PRO EXPERIMENTIS

Granulocyte Colony Formation in vitro: Enhancement by Human Placental (Umbilical Cord) Serum[1]

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Summary. Addition of human placental umbilical cord serum to bone marrow cultures reproducibly increased the number of granulocyte colonies in vitro. This stimulatory effect was significantly greater than that of fetal calf serum which was seen in cultures of human bone marrow under the conditions described.

Culture of bone marrow colony-forming units (CFU-c) has become a widely used method for in vitro studies of haemopoiesis. Proliferation and differentiation of haemopoietic progenitor cells in semi-solid media (agar, or methylcellulose) result in colonies which can be enumerated and examined morphologically. The culture system consists of: 1. a definite number of nucleated cells from bone marrow or peripheral blood; 2. a well-defined synthetic medium including 5 to 30% of serum; 3. 0.3% agar, or 0.8% methylcellulose, for partial immobilization of cells; and 4. a source of colony stimulating activity.

The quality of added serum, as well as relative activities of stimulatory and inhibitory substances present in the system, are crucial determinants. Most authors use fetal calf serum, although some other sera and combinations were described in the literature, e.g. 10% horse serum[3], 10% fetal calf serum and 5% horse serum[4], mixture of equal parts of fetal calf serum, horse serum and trypticase soya broth[5], or 15% human AB serum[6].

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Changes after replacement of fetal calf serum (FCS) by human umbilical cord serum (HUCS) in cultures of human CFU-c

Source of CFU-c	Under layer	Upper layer	No. of colonies/plate[a]	Observed change (%)
Rib	FCS	FCS	13	—
	FCS	$HUCS_A$	19	+ 46
Rib	FCS	FCS	17	—
	FCS	$HUCS_A$	19	+ 11.7
Iliac crest	FCS	FCS	65	—
	$HUCS_A$	FCS	82	+ 26.2
Rib	FCS	FCS	45	—
	$HUCS_A$	FCS	72	+ 60
Rib	FCS	FCS	31	—
	$HUCS_A$	FCS	43	+ 38.8
Rib	FCS	FCS	70	—
	FCS	$HUCS_A$	90	+ 28.6
Iliac crest	FCS	FCS	10	—
	$HUCS_A$	FCS	21	+ 110
Peripheral blood	FCS	FCS	15	—
	$HUCS_A$	FCS	20	+ 33.3
Rib	FCS	FCS	81	—
	$HUCS_B$	FCS	110	+ 35.8
Rib	FCS	FCS	50	—
	$HUCS_B$	FCS	61	+ 22
Iliac crest	FCS	FCS	20	—
	FCS	$HUCS_B$	23	+ 15
Iliac crest	FCS	FCS	65	—
	$HUCS_B$	FCS	48	— 26
	FCS	$HUCS_C$	135	+ 108
	$HUCS_B$	$HUCS_C$	169	+ 160
Iliac crest	FCS	FCS	23	—
	$HUCS_B$	FCS	58	+ 152
	$HUCS_C$	FCS	41	+ 78
	FCS	$HUCS_C$	90	+ 291
	$HUCS_B$	$HUCS_C$	102	+ 344
	$HUCS_C$	$HUSC_C$	125	+ 443
Iliac crest	FCS	FCS	98	—
	FCS	$HUCS_D$	107	+ 6.2

[a] Mean value (3–5 plates).

The selection of optimal serum is an important part of the routine in a cell-cultivating laboratory. Quality control of sera, unfortunately, depends mostly on empirical criteria, since neither active component(s), nor the mode of its (their) action are at present known.

Placenta may be a source of substances regulating fetal haemopoiesis. Furthermore, cord blood contains a substantially higher number of CFU-c when compared to adult peripheral blood[7]. Placental blood (20–40 ml) was therefore collected, under sterile conditions, immediately after scission of the umbilical cord during delivery. Serum was obtained following spontaneous coagulation at room temperature and centrifugation at $1,000 \times g$ for 15 min. Small aliquots were frozen at $-25\,^{\circ}C$ and thawed immediately before use. The control fetal calf serum (GIBCO, batch No. 245 501) was selected from 6 commercially available sera. Its quality was independently rated as satisfactory by two other laboratories[8,9].

CFU-c were cultivated essentially according to the method described by ROBINSON and PIKE[10]. Progenitor cells were suspended in 0.3% agar and McCoy 5A modified medium (upper layer). Under layers contained leukocytes isolated from peripheral blood and immobilized in 0.5% agar. To culture medium, 20% of either fetal calf serum (control plates) or human umbilical cord serum (denoted A, B, C or D, i.e. from 4 different placentas) was added. Results are summarized in the Table.

The results suggest that human umbilical cord serum significantly enhanced granulocyte colony formation. The maximum increase of the colony number was obtained when human cord serum was added to both layers of the incubating system. We observed, furthermore, that the number of cells per colony was invariably higher in cultures with human cord serum.

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A Method for Sequencing Peptides: a Co-operation of Diphenyl Phosphorazidate and 2-Mercapto- or 2-Hydroxypyridine for N-Acyldiketopiperazine Formation

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Summary. A new method for sequencing peptides is proposed. As a model experiment for this, Bz-Gly-Pro-OH and Bz-Gly-Sar-OH were conveniently converted to their diketopiperazine derivatives by a co-operation of diphenyl phosphorazidate and 2-mercapto- or 2-hydroxypyridine.

In our previous communications we drew attention to the use of diphenyl phosphorazidate (DPPA)[1] as a new reagent for the racemization-free peptide synthesis[2,3], a modified Curtius reaction[2,4,5], the ester formation reaction of some α-functionalized acetic acids[5], and the direct preparation of thiol esters from carboxylic acids and thiols[6]. During the application of DPPA to peptide sequencing, we found a remarkable effect of combination of DPPA and 2-mercapto- or 2-hydroxypyridine, known as bifunctional catalysts, for the formation of N-acyldiketopiperazines.

Exploitation of the method of peptide sequencing has been attempted[7,8] by thermal degradation of peptides to diketopiperazines. The method is attractive in that both degradation and identification could be carried out in a minute amount of peptides by g.c./m.s. methods. However, the degradative conditions are too severe to obtain diketopiperazines in good yield, and partially degradated peptides could not be used again for sequence determination.

If a diketopiperazine moiety, however, could be formed at the C-terminal residue of peptide, the mild hydrolysis of the resultant N-acyldiketopiperazine would afford diketopiperazine and the peptide, the latter would undergo the stepwise cleavage as shown in Scheme I.

Combination of this method and the selective tritium-labelling method at the C-terminal residue[9] might enable us to know which amino acid residue in the diketopiperazine is present at the C-terminal of the original peptide, and might provide a good prospect for stepwise determination of amino acid sequences.

In preparation for the work, we investigated the diketopiperazine formation from Bz-Gly-Pro-OH and Bz-Gly-Sar-OH[10]. Bz-Gly-L-Pro-OH, m.p. 106–111°, $[\alpha]_D^{20}$ −77° (c 1.2, DMF), was prepared in 60% yield by allowing an equimolecular mixture of Bz-Gly-OH and TEA in tetrahydrofuran to react with DPPA at room temperature for 2 h, followed by the addition of H-L-Pro-OH in aqueous sodium hydroxide. It is interesting that DPPA could be used for the coupling of N-protected peptides with free amino acids or peptides as well as amino acid or peptide esters. This resembles the mixed carbonic anhydride method, but the repetition of the above experiment using ethyl chlorocarbonate afforded Bz-Gly-L-Pro-OH in only 8% yield, proving the superiority of the DPPA method, at least in this case. Bz-Gly-Sar-OH, m.p. 164–166°, was also prepared from Bz-Gly-OH and free H-Sar-OH in a manner similar to above.

Intramolecular cyclization of Bz-Gly-L-Pro-OH by DPPA in the presence of a large excess of TEA in DMF was attempted to yield a considerable mixture of products detected on a thin-layer plate. Diethyl phosphorocyanidate (DEPC), a new reagent for the racemization-free peptide synthesis[11], was also proved to be fruitless. Replacement of DPPA with ethyl chlorocarbonate under the same reaction conditions afforded Bz-Gly-Pro[12] in 45% yield together with Bz-Gly-Pro-OEt in 7% yield. In an attempt to improve the yield of Bz-Gly-Pro, several cyclization reagents were investigated: phenyl chlorocarbonate (16%)[13], diphenyl phosphorochloridate (50%), triphenylphosphine and carbon tetrabromide[14] (38%). The most satisfactory results were, however, obtained by the use of an equimolecular mixture of DPPA and 2-mercaptopyridine: To a stirred mixture of Bz-Gly-L-Pro-OH (0.56 g, 2 m*M*), DPPA (0.56 g, 2 m*M*), and 2-mercaptopyridine (0.22 g, 2 m*M*) in DMF (3 ml) was added TEA (1 ml) in DMF (1 ml), and the mixture was stirred at room temperature overnight. After dilution with benzene followed by aqueous acid and alkali work-