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# THE EFFECT OF TEMPERATURE ON THE BINDING KINETICS AND EQUILIBRIUM CONSTANTS OF MONOCLONAL ANTIBODIES TO CELL SURFACE ANTIGENS

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**Abstract**—The effect of temperature on the kinetic association and dissociation binding parameters, and equilibrium constants of four monoclonal antibodies to the murine Ly-2.1 and Ly-3.1 antigens has been studied using flow cytometry. All four monoclonal antibodies were conjugated to FITC and their association to, and dissociation from, the surface of murine thymoma cells was observed at 15 sec intervals, at temperatures between 1 and 37°C. The initial association rate constant and the dissociation rate constant for each antibody at each temperature were calculated from graphs of the first-order reactions and it was demonstrated that an increase in temperature caused an increase in both association rate and dissociation rate of the antibodies. Generally the increase in association rate with temperature was less than the increase in dissociation rate. Differences between antibodies to the same antigen (Ly-2.1) suggest that changes in membrane fluidity were not solely responsible for the changes in association rate. However, the equilibrium constants ($K_{eq}$) did not always show a simple relationship of increasing temperature causing decreasing values for $K_{eq}$. For one antibody the highest value for $K_{eq}$ was seen at 17°C rather than at 37°C and differences in $K_{eq}$ between individual antibodies were greater at 1°C than at 37°C. Kinetic rate constants are usually measured at 4°C or room temperature, therefore for antibodies under consideration for *in vivo* use, measurements at 37°C are more appropriate.

## INTRODUCTION

The bond between antibody and antigen is dependent on non-covalent forces, and it is a dynamic and complex interaction. The equilibrium constant, or affinity, of an antibody can be used to describe the antigen–antibody interaction while the two are bound, the Law of Mass Action relating the equilibrium constant to the association and dissociation of antibody and antigen (Steward and Steensgaard, 1985). Thus the equilibrium constant is dependent on the rate of antigen–antibody association and also the rate of their dissociation. The rate of these reactions should vary with temperature; the thermodynamic nature of the bonds involved in antigen–antibody interactions predicts such temperature dependency. As the bond between antibody and antigen is made up of various non-covalent forces, and is dependent on physical conformations and orientation within the binding site, it cannot be assumed that the equilibrium constant for every monoclonal antibody follows a predictable pattern of increasing temperature causing decreasing affinity values. The effect of temperature changes on the kinetic parameters (association and dissociation rate constants), and affinity of antibodies has not been the subject of detailed study, but in general it appears that the dissociation rate shows greater temperature dependency than the association rate, and that differences in affinities arise mainly through variations in dissociation rates (Mason and Williams, 1980, 1986; Froese, 1968). The temperature dependency of the affinity of antibodies for haptens or antigens in solid phase may be solely confined to effects on the bonds at the binding site. In the case of antibody binding at the cell surface, temperature effects on the membrane of the cell may also affect the behaviour of bound antibody (Linden *et al.*, 1973). Accurate measurement of association and dissociation rates for antibodies binding to viable cells can be carried out using flow cytometry (Roe *et al.*, 1985). This study shows the effect of change in temperature on kinetics of antibody binding at the cell surface. Four monoclonal antibodies to the same (Ly-2.1) or related (Ly-3.1) antigens were used, and their association to and dissociation from cell surface antigens at a range of temperatures between 1 and 37°C were examined using direct immunofluorescence on a flow cytometer. The results show that variations in both association and dissociation rate constants can occur with changes in temperature and that there is not necessarily an inverse relationship between temperature and equilibrium constant.

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## MATERIALS AND METHODS

### *Monoclonal antibodies and target cells*

Monoclonal antibodies to the murine Ly-2.1 and Ly-3.1 antigens were used: 49-14.2 (14.2), IgG1; 49-11.1 (11.1), IgG2a; and 49-31.1 (31.1), IgG3 bind to Ly-2.1 (Hogarth *et al.*, 1982*a*); 5034-29.5 (29.5), IgG1 binds to Ly-3.1 (Sutton, 1984). The antibodies were affinity purified from mouse ascites fluid by Protein A Sepharose. The murine thymoma ITT(1) 75NS.E3 (E3) (Hogarth *et al.*, 1982*b*; Smyth *et al.*, 1986) which expresses both Ly-2.1 and Ly-3.1 was used as the target cell line. The cells were maintained in Dulbecco's Modified Eagle's Medium (DME) with 10% newborn calf serum (Flow Laboratories, NSW, Australia) containing 50 U/ml penicillin and 50 $\mu$g/ml steptomycin.

### *Fluorescein labelling of antibodies and activity of the labelled preparation*

Purified antibodies were conjugated to fluorescein isothiocyanate (FITC) [Sigma, Dorset, U.K. (Pimm *et al.*, 1982)] such that fluorescein to protein ratios of be tween 1:1 and 5:1 were obtained, determined spectrophotometrically (Forni, 1979). FITC–antibodies were stored in phosphate buffered saline (PBS pH 7.4) with 1.0% sodium azide at 4°C. Each preparation was shown to have activity comparable with the unlabelled preparation using competition binding assays (Robins *et al.*, 1986) on a flow cytometer (FACScan, Becton-Dickinson, Mountain View, CA, U.S.A.). The immunoreactive fractions and equilibrium binding characteristics of the particular antibody preparations used had been determined previously (Andrew *et al.*, 1990). The immunoreactive fractions were: 14.2: 52.65%; 11.1: 49.30%; 31.1: 78.41%; and 29.5: 54.28%. These figures were taken into account in calculations for absolute amounts of antibody bound per cell, corrections being made based on the assumption that only the immunoreactive fraction of the antibody had binding activity. Equilibrium binding experiments performed on the FACScan were used to determine the amount of antibody required to saturate a given number of cells, in each case 5 $\mu$g of purified antibody saturated $4 \times 10^5$ cells.

### *Association experiments*

Association of FITC–antibody to E3 cells with time was observed over a range of temperatures using flow cytometry. Cells were prepared by washing three times in PBS with 2% newborn calf serum, viability was monitored with Trypan blue; the final concentration of the cells was $4 \times 10^5$ cells per 0.5 ml. An initial fluorescence reading (time, $t = 0$) of 2000 cells was taken, then 5 $\mu$g of FITC–antibody in 200 $\mu$l PBS was added to the cells, and fluorescence readings of a mean of 2000 cells were obtained at 15 sec intervals on the FACScan. The temperature of the reaction mixture was controlled by immersion of the FACS tube in a beaker containing water at the appropriate temperature which was monitored continuously with a thermometer (the variation in temperature was within 1°C of the experimental temperature). Experiments were carried out at 1, 10, 17, 25 and 37°C.

### *Dissociation experiments*

The dissociation of FITC–antibody with time was measured using flow cytometry on E3 cells. Cells were prepared as above, and incubated with 5 $\mu$g of FITC-labelled preparations of each antibody on ice for 2 hr. An initial fluorescence reading was taken at time, $t = 0$, then a 40-fold excess of unlabelled intact antibody, taken from the same batch as the FITC-labelled preparation, was added to the tube. This large excess of unlabelled antibody ensured that there was little chance of reassociation of FITC-labelled antibody during the course of the experiment. Dissociation of FITC-labelled antibody from E3 cells was observed by taking fluorescence readings from 2000 cells at 15 sec intervals; again, the experiments were carried out a 1, 10, 17, 25 and 37°C.

For all flow cytometric observations, the FACScan was calibrated with both Calibrite beads (Becton-Dickson; Bioclone Australia Pty, Marrickville, NSW, Australia) and Quantitative Fluorescein Microbead Standards (Flow Cytometry Standards Corp., Research Triangle Park, NC, U.S.A.). The machine settings on the FACScan were standardized and the same settings were used for each experiment. The fluorescence units were calibrated, one fluorescence unit represented 2160 molecules of FITC.

### *Calculation of constants*

The association rate constant was obtained from a graph of antibody molecules bound per cell versus time, which were calculated using the mean fluorescence bound per cell, the F:P ratio and the calibration of the FACScan. The association constant,

$$k_1 = \text{gradient}/2CQN,$$

where: $C$ = molar concentration of cells [calculated from concentration of cells in mol/l = number of cells per litre/Avogadro's number (Trucco and de Petris, 1981)], $Q$ = input concentration of antibody in molecules per cell, and $N$ = number of antigenic sites per cell (Roe *et al.*, 1985). Ly-2.1 and Ly-3.1 have been shown to have $1.9 \times 10^5$ and $3.8 \times 10^5$ antigenic sites per E3 cell respectively (Andrew *et al.*, 1990).

The dissociation rate constant, $k_2$, is the slope of a graph of log $U/U_0$ vs time, where $U$ = mean fluorescence at time $t$, and $U_0$ = initial mean fluorescence (Roe *et al.*, 1985).

The equilibrium constant, $K_{eq}$, was calculated from

$K_{eq}$ = association constant,

$k_1$/dissociation constant, $k_2$.

All graphs were drawn using the simple curve of best fit obtained from Cricket Graph Software on an Apple MacIntosh personal computer. The slopes of the curves were obtained from regression analysis using the same software package.

## RESULTS

### *Variation in antibody association with temperature*

Results of association experiments carried out at various temperatures with each monoclonal antibody and E3 cells are shown in Fig. 1. The increase in fluorescence with time indicates an increase in bound antibody per E3 cell with only the initial, monovalent, association rate being measured. The association curves seen in Fig. 1 were found to be reproducible with very similar straight line curves obtained upon repetition of the experiment. For all monoclonal antibodies studied, increasing the temperature caused an increase in the rate of association of antibody to the cells.

Association rate constants for each antibody at each temperature (Table 1) show considerable variation with temperature. The temperature-related increases in association rates for individual monoclonals do not show a specific, constant pattern and there are differences in $k_1$ values between the antibody groups. The effect of temperature was considerably

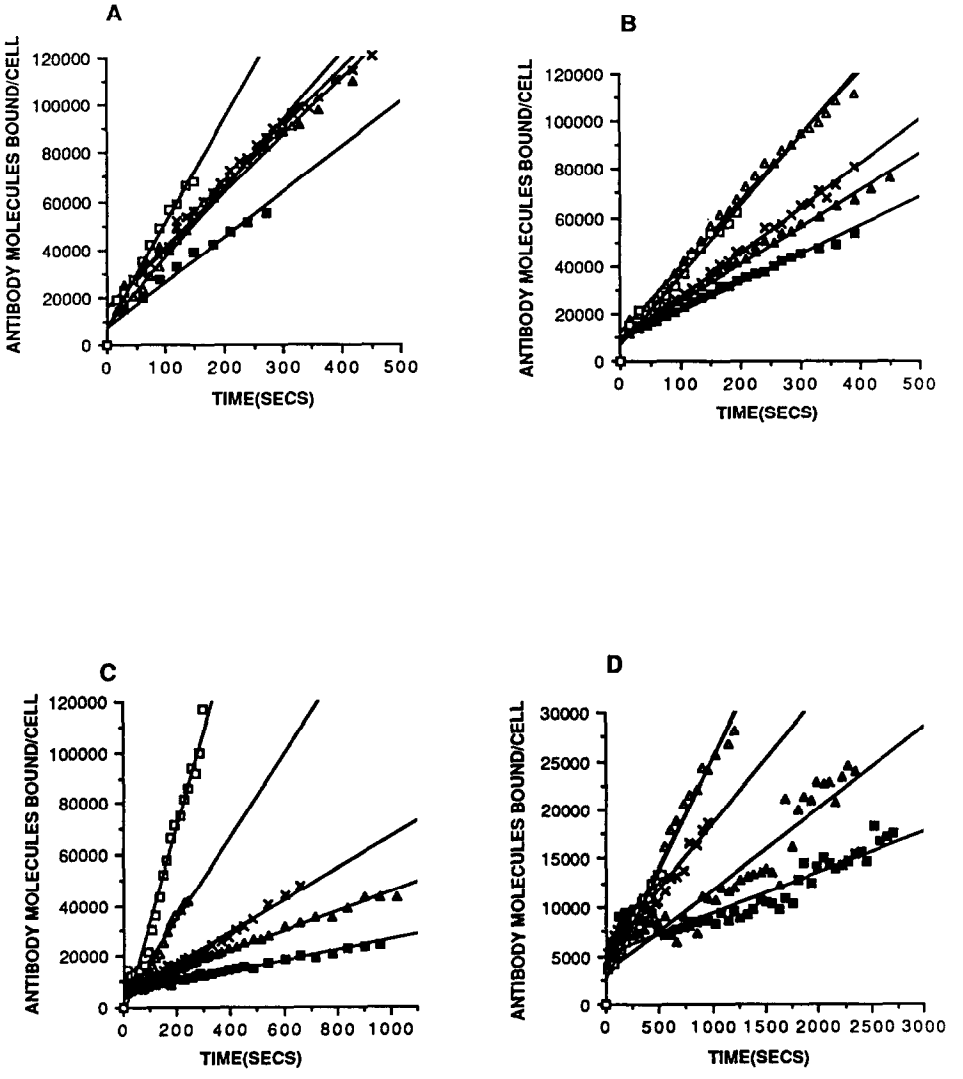


Fig. 1. Variation in antibody association rate with temperature. Molecules of antibody bound per cell (calculated from F:P ratios and flow cytometer calibration) are plotted against time (sec) for each temperature, fluorescence readings of 2000 cells were taken at 15 sec intervals. A, antibody 11.1; B, antibody 14.2; C, antibody 31.1; D, antibody 29.5. The temperatures are: 1°C (■), 10°C (▲), 17°C (×), 25°C (△) and 37°C (□). (Note: different scales for both axes in A and D.)

Table 1. Calculated values for association rate constants ($k_1$) at various temperatures

| Monoclonal antibody (subclass) | Values of $k_1$ ($\times 10^4$)l/mol/sec 1°C | 10°C | 17°C | 25°C | 37°C |
|---|---|---|---|---|---|
| 11.1 (IgG2a) | 1.407 | 1.781 | 1.866 | 2.125 | 3.241 |
| 14.2 (IgG1) | 0.944 | 1.222 | 1.472 | 2.191 | 2.320 |
| 31.1 (IgG3) | 0.104 | 0.196 | 0.312 | 0.809 | 1.881 |
| 29.5 (IgG1) | 0.018 | 0.036 | 0.057 | 0.095 | 0.098 |

greater in the case of 31.1 (Fig. 1C) and 29.5 (Fig. 1D) than on the other two antibodies (Fig. 1A, 1B). The association rate constant at 37°C for 31.1 (1.88 l/mol/sec) was 18 times that at 1°C (0.104 l/mol/sec), whereas for 14.2 and 11.1, the increase in rate constant was 2-fold between 1 and 37°C (Table 1). These latter two antibodies showed very little increase in association rate constant between 25 and 37°C (Table 1). From these results it is clear that different antibodies are affected differently by increases in temperature with respect to their association characteristics, even when they are associating to the same antigen. These differences do not appear to be subclass related, as the two IgG1 antibodies behaved differently (i.e. 14.2: $k_1 = 2.320$, 29.5: $k_1 = 0.098$, at 37°C).

Considerable variation in $k_1$ was observed between the antibodies, 29.5 having the slowest rate of association over all and 11.1 the fastest. Thus, there exists a direct relationship between antibody

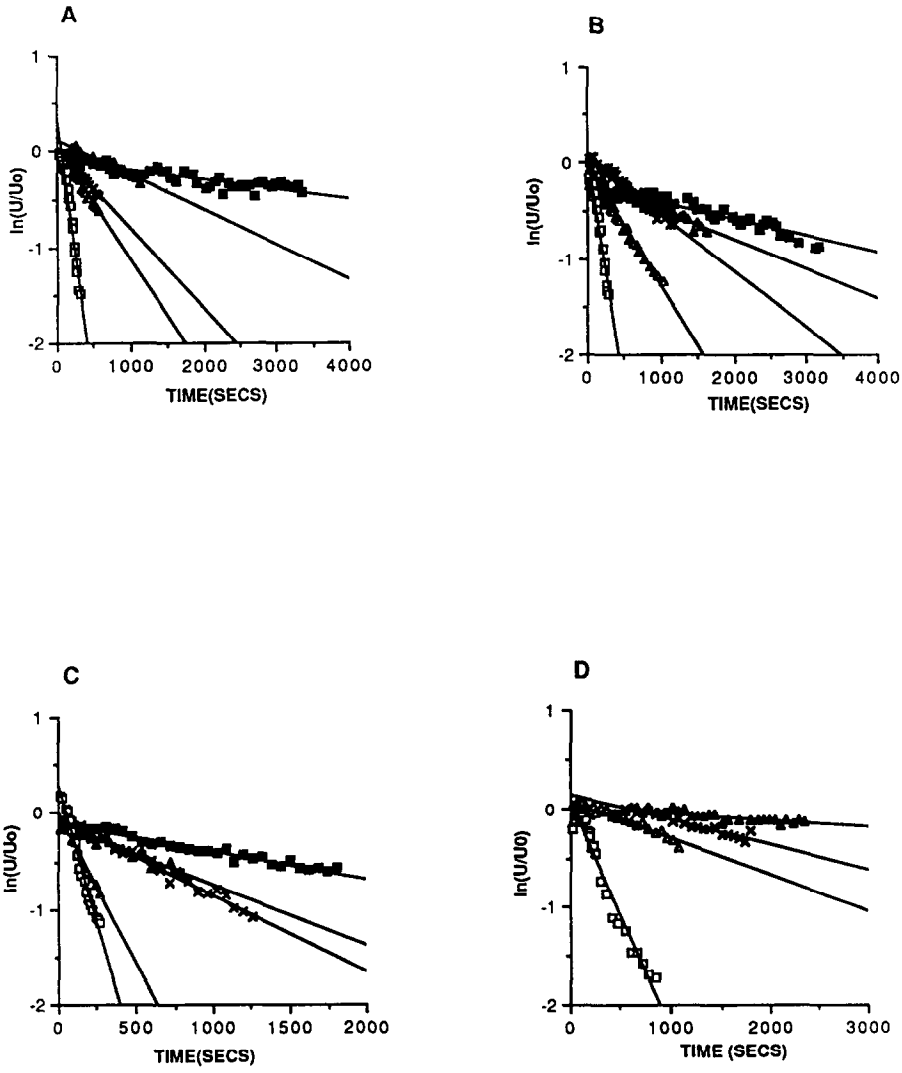


Fig. 2. Variation in antibody dissociation rate with temperature. The natural log of fluorescence values (mean of 2000 cells) divided by initial fluorescence ($\ln U/U_0$) is plotted against time; readings being taken at 15 sec intervals. A, antibody 11.1; B, antibody 14.2; C, antibody 31.1; D, antibody 29.5. The temperatures are: 1°C (■), 10°C (▲), 17°C (×), 25°C (△) and 37°C (□). (Note: dissociation of antibody 29.5 at 1°C was too slow to be accurately measured.)

Table 2. Calculated values for dissociation rate constants ($k_2$) at $t_{1/2}$ at various temperatures

| Monoclonal antibody | Values of $k_2$ ($\times 10^{-4}$)/sec, and $t_{1/2}$ min | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1°C | | 10°C | | 17°C | | 25°C | | 37°C | |
| | $k_2$ | $t_{1/2}$ | $k_2$ | $t_{1/2}$ | $k_2$ | $t_{1/2}$ | $k_2$ | $t_{1/2}$ | $k_2$ | $t_{1/2}$ |
| 11.1 (IgG2a) | 1.03 | 112 | 3.64 | 32 | 8.3 | 14 | 12.0 | 10 | 57 | 2.0 |
| 14.2 (IgG1) | 1.72 | 64 | 3.08 | 38 | 5.9 | 20 | 12.0 | 10 | 49 | 2.2 |
| 31.1 (IgG3) | 2.87 | 40 | 6.3 | 18 | 8.1 | 14 | 31 | 3.8 | 58 | 2.0 |
| 29.5 (IgG1) | *a* | *a* | 0.65 | 178 | 2.57 | 46 | 3.68 | 32 | 23.0 | 5.0 |

[a]Dissociation of 29.5 at this temperature was too slow to be reasonably assessed.

association rate constants and temperature for all of the monoclonal antibodies studied. The amount of rate increase with temperature appears to be an inherent property of the individual antibodies and is not immunoglobulin subclass related.

*Variation in antibody dissociation with temperature*

Curves showing variation in dissociation of each antibody from E3 cells with temperature are shown in Fig. 2. As the fluoresceinated antibodies dissociate from the cell, unlabelled antibodies take their place, thus decreasing the mean linear fluorescence per cell. With time, the fluorescence per cell decreases, indicating a decrease in bound fluoresceinated antibody. An increase in temperature caused an increase in the rate of dissociation of antibody from cells. The effect of temperature on dissociation was reflected in the dissociation constants, and $t_{1/2}$ (time taken for 50% of the antibody to dissociate from the cell surface) shown in Table 2. The value for $t_{1/2}$ was calculated from $k_2 = 0.693/t_{1/2}$ (Mason and Williams, 1986). For all four antibodies (Fig. 2), there was a marked increase in dissociation with an increase in temperature. At 1°C (Fig. 2), there was very little 11.1, 14.2 and 31.1 antibody dissociation from the cell surface whilst the rate of 29.5 dissociation at 1°C was too slow to allow accurate measurements. For all antibodies, $t_{1/2}$ was short at 37°C, the anti-Ly-2.1 antibodies (11.1, 14.2 and 31.1) having $t_{1/2}$ of approximately 2 min. The anti-Ly-3.1 antibody (29.5) showed very slow dissociation kinetics compared to the other three antibodies with a $t_{1/2}$ of 5.0 min at 37°C. Again, a different effect of temperature between the different antibodies was observed; antibody 11.1 was most affected with the dissociation constant at 37°C being over 50 times that at 1°C. Antibody 31.1 was the least affected by temperature with respect to dissociation rate constant, $k_2$ (1°) = 20 × $k_2$ (37°C). Comparing variations of dissociation rate constants between the various antibodies, the antibody with the slowest association rate, namely 29.5, also had the slowest dissociation rate. Thus, there is a direct relationship between temperature and the rate of dissociation for the four antibodies studied. The increase in dissociation with temperature varies between the antibodies and there appears not to be a constant pattern of dissociation rate increase.

*Variation in equilibrium constant with temperature*

The equilibrium constants for each antibody at the various temperatures are shown in Table 3. For antibodies 14.2, 11.1 and 29.5 the equilibrium constants decreased with increasing temperature, the greatest effect being seen with antibody 11.1 where $K_{eq}$ (1°C) = 24 × $K_{eq}$ (37°C). In the case of antibody 31.1 very little change in $K_{eq}$ with temperature was observed, the highest value for $K_{eq}$ occuring at 17°C; $K_{eq}$ (37°C) was slightly lower than $K_{eq}$ (1°C). The reason for this anomaly lies in the calculated equilibrium constant being taken from the association/dissociation constant; the effect of temperature on the association rate of 31.1 was considerably greater than for the other antibodies.

Generally, however, antibody–antigen binding reactions follow basic thermodynamic principles, an increase in temperature causing an increase in both association and dissociation with a resultant decrease in antibody affinity. Not all antibodies showed an inverse relationship between temperature and equilibrium constant, as with antibody 31.1 due to the nature of the derivation of $K_{eq}$.

## DISCUSSION

The major aim of the study was to examine the effect of temperature on antibody association and dissociation rates and subsequently on the affinity (equilibrium constant) of antibodies to cell surface antigens. While such studies are conventionally performed at 4°C or room temperature, the increased use of monoclonal antibodies at 37°C in *in vivo* therapy and imaging makes it appropriate to conduct *in vitro* studies at higher temperatures. This report shows that temperature has a considerable effect on antibody kinetics which are different for each antibody and are not related to immunoglobulin isotype.

Table 3. Values for equilibrium constant ($K_{eq}$) at various temperatures

| Monoclonal antibody (subclass) | Values for $K_{eq}$ l/mol | | | | |
|---|---|---|---|---|---|
| | 1°C | 10°C | 17°C | 25°C | 37°C |
| 11.1 (IgG2a) | $1.37 \times 10^8$ | $4.90 \times 10^7$ | $2.25 \times 10^7$ | $1.77 \times 10^7$ | $5.96 \times 10^6$ |
| 14.2 (IgG1) | $5.49 \times 10^7$ | $3.97 \times 10^7$ | $2.49 \times 10^7$ | $1.83 \times 10^7$ | $4.73 \times 10^6$ |
| 31.1 (IgG3) | $3.62 \times 10^6$ | $3.11 \times 10^6$ | $3.85 \times 10^6$ | $2.60 \times 10^6$ | $3.24 \times 10^6$ |
| 29.5 (IgG1) | *a* | $5.54 \times 10^6$ | $2.22 \times 10^6$ | $2.55 \times 10^6$ | $4.25 \times 10^5$ |

[a]Not calculated.

The study clearly shows that there are considerable temperature-induced variations in the kinetic binding parameters of antibodies to cell surface antigens. These variations consisted of an increase in temperature causing an increase in the rate of association and dissociation for all four antibodies studied. In all cases the initial rates of association and dissociation were studied, so that the reactions followed first-order kinetics. This not only simplified the analysis of the results but also meant that the true affinity of the (possibly bivalent) binding of intact antibody to cell surface antigens was not obtained. Thus the calculated equilibrium constants represent an "apparent affinity" and are likely to be biased towards monovalent cell surface interactions with a single binding site of the antibody. However, within this system, the results are comparable particularly as three of the antibodies react with the same cell surface antigen (Ly-2.1) and Ly-3.1 is physically associated with Ly-2.1 on the cell surface (Sutton, 1984).

This study shows that an increase in temperature caused an increase in monoclonal antibody association to, and dissociation from cell surface antigens. In the case of antibody 31.1, an 18-fold increase in association rate was observed when the temperature was increased from 1 to 37°C, however, antibody 11.1 showed only a 2-fold increase for the same temperature change. This clearly shows that although antibody–antigen reactions do follow basic thermodynamic principles, the variation in association rate with temperature is an inherent characteristic of the particular monoclonal antibody. As with association rate variations, differences in the rate of dissociation with temperature was observed between the four antibodies. Monoclonal antibody 29.5 had a dissociation rate that was so slow at 1°C, accurate and reproducible measurements could not be made. However, all antibodies showed an increase in rate of dissociation as the temperature was increased.

Using W3/25 antibody and studying dissociation from thymocyte membranes, Mason and Williams (1980) observed biphasic dissociation kinetics at higher temperatures (18 and 26°C) in experiments conducted over a longer time period (240 min) than the higher temperature experiments presented here. The antibody studies described herein were of lower affinity than those used in the previous study and had considerably faster dissociation kinetics. It may be that the low affinity antibodies follow a monophasic dissociation pattern, perhaps even showing Fab-like binding and hence biphasic binding reactions such as those observed by Mason and Williams (1980) did not occur. Further experiments including high affinity antibodies and Fab fragments may demonstrate this. In addition, these authors (1980, 1986) showed values for dissociation constants using W3/25 and 0X7 (Fab fragments) with 10–20-fold increase in dissociation seen with temperature rises of 4–26°C and 4–18°C respectively. In the current study, three of the four antibodies showed similar increases in dissociation rates (5–10-fold) up to 25°C. A large increase in rate of dissociation then occured between 25 and 37°C, there being a 4–6-fold increase in dissociation rate between these temperatures. The reason for this is unclear but it appears that this was not connected with a sudden increase in membrane fluidity or changes in state of the membrane phospholipids which have been reported to occur at characteristic temperatures (Linden *et al.*, 1973), as one anti-Ly-2.1 antibody (31.1) showed only a further 2-fold increase in dissociation rate between these two temperatures. It is possible that changes in individual epitopes may be differentially related to temperature but this is unlikely.

As well, increases in association rate with increasing temperature have also been reviewed by Mason and Williams (1986) with antibodies 0X7 and W3/25 at two different temperatures. Increases in temperature from 4 to 18°C lead to 2–3-fold increases in the values for association rate constants. In the current study, 2.5- and 2.3-fold increases in association constant ($k_1$) were obtained for antibodies 14.2 and 11.1 respectively but a 5.4-fold increase was observed for 29.5, and in the case of 31.1, the association rate at 37°C was 18 times that at 1°C. The temperatures between which the greatest change took place varied also, the greatest change being seen between 17 and 25°C for 14.2 and 31.1, but at lower temperatures for 29.5 and between 25 and 37°C for 11.1. It is unlikely therefore that the change in association rate with temperature can be accounted for by a fall in the viscosity of the surrounding medium or by simple alterations in the fluidity of the membrane which would be a characteristic of the cell.

The equilibrium constant is dependent on both association and dissociation rate constants and it has been suggested that increasing temperature may be expected to produce a decrease in the equilibrium constant (or affinity) because the small increase in association rate is more than compensated by a large increase in dissociation rate. Mason and Williams (1986) state that there is no theoretical reason to suppose this would be universally the case. We have found a graded decrease in the value for the equilibrium constant in three of the four antibodies studied. In the case of antibody 31.1 although a small decrease in $K_{eq}$ was observed between 1 and 37°C, the value for $K_{eq}$ was highest at 17°C. It is interesting to note that differences in $K_{eq}$ between the three anti-Ly-2.1 antibodies at 1°C were greater than the differences between them at 37°C. Antibody 11.1 had a $K_{eq}$ 38 times the $K_{eq}$ of 31.1 at 1°C, but only twice the value at 37°C. It is possible that when considering antibodies to the same antigen, the higher affinity antibodies are more affected by temperature changes than the lower affinity antibodies, although this could be merely a reflection on the effect of temperature on the association rate of the antibodies concerned.

The study highlights the complex phenomena occurring within the antibody–antigen binding site. In

most cases it would appear that antibody affinity does decrease with increasing temperature, but as usual with monoclonal antibodies, generalized statements cannot be made. To discount subclass associated differences, we included two antibodies of the same subclass but to different antigens; these two antibodies showed unrelated changes in kinetic binding parameters with temperature. For each of the four antibodies studied both association and dissociation rates increased with temperature, increases in association rates were less in each case than the increase in dissociation rate. Some effect of temperature was seen on antibody affinity but this was not always consistent.

The study shows that although in most cases antibody–antigen reactions follow basic thermodynamic principles resulting in increased rates of association and dissociation with temperature, not all antibodies are affected by temperature changes to the same degree. This could be an important fact when deciding which monoclonal antibody is best for clinical therapy and imaging of neoplasms. The results indicate that *in vitro* binding experiments at 37°C need to be carried out if an antibody is to be used clinically on the basis of its affinity to a particular antigen.

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