Methods. Before the experiment the tadpoles are kept for more than 24 h in sterilized well water to which antibiotics (penicillin: 100000 μ/l and streptomycin: 100 mg/l) are added. The speens are removed and placed, 2 or 3 at a time, in the disposo-trays which have been previously filled with 0.5 ml of medium. The erythroblasts are freed by fragmentation of the speen with small pincers. Then the chambers are hermetically sealed with a thin sheet of coverslip. Qualitatively and quantitatively similar results were obtained with cultures incubated in air and without air. The cultures are kept at a temperature of 18 °C and the medium is renewed every 4 or 5 days.

Observations. This culture technique allows continuous observation during the entire life of the cells. At the larval stages of development at which they are removed, the spleens are simple pouches filled with erythroid cells (Figure 1). As soon as spleen fragmentation into explants takes place, the erythroblasts spread over the bottom of the chamber. 30 minutes after the beginning of the culture, the release of a significant quantity of erythroblasts was observed.

After 15–20 h of culture, the erythroblasts which were packed in the spleen (Figure 1) have aquired a characteristic morphology (Figure 2a). It is quite remarkable that the cells thus isolated are highly homogeneous (Figure 2b). Indeed we have a majority of polychromatophil and acidophil erythroblasts. There are always cases of mitosis. The *average number* of erythroblasts thus obtained is about $5 \cdot 10^5$ cells/medium ml for 4 or 5 days of culture. It is important to note that after about 8 days of culture a part of the 'mature cells' degenerate. At 9 or 10 days, the average number of erythroblasts is about $3 \cdot 10^5$ cells/medium ml. After 12 days this number remains constant, about $2 \cdot 10^5$ cells/medium ml (table). Actually it is not possible to say whether 'mature cells' degenerate because they reach a degree of differentiation for which the conditions of culture are not adequate, or whether they must die and in vivo they would have degenerated in the same way.

The morphology, the behaviour of the remaining erythroblasts (about $2 \cdot 10^5$ cells/medium ml) continues to be normal (Figure 3). These erythroblasts thus cultivated for 4 weeks with frequent renewals of the medium, actively incorporate labelled elements in the proteins (leucine 3H) and the RNA (uridine 3H).

Conclusion. The larval erythroblast culture described above is an original for amphibians. The cells thus obtained constitute a homogeneous population, and are relatively synchronous with regard to their state of differentiation. Their numbers ($5 \cdot 10^5$ cells/ml medium on average after 4 or 5 days of culture) are sufficiently great to be biochemically studied. It should be underlined that this method of erythroblast culture makes it possible to follow their evolution during a period of several weeks.

This biological system should therefore be particularly suitable for studying the different problems raised in the introduction concerning the simultaneous analysis of biochemical and morphological red line cell differentiation.

Alternative Complement Pathway: Activity Levels in Allogeneic Pregnancy

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Summary. Classical and alternative complement pathway activities have been evaluated in sera of women in progressive stages of gestation and in pregnant mice belonging to outbred or inbred matings, as compared to suitable controls. While classical C pathway was found to be unmodified, the alternative one attained in pregnancy significantly higher activity levels. Results are discussed in the light of mother-conceptus relationships.

Foetal allografts survive in the uterus and are normally delivered notwithstanding their burden of histocompatibility antigens which partly differ from those of the mother[1,2]. Several mechanisms have been postulated which might account for this unexpected immunological behaviour, but up to date no conclusive evidence has been presented. Nevertheless, immune reactions do occur in pregnancy, as has been demonstrated either in normal or in pathologic conditions[3-5]. Among the factors which are possibly involved, the complement system is receiving increasing attention[6-10]. Thus, the early finding[6] of a significantly lower level of hemolytic activity in the sera of pregnant women has been confirmed in serum samples from late pregnancy[7], while a slight but steady increase in hemolytic potency has been recently reported in a prospective study of uncomplicated human pregnancies[8,9]. Propp and Alper[10] found augmented amounts of C3 in the serum of pregnant women at the time of parturition and their results have been widely confirmed[8,9].

Many features of the complement system have recently been clarified[11]. It is now well established that besides the classical pathway of complement activation, initiated by the assembly of the C1q–C1r–C1s complex on altered sites of the immunoglobulin Fc region, other pathways do exist which bypass the so-called early components (C1,

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4, 2) entering the classical sequence at C3 level[12]. Our interest in the alternative (properdin) complement pathway has led us to evaluate its role in several clinical situations[13,14]. In the present investigation we have examined the activity levels of such a pathway in sera of women in progressive stages of pregnancy, and in pregnant mice belonging to outbred or inbred matings, as compared, respectively, to healthy women and control animals.

Material and methods. Human serum samples, were obtained from 51 women with uncomplicated pregnancy, followed from the 10th week of gestation to 7 days post partum, and from 43 healthy (non-pregnant) women. Blood was allowed to clot no longer than 2 h and the sera stored at −70 °C.

Mouse serum samples were obtained from 45 pregnant mice belonging to inbred (I) or outbred (Swiss-Webster) strains. Females were mated with males of the same strain and examined daily for vaginal plug. Pregnant mice were isolated and, at different time intervals, blood samples were collected and sera stored as mentioned. Control non-pregnant females were kept under identical environmental and dietary conditions.

Erythrocytes. Guinea-pig red cells (for alternative C pathway) or sheep red cells (for classical C pathway) were mixed with an equal volume of Alsever's solution containing 100 µg each of penicillin and streptomycin per ml and stored at 4 °C for several days prior to use. For each experiment, an appropriate volume of the cell suspension was centrifuged and washed 3 times in the cold with veronal buffer containing 0.1% gelatin. The buffer was made 2.5×10^{-4} *M* in Mg^{++} and 1×10^{-3} *M* in Ca^{++} (VBS-Me^{++}) for classical complement pathway assay, or deprived of both ions, and made up by addition of Na-ethylenediaminetetraacetate (EDTA) to a final concentration of 0.02 *M* (VBS-EDTA) for alternative complement pathway evaluation. Each cell suspension was standardized to contain 7.5×10^7 final cell concentration.

Cobra venom factor (CVF) was purchased from Cordis Laboratories (Miami, USA). Large pools of the lyophilized product were prepared and aliquots stored at −70 °C.

Classical complement pathway (CCP), the methods described by MAYER[15] were followed with slight modifications; 1 ml of optimally sensitized sheep red cells at a concentration of 7.5×10^7 were incubated (60 min at 37 °C) with an equal volume of test serum at various dilutions. The tubes were thoroughly mixed, centrifuged and the lysates were analyzed for hemoglobin spectrophotometrically. The serum hemolytic potency was expressed in terms of CH50 units/ml.

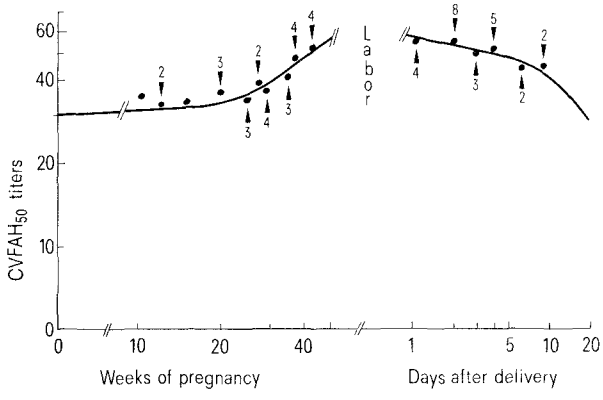
Alternative complement pathway (ACP) was investigated through a simple hemolytic assay based upon the interaction of a purified cobra venom protein with C3 proactivator and other serum factors. The extent of this interaction is reflected by lytic degree of unsensitized erythrocytes in the presence of fresh serum treated with EDTA. The procedure was carried out in 2 steps: in the first step, 0.2 ml of CVF were incubated (30 min at 37 °C) with equal volumes of test serum at various dilutions; in the second step, 0.3 ml of guinea-pig serum rendered 0.02 *M* with respect to EDTA, as source of late C components, and 0.5 ml of the standardized suspension of guinea-pig erythrocytes in VBS-EDTA were added. The reaction mixtures were incubated for 60 min at 37 °C and the tubes chilled in an icewater bath and diluted with 2.0 ml of chilled 0.15 *M* NaCl containing 0.1% gelatin. The lysates were analyzed as mentioned and the serum hemolytic potency was expressed in terms of cobra venom activable factors hemolytic units 50 (CVFAH50), according to BRAI and OSLER[16].

Results and discussion. Human sera from 43 healthy women and 51 pregnant subjects were tested for classical (CCP) and alternative (ACP) complement pathway activities. As shown in Table I, while the serum hemolytic potency referred to ACP attains in pregnancy high values which significantly differ ($p < 0.001$) from those observed in healthy subjects, the two groups do not differ with

Table I. Serum titers of alternative and classical complement pathways in human pregnancy

C-pathway	Serum donors		Student *t*-test (*p*)
	Healthy women	Pregnant women	
Alternative[a]	30.5 ± 7.9	46.7 ± 8.5	< 0.001
Classical[b]	154.6 ± 22.4	172.7 ± 38.8	> 0.3

[a] Expressed in terms of CVFAH50 units/ml. Mean ± SD.
[b] Expressed in terms of CH50 units/ml. Mean ± SD.



Relationship between alternative C pathway activity levels and gestation time course. Regression line drawn from 51 human pregnancies. Each point represents the arithmetic mean. The number of samples contributing to each value is indicated by arrows.

Table II. Serum titers of alternative complement pathway in mouse pregnancy

Groups	CVFAH50 units/ml (Mean ± SD)	Student *t*-test (*p*)
A) Pregnant animals (outbred mating SW × SW)	111.2 ± 37.9	A vs B < 0.5
B) Pregnant animals (inbred mating I × I)	106.4 ± 26.8	A vs C < 0.001
C) Non-pregnant animals	83.6 ± 18.1	B vs C < 0.001

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respect to CCP levels. A marked increase in ACP hemolytic titers was found to occur in the last few weeks of gestation (see Figure), thus suggesting that pregnancy time-course and ACP titers are closely related, as also seen in the significative correlation coefficient ($r = 0.61$; $p < 0.02$). The results of the hemolytic assays were further strenghtened by the rocket immunoelectrophoresis finding of higher C3-proactivator concentration in sera of pregnant subjects.

In an effort to extend these observations, and to reduce the variability inherent to data on hospitalized patients, we investigated ACP levels in pregnant mice. As shown in Table II, the trend in mice is fully consistent with the one found in humans. Sera from pregnant mice display a higher hemolytic efficiency which significantly differs ($p < 0.001$) from the one of the control sera. Somewhat surprizing was the likeness of the hemolytic behaviour among animals belonging to allogeneic and syngeneic pregnancy groups.

Increase in ACP and in C3-proactivator concentration, as it occurs in pregnancy, may be interpreted, from an immunologic viewpoint, as reflecting the preferential depletion of selected ACP components, namely properdin, properdin-convertase and factor D (C3PAse), as a consequence of their consumption by weak but continuous humoral or cellular immune reactions. This hypothesis suggests that antigenic differences between mother and conceptus are of relevance in pregnancy, as mainly supported by several data, such as hypertrophy of the regional uterine lymph nodes, which attest to the pregnant female's awareness of her fetuses[2]. On the other hand, it cannot be excluded, pregnancy being characterized by a different hormonal balance, that hormones more than immune reactions are responsible for the observed patterns. To cite an example, it is well established that sex-hormones influence the homeostasis of certain plasma proteins[17] among which, and to a higher extent, late-acting complement components[18,19], and acute phase reactants may attain twice the normal levels during preparturition period[20]. Consistent with this opposite view, our findings of an identical behaviour in inbred and outbred pregnancies cast doubts on whether hystocompatibility differences should be considered partially responsible for the high ACP-activation found in pregnancy. However, even in inbred pregnancy, mother and conceptus may present different antigenic specificities, due to foetal antigens and/or to sex-linked determinants.

It is difficult and unproductive, at this preliminary stage of our investigation, to relate present findings to any one of the several physiological changes which occur in pregnancy. Conclusive evidence must await more extensive studies on humans and on suitable animal strains with well defined major and minor hystocompatibility differences. The possible relevance of alternative and classical complement pathway behaviour in monitoring pregnancies, either in normal or in pathologic conditions should be emphasized.

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Mast Cells in the Pinna of Balb/c 'nude' (nu/nu) and Heterozygotes (nu/+) Mice[1]

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Summary. The relative number of mast cells in the ear lobes' skin (pinna) of nude (athymic) nu/nu and normal (thymic) nu/+ heterozygotes of Balb/c mice was similar. The results obtained contradict some suggestions about the general influence of the thymus on the number of mast cells in the skin and suggest the existence of some local factor(s) in regulation of skin mast cell numbers.

Although there is some evidence that the thymus may contain precursors of mast cells (MC)[4–6], it is hard to accept the hypothesis that the precursors of these cells are of thymic origin. Csaba et al.[7] reported a decrease of circulating MC in the blood after neonatal thymectomy of rats, but such an effect was not observed by Walker[8] in the mouse. Viklicky[9], in extensive experiments employing chimeras, showed that precursors of mouse MC are radio-resistant reticular cells. The presence of MC in athymic 'nude' mice seems to be the best evidence against the concept of thymic origin of MC[10]. It was found that in the skin of 'nude' mice there is nearly three times more MC than in normal animals[10,11]. The abundance of MC in the skin of 'nude' mice lead Viklicky et al.[10] to the conclusion that the frequency of MC is regulated in some way by the thymus. The absence of this regulation in athymic mice is, according to them, responsible for the high frequency of MC in their mice.

Searching for the role of thymus in regulating lymph node mast cell populations, we examined a number of popliteal lymph nodes of normal and athymus 'nude' Balb/c mice, but we could not demonstrate significant differences in the absolute number of MC between these animals. As an additional control, we have analyzed the relative number of MC in the 'nude' and normal Balb/c mice. This analysis was performed on the pinna (the auricle of the ear), as this organ seemed to be more uniform and thinner, thus making the quantitation of MC

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