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.

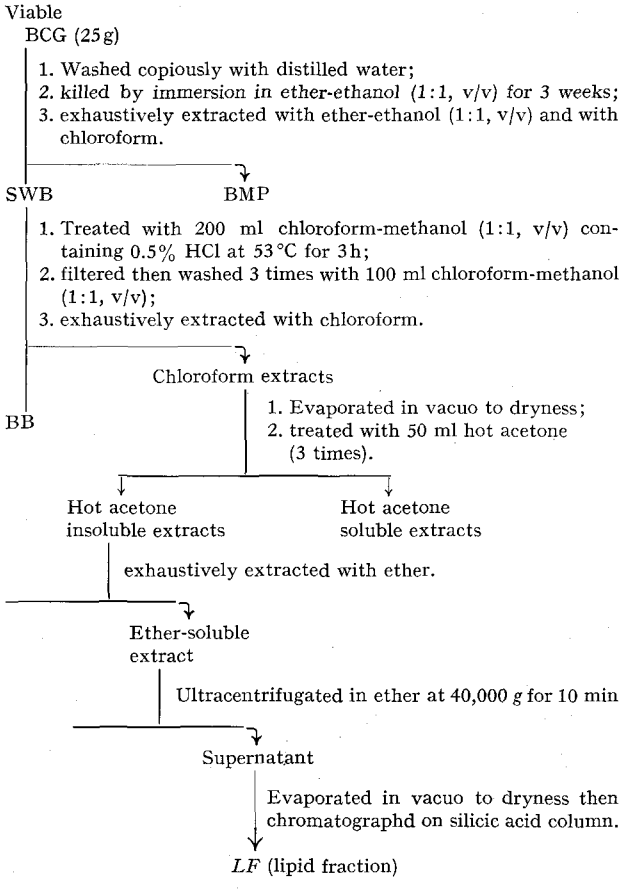
*Analytical methods.* Paper chromatography for detection of amino acids, amino sugar and neutral sugars was carried out as described previously[7]. The lipid content was determined by TAKEYA's method[8].

*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).

*Immunological tests.* Female albino guinea-pigs of 400 to 500 g were sensitized by injection into each hind footpad of 0.1 ml of a water-in-oil emulsion consisting of 1 part of paraffin oil containing LF, 2 parts of Tween 80 and 7 parts of saline containing the protein antigen, crystalline egg white albumin (CEWA). On day 21 after sensitization, the animals were skin tested by intradermal injection of various amounts of CEWA in 0.1 ml saline into the clipped flank of the guinea-pigs; the reactions were read 24 and 48 h later.

Table I. Scheme for isolation of LF, an adjuvant fraction from BCG




SWB, solvent washed bacillus; BMP, bacterial metabolic products; BB, bacterial body (BCG free from LF).

Table II. Mean diameter of induration in guinea-pig skin at sites of injection of various amounts of test antigen CEWA [a] in saline

| No. of animals | Sensitization: Sensitizing antigen (1 mg/animal) | Sensitization: Adjuvant incorporated in antigen (0.5 mg/animal) | Delayed-skin reactions: Test antigen (mg/0.1 ml) 0.1 mg | 3 μg | 0.2 μg |
|---|---|---|---|---|---|
| 6 | CEWA | LF | 17.5 (N) [b] | 5 | 0 |
| 6 | CEWA | BB–LF | 18.5 (N) | 15 | 9.5 |
| 6 | CEWA | BB | 0 | 0 | 0 |
| 6 | CEWA | None | 0 | 0 | 0 |

[a] Crystalline egg white albumin; [b] Mean diameter of induration (mm) at 48 h, (N): central necrosis. LF, lipid fraction extracted from BCG; BB, bacterial body (BCG free from LF).

F1 (DBA/2×C57Bl/6) mice weighing approximately 20 g were injected i.p. with $10^9$ sheep red blood cells (SRBC)/animal; at 15 min intervals various amounts of adjuvants in 0.1 ml of water-in-oil emulsion was given by i.p. injection. After 4 days, the animals were killed and the number of spleen cells forming or releasing antibody to SRBC was determined by the JERNE and NORDIN technique[9].

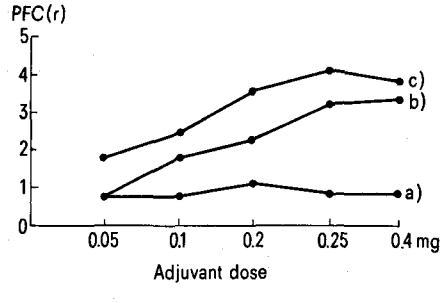
*Results.* LF is a peptidoglycolipid consisting of fatty acid esters of mucopolysaccharide with the following characteristics: $[\alpha]_D^{20}$ ($CHCl_3$) = 14°; melting point 186–189°; solubility, 3.2 g/100 ml $CHCl_3$ at 20 °C; with an approximate molecular weight of 57,000 (determined by analytical ultracentrifugation). 94 to 96% arabinose and galactose (approximate molar ratio 1:2) were found in the mucopolysaccharide moiety. Paper chromatographic analysis revealed the presence of alanine, glutamic acid, *meso*-α-α'-diaminopimelic acid and galactosamine. LF contains 40% of fatty acids mainly composed of mycolic acids (by elementary analysis and by identification with infra-red spectrum of an authentic mycolic acid specimen).

When 0.1 mg of antigen was used in skin tests, strong reactions were observed in all animals except those sensitized with antigen alone or antigen containing BB; with test antigen dose of 3 μg, skin reactions were moderately positive in the group which had received the lipid extract LF as an adjuvant. When the dose of test antigen was reduced to 0.2 μg, no visible skin reaction was observed in animals previously sensitized with LF alone, while animals sensitized with BB–LF together produced positive skin reactions (Table II).

The Figure illustrates the influence of LF and BB–LF onthe average number of plaque-forming cells (PFC) in mice 4 days after immunization with SRBC. When BB was used to adsorb ('carry') LF, the resulting BB–LF was found to be a better adjuvant of the production of immune antibody to SRBC.

*Discussion.* It has been shown that 'Wax D fractions' (i.e. chloroform soluble and hot acetone insoluble fractions) extracted from bacterial metabolic products (BMP) of the non-human BCG strain of *Mycobacteria* failed to increase either cell-mediated or humoral immune responses under conditions in which Wax D fractions from BMP of various human strains of mycobacteria exhibited a marked adjuvant effect. Inactivity of Wax D from BCG strain

[9] N. K. JERNE and A. A. NORDIN, Science *140*, 405 (1963).
[10] R. G. WHITE, L. BERNSTOCK, R. G. S. JOHNS and E. LEDERER, Immunology *7*, 54 (1958).
[11] R. G. WHITE, P. JOLLES, D. SAMOUR and E. LEDERER, Immunology *7*, 158 (1964).




Influence of various amounts of LF, BB and BB–LF on the average number of plaque forming cells (PFC) in mice 4 days after immunization with sheep red blood cells (SRBC). PFC (r): PFC per spleen (tested mice)/PFC per spleen (control). a) BB; b) LF; c) BB–LF.

was attributed to the lack of a peptide portion[10,11]. The results reported here suggest that the peptide-containing and wax D-similar active fraction, LF, from BCG was incorporated into the cellular structure in such a manner that it can only be extracted after the whole cells of BCG have been treated with diluted acid in a suitable organic solvent mixture.

The loss of adjuvant activity of BCG after extraction of LF suggests that LF is the only adjuvant fraction present in the bacillus. Nevertheless, BB–LF together are more active than LF alone. LF is a free molecule, its moderate adjuvanticity may be due in part to its rapid elimination. The BB–LF aggregate appears to be constituted with an adjuvant agent, LF, located on the bacterial body, BB. This latter has no adjuvant activity but it probably acts as an immunological carrier which protects the adjuvant molecule and delivers it to adjuvant sensitive cells.

*Résumé.* Une fraction peptidoglycolipidique peut être extraite à partir du BCG par décapage acide dans un mélange de solvants fortement lipophiles. Cette fraction appelée «LF» peut reproduire l'activité adjuvante du BCG entier dans les réactions immunitaires à médiation cellulaire et humorale. L'activité adjuvante du BCG apparaît comme liée à ce composant actif, LF, car après son extraction, le résidu bacillaire (BB) est dépourvu d'activité adjuvante. Néanmoins lorsque BB est utilisé pour adsorber («véhiculer») LF, le complexe BB–LF s'est révélé le meilleur adjuvant.

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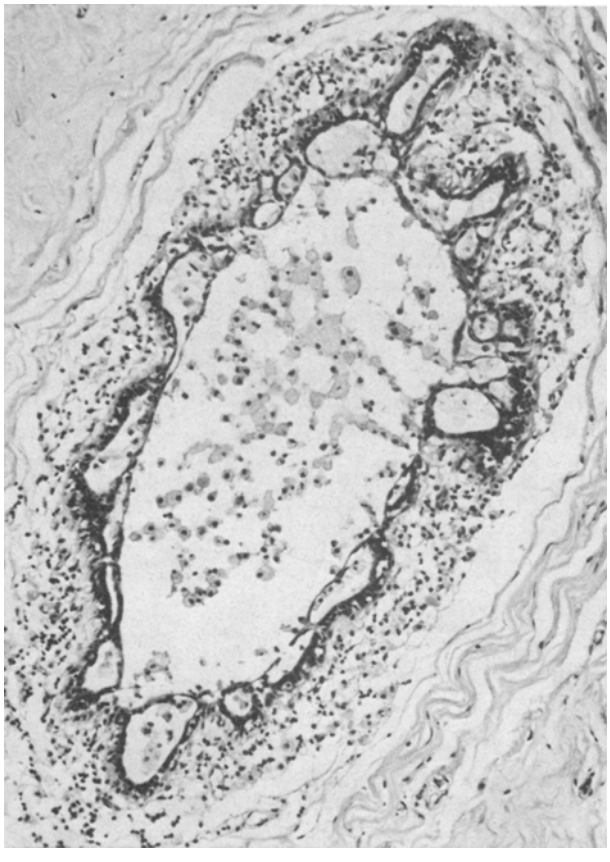
## Colostrum-Cell and Leucocyte Associations in Man

The origin of colostrum cells has been controversial since their cellular nature was first postulated in 1847[1]. Such cells possess finely vacuolar cytoplasm and sharp cell margins (Figure), and are widely found in undrained mammary secretions (colostrum)[2]. Numerous studies of the cells[3–6] in man, domestic ungulates, and laboratory rodents have yielded conflicting views as to whether the colostrum cells are derived from mammary epithelium or from infiltrating leucocytes.



Colostrum cells in duct which is surrounded by small-round-cell infiltrate. Haemalum and eosin. ×150.

Benign mammary dysplasia[7], a common disease of the breast in women, often features accumulation of undrained mammary secretion and of colostrum cells. In view of its variable morphology and the large number of lesions available for study, the condition is well suited to investigation of the relationship between colostrum cells and inflammatory cells that may be found in the lesions. The three inflammatory cell-types found in periductal tissue considered here are: small round cells[8] with inconspicuous cytoplasm, cells with ochre cytoplasmic pigment (ochrocytes)[9], and cells with prominent foamy cytoplasm lacking pigment (foam cells)[10].

An unselected consecutive 4-year series of 212 benign mammary dysplasias from women over the age of 40 years received in the University Department of Histopathology at the Bristol Royal Infirmary was studied. The tissues were fixed in buffered 10% formalin and embedded in paraffin wax. Haemalum and eosin-stained sections of lesions from which at least 2 paraffin blocks had been prepared were systematically examined in a rectilinear pattern. Each lesion was assessed for the presence or absence of colostrum cells in the ductal secretions, and independently, for the presence or absence of ochrocytes, and small round cells and foam cells in a band exceeding 0.125 mm radial width in the periductal connective tissue.

[1] B. Reinhardt, Virchows Arch. path. Anat. Physiol. *1*, 20 (1847).
[2] A. T. Cowie and J. S. Tindal, *The Physiology of Lactation* (Arnold, London 1971).
[3] C. Grégoire, Archs Anat. Histol. Embryol. *13*, 67 (1931).
[4] S. Engel, J. Anat. *87*, 362 (1953).
[5] G. Mayer and M. Klein in *Milk: the Mammary Gland and its Secretion* (Eds. S. K. Kon and A. T. Cowie; Academic Press, New York and London 1961), vol. 1, p. 47.
[6] K. H. Hollmann in *Lactation: a Comprehensive Treatise* (Eds. B. L. Larson and V. R. Smith; Academic Press, New York and London 1974), vol. 1, p. 3.
[7] R. W. Scarff and H. Torloni, *Histological typing of breast tumours* (W. H. O., Geneva 1968).
[8] J. D. Davies, J. Path. *112*, 153 (1974).
[9] J. D. Davies, Arch. Path. *97*, 369 (1974).
[10] J. D. Davies, J. Path., in press (1975).