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USE OF ISOTHERMAL TITRATION CALORIMETRY IN THE DEVELOPMENT OF MOLECULARLY DEFINED VACCINES

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Abstract

An uptake or a release of heat accompanies practically all molecular binding interactions. Therefore isothermal titration microcalorimetry is universally applicable for the characterisation of such binding processes. Calorimetric analyses do not require marker molecules or intrinsic spectroscopic reporter groups, which can modify the analysed interactions. Furthermore, measurements are carried out in solution and the adsorption of reactants to a solid phase is thus avoided. At variance with most other analytical approaches, titration calorimetry determines simultaneously enthalpy and entropy contributions of the binding interactions, as well as the binding constant and stoichiometry. In our analyses of the interactions between monoclonal antibodies and candidate antigens for vaccines *vs.* malaria and malignant melanoma, isothermal titration calorimetry has turned out to be a very valuable technique. The obtained quantitative data on biomolecular interactions can substantially support the rational design of epitope-focused vaccines.

Keywords: anti-idiotypic antibody cascade, Human Melanoma-Associated-Antigen (HMW-MMA), protein-protein binding, thermodynamic, titration calorimetry

Introduction

It is assumed that vaccines have prevented more infectious diseases than any other medical intervention, except sanitation. The greatest triumph of vaccinology is the global eradication of smallpox and today vaccination of children *vs.* infectious diseases like measles, mumps, polio, tetanus, diphtheria and pertussis is common practice. Nevertheless, 17.3 million of a global total of 52.2 million deaths in 1997 were still due to infectious diseases (WHO 1998 World Health Report). Leading causes of death from infectious diseases were acute lower respiratory tract infections (3.7 million), tuberculosis (2.9 million), diarrhoea (2.5 million), HIV/AIDS (2.3 million) and malaria (1.5-2.7 million). For many of these infections, like malaria, tuberculosis and HIV/AIDS we still lack effective vaccines and the development of new vaccines therefore remains an important goal. Today vaccine development encompasses technologies from the attenuation of pathogens to advanced genetic ma-

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nipulations. A progression from whole inactivated pathogens over purified components and recombinant proteins to molecularly even less complex immunogens is evident. The rationale behind all vaccinations is to elicit a protective immune response and to expand the number of memory lymphocytes ready to encounter the particular infectious agent, which will generate a potent secondary immune response.

In 1997 globally 6.2 million deaths were due to cancer and the field of tumour vaccines has also started to grow at a rapid rate. Unlike most vaccines for infectious diseases, cancer vaccines represent therapeutic vaccines, i.e. immune responses have to be elicited *vs.* antigens to which the immune system has already been exposed.

It has turned out to be difficult to develop vaccines *vs.* cancer and many chronic infectious diseases by using complex antigens, because these often do not direct the immune response towards antigenic determinants (epitopes), that ensure effective protection. Therefore research is moving towards a rational approach of vaccine development both for cancer and for those infectious diseases for which no suitable vaccine is yet available. Modern vaccine development is based on the detailed molecular understanding of the relevant immune effector functions and the specificity and regulation of the underlying humoral and/or cellular immune responses. Therefore, the early phase of vaccine development is now often focused on the identification of those epitopes of pathogens or tumour cells, which stimulate protective immune responses [1]. Certain undesirable epitopes may have structural similarity with host antigens and cross-reactivity of the immune response to a host antigen resembling the immunogen may provoke severe undesirable side effects. Antigenic competition between immunodominant hypervariable epitopes and less immunogenic conserved structures also speaks for the development of vaccines from which such non-protective immunodominant epitopes have been eliminated. Finally, it has become clear, that certain epitopes act as immunosuppressants, rather than stimulating the immune system in a positive way. The strategy to develop epitope-focused vaccines strives to avoid all the counterproductive and irrelevant responses *vs.* undesirable epitopes present in the pathogen or tumour cell. Since individual antigens may comprise both protective and undesirable antigenic determinants, molecularly defined vaccines may in the end not consist of entire recombinant proteins or purified components of a pathogen or tumour cell. Instead, true epitope-focused vaccines may be built of synthetic peptides or of anti-idiotypic antibodies that mimic individual epitopes. The molecularly highly defined nature of such vaccines strongly facilitates the evaluation of immune responses. The determination of antibody-antigen binding constants and kinetics is an important part of the characterization of humoral immune responses. Increased avidities and on-rates of antibodies have been postulated to provide improved effectiveness and protective activity. Some of the techniques used for the determination of binding constants are listed in Table 1. These methods either measure the ratio of the concentrations of free and complexed reactants at equilibrium or determine the kinetic dissociation (k_{off}) and association constants (k_{on}). The binding (affinity) constant (K_a) can be obtained from the concentrations of the reactants at equilibrium (K_a=[Ab–Ag]/[Ab]×[Ag]) or from the ratio of the kinetic constants ($K_a=k_{on}/k_{off}$). In the present report the use of isothermal titration calorimetry as a complementary valuable technique for the quantitative analysis of

interactions between vaccine induced antibodies and protection-relevant epitopes is described.

As practically all binding interactions are accompanied by an uptake (endothermic) or a release (exothermic) of heat, titration microcalorimetry is universally applicable for the characterisation of binding processes. Calorimetric analyses do not require marker molecules or intrinsic spectroscopic reporter groups, which can modify the analysed interactions. In our analyses of the interactions between monoclonal antibodies (mAb) and vaccine components, we have utilised isothermal titration calorimetry, which is carried out in solution, thus avoiding the adsorption of reactants to a solid phase. At variance with most other analytical approaches, titration calorimetry determines simultaneously enthalpy and entropy contributions of the binding interactions as well as the binding constant and stoichiometry [2, 3]. Many other methods aim to measure the binding constant K only, which is directly associated to the free energy of binding ($\Delta G=-RT\ln K$). As the free energy ($\Delta G=\Delta H-T\Delta S$) is composed of an enthalpy (ΔH) and an entropy (ΔS) term, microcalorimetry offers a completely different approach for the characterisation of a binding process. The enthalpy

Table 1 Methods for the determination of antibody-antigen binding constants (modified after Neri *et al*. [2])

Technique	Parameter measured	Sample requirements	Suitable for K_a range
Equilibrium dialysis	Concentration of free antigen at equilibrium	Suitable only for small antigens, which can (in free form) permeate a dialysis membrane	$10^3 M^{-1}$–$10^{12} M^{-1}$
Physical separation of bound and unbound antibody (by chromatography, ultra-centrifugation, electrophoresis)	Amount of free and bound antibody after separation	Labeling of antibody and/or antigen required. Not suitable for rapidly dissociating complexes	$10^8 M^{-1}$–$10^{12} M^{-1}$
Competition ELISA	Concentration of unbound antibody	Not suitable for rapidly dissociating complexes	$10^7 M^{-1}$–$10^9 M^{-1}$
Fluorescence quench analysis	Quenching of antibody fluorescence by binding of antigen	Pure material required. Not suitable for large antigens with intrinsic fluorescence	$10^6 M^{-1}$–$10^9 M^{-1}$
Real-time interaction analysis with optical biosensors	Refractive index changes associated with binding and dissociation	Immobilization of one reaction partner on a microsensor chip required	$10^1 M^{-1}$–$10^9 M^{-1}$

ΔH is generally considered as an indicator of the changes in intermolecular bond energies, while the entropy ΔS is an indicator for the rearrangements of the solvent molecules during the binding process.

Peptide based vaccination *vs.* malaria

In spite of intensive efforts, not a single vaccine *vs.* a human parasitic disease is currently commercially available. Malaria is the most important parasitic disease and in 1997, malaria risk of varying degrees existed in 100 countries. In 92 of these, transmission included the malignant (*Plasmodium falciparum*) form of the disease. Over 40% of the world population thus lived in areas with malaria risk. Some 1.5–2.7 million people die of malaria each year, and approximately one million deaths among children under five years of age are attributed to malaria alone or in combination with other diseases. Countries in tropical Africa account for more than 90% of the total malaria incidence and the great majority of malaria deaths (WHO 1998 World Health Report). In endemic areas natural resistance to malaria develops slowly and in young children there is high parasitaemia, morbidity and mortality until symptoms decline in later childhood. One important goal of malaria immunology therefore is the design of a vaccine, that can protect infants in endemic areas from dying of *P. falciparum* malaria. In this case restimulation of the vaccine primed immune system by infectious mosquito bites is integral part of the development of an effective immune protection. Vaccines that can protect travellers to endemic areas at least for a limited time period from malaria will presumably have to be designed in a different way.

Peptide vaccines are regarded as one ultimate goal in vaccinology, because they are ideally suited for specific targeting of desired immune responses. Furthermore, synthetic peptide vaccines are safe, easy to produce, stable and inexpensive. However, experiences with the formulation of such vaccines for use in humans are still very limited. The malaria peptide vaccine SPf66 [4, 5] developed by M. Patarroyo (Instituto de Inmunologia, Bogota) is the first synthetic peptide vaccine that has shown in phase III clinical trials to induce a partial protective immune response to an infectious disease in humans. Phase III field trials conducted with SPf66 in different epidemiological settings have yielded conflicting results [6]. Notwithstanding that vaccination with SPf66 confers only partial protection, it represents a milestone in malaria vaccine development and can serve as a unique model system that allows to study vaccine induced immunity to malaria in humans. Such studies are a prerequisite for the rational design of a more effective second generation malaria vaccine and peptide based vaccines in general.

SPf66 is a chemically synthesised 45 amino acids peptide derived from sequences of three different proteins [4]. One of the epitopes, designated 83.1, corresponds to the semiconserved region 45–53 of the well characterised parasite protein MSP-1. This parasite blood stage cell surface protein is generally regarded as a key malaria vaccine candidate [5]. The proteins containing the two other epitopes have not been cloned and characterised. We have generated panels of monoclonal antibodies (mAb) *vs.* the SPf66 building blocks and have found that all three linear peptide epitopes (35.1, 55.1 and 83.1) can induce antibodies which recognise the native antigens on the parasite cell surface (Pluschke *et al.*, manuscript in preparation).

This supports the concept of using a panel of selected peptide epitopes to build a malaria vaccine.

Currently five sequence variants of the 83.1 epitope of SPf66 have been described in *P. falciparum* isolates world-wide. We wanted to find out whether it is appropriate to incorporate such semi-conserved epitopes into a vaccine and therefore have investigated, whether an immunisation with the sequence variant incorporated into SPf66 (YSLFQKEKMVL) can induce antibodies that can also bind to the other naturally occuring variants (YSLFHKEKMIL, YGLFQKEKMVL, YGLFHKEKMIL and YGLFHKEKMLL). For quantitative analyses, we have generated 83.1 specific mAb from SPf66 immunised mice. All mAb which strongly reacted with the peptide YSLFQKEKMVL in ELISA also stained *P. falciparum* blood stage parasites in immunofluorescence assays (IFA). In IFA most antibodies did not only bind to the MSP-1 protein containing the 83.1 sequence present in the vaccine, but also to parasites expressing MSP-1 containing the variant sequences. Since already very low affinity antibody-antigen interactions can lead to parasite staining, these positive IFA results cannot be taken as a proof for biologically relevant antibody reactivities. To address this question further, we have performed quantitative antibody-antigen binding studies using isothermal titration calorimetry. Calorimetric titration experiments were performed using a MCS-ITC instrument (MicroCal, Northampton, MA). The sample cell (1.34 ml) was filled with a mAb solution (typically 2 μM) in PBS. The injection syringe (nominal volume 250 μl) was filled with a peptide solution (typically 50–100 μM) in PBS. The reference cell contained a solution of 0.01% sodium azide. During the experiments the sample solution was stirred by rotating at 400 rpm the injection syringe whose tip has the form of a paddle. After the baseline stability was better than 0.1 μcal sec^{-1}, 1–2 μl of solution were injected to remove possible air bubbles at the syringe openings. The preinjection was followed by a succession of injections of constant volume, typically between 5 and 15 μl at constant time intervals. The time intervals between two consecutive injections, typically 250 s, allowed the heat signal to come back to baseline. The instrument was equilibrated with an external circulating bath at least 5°C below the experimental temperature. Unless otherwise indicated, experiments were carried out at 25°C. Prior to each experiment sample cell and syringes were rinsed with PBS. After each experiment sample cell and syringes were rinsed first with PBS, cleaned with 200 ml of 0.1% SDS-solution (Merck, Darmstadt, Germany) and finally rinsed with at least 1 liter of double distilled water. The isothermal titration curve was registered and analysed using ORIGIN software (MicroCal) provided with the MCS-ITC instrument. At the beginning of a titration experiment antibody binding sites are available in excess in the sample cell. For a very tight binding (binding constant $>10^8 M^{-1}$) each injection then leads to an almost complete binding of the injected ligand molecules. The molar ratio of ligand to binding sites increases with each injection and therefore less and less free binding sites become available until complete saturation of the antibody binding sites is reached. At saturation the heat signal drops and only heat of dilution is measured upon further injections. Binding enthalpies ΔH are calculated from the integrated peak areas of all injections prior to saturation. The reported enthalpy values are corrected for heat of dilution obtained from the integrated peaks beyond saturation of antibody binding sites.

Clear differences in affinity of the mAb to the peptide variants were observed as demonstrated for one anti-83.1 mAb in Fig. 1 (Helg *et al.*, manuscript in preparation). The binding affinity K_A of this mAb 7.27 to the sequence YSLFQKEKMVL (1.1) incorporated into the vaccine was $1.2\cdot10^8\ M^{-1}$. With the variant YSLFHKEKMLL (1.2), which differs from the previous in two positions, the affinity was about ten times lower. Even weaker binding was observed with the variants YGLFHKEKMLL (1.3) and YGLFHKEKMIL (1.4). Finally, the affinity to the variant YGLFQKEKMVL (1.5) was too low to result in a detectable signal (Fig. 1). In spite of the lower binding affinities compared with 1.1 observed by isothermal titration calorimetry, the mAb 7.27 stained all parasite clones tested, irrespective of the expressed MSP-1 variant sequence. This shows, that reactivity of sera in IFA, which is a standard test in malaria serology, is not necessarily an indication for the presence of high affinity antibodies with biological relevance.

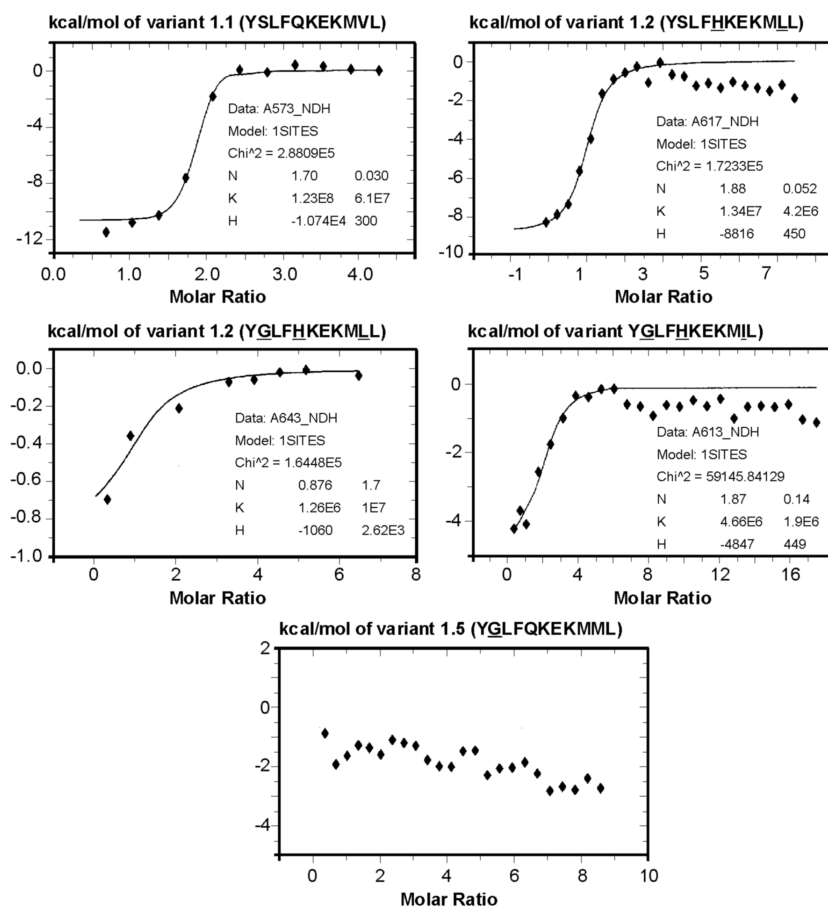

Fig. 1 Isothermal titration calorimetric analysis of the binding of the anti-MSP-1 mAb 7.27 to 83.1 peptide sequence variants. Integrated heats of binding are expressed in kcal mol^{-1} of injected peptide. The measuring temperature was 25°C

In contrast to mAb 7.27, another prototype mAb, mAb 9.22 reacted with all peptide variants tested with an affinity $>4\cdot10^6$ M^{-1} (data not shown). Based on this finding, it can be assumed that SPf66 primed memory B cells expressing this antibody on their cell surface could be activated during natural challenge by *P. falciparum* parasites expressing any of the known MSP-1 variants on their cell surface. Selection of B cells expressing somatically mutated and affinity matured antibodies in germinal centres could then lead to a fast and efficient production of high affinity antibodies *vs.* the MSP-1 of the infecting strain. These results indicate that not only conserved, but also semi-conserved sequences, such as the 83.1 peptide can be used as building block of an epitope focused vaccine.

Anti-idiotyic antibody based vaccination *vs.* malignant melanoma

According to Jerne's idiotypic network theory of the immune system [7, 8], an antigen induces antibodies (Ab1) which express an idiotype. The latter induce an anti-idiotype response (Ab2) to the Ab1 which in turn may induce an anti-anti-idiotypic response (Ab3). Since nominal antigen and Ab2 can competitively bind to the same region of an Ab1, some Ab2 carry the 'internal image' of the nominal antigen and therefore can induce immunity to it. The conformational and occasional amino acid sequence mimicry of an antigen by some of the corresponding Ab2 has led to their use as surrogate antigens in vaccine formulations. This theory has been exploited to develop active specific immunotherapy with Ab2 mAb in patients with cancer. There is mounting evidence that immunological tolerance to tumour associated antigens can be easier broken by immunisation with an Ab2 than with the nominal antigen [1].

One Ab2 that has been analysed in clinical trials with cancer patients is mAb MK2-23 [9], which mimics an epitope of the human High Molecular Weight-Melanoma Associated Antigen (HMW-MAA), which is a melanoma associated chondroitin sulphate proteoclycane [10]. In clinical trials with patients with malignant melanoma a statistically significant association was found between humoral anti-HMW-MAA immunity elicited by MK2-23 and prolongation of their survival [11, 12]. This has stimulated interest in optimising the immunisation strategy for a large clinical trial to assess in a conclusive way the clinical significance of active specific immunotherapy with mAb MK2-23. We have been exploring approaches to augment the immunogenicity of the Ab2 mAb, since we assume that an enhancement of the anti-HMW-MAA immune response may improve its beneficial effects on the clinical course of the disease. We have found that Ab2 mAb variants of the mouse IgG2a or IgG2b isotype secreted by hybridoma isotype switch variants isolated from the parental hybridoma MK2-23 [13], which secrets an IgG1 isotype are more suitable for standardisable purification than the parental mAb. The use of these variants would therefore facilitate the preparation of large amounts of well standardised immunogens for clinical trials. Another approach we have been investigating has resulted in the preparation of human-mouse chimeric Ab2 mAb [13] which we expect to induce a much lower level of anti-mouse Ig antibodies than the corresponding mouse Ab2 mAb. Anti-mouse IgG antibodies are likely to form complexes with the injected mouse Ab2 mAb and therefore may interfere with its ability to interact with the host's immune system, thus affecting its immunogenicity.

In recent studies immunoglobulin constant regions have been found to influence the functional affinity and association and dissociation constants of antibodies from the corresponding antigens [14–18]. These findings raised the possibility that chimeric and isotype switch variants of mAb MK2-23 may differ from the parental mAb in their immunogenic properties [19]. Such differences would be expected to be paralleled by differences in the interactions of the Ab2 variants with the Ab1 (mAb 763.74), *vs.* which the Ab2 MK2-23 was generated. Employing high sensitivity titration calorimetry we have therefore probed the interactions of the Ab2 variants (mouse/human chimeric, mouse IgG1, IgG2a and IgG2b) with the Ab1 [13].

Calorimetric titration experiments were performed essentially as described above for the analysis of peptide-antibody interactions. Antibody-protein antigen interactions are characterised by large binding constants and are usually driven by enthalpic forces (van der Waals interactions and hydrogen bonds with occasional ion pairs), with varying degrees of favourable or unfavourable entropic contribution [13]. Similar thermodynamic properties were found also in the id to anti-id human HMW-MAA system. Interactions of Ab2 with the anti-HMW-MAA Ab1 were characterised by large binding constants (larger than $6\cdot10^9$ M^{-1}) and a large exothermic binding enthalpy. Entropic contributions to the binding process played only a minor role and binding thus was almost completely driven by enthalpic forces.

Figure 2 illustrates typical results of a calorimetric titration (binding of the Ab1 with the Ab2 variant of the heavy chain class IgG2b). Aliquots of Ab2 solution were automatically injected into Ab1 solution. Each injection resulted in an exothermic peak of equal height until with the 11th injection the Ab1 mAb binding sites were saturated. Additional injections led to the practically same endothermic heat signal because only heat of dilution was measured. The lower part of Fig. 2 shows the integrated area of each peak corrected for heat of dilution and expressed in kcal mol^{-1} of injected Ab2. The almost rectangular shape of the binding curve indicates a very

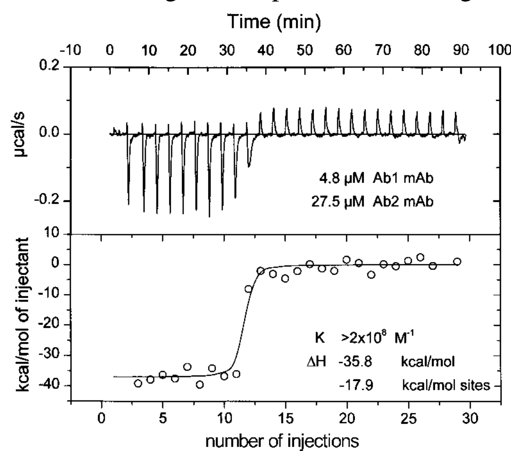

Fig. 2 Typical isothermal titration calorimetric profile of the binding of the Ab1 to the Ab2 of the IgG2b heavy chain subclass (upper curve) and integrated heats of binding expressed in kcal mol^{-1} of injected Ab2 (lower curve). Aliquots of 10 μl solution of Ab2 (27.5 μM) were automatically injected into a solution of 1.34 ml Ab1 (4.8 μM). The measuring temperature was 25°C

tight binding effect. As antibody molecules are equipped with two identical binding sites, it is convenient to express binding enthalpies in kcal per mole of antibody binding sites (n=2). The binding enthalpy ΔH=–17.9 kcal mol^{-1} is then given by the ratio of the peak area to the number of moles of Ab2 mAb binding sites added per injection. If not stated otherwise, this convention for ΔH is used throughout the text. The rectangular shape of the binding isotherm did not allow an accurate determination of the binding constant K from a deconvolution of the binding isotherm (Fig. 2). For a reliable deconvolution of the binding curve, the parameter $c=KM_{tot}n$ should be in the range from 1 to 1000, where M_{tot} is the concentration of Ab1 mAb in the cell and n the number of binding sites [20]. In the present experiment, M_{tot} was 4.8 μM and n=2, which sets the upper limit for an accurate determination of K to $1\cdot10^8$ M^{-1}.

Since the binding constant exceeded the upper analytical limit of titration calorimetry we performed experiments at higher temperatures to determine whether the binding constant would drop into the analytical window (Fig. 3). The influence of the temperature on the binding constant K, when varying the temperature, can be calculated using a formula derived from the Gibbs-Helmholz equation [21]. The heat capacity change ΔC_p is measured from the slope of the temperature dependence of the binding enthalpy $\Delta H(T)$. Figure 3 shows the titration of Ab1 with 20×10 μl of an Ab2 solution at 25, 37 and 50°C. The binding enthalpy ΔH shows a linear decrease from –16.8 to –25.7 kcal mol^{-1} with an increase of the temperature from 25 to 50°C. The calculated change in the specific heat capacity ΔC_p showed a negative value of –357 cal mol^{-1} K^{-1} (per mol binding sites). However, even at 50°C the binding curve showed an almost rectangular shape indicating a binding constant K above the upper analytical limit of $3.7\cdot10^8$ M^{-1} for this experiment (M_{tot}=2.7 μM). Inserting the corresponding experimental values of ΔH and ΔC_p into the Gibbs-Helmholz equation demonstrates that an increase of the temperature from 25 to 50°C reduced the binding constant approximately 16 fold. The larger than $3.7\cdot10^8$ M^{-1} value of the binding constant K at 50°C indicates a value of $6\cdot10^9$ M^{-1} for the lower limit of the binding constant at 25°C.

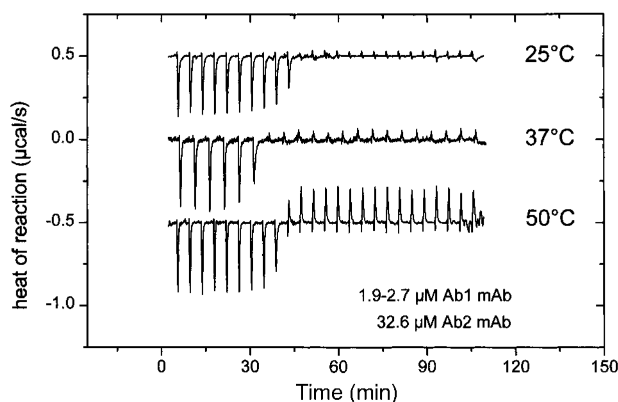

Fig. 3 Isothermal titration calorimetric profiles of the binding of the Ab1 to the Ab2 of the IgG2b heavy chain subclass recorded at 25, 37 and 50°C. Injections of 10 μl of Ab2 mAb solution (32.6 μM) were made into Ab1 mAb solution (1.9–2.7 μM)

Table 2 Binding enthalpies ΔH for binding of Ab2 variants to the anti-HMW-MAA Ab1 at 25°C

Type of Ab2	ΔH/ kcal per mol binding sites
Mouse IgG1*	–15.8, –15.4
Mouse IgG2a*	–16.2, –17.3
Mouse IgG2b	–17.9
Chimeric (human IgG1)	–15.1

* Two independent measurements

To determine whether Ig constant regions influence Ab1 to Ab2 interactions in the HMW-MAA system, the thermodynamic parameters that characterise the interactions of the Ab1 with the four variants of the Ab2 described above, were compared. The binding enthalpies of the four Ab2 mAb variants to the Ab1 mAb did not significantly differ from each other, as they ranged from –15.1 to –17.9 kcal mol^{-1} at 25°C (Table 2). These results indicated, that Ig constant regions do not significantly influence Ab1 to Ab2 interactions in the anti-HMW-MAA system. As therefore expected, subsequent immunogenicity studies showed, that the four Ab2 variants in fact also do not differ significantly in their ability to elicit anti-HMW-MAA antibody responses in mice (O'Reilly *et al.*, manuscript in preparation). Both the IgG2a and IgG2b isotype switch variants, as well as the chimeric variant could thus be used to replace the original Ab2 of the IgG1 subclass in large scale preparations for clinical trials.

To determine the extent of binding of the Ab1 to the heavy or light chain variable regions of the Ab2, either its heavy or its light chain variable region was replaced with the corresponding regions of the unrelated mAb F5-444. The two Ab2 derivatives are referred to as MK_{light} and MK_{heavy}, respectively. The Ab1 was titrated with these two derivatives. The calorimetric traces for the titrations are indicated by the lower curves in Figs 4 and 5, while the corresponding heats of dilution are shown by the upper curves. The mAb MK_{light} with the light chain variable region of the Ab2 showed no significant net effect (Fig. 4), while mAb MK_{heavy} with the heavy chain variable region of the Ab2 displayed a very low net heat effect (Fig. 5). These results indicate that the heavy and light chain variable region of the Ab2 are both required for the expression of the idiotope recognized by the anti-HMW-MAA Ab1. The observed lack of Ab1 mAb defined HMW-MAA epitope expression by isolated heavy and light chain variable regions of the Ab2 mAb and the lack of amino acid sequence homology (Pluschke *et al.*, manuscript in preparation) between its CDRs and the MCSP core protein of HMW-MAA [10] suggest that the antigen mimicry by the Ab2 mAb requires the involvement of several complementarity determining regions (CDRs). These results suggest that a combination of both variable regions of the Ab2 is required to elicit an anti-HMW-MAA immune response. This was reconfirmed by immunogenicity studies in mice, which showed, that the two mAb MK_{light} and MK_{heavy} in contrast to the four Ab2 variants described above, do not elicit antibodies which bind to HMW-MAA expressing human melanoma cells (O'Reilly *et al.*, manuscript in preparation).

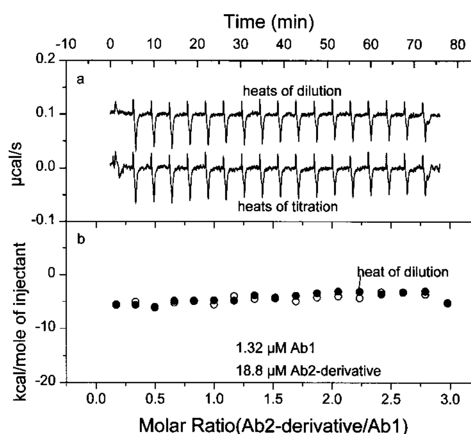

Fig. 4 Isothermal titration calorimetric profiles of the binding of the Ab1 to the Ab2 derivative mAb MK_{light} generated with the heavy chain variable region replaced by the corresponding region of the unrelated mAb, F5-444. The upper curve shows the corresponding heat of dilution profiles obtained from injection of mAb MK_{light} into buffer solution. Aliquots of 15 µl of the Ab2 derivative (18.8 µM) were automatically injected into a solution of 1.34 ml Ab1 (1.3 µM)

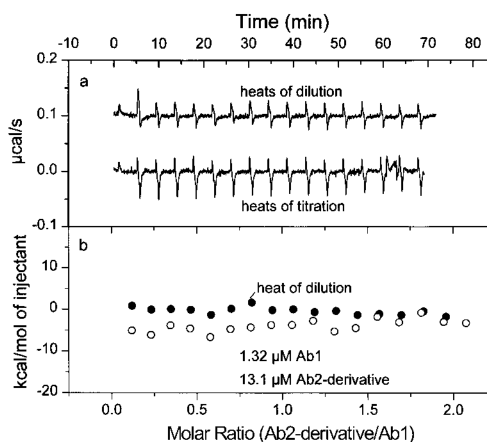

Fig. 5 Isothermal titration calorimetric profiles of the binding of the Ab1 to the Ab2 derivative mAb MK_{heavy} generated with the light chain variable region replaced by the corresponding region of the irrelevant mAb F5-444. The upper curve shows the corresponding heat of dilution profiles obtained from injection of mAb MK_{heavy} into buffer solution. Aliquots of 10 µl of the Ab2-derivative (13.1 µM) were automatically injected into a solution of 1.34 ml Ab1 (1.3µM)

The thermodynamic parameters found for the binding of the Ab2 variants to the Ab1 are comparable to previously published values for interactions between antibodies and protein antigens [13]. The binding enthalpy of various protein antigen – antibody interactions is in the range from –7 to –23 kcal mol^{-1} with binding con-

stants ranging from 0.1 to $400 \cdot 10^8$ M^{-1}. Values for the heat capacity change vary between –150 and –650 cal mol^{-1} K^{-1}. The value for ΔC_p we have measured for Ab1 to Ab2 binding in the HMW-MAA system is also similar to the theoretical value of –356 cal mol^{-1} K^{-1} that Hibbits *et al.* [22] had calculated from structural data of the buried polar and apolar areas of hen egg lysozyme and the mAb HyHEL-5 system. In contrast to protein-protein interactions in general, antibody – protein-antigen interactions usually exhibit large exothermic binding enthalpies. These findings show that enthalpic forces always play an important role in the binding process. This rule is also followed by the interaction we have analyzed in the present study.

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