Protein Synthesis in Human Leukocytes and Lymphocytes:  
1. Effect of Steroids and Sterols

By Seymour Werthamer, Carl Hicks and Leonard Amaral

The in vitro culture of rabbit lymphoid cells in a cortisol containing medium has been demonstrated to result in the inhibition of lymphoid protein and RNA synthesis. In liver cells, however, this hormone increases both RNA and protein synthesis, while inhibiting the synthesis of DNA, thus suggesting the target cell specificity of this hormone. In man, administration of cortisol or cortisol analogs, have been shown to result in lymphopenia. Inasmuch as cortisol is well known to be a "lympholytic agent," the possibility that lymphopenia results from the inhibition of lymphocytic protein synthesis and that this inhibition ultimately results in the destruction of the lymphocyte, seemed feasible. Furthermore, cortisol is found to exist in the plasma at a specific level and in equilibrium with its binding plasma protein, transcortin. This latter point would suggest that if cortisol does inhibit the protein synthesis of human lymphocytes, its normal level in the plasma may act as regulator in the control of the number of lymphocytes. This regulation perhaps, may be the result of a shift of the ratio of plasma levels of cortisol to its binding protein. We have therefore undertaken the investigation of the in vitro effect and mechanism of action of cortisol and related steroids and sterols on protein synthesis of human mixed leukocytes and purified lymphocytes. This paper presents the results of the first phase of our investigation.

Materials and Methods

Materials

Cortisol, Prednisolone and Testosterone were purchased from Sigma, Sigma and Vitarine, respectively. 3-Methyl Cholanthrene and Cholesterol were generously provided by Drs. Leo Sokind and Julius J. Carr, respectively. C-14-L-leucine, specific activity of 240 mC/mM, was purchased from International Chemical Nuclear Corporation. Eagle's TC 199 was purchased from Difco.

Preparation of Suspensions of Normal Human Leukocytes and Lymphocytes

All glassware used for preparations and incubations was siliconized and sterilized. Whole human blood, donated by healthy employees of Methodist Hospital, was heparinized (20 units per ml. of whole blood) and allowed to sediment for a period of two hours at 37 C. in 5 ml. pipettes. After the sedimentation period, the upper two thirds of the sedimented plasma was siphoned off, pooled, and centrifuged at 150 g. for 10 minutes. The supernatant plasma was gently removed with a Pasteur pipette—leaving just enough plasma to
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Cover the cap of leukocytes. The plasma was subsequently centrifuged at 1500 g. for 20 minutes. This step was sufficient to remove all the platelets from the plasma. For the cultures to contain leukocytes, the cap of leukocytes was gently suspended in a volume of platelet-free autologous plasma which would result in a cell number of $11 \times 10^6$ per ml. For pure lymphocyte cultures the cap of cells was gently suspended into a final volume of 3 ml. of platelet-free plasma, and a pure suspension of lymphocytes, slightly contaminated by red blood cells was obtained according to the procedures described by Rabinowitz. The eluted lymphocyte suspensions were adjusted to $6.0 \times 10^6$ cells per ml. of plasma. The use of a single sample of 80 ml. of whole blood yielded enough cells for any one experiment. For those experiments involving the use of saline-washed cells resuspended in plasma or cells cultured in the absence of plasma, the following procedures were used:

After obtaining the adjusted lymphocyte suspensions, 0.6 ml. of the suspension was placed into culture tubes and 2 ml. of saline was added. The culture tubes were then centrifuged at 200 g., the supernatant discarded, the cap of cells suspended and washed with 2 ml. of saline, centrifuged at 200 g., the supernatant subsequently discarded. This wash procedure was repeated once. For the cultures involving the use of saline washed cells resuspended in plasma, platelet-free plasma was added to the culture tubes to a final volume of 0.6 ml. For those cultures involving the absence of plasma, the final volume of 0.6 ml. was achieved with saline. The cells for both cultures were subsequently suspended and incubated under the conditions to be described. All experiments were performed in duplicate or triplicate cultures.

Description of Culture Composition and Incubation Period

The following are the components of a final 3 ml. culture: 2.0 ml. of TC 199 (containing 300 units of penicillin and 3 µg of 14C-leucine), 0.4 ml. of hormone or sterol (previously dissolved in saline — 2 per cent ethanol—to yield a final concentration per culture of $10^{-4}$, $10^{-5}$, or $10^{-4}$ M. The controls received 0.4 ml. of saline — 2 per cent ethanol), and 0.6 ml. of leukocyte or lymphocyte suspension. These cultures were incubated at a 45° angle for a period of 2 hours at 37, 25 and 4 C. This 2 hour period of incubation represents the earliest response of the cells to the experimental agents used at 37 C. For those cultures involving the use of two agents, the final concentration of each was $10^{-5}$ M.

Determination of Viability

The use of the trypan blue exclusion test for viability was routinely carried out after each of the above steps (i.e., before column, after column, after centrifuging and after incubation). After each of the procedural steps, the viability, as judged by the criterion of trypan blue exclusion, remained upwards of 98 per cent, demonstrating that little detectable injury had occurred.

Procedures Used After the Incubation of Cultures

At the end of the incubation periods, the cultures were placed in an ice bath and cooled to 0–4 C., centrifuged at 200 g. for 10 minutes, the supernatant discarded, the pellets gently suspended in 2.0 ml. of 1 per cent acetic acid to disrupt the erythrocytes present, centrifuged at 200 g. for 10 minutes and the supernatant discarded. Three successive saline washes and centrifugations (as before) followed. The pellets of cells were resuspended in 1.0 ml. of 0.1 N NaOH resulting in the dissolving of the cells. These were then neutralized with the equivalent amount of 0.1 N HCl, placed in an ice bath, the total protein precipitated with a final concentration of 5 per cent TCA, the precipitate subsequently collected and washed on Millipore filters (0.45 µ pore size). These filters were then mounted on 2 inch planchette and dried under infrared lights. The radioactivities of the above were obtained by the use of a Tracerlab 132 M Scaler gas flow counter (efficiency of 38 per cent). The data presented are the per cent alterations of the average total counts per minute per set of duplicate or triplicate cultures to that of their controls. In all cases the variations in CPM within triplicate cultures did not exceed 4 per cent.
Fig. 1.—The effect of steroids and sterols on the incorporation of $^{14}$C leucine into human leukocyte total protein. An average of $6.5 \times 10^6$ leukocytes suspended in 0.6 ml. of plasma were incubated at 37 C. under conditions described in the text. The percent inhibitions noted are the averages obtained from cultures of 9 different cell donors. All incubations were performed in triplicate. The final molar concentration of each agent in the culture was $10^{-5}$, p < .001 for each series of experiments. Incubations performed at 25 and 4 C. resulted in no significant inhibitions.

Fig. 2.—The effect of sterols and two concentrations of steroids on the incorporation of $^{14}$C leucine into lymphocyte total protein. Incubations are described in text. Results obtained at $10^{-5}$ M and $10^{-4}$ M of steroid are presented as upper and lower bars, respectively. The per cent inhibitions noted are the averages obtained from cultures of 11 different cell donors. All incubations were performed in triplicate. p < .001 for each series of experiments.

RESULTS

The effects of the hormones (cortisol, prednisolone, testosterone) and the sterols (3-methyl cholanthrene, cholesterol) on the incorporation of $^{14}$C-leucine into leukocyte total protein are inhibitory at 37 C. (Fig. 1). No inhibitions were observed when incubations were performed at 25 and 4 degrees centigrade. Inasmuch as the leukocyte population is composed of various cell types, the above inhibitions may be the result of a summation of unknown responses of each cell type thus leading to the possibility that distinct members of the leukocyte population may each respond in a different manner to the agents employed. To test this possibility, pure suspensions of lymphocytes, obtained by the methods of Rabinowitz,6 were incubated under the conditions described in the text. The results presented in Figure 2 indicate that the
agents inhibited amino acid incorporation into lymphocyte total protein. Increasing the concentrations of the steroids resulted in no increase in the percent inhibitions of amino acid incorporation. A comparison of Figures 1 and 2 indicate that the inhibitory responses of the lymphocytes to testosterone and 3-methyl cholangrene are of a greater magnitude than those produced in the leukocyte incubations. This comparison suggests that some specific cell type of leukocyte population must be more resistant to the action of these two agents than lymphocytes. By far, the largest portion of this normal non-lymphocyte, leukocyte population is the neutrophil (90 per cent).

The procedures described by Rabinowitz would afford the separation of a pure suspension of neutrophils which could be cultured under the conditions employed for both leukocytes and lymphocytes. However, it has been our experience (data to be presented elsewhere) that the elution of neutrophils from glass bead columns by EDTA results in a gross alteration in the ability of such eluted cells to incorporate the \(^{14}\)C amino acid into protein. Although no direct method is presently suitable for the determination of the response of neutrophils to the agents employed in our study, the use of the following procedures and mathematical calculations affords the deduction of such response.

A prepared leukocyte suspension was divided equally into two parts, A and B. The total number of leukocytes and a cell differential analysis was determined for A. Triplicate control and experimental leukocyte cultures of known and equal cell number were prepared. The cells of part B were used for the preparation of triplicate control and experimental lymphocyte cultures of known and equal cell number. Incubations and determination of the response of the neutrophils of the leukocyte cultures to the agents employed was determined by the following mathematical calculations:

\[
P = \frac{\text{CPM of lymphocyte total protein}}{\text{lymphocyte}}.
\]

\[
Y = \text{The number of lymphocytes in the leukocyte culture which equals the per cent of lymphocytes } \times \text{ leukocyte number divided by 100}.
\]

\[
P \times Y = Z \quad \text{which equals the total counts per minute of total protein contributed by the lymphocytes present}.
\]

\[
W = \text{The counts per minute of total leukocyte protein of that set}.
\]

\[
W - Z = X \quad \text{The counts per minute of total protein of the non-lymphocyte}.
\]

Performing these calculations for each of the leukocyte and lymphocyte culture controls and their corresponding experimental sets allows one to deduce the effects of the agents on the non-lymphocyte (neutrophils) portion of the leukocyte population. The results of such experiments and calculations are presented in Figure 3. A comparison of the Figure to Figure 2, suggests that in reference to testosterone and 3-methyl cholangrene, the inhibitions noted in the lymphocyte population are more marked than that observed in the non-lymphocyte (mainly neutrophil) population. Table 1 serves to readily illustrate this comparison.

Inasmuch as cortisol, testosterone, 3-methyl cholangrene and cholesterol bind to plasma proteins, the possibility was investigated that the inhibitions of amino acid incorporation produced by these agents is dependent
Table 1.—Comparison of the Effects of Agents Tested on the Incorporation of 14C Leucine into Total Protein of Lymphocytes and Neutrophils

<table>
<thead>
<tr>
<th></th>
<th>Lymphocyte</th>
<th>Neutrophil</th>
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<tbody>
<tr>
<td>Control</td>
<td>21.4±1.4</td>
<td>22</td>
</tr>
<tr>
<td>Cortisol</td>
<td>14.6±1.7</td>
<td>17.0±4.3</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>17.8±1.9</td>
<td>9</td>
</tr>
<tr>
<td>Testosterone</td>
<td>17.0±2.5</td>
<td>8</td>
</tr>
<tr>
<td>3-Methyl Cholanthrene</td>
<td>22.0±4.3</td>
<td>21</td>
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Fig. 3.—The deduced effect of sterols and steroids on the incorporation of 14C leucine into neutrophil total protein. Methods involved in the determination of the data presented are the averages obtained from the triplicate incubations of cells from 3 different individuals.

Fig. 4.—The effect of sterols and steroids on the incorporation of 14C leucine into total protein by normal human lymphocytes cultured in the absence of plasma. Conditions of culture are described in text. The results presented are the averages obtained from triplicate cultures of cells from 3 different individuals.

upon the presence of plasma. The results presented in Figure 4 suggest that the inhibitory actions of the three hormones on the incorporation of amino acid into lymphocyte total protein are totally dependent upon the presence of plasma. The sterols, 3-methyl cholanthrene and cholesterol, under these conditions, still inhibit amino acid incorporation. However, in the case of
cholesterol, the magnitude of inhibitions is not as great as that observed in the presence of plasma (compare to Figure 2).

To eliminate the possibility that in these experiments the washing of the cells in saline results in the loss of the response to the action of the hormones, lymphocytes were washed in saline as those of the above experiment and subsequently cultured in a medium containing plasma. The results presented in Figure 5 illustrate that the washing of the lymphocytes in saline does not prevent the inhibitory actions of the hormones. Instead, saline washes appear to result in an increase in the magnitude of the inhibitions of amino acid incorporation.

Because the manner by which mediation of the inhibition of lymphocyte amino acid incorporation is different between the hormones and sterols, the possibility was investigated that their site of inhibition may be different. The hypothesis tested in this experiment is that since the increase in the concentration of the hormones which has been observed to result in no increase in the per cent inhibition produced (see Figure 2), any significant increase resulting from the simultaneous use of any two agents whose final concentrations in that culture were those previously tested \(10^5 \text{ M}\) over that produced by each alone indicates a difference in their site of action. The results presented in Figure 6 suggest that 3-methyl cholanthrene differs from all the others in its site of action as only 3-methyl cholanthrene, in conjunction with either cortisol or testosterone, results in an increase in the per cent inhibition. Since these increases are about equal to the sum of the per cent inhibitions expected (about 38 per cent and 35 per cent for 3-methyl cholanthrene and cortisol and 3-methyl cholanthrene and testosterone respectively), the inhibitory site of action of 3-methyl cholanthrene must be different from that of the hormones.

**DISCUSSION**

The results presented in this paper suggest that the hormones, cortisol, its analog prednisolone and testosterone, and the sterols, 3-methyl cholanthrene, and cholesterol, inhibit the incorporation of amino acid into human lymphocyte and leukocyte, total protein. That these are indeed inhibitions
and not the loss of radioactivity resulting from cell injury, death or lysis, caused by either the agents employed or the manner of handling, is evident from the following:

1) Trypan blue exclusion tests and cell counts of the cultures following the incubation periods indicate that the agents employed were neither injurious or cytolytic.

2) TCA-tungstate precipitation of all cell washes normally discarded including the 1 per cent acetic acid supernatant yielded no activities indicating no detectable loss of labelled protein during these procedures.

Previous studies have demonstrated that cortisol and hydrocortisone inhibit protein synthesis of lymphoid cells. Furthermore, prednisolone as well as hydrocortisone inhibits the responses of the lymphocyte to phytohemagglutinin. The results obtained in our study are, in this respect, in agreement with those cited. However, whereas Makman et al. found no dependence upon serum for the inhibitory effect produced by cortisol we have observed a total dependence. Interestingly, in our studies, both testosterone and the steroid precursor, cholesterol, inhibit the synthesis of lymphocyte protein suggesting the possibility that all sterols whose nuclear structure is not too dissimilar may have the same inhibitory effects. This suggestion appears plausible in that 3-methyl cholangrene also has been noted in our studies to have such an effect (although it apparently acts at a different site).

Transcortin, a cortisol binding protein exists at a molar concentration of about 10^{-7} M in normal plasma (calculated from the data presented by Seal and Doe). Their studies have shown that this protein binds the hormone at
equimolar concentrations. From the binding constant provided by these and other studies, the final concentration of cortisol required for the saturation of binding is of the order of $10^{-3}$ M. At a concentration of $10^{-4}$ M, cortisol did not enhance the inhibition value observed at $10^{-5}$ M. Since cortisol, in the absence of plasma, did not produce inhibition of amino acid incorporation, our results suggest, in the light of the transcortin data, that the inhibition of amino acid incorporation by lymphocytes is mediated via a transcortin-cortisol complex. A similar mechanism may also apply for prednisolone and testosterone.

Testosterone has been demonstrated to bind with different affinities to different plasma proteins. Whether or not cortisol and testosterone compete for transcortin binding sites is not presently known. However, if the two steroid protein complexes were to inhibit at different sites, it would be expected that since an increase in either steroid results in no increase in its inhibitory effect, any increase noted when both steroids are present, would suggest a difference in their site of action. Our results suggest a common site of action since the per cent inhibition in these cultures never exceeded that produced by the more potent inhibitor. Unlike these results, 3-methyl cholanthrene when present in conjunction with either of the above steroids, resulted in an increase in the present inhibition. 3-methyl cholanthrene was also observed in our studies to inhibit amino acid incorporation in the absence of plasma. These results therefore suggest that 3-methyl cholanthrene inhibits incorporation by means of a different mechanism and at a different site of the lymphocyte.

In conclusion, the in vitro inhibition of lymphocyte protein synthesis by a cortisol-plasma protein complex suggests that such a complex, normally present in the human vascular system, may act as a regulator of lymphocyte protein synthesis. The molar amount of the cortisol binding protein is normally higher than that of cortisol, therefore, administration of cortisol would result in the increased formation of such complexes and the condition of lymphopenia would occur as the result of the inhibition of lymphocyte protein synthesis.

**Summary**

The in vitro effects of sterols, cholesterol and 3-methyl cholanthrene and steroids, cortisol, prednisolone and testosterone on protein synthesis in separate populations of human lymphocytes and leukocytes has been investigated. It has been shown that all agents used result in the inhibition of protein synthesis under these conditions. It has also been shown that the inhibitory mechanism of the steroid hormones requires the presence of plasma, presumably as a protein binding factor in order to achieve its effect. The sterol, cholesterol and 3-methyl cholanthrene, in the absence of plasma, still inhibit amino acid incorporation. However, in the case of cholesterol, the magnitude of inhibition is lower than that observed in the presence of plasma, perhaps indicating a partial plasma dependence. The results presented therefore support the hypothesis that the inhibition of lymphocyte protein synthesis by steroid hormones occurs only when the steroid is bound to a plasma protein. The physiologic role of the plasma protein-cortisol complex and its relation to the condition of lymphopenia in man is discussed.
SUMMARIO IN INTERLINGUA
Esseva investigate le effectos in vitro de steroles—cholesterol e 3-methyl-cholanthrena—e de steroides—cortisol, prednisolona, e testosterona—super le synthese de proteina in separate populationes de lymphocytes e leucocytes human. Esseva mostrare que omne le agentes usate resultava in le inhibition del synthese de proteina sub iste conditiones. Esseva etiam monstrare que le mechanismo inhibitori del hormones steroide require le presentia de plasma, presumitemente como un factor de ligation proteinic pro effectuar iste resultato. Le sterol—cholesterol e 3-methyl-cholanthrena—in le absentia de plasma inhibiva ancora le incorporation de amino-acido. Tamen, in le caso de cholesterol, le magnitude del inhibition esseva plus basse que illo observate in le presentia de plasma, possibilemente in consequentia de un partial dependentia ab le plasma. Le resultatos hic presentate supporta consequentemente le hypothese que le inhibition del synthese lymphocytic de proteina per hormones steroide occurre solo quando le steroide es ligate a un proteina de plasma. Le rolo physiologic del complexo de proteina plasmatic e cortisol e su relation con le condition de lymphopenia in humanos es commentate.

ACKNOWLEDGMENTS
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