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Calculation of the affinity constant K_{ASS} for solid phase antigen

A model system using monoclonal antibodies against the cat allergen *Fel d I*

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In this report a procedure is described to calculate the affinity constant of an antibody for solid-phase Ag. K_{SP} (K_{ASS} solid phase) was defined as the reciprocal of the concentration of Ag required for half saturation of the Ab-binding sites, extrapolated to an infinitely small concentration of Ab (semi-saturation plot). Using this procedure, the affinity of IgE antibodies can be measured without interference from 'invisible' IgG antibodies. As a model system, mAbs against the cat allergen *Fel d I* were used. Serial dilutions of *Fel d I*-Sephacrose were incubated with serial dilutions of mAb Fd1a and Fd1b, with or without rabbit antibodies as 'invisible' antibodies. The Ab-binding capacity of Sephacrose-coupled *Fel d I* was low: 4.15% and 2.13% of the expected value for mAb Fd1a and Fd1b, respectively, and this must be taken into account when calculating K_{SP} . The K values of mAb Fd1a and Fd1b, calculated from the y axis intercept of the semi-saturation plot, were $85 \text{ (pmol/test)}^{-1}$ and $65 \text{ (pmol/test)}^{-1}$ respectively. Using the semi-saturation plot, K_{SP} of mAb Fd1a was not affected by the presence of rabbit antibodies against *Fel d I*, confirming the applicability of the procedure for measuring the K_{SP} of IgE in patient sera. For one cat-allergic patient the K_{SP} of IgE and IgG4 for *Fel d I* were calculated and found to be $62 \text{ (pmol/test)}^{-1}$ and $147 \text{ (pmol/test)}^{-1}$ for IgE and IgG4 respectively.

Key words: Affinity constant; Solid-phase antigen; *Fel d I*; Monoclonal antibody

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Abbreviations: *Fel d I*, *Felis domesticus* allergen 1; U, unit (1 U *Fel d I* = 2 μg); Ag, antigen; Ab, antibody; B, Ab bound; K_{ASS} , association constant; K_{SP} , K_{ASS} solid-phase Ag; K_{FP} , K_{ASS} fluid-phase Ag; mAb, monoclonal antibody; RAST, radioallergosorbent test; PBS-AT, phosphate buffered saline pH 7.4 containing 10 mM EDTA, 0.3% BSA, 0.1% Tween 20 and 0.05% NaN_3 .

Introduction

A number of experimental methods are available to determine the absolute affinity constant of an antibody (Ab). In most assays equilibrium between Ab and antigen (Ag) is reached with both components in the fluid phase. Complexed Ag can then be separated from free Ag by precipitation techniques with ammonium sulfate (Farr, 1958), polyethylene glycol (Heyningen van et al.,

1983) or anti-immunoglobulins (Jacobsen et al., 1982). Relative antibody affinity may also be determined with a solid-phase Ag. In these experiments Ag is coated onto microtiter plates, Ab is added, and increasing concentrations of free Ag are used to competitively inhibit binding of the Ab to the solid-phase Ag. The concentration of soluble Ag required for 50% inhibition is taken as a relative measure of affinity (Nieto et al., 1984; Rath et al., 1988; Devey et al., 1988, 1989; Dam van et al., 1989; Persson et al., 1988; Hetherington, 1990). Relative affinity has also been measured using mild denaturing or chaotropic agents to inhibit antibody binding (Inouye et al., 1984; Devey et al., 1988).

In this report a procedure is described for calculating the affinity constant K_{ASS} for solid-phase Ag (K_{SP}). The aim of this study was to develop a procedure to determine the K_{SP} of IgE antibody in a patient serum using a radioallergosorbent test (RAST). Using solid-phase Ag, we met with three difficulties: (1) not all Ag coupled to Sepharose was available for antibody binding, (2) using whole serum, isotypes other than the isotype measured (i.e., IgE) also bound to the solid-phase Ag (so called 'invisible' antibody) and (3) the Ag and Ab concentrations did not obey to the classical equation, because the apparent K_{SP} was dependent on the test volume. A procedure was developed in which K_{SP} was defined as the concentration of Ag required for saturation of half of the Ab-binding sites when an infinitely small concentration of Ab was added. As a model system, the K_{SP} of mAbs against the major cat allergen *Fel d* I (De Groot et al., 1988) were measured. Serial dilutions of Ag-Sepharose were incubated with suitable dilutions of mAb and IgG bound to the Sepharose was detected with radio-labelled rat anti-mouse IgG. The procedure of affinity measurement was performed as follows: (1) radioactivity bound to the Sepharose was converted into fmol of Ab bound; (2) maximum Ab binding was estimated by linear extrapolation of a double reciprocal plot $(\text{Ab bound})^{-1}$ versus $(\text{mg Ag-Sepharose added})^{-1}$, which is an approximation of the Steward and Petty plot (Steward and Petty, 1972); (3) the amount of Ag-Sepharose available for binding Ab (Ab-binding capacity) was estimated by determining the maximum

amount of Ab bound by a low dose of Ag-Sepharose; (4) the antigen concentrations required for half-maximum binding (Ag_{50}) were estimated at different Ab concentrations by interpolation and these Ag_{50} values were plotted against the concentration of Ab added (semi-saturation plot). By an extrapolation procedure Ag_{50} was estimated for an infinitely small Ab concentration.

In the presence of 'invisible' IgG antibodies, the K_{SP} of IgE (1) and the K_{SP} of IgG (2) can be expressed as follows:

$$K_E = \frac{B_E}{(\text{Ab}_E - B_E) \times (\text{Ag} - B_E - B_G)} \quad (1)$$

$$K_G = \frac{B_G}{(\text{Ab}_G - B_G) \times (\text{Ag} - B_G - B_E)} \quad (2)$$

From these two equations it follows that:

$$B_G = \frac{\text{Ab}_G}{\frac{K_E}{K_G} \times (\text{Ab}_E - B_E) \times \frac{1}{B_E} + 1} \quad (3)$$

$$\text{Ag} = \frac{B_E}{K_E} \times \frac{1}{(\text{Ab}_E - B_E)} + B_E + B_G \quad (4)$$

When $\text{Ag} = \text{Ag}_{50}$, $B_E = \frac{1}{2} \text{Ab}_E$. Then B_G and Ag_{50} are defined as follows:

$$B_G = \text{Ab}_G \times \frac{K_G}{K_E + K_G} \quad (5)$$

$$\text{Ag}_{50} = \left(\frac{1}{2} + \frac{\frac{\text{Ab}_G}{\text{Ab}_E} \times K_G}{K_E + K_G} \right) \times \text{Ab}_E + \frac{1}{K_E} \quad (6)$$

In the semi-saturation plot, Ag_{50} values are plotted against the concentration of Ab added (Ab_E). Thus, using this procedure, $1/K_{SP}$ is defined by the y axis intercept and is independent of K_G and of Ab_G . To confirm this theory, the effect of 'invisible' rabbit antibodies (anti-*Fel d* I) on the affinity measurement of mAb Fd1a was studied.

For one cat-allergic patient, the K_{SP} of IgE and IgG4 for the cat allergen *Fel d* I were measured using the semi-saturation plot.

Materials and methods

Preparation of solid-phase antigen

To 200 mg of CNBr-activated Sepharose 4B (Pharmacia) 50 U (2.86 nmol) of affinity purified *Fel d* I were coupled, according to the manufacturer's instructions, in 0.1 M NaHCO₃ pH 8.5, containing 0.5 M NaCl. After an overnight incubation at 4°C, remaining binding sites were blocked with 0.5 M glycine pH 8.5, and the Sepharose was suspended in 100 ml PBS, containing 10 mM EDTA, 0.3% BSA, 0.1% Tween 20 and 0.05% NaN₃ (PBS-AT).

Monoclonal antibodies and rabbit antibodies

Fel d I-specific mAbs CLB-Fd1a and CLB-Fd1b were obtained by immunizing BALB/c mice with cat dander extract (De Groot et al., 1988). MAb Fd1a was a protein A purified IgG1 preparation from mouse ascitic fluid (Pharmacia Fine Chemicals, Uppsala, Sweden). The concentration of IgG was 1.2 mg/ml, derived from A_{280} (upon iodination, 80% of the protein-bound radioactivity was bound by an excess of adsorbent). An excess of *Fel d* I-Sepharose was tested with serial dilutions of mAb Fd1a and used as a standard curve to determine the concentration of *Fel d* I-specific IgG in mAb Fd1b preparations (unpurified mouse ascites). The concentration of *Fel d* I-specific IgG in mAb Fd1b was 7.2 mg/ml. The affinity constants of the mAbs with fluid-phase, iodinated Ag (K_{FP}), were $6.7 \times 10^9 \text{ M}^{-1}$ for mAb Fd1a and $1.7 \times 10^9 \text{ M}^{-1}$ for mAb Fd1b, as determined by Scatchard analysis.

Polyclonal antibodies against *Fel d* I were obtained by immunizing a rabbit with affinity purified *Fel d* I (De Groot et al., 1988). The K_{FP} value of this rabbit serum was $65 \times 10^9 \text{ M}^{-1}$, as determined by Scatchard analysis. The concentration of *Fel d* I-specific Ab was 0.14 mg/ml, also derived from Scatchard analysis.

Patient serum

The serum of a cat-allergic patient with a high level of both IgE and IgG4 antibodies against *Fel d* I, was selected for this study. The patient did not receive immunotherapy. The K_{FP} values were $27 \times 10^9 \text{ M}^{-1}$ and $66 \times 10^9 \text{ M}^{-1}$ for IgE and IgG4 respectively, as determined by Scatchard analysis.

Allergen sources

Cat dander extract (10%, w/v) was obtained from HAL (Haarlems Allergenen Laboratorium, Haarlem, Netherlands), batch no. 99337; it contained 160 U/ml *Fel d* I. *Fel d* I (230 U/ml) was isolated from house dust by affinity purification with mAb Fd1a (Chapman et al., 1988).

Radioimmunoassay (RIA) procedures

Binding of mAb Fd1a and Fd1b to Sepharose-coupled *Fel d* I

50 μ l volumes of serial dilutions of mAb Fd1a and Fd1b were incubated overnight, using end-over-end mixing with *Fel d* I-Sepharose in a final volume of 500 μ l PBS-AT. After washing the Sepharose 1.2 ng of ¹²⁵I-rat anti-mouse IgG (specific activity 0.37 MBq/ μ g) and 500 μ l of PBS-AT were added. After another incubation overnight the Sepharose was washed and the radioactivity bound to the Sepharose was measured. To study the effect of the test volume on Ab binding, additional PBS-AT was added. To ensure equilibrium in a larger reaction volume, the first incubation step was extended to 48 h.

IgE and IgG4 RAST

50 μ l dilutions of serum were incubated with *Fel d* I-Sepharose in a final volume of 500 μ l. IgE and IgG4 bound to the Sepharose were detected using 1 ng of ¹²⁵I-sheep anti-human IgE (M1294, CLB, Amsterdam, Holland) and 1 ng of ¹²⁵I-mouse anti-human IgG4 (M1278, CLB) respectively.

Competitive RIA

50 μ l of mAb Fd1a (15.9 fmol) and mAb Fd1b (25.8 fmol) were preincubated with serial dilutions of cat dander extract. After 2 h *Fel d* I-Sepharose (0.13 mg) was added in a final volume of 500 μ l of PBS-AT and the mixture incubated overnight. The rest of the procedure was performed as previously described.

Calculation of affinity constant K_{SP}

Serial dilutions of *Fel d* I-Sepharose were incubated with serial dilutions of mAb Fd1a and Fd1b. The binding percentages of ¹²⁵I-rat anti-mouse IgG were converted into fmol of mAb

TABLE I

CONCENTRATIONS OF mAb BOUND (fmol/TEST) FOR SERIAL DILUTIONS OF *Fel d* I-SEPHAROSE (fmol/TEST) AND SERIAL DILUTIONS OF mAb Fd1a AND mAb Fd1b (fmol/TEST)

For *Fel d* I-Sepharose, both the nominal concentration of Ag and the concentration of Ag available for binding mAb Fd1a and Fd1b are given.

Ag fmol/test nominal available	1784	892	446	223
Fd1a fmol/test	74	37	18.5	9.25
49.5	47.6	28.9	16.2	8.6
24.7	23.8	16.5	13.5	7.6
12.4	12.7	9.7	6.8	3.8

Ag fmol/test nominal available	3568	1784	892	446
Fd1b fmol/test	76	38	19	9.5
100.6	86.2	37.3	14.9	8.6
50.3	51.9	27	11.4	6.5
25.1	24.6	19.5	9.5	6.5

bound to the Sepharose (Table I). *Fel d* I-Sepharose (0.25 mg/test) with mAb Fd1a was used to generate a standard curve. The Ab concentration of mAb Fd1a was known and the dose-response curves of mAb Fd1a with two-fold and four-fold diluted *Fel d* I-Sepharose overlapped (Fig. 1), which indicated that all mAb added were bound to the Sepharose. To estimate the molar concentration of *Fel d* I available on the Sepharose (Ab-binding capacity), the maximum amount of mAb bound by a low concentration of

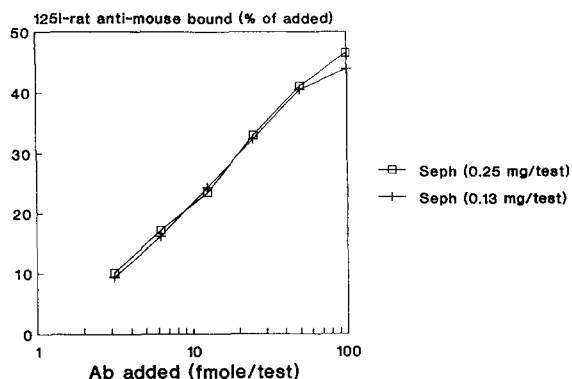


Fig. 1. Dose-response curves of mAb Fd1a with two-fold and four-fold diluted *Fel d* I-Sepharose.

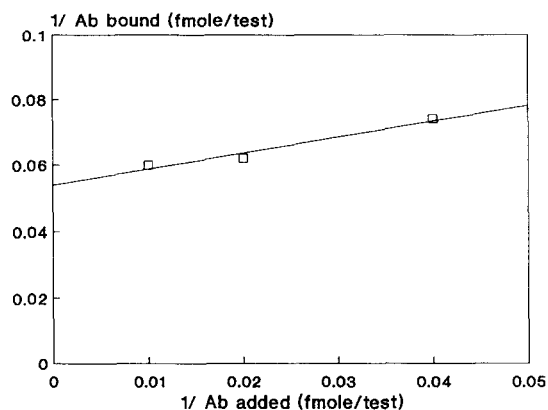


Fig. 2. Ab binding capacity of *Fel d* I-Sepharose for mAb Fd1a. *Fel d* I-Sepharose was diluted 1/16 (and tested with serial dilutions of mAb Fd1a). Binding percentages of ^{125}I -rat anti-mouse IgG were converted into fmol of IgG bound and plotted versus fmol of Ab added, in a double reciprocal plot. By linear extrapolation the maximum concentration of Ab binding to the Sepharose was estimated.

Fel d I-Sepharose was determined. By linear extrapolation of a double reciprocal plot, the maximum amount of mAb bound by the appropriate Sepharose dilution was estimated (Fig. 2). The K_{SP} was calculated by determining at different Ab concentrations the concentration of solid-phase Ag, required for binding half of the Ab-binding sites (semi-saturation plot). Bound Ab was plotted against the amount of *Fel d* I-Sepharose added. Knowing the concentration of Ab added, the concentration of Sepharose-coupled Ag binding half this concentration (Ag_{50}) was found by interpolation. These Ag_{50} values were plotted versus the concentration of Ab added. The dissociation constant K_{DISS} (fmol/test) was defined by the y axis intercept: the concentration of Ag required to bind half of the Ab-binding sites, when an infinite concentration of Ab is added. K_{SP} was expressed as $(\text{pmol/test})^{-1}$.

Effect of additional rabbit serum on the affinity of mAb Fd1a, calculated by the semi-saturation plot

In order to determine the effect of 'invisible' antibody on the affinity measurement of mAb Fd1a by the semi-saturation plot, rabbit serum anti-*Fel d* I (933 fmol/test) was added to mAb Fd1a (49.5 fmol/test). Serial dilutions of this Ab mixture were made in PBS-AT. The rest of the

procedure was performed as previously described.

Results

The antibody binding capacity of Sepharose-coupled *Fel d* I was estimated, by determining the maximum concentration of mAb, bound by a low concentration of *Fel d* I-Sepharose. For mAb Fd1a, the maximum concentration bound by a 1/16 dilution of *Fel d* I-Sepharose (446 fmol), was 18.5 fmol/test (femto = 10^{-15}), as estimated by linear extrapolation of a double reciprocal plot (Fig. 2). With the starting assumption that under the experimental conditions antigen-antibody binding resulted in a 1:1 complex, the Ab-binding capacity of *Fel d* I-Sepharose was $(18.5/446) \times 100\% = 4.15\%$. Of mAb Fd1b, 19 fmol was bound by a 1/8 dilution of *Fel d* I-Sepharose (892 fmol), resulting in an Ab-binding capacity of 2.13%.

Using the law of mass action, $K = [B]/([Ab] - [B])([Ag] - [B])$, K is dependent on the concen-

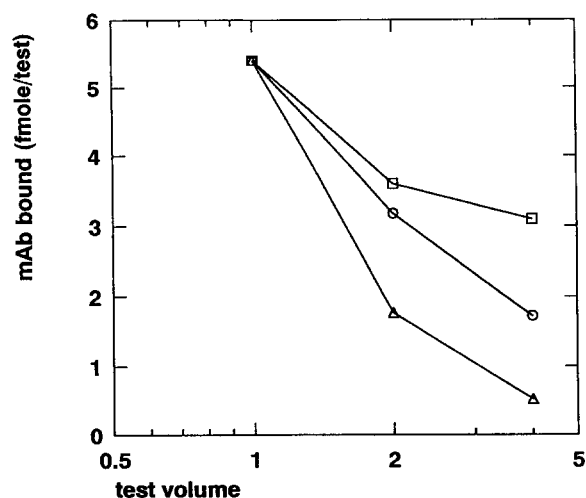
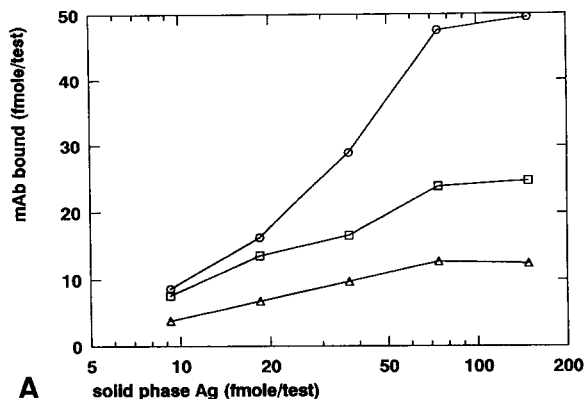
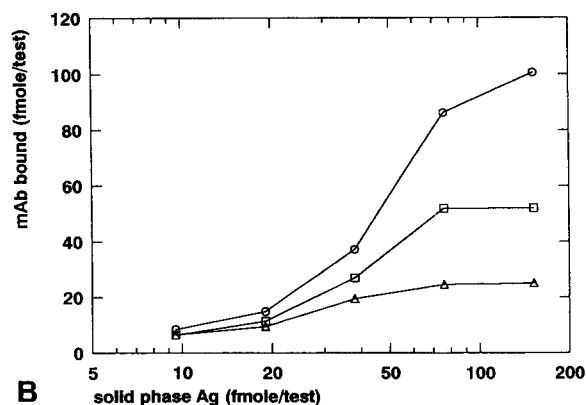


Fig. 3. Effect of the test volume used, on Ab binding to solid-phase Ag. 200 μ l of *Fel d* I-Sepharose (0.031 mg; 446 fmol Ag) were incubated with 50 μ l of mAb Fd1a (25 fmol), using three test volumes. The open squares represent the binding values found; the open triangles represent the calculated binding, lowering the amounts of Ag and Ab by a factor 2 and 4; the open circles also represent the calculated binding, but by lowering only the amount of Ab, and keeping the amount of Ag constant.



A



B

Fig. 4. Binding results for three concentrations of mAb with serial dilutions of Ag-Sepharose: calculation of Ag_{50} . Three concentrations of mAb Fd1a (A: 49.5 fmol/test = circles; 24.7 fmol/test = squares; 12.4 fmol/test = triangles) and mAb Fd1b (B: 100.6 fmol/test, 50.3 fmol/test and 23.1 fmol/test) were incubated with serial dilutions of *Fel d* I-Sepharose. The mAb bound to the Sepharose (fmol/test) was plotted against the concentration of *Fel d* I-Sepharose added. For all three Ab concentrations, the Ag_{50} was determined by interpolation.

trations of Ab and Ag added and is expressed as M^{-1} . However, using solid-phase Ag, K_{SP} appeared to be dependent on the test volume used! Enlargement of the test volume resulted in a smaller decrease of Ab binding than was predicted from the law of mass action (Fig. 3). Apparently, using solid-phase Ag, the antigen activity does not vary directly with the test volume. Therefore, Ag was expressed as mol/test and, consequently, K_{SP} was expressed as $(\text{mol/test})^{-1}$.

From the concentrations of Ag added and the concentrations of complex formed, the affinity

constant K_{SP} was calculated, using the semi-saturation plot.

Fig. 4 shows the binding results for three concentrations of mAb Fd1a, using serial dilutions of *Fel d* I-Sepharose. For each Ab concentration, Ag_{50} was determined by interpolation and plotted against the concentration of Ab added (Figs. 5A and 5B). The affinity constant K_{SP} was determined by the y axis intercept: K_{SP} mAb Fd1a = $1/11.8 \times 10^{-15}$ mol/test = 85 (pmol/test) $^{-1}$ and K_{SP} mAb Fd1b = $1/15.4 \times 10^{-15}$ mol/test = 65 (pmol/test) $^{-1}$. Calculation of the K_{SP} of mAb Fd1a was performed 4 times, and K values ranged from 85–220 (pmol/test) $^{-1}$. To validate

the semi-saturation plot for measuring an affinity constant, K_{SP} found by this procedure was compared with K_{SP} calculated from the concentrations of Ab and Ag added and the concentration of Ab bound, using the equation $K = B/(Ab - B)$ ($Ag - B$); K_{SP} of mAb Fd1a was 78 (pmol/test) $^{-1}$ and of mAb Fd1b 59 (pmol/test) $^{-1}$.

The effect of 'invisible' antibody on the affinity of mAb Fd1a, using the semi-saturation plot, was studied by adding high affinity rabbit antibodies anti-*Fel d* I to mAb Fd1a. In the presence of rabbit antibody, more Ag was required for half maximal binding of mAb Fd1a. In this experiment K_{SP} of mAb Fd1a were 176 (pmol/test) $^{-1}$

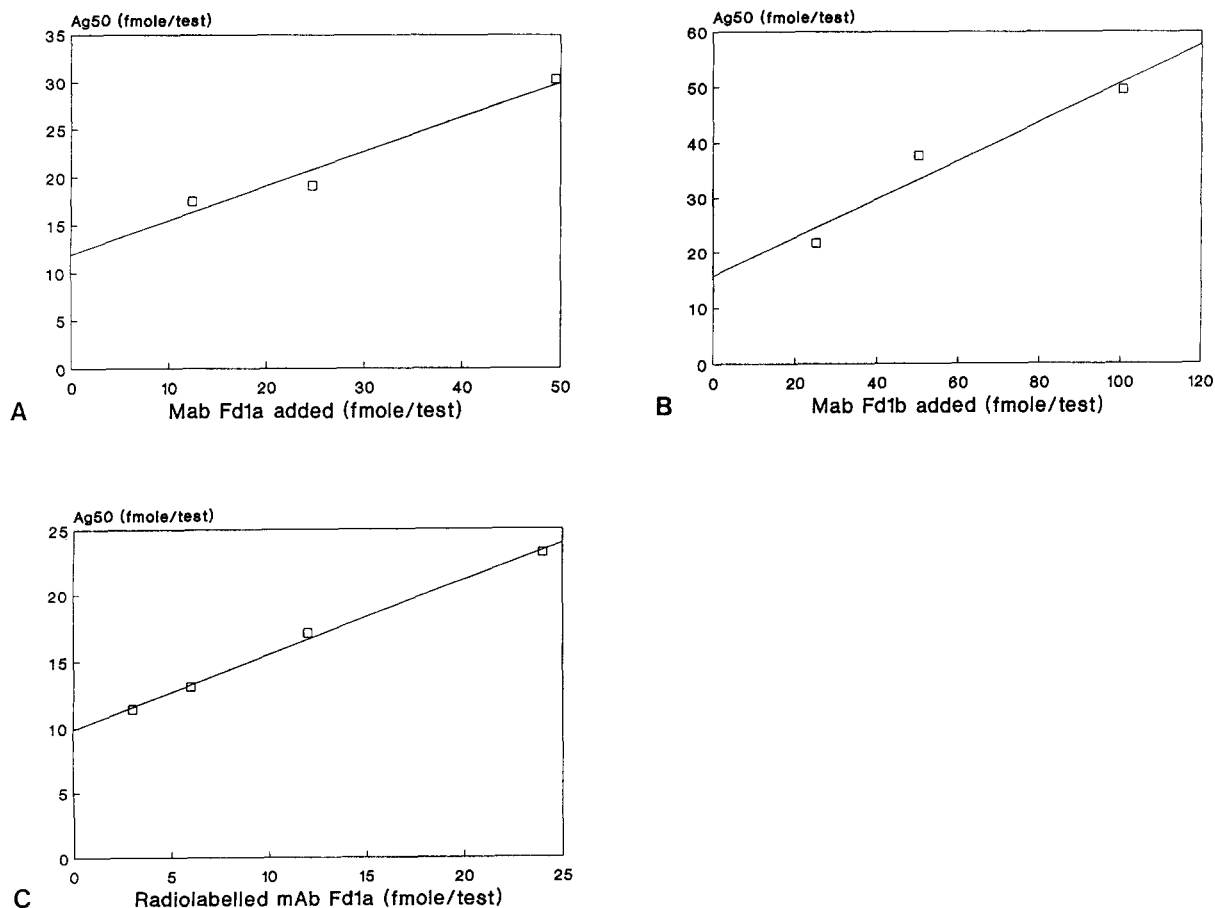


Fig. 5. Calculation of K_{SP} , using the semi-saturation plot. The Ag_{50} values of mAb Fd1a (A) and mAb Fd1b (B), found for three concentrations of mAb, were plotted against the concentration of mAb added. The Ag_{50} values of mAb Fd1a, found in a RIA with radiolabelled mAb, were also plotted against the concentrations of Ab added (C). The concentration of Ag required for half-maximal Ab binding when the concentration of Ab becomes infinitely small was defined by the y axis intercept: $1/K_{SP}$.

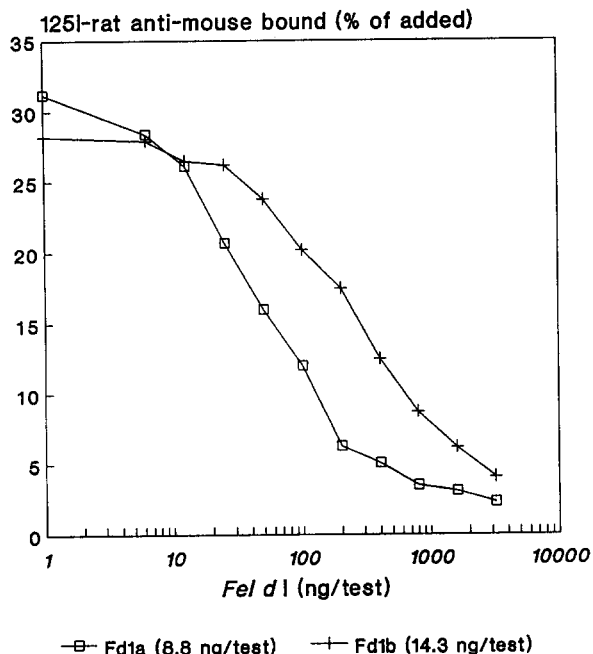


Fig. 6. Competitive inhibition curves for mAb Fd1a and Fd1b. 50 μ l of MAb Fd1a (15.9 fmol/test) and mAb Fd1b (25.8 fmol/test) were preincubated with serial dilutions of cat dander extract. After 2 h four-fold diluted *Fel d I*-Sephacrose was added and after an overnight incubation IgG bound to the Sepharose was detected with ^{125} I-rat anti-mouse IgG.

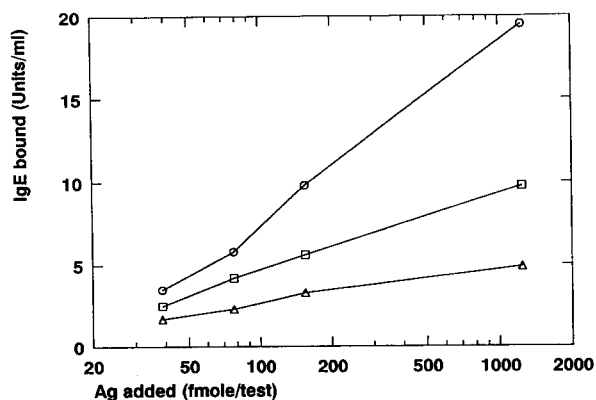


Fig. 7. IgE binding to *Fel d I* for three concentrations of serum. Three concentrations of serum (25 μ l = circles; 12.5 μ l = squares and 6.25 μ l = triangles) were incubated with serial dilutions of *Fel d I*-Sephacrose and IgE bound to the Sepharose was detected with ^{125} I-anti-IgE. Binding percentages were converted into units of IgE bound, and plotted against the log of the concentration of *Fel d I* added. For each serum concentration the Ag_{50} was determined by interpolation.

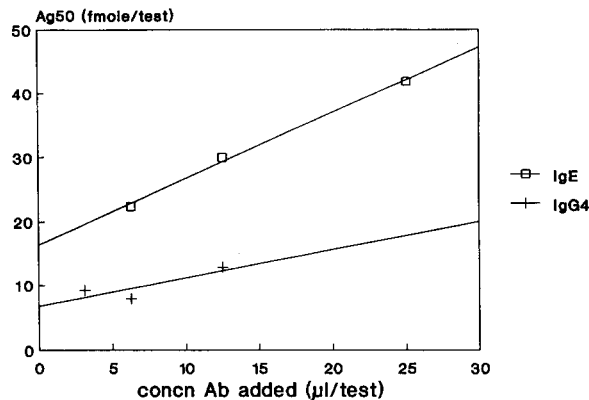


Fig. 8. Calculation of K_{SP} of patient sera, using the semi-saturation plot. The Ag_{50} values found in Fig. 5A and 5B were plotted against the concentration of serum added. The concentration of Ag, required for binding half of the Ab added, when an infinitely small concentration of serum was added, was defined by the y axis intercept.

and 220 (pmol/test) $^{-1}$, without and with additional rabbit antibodies, respectively.

The relative affinities of mAb Fd1a and Fd1b for solid-phase *Fel d I* were also determined in inhibition experiments (Fig. 6).

For one cat-allergic patient, K_{SP} of IgE and IgG4 for *Fel d I* were calculated, using the semi-saturation plot. Fig. 7 shows the IgE binding curves for three concentrations of serum and in Fig. 8 the Ag_{50} values of IgE and IgG4 are plotted against the concentration of serum added.

Discussion

In this report a procedure is described to calculate the affinity constant K_{SP} , using solid-phase Ag. Measuring K_{SP} of serum IgE for solid-phase Ag in a RAST, isotypes other than the isotype measured (i.e., IgE) also bound to solid-phase Ag ('invisible' antibody). Moreover, the Ag and Ab concentrations did not behave according to the classical equation, and thus K_{SP} was dependent on the test volume used. A procedure was developed to measure the K_{SP} of an isotype, independent of invisible antibody. K_{SP} was expressed as (mol/test) $^{-1}$, making it independent of the test

volume used. K_{SP} was defined by the concentration of Ag required for binding half of the Ab added when an infinitely small concentration of Ab was added. As a model system mAbs against the cat allergen *Fel d* I were used. *Fel d* I was coupled to Sepharose at low Ag concentration (50 μ g per 100 mg of Sepharose), favoring monovalent binding which is more affinity-dependent (Griswold, 1987; Holland and Steward, 1991; Werthen and Nygren, 1988; Lew, 1984; Nimmo et al., 1984). Serial dilutions of Ag-Sepharose were prepared and incubated with mAb. IgG bound to the Sepharose was detected with ^{125} I-rat anti-mouse IgG and binding results were converted into fmol IgG bound per test, using *Fel d* I-Sepharose (0.25 mg/test) with mAb Fd1a to generate the standard curve.

First, the Ab-binding capacity of Sepharose-coupled *Fel d* I was determined. The Ab-binding capacities were 4.15% and 2.13% for mAb Fd1a and Fd1b, respectively. This poor availability of Sepharose-coupled *Fel d* I can be caused by destruction of the epitopes during the coupling procedure or by the solid surface sterically hindering some of the epitopes from reacting with the antibody. The difference in Ab-binding capacity for mAb Fd1a and Fd1b could be caused by bivalent binding of mAb Fd1a to the Sepharose or by selective destruction of the epitope recognized by mAb Fd1b. Moreover in ELISA experiments account has to be taken of the poor availability of Ag coated to microtiter plates.

Using solid-phase Ag, Ag activity did not vary directly with the test volume (Fig. 3). Apparently the solution was not homogeneous and there was a concentration of Ab near the solid-phase Ag.

K_{SP} of mAb Fd1a and mAb Fd1b, using the semi-saturation plot, were 85 (pmol/test) $^{-1}$ and 65 (pmol/test) $^{-1}$, respectively. To validate these K_{SP} values, for both mAbs K_{SP} was calculated from the concentrations of Ag and Ab added and the concentration of Ab bound, using the equation $K = B/(Ab - B)(Ag - B)$. The K_{SP} values found by the two procedures fitted reasonably well, confirming the correctness of the semi-saturation plot. Also for radiolabelled mAb Fd1a, K_{SP} was calculated using the semi-saturation plot, yielding an affinity constant of 100 (pmol/test) $^{-1}$ (Fig. 5C). The K_{SP} values of mAb Fd1a found in

three experiments were 85, 100 and 176 (pmol/test) $^{-1}$, showing some variation between the experiments. The K_{SP} value of mAb Fd1a, determined by the semi-saturation plot, was not affected by the presence of and overload of 'invisible' rabbit antibodies, confirming the applicability of this procedure for measuring the K_{SP} of IgE antibodies in a RAST.

For one cat-allergic patient, the affinities of IgE and IgG4 for *Fel d* I were calculated using the semi-saturation plot. The K_{SP} values found were 62 (pmol/test) $^{-1}$ and 147 (pmol/test) $^{-1}$ for IgE and IgG4 respectively.

The affinity constants determined for mAb Fd1a and Fd1b, were compared with the relative affinities of the mAbs determined in inhibition experiments with *Fel d* I-Sepharose. The concentrations of free Ag, required for 50% inhibition of Ab-binding to solid-phase Ag were 380 fmol and 1980 fmol for mAb Fd1a and Fd1b respectively, again confirming the higher affinity for mAb Fd1a (Fig. 6). However, the affinity of an Ab for solid-phase Ag can differ from the affinity for the same Ag in the fluid phase and this can give rise to problems in inhibition studies. For example, mAb Fd1d, raised against denatured *Fel d* I, strongly bound to solid-phase Ag in a RIA and in an ELISA, but this binding was only weakly inhibitable by fluid-phase Ag. Moreover using Scatchard analysis with radiolabelled Ag, maximum binding of the label was low, probably by removing the Ag during the washing procedure. These observations point to the high affinity of this mAb for solid-phase Ag but a low affinity for fluid-phase Ag (data not shown).

We conclude that the semi-saturation plot is an acceptable procedure for calculating the K_{SP} of an Ab for solid-phase Ag, even in the presence of 'invisible' antibody. However, the Ab-binding capacity of the Sepharose may present a problem. With a low Ab-binding capacity the maximum binding of the Ab has to be estimated by an extrapolation procedure. Alternatively, Ag_{50} can be measured by determining the concentration of Ag required for binding half of the Ab and measuring the unbound Ab in the supernatant after the first incubation step. This procedure can also be used when the ELISA detection system is used.

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