

Estimation of Affinity and Quantity of Human Antigen-Specific Serum IgG (Blocking Antibodies)

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An assay for estimation of quantity and quality of antigen-specific IgG against two purified antigens from cow hair and dander is described. The substance concentration of antigen-specific IgG was more than 10 times higher in serum from immunized patients than in serum from non-immunized patients. Almost the same K_A values of 5×10^9 l/mol were calculated for IgG in sera from immunized and non-immunized patients with allergic asthma to cow hair and dander.

Key words: blocking antibodies; double antibody assay; cow allergens.

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CLINICAL ASPECTS

The relief from symptoms following desensitization is partially correlated to an increase in antigen-specific IgG. This effect is dependent on antibody affinity and number of antibody combining sites. It is therefore important to be able to estimate both of these characteristics when one wants to study blocking antibodies.

Immunotherapy with antigen extracts is a well documented way to treat patients with allergic asthma and rhinitis. One of the immunological changes following this therapy is an increase in antigen-specific IgG antibodies in serum – the so-called “blocking antibodies”. This is the only immunological change to date to show some, albeit rough, correlation with symptom scores (5). The results from investigations dealing with the clinical importance of blocking antibodies are conflicting. A reason for this may be the diversity of the methods used so far. Some have mainly characterized the quality of blocking antibodies (inhibition of histamine release) (4), others the quantity (double antibody assay) (6, 12) and only a single study has considered both quantity and quality (1).

The purpose of the present work is to describe an assay for quantity and affinity of antigen-specific IgG. The double-antibody radioimmunoassay developed has been used on sera from healthy individuals with and without

exposure to cattle, sera from patients with cow dander-induced asthma and sera from patients with eczema after contact with cattle. All groups except normal controls contained measurable amounts of serum antigen-specific IgG against two major allergens of extract from cow hair and dander. The affinities were alike, whereas the quantity was 10 times higher in desensitized patients as compared to untreated patients.

MATERIAL AND METHODS

The allergens

Two major allergens (Ag 1 and Ag 3) from cow hair and dander identified and purified according to Prahl et al. were used (8, 9). The activity of the antigens was ensured by RAST and prick test. Judged from quantitative immunoelectrophoresis and from polyacrylamide gel electrophoresis, the antigens contained less

than 1% of impurities. The same antigen batch was used for all experiments. The substance concentration of the antigens used for iodination was determined by quantitation of protein content from the optical density at 215 and 225 nm (13) on a Unicam SP 1800-spectrophotometer and the relative molecular mass of the antigens (Ag 1 = 24500, Ag 3 = 21000) (9).

Rabbit antibodies

The second antibody for the double-antibody assay was rabbit anti-human IgG. The antibody specificity of anti-IgG was deduced from the identical results obtained for four human sera tested directly or after removal of IgE by Sepharose coupled anti-IgE. Identical results were also obtained in the double-antibody assay when the four sera were tested directly or after precipitation with rabbit anti-human IgA and IgM. All rabbit antibodies were obtained from Dako (Dako, Copenhagen, Denmark).

Preparation of radiolabelled antigens

Ag 1 and Ag 3 were labelled with sodium ^{125}I by the chloramine-T technique according to Hunter (3). Briefly, 0.2×10^{-9} mol purified antigen were oxidized by 10×10^{-9} mol chloramine-T in the presence of 1 mCi sodium ^{125}I in a total volume of 120 μl . After 40 s of agitation 52×10^{-9} mol of sodium metabisulphite in 20 μl was added and 15 s later the reaction mixture was diluted with 110 μl 0.1 mol/l sodium phosphate buffer, pH 7.5, containing 14.5×10^{-6} mol/l serum albumin as carrier protein and potassium iodine 0.03 mol/l. Non-protein bound iodine was removed by gel-filtration on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden). Bed-volume was 50 ml and flow rate 40 ml/h. The elution was performed with a phosphate buffer (pH 7.5, containing human serum albumin 14.5×10^{-6} mol/l and sodium chloride 0.1 mol/l).

Fractions of 600 μl were collected. Pooling of the iodinated protein was based on precipitability by a serum sample (G.E.) containing significant amounts of IgG against antigens of cow hair and dander. The pooled ^{125}I -labelled anti-

gens were 95–100% precipitable by trichloroacetic acid 0.6 mol/l. Specific activities achieved were about 2×10^{18} cpm/mol for Ag 1 and about 1.5×10^{18} cpm/mol for Ag 3.

Radioimmuno-precipitation technique

The assay used was a double antibody assay. The procedure was as follows: 100 μl of serum diluted between 1 + 15 and 1 + 119 (depending on the amount of antigen-specific IgG) in test buffer (sodium borate 55 mmol/l, sodium chloride 100 mmol/l, human serum albumin 14.5×10^{-6} mol/l, sodium azide 0.015 mol/l, pH 8.0) and 100 μl ^{25}I -labelled antigen were allowed to react for 3 h at room temperature, followed by incubation overnight at 4°C with 100 μl rabbit anti-human IgG (Fc-specific, Dako, Copenhagen, Denmark) diluted 1 + 1 with test buffer. To remove non-bound activity the precipitates were washed four times with 2.5 ml test buffer. Centrifugation was performed for 15 min at 3000 rpm. The bound activity was measured in a gammacounter. Non-specific binding in the assay was evaluated by incubating the sera in excess of unlabelled antigen. Serum dilutions above 1 + 15 were performed in test buffer containing normal serum diluted 1 + 15. The optimal amount of anti-IgG for precipitation was ensured by examining the supernatants from a patient with a high total IgG level and from a 1000 donor pool according to Sewel (11). The non-specific binding was subtracted from all assay results and the net cpm used for calculations. The assay was performed in duplicate.

Binding analysis

Sera were studied by analysis of their ability to bind from 0.36 to 170×10^{-9} mol/l of antigen. In most assays the range of bound to total antigen added varied from 5–70%. The average equilibrium constant K_A was calculated by Scatchard analysis:

$$\frac{B}{F} = K_A (A_{\text{t}} - B) \quad (10)$$

where B and F are the molar concentration of the bound (B) and free (F) antigen and A_{t} is the

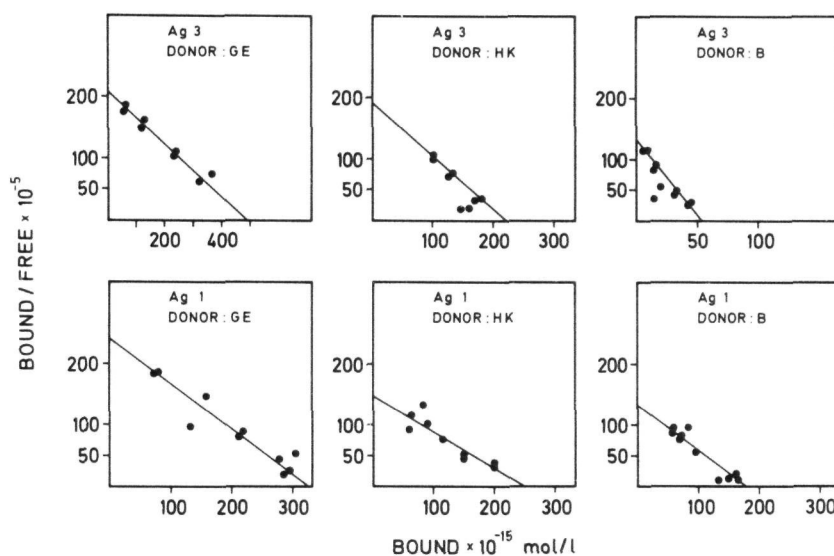


Fig. 1. Scatchard analysis of antigen-binding data for two allergen systems. Binding curves for three sera with two purified major allergens from an extract from cow hair and dander (Ag 1 and Ag 3) are shown.

Donors: G.E.: Patient with allergic asthma from cow, immunized with cow epithelial allergens for 6 years.

H.K.: Non-immunized patient with allergic asthma from cow.

B.: Non-immunized patient with eczema due to contact with cow pelt.

The average equilibrium constant (K_A) calculated from the lines as well as the serum substance concentration of antibody combining sites are indicated in Table 1.

molar concentration of binding sites on the antibody molecules at the dilution of antiserum employed. A graph of B/F against B represents a line in which K_A is determined by the slope and Ab_t by the intercept on the x-axis (Fig. 1).

Total serum IgG was quantitated by quantitative immunoelectrophoresis according to (14).

Total IgE and allergen-specific IgE were measured by PRIST (2) and RAST (15) assays, respectively.

Donors of sera

The following groups of subjects were investigated. The diagnosis was based upon anamnesis, bronchial provocation test, RAST and prick test (16).

Patients with allergy to cow ($n = 26$):

Immunized patients ($n = 6$): Four had been immunized with only extract from cow hair and dander (three for 2 years – M.K., L.T. and L.H.H. and one for 6 years – G.E.). One (H.G.H.) had been immunized for 2 years with an epithelial extract consisting of equal amounts of cow, horse and rabbit. One (A.M.P.) had been

immunized for 2 years with equal amounts of extracts from cow, horse and swine. All donors had been given a maintenance dose for more than 1 year. *Non-immunized patients* ($n = 11$). *Veterinary surgeons* ($n = 9$) with contact eczema due to contact with cow pelt and cow amniotic fluid.

Controls ($n = 8$):

Normal donors ($n = 4$) without exposure to cattle and a 1000 donor pool. *Normal donors* ($n = 4$) with daily exposure to cattle for more than 10 years.

RESULTS

Methodological evaluation

The antibody-reaction reached equilibrium within 3 h for both antigens. No increase in non-specific binding was seen during this time interval.

The coefficient of variation for the results was 20% as estimated from eight sera tested

Table 1
Serum antigen Ag 1- and Ag 3-specific IgG antibodies – equilibrium constant and substance concentration

Antigen	Immunized (asthma)			Non-immunized (asthma)			Non-immunized (contact eczema)		
	Patient	K _A 10 ⁹ l/mol	Binding sites 10 ⁻⁹ mol/l	Patient	K _A 10 ⁹ l/mol	Binding sites 10 ⁻⁹ mol/l	Patient	K _A 10 ⁹ l/mol	Binding sites 10 ⁻⁹ mol/l
Ag 1	G.E.	7.2	40.0	K.B.H.	1.8	0.6	F.N.	4.7	3.2
	M.K.	3.4	0.3	H.K.	6.1	11.7	A	6.6	2.5
	L.T.	4.5	75.0	M.P.	8.4	10.2	B	4.8	8.2
	L.H.H.	4.8	43.0	K.L.	3.8	2.0	C	3.3	1.0
	A.M.P.	13.0	0.1	K.M.N.	6.8	0.1	D	2.4	1.0
	A.G.H.	3.4	3.4	H.E.J.	4.7	1.0	E	3.3	0.5
				B.A.	1.3	0.8	G	**	**
				Sv.T.	*	*	I	1.4	0.5
				M.D.	**	**	J	1.4	0.6
				S.K.	*	*			
				O.D.	*	*			
	Median	4.7	21.7		4.7***	0.6***		3.3***	1.0***
Ag 3	G.E.	3.0	55.2	K.B.H.	2.7	2.6	F.N.	8.5	13.0
	M.K.	5.2	29.4	H.K.	9.4	10.8	A	5.2	8.2
	L.T.	7.5	15.9	M.P.	8.3	8.2	B	24.0	2.6
	L.H.H.	1.6	36.1	K.L.	*	*	C	13.0	20.0
	A.M.P.	12.5	21.4	K.M.N.	**	**	D	13.2	9.0
	A.G.H.	5.2	11.2	H.E.J.	4.5	4.6	E	**	**
				B.A.	**	**	G	6.6	4.0
				Sv.T.	*	*	I	8.7	5.7
				M.D.	*	*	J	13.0	4.2
				S.K.	*	*			
				O.D.	2.5	2.5			
	Median	5.2	25.9		4.5***	*		9.7***	8.2***

* Detectable specific binding, but too low for quantification.
** Non-detectable binding.
*** Of the values quantificated.

2–3 times, whereas the mean coefficient of variation within experiments was 5 % (*n* = 48). The immunological reactivity of the labelled antigens was the same as that of unlabelled antigens as determined by dilution curves.

Non-specific binding was about 0.5 % of the total activity added.

Binding analysis

Quality of allergen-specific IgG. The Scatchard plots obtained for Ag 1 and Ag 3 from one of each patient category are shown in Fig. 1. The data for each experiment fit a straight line, thus suggesting antibody homogeneity. Almost the same *K_A* values of approximately 5 × 10⁹ l/mol were shown for Ag 1 in sera from all three

patient categories. Similar results were obtained for Ag 3 in sera from asthmatic patients. However some of the patients with eczema proved to have IgG antibodies with an affinity constant of more than 10 × 10⁹ l/mol against Ag 3.

The normal controls with and without contact with cattle contained no measurable amounts of Ag 1- or Ag 3-specific IgG except for one (F.C.), a 35-year-old man, who had worked with cattle for 15 years without showing any symptom of allergy to cow. *K_A* value for Ag 3 was 1.6 × 10⁹ l/mol but not measurable for Ag 1.

The number of antibody combining sites

Eight of nine sera from patients with eczema from contact with cow contained antigen-spe-

cific IgG that could be quantitated, whereas this was only the case in eight of 11 sera from non-immunized patients with allergic asthma.

Serum antibody substance concentrations estimated from the Scatchard plots are presented in Table 1. Immunized patients showed by far the greatest substance concentration of cow antigen-specific IgG, which was about 10 times as much as that in non-immunized patients. Immunization increased the substance concentration of Ag 3-specific IgG antibodies in all patients to approximately the same level, whereas the substance concentration of Ag 1-specific IgG varied from 0.1 to 75×10^{-9} mol/l.

Serum from four donors who had worked with cattle for more than 10 years contained some IgG against Ag 3 and one of them (F.C.) against Ag 1 too, but not enough to quantitate exactly. Normal controls without contact with cattle, and a 1000 donor pool, contained no Ag 1- or Ag 3-specific IgG. All sera contained a normal amount of IgG. In all sera specific IgG (mol/l) against Ag 1 and Ag 3 made up less than 0.05% of total IgG (mol/l).

DISCUSSION

We have used a double-antibody radioimmunoassay combined with binding analysis to calculate the average equilibrium constant and the number of antibody-combining sites from the plot of the Scatchard equation. The antigens were two purified major allergens from an extract of cow hair and dander. The sera were from cow-allergic human donors and from normal controls with and without contact with cattle. The average equilibrium constant did not vary markedly (1 to 10×10^9 l/mol). Only in one patient did the immunization with extract from cow hair and dander cause the formation of IgG antibodies with K_A values greater than among non-immunized patients. Nine patients, who developed an itching eczema with urticaria within half an hour after contact with cow were examined. Four of these had Ag 3-specific IgG with K_A values from 13 to $24 \times 10_9$ l/mol.

The substance concentration of the specific-IgG varied from 0.5 to 75×10^{-9} mol/l. Immu-

nization with extract from cow hair and dander seemed to enhance the formation of specific IgG antibodies more than it did the equilibrium constant.

Our results are in agreement with those of Adkinson et al. who examined the affinity and number of binding sites of human serum IgG against honeybee phospholipase A, ragweed antigen Ra 5 and benzylpenicilloyl (1). They also found moderate variation of affinity constants, which ranged from 3 to 30×10^9 in sera from donors with allergic symptoms due to the allergens mentioned.

Considering the positive correlation between the amount of antigen-specific IgG and the relief from symptoms following immunotherapy it is important to be able to measure these antibodies in an exact and reproducible way. Until recently antigen-specific IgG has been quantitated by its ability to inhibit antigen-induced histamine release from sensitized basophil leucocytes. As this is a laborious and costly method the simple assay presented in this study is advantageous. Using the double-antibody radioimmunoassay it is possible to give an exact estimation of specific IgG substance concentration and of the affinity constant for equilibrium of these antibodies. We believe that this assay combined with a similar assay for IgE (7) will be valuable tools for the evaluation of different hyposensitization schemes.

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