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GLUCOCORTICOID RECEPTORS, DNA SYNTHESIS, MEMBRANE ANTIGENS AND THEIR RELATION TO DISEASE ACTIVITY IN CHRONIC LYMPHATIC LEUKEMIA

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SUMMARY

Chronic lymphatic leukemia (CLL) has a very variable clinical course. Laboratory parameters which may be of prognostic value are badly needed. We have analysed chronic lymphatic leukemia cells from 27 patients for the following parameters: cytoplasmic glucocorticoid receptors (GR), cell surface immunoglobulin, complement and sheep red blood cell receptors, concanavalin A (ConA) agglutinability and for proliferative activity with tritiated thymidine (^{3}H-Tdr) uptake. The two latter parameters have been considered by others to be indicative of aggressive disease.

All CLL cases except one were of B-lymphocyte origin as judged from the surface markers. Relatively few cases (2/9) had ConA agglutinable cells. Cells from 17 patients showed significant levels of GR. Patients lacking GR generally showed no disease progression and comparatively low labelling index for ^{3}H-Tdr. Seven cases could be followed by serial blood samples during various phases of the disease and during glucocorticoid-treatment. In GR containing cells, the therapy resulted in decreased ^{3}H-Tdr uptake but only the 5 patients with an active disease showed an objective remission.

INTRODUCTION

Chronic lymphatic leukemia (CLL) shows a variable clinical course. Methods are needed which could predict the course of the disease and the correct timing of antileukemic therapy. Several laboratory techniques have been suggested but their relevance is presently unknown. We have initiated a multiparameter investigation on unselected patients [1, 2]. Apart from clinical evaluation and routine haemotology evaluation, cell surface markers (immunoglobulin (Ig), complement (C) receptor, Fc receptor and sheep red blood cell (SRBC) receptor), agglutinability with concanavalin A (ConA) and cytoplasmic glucocorticoid receptor (GR) were measured. The proliferative cell fraction was analysed with ^{3}H-thymidine (^{3}H-Tdr) uptake.

Several of these parameters were also studied in relation to glucocorticoid treatment.

MATERIAL AND METHODS

Subjects

Twenty-seven unselected cases of CLL were studied. The main criteria for diagnosis were a peripheral mature lymphocyte count over 5000/mm^3 and that at least 30% of the non-erythroid nucleated cells in the bone marrow were mature lymphocytes. Six patients were classified as having active (progressive) disease as defined by the NCI committee for studies on CLL [3].

At the time of analysis none of the patients was treated with cytostatics and 22 of the 27 patients had never received such treatment. In patients under treatment with prednisolone (11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione), the steroid was withdrawn 24 h before the blood sample was taken.

A clinical response to glucocorticoid therapy was considered to be present when peripheral white blood cell counts, haemoglobin concentration and general symptoms changed in a favourable direction.

Cell surface markers

(For details see ref. 1)

Cell surface immunoglobulin (S-Ig) was detected by indirect immunofluorescence using rabbit antihuman serum and fluoresceine-labelled porcine antirabbit serum. Complement (C) receptors were assayed with C coated sheep red blood cells. A rosette technique was used for the detection of SRBC receptors. Concanavalin A induced agglutination was analysed in a celloscope and the percentage cells trapped in agglutinates was estimated.

Thymidine uptake

Triplicate cultures of 2×10^6 cells in 2 ml prewarmed (+37°C) F-10 medium supplemented by 10% post-natal calf serum and 2 μCi of ^{3}H-Tdr (S.A. 5 Ci/mmol, Radiochemical Centre, Amersham, England) were incubated for one h at 37°C in a 5% CO_2-in-air atmosphere. The ^{3}H-Tdr incorporation was assayed by liquid scintillation as described by Ghetie *et al.* [4] or by autoradiography. Then, 1 ml of the cell suspension was centrifuged onto microscopic slides

by a Cytocentrifuge (Shandon Scientific Ltd., London). After being dried in air, washed in PBS and fixed in methanol–acetic acid (3:1 v/v) the slides were coated by Kodak AR 10 stripping film. After exposure for 8 days at +4°C, films were developed in Kodak D-19B. The coated slides were finally stained in 0.05% (w/v) toluidine blue for 5 min. ^{3}H-Tdr incorporation was estimated by counting 3000 cells.

Glucocorticoid receptor studies

Peripheral blood was defibrinated and transferred into 100 ml tubes. The tubes were cooled to +4°C and all subsequent operations were done at that temperature. Dextran T500 (Pharmacia, Uppsala, Sweden) was added to a final concentration of 5% (w/v). The tubes were kept in vertical positions for 60 min. The sedimented erythrocytes were discarded and the supernatant containing the leukocytes was spun at 600 *g* for 10 min. The supernatant was discarded and the pellet was resuspended in 40 ml 0.2% NaCl, rapidly followed by additional 40 ml 1.6% NaCl. The mixture was spun down again and the whole procedure was repeated once. This treatment lysed almost all contaminating erythrocytes. (Smears showed, for this receptor study, an insignificant admixture of granulocytes).

The pellet containing >70% lymphocytes was homogenized in an all-glass homogenizer with 3 vol. of a buffer (0.01 M Tris, 0.0015 M EDTA, 0.25 M sucrose and 0.01 M dithioerythritol of pH 7.4). The homogenates were centrifuged at 130,000 g_{av} for 1 h. The supernatant (= the cytosol) was obtained by punching the tubes and was stored at −70°C until analysed for receptor content. Protein was determined with the Folin reagent according to Lowry *et al.* [5].

Incubations for receptor assay were performed in 1 ml glass tubes. The tubes contained 100 μl of incubation buffer (the homogenization buffer supplemented with 10% glycerol), ^{3}H-dexamethasone (NEN Chemicals, Dreieichenhain, Germany) (10^{-9} M final concentration) and non-radioactive dexamethasone (Sigma, St. Louis, Mo., U.S.A.). Cytosol (25 μl) was added to the tubes and the mixture was incubated for 16–17 h at +4°C. The incubation was terminated by adding 100 μl of a charcoal suspension (0.5% Norite A (Sigma Co.) and 0.05% Dextran 10 (Pharmacia) in incubation buffer) which binds free steroid. The tubes were shaken on a vortex mixer, allowed to stand for 5 min in the cold and finally centrifuged for 30 s in a Microfuge (Beckman, Palo Alto, Calif., U.S.A.). One hundred μl of the clear supernatant was withdrawn and the content of radioactivity was measured. All runs included blanks without cytosol and blank values were substracted from experimental values, thus giving receptor bound steroid. All cytosols were incubated with logarithmic dilutions of unlabelled dexamethasone, generally 10^{-10}, 3×10^{-10}, 10^{-9}, 3×10^{-9}, 10^{-8}, 10^{-7} and 10^{-6}M. The results were plotted on semi-logarithmic plots. The binding of dexamethasone reaches a saturation plateau at 10^{-7}–10^{-6} M and residual binding at those concentrations is considered non-specific. The concentrations at half-maximum inhibition (ED_{50}) and the maximum suppressible binding was calculated from the plots.

RESULTS

The tests for surface markers indicated that all investigated cases except one were of B-cell origin. The exceptional case was also glucocorticoid receptor negative and showed no disease activity. This patient is not included in the following.

Analysis for glucocorticoid receptors was performed on cytosol derived from isolated leukocytes. Incubation with a fixed contraction of radiolabelled dexamethasone and increasing amounts of nonlabelled substance allows the determination of the ED_{50} (which will approximate the K_D) and the binding capacity. The mean apparent K_D for the 17 receptor positive leukemias was 3×10^{-9} M (range $3 \times 10^{-10} - 8 \times 10^{-9}$M). Fig. 1 gives an example of one receptor positive cytosol (Patient A.T.) and one receptor negative cytosol (Patient J.J.). In order to check that all parameters were kept constant for the experiment, a rat thymus cell cytosol was always run simultaneously.

The specificity of the binding was that expected for a glucocorticoid receptor. Thus, with the relative affinity of dexamethasone = 1, prednisolone was 0.04, hydrocortisone 0.03 and epi-cortisol 0.001. This is comparable to their relative glucocorticoid activity (see e.g. ref. 6).

Evidence for the presence of glucocorticoid receptors was obtained in 17 of the total of 22 tested cases B-cell derived leukemias. One patient gave a marginal value and was excluded from the receptor positive and receptor negative groups. Only 4 cases out of 19 showed an increased labelling index (>0.2%). The relation between receptor positivity, disease activity and increased labelling index is shown in a Venn diagram (Fig. 2). It is clear that every case with disease activity had glucocorticoid receptor and that several

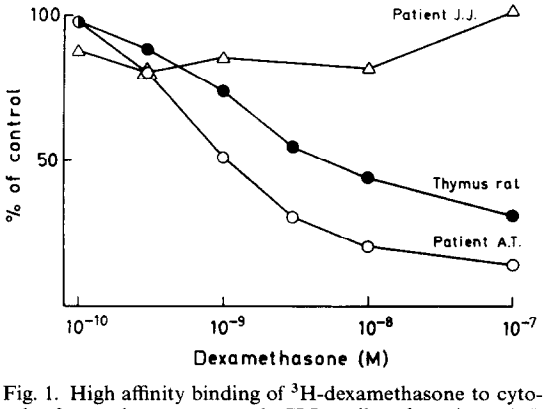

Fig. 1. High affinity binding of ^{3}H-dexamethasone to cytosol of rat thymocytes and CLL cells of patient A.T. Absence of such binding to CLL cells of patient J.J.

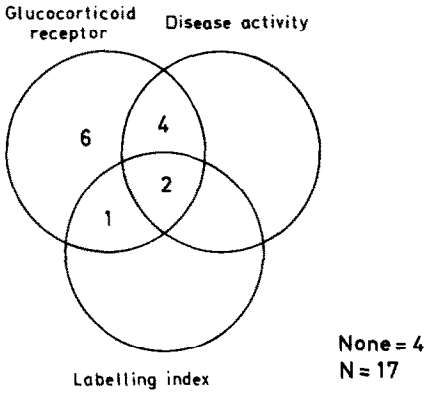

Fig. 2. Venn diagram showing distribution of CLL cases with respect to presence of glucocorticoid receptor, active disease and increased labelling index.

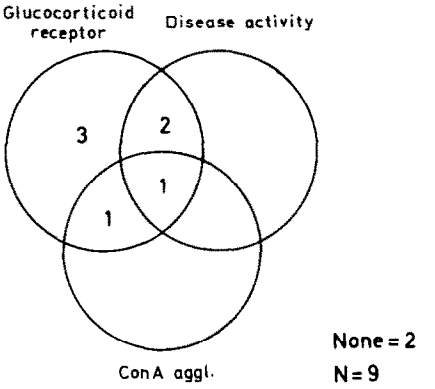

Fig. 3. Relation between glucocorticoid receptor, disease activity and increased ConA agglutinability.

cases with disease activity showed low labelling index. Similarly heterogeneity was observed among 9 patients analysed for ConA agglutinability. Only 2 showed values above the normal range. Both these cases had glucocorticoid receptor and one showed disease activity (Fig. 3). In 4 untreated patients, blood samples were obtained on more than one occasion with similar findings reached each time.

In six patients, the progress of the disease was such that glucocorticoid therapy was started. In these cases a blood sample was obtained before any treatment began. Then treatment with 30 mg prednisolone/day for about 3 weeks, 15 mg, 10 mg and finally 5 mg, each dosage regimen for about 2–3 weeks followed. A second blood sample was usually obtained under the 15 mg/day regimen and a third blood sample after the prednisolone treatment period had expired. Two patients were studied twice and three times, respectively. The clinical response was compared with the effect on DNA-synthesis (Fig. 4). A clinical response and a reduction in labelling index was observed in 4 of the 6 cases. One case did not respond at all.

When DNA-synthesis was analysed by impulse counting and this was used as the biochemical parameter for a response, there were 4 of the 6 cases which showed a response by both parameters and 2 which

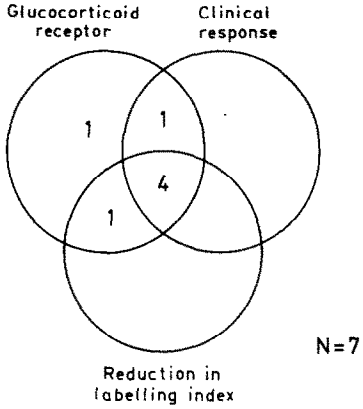

Fig. 4. Response to prednisolone therapy in CLL cases. The presence of glucocorticoid receptor at the start of therapy related to the clinically observable response and the reduction of labelling index.

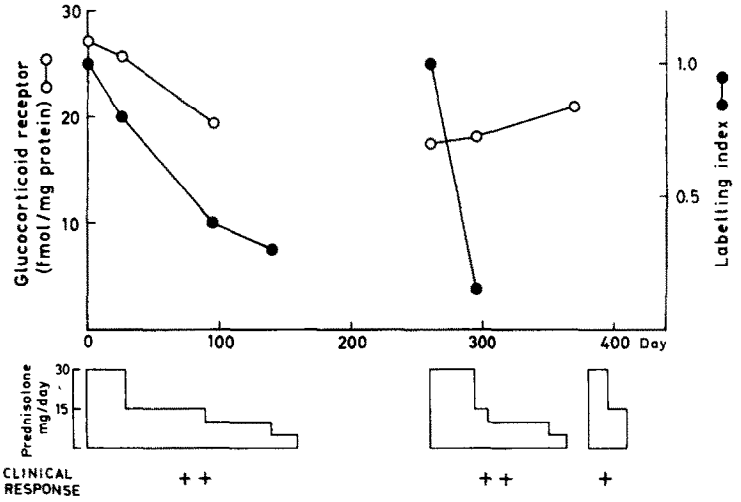

Fig. 5. The clinically observable effect of prednisolone therapy related to glucocorticoid receptor levels and labelling index of CLL cells from a single patient (L.A.).

only showed a reduced DNA-synthesis, (not shown in the Fig.). One patient (not shown) did not respond on prednisolone but responded when the treatment was supplemented with 2 single doses of chlorambucil 1 week apart.

One patient has been subjected to serial sampling on 9 different occasions. Some of the results obtained under prednisolone therapy are shown in Fig. 5. In each treatment session there was a clinical response and a reduction in labelling index (labelling index for 3rd session not shown). The levels of glucocorticoid receptors were rather similar during the whole observation period, which was also observed for the other patients.

DISCUSSION

The unpredictable course of CLL has prompted several investigators to study various laboratory parameters to characterize the disease. In accordance with data in the literature [7] we observe that CLL cells usually are of B-cell origin. Our findings here therefore only relate to B-cell derived CLL. It has been claimed that high agglutinability by ConA should be indicative of malignancy [8]. Our data (Fig. 3) do not support this hypothesis and are in line with findings by Glimelius *et al.* [9, 10]. They suggest that within most if not all groups of human tumors a spectrum of ConA agglutinability is found and that there is no absolute correlation between this parameter and malignancy. A high proliferative activity of the peripheral cells has also been considered to be indicative of disease activity [11]. This does not seem to hold in our series, although of 3 cases with increased labelling index 2 showed disease activity (Fig. 2).

Lymphocytes from 16 of the 27 tested patients had a measurable, saturable dexamethasone-binding, indicating the presence of a glucocorticoid receptor. The hormone concentration giving half saturation (ED_{50}) of the receptor is of the same order as observed for other glucocorticoid responsive cells [6]. The structural specificity of the binding is also that expected for a glucocorticoid receptor. Similar findings have been obtained by Simonsson on intact normal lymphocytes [12]. Gailiani *et al.* [13] studying 8 patients could not find any specific glucocorticoid binding in CLL cell cytosol. The most likely explanation for the discrepancy lies in the careful homogenization used here and the high sensitivity of our method. Homo *et al.* [14] could also demonstrate receptor in 19 cases of CLL. They used a whole cell technique and found the receptor levels to be generally lower than in normal lymphocytes. The importance of glucocorticoid receptor occurrence is uncertain at present, but our data indicate a lower disease activity in the patient lacking glucocorticoid receptor. It is obviously not yet possible to draw a conclusion about the prognostic value of this parameter, but the question is interesting since Terenius *et al.* [15] studying breast cancer patients found a higher mortality among pre- and peri-menopausal women with oestrogen receptor positive tumors and among post-menopausal women with receptor negative tumors.

The therapeutic effect of glucocorticoids in CLL is well documented [16, 17]. Taken as a group these patients, however, are heterogenous in their response to glucocorticoids since some recover while others are unaffected. Since the side-effects of glucocorticoid treatment are considerable it would be of great value to be able to predict the response of the individual patient. At present this is not possible

All the patients who were subjected to predisolone theraphy had receptor positive cells (Fig. 4). At present we can therefore not evaluate the results of therapy on a receptor negative case. In 6 cases a clinical response was observed and in 4 of these cases a reduction in labelling index was also present. Two cases did not respond according to the clinical criteria but interestingly, one of these cases showed a reduction in labelling index (Fig. 4) while the other showed a reduced DNA synthesis as measured by impulse counting of incorporated ^{3}H-Tdr. This observation is probably very important, since it indicates that although the cells are responding, the therapy might only have been successful had a minor change in the dosage regimen been carried out. One possible reason for a failure could be a big total tumor mass. In fact, one patient (not included in the data above) with glucocorticoid receptor positivity, who did not respond to glucocorticoids alone, went into remission when rather mild adjuvant therapy was installed. A very convincing relationship between clinical response and reduction of labelling index was observed in patient L.A. from whom 9 serial blood samples were obtained (Fig. 5). During the observation time, the apparent receptor content remained fairly stable which was a general observation.

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DISCUSSION

Munck. Some brief questions on methodology. We've had a lot of trouble, especially with homogenisation, determining whether or not lymphocytes break, and I would like you to comment on what checks you make that you get complete breakage of cells after glass homogenisation. The second point, is whether you check for the influence of endogenous steroid in the measurements you make particularly with treated patients. The third point is whether you have considered measuring nuclear as well as cytoplasmic receptors, since with lymphocytes it is very easy to incubate the cells at 37°C with tritiated steroids to carry out assays for both. That would provide you with the kind of information that often is thrown away.

Terenius. To answer your first question about methodology, we are using an all glass homogeniser and we have found that to be the most efficient way to disintegrate the cells without losing receptor activity. If you use a teflon/glass homogeniser you will get too little cell breakage. As to your second question we do not make any measurements on either endogenous or exogenous glucocorticosteroids. Hopefully, some of these are removed during the isolation of the lymphocytes. To your third question, we have done some studies on intact cells but this has not been done systematically mainly because of the amount of work that implies, but I think that is a very good suggestion. Of course, one has very little cytosol in these cells, and one might question what one really gets in the supernatant after homogenisation.