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Electrokinetic studies on concanavalin A as a tool to probe the surface characteristics of differentiated lymphoid cells

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Abstract. The redistribution of surface receptors induced by the binding of concanavalin A to different types of lymphoid cells was studied by the techniques of cell electrophoresis and fluorescence microscopy. The cells studied included, splenic lymphocytes from normal healthy as well as terminally leukaemic mice, thymocytes from mice of varying ages from newborns to adults and antigen sensitised or educated lymphocytes. These cells were in different stages of growth and/or differentiation. The nature and especially the behaviour of surface receptors in response to treatment with concanavalin A under capping conditions differed markedly but appeared to be dependent on the differentiational status of the cells. On this basis, the adult thymocytes were found to consist of two sub-populations differing in their proliferative and differentiational status. The proportions of these varied during their ontogenic development. Lymphocytes specifically committed to an antigen bound concanavalin A but were found to be incapable of bringing about the redistribution of the surface receptor-ligand complexes.

Keywords. Lymphocyte differentiation; concanavalin A; electrophoretic mobility; capping.

Introduction

The lectin concanavalin A (Con A) has proved to be a very useful probe in elucidating differences between the surface architecture of normal and malignant cells (reviewed in Nicolson, 1976; Brown and Hunt, 1978). Its specific mitogenic action has also been employed to assess the functional status of a sub-population of lymphocytes *i.e. T*-(thymus derived) cells (Greaves and Janossy, 1972; Stobo *et al.,* 1972). Furthermore, its ability to induce redistribution (patching, capping and endocytosis) of receptor-ligand complexes on cell-surfaces leads to alteration in their surface charge density (Yamada and Yamada, 1973; Wioland *et al.,* 1976; Sainis *et al.,* 1979a) which are measurable by the biophysical technique of cell-electrophoresis. On the basis of the biphasic changes in the electrokinetic behaviour of normal mouse lymphocytes we have clearly demonstrated the existence of two different types of receptor sites for Con A (Sainis *et al.,* 1979a, b; Sainis and Phondke, 1980). These sites differ in their mobility in the plane of the cell membrane and the binding of the lectin to these sites results in quantitatively distinct modulations of electrophoretic mobility of normal splenic lymphocytes.

Abbreviations used: Con A, Concanavalin A; EPM, electrophoretic mobility measurement; FITC-Con A, Fluoresceinated Con A; TRITC-Con A, tetramethyl rhodaine labelled Con A; α-MM, α-methyl mannoside; PNA, peanut agglutinin; DNFB, dinitrofluorobenzene.

On the other hand splenic lymphocytes from spontaneously leukaemic mice of the same AKR strain display, only a single type of receptor sites for the lectin (Sainis *et al.,* 1982). Yet, these sites differ from any of the types on the normal lymphocytes in their response to high concentrations of the lectin (Sainis *et al.,* 1982). Since it has been suggested that the malignant development involves an arrest in cellular differentiation (Nakamura and Metcalf, 1961; Metcalf, 1962), it was interesting to explore whether the Con A — induced modifications of electrophoretic mobilities could serve as a structural probe for studying lymphoid growth and differentiation. In the present investigations we have, therefore, examined the interaction between Con A and lymphoid cells of growing AKR mice and also the antigen sensitised lymphocytes of the adult mice.

Materials and methods

Lymphocytes from spleens, lymph nodes and thymus were obtained from normal healthy AKR mice (8 weeks old) as well as from young mice of various ages. Cell suspensions were prepared in standard saline solution (0.146 M NaCl + 0.01 M KCl, pH 7.2). All electrophoretic mobility (EPM) measurements were made with the cylindrical cell electrophoresis apparatus described by Bangham *et al.* (1958), at 5° C or 25° C (Sundaram *et al.,*1967). Treatments with Con A were carried out in various schedules as described previously (Sainis *et al.,*1979a, b) . For fluorescence studies, different preparations of the lectin *viz.* fluoresceinated Con A (FITC—Con A) and tetramethyl rhodamine labelled Con A (TRITC—Con A) were prepared according to the method of de Petris (1975).

Results

The responses of the splenic lymphocytes of normal and leukaemic AKR mice to their interaction with Con A provide the basis for subsequent studies on differentiating lymphoid cells. Though the details of these interactions are described elsewhere (Sainis *et al.,* 1979a, b, 1982; Sainis and Phondke, 1980) the salient features may be briefly stated as follows:

Interaction of Con A with normal splenic cells

The treatment of normal lymphocytes with the lectin under capping conditions *(i.e.* 22° C or 37° C for 1 h) results in biphasic changes in their electrophoretic mobility (EPM). These consist of an increase in the EPM at low concentrations of Con A and reduction in the same below that of the untreated cells at higher (≥ 15 μg/ml) concentrations of the lectin. Analysis of this profile clearly established that these changes result from a step-wise, sequential interaction between the lectin and two types of receptor sites on the surface of the same cell (Sainis *et al.,* 1979a, b; Sainis and Phondke, 1980). These receptor-types are:

Type I receptors: These sites are the first to interact with Con A. Binding of the lectin to these sites, by itself, does not alter the electrophoretic mobility. They are induced to undergo redistribution i.e., capping at low concentrations of Con A. This process is accompanied by an enhancement in the cellular EPM (figure 1A).

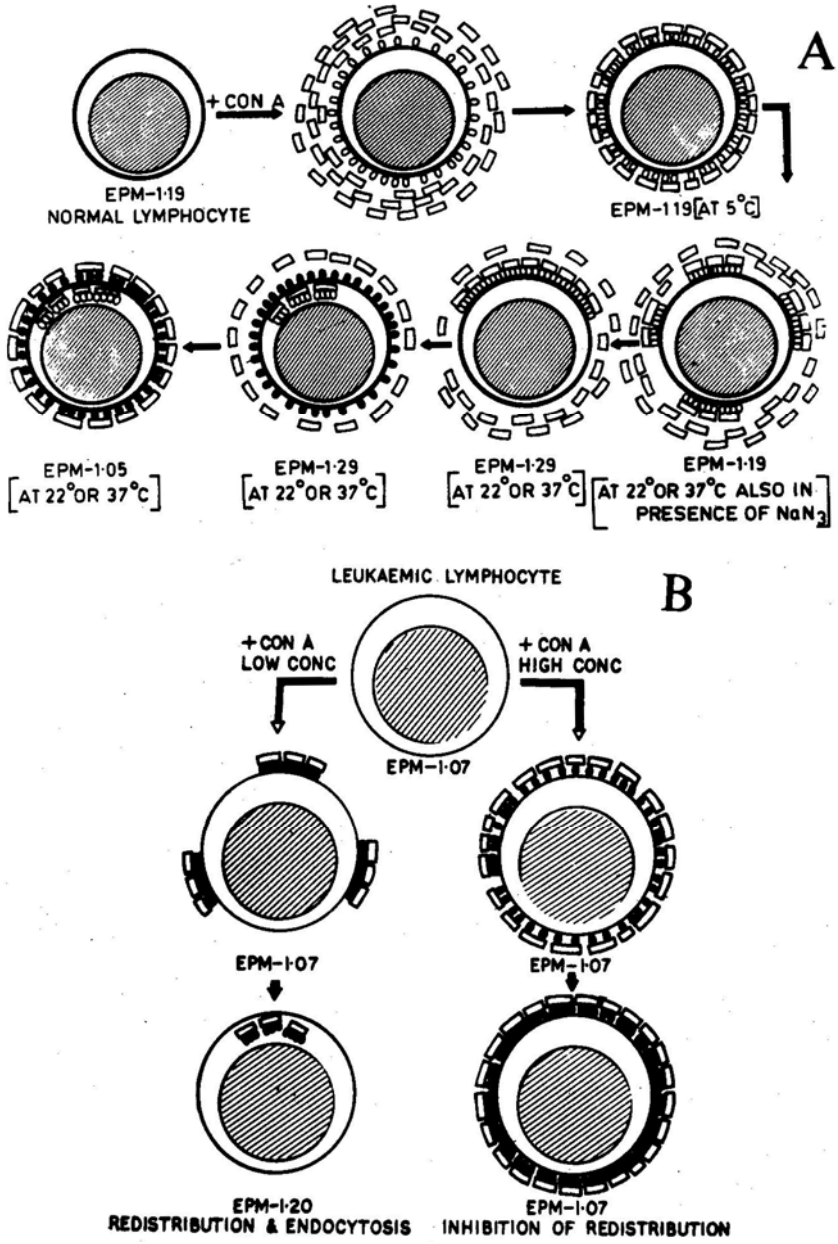

Figure 1. Pictorial representations of the electrophoretic status and the interaction of surface receptors to Con A on lymphocytes of AKR mice.
A. Normal lymphocytes. (□), Con A molecule; (O), type I receptors; (●), type II receptors. **B.** Lymphocytes of leukaemic mice. (□), Con A molecule; **(●) ,** receptors for Con A.

Type II receptors: These emerge only as a consequence of the redistribution of receptors of type I. Mere binding of the lectin to these sites reduces the EPM of the lymphocytes below that of the untreated cells. They fail to undergo redistribution (figure 1A). Fluorescence studies clearly show that full endocytosis of type I sites is

not necessary for the emergence of the second type of receptors. Even capping or macropatching are sufficient (Sainis and Phondke, 1980).

Using lymphocyte sub-populations separated by their differential adhesion to nylon, it is seen that these two types of receptor sites are present on a sub-population of the *T*-lymphocytes (Sainis *et al.,* 1979a).

Receptors for Con A on leukaemic cells

The essential features of the behaviour of Con A-treated splenic lymphocytes from AKR mice having spontaneous lymphoblastic leukaemia are depicted in figure 1 B. The leukaemic lymphocytes undergo an increase in EPM at low concentrations of the lectin (2–7.5 μg/ml). However, at higher concentrations (≥10 μg/ml) their mobility is the same as that of the controls. This is attributed to the inhibition of the laternal mobility of their lectin-binding sites by high concentrations of the lectin (figure 1B; Sainis *et al.,* 1982). Such an inhibition is not observed in the normal cells. Thus the receptors for Con A on the leukaemic lymphocytes are qualitatively distinct from those on the normal cells in their inability to undergo capping at high concentrations of the lectin (Sainis *et al.,* 1982). This can ensue from the preponderence of proliferating cells (Blomgren and Andersson, 1969).

Interaction with Con A during lymphoid growth and differentiation

The characteristic differences in the electrokinetic profiles and properties of receptors for the lectin among Con A—treated normal and leukaemic cells prompted us to examine whether in a particular lymphoid tissue, various stages during growth and differentiation could be associated with characteristic profiles of interaction with Con A. Comparative assessment of these interactions was first carried out in the thymocytes of adult and developing AKR mice. Thymus is a very crucial organ in immunological development. It is a seat for cell recruitment and traffic. Stem cells from bone marrow come under the differentiational influence in the thymus before migrating out to the peripheral lymphoid organs as mature T-lymphocytes. There are several reports of cellular heterogeneity within the thymus (Boyse *et al.,* 1968; Leckband and Boyse, 1971; Reisner *et al.,* 1976; Papiernik *et al.,* 1977; Shortman *et al.,* 1977).

Interaction of Con A with adult thymocytes:

Figure 2 shows the electrophoretic mobilities of adult thymus cells treated at various concentrations of Con A under capping conditions. The profile was bimodal. There was an initial increase in EPM at 5 μg/ml of Con A. The EPM further increased at moderately high concentrations (15-25 μg/ml) of the lectin. At very high concentrations of Con A, the mean EPM remained unaltered (figure 2).

The close relationship between increase in EPM and the extent of redistribution (Sainis *et al.,* 1979a) suggested that at concentrations between 15 and 25 μg/ml of Con A, a higher proportion of thymocytes should be undergoing capping. However, as shown in figure 3, the proportion of thymocytes displaying redistribution progressively decreased with increasing concentration of FITC–Con A. At very high concentrations of the fluorescent lectin very few thymocytes showed capping (figure 3). At no time/no concentration of the lectin more than 40% of the thymocytes showed capping.

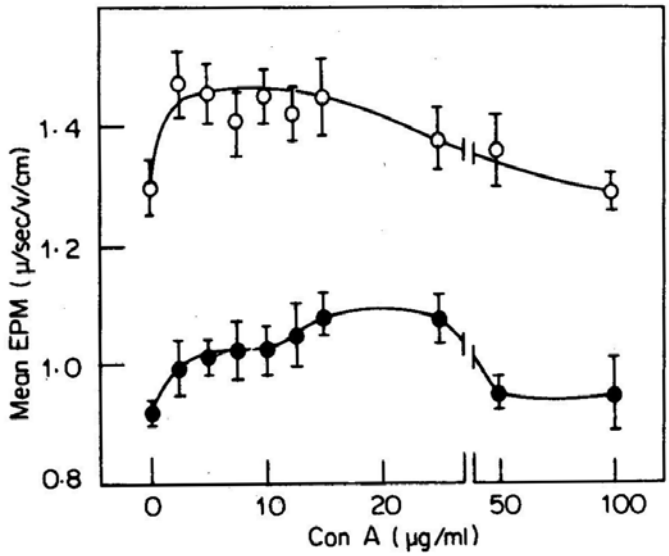

Figure 2. Electrophoretic mobilities of thymocytes and hydrocortisone resistant thymocytes of adult AKR mice after treatment with Con A. 5 × 10^6 cells/ml were incubated with various concentrations of Con A at 22° C for 1 h. They were washed and their electrophoretic mobilities were measured at 25°C in a cylindrical cell microelectrophoresis apparatus in standard saline solution. Each point shows the mean EPM and bars indicate 95% confidence limits.

(●), Adult thymocytes; (O), hydrocortisone resistant thymocytes.

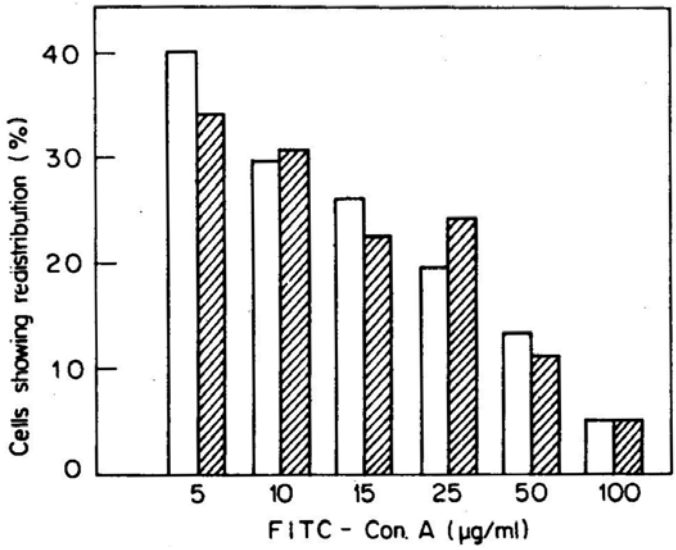

Figure 3. Extent of redistribution of Con A receptors on adult thymus cells and hydrocortisone resistant thymus cells.

5 × 10^6 Cells/ml were incubated with 5 to 100 μg/ml FITC-Con A at 22° C for 1 h. They were washed and fixed with 4% formaldehyde for 20 min. All the cells bound FITC-Con A. Total number of cells showing patching, capping and endocytosis were counted after observation under a Zeiss-epiillumination microscope and expressed as per cent of all thymocytes.

(❑), Adult thymocytes; (▨), hydrocortisone resistant thymocytes.

The increase in the EPM of thymocytes was observed to be due to capping induced by the lectin, as it was inhibited by sodium azide. The unmodified EPM and poor proportion of capped cells at very high concentrations was observed throughout the incubation period. It was possible that this was due to the inhibition of redistribution of receptors by high concentrations of the lectin. To resolve this point the thymocytes were first incubated with high concentrations of Con A. When these cells were subsequently treated with optimum concentrations of α-methyl mannoside (α-MM), they showed enhanced proportion of capping and increase in EPM (N. N. Johsi, K. B. Sainis and G. P. Phondke, unpublished observations). Inhibition of receptor redistribution on thymocytes by high concentrations of lectin was thus confirmed. Similar observations were made earlier in leukaemic lymphocytes (Sainis *et al.,* 1982).

The next question concerned with this bimodal electrophoretic profile was whether it represented increasing alteration in the same set of cells or changes in the receptor distribution on different sub-populations of cells. If all the cells capped at a low concentration (5 μg/ml) of Con A and a major fraction of them possessed the second set of receptors which bound Con A post-redistributionally, it could lead to a smaller proportion of cells showing redistribution at moderate concentration of the lectin. Thymocytes were, therefore, pulsed at low as well as at moderate concentrations (25 μg/ml) of FITC Con A at 5°C. The excess of lectin was removed and the cells were allowed to undergo capping (Sainis *et al.,*1979b). Thirty eight per cent of total thymocytes showed capping on pulse-treatment at 5 μg/ml, while only 28 % of the total thymocytes capped when pulsed with FITC Con A at 25 μg/ml (table 1). This compared well with 40% and 20% of cells show-

Table 1. Capping and binding of Con A to second type of receptors in adult thymocytes.

Conc. of FITC-Con A μg/ml	Type of treatment	Cells with FITC-Con A - caps[a]	Cells with TRITC-Con A free region [a]	Cells showing second type of receptor [b]
5	Pulse	38%	N.D.	—
5	Direct	40%	35.4	83%
25	Pulse	28%	N.D.	—
25	Direct	20%	1%	5%

[a] Per cent of total thymocytes

[b] Per cent of cells showing FITC-Con A caps.

N.D. Not determined.

Adult thymocytes (5×10^6) were first incubated separately with 5 μg/ml or 25 μg/ml of FITC-Con A at 5°C (Pulse) or at 22° C (direct). Pulsed cells were washed in cold and reincubated at 22° C. After incubation at 22° C for 30 min cells were washed and fixed in formaldehyde. Fixed cells were reincubated with 20 μg/ml of TRITC-Con A for 30 min at 22° C. They were washed and observed in a Zeiss-epiillumination microscope for FITC-Con A caps and the binding of TRITC—Con A to these capped cells, in an area of membrane free of the FITC—Con A cap (to second type of receptor sites as described in ref. Sainis and Phondke, 1980).

ing redistribution after direct incubation with these concentrations of FITC–Con A respectively (figure 3). It, therefore, appeared that the cells undergoing redistribution at low and moderate concentrations of the lectin probably belonged to different sub-populations within the thymus.

Such a possibility implied that the population undergoing redistribution at low concentrations of Con A might possess second set of receptor sites like the normal T-cells. To confirm this possibility thymocytes were first allowed to cap the recaptors bound to FITC – Con A at the concentrations of 5 and 25 μg/ml respectively. They were fixed with formaldehyde and were reincubated with a Con A preparation labelled with different fluorochrome (TRITC – Con A; Sainis and Phondke, 1980) and examined. Most of the cells which capped FITC-Con A (5 μg/ml). showed exclusive labelling with TRITC – Con A (>80% of the capped cells). Thus almost all the thymocytes which capped at low concentration of Con A possessed two types of receptors for Con A as in normal T-cells (table 1).

In contrast, very few of the cells which capped at moderate concentrations (25 μg/ml) of FITC-Con A displayed such an exclusive double-labelling, suggesting that they lacked the two types of receptor sites as in normal T-cells (table 1). Thus it appeared that the thymocytes consist of two sub-populations differing in their ability to undergo Con A-induced receptor redistribution. These sub-populations would have the following characteristic features.

Sub population A: It was induced to cap by low concentrations of Con A but the resultant alteration in EPM was relatively low in magnitude (figure 2). It constituted approximately 40% of the thymus cells and like the normal T-cells displayed the two types of sequentially interacting receptors for the lectin. It is possible that these cells display high affinity for Con A. At very high concentrations of the lectin the lateral mobility of the receptors on this sub-population was inhibited.

Sub population B: It constituted nearly 20% of thymocytes. These cells probably displayed Con A receptors of low affinity as it underwent redistribution at moderately high concentrations (25 μg/ml) of Con A. The net increase in EPM brought about by this process was, however, higher in magnitude as compared to that in sub-population A. They seemed to possess only a single type of receptors, inhibitable by very high concentrations of the lectin as also observed in the case of the leukaemic lymphocytes (Sainis *et al.,* 1982).

Identification of thymocyte sub populations

In order to relate the above sub-populations with differentiationally distinguishable thymocytes hitherto reported (Boyse *et al.,* 1968; Leckband and Boyse, 1971; Reisner *et al.,* 1976; Papiernik *et al.,* 1977; Shortman *et al.,* 1977) these studies were repeated using hydrocortisone resistant thymocytes (Papiernik *et al.,* 1977) and thymocyte sub-populations fractionated by agglutination with peanut agglutinin (PNA; Reisner *et al.,* 1976). The cortisone resistant cells are considered to be mature cells and resemble the peripheral T-cells, electrophoretically (Wioland *et al.,* 1972; Phondke *et al.,* 1975). When these cells were treated with Con A under capping conditions, the enhancement in the mean EPM was recorded over a broader range of concentrations of Con A (2.5 to 25 μg/ml) as shown in figure 2. In peripheral T-cells this effect was seen only between 2.5 to 12.5 μg/ml of the lectin (Sainis *et al.,* 1979a). Likewise, the redistribution induced by FITC-Con A was

observed over a similar range of lectin concentration. The unaltered EPM of these cells at high concentrations of the lectin was attributable to the inhibition of redistribution (N. N. Joshi, K. B Sainis and G. P. Phondke, unpublished observations). Furthermore, since their proportion within the thymus is very small (~5%, Papiernik *et al.,* 1977) they were unlikely to be the major constituent of the sub-populations.

Another possibility could have been that the two sub-populations represented the mature and immature thymocytes as defined by Reisner *et al.* (1976) on the basis of the agglutination brought about by PNA. However, when thymocytes were separated into PNA—agglutinable and non-agglutinable fractions, signifycant differences in their profiles of Con A—induced redistribution were not seen.

Interaction of Con A with developing thymocytes

Ontogenic development represents an interplay of proliferation and differentiation. The electrokinetic profiles of Con A treated thymus cells from new born (0–18 h age), 9 days old and 21 days old AKR mice are shown in figure 4 as representative examples. The thymocytes from new born mice showed enhancement in EPM at low concentration of Con A (figure 4) . At higher concentration the mean EPM was marginally reduced. In contrast, thymocytes of 9 days old and 21 days old mice showed typically bimodal profiles as a function of lectin concentration (figure 4). It is possible that the two peaks in these profiles emanate from the interaction of the lectin with the two sub-populations in the thymus.

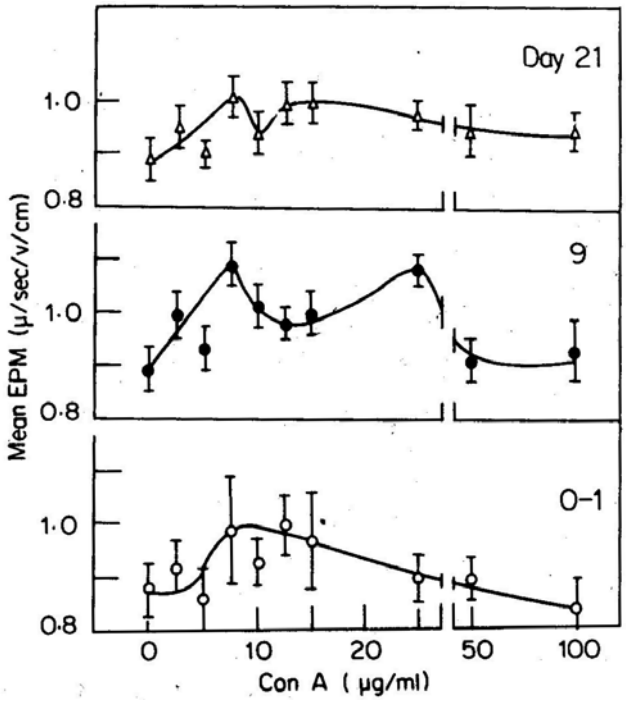

Figure 4. Electrophoretic mobilities of Con A – treated thymocytes from developing AKR mice (0–21 days old). Cells were treated under capping conditions and EPM were measured at 25° C. Each point shows mean EPM and the bars show 95% confidence limits.

Thymocytes from: (O), 0–1 day old mice; (●), 9 days old mice and (Δ), 21 days old mice.

Interaction of Con A with lymphocytes following antigen-induced differentiation

Though peripheral T and B lymphocytes represent terminal stages of normal lymphoid ontogeny, these cells can further proliferate and differentiate in response, to an antigenic stimulus. To study the effect of Con A on the electrokinetic properties of antigen specific differentiated cells, AKR mice were immunized with sheep erythrocytes (5×10^8 SRBC *i.p.)* or contact sensitized to dinitrofluorobenzene (DNFB, Phanupak *et al.,* 1974). The immune lymphocytes were treated with Con A in the same way as the normal lymphocytes. In either case biphasic changes in the EPM were observed like those in normal lymphocytes (figure 5). Only the magnitudes of change in EPM at a given concentration of the lectin were different in the normal and immune lymphocytes (figure 5). The proportions of cells undergoing capping were also similar (table 2).

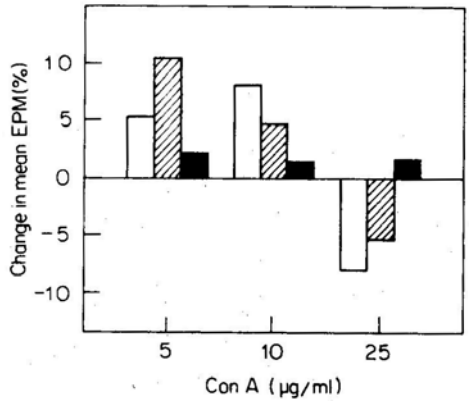

Figure 5. Extent of change in the electrophoretic mobilities of normal lymphocytes, immunised lymphocytes and educated T-cells following interaction with Con A under capping conditions. Change in EPM was calculated at each of the concentrations of the lectin and expressed as per cent of the EPM of untreated cells.

(❑), Normal splenic lymphocytes; (▨) splenic lymphocyte of mice immunised with sheep erythrocytes (primary response); (■), educated T-cells.

Table 2. Redistribution of Con A receptors on antigen – stimulated lymphocytes,

Cell type	Cells showing redistribution (%)
Unimmunised lymphocytes	34
SRBC immunised lymphocytes	30
DNFB sensitised lymphocytes	30
Educated T-cells	8

Normal and immune lymphocytes were pulsed with 50 µg/ml of FITC-Con A at 5°C and reincubated at 22° C for 30 min. This treatment is equivalent of direct incubation at low concentrations of Con A (Sainis *et al.,* 1979b). It also eliminates the effects of post-redistributional binding of the lectin to the second type of receptor sites.

It was possible that due to the small number of specifically differentiated cells in the spleen or lymph nodes of the immunised mice, the effect of Con A on these cells was marked by the changes in the larger population of antigenically uncommitted cells. This point was validated by the electrophoretic and fluorescence

measurements on a population of lymphocytes enriched in specifically antigen-stimulated cells. These cells were obtained as educated T-cells by the method described by Mitchell and Miller (1968). The antigen used was SRBC and these cells are considered to be helper T-cells (Hunter *et al.,* 1972).

The. educated T-cells did not show very significant changes in their EPM upon treatment with Con A under capping conditions (figure 5). Furthermore, they were unable to undergo any appreciable degree of ligand-induced redistribution (8%) when incubated with low concentration of FITC-Con A (table 2). In this respect they differ from the leukaemic cells or sub-population B in the thymus which show capping at low and moderate concentrations of Con A respectively but the high concentrations of lectin restrict the lateral mobility of their receptors.

Discussion

The foregoing data demonstrated that normal and developing cells-differ in the nature and lateral mobility of their receptors to Con A. These alterations are reflected in their characteristics electrokinetic profiles as also in microscopically discernible receptor redistribution patterns. Whereas normal lymphocytes displayed two behaviourally distinct types of receptor sites (Sainis *et al.,* 1979a, b; Sainis and Phondke, 1980), the highly proliferating leukaemic cells of the same strain of mice displayed a single set of receptor sites for Con A. The lateral mobility of these sites was restricted by high concentrations of Con A (Sainis *et al.,* 1982).

Cells undergoing normal growth and differentiation were marked for their cellular heterogeneity on account of Con A-induced receptor redistribution. This cellular heterogeneity observed in the adult and developing thymus cells was based on the presence of two major sub-populations. One of them shows receptor characteristics similar to those in adult peripheral T-lymphocytes, while the other largely resembles proliferating leukaemic cells. Several workers have earlier shown mouse thymocytes to be heterogenous with respect to cortisone resistance (Papiernik *et al.,* 1977) PNA-agglutinability (Reisner *et al.,* 1976), mitogen responsiveness, location (Shortman *et al.,* 1977) and surface markers (Boyse *et al.,* 1968; Leckband and Boyse, 1971). Our present investigation has brought to light yet another parameter for cellular heterogeneity *viz.* Con A – induced receptor redistribution and resultant changes in EPM.

The peripheral T-lymphocytes are considered to be at the end of the differentiation pathway. In adult unimmunised mice they will represent a differentiated non-proliferating population of cells. The leukaemic cells on the other hand, are highly proliferating (Joshi *et al.,* 1983) and are not terminally differentiated. In the adult thymus only a small fraction of cells have been observed to undergo active proliferation (D. S. Joshi, M. Rajadhyaksha and G. P. Phondke, unpublished observations). The cells of sub-population *A* within the adult thymus which undergo Con A – induced redistribution at low concentrations of the lectin have receptor sites which share the properties of differentiated, non-proliferating peripheral T-cells. However, they differ from the latter in that the thymic cells were inhibited from Con A induced redistribution by high concentrations of the lectin. The cells of sub-population *B* in the adult thymus show receptor characteristics similar to those of the highly proliferating but undifferentited leukaemic cells. Here again, the main difference was only in the concentration at which the leukaemic cells and

those of sub-population *B* showed capping (Sainis *et al.,* 1982, figure 3). On the basis of the profiles of Con A-induced receptor-redistribution and the nature of the receptors, it may thus be possible to ascribe differentiational status to various sub-populations of lymphoid cells. In the present system, sub-population *A* in the thymus would then be considered more differentiated than sub-population *B,* but less differentiated than the peripheral T-cells.

Since the hydrocortisone-resistant thymocytes also retain the property of restricted mobility of surface receptors bound to high concentrations of Con A, they may also be considered less differentiated than the peripherl T-lymphocytes.

In the thymuses of growing mice there is a considerable amount of cellular traffic. There is also considerable higher degree of cell proliferation and differentiated cells continue to migrate out to peripheral lymphoid organs. This leads to cellular heterogeneity which can arise from the two sub-populations described in adult thymocytes. In the developing thymus the proportions of the two sub-populations at different levels of differentiation will also vary. This is borne out by pronounced bimodal EPM profiles following treatment with Con A. The proposition that differentiational status is related to the nature and lateral mobility of the receptors for Con A, derives further support from the observation that lymphocytes differentiated in response to a specific antigen show restriction of the mobility of receptors to polyclonal ligands like Con A even at low concentrations (figure 5; table 2).

Our data thus indicate that electrokinetic measurements on the interaction of Con A could be used as a structural probe for alterations in lymphoid cell surfaces following normal growth and differentiation and abnormal proliferation or specific maturation.

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