FOLKE RØNNIKE

THE INHIBITION OF EHRLICH ASCITES TUMOUR CELL GROWTH BY HUMAN SERUM ESPECIALLY SERUM FROM WOMEN IN PREGNANCY AND THE EARLY PUERPERIUM

Det Kongelige Danske Videnskabernes Selskab
Biologiske Meddelelser **24, 3**

Kommissionær: Munksgaard
København 1971

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BIANCO LUNOS BOGTRYKKERI A/S

1. Introduction

Addition of serum to synthetic cell culture media promotes cell growth, and is, in fact, essential in the great majority of human and animal cell culture methods of to-day.

In general it is not clear which type of serum—autologous, homologous or heterologous—is most effective, nor is the mechanism of action fully understood. It is probable that high molecular weight serum proteins with their physico-chemical properties are effective detoxicants, but low molecular weight substances also give some unknown, but necessary, character to the culture medium (Foley & Epstein 1960, Levintow & Eagle 1961).

Human and animal sera also contain low molecular weight substances which modify growth of cells from higher plants (Rønnike 1961, 1967, 1969, 1970), but, again, the reasons for this are unknown. Generally, however, plant cell cultures differ from animal cell cultures, in that they can be maintained on wholly synthetic media, without addition of serum proteins or other high molecular weight substances (Larsen 1962).

The author has demonstrated that serum from pregnant women, women in the puerperium, and children, has effects on plant cell cultures different from other human serum (Rønnike 1969). The effect of serum from pregnant and non-pregnant women on human and animal cell cultures has not been much studied. Meyer, Penttinen & Saxén (1964) found "... that it is obvious that pregnancy has an effect on the growth-controlling capacity of the serum. The changes in the serum which occur during pregnancy and are responsible for the differences in the growth behaviour of cells in cell culture are unknown. The more frequent clumping of cells at the end of pregnancy could be connected with the increase in e.g. beta-lipoproteins at that time.

The obvious disappearance of the clumping phenomenon after the first weeks of pregnancy remains unsolved . . .".

Rejnek, Bednarik, Rerábková & Dolezal (1963) observed some differences in HeLa cell culture growth in the presence of, respectively, pregnant and non-pregnant serum, but no author has made further investigation of this interesting phenomenon.

This author, inspired by his basic plant physiology studies, undertook examination of the effect of fresh human serum on the growth of Ehrlich ascites tumor cells in a culture already optimal as regards its serum component (i.e. 10 % embryonic bovine serum was added to the basic medium, and the Ehrlich cells were adapted to this milieu over a considerable time before the studies).

The categories of serum studied were obtained from: 1) healthy young men, 2) healthy young women, 3) healthy pregnant women, and 4) healthy women early in the puerperium. An inhibitory effect on the Ehrlich cell growth was observed, increasing from category 1) to category 4) serum; i.e. women's serum is more toxic to the Enrlich cells than men's and still more so when the women are pregnant or in the early puerperium.

The following report documents these observations.

2. Methods

a. *Cell type and culture medium.* Throughout this study, the common standard culture technique with cells in suspension was used.

The Institute of Pharmacology of the University of Bonn provided the Ehrlich ascites tumor cell suspension (Karzel 1965, Karzel & Breull 1968, Karzel & Schmid 1968) from a continous culture.

The standard culture medium, to which sera were added to obtain test preparations, was "FIB 41 B" (Briand 1969), to which was added 10 % embryonic bovine serum (Flow Laboratory, Ltd., Irvine, Scotland/Dansk Mikrobiologisk, Skoletoften 16, Grundfør pr. 8332 Hinnerup Danmark). Control tubes contained this standard medium only.

b. *Cell suspensions.* Fresh culture medium was added daily after disposal of between 50 % and 80 % of the culture. From one

to four times monthly, cells were centrifuged down (300 G for 5 mins.) and the culture medium completely replaced.

c. *Preparation and treatment of human serum.* Following venepuncture, the blood was allowed to stand for at least one hour. Coagulated blood was thereafter freed from the test tube sides with a sterile metal probe, and the whole centrifuged at 3,000 G for 10 minutes. The serum was drawn off with a sterile disposible syringe. Serum dilutions were always made on the day of venepuncture, and if the preparation was not used the same day, it was stored under sterile conditions at ≤ 20 °C.

d. *Experiment technique.* In preparation of the cell suspension before addition of serum (or the control solution), a greater or lesser number of cells were centrifuged down (300 G for 5 min.), and the cell concentration adjusted by the addition of a greater or lesser quantity of the basic culture medium. While the cell suspension was being added to the serum preparation, or the control solutions, even cell distribution was ensured by magnetic stirring. In general, 1 ml cell suspension, containing approximately 60×10^4 cells, was added to 1 ml of the serum preparation, or control solution.

The experimental cultures themselves were conducted in radiation sterilized reagent tubes, 100×13 mm with lids ("Nunclon"®, Algade 8, Roskilde, Danmark). Standard sterile cell culture technique with sterile disposible syringes and needles was used. All procedures involving cell nutrition and transfer were conducted in locked containers with front walls of glass. Glass ware, stoppers etc. were cleaned in "7 X" fluid (Gateway International 107, N. Virgil Ave. Los Angeles, California 90004/Bjellekjær Madsen, Vesterbrog. 69, København, Danmark). Utensils were autoclave, dry air, or radiation sterilized. (Radiation sterilization by Radest, Formervangen 16, 2600 Glostrup, Danmark).

Cell growth took place at 37 °C in a thermostat with forced circulation. All changes of nutritional medium, and cell transfers, were conducted at room temperature.

Generally, each *E*xperiment comprised 5 test tubes for each of some 20–25 serum admixtures in addition to 10 control tubes. The arrangement of these tubes in the thermostat was randomized.

Whenever the word "*E*xperiment" is written with a capital *E*, such an experimental run is meant.

e. *Cell counts.* Bürger-Türck counting chambers were used. The cells were counted without staining or after addition of a few drops of Lissamine Green to each 2 ml. sample. The counted numbers of cells for each tube is denoted *N*, which lay between 0 and 200 (approximately). The total number of cells in each tube (2 ml suspension) is obtained by multiplying by 10^4.

(From subsequent studies (HILDEN, BIRGER JENSEN & RØNNIKE) it seems probable that approximately 1/10 of the variation between the reagent tubes with identical serum admixture (replicates) can be explained by count variation, except for very low *N* (less than 10), where counting variability is the major source of variation. This will affect the standard errors of the means (SEM) recorded in Figures 1, 2, 3, 4, 6, 8 and 9. This counting variation, taking into account other fluctuations inherent in the investigation, is of an acceptable order).

3. Results

a. *Control cultures.* The variation in numbers of cells in the control cultures after 24 hours growth is shown in Figs. 1 & 10. It is apparent that the initial number of cells was a significant factor (Fig. 10: positive regression between the initial number of cells and the number after 24 hours growth), but attempts to hold the control rates of growth constant by regulating the initial number of cells were not successful (Fig. 1). The marked variations depend, therefore, on factors which cannot be adequately controlled even by the present, highly standardized technique.

b. *Human serum concentration.* After addition of human serum to the cultures (which already contained embryonic bovine serum 10 % v/v), cell growth was invariably inhibited after a period of 24 hrs. (Fig. 2). It is seen that both a pooled serum preparation from 10 healthy donors and a serum preparation from one donor had marked inhibitory effect at 15–20 % serum dilution, while growth in a 10 % dilution was between 30–40 % of control culture growth. At higher dilutions the serum inhibitory action decreased rapidly. Slight differences in inhibitory effects could accordingly be best identified at about 10 % dilution. This level of dilution was therefore chosen as the standard for tests comparing the various serum categories. (In plant physiology studies of comparable principle, the optimal dilution was 1 % v/v).

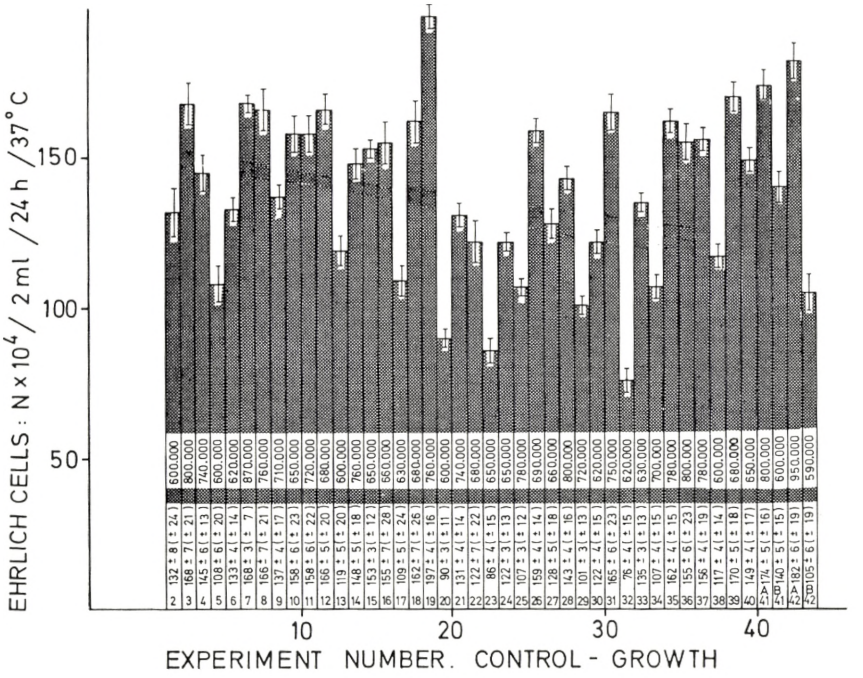

Fig. 1.

Control culture growth. March-August 1970.

Consecutive values of optimal growth in control cultures ("Fibiger 41 B" + foetal bovine serum 10 % v/v). At the column peaks SEM is shown.

Under the columns are shown: 1) Experiment numbers. 2) Number of cells in colonies ± SEM (± SD between test tubes) × 10^4 after 24 hours ("Time 24"). 3) Number of cells at the beginning of the experiment "Time 0").

Ordinate: No. of Ehrlich cells/2 ml after 24 hrs. culture at 37 °C.

Conclusion: Wide variation of growth in control cultures during Experiment period.

c. *Serum from men compared with serum from women in the early puerperium.* It became early apparent that there was considerable overlapping variation in the inhibitory action of the 10 % serum dilutions from the different donor categories, as also observed in plant cell studies. Thus, to identify any differences in effect between serum from the different donor groups, it was necessary to study many members within the same group. Because of the limited number of hours in a working day p. p., several Experiments were often necessary to secure this. In order to combine figures from several Experiments the following formula was applied:

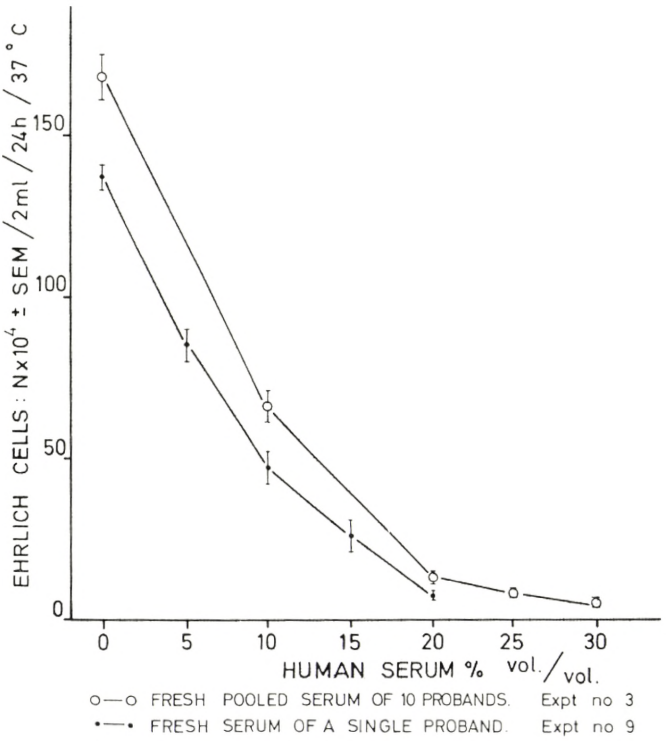

Fig. 2.

Growth in dilutions of human serum.

Abscissa: Concentration of human serum in the control solution ("Fibiger 41 B" + foetal bovine serum 10 % v/v).

Ordinate: No. of Ehrlich cells/2 ml after 24 hrs. incubation at 37 °C. The vertical lines denote SEM.

Conclusion: 30–40 % growth inhibition by 10 % dilution of human serum.

$$t = \frac{\left[\left(\frac{(\Sigma r)_1}{M+1}\right)_{\mathrm{I}} + \left(\frac{(\Sigma r)_1}{M+1}\right)_{\mathrm{II}} + \ldots\right] - \left[\left(\frac{m_1}{2}\right)_{\mathrm{I}} + \left(\frac{m_1}{2}\right)_{\mathrm{II}} + \ldots\right]}{\sqrt{\frac{1}{12}\left[\left(\frac{m_1 \cdot m_2}{M+1}\right)_{\mathrm{I}} + \left(\frac{m_1 \cdot m_2}{M+1}\right)_{\mathrm{II}} + \ldots\right]}}$$

[D.F.: ∞]

in which Roman numerals identify the quantities relating to each individual Experiment. Within each individual Experiment, $(\Sigma r)_1$ denotes the Wilcoxon rank sum pertaining to the first proband group, while $m_1(m_2)$ is the number of probands in the first (the second) group; finally, $M = m_1 + m_2$.

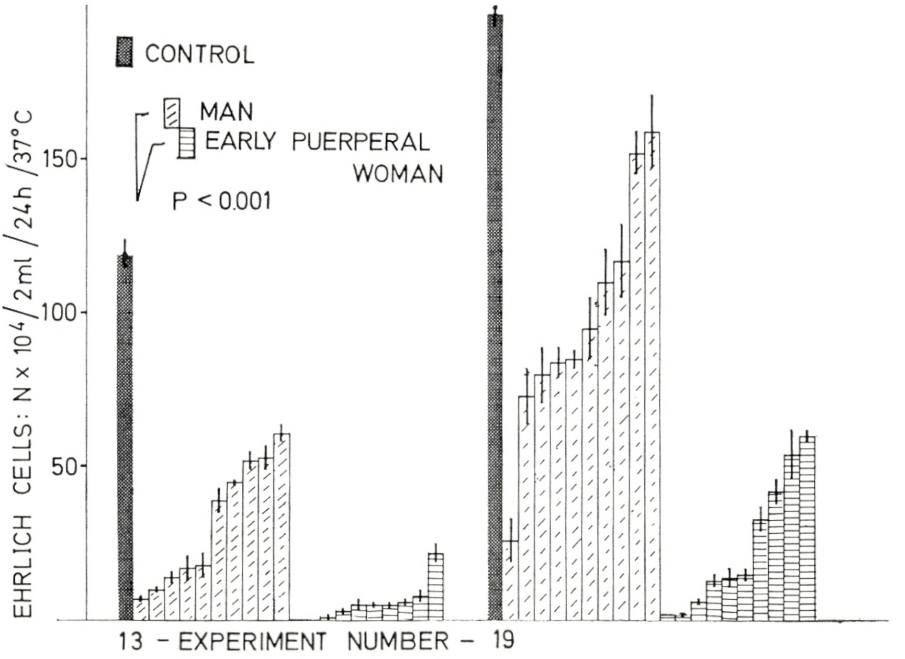

Fig. 3.

Cell growth inhibition by serum – from men, and from early puerperal women.

Human serum dilution: 10 % v/v in the control medium ("FIB 41 B" + 10 % foetal bovine serum).

Independent Experiments Nos. 13–19. For each is given: 1) Control growth ± SEM. 2) Growth in 10 % dilution of human serum from each of 20 donors (10 men and 10 early puerperal women) ± SEM.

The probability of significant difference between the two categories (Experiment 13 and 19 combined) is shown on the graph.

Conclusion: A: Early puerperal serum markedly more inhibitory than male serum.
B: High growth in control cultures apparently paralleled by high growth in human serum cultures (Experiment 19 growth higher than Experiment 13 growth – Compare Fig. 4).

Fig. 3 shows that the difference in inhibitory effect between serum from men and serum from women in the early puerperium is established at the 0.1 % significance level. Comparison of values found in Experiments 13 & 19 further shows that higher initial cell counts are correlated with greater growth in sera, whether this be slightly inhibiting male serum or strongly inhibiting early puerperal serum.

d. *Control growth – serum growth* (1). That higher control values were associated with higher growth in serum seems also to be apparent from the test described in section f (Fig. 6). Results

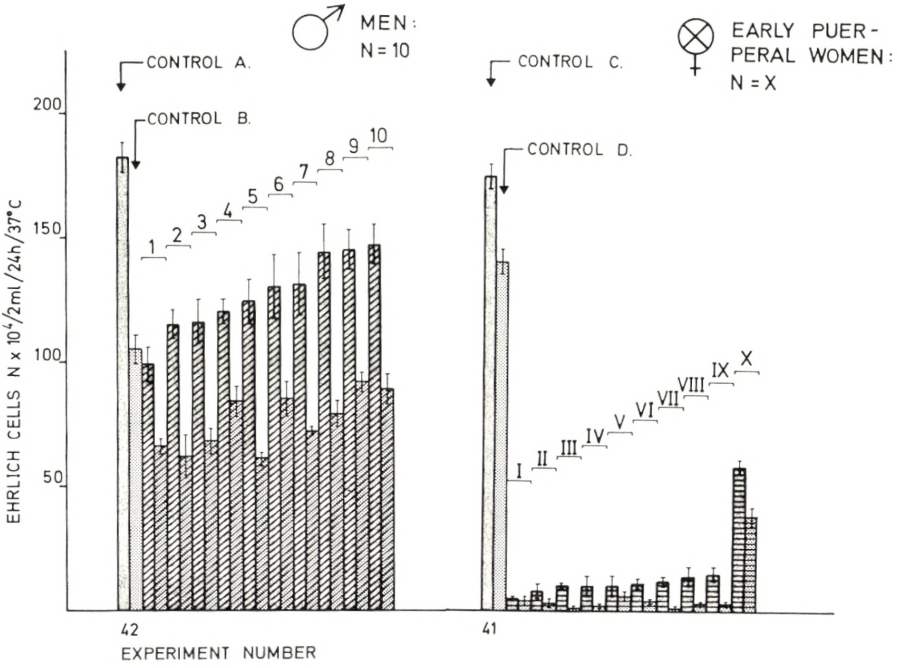

Fig. 4.

Relationship between control and human serum growth.

Duplicate tests of serum from 10 men (1-10) (Experiment 42) and serum from 10 puerperal women (I-X) (Experiment 41),– first test initial number of cells "large", second test initial number of cells "few".

When the initial number of cells was large, growth in both control and serum dilutions was higher than when the initial number of cells was few.

A + C: initial number of cells in control solutions: "large".

B + D: initial number of cells in control solutions: "few".

Conclusion: High control growth parallelled by higher growth in human serum.

of Experiments to confirm these observations appear in Fig. 4. Cells were added to serum preparations obtained from each of 10 male donors, and from each of 10 female donors in the early puerperium. A control test was also run using the control preparation. Growth after the addition of both many and few cells was studied. In all tests growth was higher after 24 hrs. when the initial number of cells added was large. This was particularly marked in the puerperal serum trial, where the increase was more than proportional to the control trial increase.

e. *Control growth – serum growth* (2). In the previous section d, the relationship between control and serum growth was analysed, and it was found that high control growth was associated

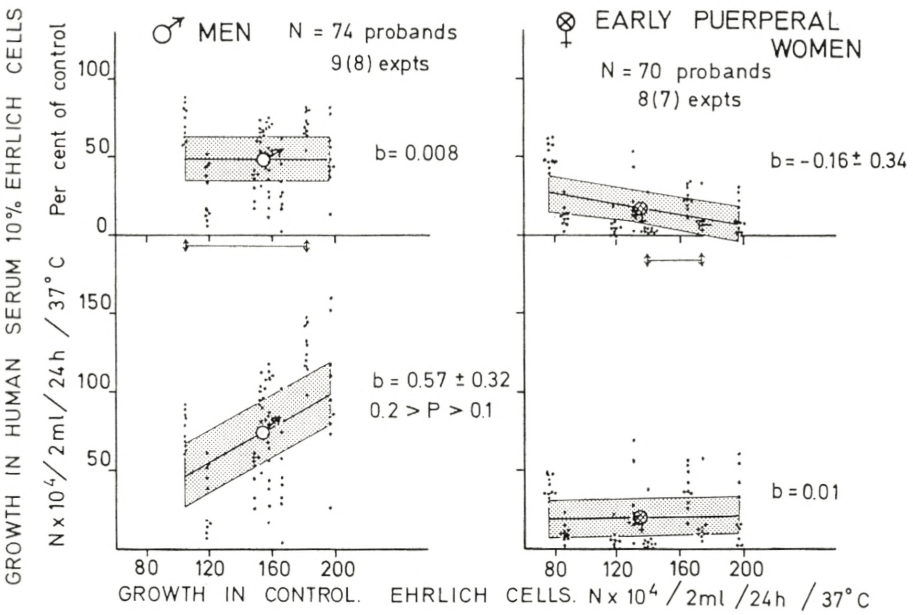

Fig. 5.

Relationship between growth in control medium and growth in male serum and early puerperal serum.

Two sets of male probands and two sets of early puerperal probands (marked "↕") are identical in each of two Experiments. Their Experiment results are also included in Fig. 4. None of the regression coefficients varies from 0 – i. e. no correlation is apparent between control and human serum growth (see Fig. 4, the analysis of variance in Table 1, and text).

Conclusion: The interexperimental variability of this figure is so large that the positive correlation between control and human serum growth demonstrated in Fig. 4 is not apparent.

with high serum growth. It may be noted that this finding depends on an experimental procedure which avoids interexperimental variability. Otherwise, even though the results of various tests are in fact consistent, this can be obscured. Fig. 5 records the results of cell growth: in serum from 74 men (9 tests): in serum from 70 early puerperal women (8 tests); and in the control preparation. In no instance was regression certainly positive. Accordingly, the analysis of variance (Table 1) indicates that the variation between Experiments around the regression line is too great to be attributed to the variability of the means of each individual Experiment ($P < 0.001$). Considerably more Experiments than recorded in Figure 5 (9 and 8 Experiments, resp.) would be necessary to display clearly the connection which is evidenced by the above-

TABLE 1. Analysis of variance. Growth in serum from men. Actual values from Fig. 5.

Type of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Variation explained by linear regression	1	21 539.6	21 539.6
Deviations of the nine experiments from their common regression line	7	540 473.3	72 210.5***
Between subjects, within experiments	75	46 663.6	622.2

$F_{(7,75)} = 116.1 \quad P < 0.001$
$F_{(1,7)} = 0.298 \quad P > 0.1$

explained experimental set-up (Fig 4), in which the trend is not obscured by inter-experimental variation.

It is this large interexperimental variability that necessitates the experimental procedure employed in the studies described in sections *c*, *f*, *g*, and *h*.

f. *Cell growth in human serum:* α) *Men's serum, women's serum – non-pregnant women, pregnant women, early puerperal women.* β) *Additional indicated studies. The influence of oral contraceptives. Serum from children under* 2 *yrs., and from post-menopausal women.*

α) Fig. 6 shows findings from comparison of successively more inhibitory serum categories. Thus the least inhibitory men's serum is compared with more active non-pregnant women's serum (Experiments 11 + 15, $P < 0.001$ by the formula of section c); non-pregnant women's serum is compared with early and late pregnant women's serum (Experiments 16, 26, 27, 28 combined: $P < 0.001$), and finally late pregnant women's serum is compared with early puerperal women's serum (Experiments 31 + 32: $P < 0.001$), which was the most actively inhibitory.

β) Many of the young non-pregnant women took oral contraceptives (50 %), but this was not apparently a factor in serum inhibitory activity (Fig. 6, Experiments 33 + 34). Serum from children under 2 yrs., and from post-menopausal women was also studied in a single pilot Experiment and was found to have

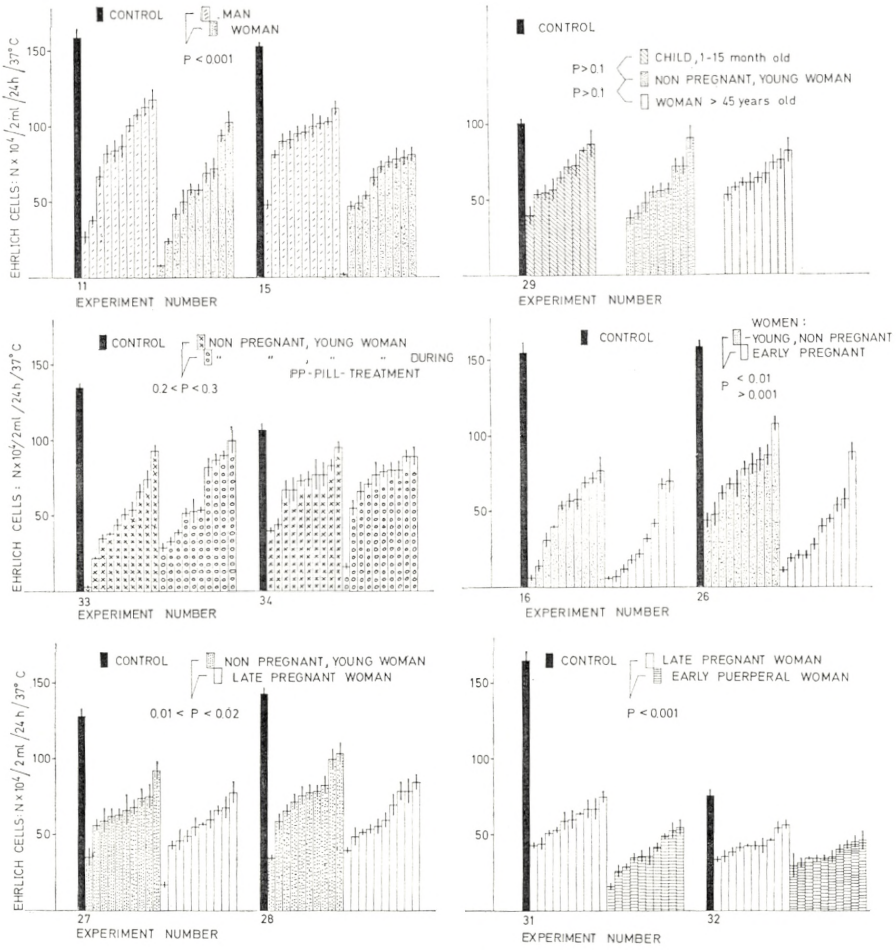

Fig. 6.

Cell growth in human serum of different categories.

Basic categories: 1) Men (Experiment 11 and 15), 2) healthy non-pregnant women (Experiments 11, 15, 16, 26, 27 and 28), 3) women – early (Experiments 16 and 26), and late (Experiments 27, 28, 31 and 32) pregnant, 4) early puerperal women Experiments 31 and 32).

Additional categories: 1) Serum from women taking oral contraceptives (Experiment 33 and 34), 2) Serum from children under 2 yrs. old and from post-menopausal women – compared with serum from non-pregnant young women (Experiment 29).

Serum from, in all, 226 individuals tested. Each column represents cell growth in freshly prepared 10% v/v human serum. Control cultures were run for each test. ± SEM are shown at the column peaks. Level of significance is shown over the columns. The diagrams are presented in order of increasing inhibitory activity of the serum categories.

Conclusion: From category 1 (men) to category 4 (puerperal women) there is progressively increasing serum inhibitory activity.

Oral contraceptives are without significance. Serum from children under 2 yrs. and from post-menopausal women, has activity similar to serum from young non-pregnant women (category 2).

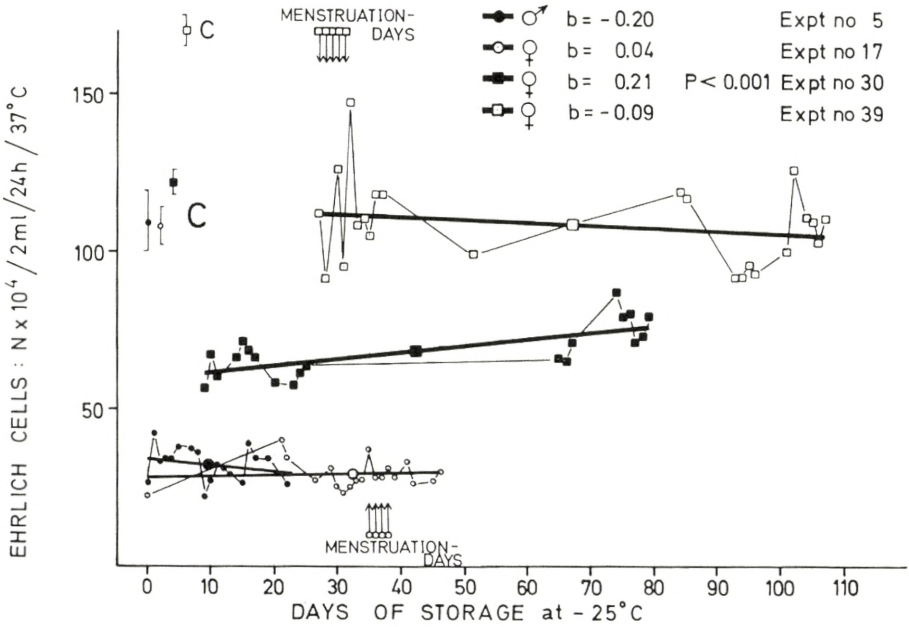

Fig. 7.

Storage of serum.

The influence of storage up to 108 days on serum from three women and one man. Four independent Experiments. The regression line for each proband is shown.

Conclusion: No relevant influence of storage at ≤ 20 °C on activity of serum from 4 probands. Two of the women menstruated during the periods of blood-sampling. No change in serum activity was evident.

inhibitory activity similar to that of the young non-pregnant womans category (Experiment 29).

g. *Storage of serum.* Fig. 7 gives the results of 4 experiments with sera from 4 different individuals, obtained by repeated blood-sampling and stored for up to 108 days. Only in one case was there difference in inhibitory capacity—Experiment no. 30, $b = 0.21 \times 10^4$ cells/day, $P < 0.001$, mild fall off of inhibition. The experimental design does not allow differentiation between an effect of storage, and an "in vivo" change in the properties of the individual's serum but the absence of a similar trend in the other 3 subjects speak in favour of an "in vivo" change.

Variation in results of Experiments with serum with weak inhibitory properties (Experiment 39) was greater than in serum with strong inhibitory properties (Experiments 5 + 17), but this difference in variation could be predicted from the curve in Fig. 2.

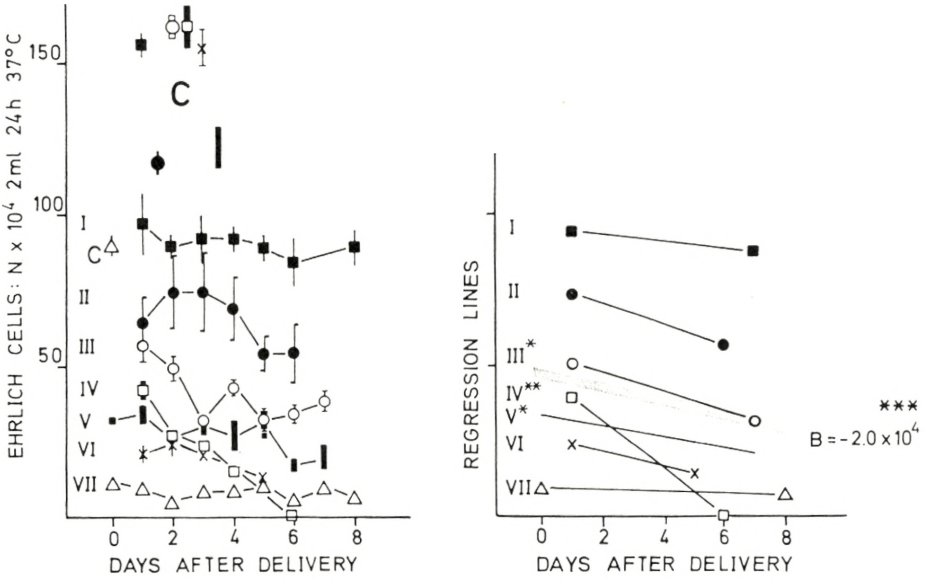

Fig. 8.

Inhibition of cell growth by serum from women in the first eight days of the puerperium.

Left of the Figure: Individual findings in each of 7 probands (I–VII) ± SEM with respective control values ± SEM.

Right of the Figure: Regression lines corresponding with graphs on left of Figure, and the combined regression line.

Conclusion: Inhibitory activity of serum increases in the first 8 post partum days.

Two donors had menstruation as marked in Fig. 7. No difference in serum activity was detected.

h. *Growth in early and late puerperal serum.* As recorded in Fig. 6 the serum with the greatest inhibitory effect was from women in the early puerperium, followed by serum from pregnant women. Figs. 8 & 9 demonstrate the increasing of the inhibitory activity from delivery over the first 8 days, and also the falling off of this activity during the first 3–4 post partum weeks. The graph on the left of Fig. 8 shows the individual growth values ± SEM for each of the seven women (I–VII) from delivery to the eighth day of the puerperium. Results of the controls run concurrently with each specific serum test are shown uppermost on the graph. On the right of Fig. 8 the regression lines corresponding to the curves on the left side are appended. Only three of the curves slope significantly (*: $P < 0.05$, **: $P < 0.01$) while the total regression line "summing" the 7 regression lines (the broad line

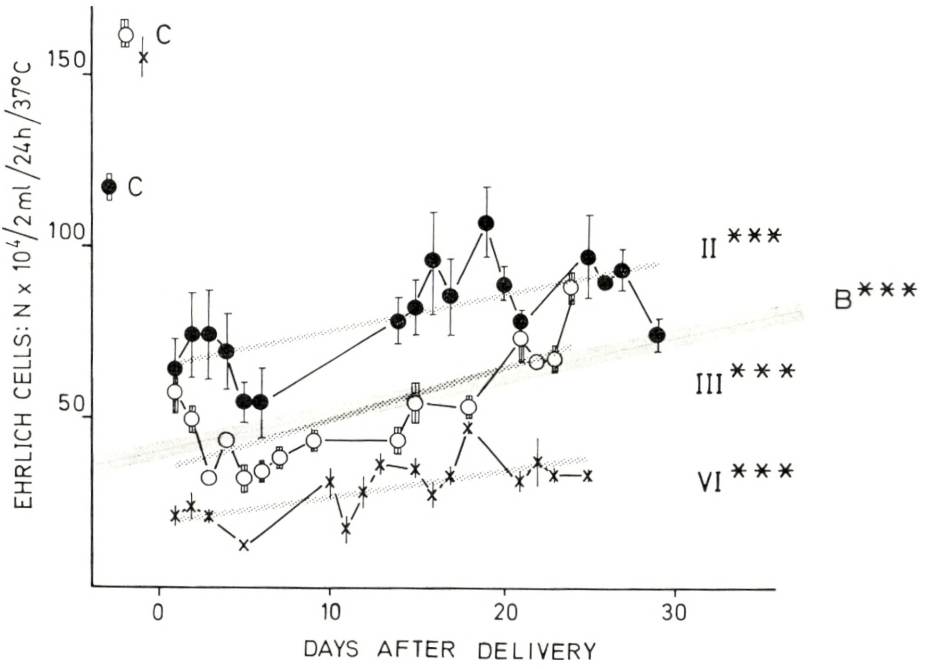

Fig. 9.

Inhibition of cell growth by serum taken during the first 4 weeks post partum.

Conclusion: Serum inhibitory activity, having increased during the first 8 post partum days (Fig. 8), reduced markedly 24–28 days post partum.

Three probands studied. Regression lines for each, and the combined regression line are shown.

on the Figure) has value $B = -2.0 \times 10^4$ cells/day ($P < 0.001$) indicating that serum's inhibitory activity increases during the first 8 days post partum in these 7 women. This statement can be extended to puerperal women in general ($P \leq 1/64$).

Figure 9 shows cell growth in serum from the first 29 post partum days. Results of three tests together with regression line for each and the shared regression line are recorded. Despite the fact that the data obtained from study of serum during the first 8 days is included, where fall in growth was noted, all lines demonstrate increase in growth (reduced inhibition) $P < 0.0005$, $B = 1.27 \times 10^4$ cells/day. Falling off of inhibitory serum activity is, therefore, marked from the 2nd to 4th post partum weeks in these 3 women, and fairly certainly in all women.

It is not possible to judge from the curves in Fig. 9 how long after pregnancy the activity of serum falls to the level found in the non-pregnant group.

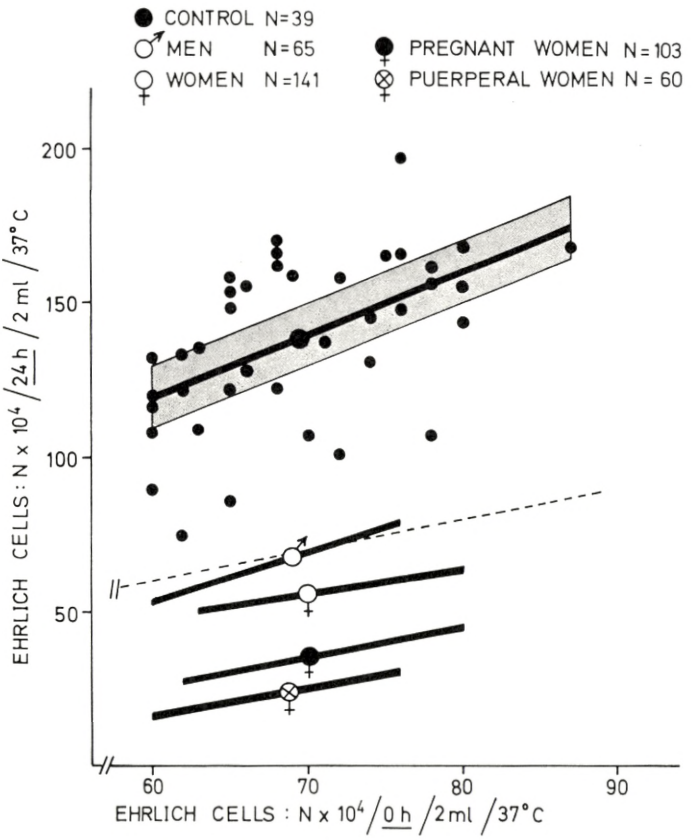

Fig. 10.

Cell counts at the beginning and completion of experiments.

Abscissa: Initial cell counts.

Ordinate: Cell counts after 24 hours growth in: 1) Control culture medium. 2) 10% v/v serum dilution from 65 men. 3) 10% v/v serum dilution from 141 non-pregnant women. 4) 10% v/v serum dilution from 103 pregnant women. 5) 10% v/v serum dilution from 60 puerperal women. 6) The dotted line in the middle of the graph denotes no net growth.

In the control study, individual Experiment values, the grand average, the regression line and the confidence interval for this line are shown. For the human serum Experiments the grand average and regression lines are given.

Conclusion: Number of cells in control colonies doubled after 24 hrs. growth. No net growth of cells in mens' serum dilution.

Net cell death in womens' serum dilution – most marked when serum was from women in the early puerperium.

i. *Cell counts – initial and final. α) Grand averages and regressions for all control and serum category studies.* Fig. 10 gives the initial (time 0), and final (time 24), cell counts in the control and category studies (the categories comprised, respectively, 65 healthy

men; 141 healthy women; 103 healthy pregnant women, and 60 healthy women in the early puerperium). The control values were also discussed in section *a*. The grand average of the 39 control values $\bar{y} = 139 \pm 4$ $(\pm 28) \times 10^4$, $s_{Y.X} = 22.2 \times 10^4$. The initial number of cells $\bar{x} = 70 \times 10^4$ (the factor 10^4 is omitted in the following equations).

For the 65 men: $\bar{y}_{\text{total}} = 68 \pm 4$ (± 35), $s_{Y.X} = 33.4$; for the 141 women $y_{\text{total}} = 56 \pm 2$ (± 25), $s_{Y.X} = 24.3$ ($P_{\text{men-women}}$: < 0.005, > 0.001); for the 103 pregnant women $\bar{y}_{\text{total}} = 37 \pm 2$ (± 24), $s_{Y.X} = 23.5$, and for the 60 early puerperal women $\bar{y}_{\text{total}} = 24 \pm 2$ (± 18), $s_{Y.X} = 17.1$. It can be seen that the large spread gives rise to considerable overlap between the different serum categories.

The regression line for the control values increases significantly, but none of the lines for the serum category values slopes significantly. As discussed in section *e*, the large interexperimental variation is again an important factor. Nevertheless the difference between the average in all 4 categories is clear. The graduated increase in serum's inhibitory effect from male to early puerperal women is again apparent from comparison of the grand averages for each serum category.

β) Interpretation in terms of growth acceleration, growth inhibition and cell destruction. The stippled line in the middle of the graph in Fig. 10 gives a base line of no growth after 24 hrs. Generally speaking, as previously mentioned, growth in the controls doubled the initial number of cells in the colony. The male serum line comes near to the stippled line suggesting total growth inhibition, whereas the lines of the other serum categories fall progressively more below the stippled line, suggesting not only inhibited growth but a net cell mortality.

4. Discussion

Earlier studies on the effect of serum from pregnant women on animal and human cell growth have been few.

Meyer, Penttinen & Saxén (1963) in Finland, and Rejnek, Bednarik, Rerabkova & Dolezal (1963) in Czechoslovakia, found that fresh serum from pregnant women modified growth

of HeLa cell culture growth. The difference in technique make it difficult to relate the above authors' findings with those of this study. MEYER et al. found differences in activities of serum from early and late pregnancy, and also that "delivery" serum, 6 days post partum serum and complicated pregnancy serum, relatively often gave rise to a "clumping" phenomenon. Both the Finnish and Czechoslovakian studies lack probability calculations to support the presented data and conclusions. REJNEK et al. ascribe the effects of pregnancy serum to an abnormal alpha-lipoprotein.

In this study here presented, it is demonstrated that freshly prepared serum contains one or more substances which inhibit cell growth in cultures which are otherwise optimal. The serum categories tested—male, female, pregnant women's, early puerperal women's—evidenced increasing inhibition capacity in the foregoing order.

In the author's comparable plant cell studies (men's serum not tested), an opposite phenomenon was observed—i.e. that length of root cell growth was most inhibited by non-pregnant women's serum.

If plant cells from the above-earth part of a plant—e.g. koleoptile cells—are considered, observed effects are comparable with those in this Ehrlich cell study.

In further contrast, the effect of serum from young children (2/12—1 yr.) in plant studies was comparable with the effects of pregnancy and early puerperal serum, but in the Ehrlich cell study comparable with only non-pregnant women's serum (Fig. 6, Experiment 29).

This may well indicate that the active serum component(s) are not the same in the two studies.

Fig. 10 shows that men's serum holds cell cultures static (i.e. no net growth), whereas in increasing degree, there was a net mortality when women's, pregnant women's and puerperal women's sera were tested.

It could be that men's serum has a purely inhibitory effect, while women's is to degrees lytic, but the individual records rule out this possibility. On occasion, there was good cell growth in men's serum, but there was also net cell mortality in some of the individual Experiments (e.g. no. 13, Fig. 3), with the exception of one individual proband where there was no net growth. Basic

difference between men's and women's serum may therefore be quantitative and not qualitative.

Comparisons of repeated blood-samplings from the same proband over a period of time indicate that storage does not affect serum activity. Fig. 7 records the results of a study of 3 individuals in which no difference in serum inhibitory activity could be demonstrated after storage. In a fourth individual a slight falling off of serum inhibition was noted, but it would seem that storage of serum at < 20 °C has no effects on serum's inhibition of Ehrlich cell growth (see: PENTTINEN & SAXÉN 1957). Combined significance was not tested because the degree of inhibition, and times of storage were very different in each case. It is noted that in plant cell studies, the effects of serum storage were also negligible (RØNNIKE 1967).

Seven early puerperal probands were individually studied daily for 7 (8) days after delivery (Fig. 8). All the regression lines, shown on the right of the Figure, slope down. The combined regression coefficient $B = -2.0 \times 10^4$ cells/day is certainly negative.

One of the seven (no. IV, Fig. 8) was qualitatively completely different from the others. There was total cell lysis in her serum after 6 days. Unfortunately, it was not possible to continue the studies with her.

The falling off of serum's inhibitory effect from the 6–8th day to the 25–30th day of the puerperium (Fig. 9 – 3 studies) was apparent in the linear regression but there was no clear curving. At what point effects return to a non-pregnant niveau is, therefore, obscure. Fig. 10 shows the average difference between early puerperal and non-pregnancy serum to be about 30 ordinate units. Probably, therefore, the low early puerperal values, as represented by the regression coefficients, return to "normal" over 3–4 weeks i.e. (56 – 24): 1.27 days. In plant studies the comparable period was 3 weeks after delivery (RØNNIKE 1967).

In plant studies, early pregnancy and late pregnancy sera were found to have equivalent effects on root cell growth (RØNNIKE, not published). This was also apparent in the Ehrlich cell study. If there is, in fact, a difference, the tests used are not sufficiently sensitive to demonstrate it. Extended further studies were not made. Nevertheless, MEYER et al., as mentioned, record difference in the "clumping" activity of serum from early and late pregnancy. The

inhibitory activity of serum during menstruation was studied in only two probands (Fig. 9): one serum had constant marked inhibitory capacity, while the other was only mildly inhibitory. Menstruation produced no observable changes. If this proves to be a constant finding, it may be reasonable to assume that the steroid hormones are not involved in serum inhibition of Ehrlich cell growth.

While no definite conclusions can be drawn at present, current studies indicate that the active serum factor(s) is probably a protein. It is thermolabile, inactivated at 56 °C, and is not dialysable. Sephadex G-200 fractionation has shown that the biologically active proteins are among the first eluates, and therefore the molecular weights must be high.

This characteristic is the opposite of that found for serum and urine substances active in plant cell growth.

The puerperal serum factor inhibiting Ehrlich cell growth can be inactivated by repeated absorption with washed concentrated cell suspensions of the serum dilution, which may indicate that the inhibition is due to an antigen/antibody reaction.

Observation of comparable cell cultures in male serum, and in puerperal serum, has thown that proportionately more cells in the latter cultures take up Trypane Blue stain after one hours incubation at 37°C. This indicates a higher proportion of dead cells.

Biological testing of the serum protein fractions after separation on Sephadex and Sepharose columns are in progress.

Pilot experiments indicate, that some coagulation factors in the blood may be significant.

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6. Summary

A. Freshly prepared human serum inhibits growth of Ehrlich ascites tumour cells in otherwise favorably nutrient suspension cultures.

B. Serum from men is less active than serum from women. Serum from non-pregnant women is less active than serum from pregnant women, which is, in turn, less active than serum from women in the early puerperium.

C. Three weeks into puerperium inhibitory activity of serum is declined.

D. Storage of serum at $\leqq 20$ °C does not affect inhibitory properties.

E. Influence of pregnancy serum on Ehrlich cell growth and plant cell growth, is compared.

F. Earlier studies of possible effects of pregnancy serum on cell growth (MEYER, PENTTINEN & SAXÉN, 1963; REJNEK, BEDNARIK, RERÁBKOVÁ & DOLEZAL, 1963) are compared with this study.

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