

EFFECT OF LOW MOLECULAR WEIGHT HUMAN SERUM FACTORS AND HUMAN SOMATOMEDIN PEPTIDES ON HUMAN LYMPHOCYTE CULTURES

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SUMMARY

Using [³H]thymidine incorporation into DNA of human lymphocytes in culture, we have shown that human serum contains an ultrafiltrable factor(s) that sensitizes the activation of lymphocytes by phytohaemagglutinin. This (these) factor(s) can replace either human serum or foetal calf serum in the culture. In addition, it permits the expression, and therefore the assay, of human somatomedin A peptides. In somatomedin assays we have shown that the assay using lymphocytes is a much more sensitive test than the assay using sulphation of chick embryo cartilage.

INTRODUCTION

Somatomedins are peptides acting as cell growth factors that are growth-hormone-dependent. Their activities were measured by different techniques such as ³⁵SO₄ incorporation in cartilage of different animal species (Salmon & Daughaday, 1957; Salmon & Du Vall, 1970; Hall, 1970; Van den Brande & du Caju, 1974) or in isolated chondrocytes (Corvol *et al.* 1978); by [³H]thymidine incorporation in different cells (Garland, Lottes, Kozak & Daughaday, 1972; Daughaday & Reeder, 1966; Jennings, Buchanan, Freeman & Garland, 1980; Rechler *et al.* 1977) by mitogenic activity (Weidman & Bala, 1980).

Recently, Thieriot-Prevost & Schimpff (1980), showed that somatomedin activity could be measured by [³H]thymidine incorporation in human lymphocytes activated by phytohaemagglutinin (PHA). Heulin *et al.* (1981) showed the presence of an ultrafiltrable factor in human serum, with a molecular weight between 300 and 500, which stimulated somatomedin activity measured by chick embryo cartilage sulphate uptake.

In this work, we confirm that a human serum ultrafiltrable factor could stimulate

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somatomedin activity as measured by [^3H]thymidine incorporation in activated human lymphocytes.

MATERIALS AND METHODS

^{35}S incorporation into chick embryo pelvic cartilage was measured by a modified version (Schimpff & Donnadieu, 1973) of Hall's (1970) method.

[^3H]thymidine ([^3H]dThd) incorporation in PHA-activated human lymphocytes was measured by Schimpff's method (Thieriot-Prevost & Schimpff, 1980). In the present experiments, incubations were carried out in 200 μl containing 50 $\mu\text{g}/\text{ml}$ gentamycin: 100 μl of lymphocyte preparation (200 000 lymphocytes) in Eurobio medium (medium 199), 50 μl PHA at 40 $\mu\text{g}/\text{ml}$; the substances to be assayed were added in the last 50 μl at a final concentration in the total incubation volume as specified for each experiment. When necessary, the samples were first brought to suitable concentrations by dilution in incubation medium. The lymphocytes were incubated in microplates for 72 h at 37 °C and 0.25 μCi of [^3H]dThd (sp. act. 26 Ci/mmol) were added for the last 18 h. Each dilution was tested in triplicate.

Evaluation of cell viability was by the blue trypan exclusion test (Philipps, 1973). Viable cells do not take up the dye, but unviable cells do.

Statistical evaluation of assays: the results were given either as the ratio of the assays to the controls (lymphocytes activated by PHA), or as c.p.m. incorporated. Statistical evaluation of the assays was done by Student's *t*-test.

Preparation of ultrafiltrate 1000 (UF 1000): this was prepared from a pool of normal human serum. Pooled serum (30 ml) was ultrafiltered in an Amicon 52 cell, through Millipore PSAC (NMWL 1000) membrane. The ultrafiltration was stopped when 10 ml of 'retentate' remained in the cell. UF 1000 was used without further preparation; the retentate was washed and ultrafiltered several times with 0.15 M-NaCl and could be used as a somatomedin standard solution.

Somatomedin: the somatomedins used in the assays were either a serum retentate, or partially purified somatomedins of high (Curling, 1980) and low (Straczek, 1980) molecular weight, or purified somatomedin C (Svoboda *et al.* 1980).

RESULTS

First we verified that during 72 h of incubation the lymphocytes remain viable (three separate assays were done). After 3 days of culture 18% of lymphocytes cultivated in medium alone are non-viable, but when 5% foetal calf serum (FCS) or 5% UF 1000 are added to the medium only 10% of lymphocytes are non-viable. The presence of UF 1000 is as active as FCS in preventing cell damage.

Before studying the effect of UF 1000 in the presence of somatomedin, we checked that UF 1000 does not influence the activation process of lymphocytes by PHA. Thus the [^3H]dThd uptake in PHA-activated or non-activated lymphocytes was measured, the lymphocytes being cultivated without either somatomedin, FCS or human serum. Fig. 1 shows the results obtained: increasing amounts of UF 1000 significantly improve the [^3H]dThd incorporation in PHA-activated and non-activated lymphocytes. However, even though the level of [^3H]dThd uptake in PHA-activated lymphocytes is 60 to 80 times higher than in non-activated lymphocytes, the ratio (c.p.m. in activated lymphocytes over c.p.m. in non-activated lymphocytes) remains nearly constant.

Fig. 2A shows, however, that the [^3H]dThd uptake in PHA-activated lymphocytes in the presence of increasing amounts of UF 1000 alone is much lower than that in the presence of increasing amounts of normal human serum (containing, by convention,

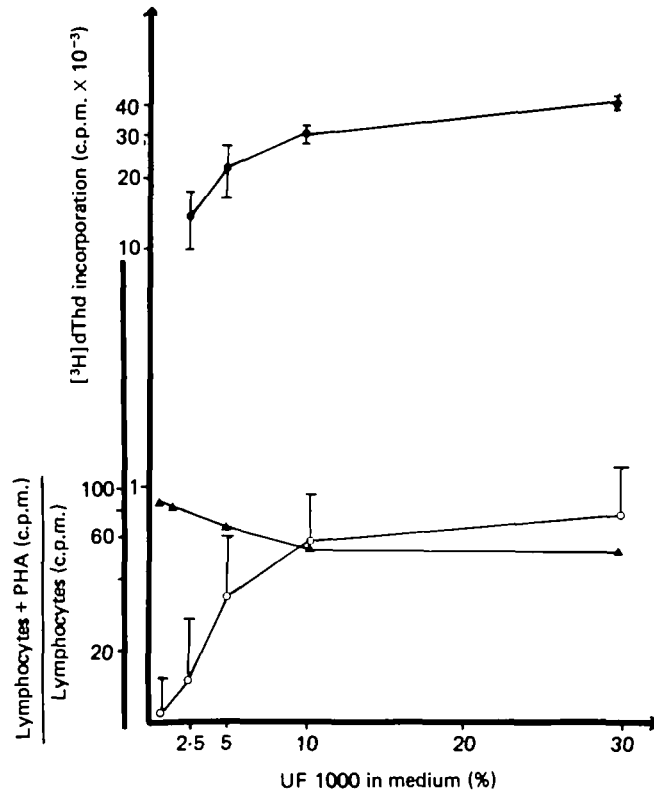


Fig. 1. Effect of UF 1000 on $[^3\text{H}]\text{dThd}$ incorporation in non-activated lymphocytes ($\text{O}-\text{O}$); in PHA-activated lymphocytes ($\bullet-\bullet$); ratio of PHA-activated lymphocytes *versus* non-activated lymphocytes ($\blacktriangle-\blacktriangle$).

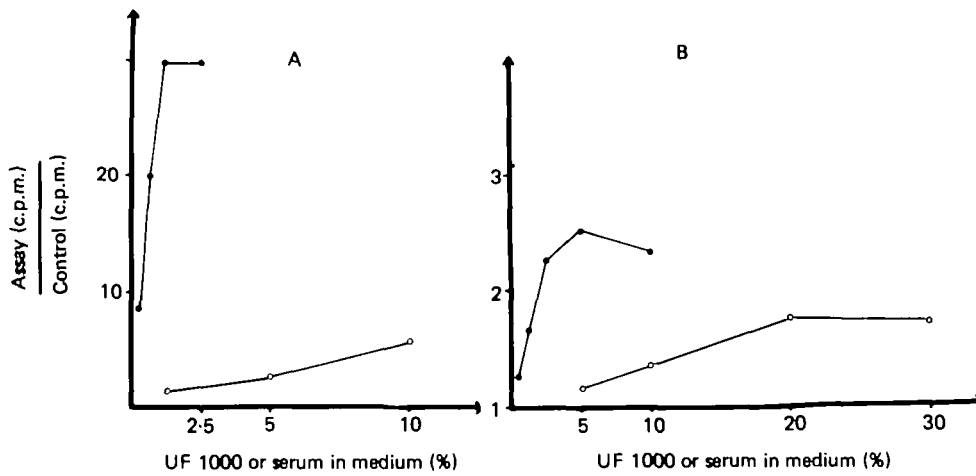


Fig. 2. Effect of UF 1000 ($\text{O}-\text{O}$) and serum ($\bullet-\bullet$) on $[^3\text{H}]\text{dThd}$ incorporation in PHA-activated lymphocytes (A) and on ^{35}S incorporation in chick embryo pelvic cartilage (B).

a somatomedin activity of 1 unit/ml). This fact has already been noticed in measuring $^{35}\text{SO}_4$ uptake by chick embryo pelvic cartilage (Fig. 2B).

Fig. 3 represents the results of the experiment proving that the greatest activity of all is due not only to the presence of peptidic growth factors but also to one (or several) ultrafiltrable factor ($M_r < 1000$). Normal human serum was ultrafiltered through membrane at a cut-off of 1000 M_r and the retentate was washed several times. The

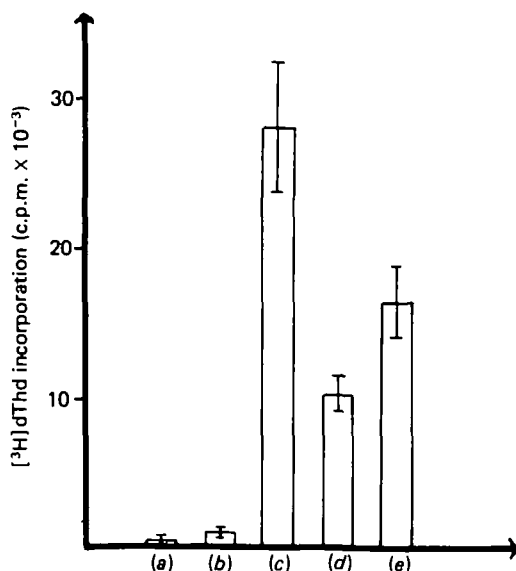


Fig. 3. Effect on $[^3\text{H}]\text{dThd}$ incorporation in PHA-activated lymphocytes of: (a) control; (b) 5% UF 1000 alone; (c) 5% whole serum; (d) 5% washed retentate; (e) 5% reconstituted serum (1/3 washed retentate + 2/3 UF 1000). (c) and (d) are significantly different $P < 0.05$; (d) and (e) are significantly different $P < 0.05$; (a) and (b) are not significantly different.

activities of the whole serum, the washed retentate and the reconstituted serum were assayed: the UF 1000 alone was not very active, the retentate had lost about 70% of the initial activity of the serum and restored serum had recovered 60% of this activity.

During somatomedin purification, it is often very difficult to estimate the activity of purified fractions. It is this observation that led us to search in the serum for a cofactor essential for the measurement of somatomedin activity. Fig. 4 shows that $[^3\text{H}]\text{dThd}$ incorporation in PHA-activated lymphocytes cannot be used for the measurement of somatomedin activity of purified fractions when the assay is done in incubation medium alone. Indeed, in this case whichever purified somatomedin form was used (low or high molecular weight) and whatever concentration was used, the results were always lower than the controls. On the contrary, when UF 1000 was added at final concentration of 10% to the incubation medium, one observed that, at high concentrations, both somatomedin forms were inhibited, but at high dilutions (from 10^{-4} to 10^{-6}) the $[^3\text{H}]\text{dThd}$ uptake became proportional to the somatomedin levels.

The lymphocytes were usually incubated in medium containing human serum or

FCS, which provided components essential for cellular life. We compared the reciprocal effects of UF 1000, FCS and human serum. In the first experiment the activity of the retentate on $[^3\text{H}]$ dThd incorporation in PHA-activated lymphocytes was determined in the presence of either 10% UF 1000 alone, 10% FCS alone, or

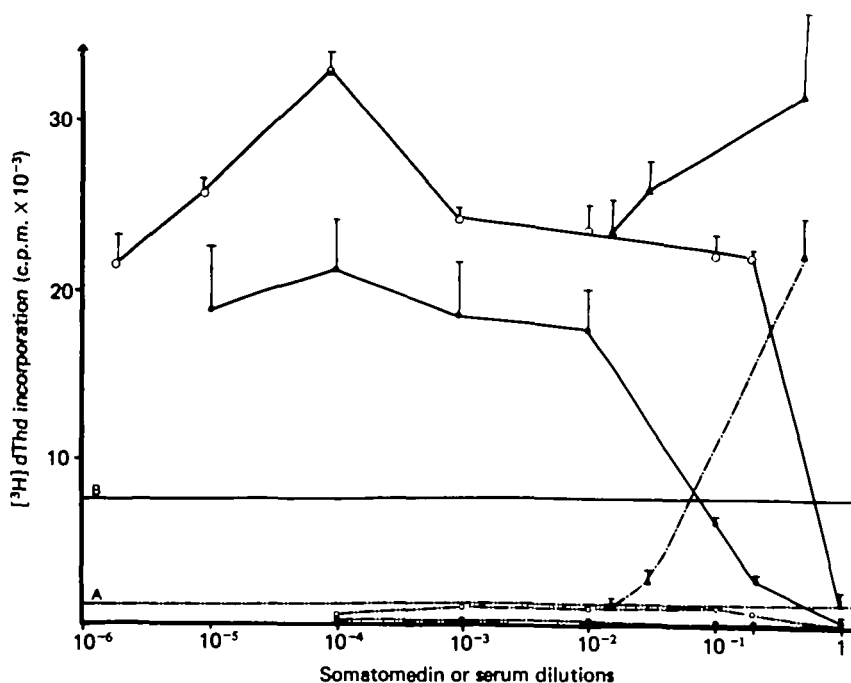


Fig. 4. $[^3\text{H}]$ dThd incorporation in PHA-activated lymphocytes with and without 10% UF 1000. B and A represent the respective controls. Addition of a normal human serum: (\blacktriangle - \blacktriangle) with and (\blacktriangle - \cdots - \blacktriangle) without 10% UF 1000. Addition of a somatomedin of high molecular weight; the first dilution assayed (= 1) contains 0.580 mg protein per ml (1.5 units somatomedin/mg protein in the sulphation test): (\circ - \circ) with and (\circ - \cdots - \circ) without 10% UF 1000. Addition of a somatomedin of low molecular weight; the first dilution assayed (= 1) contains 0.891 mg protein per ml (2.6 units somatomedin/mg protein in the sulphation test): (\bullet - \bullet) with and (\bullet - \cdots - \bullet) without 10% UF 1000. All diluted samples were added to the incubation medium at 5% of final concentration.

10% UF 1000 and 10% FCS added simultaneously. Fig. 5 shows that the retentate is seven times more active with UF 1000 than with FCS. In addition, the latter is an inhibitor, since, when it is added with UF 1000, it decreases the action of UF 1000.

In the second assay the activity of highly purified somatomedin C was assayed in the presence of either UF 1000 or human serum. The activity was as high with UF 1000 as with human serum at the same concentration, and when the UF 1000 level was increased the activity still rose. Somatomedin alone was not very active (Fig. 6).

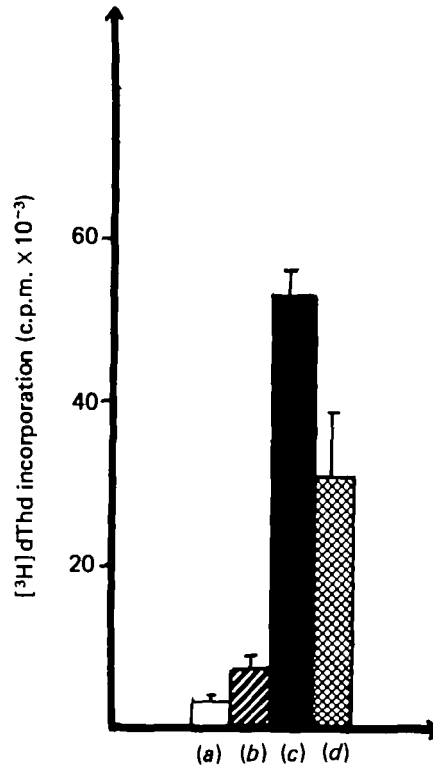


Fig. 5. Effect of serum retentate on [³H]dThd incorporation in PHA-activated lymphocytes in the presence of UF 1000 or (and) FCS: (a) 5% retentate; (b) 5% retentate + 10% FCS; (c) 5% retentate + 10% UF 1000; (d) 5% retentate + 10% FCS + 10% UF 1000. (a) and (b) are significantly different, $P < 0.05$. (b) and (c) are significantly different, $P < 0.05$. (c) and (d) are significantly different, $P < 0.05$.

DISCUSSION

Heulin *et al.* (1981) demonstrated the presence in human serum of an ultrafiltrable factor increasing somatomedin activity as measured by ³⁵SO₄ uptake by pelvic cartilage of chick embryos.

Using the [³H]dThd incorporation in PHA-activated human lymphocytes as a test for the evaluation of somatomedin activity (Thieriot-Prevost & Schimpff, 1980), we have shown that human serum also contains an ultrafiltrable factor that enhances this biological activity. This fact has been verified with partially purified somatomedins of low (M_r 6000–7000) and high ($M_r \geq 60000$) molecular weight, and with highly purified somatomedin C of low molecular weight.

We have verified that the ultrafiltrate (UF 1000) is not active by itself in the PHA activation process in lymphocytes. Effectively, if [³H]dThd incorporation is higher in the presence of UF 1000, the ratio of [³H]dThd incorporation in activated lymphocytes to [³H]dThd incorporation in non-activated lymphocytes is constant. UF 1000 only sensitizes the activation but does not interfere with the activation process. The slight, but significant, increase in [³H]dThd incorporation in non-activated lympho-

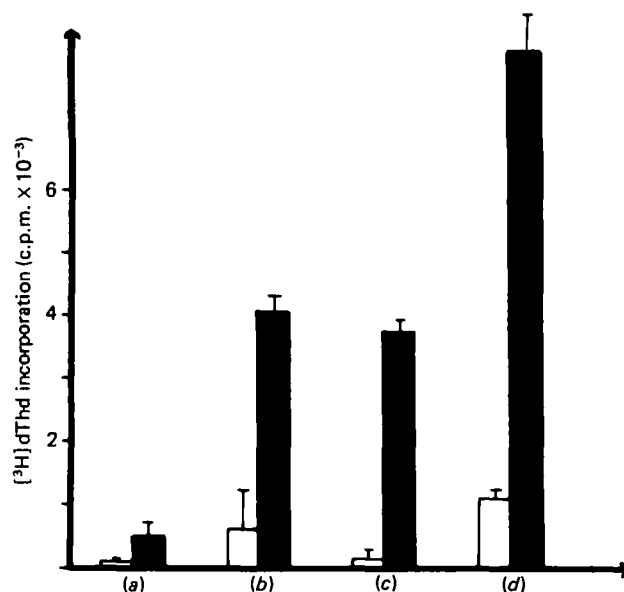


Fig. 6. Effect of purified somatomedin C (SMC) on [³H]dThd incorporation in PHA-activated lymphocytes (c.p.m. of assays minus c.p.m. of controls): (a) SMC; (b) SMC + 2.5% human normal serum; (c) SMC + 2.5% UF 1000; (d) SMC + 5% UF 1000. (□) 0.1 ng SMC; (■) 1 ng SMC.

cytes in the presence of UF 1000 can be interpreted as the action of UF 1000 on a pre-existing mitogenic factor in the cells that is present at a very low level.

We have not shown whether the two factors are identical or not, since they have not been purified. Furthermore, Schimpff, Thieriot-Prevost & Job (1981) think that the factors with thymidine activity in human lymphocytes are probably different from those with sulphation factor activity.

We can only say that the same purified or partially purified fractions have simultaneous thymidine and sulphation activity and that the two activities are stimulated by an ultrafiltrable factor.

However, the thymidine assay is much more sensitive than the sulphation assay. The same sample must be assayed at 10^{-4} to 10^{-6} dilutions in the first assay, and 3×10^{-1} to 2.5×10^{-2} in the second assay. It is necessary to consider that in the second assay, carried out according to Schimpff's (1973) method, the pre-incubation and incubation sequences were perhaps not optimized (Jennings *et al.* 1980).

The measurement of serum somatomedin activity by bioassay is relatively easy, but difficulties occur when purified fractions are tested and several authors have proposed the addition of animal or human serum to the incubation medium (Froesch *et al.* 1976; Van Buul-Offers & Van den Brande, 1979; Rechler *et al.* 1977; Stiles *et al.* 1979).

We have shown that UF 1000 at the same concentration as whole serum, allows the assay to be made: in our assays, purified somatomedin peptides added alone to the medium gave lower values than the control PHA-activated lymphocytes; in the presence of UF 1000, the activity could be expressed.

Use of UF 1000 instead of whole serum ensures better control of the composition of the medium and avoids interference in the assay from additional serum factors and in UF 1000 there were as many living cells as in FCS.

In our experiments we have shown that lymphocytes could be kept alive only in the presence of UF 1000 for the duration of the test and without FCS. This is important since FCS contains some inhibitors of human cells. De Luca (De Luca, Habeeb & Tritsch, 1966) has already shown that some fractions obtained from FCS are toxic to cultured human cells.

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