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Short Communication

Sex steroid binding to human lymphocytes plasma membrane

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Sex steroid hormones such as testosterone and oestradiol induce immunomodulation of the lymphoid system *in vivo* and *in vitro*, (Clemens *et al.*, 1979; Fuja Kotane, 1975; Kevorkov & Shvetsov, 1979) and cytosolic binding has been demonstrated in normal and pathological human lymphocytes, (Danel *et al.*, 1981). How the hormones enter the cells is controversial: a "passive" diffusion as described by Peck *et al.* (1973) or a "facilitated" diffusion as postulated by Milgrom *et al.* (1973).

Specific binding sites for steroid have been demonstrated on the plasma membrane of target cells (Nenci *et al.*, 1980; Pietras & Szego, 1977, 1980) and steroid binding on their membrane "receptors" induced cellular functions such as cAMP uptake (Chew *et al.*, 1974).

In the present study, human peripheral lymphocytes were characterized with regard to oestradiol membrane binding sites. In order to eliminate diffusion through the membrane and to study external binding, steroid was covalently bound to bovine serum albumin (BSA) to constitute a macromolecular complex which could not diffuse into the cells and this complex was fluoresceinated.

Human peripheral blood from 30 healthy adult donors and from 23 adult patients with chronic or acute leukaemia from the Haematological Department of the Institut J. Paoli-I. Calmettes (Marseille) was collected in heparin. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation, (Boyum, 1968). From healthy donors, lymphocyte subpopulations were isolated by first removing phagocytic cells with carbonyl iron and second by a Ficoll-Hypaque gradient on rosetted (R) lymphocytes with sheep red blood cells (SRBC) (Fournier & Bach, 1976). The two isolated cells subpopulations R+ cells (T lymphocytes) and R− cells (mostly B lymphocytes) were characterized by SRBC rosettes, surface membrane immunoglobulin (SIg) (Papamichail *et al.*, 1971) and morphological identification by light microscopy after May Grumwald Giemsa Staining.

These characterization techniques yielded the following results: R− cells contained >90% lymphocytes, <5% monocytes and <4% polynuclear cells; 80–90% of these cells were SIg^+ and <5% re-rosetted with SRBC.

R+ cells contained >95% lymphocytes <2% monocytes and <2% polynuclear cells. Less than 5% were SIg^+ and >80% re-rosetted with SRBC. R+ cells were tested after dissociation of rosettes by ammonium chloride. Viability was >95% in both groups.

R− cells were also tested after incubation with ammonium chloride to eliminate its role in oestradiol membrane binding.

In patient donors with acute leukaemia, poorly differentiated lymphoma or chronic lymphocytic leukaemia, the cells were isolated from peripheral blood before chemotherapy and characterized by the following tests: R and EAC rosette formation, (Bianco *et al.*, 1970), identification of rosetting cells by spinning and staining with May Grumwald Giemsa, cytotoxic assay with an anti-T lymphocyte rabbit antiserum (Touraine *et al.*, 1974) and determination of SIg.

For oestrogen binding assays we used oestradiol covalently linked to BSA (steraloids). Each BSA molecule carried on the average 22–25 oestradiol molecules. 1, 3, 5 (10) Estratrien 3,17 β diol 6 one 6 CMO-BSA was fluoresceinated, (E_2-BSA-FITC) (Walter *et al.*, 1978).

Cells (10^6) pre-incubated with latex particles in 100 μl of Hank's medium, were incubated for 30 min at 4°C or 37°C with E_2-BSA-FITC at different concentrations, ranging from 0.1 to 4×10^{-5} M for the conjugate. In some experiments E_2-BSA-FITC was incubated in the presence of dihydrotestosterone (DHT) (10^{-5} M) in the medium. At the end of incubation tubes were centrifuged 10 min at 600 *g* at 4°C and the pellet was washed twice in Hanks solution. After the last wash, the cells were placed on slides, dried in air, fixed with 1% glutaraldehyde, or ethanol; the slides were then mounted in glycerol and examined under an Orthoplan Leitz microscope. The percentage of fluorescent cells was obtained by counting 200 latex

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negative cells from each preparation. Controls were incubated with BSA-FITC. Viability of cells was checked at the end of each assay by trypan blue exclusion and was invariably >90%. Each test was run in triplicate and most experiments repeated at least 5 times.

Carbonyl iron-treated peripheral blood mononuclear cells (PBMC) from 30 normal male and female subjects were incubated for 30 min at 4°C or 37°C with E_2-BSA-FITC at a concentration of 4×10^{-5} M: the average percentage of fluorescent cells was 5.2% (range 0–16%). Fluorescence was bright and distributed over the cell surface. The same test performed on the isolated R+ and R− lymphocyte sub-populations gave significantly different results. In 25 different R− cell populations, the average percentage was 20% (range 8–32), 18% (2–20) in males and 22% (9–32) in females. In contrast, no binding occurred in R+ cell populations. The controls performed with BSA-FITC alone were always negative. In the presence of DHT, similar results were obtained thereby ruling out the binding of E_2-BSA-FITC to plasma protein absorbed on the cell membrane. In order to identify the E_2-BSA-FITC fluorescent cell sub-population, the same cells were incubated with E_2-BSA-FITC and rhodamine goat antibody (Fab'2) to human Ig. (Biolyon). The incubation was performed at 4°C for 30 min to avoid capping of Ig. Interestingly, the cells which were fluorescent for FITC also fluoresced for rhodamine showing that E_2-BSA-FITC binding cells were B cells, but when cells were first incubated at 37°C for 180 min with rhodamine Fab'2 antibodies to human Ig and secondly with E_2-BSA-FITC, we observed rhodamine capping and diffusely distributed fluorescein on the same cells (Figure 1) E_2-BSA-FITC did not interfere with Ig capping mechanisms.

E_2-BSA-FITC binding was tested in 23 monoclonal cell populations of chronic and acute lymphocyte leukaemia (Table I). No binding was observed in T or "null" leukaemia. Significant binding occurred only in some B proliferations. In two cases of B acute lymphocytic leukaemia, 70% and 100% of the cells bound E_2-BSA-FITC. Consequently normal R− cells or leukaemia B cells were used to characterize the steroid binding. The percentage of E_2-BSA-FITC binding cells from the same donor did not change when blood was collected at different times. Furthermore, the freezing time which ranged from one month to one year did not affect binding since it varied only from 27–34% in a 15 test control experiment.

Saturation binding curve Cells (10^6) were incubated with increasing concentrations of E_2-BSA-FITC from 0.1 to 4×10^{-5} M. Typical results are shown in Figure 2 where the percentage of fluorescent cells was enhanced from 6% at 0.1×10^{-5} M to 20% at

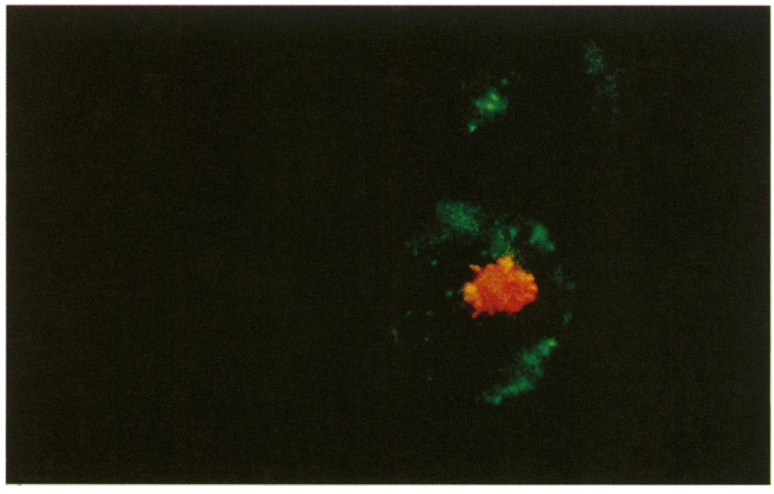

Figure 1 Rhodamine-FITC membrane staining on the same cell. Photo-micrography of human lymphocytes showing rhodamine fluorescence in a cap and diffuse plasma membrane FITC fluorescence. This double staining was obtained by a double incubation as follows. First R-lymphocytes were incubated with rhodamine-conjugated fragments (Fab'2) antibodies to human Ig for 180 min at 37°C to obtain a rhodamine cap and second, with E_2-BSA-FITC 10^{-5} M for 30 min at 37°C. After two washes viability was checked by trypan blue exclusion and exceeded 90%. The cells were examined with an Orthoplan Leitz microscope using Block I_2 (Leitz) for FITC and Block N_2 for rhodamine. The photography was obtained by a superimpression of the both photographs of the same cells.

Table I Sex steroid binding to pathological cell populations

Disease	Patients Identification	Sex	Age	Immunological characterization % of pathol. cells	Lymphocyte subset	From total cell population % E_2-BSA fluorescent cells
	RIC	M	60	60	B ($\gamma\kappa$)	9
	EYM	F	50	100	B ($\mu\kappa$)	74
	CUC	M	60	75	B ($\mu\delta\kappa$)	3
	HOL	M	65	100	B ($\mu\kappa$)	27
	JAU	M	42	100	B ($\mu\kappa$)	29
CLL	LIM	F	50	100	B ($\mu\kappa$)	32
	ZER	F	65	60	B (κ)	0
	DER	F	70	80	B ($\mu\kappa$)	11
	DIL	M	50	100	B ($\delta\lambda$)	36
	BER	M	60	50	B ($\gamma\kappa$)	32
	PAC	F	50	80	B ($\gamma\kappa$)	11
	DEH	F	27	100	T	0
	BES	M	34	100	T	0
	LAL	F	17	86	Not T nor B	2
	BRE	M	16	100	Not T nor B	3
ALL	CAR	F	72	67	Not T nor B	5
	DEB	M	64	100	Not T nor B	0
	HAN	M	17	96	Not T nor B	0
	HEL	M	14	70	Not T nor B	0
	LOU	F	16	90	Not T nor B	0
PDL	LAT	M	15	80	B ($\mu\kappa$)	4
	EST	M	48	95	B ($\mu\gamma\kappa$)	9

Peripheral blood cells from patients with CLL (chronic lymphocytic leukaemia), ALL (acute lymphocytic leukaemia) or PDL (poorly differentiated lymphoma) were isolated as described in the text.

They were tested by usual immunological methods and identified as:

- B lymphocytes by determination of SIg,
- T lymphocytes by R rosette and/or using an heterologous rabbit antiserum,
- neither T nor B lymphocytes when these tests were negative.

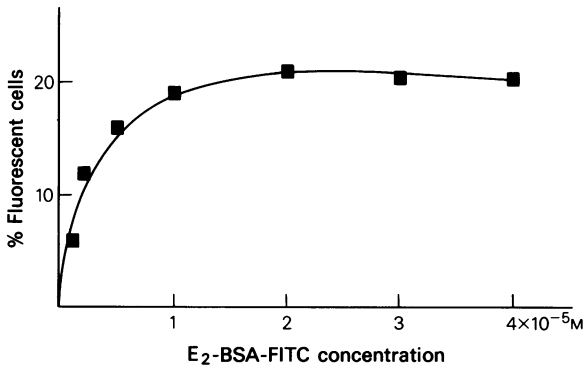

Figure 2 Saturation binding curve. Percent of fluorescent cells obtained when 10^6 human normal R-lymphocytes (isolated as described in **Figure 1**) were incubated in Hanks medium with E_2-BSA-FITC at concentrations ranging from 0.1 to 4×10^{-5}M for 30 min at 37°C. Each E_2-BSA-FITC concentration was run in triplicate, the mean values of which are represented by the points shown.

1×10^{-5}M, and remained stable until 4×10^{-5}M. Similar binding curves were obtained with several cell populations.

Determination of binding dissociation Cell preparations were first incubated with a saturation concentration of E_2-BSA-FITC (4×10^{-5}M) for 30 min at 37°C. At the end of incubation, the number of fluorescent cells was counted and regarded as equivalent to 100%. After two washes the second step was performed by incubating these cell preparations with non-fluorescent E_2-BSA at different concentrations (from 4×10^{-6}M to 1.5×10^{-4}M) for 30 min at 37°C. A decrease in the percentage of fluorescent cells was obtained as a function of the concentration of non-fluorescent E_2-BSA added (Figure 3).

Specificity of E_2-BSA-FITC binding Coincubation of E_2-BSA-FITC at saturation concentration with non-fluorescent competitors at a 100-fold excess concentration was not possible because these

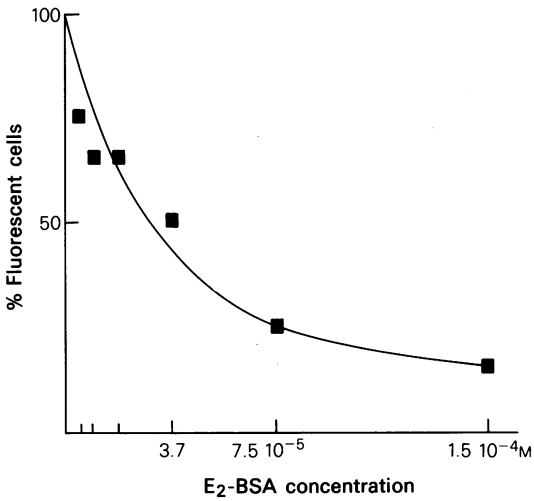

Figure 3 Dissociation binding curve. Percentage of fluorescent cells obtained after two incubations: *First:* R-lymphocytes suspended in Hanks medium were incubated with E_2-BSA-FITC at 10^{-5}M (saturation concentration) for 30 min at 37°C and washed twice. The percentage of fluorescent cells determined used as 100%. *Second:* After the two washes, the cells were incubated with non-fluorescent E_2-BSA at different final concentrations from 4×10^{-6}M to 1.5×10^{-4}M. Each incubation was performed for 30 min at 37°C. At the end of each experiment the percentage of fluorescent cells was determined. *Controls:* the cells were exposed to Hanks medium alone, during the second incubation. Each point represents mean of triplicate determinations.

complexes dissolved poorly in our buffer at these concentrations. Therefore cells were incubated for 30 min at 37°C with non-fluorescent competitors steroid-BSA (1.5×10^{-4}M), then E_2-BSA-FITC was added (1×10^{-5}M) and the incubation was continued for a further 30 min at 37°C. The percentage of fluorescent cells from this double incubation was compared with that obtained after a single incubation with E_2-BSA-FITC. The non-fluorescent steroid-BSA conjugates used were: 1,3,5, (10) Estratrien 3,17β diol 6 CMO-BSA (E_2-BSA) −1,3,5, (10) Estratrien 3 ol-6,17 dione 6-CMO-BSA (E_1-BSA)-4 androsten 17β ol 3 one 3 CMO-BSA (Testo-BSA)-4 androsten 3,17 dione, 3 CMO-BSA-4 pregnen 3,20 dione 3 CMO-BSA. These molecules are the same as E_2-BSA except in C 17 or C 3 and on A cycle.

Under our experimental conditions, 90% of E_2-BSA-FITC binding was inhibited (77–100) by E_2-BSA. 50% (45–55) by E_2-BSA and less than 20% (0–20) by Testo-BSA and 4 androsten 3,17 dione-BSA as well as pregnen 3,20 Dione-BSA. BSA did not prevent E_2-BSA-FITC binding.

Time temperature and pH dependence of binding As shown in Figure 4 E_2-BSA-FITC binding was maximum after 10 min of incubation at 37°C and was stable up to 360 min. When the steroid was used up to and at saturation concentration (10^{-5}M) similar binding kinetics and levels were obtained at 4°C and 37°C. However when the concentration of E_2-BSA-FITC used was less than saturation concentration, the binding kinetic was temperature dependent as indicated in Figure 4. The maximum binding was the same at both temperatures but delayed at 4°C. The pattern of immunofluorescence over the cell membrane was studied at 4°C and 37°C. A temperature-dependent phenomenon of capping is known to occur with multivalent ligands bound to plasma membrane of freely suspended cells. This process did not occur with the binding of E_2-BSA-FITC to the plasma membrane of lymphocytes.

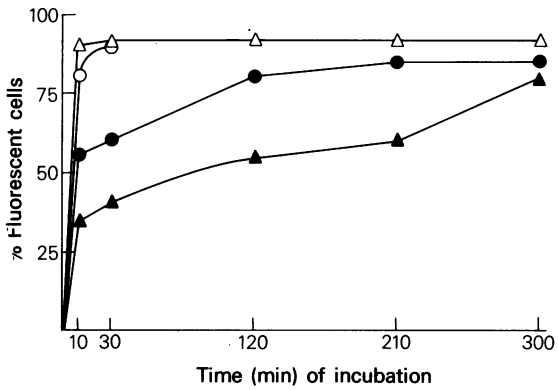

Figure 4 Time temperature dependence of binding. Percentage of fluorescent cells obtained after incubation of CLL cells with E_2-BSA-FITC from 10 to 300 min at 4°C or 37°C. E_2-BSA-FITC was used at saturation concentration (10^{-5}M) at 4°C and at 37°C and at a concentration of 5×10^{-7}M at 4°C and at 37°C. Each point represents mean of triplicate determinations.

Changing the buffer pH from 6.2 to 8.2 which is within the range of cell viability did not noticeably affect binding.

Effect of enzyme digestion A preliminary attempt to characterize the E_2-BSA-FITC binding sites on lymphocytes, was performed by exposing the cells to enzymes that commonly modify cell membrane receptors: SIg^+ lymphocytes were incubated 30 min at 37°C in Hank's medium with trypsin (0.25%) or neuraminidase (10 μg ml^{-1}). The variability checked after each incubation was always >90%. Binding with E_2-BSA-FITC was checked as previously stated: neuraminidase had no effect whereas trypsin

inhibited 100% of E_2-BSA-FITC binding. Recovery of sex steroid binding after exposure to trypsin, was tested by incubating cells in the presence or absence of cycloheximide ($50\,\mu g\,ml^{-1}$) (inhibitor of protein synthesis) in culture medium (RPMI + 20% foetal calf serum) at 37°C in CO_2 atmosphere. After 3 h, cells incubated alone recovered 20% of their initial E_2-BSA-FITC binding and 100% after 18 h. Cells incubated in the presence of cycloheximide recovered $<10\%$ of their binding after 18 h although cell viability was $>80\%$.

Our experiments show that sex steroid macromolecular complex constituted by oestradiol covalently linked to bovine serum albumin binds to the plasma membrane of a sub-population of human B lymphocytes regardless of the sex of the donor. Similar binding occurred on some B acute and chronic leukaemia cells. The macromolecular complex E_2-BSA did not bind to T cells; BSA by itself bound to neither B nor T cells. Given the limits of our fluorescent technique, accurate characterization of the binding is impossible. However, we can draw some conclusions: the binding occurred at the plasma membrane of the cells and was saturable, rapid, reversible, partly temperature dependent and steric specific. Moreover, it was insensitive to sialic acid digestion with neuraminidase but was markedly reduced by proteolysis with trypsin. This effect was reversed by an 18 h incubation in serum containing culture medium but not when an inhibitor of protein synthesis was added.

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