A STUDY OF THE INTERACTIONS OF AN IMMUNOGLOBULIN λ LIGHT CHAIN WITH ARTIFICIAL AND B-LYMPHOCYTE MEMBRANES.

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ABSTRACT

The binding of an immunoglobulin λ light chain (IgLC) to synthetic and biological membranes was monitored in real-time using a recently developed, time-resolved fluorescence technique. λ IgLC purified from the urine of patients with multiple myeloma, were used in studies of protein-membrane interactions. The association of the λ IgLC dimer with B-lymphocytes was shown to be stabilised predominantly by non-polar interactions.

Furthermore, it was found that following binding to synthetic phospholipid membranes, a reorientation of the light chain occurred which resulted in a change in the distribution of charged residues at the lipid-water interface. The rate constants associated with the binding event were calculated, and appear to comprise both temperature insensitive and sensitive components. The calculated activation energies of the binding and reorientation events were found to be 13.53 KJmol⁻¹ and 87.89 KJmol⁻¹, respectively. The large activation energy associated with the reorientation phase suggests the movement of large protein domains, possibly involving a whole immunoglobulin domain. The binding and reorganisation of the IgLC upon the phospholipid membrane may confer novel biological functions to the bound protein and potentially contribute to such phenomenon as myeloma-associated immuno-suppression.

INTRODUCTION

It is well known that the light chain components of human immunoglobulins may become a pathogenic agent in immuno-proliferative disorders such as multiple myeloma (1, 2). The overproduction of monoclonal IgLCs may induce many clinical complications (3) which include systemic deposition of amyloid cast formation especially in the kidneys (4, 5, 6, 7), an elevated erythrocyte sedimentation rate and serum viscosity (8) and in some cases a generalised immuno-suppression (9, 10). The mechanisms which give rise to these effects are uncharacterised, and the possibility of a specific "ligand-receptor" type interaction also remains to be identified.

It has been demonstrated that the properties of free IgLCs, include possible protease activity similar to that of serine proteases (11, 12); the ability to activate the complement cascade via the alternative pathway (13); to bind chemotactic factors (14) and opioid peptides (15); antigen binding activity in the monomeric form (16, 17) as well as species and antigen cross reactivity (18). Perhaps the most important, potential of IgLCs is to modulate the immune system by functioning as an anti-idiotypic antibody (19), or possibly by induction of natural immuno-suppressive agents.

All these studies reaffirm the importance of the interactions of IgLCs, and imply that monomeric forms of IgLCs can act in a specific or multi-reactive manner. Presumably this occurs by the high-affinity associations which involve residues in the three complementarity-determining domains in the variable region of the IgLC. In this respect, the reactivity of monomeric IgLCs is unexpected. On the other hand, a far more favourable situation can be envisaged with the dimeric forms, which have been shown by X-ray crystallography to resemble the Fab region of whole Igs. In such a configuration, the two variable regions associate to form a pseudo-antigen binding cleft (20).

An excess of monoclonal IgLCs associated with multiple myeloma has been found to affect the immuno-competency of patients, many of whom develop systemic immuno-suppression (9, 10). This was investigated by Wearne and co-workers (21, 22, 23, 24), who expounded the phenomenon as Light Chain Isotype Suppression (LCIS), due to the specific suppression of normal Igs containing the light chain of the same isotype as the paraprotein. This interesting suggestion was criticised by King et al (25) who in a study of 25 multiple myeloma patients found no evidence of LCIS. However, in light of the data presented in recent reports (16, 17, 18, 19), the possibility exists that IgLCs may contribute to an immuno-suppressive mechanism. The means by
which this may be achieved may include: suppression mediated via an anti-idiotypic network; by displaying specificity to, and thereby inactivation of a membrane antigen involved in lymphocyte proliferation, differentiation or Ig synthesis; or by inducing the production of natural immuno-suppressive cytokines, by an as yet unspecified mechanism.

The immuno-suppressive effects of IgLCs, by virtue of induction of suppressive cytokines, may be related to the immuno-modulatory properties of intravenous immunoglobulin (IvIg), (26). These studies demonstrated a decrease in all lymphocyte subsets and a 41% decrease in the total peripheral blood lymphocyte count. The mode of action of this treatment is still unknown and is undergoing investigation. Ling et al (27), have assessed the effects of IvIg treatment on interferon γ and interleukin-6 in patients with secondary generalised epilepsy, and have shown a rapid increase in the plasma concentrations of both cytokines. The induction of these two proteins may be an important factor. It has been demonstrated that interferon γ causes a down regulation of interleukin-6 receptors on human B-lymphocytes (28, 29) and in synergy with interleukin-7 (which is abundant in the bone marrow stem-cell environment), arrests proliferation and differentiation of murine pre-B cell lines and induces apoptosis in these cells (30).

The interactions of IgLCs with a membrane surface has received little attention. A low-affinity, high capacity, receptor for IgLCs has been demonstrated in the rat and human renal proximal tubular brush-border cells (31, 32). This receptor however, is thought to be an endocytic scavenger involved in the resorption and catabolism of free light chains and small molecular weight proteins (31, 33). The possibility that a membrane-IgLC interaction can occur in the absence of a classical proteinacious receptor has, until now, not been considered. In light of the multi-functional potential of IgLCs, in this report we examined the biochemical aspects of interactions of these molecules.

MATERIALS AND METHODS

Theory: The use of FPE to monitor membrane-protein interactions.

The present study makes use of the fluorescent probe, fluorescein-phosphatidylethanolamine (FPE) which can be securely incorporated into liposome and cell membranes for reporting changes in the membrane surface potential, in response to protein-membrane interactions. The principles of this strategy are described by (34, 35, 36). A related technique described by Bergers et al (37) measured changes in the zeta potential associated with a pH titration of globular proteins bound to liposomes. This latter technique, however, is in principle less accurate than those obtained with FPE (34, 38, 39, 40).

Preparation of FPE labelled liposomes.

Negatively charged large unilamellar vesicles (LUVs) (41), with a diameter of 100nm were prepared using 85 mole% Palmitoyl-oleoyl-phosphatidylcholine (PC); 14 mole% Palmitoyl-oleoyl-phosphatidylserine (PS) : 0.5 mole % FPE. The method of preparation is essentially as described by Hope et al (42). Briefly, the phospholipid (stored in methanol: chloroform, 1:1 v/v) and the FPE (stored in methanol: chloroform, 1:5 v/v) were mixed in a round bottomed flask and dried under a stream of oxygen free, argon gas by rotary evaporation until a thin film was formed. This was best achieved by repeating the drying process after resolubilising the lipid mixture in pure chloroform. The lipid film was rehydrated using 1ml of the appropriate buffered solution and left to stand in the dark. The resulting multilamellar vesicle suspension was then freeze-thawed five times in liquid nitrogen (43) followed by aspiration, ten times, with a Pasteur pipette before being transferred to a device (Sciema Technical Services Ltd., Richmond, BC) for extrusion through standard 25mm diameter polycarbonate filters with a 100nm pore size (Nucleopore Filtration Products, CA, USA). The vesicles were injected into a chamber above the filter and nitrogen pressure applied from a gas cylinder fitted with a pressure regulator. The vesicles were extruded ten times under positive pressures of 250-550 lb/in². The resulting unilamellar vesicles, made up a homogenous population with 90% having a diameter of 100nm and another 10% of 100 nm ± 10nm. The sample was collected and stored under argon, in the dark at 4°C until required for the experiments.

Vesicles were prepared and suspended in one of the following solutions: 280mM sucrose; 100mM potassium chloride; and 100mM potassium thiocyanate. All solutions contained 5mM Tris-base as a buffer with the pH set at 7.5 using HCl/KOH, at 20°C.

Culturing and FPE labelling of HOM-2 lymphocytes.

The Epstein-Barr virus transformed human lymphoblastoid cell line, HOM-2 (HLA phenotype A3, B7, DR1, DQw1, DPw4), was cultured in RPMI 1640 (Life Technologies, USA) supplemented with 10% foetal calf serum. The cells were harvested and washed by two centrifugation steps at 2500xg for 5-10 seconds in PBS (150mM NaCl, 5mM Na₂HPO₄, pH 7.5). The cell number was determined by hemocytometer counting of a minimum of 100 cells. Cell viability was determined, by trypan blue exclusion (Sigma Chemical Co, St Louis, USA, application note). Cells were labelled with FPE
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according to the ratio, 10µg FPE : 3×10⁶ cells. FPE was prepared for addition by removal of the chloroform-methanol solvent under a stream of argon gas followed by re-suspension in 15µl of 95% ethanol, to which the cell suspension was added in a maximum volume of 3ml. The mixture was gently agitated before incubation in the dark for 1 hour at 37°C. Unincorporated FPE was removed by three serial centrifugations at 2500xg for 10 seconds in the appropriate iso-osmotic buffered solution. The cells were stored in the dark at 4°C and used within 48 hours of preparation.

Agarose Gel Electrophoresis.

An estimation of the net charge of λRG57, as well as the purity of protein samples were estimated by agarose gel electrophoresis. Electrophoresis was performed using either pre-cast, eight well, 1% agarose gels, prepared using sodium barbitone, pH 8.6 (Ciba Corning Diagnostics, Switzerland). Alternatively, 1% agarose gels at pH 7.5 and 9.5, in the same buffer, were cast in our laboratory. Samples were loaded onto the gel using plastic applicator tubes (Ciba Corning) which allowed a standard volume of 4µl to be accurately applied. Electrophoresis was performed using barbital buffer (50mM sodium barbital, 1mM EDTA, at pH 8.6) as the electrolyte, for 35-40 minutes at a constant voltage of 90mV. The gels were stained by immersion in a 0.2% w/v Amido black solution for approximately 10 minutes. Then, the gels were air dried overnight prior to destaining with a 5% v/v acetic acid solution.

Multiple Myeloma patients.

Urine from a patient with multiple myeloma was obtained with an informed consent from Severalls Hospital, Colchester UK, and urine was kept frozen at -70°C until used. The patient presented with a high level of a monoclonal paraprotein in the serum and urine. The paraprotein was a Bence Jones protein of λ isotype and presented predominantly as a dimer in the urine, which was designated λRG57. The amount of, λRG57 in the urine was 6mg/ml λ light chain. The amount of urine creatinine and serum levels of other immunoglobulins were normal.

Isolation of the myeloma paraprotein.

The myeloma paraprotein was isolated according to well established methods (44, 45), involving a concentration step followed by chromatographic purification.

The protein was concentrated in one of two ways:-

1) Ammonium sulphate precipitation, by adding ammonium sulphate to a final concentration of 50% (w/v), followed by incubation at 4°C whilst continuously stirring for 48 hours. The precipitate so formed was isolated by centrifugation at 10 000xg for 20 mins. The precipitate was re-dissolved in a small volume of 5PB at pH 7.5 and stored in 5ml aliquots at -70°C until used.

2) Size exclusion concentration employing, an Amicon™ concentrator and filter (Diaflow, UK) with a cut-off size for proteins greater than 10kD. The urine was forced through the filter under pressure using nitrogen gas at 200lb/in². This was carried out in the dark at 4°C. The filtration continued until a twenty fold concentration was achieved. The resulting turbid protein solution was dissolved in a small volume of PB at pH 7.5 and stored in 5ml aliquots at -70°C until used.

The protein was isolated using G-75 (Sigma) size exclusion chromatography, and further purified by DE52 (Whatman, UK) anion exchange chromatography. After each procedure the purity of protein was monitored with agarose gel electrophoresis.

The protein concentration was monitored after each step essentially as described (46). Determinations of the absorbances of the protein solution at 235nm and 280nm against a suitable blank, were made, and the following equation employed:

\[
\text{Protein conc. (mg/ml)} = \frac{\text{Abs}_{235nm} - \text{Abs}_{280nm}}{2.51}
\]

Spectroscopic Analysis.

Circular dichroic spectra were obtained in PBS (pH 7.5) and sucrose solution (pH 7.5), in the presence and absence of FPE-free PC/PS phospholipid vesicles (PLVs), as indicated. In all cases, λRG57 was at 2.17µM and the PLV sample contained 128µM lipid. This provided a protein: lipid ratio of 1 : 59 on a molar basis. Circular dichroic measurements were performed at room temperature, taking measurements at UV wavelengths, between 200nm to 260nm. Samples were analysed in a silica quartz cell with a 0.5mm pathlength on a Jasco J600 spectropolarimeter.

Fluorescence measurements were performed using a Perkin Elmer LS-50 spectrofluorimeter (Perkin Elmer, UK) with the excitation and emission wavelengths set at 490nm and 516nm respectively. Stopped-flow rapid mixing experiments were performed using an AppliedPhotophysics system (Leatherhead, UK), employing a 500nm cut-off filter, with the excitation wavelength set at 490nm. The time resolution of this apparatus was to within a few milliseconds. The data were imported into a data analysis package (Ultrafit, BIOSOFT, Ferguson, MO, USA) and the rate
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constants determined by fitting the curves to either a single or double exponential decay with offset, employing the following equations,

single exponential with offset:
\[ y = y_0 \cdot e^{k_1 \cdot t} + c \]

double exponential with offset:
\[ y = y_{01} \cdot e^{k_1 \cdot t} + y_{02} \cdot e^{k_2 \cdot t} + c \]

Where \( y \) is the intensity of the fluorescence signal, \( y_0 \) and \( y_{02} \) are the initial fluorescence signals determined at \( t = 0 \), for rates \( k_1 \) and \( k_2 \) respectively, \( t \) is the time and \( c \) the value of the offset, which represents the endpoint of the exponential decay at \( t = \infty \). The fitting used 95% confidence limits and was carried out using the Gauss-Newton-Marquardt algorithm with robust weighting which rejected outliers with a rejection factor of 6. The corresponding residuals were calculated and the "goodness of fit" was expressed as a \( \chi^2 \) value.

RESULTS

A qualitative measurement of the net charge of \( \lambda \)RG57 was determined by agarose gel electrophoresis performed at pH values of 8.6, 7.5 and 9.5 (Fig. 1). At pH 8.6, the protein demonstrated slight electrophoretic mobility towards the cathode, suggesting that its net charge at this pH is positive, implying a \( pK \approx 8.8 \). The protein therefore possesses a net positive charge at pH 7.5, and a net negative charge at pH 9.5 (Fig. 1).

The interaction of \( \lambda \)RG57 with PC and PC/PS PLVs, at pH 7.5, generally resulted in a decrease in the steady state fluorescence intensity of the membrane bound FPE (Fig. 2). The exceptions to this rule were found when PLVs were suspended in a medium containing 100mM KSCN. The changes in the fluorescence intensity of FPE, associated with the addition of \( \lambda \)RG57 to PC and PC/PS PLVs, are summarised in table 1.

The presence of 100mM KSCN in the suspending medium completely abrogates any change in the fluorescence intensity upon the addition of \( \lambda \)RG57 to either PC or PC/PS PLVs (Fig. 2A & Table 1). In contrast, the fluorescence intensity of FPE labelled PLVs suspended in 100mM KCl, was found to decrease upon the addition of \( \lambda \)RG57 (Fig. 2D & Table 1). The interaction of \( \lambda \)RG57 with PLVs suspended in either 150mM NaCl or 280mM sucrose solutions, resulted in a similar decrease in the fluorescence intensity. The change in the fluorescence intensity, in these cases, appeared to be independent of the charge carried by the PLV, as demonstrated by the parity of the signals for PC and PC/PS PLVs (Fig. 2A and C).

Table 1. Summary of the mean changes in the fluorescence intensity of FPE incorporated into PC and PC/PS PLVs, upon the addition of \( \lambda \)RG57.

<table>
<thead>
<tr>
<th>PLV composition</th>
<th>Suspending medium (containing 5mM Tris at pH 7.5)</th>
<th>Mean % change in fluorescence intensity ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>100mM KCl</td>
<td>-4.3 ± 0.8</td>
</tr>
<tr>
<td>PC/PS</td>
<td>100mM KCl</td>
<td>-8.0 ± 2.4</td>
</tr>
<tr>
<td>PC</td>
<td>100mM KSCN</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>PC/PS</td>
<td>100mM KSCN</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>PC</td>
<td>280mM sucrose</td>
<td>-7.3 ± 1.6</td>
</tr>
<tr>
<td>PC/PS</td>
<td>280mM sucrose</td>
<td>-8.8 ± 2.6</td>
</tr>
<tr>
<td>PC</td>
<td>150mM NaCl</td>
<td>-8.2 ± 2.3</td>
</tr>
<tr>
<td>PC/PS</td>
<td>150mM NaCl</td>
<td>-6.2 ± 0.7</td>
</tr>
</tbody>
</table>
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Fig. 2 The interaction of λRG57 with PC and PC/PS PLVs.
PLVs were suspended at a concentration of 80μM lipid, in either, 280mM sucrose (A), 100mM KSCN (B), 150mM NaCl (C) or 100mM KCl (D), all supplemented with 5mM Tris at pH 7.5 and 20°C. Single additions of λRG57 were made to the cuvette, after approximately 30 seconds, to give a final concentration of 39μM. The emission at 518nm was measured whilst exciting at 490nm.

A decrease in the fluorescence intensity of the membrane bound FPE, suggests that Ψs is becoming more electronegative due to the association of λRG57 with the membrane surface. This observation appears to disagree with the prediction of the net charge of the protein shown by its electrophoretic mobility on an agarose gel (Fig 1). Considering the apparently conflicting fluorescence and electrophoretic mobility data, it is clear that the interaction of λRG57 with the PLV membrane appears not to be a simple adsorption event. In other words, the apparent charge of the protein on the membrane surface differs from that in solution.

Finally, in an attempt to resolve in time, the events associated with the interaction of λRG57 with charged PC/PS membranes system, a series of stopped flow rapid mixing experiments were undertaken. Mixing of the protein and liposomes results in a rapid increase in the fluorescence intensity (Fig. 3). A second phase which was slower and a decrease in the fluorescence intensity was also observed (with a half-time of ca. 20 seconds). An important feature of these data is the time over which these two events occur, the binding phase is rapid and is described by a single exponential phase with a calculated rate constant of k = 30.9 ± 1.2 sec⁻¹. The
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Fig. 3 Time resolution of the series of events associated with the interaction of λRG57 with PC/PS vesicles. Rapid mixing experiments were performed by combining solutions containing 0.2mg/ml (4.34mM) λRG57 and 0.2mg/ml (256µM) PC/PS vesicles in sucrose solution (280mM sucrose, 5mM Tris, pH 7.5) were prepared to allow for the dilution effect associated with the mixing process in the stopped flow apparatus. The excitation wavelength was set to 490nm and a 500nm cut-off filter allowed the acquisition of all light above this wavelength. Eight scans were taken and the average signal displayed, the data were analysed as described in Materials and Methods. A) The fast, binding phase. B) The slow reorganisation event. The residuals of the analysis are shown below the experimental data.

Identical binding experiments were performed at increasing temperatures up to 37.8°C (Fig. 4). The extent of binding, defined as the difference between the starting fluorescence intensity and that at equilibrium, decreased markedly as the temperature increased. This effect was accompanied by an increase in the rate constants (Table 3). Experiments performed at 2.8°C and 11.8°C (Figs. 5 and 6) once fitted to double exponentials made it clear that the second rate, associated with the binding process, remained constant, at 1.5 mol⁻¹sec⁻¹. From this observation, k₂ was deemed to be temperature insensitive and was fixed at 1.5 whilst the data taken at 24.8°C and 37.8°C were fitted to a double exponential decay. In order to determine whether the binding phase of λRG57 to PC/PS vesicles was indicative of a single or double exponential process with a temperature insensitive second rate, both equations were used to fit the data and the residuals and χ² values for the goodness of fit were noted. The unweighted residuals for the data taken at 24.8°C were very similar when the data were fitted using either a single or double exponential (Fig. 7), and the χ² values were almost identical (Table 2). This suggests that the data can be adequately represented by a double exponential function. An investigation was undertaken as to whether the data collected at the two lowest temperatures could be fitted equally well, to a single exponential equally well, was undertaken (Figs. 5 and 6). It can be clearly seen from the residuals and fitted curves, that a fit to a single exponential was far less satisfactory than that to a double exponential function. This is borne out by a comparison of the χ² values for the residuals shown in Table 2.

Table 2. Statistical results for the "goodness of fit", the data represents the binding, fast phase, of the interaction of λRG57 with PC/PS liposomes in a low ionic strength solution, at the indicated temperatures.

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>χ² for Double Exponential</th>
<th>χ² for Single Exponential</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.8</td>
<td>2.55E-4</td>
<td>4.86E-4</td>
</tr>
<tr>
<td>11.8</td>
<td>2.41E-4</td>
<td>4.93E-4</td>
</tr>
<tr>
<td>25.8</td>
<td>1.26E-4</td>
<td>1.71E-4</td>
</tr>
</tbody>
</table>
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Table 3. The rate constants determined by stopped flow technique, for the interaction of λRG57 with PC/PS PLVs. Experiments were performed as described for Fig. 3, at increasing temperatures. The $k_1$ was fixed at 1.5 before fitting the data set.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$k_1$ (mol$^{-1}$ sec$^{-1}$)</th>
<th>$k_2$ (mol$^{-1}$ sec$^{-1}$)</th>
<th>$k_{-}$ secondary event (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.8</td>
<td>1.5</td>
<td>20.7</td>
<td>$7.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>11.8</td>
<td>1.5</td>
<td>26.0</td>
<td>$1.8 \times 10^{-3}$</td>
</tr>
<tr>
<td>24.8</td>
<td>1.5</td>
<td>46.0</td>
<td>$3.1 \times 10^{-2}$</td>
</tr>
<tr>
<td>37.8</td>
<td>1.5</td>
<td>98.4</td>
<td>12.2</td>
</tr>
</tbody>
</table>

Fig. 4 Temperature dependence effects on the binding phase of λRG57 with PC/PS vesicles. Additions of λRG57 were made to PC/PS vesicles as described in figure 3, at the indicated temperatures.

The calculated rate constants for the double exponential analysis of the interaction of λRG57 with PC/PS PLVs, at all temperatures are summarised in Table 3. Also included are the calculated rate. The presence of liposomes and a high and low ionic constants for the secondary, slower event, at each temperature. The secondary event is adequately described by a single exponential decay at all the experimental temperatures studied, with a mean $\chi^2$ value of $1.4 \pm 0.4 \times 10^{-4}$ for all four temperatures studied.

The data in Table 3 were used to construct an Arrhenius plot (see e.g., 47) using the following equation,

$$k = A \exp \left( - \frac{E_a}{RT} \right)$$

where, $k$ is the rate constant, $A$ and $E_a$ are the pre-exponential factor and the activation energy (KJmol$^{-1}$) respectively, R and T have their usual meanings. For relatively simple processes, a plot of $(\ln k)$ against $1/T$ is anticipated to be linear, the gradient of which yields the activation energy. The results of an Arrhenius plot of λRG57 binding to PC/PS vesicles in a medium of low ionic strength are shown in Figure 10. The temperature sensitive rate associated with binding was fitted by linear regression with an $R^2$ value of 0.93 (1 is a perfect fit). Accordingly, the calculated energy for the temperature sensitive process was $13.53$ KJmol$^{-1}$. The secondary event yielded an activation energy of $87.89$ KJmol$^{-1}$ with an $R^2$ value of 0.98 (Fig. 8). The presence of liposomes and a high and low ionic strength bulk phase on the secondary structure of λ RG57 was assessed by circular dichroic measurements. No change in the secondary structure was witnessed under these conditions. As shown in Fig 9, the protein remaining in its predominantly β-sheet configuration as indicated by the lack of change at the 218nm minima.
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Fig. 5 Comparison of the single and double exponential analysis for the binding phase of \( \lambda \)RG57 to PC/PS vesicle at 2.8°C. Experiments were performed as described in figure 3, the temperature was set to 2.8°C. The double (solid line) and single (dotted line) exponential analyses of the data are shown together with the double exponential residuals (upper panel), the single exponential residuals (dotted line) are overlaid in the bottom panel.

An approximation of the isoelectric point of \( \lambda \)RG57 was determined by agarose gel electrophoresis at three different pH values (Fig. 1). At pH 8.6, \( \lambda \)RG57 carries a slight positive net charge as shown by its’ electrophoretic movement to the cathode (Fig. 1). At the experimental and physiological pH of 7.5, the protein is unequivocally cationic and similarly at pH 9.5 \( \lambda \)RG57 is anionic (Fig. 1). Although this is a crude representation of the pI of the protein, it provides sufficient information for the interpretation of their binding to membranes, as revealed by the changes in the fluorescence intensity of FPE.

The interaction of \( \lambda \)RG57 with FPE labelled PC and PC/PS PLVs in various suspending media generally resulted in a decrease in the fluorescence under the experimental conditions investigated (Fig. 2). PLVs suspended in 100mM KSCN however, exhibited no change in the fluorescence intensity upon the addition of \( \lambda \)RG57.

The role of the hydrophobic effect in the interaction of \( \lambda \)RG57 with PLV membrane surfaces has been investigated using the chaotrope KSCN (48). KSCN exhibits a high entropy of hydration and thereby, interferes with hydrophobic interactions (Fig. 4). The partial molar entropies of hydration of SCN\(^-\) and Cl\(^-\) have been quoted as -33.92 and -76.20 Jmol\(^-1\)K\(^-1\), respectively (49). Binding was completely abrogated in 100mM KSCN, with respect to 100mM KCl implying that the hydrophobic effect, appears to play a major role in the binding process.

**DISCUSSION**

It has previously been demonstrated that the fluorescent probe, FPE, may be stably incorporated into biological and synthetic membranes, allowing monitoring of the binding of proteins (34, 35, 36). After the initial protein-membrane contact has taken place it is then possible to characterise any protein reorientation which may follow. In order to investigate the potential physiologic effect of Bence-Jones proteins, the interaction of \( \lambda \)RG57 with cell membranes was studied. The binding of \( \lambda \)RG57 to lymphocyte and synthetic membranes was shown to be comprised of two distinct events. The first event presumably represents a collision limited binding process followed by a slower reorganisation event. The secondary event is believed to be associated with the maximisation of coulombic attractive forces between the protein and the membranes, as well as entropically (and/or enthalpically) driven
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Fig. 6 Comparison of the single and double exponential analysis for the binding phase of λRG57 to PC/PS vesicle at 11.8°C. Experiments were performed as described in Figure 3, the temperature was set to 11.8°C. The double (solid line) and single (dotted line) exponential analyses of the data are shown. The double exponential residuals are given (upper panel) upon which the single exponential residuals (dotted line) have been overlaid in the bottom panel.

interaction as a result of the hydrophobic effect. The technique of using FPE as a probe for monitoring changes in Ψₘ facilitates the real-time visualization of the proteins charged moieties when they come into close proximity to the membrane surface (Fig. 3). Once the protein-membrane contact has been made, FPE may report any protein reorganisation events which are accompanied by a modification of the charge profile present at the membrane-water interface (34, 35, 36).

The lack of interaction shown in the KSCN media, together with the parity of the results obtained in the other experimental media suggest that the interaction of λRG57 with the PLV membrane is governed predominantly by electrodynamic forces, rather than Coulombic attraction. In simpler terms, the hydrophobic effect, is thought to be the major force directing the binding of λRG57 to the PLV membrane.

This conclusion is reached on the basis that in a medium where the major anion is SCN⁻ little binding is observed. It is believed that the high entropy of hydration of SCN⁻ (49) disrupts the non-polar properties of the phospholipid bilayer thereby modifying/removing the λRG57 binding site. This residuals (dotted line) are overlaid in the bottom panel. Ultimately results in the observed loss of binding of the protein in this medium (Fig. 2 & Table 1).

The observation that an addition of λRG57 results in a decrease in the fluorescence intensity of FPE suggests that Ψₘ is becoming more electronegative. It has been shown however, that the net charge carried by λRG57 at this pH is positive (Fig. 1). These observations may be reconciled in light of the mode of action of FPE (34), which is only responsive to charged species residing within the immediate vicinity of the lipid-water interface, as dictated by the Debye length. However, if the charged groups move away from the membrane surface into the bulk, or become buried within the lipid phase of the membrane, as a result of binding, a reversal of the apparent net charge may occur (34). If this interpretation is valid it would be expected that the interaction of λRG57 with the membrane surface involves more than just a simple surface-binding event. The multi-component interaction model for λ RG57, is given support by the results of the stopped-flow experiments, discussed below (Fig. 3).

The far-UV CD spectra of λRG57, under all experimental conditions tested, are characterised by single (negative) minimum at approximately 218nm
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Fig. 7 Comparison of the single and double exponential analysis for the binding phase of λRG57 to PC/PS vesicle at 24.8°C. Experiments were performed as described in Figure 3, the temperature was set to 25.8°C. The double exponential analysis of the data is shown together with the corresponding residuals (upper panel), the single exponential binding, circular dichroic studies indicated no change in the secondary structure of λRG57, and the protein remained in its predominantly β-sheet configuration, as shown by the single minimum at 218nm, in both high and low ionic strength media, as well as in the absence or presence of liposomes (Fig. 9).

The effect of temperature on the fast phase of the λRG57-membrane association (Fig. 4 and Table 3), believed to be the binding event, suggests that there are two effects which may be responsible for this process. The first, a slow event with a temperature insensitive rate constant of 1.5 mol⁻¹sec⁻¹, combined with a rapid event which increases dramatically over the temperature ranges studied. It is interesting to postulate that these two components represent the electrostatic and hydrophobic components respectively. This is in accordance with the data for λRG57 binding in 100mM KSCN (Fig. 2) which indicates that the major factor determining the association of the protein with a lipid membrane is a hydrophobic interaction. Furthermore, the estimated activation energy which corresponds to the temperature sensitive, kinetic phase is 13.53 KJmol⁻¹.

This event is accurately described by a single exponential decay, the rate of which increases from 7.1 × 10⁻⁴ sec⁻¹ at 2.8°C to 12.2 sec⁻¹ at 38.3°C (Table 3). The activation energy associated with this process is calculated to be 87.78 KJmol⁻¹ (Fig. 8 & Table 3). This molecular process therefore, appears to require 6.5 times more energy than the binding process, which is consistent with the view that the reorganisation of large domains of the protein is occurring.

These data show very clearly a rapid increase in fluorescence intensity over the first 500 milli-seconds, which is believed to be the binding process which results in Ψs becoming increasingly electropositive. This phase is then followed by a sustained decrease in fluorescence intensity, or an increase in the electronegativity of Ψs, which extends over a period of more than 50 seconds.

The binding of IgLCs to synthetic and B-cell membranes appears to involve a complex series of events, rather than simple adherence due to electrostatic attraction. It seems likely therefore, that the biological effects of these proteins are linked to many inherent factors, and not just to the pI of the protein, a relationship which has been previously criticised (51). Indeed, it has been seen that there is little correlation between the net charge of an
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Fig. 8 Arrhenius plot for the temperature sensitive rate of the binding of λRG57 to PC/PS vesicles. Arrhenius plot of the binding of λRG57 to PC/PS vesicles. Constructed using the rate constants shown in table 4. The data were fitted by linear regression with. The calculated value of $E_a$ for the binding phase is 13.53 KJmol⁻¹, and that for the slower reorganisation event is 87.79 KJmol⁻¹.

IgLC and the efficiency of binding to simple, model membranes, as shown in this study. If any attempts are to be made to limit the binding of IgLCs to normal cells in a clinical situation, or to prevent high order aggregates forming and becoming deposited systemically, then it appears more likely that this will be achieved by the abrogation of the hydrophobic forces which are believed to govern these processes.

The reorientation of the IgLC upon its binding to the membrane surface may facilitate its interaction with integral membrane proteins. Such interactions may lead to the initiation of transmembrane signals via membrane protein aggregation. Alternatively, the membrane-bound IgLC may adopt a configuration which initiates, or enhances the formation of light chain fibrils, associated with amyloidosis. The role of the membrane surface in the aetiology of amyloidosis is unclear but its involvement is undergoing investigation (unpublished data). Furthermore, the reorientation event induced by the IgLC-membrane surface interaction, raises the question as to whether other proteins use non-specific binding mechanisms as the first step of interaction with cell membranes. This may also be a means to re-configure their structure in such a way as to facilitate a specific ligand-receptor interaction or transmembrane insertion and thereby possibly mediate a cellular response.

Fig. 9 Circular dichroic study of λRG57 in high and low ionic media and in the presence and absence of PC/PS vesicles. Circular dichroic spectra were taken in PBS (150mM NaCl, 5mM Na₂HPO₄) and sucrose solution (280mM sucrose, 5mM Tris, pH 7.5), in the presence and absence of PC/PS vesicles without FPE, as indicated. In all cases λRG57 was at 0.1mg/ml (2.17mM), the liposome sample contained 0.1mg/ml (128µM) lipid. This provided a protein to lipid ratio of 1: 58 on a molar basis.

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