**Introduction**

Histocompatibility molecules become functional transmembrane receptors following peptide binding and display at the cell surface of an antigen presenting cell (APC) (Rotzschke et al., 1990). This is followed by a ligand-binding interaction with a responding T cell. This reaction is essential for the initiation of specific immunity and requires activation of a small subset of responding T cells by a given MHC subset on an APC (Sakihama et al., 1995; Clark and Ledbetter, 1994; Janeway 1992). Secretion of cytokines by the reacting T cell further amplifies the response by upregulating and possibly redistributing the MHC receptors and thus enhancing the recruitment of T cells (Abbas et al., 1994; Davis and Chien, 1993). It is conceivable that the cell-surface density of MHC molecules, their relationship with other membrane-bound proteins in the correct orientation and their homo- and heterotypic aggregation state play a crucial role in effective T cell recognition (Dustin and Springer, 1989; Fernandez et al., 1992).

A major advance in understanding the molecular basis of MHC and peptide interactions was the determination of the high resolution crystal structures of the human HLA A2 and HLA DR1 which have provided information on the 3-D structure of the MHC molecules and their peptide binding grooves (Bjorkman et al., 1987; Brown et al., 1993). However, a major limitation in the understanding of the way MHC molecules activate T cells remains the resolution of molecular interactions at the cell surface on single cells on a nanometer scale. In the case of MHC class I molecules, evidence has been obtained for receptor clustering and interactions with other cell surface receptors (Chakrabarti et al., 1992; Capps et al., 1993; Matko et al., 1994, 1995; Damjanovich et al., 1995; Philpott et al., 1995; Bene et al., 1994; Edidin, 1990). Association of receptors is likely to be a dynamic process, possibly playing an important role in T cell activation (Sakihama et al., 1995; Kupfer and Singer, 1988). Dynamic associations in turn depend upon mobility of receptors in the plane of the membrane and their relationship with the cytoskeleton (Stossel, 1994).

The recently developed methods of single particle tracking (SPT) provide a powerful approach to observing the movement of receptors at high spatial resolution. These methods employ either small fluorescent particles (Gross and Webb, 1988; Anderson et al., 1992; Wang et al., 1994; Ghosh and Webb, 1994; Hicks and Angelides, 1995) or colloidal gold particles.
otics and 10% (v/v) foetal calf serum at 37°C in a 7% CO₂ humidified atmosphere. Virus particles bound to their receptors on fibroblasts (Anderson et al., 1992). Evidence for the confinement of receptors by barriers has also been obtained by an elegant combination of SPT and laser tweezer techniques (Edidin et al., 1994; Sako and Kusumi, 1995).

We have previously performed SPT measurements with fluorescence-labeled low density lipoprotein (LDL) and influenza virus particles bound to their receptors on fibroblasts (Anderson et al., 1992). We applied this technique to the analysis of HLA class II molecules, a subset of the MHC primarily expressed on APCs of the immune system. We have developed a set of experimental conditions to label HLA-DR specific monoclonal antibodies with the phycobiliprotein, R-phycocerythrin (Smith et al., 1996). These light-harvesting proteins have a fluorescence yield equivalent to that of up to 30 fluorescein molecules (Oi et al., 1982) yet are only about 12 nm × 6 nm in size (Glazer, 1983). We have shown that these small fluorescent particles can be sequentially imaged with a cooled slow-scan charge-coupled device (CCD) camera and can thus be used in SPT experiments. We report the results of such experiments performed with human MHC negative fibroblasts transfected with HLA DR alpha and HLA DR beta genes (Dodi et al., 1994). These studies are relevant to the intrinsic capacity of MHC class II molecules to trigger T cells by rapid polarised cross-linking of the T cell receptor (TCR). This approach is also relevant to determining the role of MHC mobility in forming heterotypic associations with accessory molecules in the plane of an APC membrane.

**MATERIALS AND METHODS**

**Cells**

HOM-2 a normal B-lymphoblastoid cell line (DR1.1;DQw1.1;DPw4) was obtained from the Department of Immunology, The Royal London Hospital. The cells were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, streptomycin/ampicillin antibiotics and 10% (v/v) foetal calf serum at 37°C in a 7% CO₂ humidified atmosphere.

The human transfantant fibroblast M1DR1 expressing cell surface HLA-DR alpha and beta molecules was a kind gift from R. Lechler (Royal Postgraduate Medical School, Hammersmith Hospital, London). The cells were maintained as described above. For imaging experiments, trypsinised cells were seeded onto 8-well Lab-Tek slides (Gibco) at a density of 5,000 cells/well and cultured for a further 72 hours before use.

**Monoclonal antibody purification**

The hybridoma cell line HB55 secreting antibody specific for monomorphic HLA-DR determinants was obtained from the American Type Culture Collection (Bethesda, MD). Ascites fluid was centrifuged at 20,000 g for 30 minutes to remove insoluble material and loaded onto a HiTrap Protein-A column (Pharmacia). Briefly, the HiTrap column was equilibrated with 20 mM sodium phosphate buffer, pH 7.0, and the bound immunoglobulin eluted with 0.1 M citric acid buffer as previously described (Smith et al., 1996). Upon elution the pH was adjusted to 7.0 with 1 M Tris-HCl, pH 9.0, and the amount of IgG quantified using the mouse IgG extinction coefficient as described previously (Harlow and Lane, 1989).

**Labeling with R-phycocerythrin (PE)**

Aliquots of purified IgG were labeled with PE pyridyl disulphide derivative (Molecular Probes, USA) essentially as described (Smith et al., 1996). Briefly, 2.6 mg of IgG was dialysed into 100 mM Na₂HPO₄, 100 mM NaCl, pH 7.5 (phosphate buffer), and concentrated to 5 mg/ml using Amicon 30 microconcentrators. Thiol-reactive maleimide residues were introduced into IgG by incubating with 10 molar equivalents of 5 mM succinimidyl trans-4-[(N-maleimidylmethyl) cyclohexan-1-carboxylate (SMCC) in dimethyl sulphoxide for 2 hours at room temperature. Excess SMCC was removed by extensive dialysis against phosphate buffer. In parallel, 2.0 mg of PE pyridyl disulphide derivative (average 2.2 pyridyl residues per molecule) was incubated with 50 mM dithiothreitol (DTT) for 15 minutes at room temperature. Excess DTT was removed by dialysis with phosphate buffer. The PE was then incubated with the IgG for 20 hours at 4°C in the dark. To prevent IgG disulphide reduction or oxidation to dimers, a 20 molar excess of N-ethylmaleimide was added.

**Purification of PE Labeled IgG**

PE labeled IgG was purified from free PE and unlabeled IgG on an XK 16/70 column packed with Sephacryl S-300 HR (Pharmacia). The crude labeled IgG was loaded onto the column, eluted with phosphate buffer and 1 ml fractions collected. The elution profile was monitored spectrophotometrically at 280 nm.

Analytical size exclusion chromatography was performed on a Bio-Rad 5000F HRLC controlled by a 486 PC. PE labeled IgG was loaded onto a Bio-Select SEC 250-5 column equilibrated with 50 mM Na₂HPO₄, 150 mM NaCl, pH 6.8, at 1.0 ml/minute. Integration was performed using ValueChrom² integration analysis software (Bio-Rad). PE labeled IgG concentration was determined using a molar extinction coefficient of 1.96×10⁶ M⁻¹cm⁻¹ for PE.

**Flow cytometry**

HOM-2 cells were harvested by centrifugation at 80 g for 5 minutes at room temperature and washed three times with phosphate buffered saline (PBS) supplemented with 0.02% bovine serum albumin and 0.02% sodium azide (Buffer A). PE-labeled IgG was diluted to 2 pmol/100 μl in Buffer A and incubated with the cells at this concentration for 45 minutes at room temperature. The cells were then washed three times with Buffer A, once with PBS and then examined for fluorescence using an EPICS CS flow cytometer (Coulter Corporation), counting 10,000 cells/sample.

**Fluorescence digital imaging microscopy**

M1DR1 cells cultured on Lab-Tek slides were labeled with PE-IgG as follows. The chilled cells were washed three times with cold PBS and then once with cold RPMI 1640 supplemented with 2 mg/ml sodium bicarbonate (Buffer B). The PE-IgG labeled antibody probe was then diluted to 1-2 pmol/100 μl in Buffer B and the cells incubated with PE-IgG at this concentration for 30 minutes at 4°C. They were then washed three times with cold PBS. After washing once more in Buffer B, the slide chamber was then covered and transferred to the microscope stage, maintained at room temperature, for analysis. Non-specific binding was checked by incubating cells with PE-IgG in the presence of a 10-fold excess of unlabeled IgG.

Polylysine-coated slides were prepared by cleaning glass microscope slides with 70% (v/v) ethanol containing 3% (v/v) concentrated HCl solution for 1 hour, rinsing with distilled water, and incubating for 10 minutes in 0.01% poly-L-lysine solution at room temperature. After incubation, slides were drained on Whatman number 1 filter paper and dried in an oven; 1-2 pmol of PE-IgG probe was then allowed to settle on the slide for 5 minutes, rinsed with distilled water and the coverslip mounted using a silicone grease ring and distilled water.

Fluorescence digital imaging microscopy was performed using a...
Nikon Diaphot inverted fluorescence microscope. The objective was a x40 phase contrast lens with numerical aperture 0.55. Illumination was by a 50 W mercury lamp, and wavelengths were selected using Omega Optical Inc. filters and dichroic mirrors; 525 nm and 575 nm filters were placed in the excitation and emission beams, respectively, and the dichroic mirror cutoff was 545 nm. A Wright Instruments CCD camera was attached to the video port of the microscope and the image focused on to an EEV P8603 detector (576x384 pixels). This device has a maximum quantum efficiency of around 35% and a mean readout noise equivalent to 7 electrons/pixel. Image acquisition, storage and display were performed using the Wright instruments AT1 image control software, running on a 486 PC. Images were typically recorded every 1 or 2 minutes with an exposure time of 5 seconds.

Data analysis – tracking

The procedures required to track fluorescent particles through time-lapse images have been given in detail elsewhere (Anderson et al., 1992). The particles appear as diffraction limited spots covering a number of pixels; their approximate positions are identified by a simple image analysis algorithm, and then quantified by least-squares fitting the pixels in this immediate area with a 2-dimensional Gaussian function. The spot positions are thus obtained to within a standard error value determined by the fit algorithm (Bevington, 1969), which is carried through all the succeeding mobility calculations. In addition, this fit procedure gives values for the spot widths, which can be used for artefact rejection (only spots near to the diffraction limited width are accepted, and spots with large deviations in the width or high residual variance values are likely to be ‘close doubles’ and are rejected). The spot intensity above local background then helps to identify particles as monomers, dimers or larger cluster sizes (Morrison et al., 1994).

After quantification of the spots, they are linked through the time-lapse images by a ‘nearest spot with similar intensity’ probability method. In the images obtained in this work, the observed particle densities were low, so that distances between spots were typically >2 μm. The intensity probability is only important in crowded areas; and since the algorithm must accept variations caused by photobleaching, and single PE-labelled molecules have only small variations in fluorescence intensity, the distance probability provides the most important part of the tracking algorithm. All tracks are operator checked and any of doubtful validity are eliminated from subsequent analysis.

The particle tracks thus obtained may be affected by ‘creep’ of the slide with respect to the microscope optics, by thermal or mechanical effects. This can be evaluated by tracking off-cell particles (dust or antibodies bound to the glass surface), and selecting a uniform subset of these spots to provide a background movement correction factor.

Data analysis – mobility

The analysis of single particle tracks to obtain mobility characteristics has been the subject of considerable discussion recently (Qian et al., 1991; Saxton 1994, 1995; Simson et al., 1995). The method adopted here is to use simple mean-square-displacement analysis of individual particle tracks (Anderson et al., 1992) and then apply other methods to the overall results obtained for each cell.

For each track through m images, the mean-square-displacement \( \langle r_n^2 \rangle \) is calculated for different time intervals \( n \delta t, n = 1, 2, \ldots \), being the number of images between which the displacement \( r \) is computed, and \( \delta t \) is the time interval between images. These calculations can be made up to \( n = m - 1 \), but values for \( n > m/2 \) are not useful as there is insufficient averaging – all segments of the track overlap. The \( \langle r_n^2 \rangle \) values are plotted as a function of \( n \delta t \), and the particle mobility assigned as simple diffusion with coefficient \( D_{lat} \) if the plot is linear (equation 1a); directed motion with velocity \( v \) if the plot curves upwards (equation 1b); or domain diffusion if the plot curves down to an asymptotic value \( r_{max}^2 \) (equation 1c):

\[
\langle r_n^2 \rangle = 4D_{lat}(n\delta t); \quad (1a)
\]

\[
\langle r_n^2 \rangle = v^2(n\delta t)^2; \quad (1b)
\]

\[
\langle r_n^2 \rangle > r_{max}^2[1-\exp(-D_{lat}(n\delta t)r_{max}^2)]. \quad (1c)
\]

An exact solution of the diffusion equation for this latter situation has been published (Saxton, 1993), but the accuracy of the data do not yet justify the extra computational complexity, and we use an empirical equation (Anderson et al., 1992; equation 6) to obtain the diffusion coefficient and determine the domain diameter from \( r_{max} \) (Saxton, 1993). Individual tracks cannot be assigned unambiguously to non-random diffusion since there is always a finite probability that any track occurs by random movements (Qian et al., 1991; Saxton, 1994). One approach to this problem is to select tracks for which the probability of their arising by random diffusion is very small. This is likely to overestimate the number of tracks arising from random diffusion. The method used here is to classify tracks according to whether they are more likely to correspond to one or other type of motion. The criterion for classification is simply the goodness of fit parameter for the 3 types of motion. Whilst this analysis will not assign all tracks correctly, it provides partially ‘purified’ populations from which to extract parameters for the different types of motion. Further tests are then carried out on the total data set for each cell as described below in order to validate the simple analysis.

Data analysis – distance histograms

A useful technique for obtaining the mean mobility behaviour of all the particles on a cell or part of a cell, is to create a probability distribution of the distance moved (\( r \)) by all spots for a time interval \( n \delta t \) (Anderson et al., 1992). Such distributions can be used in two ways: (a) individual distributions can be fitted by the random diffusion model

\[
P(r)dr = r(2D_{qn}\delta t)\exp[-r^2/(4D_{qn}\delta t)]dr, \quad (2)
\]

to obtain a quasi-diffusion coefficient \( D_q \). In cases where a major fraction of particles are not randomly diffusing, this value will vary with the time interval.

(b) A number of distributions with different values of \( n \) can be fitted by global analysis (Morrison et al., 1994) to a mobility model. This model can combine two types of behaviour, e.g. random diffusion with directed motion. In this case, the quasi diffusion coefficient becomes a true diffusion coefficient \( D_{lat} \), and the equation includes an additional term for directed motion; a fraction \( C_d \) of the jumps in a distribution are considered to have a normal distribution of velocities around a mean value \( v \), with width \( s_v \), so that after time \( n \delta t \) the distance moved has mean value \( vn\delta t \) and width \( s_vn\delta t \). Then:

\[
P(r)dr = C_{dr}(2D_{lat}\delta t)\exp[-r^2/(4D_{lat}\delta t)]dr + \frac{C_d}{(2\pi s_v^2)}\exp[-(r-vn\delta t)^2/2s_v^2]. \quad (3)
\]

\( C_d \), the fraction of jumps showing diffusion, and \( C_{dr} \) are both normalised to reflect the total number of jumps in the \( n \)th distribution. The fit will then use \( D_{lat}, v, s_v, C_d \) and \( C_{dr} \) as global variables common to all the distributions, while the normalising factors are individual.

When domain diffusion dominates, the model can combine a global random diffusion coefficient \( D_{lat} \) with a second individual quasi diffusion coefficient, \( D_q \) that will simulate the domain diffusion:

\[
P(r)dr = C_{dr}(2D_{lat}\delta t)\exp[-r^2/(4D_{lat}\delta t)]dr + \frac{C_q}{(2\pi s_v^2)}\exp[-r^2/(4D_q\delta t)]dr. \quad (4)
\]

\( D_{lat} \) is a global variable across the distributions with different \( n \delta t \) values as will be \( C_d \) and \( C_{dr} \); the individual variables will be \( D_q \) and the normalising factors for the \( n \)th distribution.

RESULTS

PE labeled IgG probe

PE-IgG will have a molecular mass considerably higher than
Fig. 1. Purification of PE-IgG probe by gel filtration chromatography. The crude labeled IgG was loaded onto a XK 16/70 column packed with Sephacryl S-300 HR and equilibrated with 100 mM sodium phosphate, 100 mM sodium chloride, pH 7.5, at 0.25 ml/minute. The labeled IgG species are eluted first (peak A), followed by the free PE and unlabeled IgG (peak B). The solid bar indicates the labeled IgG fractions that were collected and used in all later experiments.

Fig. 2. Analytical HPLC size exclusion chromatography of phycoerythrin (PE) labeled IgG fractions collected as indicated in Fig. 1. Labeled IgG (A), unconjugated PE (B) and unlabeled IgG (C) were loaded onto a Bio-Select SEC 250-5 column (Bio-Rad) and equilibrated with 50 mM sodium phosphate, 150 mM sodium chloride, pH 6.8, at 1.0 ml/minute. PE-IgG elutes at a retention time corresponding to a molecular mass of 400 kDa (peak 1); PE elutes at 180 kDa (peak 2) and IgG elutes at 153 kDa (peak 3).

HLA-DR dynamics on transfected fibroblasts
The PE IgG probe was then used to study the cell-surface dynamics of HLA-DR receptors. For these experiments we utilised M1DR1, a human fibroblast transfected with HLA-DR alpha and beta genes. Fig. 5A shows part of a fluorescent image obtained for PE-IgG bound to transfected fibroblast; 5B,C,D show a small area area of this cell at two minute intervals, and serve to illustrate the size of the frame-to-frame spot displacements. Non-specific binding was tested as described in Materials and Methods; a typical area of a cell used in such a test appears in Fig. 5E.

Many of the fluorescent patches observed in this and other images are too wide and intense to be individual receptors or small clusters. Those fluorescent spots that are close to the diffusion limited width (calculated to be 2.98 pixels for the objective lens employed for these images) were analysed as described, and tracked through time-lapse images; typically 20 images at 60 second intervals were obtained. A group of tracks found in part of the cell shown in Fig. 5 (cell a) are displayed in Fig. 6; the circles signify the starting positions of the spots and indicate the inter-spot distances, while the tracks have been expanded by 2.5 for clarity. It should be noted that this figure only shows a selection from the total number of analysed tracks in this area; it also does not include fluorescent patches and some spots that photobleached excessively. Tracked spots were found to bleach with a time constant of 240±70 seconds exposure time, assuming single exponential characteristics. This resulted in a 40% loss of intensity over 20 images.

Analysis of all the data for cell a by the methods detailed in equations (1a-c) suggests that ~30% of the tracks are best described by directed motion, with velocities calculated using equation (1b) giving a mean value 2.2±0.8 nm second⁻¹; the remaining particles were mostly random diffusion. A correlation diagram of diffusion coefficient against fluorescence
intensity, for the particles showing random or domain diffusion, displays the range of values obtained (Fig. 7A), and Fig. 7B is a histogram of the corresponding fluorescence intensities.

A second cell of this type (cell b) gave very different results. Most of the particles were characterised as being domain constrained, and the remainder showed random diffusion. Values of the diffusion coefficients obtained are displayed in Fig. 7C as a function of the spot fluorescence intensity, which is also given as a histogram in Fig. 7D. Domain sizes calculated from equation (1c) give a mean diameter of 380±160 nm. The limiting value of $D_{lat}$ that can be found using this methodology was assessed using the set of off-cell spots employed for 'creep' correction. Individual tracks in this group were analysed for random diffusion, with the remaining tracks providing the creep correction. This suggests that a diffusion coefficient of $1.00\times10^{-13}$ cm² second⁻¹ or less is indistinguishable from an immobile particle.

The second type of analysis using the distance probability distribution described in Materials and Methods, was also applied to these data sets. Tracks are not identified as directed motion or diffusion types, but all jump distances for all tracks over a set time interval are combined into the distributions shown in Fig. 8. The histograms were fitted by equation (2) to give the quasi-diffusion coefficients shown in Table 1 and displayed as dashed lines in Fig. 8.

The histograms for cell a were also fitted to the combined diffusion and directed motion model (equation 3) by global analysis to give:

$$D_{lat} = 1.24(\pm0.02)\times10^{-12} \text{ cm}^2 \text{ second}^{-1}, C_p = 63(\pm1)\%;$$

$$\nu = 2.32(\pm0.06), s = 1.67(\pm0.08) \text{ nm second}^{-1}, C_v = 37(\pm2)\%,$$

and the fits are shown as solid lines in Fig. 8A-C.

For cell b, the preponderance of domain diffusion type tracks shows as a decreasing quasi-diffusion coefficient, and so the data were analysed according to equation (4), to give quasi-diffusion coefficients similar to those in Table 1, and a global diffusion coefficient $D_{lat} = 0.83(\pm0.05)\times10^{-12}$ cm² second⁻¹ (Fig. 8D-F, solid lines) with $C_p = 47(\pm2)\%$.

**DISCUSSION**

Immune recognition involves the formation of larger assemblies of receptors and co-receptors with the potential capacity to redistribute in the lipid bilayer of the cell membrane. In specific immunity, for example, the activation of the antigen presenting cell results in the formation of the ternary complex MHC-peptide-TCR to form a functional lattice. Additional molecules are also required such as the co-receptors CD4, CD8 and CD3 that form serially engaged polypeptide chains. A detailed understanding of the the interactions of cell surface receptors, including the dynamics of such associations, is thus an important aspect in elucidating the molecular basis of immune recognition.

A number of techniques which have been used to give information on receptor associations in cells have been reviewed by Edidin (1990). Although these approaches have been of considerable value, the information that they provide tends to be mainly qualitative. Ideally, one would wish to be able to quantify receptor movements in the cell membrane and to observe the dynamics of receptor associations in real time in living cells. The recent development of techniques for imaging small particles bound to individual receptors offers the possibility of making significant progress in this direction.

Here we describe the development and initial application of phycobiliprotein-labeled antibodies for studying MHC molecules in cell surface membranes. Phycobiliproteins have a number of advantages for the studies that we wish to pursue.
They are of relatively small size (phycoerythrin is 12 nm × 6 nm) (Glazer, 1983) which minimises the risk of perturbation by attachment of the particle to the receptor. Secondly, they can be coupled to antibodies in a 1:1 mole ratio to provide a well-defined probe. Thirdly, they contain a defined number of fluorophores and thus have a uniform fluorescence. Not all these advantages are critical for the particle tracking experiments described in this initial report. They will, however, be crucial for our long-term aim of studying both associations and dynamics of MHC molecules and related receptors.

Characterisation of PE-IgG complexes

As shown in Figs 1 and 2, we have successfully used the pyridyl sulphide derivative of PE to produce a pure preparation of individually labeled antibodies. The results of flow cytometry assays shown in Fig. 3 demonstrate that the binding activity of the antibody is largely retained after labeling. PE on its own has almost no binding affinity for the cell surface.

To calibrate the imaging system, the fluorescence intensity of the PE-IgG complex on a glass surface was measured. The glass was first coated with polylysine to ensure stable adhesion. The distribution of intensities (Fig. 4) is not as wide as was found with heterogeneously labeled LDL (Morrison et al., 1994). In principle, PE particles should have uniform intensity; the observed width is probably caused by a combination of detector non-uniformity and focal imperfections (both small) and photon-counting effects, i.e. a measure of the probability of emitted photons entering the detection optics. There may also be some broadening from PE photodamage, if the damage does not cause complete destruction of the fluorophores in an individual particle.

SPT experiments with transfected fibroblasts

For our initial SPT experiments, we have chosen to work with a human fibroblast cell line designated M1DR1. Fibroblasts are a type of connective-tissue cell, found in almost all vertebrate organs, they have a fast doubling-time and proliferate in vitro as cells attached to surfaces. The large, approximately flat, areas facilitate optical imaging and tracking. These cells also migrate and proliferate during inflammatory processes and wound healing (Lodish et al., 1995); in specific immunity fibroblasts have the in vivo (and in vitro) potential to become an APC following antigen-specific stimulation and cytokine secretion (Lightstone et al., 1995). The untransfected M1DR1 fibroblast is devoid of detectable HLA class II expression. This phenotype can be reversed by co-transfection of exogenous HLA DR alpha and HLA DR beta genes which generates fully assembled HLA class II transmembrane heterodimers, as shown by Dodi et al. (1994).

A representative image of an M1DR1 cell in which cell-surface HLA DR molecules are labeled with the PE-IgG probe is shown in Fig. 5. There are a significant number of large fluorescent patches which are indicative of clustering of the antibody-MHC complexes. Whether these patches correspond to pre-existing clusters or are induced by antibody crosslinking cannot be ascertained at present. We will address this issue in future experiments by preparing PE-labeled Fab to obviate crosslinking. For the present, we focus attention on the many small fluorescent spots whose intensity profiles indicate that they are diffraction-limited images. These may correspond to single particles or clusters of a few particles: we will return to this question later in the discussion.

Although the small fluorescent spots are of low intensity, PE is sufficiently photostable for us to obtain a sequence of images and hence to determine particle tracks, as illustrated in Fig. 6. An analysis of these particle tracks to provide information on receptor mobility has been attempted using two different methods. The first method, analysis of individual particle tracks,
has the advantage of sampling discrete particles, but the stochastic nature of diffusion makes it difficult to distinguish between different models of mobility. It has been shown by Monte Carlo methods (Saxton, 1993) that a theoretical pure diffusion track can appear to mimic directed motion, domain diffusion etc., in a significant number of cases. Thus, we have also analysed the data from a number of particles by a model-independent method, using distance probability distributions (see below).

The data analysis shown in Fig. 7 and Fig. 8 reveals different behaviour of the two cells presented here. Cell a gives reasonably constant quasi-diffusion coefficients for different time intervals (Table 1), but the distributions are distorted at higher time intervals. This can be fitted by the extra term for directed motion; the model equation (3) used to describe this does not assume that particles move with constant velocity over all the length of their track, but that a range of velocities exist that cover a normal distribution. Alternative models are possible but would merely produce a different value for the width of this distribution \( s_v \), and are unlikely to alter the fraction of jumps caused by directed motion, \( C_v \). The interesting point about this...
analysis is that the value of $C_v = (37 \pm 1\%)$ is similar to the fraction of tracks thought to be displaying directed motion (30%) obtained from analysing individual tracks, suggesting that the single particle track selection procedure is fairly reliable. Moreover, the mean of the velocities obtained from the distance probability distribution (2.3 nm second$^{-1}$) is very similar to that obtained from the analysis of individual tracks (2.2 nm second$^{-1}$). This suggests that a large proportion of the jumps which contribute to $C_v$ arise from the tracks which were assigned as displaying directed motion and hence provides further support for the particle track selection procedure.

Cell b gives much smaller quasi-diffusion coefficients which also decrease at higher time intervals (Table 1). This suggests that long distance diffusion is obstructed, and hence that domain diffusion is dominant. This is also the conclusion of the single particle track analysis. It must also be noted that the total observation time (~1,500 seconds) is sufficient to give a good probability that a particle will explore a domain and hence be identified as undergoing restricted diffusion; according to Saxton (1993), the cumulative probability of a random walk staying within a region of radius $R$ is:

$$\log \psi = 0.2048 - 2.5117(D_{lat}/R^2),$$

which for the mean values $D_{lat}=10^{-12}$ cm$^2$ second$^{-1}$ and $R = 250$ nm gives $\psi = 0.0000015$.

For the shorter time intervals of 60 and 180 seconds, the cumulative probabilities are about 0.9 and 0.3, respectively. This is consistent with the data in Table 1 where a significant decrease in the quasi-diffusion coefficient occurs after 60 seconds. The approximately constant quasi-diffusion coefficient occurring after 420 seconds suggests that for these long times, diffusion is determined by hopping between domains.

Sako and Kusumi (1994) previously found a mean residence time within domains of 29 seconds for transferrin and alpha-macroglobulin receptors in rat kidney fibroblasts at 37°C. Because of the limited time resolution of the present experiments, we cannot exclude the possibility that MHC molecules are moving much more rapidly within domains than indicated by the diffusion coefficients that we determine.

It is noticeable that for cell b, the fits to equation (2) are increasingly poor at longer time intervals. This might be expected if there is a mixture of domain-limited diffusion and random diffusion. We have therefore extended the analysis as described in Materials and Methods to include a random diffusion component. This component has a diffusion coefficient of 85 nm$^2$ second$^{-1}$, which is reasonably consistent with the data in Fig. 7. The proportion of jumps assigned to random diffusion is 47%, compared with 33% for the individual track analysis. The distance distribution analysis does not give a measure of domain size. Qualitatively, however, it is clear from Fig. 8 that the constraints on random diffusion appear when the particles have moved a mean distance on the order of 200 nm. This is comparable to the $r^2_{max}$ values which we obtain from individual tracks which are assigned to domain-limited diffusion.

Whilst the investigation of receptor associations was not the primary focus of the present paper, the fact that the intensities of the particles observed on cells a and b differ by a factor of about two (Fig. 7) deserves comment. Exact comparison with the peak intensity value found for the particles dispersed on polylysine-coated glass (Fig. 4) is difficult; focusing of the microscope is performed by taking a number of images of the fluorescent spots, and so there will be different amounts of photobleaching on different samples. Nevertheless, it appears that the spots on cell a may be single particles, while the intensities of the spots on cell b are closer to the value expected for doubles, which would suggest the existence of dimers or small clusters of HLA class II heterodimers. The formation of such structures has been suggested for the mouse MHC class I (Capps et al., 1993) and MHC class II molecules (Schafer and Pierce, 1994). However, it will require considerably more work to ascertain whether there really is a relationship between the association state of the MHC molecules and their mobility. It is conceivable that homotypic aggregation of HLA class II receptors at the cell surface facilitates encounters with responding T cells. It might be therefore of physiological advantage for HLA class II to form self-associated micro-clusters in order to maximise antigen presentation by increasing the avidity of the MHC+peptide to bind the T cell receptor. We are currently undertaking a detailed study of the association of HLA class II molecules on M1DRI acting as APCs of selected immunogenic peptides.

### Concluding remarks

The present results demonstrate that phycobiliprotein-labeled antibodies can be used for single particle imaging studies on structures that act as transmembrane receptors of the immune system. Thus we have generated the basis for analysis of MHC molecules and their behaviour in living cells and therefore in their intact native configuration. Single particle tracking experiments indicate that MHC class II molecules in a human transformed fibroblast exhibit domain-limited diffusion and directed motion. Future studies will exploit the ability of fluorescent particle imaging to detect self-associations; experiments for which the phycobiliprotein probes are particularly well-suited. In this way, it should be possible to develop a detailed picture of the organisation and dynamics of MHC molecules at the cell-surface of APCs and to investigate how these properties change in response to various immunological stimuli.

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### REFERENCE

