AN INTERACTION BETWEEN BLEOMYCIN AND HUMAN SERUM

THE REACTION OF EHRLICH CELLS AND *TRITICUM* PLANT ROOTS TO SOME ANTINEOPLASTIC DRUGS ALONE AND IN COMBINATION WITH HUMAN SERUM

JØRGEN HILDEN, FOLKE RØNNIKE and HENNING SCHOU

Institute of Human Genetics, University of Copenhagen; Department of Clinical Physiology, of Clinical Chemistry and of Radiotherapy, The Finsen Institute; The Fibiger Laboratory; and The Deaconess Hospital. Copenhagen, Denmark

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In two growth tests, one employing EHRLICH ascites tumor cells in suspension culture, the other employing intact roots of wheat (*Triticum sativum* LAM.), the growth inhibiting action of bleomycin (BLM) was shown to be intensified by human serum. Practical consequences of the finding are suggested. No such serum-drug interaction has been demonstrated with other anti-tumor compounds, including methotrexate (MTX) and threosulphan (TRSF). Isolation of the active factor(s) in serum has not yet been attempted.

Bleomycin (BLM), an antitumor and antibacterial agent, which contains a number of separable, but similar glycopeptides, was isolated by UMEZAWA *et al.* in 1966 from cultures of *Streptomyces verticillis*[1]. The antitumor action of BLM is very complex and interaction with many compounds is known to take place[2].

The present paper demonstrates an intensifying effect of native, *i.e.* untreated, human serum on the cell growth inhibiting effect of BLM. This interaction of BLM with serum is specific, in the sense that such interaction is not necessarily found with other antitumor compounds; this is evidenced by our negative results with methotrexate (MTX), *i.e.* the folic acid antagonist 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid, and treosulfan (TRSF), *i.e.* dihydroxybusulphan=L-threitol 1,4-bismethanesulphonate.

Materials and Methods

1. The EHRLICH ascites tumor cells were cultured in suspension culture[3,4]. Daily changes of medium served to keep the cells in active proliferation. The standard synthetic culture medium was a modified EAGLE's medium, MEM (minimal essential medium), supplemented with 10 % v/v 56°C 1/2 h denatured fetal bovine serum. Control tubes contained this standard medium. The human serum preparations were diluted to 10 % v/v with the standard medium, which was also used for diluting the drugs to specified concentrations. EHRLICH cells, 50~90 $\times 10^4$, suspended in 1 ml of the standard medium, was added to 1 ml of the human serum (final amount, 5 % v/v) and/or drug preparation, or—in the case of control tubes—to one ml of the medium. The cells were grown for 24 hours at 37°C. (The small inoculum was used in those experiments that did not involve human serum so as to obtain roughly equal amounts of cells in those tubes that will be represented by columns C (left) and S in each of Figs. 1, 4 and 6). Prior to counting in Bürker-Türck counting chambers trypanblue was added in order to distinguish between live and dead cells. The dead cells amounted to approximately 10 % of all cells. The counted number of cells for each tube ranged between 0 and 200. The total number of cells in each tube (2 ml suspension) is obtained by multiplying by 10^4. Each serum and/or

drug admixture was run in at least 10 tubes. Each value reported is therefore the average of 10 or more counts. The amount of intertube variability is expressed as the standard error of the mean, SEM, in our figures. The tubes were arranged in a randomized fashion in the incubator and were counted before the code was broken.[5]

2. The plant method. The principle of the quantitative method is the simple one of measuring the root length increments in *Triticum sativum* LAM., variety "Starke"[6,7] (from Weibull's, Landskrona, Sweden). Only the central, positively geotropic trunks of the seminal roots of the intact plants are measured. The human serum preparations of the plant experiments were diluted to 1 per cent v/v with a phosphate buffer 1/150 M at pH 6.0 with 5 mM $Ca(NO_3)_2$; this buffer was also used for the drug dilutions. The serum and drug dilutions were prepared immediately prior to use. Growth in salt solution without serum served as control. Each value reported is the average (with standard error) of five growth cups; the value for each individual cup is itself the average of ten plants. The cups were coded and randomized as above.

3. The preparation of the serum and drug samples. The blood samples were usually taken in the morning. The samples were left for a few hours and then centrifuged; the serum was removed with a pipette. On many occasions the blood serum was kept undiluted for some days at −25°C. Such storage has no influence on the obtained values[6,8]. All the sera tested were pooled sera from at least 10 healthy donors.

The bleomycin dilutions were prepared from "Bleomycin Lundbeck ad injectionem" Bleomycin sulf. respond. Bleomycin (stand. NIHJ) D. sp. nr. 2883 N. sp. nr. 5577 IS. sp. nr. 1304. H. Lundbeck & Co A/S, Copenhagen. Methotrexate was prepared from "Methotrexate U.S.P. 81.5 %" Lederle Lab. Div., Am. Cyanamid Corp., Pearl River, N.Y. 10965. The dilutions of threosulphan were prepared from a preparation by Leo Pharmaceutical Products, Copenhagen: "L-Treitol-dimetansulfonat til opløsning i . . . sterilt vand til intravenøs anvendelse". L 8225 G. The dilutions of PCIB, *p*-chlorophenoxyisobutyric acid, was made from a preparation from O-Calbiochem., San Diego. Lot 30377.

Abbreviations in the Figs.: C, control: S, serum: 1, 2, . . . : drug concentration of 10^{-1}, 10^{-2} units, . . . , the unit being g/liter for BLM, but mol/liter for the other compounds.

Results

I. Bleomycin Experiments

1. EHRLICH growth.

The interaction between BLM and serum is evident from Fig. 1. The left half of the figure shows the effect of BLM alone. It is seen that concentrations of BLM higher than 10^{-3} g/liter inhibit the growth. The right half of the figure shows the usual inhibition by 10 % v/v human serum[8], *i.e.* the difference in height between the first and the second column. As increasing doses of BLM are added the cell count falls off more rapidly than in the left half of the figure. More precisely, serum seems to intensify the effect of BLM 10∼100 times. Actually, net cell death is seen with serum and 10^{-2}∼10^{-1} g/liter BLM.

2. *Triticum* Root Growth.

BLM does not inhibit root growth except in the very high concentration of 10^{-1} g/liter, (Fig. 2, left half). As to the right half of the figure, the well-known serum inhibition effect is evident[6] from the difference between the heights of the control column (without serum) and the adjacent columns. It is also seen, that BLM concentrations of 10^{-2} or 10^{-1} g/liter cause an additional growth inhibition: with human serum the effect of BLM appears to be intensified at least 10 times.

In Fig. 3 the interaction phenomenon between human serum and BLM is further elucidated by a 2^3-factorial arrangement, used in two experiments 27~28/XI and 19~20/XII 1974, identical except for different BLM concentrations, 10^{-2} and 10^{-1} g/liter respectively. The three factors were: BLM, human serum 1 %, and the anti-plant hormone PCIB 10^{-5} M. PCIB 10^{-5} M has the effect of highly promoting the length growth of plant roots[9]. This compound was added because the differential effect of various growth-inhibiting substances is often more clear-cut under these conditions.

The left half of the figure corroborates the findings of Fig. 2. When PCIB 10^{-5} M was added (the right half of Fig. 3), the length growth was more rapid, but the combined effect of serum and BLM followed the same pattern as before, except that BLM 10^{-2} g/liter was sufficient to depress growth slightly. Nevertheless growth with both serum and BLM was on each occasion lower than that what could be expected from the hypothesis of a purely additive effect.

II. Methotrexate Experiments.

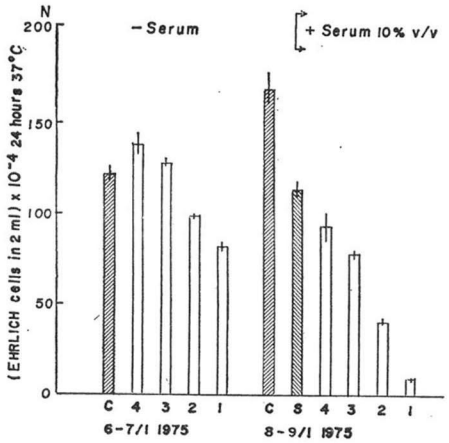
1. EHRLICH growth.

Fig. 4 corresponds to Fig. 1 except that

Fig. 1. EHRLICH cell growth inhibition by BLM without and with addition of native human serum.

Two independent experiments 6~7/I and 8~9/I 1975. Control medium: MEM+10 % v/v inactivated fetal bovine serum.

Ordinate: No. of EHRLICH cells/2 ml after 24-hour incubation at 37°C. The vertical lines on the top of each column denote SEM.

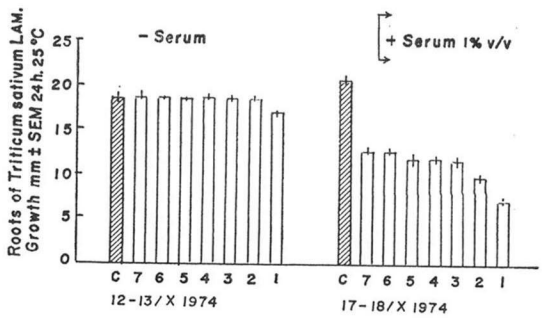


Conclusion: Human serum intensifies the inhibition caused by BLM.

Fig. 2. Inhibition of *Triticum* root growth by BLM without and with addition of native human serum.

Two independent experiments 12~13/X 1974 and 17~18/X 1974.

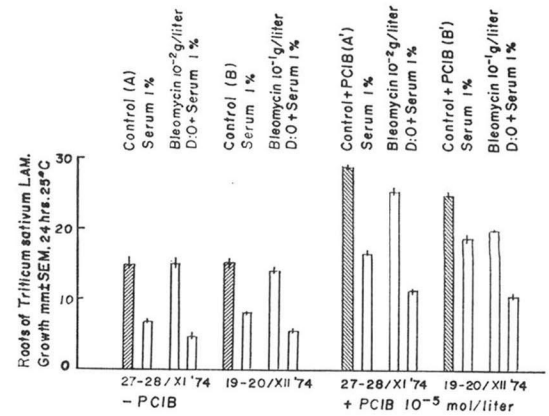
Control medium: 1/150 M phosphate buffer at pH 6.0 with 5 mM $Ca(NO_3)_2$. SEM is shown at the top of each column.



Conclusion: Human serum intensifies the inhibition caused by BLM.

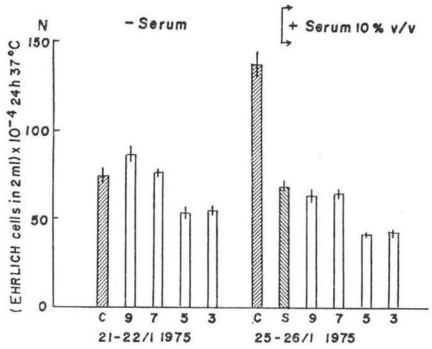
Fig. 3. Inhibition of *Triticum* root growth by BLM without and with addition of native human serum 1 % v/v and of the antiauxin PCIB 10^{-5} mol/*l*.

Two 2^3-factorial experiments: AA′ (27~28/XI 1974) with BLM 10^{-2} g/*l*, and BB′ (19~20/XII 1974) with BLM 10^{-1} g/*l*.



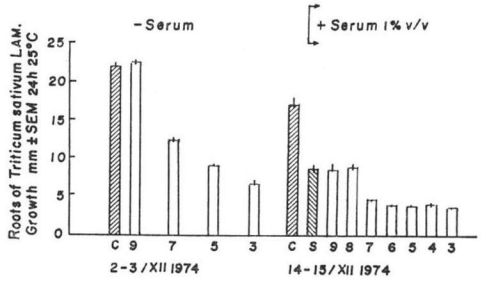
Conclusion: Also after growth stimulation with PCIB human serum seems to intensify the inhibition caused by BLM.

Fig. 4. EHRLICH cell growth inhibition by MTX ($10^{-9}\sim10^{-3}$ M) without (21~22/I 1975) and with (25~26/I 1975) addition of native human serum.



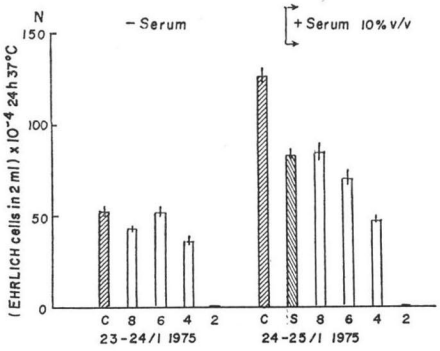
Conclusion: No interaction between MTX and human serum.

Fig. 5. Inhibition of *Triticum* root growth by MTX ($10^{-9}\sim10^{-3}$ M) without (2 ~ 3/XII 1974) and with (14~15/XII 1974) addition of native human serum.



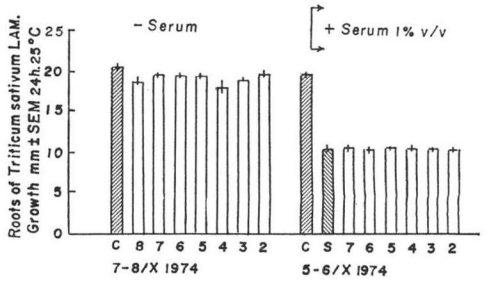
Conclusion: No interaction between MTX and human serum. Plant test more sensitive than EHRLICH ascites mouse cell test.

Fig. 6. EHRLICH cell growth inhibition by TRSF ($10^{-8}\sim10^{-2}$ M) without (23~24/I 1975) and with (24~25/I 1975) addition of native human serum.



Conclusion: No interaction between TRSF and human serum.

Fig. 7. *Triticum* roots exposed to TRSF ($10^{-8}\sim10^{-2}$ M) without (7~8/X 1974) and with (5~6/X 1974) addition of native human serum.



Conclusion: The plant roots are resistant to TRSF whether serum is added or not.

MTX is studied. Only concentrations of MTX of 10^{-5} M or more are inhibitory, whether serum is present or not. No interaction seems to exist.

2. *Triticum* Root Growth.

Fig. 5 shows a comparable experimental arrangement with *Triticum* roots. The plant root cells seems to be more sensitive to MTX, as dilutions down to 10^{-7} M are inhibitory. No interaction between human serum and MTX is discernible; only additive effects are recognized.

III. Threosulphan Experiments

1. EHRLICH growth.

TRSF is only slightly inhibitory in a concentration of 10^{-4} M, whereas the 10^{-2} M solution is maximally toxic (Fig. 6). No interaction between human serum and TRSF is seen.

2. *Triticum* Root Growth.

As opposed to EHRLICH cells, the *Triticum* roots are resistant to TRSF, Fig. 7, both without and with addition of human serum.

Discussion

Three anti-tumor compounds, BLM, MTX, and TRSF, have been compared in nearly identical experimental designs in two *in vitro* tests, an animal tumor test and a plant root cell test, (Figs. 1~7). The growth in the anti-tumor compound dilutions took place in media without and with human serum. The three anti-tumor compounds all showed different characteristics. MTX was inhibitory in both tests, although the two tests were not equally sensitive (Figs. 4~5). TRSF was inhibitory only against the EHRLICH cells, the *Triticum* roots being resistant (Figs. 6~7). This resistance is contrary to the sensitivity of onion roots to TRSF[10,11]. One plausible explanation (suggested by Dr. P. W. FEIT) is that TRSF is not converted into the active compound diepoxithreitol at the low pH of the medium employed[12].

Neither MTX nor TRSF shows any interaction with human serum. Nor do cyclophosphamide and vincristine (preliminary experiments; data not presented).

BLM inhibits the growth of both the EHRLICH cells and the *Triticum* roots, and in both cases the effect is intensified by human serum, (Figs. 1~3). More precisely, the effect is stronger than what could be expected if the effects of serum and BLM were additive.

Concerning the mode of action of BLM itself, a lot of information is available[2,13~22]. BLM is a mixture of several related glycopeptides, whose distribution, mode of action, *etc.*, could differ significantly. BLM seems to act by forming a complex with DNA causing thymine to be hydrolyzed off (leaving a free aldehyde group on desoxyribose); subsequently the DNA strand may break. The BLM action is potentiated by certain redox compounds such as sulphydryl compounds (dithiothreitol and 2-mercaptoethanol), hydrogen peroxide, and FAD (flavine adenine dinucleotide)[13,14,22]. The BLM effect is weakened by DNA repair mechanisms and by certain BLM-degrading enzymes, whereas the effect is more pronounced in cells that are actively transcribing or replicating their DNA. Thus, the finding that BLM is very potent against carcinomas of the skin yet has little effect in lymphoid tissues (no immunosuppressive effect) is thought to be due to unequal occurrence of the BLM-degrading enzymes. These enzymes appear to comprise an enzyme responsible for hydrolyzing a carboxamide group of the β-aminoalanine moiety present in the BLM molecule[24]. In a recent report ONISHI *et al.*[23] were able to explain variations in the rate of DNA degradation and also the rate of BLM inactivation entirely in terms of varying ascorbic acid concentrations in the preparations that they tested. Their findings suggest that a triple complex DNA-BLM-ascorbate is involved in both processes.

Cells exposed to BLM primarily show reduced syntheses of RNA and proteins and the cell reproduction is blocked premitotically (late S or G_2 phase); at higher doses the ability to synthesize DNA is impaired[18,21].

The possibility of an interaction between BLM and human serum seems not to have been examined before. The intensification of the BLM action by native human serum, as demonstrated in this paper, might be quite unspecific, but at any rate MTX and TRSF (and cyclophosphamide and vincristine too) do not possess this property. Moreover the fact that the interaction occurs in cells as disparate as those studied in the present paper suggests a mechanism common to all cells.

The effect of serum on EHRLICH cells is complex. It seems to be a combination of an initial cell kill followed by normal proliferation of those cells that survive the first two hours of incubation. The growth of these cells may even be accelerated owing to the well-known growth promoting effect of human serum, which can be demonstrated after heat treatment or by fractionation[5,23]. Therefore, from what has been said above concerning the mode of action of BLM, it is not surprising to find a serum-BLM interaction, whether it is because the cells are put into a state of accelerated growth by serum or because serum contributes redox compounds, —to mention two simple explanations. In this connection it should be recalled that the EHRLICH growth medium always contains fair amounts of mammalian serum constituents due to the presence of heat-inactivated fetal bovine serum. As regards ascorbic acid, the concentration of this compound in human serum is far below the tissue concentrations reported

by ONISHI *et al.*[23]

Using various mammalian cells some authors[26,27] have studied the relative sensitivity of resting plateau cells and exponentially growing cells to BLM; hence it may be pertinent to mention that our EHRLICH cells were taken from a stock culture held in permanent exponential growth.

As regards the effect of human serum on plant roots, a 1% dilution typically reduces growth by some 40 per cent, but the mechanism is equally poorly understood[6,7,28,29]. Therefore it is not possible to offer specific explanations of the serum-BLM interaction in the *Triticum* test, nor to cite direct evidence in favour of the supposition that the underlying mechanism is identical to the one operating in the EHRLICH test.

It might be interesting to try to identify the serum components that are responsible for these interactions by means of fractionation procedures, but this has not been done as yet. Whether its cause is uncovered or not, the serum-BLM interaction may have certain practical implications. Almost all the cells of a living organism are in contact with serum, or with tissue fluids that share components with serum. Therefore it may be preferable to add untreated serum or serum fractions routinely to the growth media, if the outcome of BLM assays *in vitro* is to be interpreted in a clinical context.

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