Broken line denotes control values. Solid line denotes experimental values. Each point represents the mean of 5 cultures. Bars indicate SD.

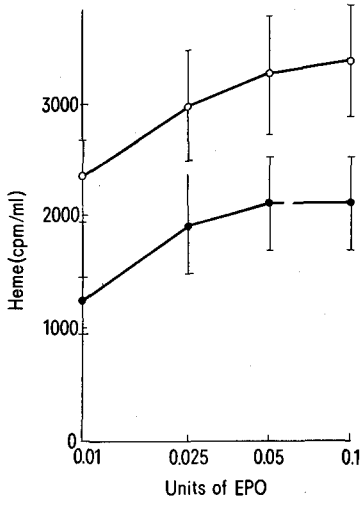

Fig. 2. EPO dose-response curves comparing the effects of 2 lots of fetal calf serum on the 4-hour rate of heme synthesis of rat bone marrow cells. Cells were cultured for 24 h; ^{59}Fe was added 4 h before termination. Counts per min per ml of cyclohexanone are plotted against EPO dosage. Initial concentration was 10×10^6 nucleate cells per ml. ■, Lot R2230U; ●, Lot C2220L. Each point represents the mean of 5 cultures. Bars indicate SD.

1 ml of rat marrow cell suspension ($10–30 \times 10^6$ nucleate cells) was dispensed into each 35-mm diameter glass Carrel flask. Cultures were incubated at 37 °C in a humidified atmosphere of 10% CO_2 and 90% air. 100 μl of EPO or cAMP solution were added to each culture at initiation. Rat serum was labeled by incubating it with $^{59}FeCl_3$ (15.1–16.5 μCi per mg Fe) for 30 min at 37 °C. 50 μl of this transferrin (approximately 1.0 μCi) were added to each culture 4 h before its termination.

Cells were loosened from the surface of each flask by the addition of 1.0 ml of 0.25% trypsin in Ca^{++}-, Mg^{++}-free phosphate-buffered saline solution (PBS)[10]. After 15 min further incubation at 37 °C the cells were harvested by gently flushing each flask with 30 ml PBS in 5-ml aliquots and collected by centrifugation. The cell pellets were resuspended in 5 ml of PBS and re-collected by centrifugation. The cells were lysed by 1.0 ml of DRABKIN's[11] solution. The lysate was acidified with 0.2 ml of 1.0 *N* HCl. To each acidified lysate 2.0 ml of cyclohexanone were added. The radioiron in 1.0 ml of the organic phase was counted by a spectrometer with a 3-in NaI(Tl) crystal. Values of control and experimental samples were compared by Student's *t*-test.

Results and discussion. Figure 1 illustrates a range of cAMP's effects on in vitro heme synthesis of rat marrow cells. At 10^{-2} *M* cAMP inhibited ($p < 0.001$) heme synthesis. The apparent optimum at 10^{-4} *M*, although higher than physiologic levels[12,13], is within the range for enhanced δ-aminolevulinic acid synthetase activity in rabbit marrow cells in vitro[6]. Surprisingly, when this experiment was conducted with another lot of fetal calf serum from the same supplier, cAMP had no effect.

Heeding GOLDWASSER's[7] advice, I compared the lots to see how well they sustained heme synthesis (Figure 2). Control and experimental rates of heme synthesis of the same cell population differed markedly ($p < 0.005$) at all EPO concentrations tested. The deviation from linearity

[10] L. M. RINALDINI, Expl. Cell Res. *16*, 477 (1959).
[11] D. L. DRABKIN and J. H. AUSTIN, J. biol. Chem. *98*, 719 (1932).
[12] J. G. HARDMAN, G. A. ROBISON and E. W. SUTHERLAND, A. Rev. Physiol. *33*, 311 (1971).
[13] G. A. ROBISON, R. W. BUTCHER and E. W. SUTHERLAND, *Cyclic AMP* (Academic Press, New York 1971), p. 29.

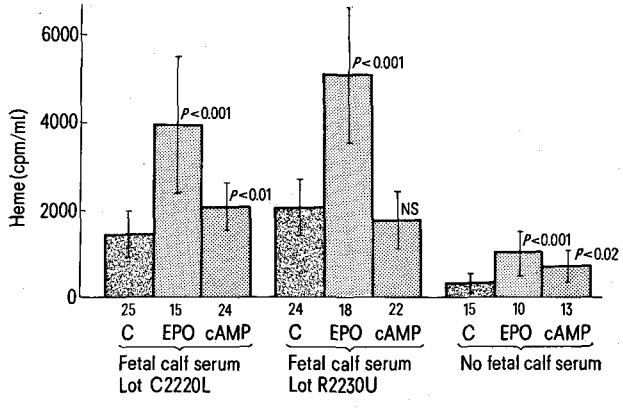

Fig. 3. Effect of fetal calf serum on heme synthesis of rat bone marrow cells. Cells were cultured for 24 h in media with different lots of fetal calf serum or medium with 0.5 μg insulin per ml; ^{59}Fe was added 4 h before termination. Mean ± SD cpm per ml of cyclohexanone are shown for the various culture conditions. Initial concentration was $13.4 \pm 1.99 \times 10^6$ nucleate cells per ml. *n*'s are indicated on the abscissa.

at higher concentrations of EPO does not invalidate the comparison and has been attributed by GOLDWASSER[7] to impurities.

A comparison of the effects of fetal calf serum on cAMP- and EPO-stimulated heme synthesis is presented in Figure 3. Fetal calf serum alters the direction and degree of stimulation. Oddly, Lot C2220L, which gave lower EPO rates, sustained higher cAMP rates; and in Lot R2230U, which produced higher EPO rates, cAMP had almost no effect. Although medium without fetal calf serum sustained control and EPO-induced heme synthesis almost 5 times less efficiently than both other media, in this serum-free medium cAMP stimulated a rate of heme synthesis almost 2 times greater than the control level.

The observation that some lots of fetal calf serum are inhibitory to cAMP stimulation of heme synthesis may explain the contradictions in the literature about cAMP's effect on marrow cells. The active constituent of serum responsible for this effect is unidentified. The effect may be related to phosphodiesterase activity[14] or to calcium ion concentration[15].

Because cAMP affects mitosis[16] and postconfluence inhibition of cell division[17] in nonhematopoietic cell systems, stimulated heme synthesis could be a direct manifestation of these phenomena; therefore, cAMP may be indirectly related to erythropoiesis in vitro. On the other hand, CHANG et al.[18] have shown that EPO exerts its effect from outside the cell; additional experiments must be conducted to demonstrate if cAMP plays a role, as in other hormone systems[12] or otherwise.

Nevertheless, the fact that some component of fetal calf serum modifies cAMP's effect on heme synthesis in rat marrow cells illustrates the need for a chemically defined medium for accurate assessment of the function of cAMP in erythropoiesis in vitro.

Summary. An investigation of the effect of cAMP on heme synthesis of rat bone marrow cells revealed that at 10^{-2} *M* this cyclic nucleotide inhibits heme synthesis and that optimum stimulation occurs at 10^{-4} *M*. Some unidentified constituent of fetal calf serum in the culture medium modifies the direction and degree of cAMP's effect.

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[14] S. E. GRABER, M. CARRILLO and S. B. KRANTZ, J. Lab. clin. Med. *83*, 288 (1974).
[15] A. D. PERRIS and J. F. WHITFIELD, Can. J. Physiol. Pharmac. *49*, 22 (1971).
[16] J. OTTEN, G. S. JOHNSON and I. PASTAN, Biochem. biophys. Res. Commun. *44*, 1192 (1971).
[17] J. R. SHEPPARD, Nature New Biol. *236*, 14 (1972).
[18] S. C.-S. CHANG, D. SIKKEMA and E. GOLDWASSER, Biochem. biophys. Res. Commun. *57*, 399 (1974).
[19] The author thanks Dr. EARL BARNAWELL of the University of Nebraska-Lincoln for donating the zinc-free insulin, a gift to him from Dr. WILLIAM BROMER of Eli Lilly; Dr. EUGENE GOLDWASSER of the University of Chicago for his gift of EPO to the Laboratory of Experimental Hematology; SPARROW BUSH of Austin College for preparing the illustrations; Mr. LARRY PAULK for computer programming; and JANETTE L. FORGY for editorial advice and preparation of the manuscript.

Mycobacterial Adjuvant and its Carrier

Freund's complete adjuvant (FCA) has been widely used in experimental immunology to influence immune responses[1-3]. *Mycobacteria* are the essential constituents of FCA[4-6]. The increase of immune responses induced by an antigen in FCA is attributed to the presence of Mycobacteria.

This paper reports the isolation of a lipid fraction from *Mycobacteria* of a bovine strain, BCG. Inclusion of this fraction, called 'LF', in antigen injection resulted in strong skin reactivity of delayed type hypersensitivity (DTH). LF exhibited an adjuvant effect also on the production of immune antibody to sheep red blood cells (SRBC). The ability of BCG to act as an adjuvant appears to be related to this active component, LF, since a complete disappearance of adjuvant activity of the bacterial body (BB) is observed when LF is extracted. Nevertheless, when BB was used to adsorb ('carry') LF, the resulting 'BB–LF' was found to be a better adjuvant than free LF.

Materials and methods. Extraction. The bacteria used in the present investigation were *Bacillus* of CALMETTE and GUÉRIN (BCG) from the Pasteur Institute, Paris. The organism, grown in Sauton's medium for 17 days at 37 °C, was collected by filtration, washed copiously with distilled water and killed by immersion in ether-ethanol (1:1, v/v) for 3 weeks. Bacterial metabolic products (BMP) were removed from the killed BCG by exhaustive extraction with ether-ethanol (1:1, v/v) and with chloroform. From the solvent washed bacillus (SWB) thus obtained, an adjuvant fraction called lipid fraction (LF) was isolated according to the procedure shown in Table I.

Purification of the extract by ultracentrifugation and column chromatography. Purification of LF was achieved by ultracentrifugation in ether at 40,000 *g* for 10 min and chromatography of the supernatant on a silicic acid column eluted with chloroform-methanol (95:5, v/v). The substance was obtained in pure form as determined by thin-layer chromatography on Silica-gel GF-254.

Analytical methods. Paper chromatography for detection of amino acids, amino sugar and neutral sugars was carried out as described previously[7]. The lipid content was determined by TAKEYA's method[8].

Preparation of BB–LF. Into a 25 ml 'Quick-Fit' flask 200 mg of BB (bacterial body, see Table I) was taken up in 15 ml ethanol. The flask was then connected to a nitrogen source and stirred magnetically after flushing with nitrogen. After 3 h, ethanol was removed by centrifugation and 200 mg of LF in 15 ml ether were added. The mixture was then stirred magnetically under nitrogen atmosphere for 6 h and centrifuged. The residue (BB–LF) was washed 3 times with ether, then dried in vacuo.

[1] K. LANDSTEINER and M. W. CHASE, J. exp. Med. *71*, 237 (1940).
[2] F. PARASKEVAS, K. B. ORR and S. T. LEE, J. Immun. *109*, 1254 (1972).
[3] I. J. HIU and J. L. AMIEL, J. gen. Microbiol. *66*, 239 (1971).
[4] J. FREUND, J. CASAL and E. P. HOSMEN, Proc. Soc. exp. Biol. Med. *37*, 509 (1937).
[5] J. FREUND and K. MCDERMOTT, Proc. Soc. exp. Biol. Med. *68*, 373 (1942).
[6] J. FREUND and M. V. BONANTO, J. Immun. *52*, 231 (1946).
[7] I. J. HIU and J. L. AMIEL, Revue Etud. clin. biol. *16*, 55 (1971).
[8] K. TAKEYA, K. HISATSUNE and Y. INOUE, J. Bact. *85*, 24 (1963).