ROTATIONAL DIFFUSION OF LECTINS BOUND TO THE SURFACE MEMBRANE OF NORMAL LYMPHOCYTES

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1. Introduction

Lectin [1] are proteins which can bind to specific saccharide sites on the cell surface membrane. The lectin concanavalin A (Con. A) [2], which binds to glucose or mannose-like sites [3], can activate lymphocytes to undergo DNA synthesis and the formation of blast cells [4–6]. On the other hand, wheat germ agglutinin (WGA) [7, 8] and soybean agglutinin (SBA) [9], are lectins that bind to sites containing *N*-acetyl glucosamine and *N*-acetyl galactosamine, respectively, and do not induce lymphocyte activation [6]. Activation of lymphocytes by Con. A is associated with mobility of Con. A binding sites on the surface membrane [10–13], which allows their cross-linking and redistribution to form caps [10, 11, 14].

The present study was undertaken to directly determine the degree of mobility of Con. A, WGA and SBA bound to the surface membrane of normal lymph node lymphocytes, by fluorescence polarization of fluorescein isothiocyanate conjugates of the three lectins (F-Con. A, F-WGA and F-SBA). We have defined a quantitative term for the degree of mobility that extends from 0, the immobilized state, to 1, the fully mobile state. Our results show that the degree of mobility of F-Con. A, F-WGA and F-SBA, are 0.83, 0.37 and 0.43, respectively. F-WGA and F-SBA, in contrast to F-Con. A, do not induce the formation of caps. Our results, therefore, support the assumption that lectin activation of lymphocytes is associated with a dynamic redistribution of membrane components upon binding of the lectin.

2. Materials and methods

The normal lymphocytes were obtained from lymph nodes of 6–8 week-old male CR/RAR rats. The cells were collected by tearing the tissue apart in phosphate-buffered saline, pH 7.2 (PBS) and allowing the pieces to sediment. The cells were then suspended and washed three times in PBS. Fluorescein isothiocanate conjugates of purified Con. A, WGA and SBA were obtained from Miles-Yeda and were stored in PBS at -20°C. The concentrations of cells and lectin used for the experiments, were chosen to give a maximum fluorescent signal with a minimum contribution of scattered light. One ml of the cell suspension (5×10^7 cells/ml) were saturated with 1 ml fluorescent lectin solution diluted in PBS (500 µg/ml) and incubated for 15 min at 37°C. The cells were then washed three times and diluted in PBS to give a final concn. of 2.5×10^7 cells/ml. The formation of caps on the cell surface membranes was observed with a Leitz-Ortholux fluorescence microscope.

Fluorescence polarization and fluorescence intensity were measured with an instrument which was described previously [15]. A 436 nm band of a 500 W mercury arc, which was passed through a Glan–Thompson polarizer, was used for excitation. The emission was detected in two independent channels after passing through a Glan–Thompson polarizer and a 20% sodium bichromate solution used as a cut-off filter. The emission intensities polarized parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the direction of polarization of the excitation beam, were obtained by a simultaneous measurement of $I_{\parallel}/I_{\perp}$ and $I_{\perp}$. The fluorescence

anisotropy r, and the fluorescence intensity, F, relates to $I_{\parallel}$ and $I_{\perp}$ by

$$r = \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + 2I_{\perp})}; \qquad F = I_{\parallel} + 2I_{\perp} \tag{1}$$

Prior to each reading with the cell suspension, the solutions were gently mixed. Correction for scattering artifacts were carried out with fluorescein free systems as previously described [16]. Excited state mean lifetimes, τ, were measured with a pulse sampling instrument [17] using a convolution analysis of the fluorescence decay curves.

3. Results and discussion

In agreement with previous results [10, 11] the final distribution of F-Con. A on the surface membrane of lymphocytes, observed with the fluorescence microscope, showed that 30% of the single stained cells had a polar fluorescence cap which covers about half of the cell surface. However, nearly all F-WGA and F-SBA stained cells display a ring of fluorescent clusters over the cell periphery and there were no caps. Cap formation with F-Con. A, but not with F-WGA and F-SBA, suggests that the degree of mobility of Con. A binding sites is greater than that of the WGA or SBA sites. In order to obtain direct evidence for this assumption, the rotational relaxation times of the fluorescent lectins bound to lymphocytes were determined.

The relation between fluorescence depolarization and Brownian rotation is given by the Perrin equation

$$\frac{r_0}{r} = 1 + \left(\frac{3\tau}{\rho}\right) \tag{2}$$

where r_0 is the fluorescence anisotropy when no rotations take place during the excited state lifetime, τ, and ρ is the rotational relaxation time [18]. For non-spherical structures, ρ is an harmonic mean of the relaxation times about the symmetry axes [18, 19]. The apparent value of ρ in fluorescence labeled proteins results from a combination of internal rotations of the fluorescence probe about the bonds which attach it to the protein core, and the rotations of the protein molecule [20, 21]. ρ is proportional to the viscosity, η, and inversely proportional to the absolute temperature, T.

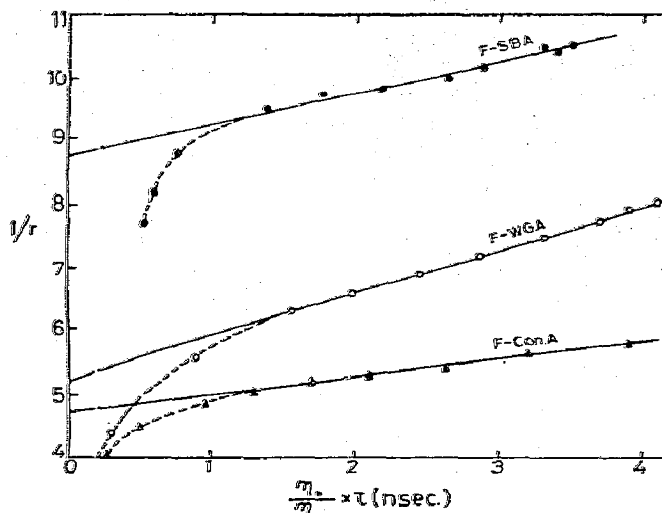

Fig. 1. Perrin plots for F-Con. A (▲—▲—▲), F-WGA (o—o—o) and F-SBA (●—●—●) in PBS at 24°C.

To evaluate the relaxation times of F-Con. A, F-WGA and F-SBA in the absence of cells, the values of r and τ were measured simultaneously in a series of solutions containing 20 μg/ml lectin and increasing amounts of sucrose (0 to 70% w/v) in PBS at 24°C. The results are given in fig. 1 as $1/r$ versus $\eta_0\tau/\eta$. η_0, the viscosity in PBS without sucrose, was introduced in order to relate the data to rotational diffusions in PBS. The non-linear Perrin plots of the three lectins are typical of cases where both the internal rotations of the fluorescence probe and the rotations of the protein molecule can contribute to fluorescence depolarization [18, 20, 21]. By an appropriate mathematical analysis of the non-linear Perrin plot the relaxation time of these rotations can be evaluated [20, 21]. However, because of the large difference in molecular dimensions between the probe and the lectin molecules it is reasonable to assume that for all three lectins the rotational relaxation time of the protein core is much longer than that of the fluorescein moiety. This difference permits the division of the Perrin plot into distinct regions [18]. At low $\eta_0\tau/\eta$ values the protein molecule retains its orientation during the excited state lifetime and fluorescence depolarization is caused almost exclusively by rotations of the fluorescence probe relative to the protein. As $\eta_0\tau/\eta$ increases the fluorescence depolarization resulting from internal rotations of the probe approaches its proper

Table 1
Fluorescence polarization characteristics (λ_{ex} = 436 nm) of fluorescein labeled lectins at 24°C.

Lectin	In PBS τ (nsec)	r	r'_0	ρ_0 (nsec)	Bound to lymphocytes r	ρ (nsec)	ρ_0/ρ
F-Con. A	[illegible].9	0.174	0.209	58	0.179	70	0.83
F-WGA	[illegible].1	0.124	0.193	22	0.160	59	0.37
F-SBA	[illegible].5	0.095	0.114	53	0.105	123	0.43

τ is the excited state lifetime, r is the fluorescence anisotropy; ρ_0 and ρ are the estimated rotational relaxation times of the lectin molecule in PBS and when bound to lymphocytes, respectively.

limit and rotations of the protein molecule start to contribute to fluorescence depolarization. Thus, at relatively high values of $\eta_0\tau/\eta$ the Perrin plot is expected to be linear since it relates to the rotations of the protein only. For the three lectins this region is at $\eta_0\tau/\eta > 2$ and is displayed by the straight lines given in fig. 1. These lines extrapolate to the values of $1/r'_0$ which correspond to situations where during the lifetimes of the excited state, the fluorescein label rotates freely but the protein molecule stays fixed. By substituting in eq. 2 the values of r and τ in PBS and r'_0, the corresponding relaxation times, ρ_0, were evaluated. Practically identical ρ_0 values are obtained from the slope of the lines. These and other fluorescence polarization parameters of the fluorescein conjugated lectins in PBS are summarized in table 1.

Addition of 0.1 M α-methyl mannoside, *N*-acetyl glucosamine and *N*-acetyl galactosamine to 20 μg/ml F-Con. A, F-WGA and F-SBA, respectively, in PBS results in a 1–2% increase in relaxation time. This increase can be accounted for by the change in molecular weight of the lectin–saccharide complex, and it indicates that the binding of ligands to these lectins has no effect on the internal rotations of the fluorescein moiety (r'_0 is unchanged). Moreover, since r'_0 was deduced from solutions containing high concentrations of sucrose, this value corresponds to the lectin–sucrose complex. It was therefore assumed, that upon binding of the lectins to the surface membrane of lymphocytes the values of r'_0 and τ remain unaltered and that the thermal mobility of the bound lectin is directly reflected in the determined value of r.

The corrected r values of the fluorescein labeled lectins bound to lymphocytes and their corresponding ρ values in PBS at 24°C are given in table 1. In order to compare the motional freedom of lectins bound to the cell surface membrane to those free in solution, we have defined the parameter ρ_0/ρ, the "degree of mobility", which extends from 0 (immobilized state) to 1 (fully mobile state). The data given in table 1 show, that F-Con. A bound to lymphocytes has a high degree of mobility close to that of free Con. A in PBS, and that this is about twice that of F-WGA and F-SBA.

Previous studies have shown that activation lymphocytes, resulting in thymidine incorporation and the formation of blast cells, is induced by Con. A but not by WGA or SBA [6]. In the present study we have observed that in contrast to F-Con. A, F-WGA and F-SBA do not induce cap formation on lymph node lymphocytes, and that there is a higher degree of mobility of the Con. A binding sites compared to the binding sites for WGA and SBA. It is suggested that a high mobility of sites is also required for the activation of lymphocytes by antigens.

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