

Immunogenic Properties of Two Types of Sea-Squirt Antigens¹

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Received for publication, October 5, 1981

Two antisera were prepared in rabbits against substantially purified preparations of sea-squirt antigens, Gi-rep and Ei-M, which were apparently discriminated from each other in antigenic behavior in radioimmunoassay experiments. Radioimmuno-metric analysis of the interaction between the two anti-sea-squirt sera and the two antigens revealed that Gi-rep induced in rabbit only one type of antibody (type *A*) which reacted with Gi-rep and Ei-M in common, while Ei-M induced not only type *A* antibody having the common reactivity but also another antibody (type *B*) which was strictly specific to Ei-M. This observation supported the preceding suggestion that Gi-rep and Ei-M carried a common antigenic determinant (type α) but that Ei-M had an additional determinant (type β) specific to Ei-M. In this connection, in view of the similarity of type *A* antibody in specificity to the blocking antibody induced in asthmatic patients with sea-squirt allergy on hyposensitization therapy with these antigen preparations, it was suggested that the presumed allergenic substance in sea-squirt also carries the type α determinant.

Previously, two antigen preparations, G-2 and E-2, were separated from sea-squirt, *Styela plicata*, by gel chromatography as the fractions active to skin tests on asthmatic patients with sea-squirt allergy (1, 2). Subsequently, G-2 and E-2 were both partially purified by treatment with an anion exchanger, QAE-Sephadex A-25, giving the preparations Gi-2 and Ei-2, respectively (3, 4). In spite of their high specificity to asthmatic patients as

examined by skin tests, the preparations did not stimulate asthmatic attack. Thus, they were considered to be antigens, but not allergens, specific to sea-squirt allergy (2-4).

On the other hand, those antigen preparations were all very effective in hyposensitization therapy of the asthmatic patients. In the therapy, it was also recognized that any one of the preparations could induce a certain blocking antibody which competed with the reaginic antibody, specific IgE, for the presumed allergen, resulting in a very high efficiency of the therapy (2-4). The occurrence of such a blocking antibody was also supported by

¹ This work was supported in part by a Grant-in-Aid for Scientific Research (No. 56430030) from the Ministry of Education, Science and Culture of Japan.

the results of subsequent radioimmunometric analyses of the sera of the treated patients (5-7).

Recently, by introducing radioimmunoassay (RIA) as a method for monitoring the antigenic activity, two antigen preparations, Gi-rep and Ei-M, were newly isolated from sea-squirt as the principal antigenic components in the previous preparations, Gi-2 and Ei-2, respectively (8, 9). Gel chromatographically, both of them were fairly homogeneous and the weight-average molecular weights were estimated to be 1.1×10^5 for Gi-rep and 2.3×10^4 for Ei-M by sedimentation analysis. Both preparations were shown to be acidic glycoproteins which contained considerable amounts of sulfate and phosphate.

Antigenically, Gi-rep and Ei-M both were highly specific to the asthmatic patients as examined *in vivo* by skin tests. However, they were apparently different from each other in the mode of immunological reaction against a rabbit anti-sea-squirt serum, anti-Gi-2 (238), when examined *in vitro* by radioimmunity. The *in vitro* observation also suggested that anti-Gi-2 serum contained at least two types of antibodies, *i.e.* one (type *A*) that reacted with Gi-rep and Ei-M in common, and another (type *B*) that was strictly specific to Ei-M (8, 9).

The rabbit antibody of type *A* having the reactivity against Gi-rep and Ei-M in common appeared to be similar in specificity to the blocking antibody induced in the patients by the hyposensitization therapy. However, the reason for such multispecificity of the rabbit antiserum and the sera of the treated patients remained to be elucidated, because there could be Ei-M type antigen contamination in the preparation of Gi-2 which was employed in immunizing the rabbit and in treating the patients (8, 9). Therefore, in the present study, two rabbit antisera were prepared against the substantially purified antigen preparations, Gi-rep and Ei-M. Then, the interaction between the antisera and the antigens was analyzed *in vitro* by radioimmunometric method to characterize the sea-squirt antigens in regard to immunogenic as well as antigenic properties.

MATERIALS AND METHODS

Sea-Squirt Antigens—Substantially purified antigen preparations termed Ei-M (8) and Gi-rep

(9) were employed in the present study.

Anti-Sea-Squirt Sera—Two antisera, anti-Gi-rep (L 304), and anti-Ei-M (L 305), were prepared by immunizing rabbits with the corresponding antigens with the aid of Freund's complete adjuvant according to the procedure used in the previous studies (4, 8). Anti-Gi-2 (238) serum used in the previous studies (4, 8, 9) was also employed as a reference.

Radiolabeled Antigens—Each (10 μ g) of Gi-rep and Ei-M was iodinated with 1 mCi of ^{125} I to prepare the corresponding radioactive tracer according to the procedure used before (8, 9). The specific radioactivities of labeled Gi-rep and Ei-M were 19.8 and 25.2 μ Ci/ μ g, respectively.

Since the radiolabeled antigens were inevitably contaminated with some radioactive iodide which was liberated spontaneously from the labeled substance (10), the fraction of net radioactivity emitted from the radiolabeled antigen (*a*) was determined by equilibrium dialysis at a time synchronized with the subsequent radioimmunometric experiments. The dialysis was carried out against the dilution buffer for the labeled antigen in a dialysis cell, model 260, Technilab Instruments, Pequannok, New Jersey, equipped with two 1 ml-chambers separated by a sheet of cellophane. The values of *a* were 0.983 for labeled Gi-rep and 0.978 for labeled Ei-M at the time of the present experiments.

Buffer Solutions—A phosphate buffer (0.05 M, pH 7.6) containing 1% (w/v) of NaCl and 0.025% (w/v) of $\text{Na}_2\text{S}_2\text{O}_3$ was used for diluting the unlabeled antigen samples. The buffered saline was supplemented with 0.1% (w/v) crystallized ovalbumin for diluting the radiolabeled antigens and with 5% (v/v) of bovine serum for diluting the antisera.

Radioimmunity—The procedure of radioimmunity was essentially the same as that described previously (4, 8, 9) except that the amounts of antiserum and bovine serum as a carrier were reduced to one-half of the amounts used in the previous radioimmunoassay (RIA).

A 50 μ l portion of a diluted antiserum (1 : 200) containing 5% (v/v) of bovine serum was reacted with 100 μ l of a diluted radiolabeled antigen (*ca.* 30,000 cpm) and 50 μ l of an unlabeled antigen of known concentration (0.06-500 μ g/ml) at room temperature. After 16 h incubation, the reaction mixture was salted out by addition of

400 μ l of 20% Na_2SO_4 , followed by centrifugation. Then, the precipitate was counted for radioactivity (CB_{obs}) after aspirating the supernatant off.

Since 3.6% of the supernatant, on average, is usually retained in the precipitate, the net radioactivity of the antibody-bound labeled antigen (CB) is given by Eq. 1,

$$CB = CB_{obs} - 0.036(CT - CB_{obs}) \quad (1)$$

where CT is the total radioactivity in the reaction mixture. On the other hand, the total effective radioactivity of the radiolabeled antigen is given as the product of CT and the value of α determined by equilibrium dialysis. Thus, the net radioactivity of the free radioactive antigen (CF) is given by Eq. 2,

$$CF = \alpha CT - CB \quad (2)$$

After the above-mentioned calculation, the ratio of CB/CF was employed as an index representing the equilibrium between the antibody-bound labeled antigen and the free one.

RESULTS

Interaction between the Antisera and the Antigens—The immunological reactivities of anti-Gi-rep, anti-Ei-M, and anti-Gi-2 against Gi-rep and Ei-M were examined. Each of the three antisera was reacted with a minute amount (*ca.* 30,000 cpm) of radiolabeled Gi-rep or Ei-M in the presence of varying amounts of the corresponding unlabeled antigen. Then, CB/CF , the ratio of antibody-bound labeled antigen to free one in radioactivity, was determined and plotted against the concentration of the unlabeled antigen on a logarithmic scale.

In Fig. 1, the value of CB/CF significantly decreases with increase in the concentration of unlabeled antigen in every combination of the antisera and the antigens. This indicates that all three antisera are reactive to both Gi-rep and Ei-M.

On the assumption that a radiolabeled antigen is essentially the same as the corresponding unlabeled antigen in reacting behavior against a given antiserum, the value of CB/CF is defined by Eq. 3,

$$CB/CF = b^*/f^* = (B + b^*)/(F + f^*) \quad (3)$$

where B and F are the concentrations of antibody-bound unlabeled antigen and free one, respectively,

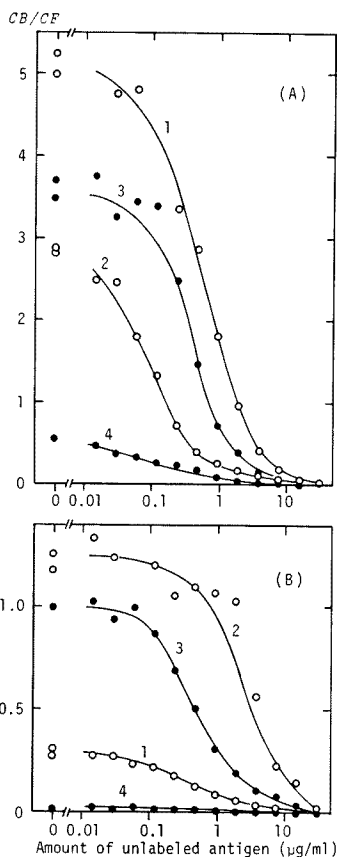


Fig. 1. Inhibition of antibody-binding of radiolabeled antigen by the corresponding unlabeled antigen. A: Experiments with radiolabeled Gi-rep and unlabeled Gi-rep. B: experiments with radiolabeled Ei-M and unlabeled Ei-M. Curves 1, 2, and 3 are the experiments with anti-Gi-rep, anti-Ei-M, and anti-Gi-2, respectively. Curve 4 is the blank experiment without antiserum but with bovine serum as a carrier.

and b^* and f^* are the concentrations of antibody-bound radiolabeled antigen and free one, respectively.

If the interaction between the antigen and the antibody obeys the law of mass action, the equilibrium constant (K) as an index of affinity between antibody and antigen is expressed by Eq. 4,

$$K = (B + b^*)/(F + f^*)(B_m - B - b^*) \quad (4)$$

where B_m is the maximum concentration of the antigen which can be bound by the antibody in the reaction mixture.

In the control experiment without unlabeled antigen but with labeled antigen alone ($B=0$ and $F=0$), the value of K is given by Eq. 5,

$$K = (b_0^*/f_0^*)/(B_m - b_0^*) \quad (5)$$

and

$$K(B_m - b_0^*) = b_0^*/f_0^* = CB_0/CF_0 \simeq KB_m \quad (6)$$

where the subscript '0' indicates that those values are expected or observed in the control experiment.

In Eq. 6, if the concentration of radiolabeled antigen was reduced and b_0^* approached zero, the value of CB_0/CF_0 would approach a constant value, KB_m . In Fig. 1, it appears that the CB_0/CF_0 values observed in all of the control experiments have practically reached the KB_m values. Thus, the value of KB_m is considered to be approximated by the corresponding CB_0/CF_0 value observed in the control experiment.

On the other hand, when the total concentration of antibody-bound unlabeled antigen and labeled one becomes equal to 50% of B_m , ($B + b^* = 0.5 B_m$), in the presence of a certain concentration of unlabeled antigen ($T_{0.5}$), the equilibrium constant, K , is expressed by Eq. 7,

$$K = (B_{0.5} + b_{0.5}^*)/(F_{0.5} + f_{0.5}^*)/(B_m - 0.5 B_m) = 1/(F_{0.5} + f_{0.5}^*) \quad (7)$$

or from Eq. 3,

$$K = (CB_{0.5}/CF_{0.5})/0.5 B_m \quad (8)$$

and from Eqs. 8 and 6,

$$CB_{0.5}/CF_{0.5} = 0.5 K B_m \simeq 0.5 (CB_0/CF_0) \quad (9)$$

where the subscript '0.5' indicates that those values are expected at the unlabeled antigen concentration of $T_{0.5}$. Equation 9 indicates that the value of $T_{0.5}$ can be estimated as the concentration of unlabeled antigen which is required to bring the observed CB/CF value to 50% of CB_0/CF_0 .

Then, the value of K is calculated again using the values of $T_{0.5}$, CB_0/CF_0 and the total concentration of radiolabeled antigen (t^*) as follows,

$$\begin{aligned} \frac{(T_{0.5} + t^*)}{(F_{0.5} + f_{0.5}^*)} &= \frac{(F_{0.5} + f_{0.5}^*)}{(F_{0.5} + f_{0.5}^*)} + \frac{(B_{0.5} + b_{0.5}^*)}{(F_{0.5} + f_{0.5}^*)} \\ &= 1 + CB_{0.5}/CF_{0.5} \\ &\simeq 1 + 0.5 (CB_0/CF_0) \end{aligned} \quad (10)$$

from Eqs. 7 and 10,

$$\begin{aligned} K &= [1 + CB_{0.5}/CF_{0.5}]/(T_{0.5} + t^*) \\ &\simeq [1 + 0.5 (CB_0/CF_0)]/(T_{0.5} + t^*) \end{aligned} \quad (11)$$

and from Eq. 9,

$$B_m = 2 (CB_{0.5}/CF_{0.5})/K \simeq (CB_0/CF_0)/K \quad (12)$$

The values of $T_{0.5}$ were estimated from the inhibition curves in Fig. 1 for the combinations of antisera and antigens. The estimated t^* values were 0.0049 and 0.0038 $\mu\text{g/ml}$ for 30,000 cpm of radiolabeled Gi-rep and Ei-M, respectively, as calculated from their specific radioactivities. Then, the values of K and B_m were calculated by means of Eqs. 11 and 12, respectively, as summarized in Table I.

TABLE I. Interaction between antisera and antigens. K is an index of affinity of an antibody for a given antigen. B_m is the maximum concentration of the antigen which can be bound by the antibody in the reaction mixture which contains 1.25 $\mu\text{l/ml}$ of the corresponding antiserum. See the text for the procedure for calculation of these values.

Antiserum	Antigen	$\frac{CB_0}{CF_0}$	$T_{0.5}$ ($\mu\text{g/ml}$)	K ($\text{ml}/\mu\text{g}$)	B_m ($\mu\text{g/ml}$)
Anti-Gi-rep (1 : 800)	Gi-rep	5.13	0.540	6.54	0.784
	Ei-M	0.29	0.395	2.87	0.101
Anti-Ei-M (1 : 800)	Gi-rep	2.86	0.097	23.8	0.119
	Ei-M	1.21	2.68	0.598	2.02
Anti-Gi-2 (1 : 800)	Gi-rep	3.60	0.394	7.01	0.512
	Ei-M	1.04	0.422	3.56	0.291
Bovine serum (1 : 80)	Gi-rep	0.56	0.116	10.5	0.052
	Ei-M	—	—	—	—

It is of interest that the B_m values of the antisera against Gi-rep were in the order anti-Gi-rep > anti-Gi-2 > anti-Ei-M, while, in contrast, against Ei-M the order was anti-Ei-M > anti-Gi-2 > anti-Gi-rep. Although bovine serum used as a carrier also reacted with Gi-rep to some extent, no correction was obtained for the data in Fig. 1A, because the B_m value of this non-specific reaction was very low.

Antibody Composition in the Antisera—The apparent common reactivities of the three antisera against Gi-rep and Ei-M were further analyzed. In this case, the reaction between a minute amount of a radiolabeled antigen and each of the antisera was inhibited by various concentrations of another unlabeled antigen and the values of CB/CF were determined.

In Fig. 2A, the reactions of the three antisera against labeled Gi-rep are all significantly inhibited by unlabeled Ei-M, indicating that the Gi-rep-reactive antibodies in the antisera also react with Ei-M. In this case, when compared with Fig. 1A, the corresponding inhibition curves are significantly shifted to the right; the values of $T_{0.5}$ are 6 to 10 times higher than those in Fig. 1A. Such a shift of the inhibition curves is ascribable to the lower affinity of Ei-M for the antisera (K , in Table I) as compared with that of Gi-rep.

In Fig. 2B, curve 1 shows that the reaction of anti-Gi-rep with labeled Ei-M is also completely inhibited by unlabeled Gi-rep, giving a $T_{0.5}$ value of only one-third of that given by Ei-M of lower affinity. Thus, the Ei-M-reactive antibody in anti-Gi-rep is considered to be identical with the Gi-rep-reactive antibody observed in the same antiserum (curve 1 in Fig. 2A). The antibody which reacts with Gi-rep and Ei-M in common is termed *type A*.

On the other hand, curves 2 and 3 in Fig. 2B show that the reactions of anti-Ei-M and anti-Gi-2 with labeled Ei-M were hardly inhibited by unlabeled Gi-rep. The resistance of their reactions to inhibition by Gi-rep indicates that the major portion of the Ei-M-reactive antibody in these antisera is strictly specific to Ei-M and does not react with Gi-rep. Thus, the Ei-M specific antibody is termed *type B* to discriminate it from *type A*.

However, curves 2 and 3 in Fig. 2A still show that anti-Ei-M and anti-Gi-2 both contain *type A*

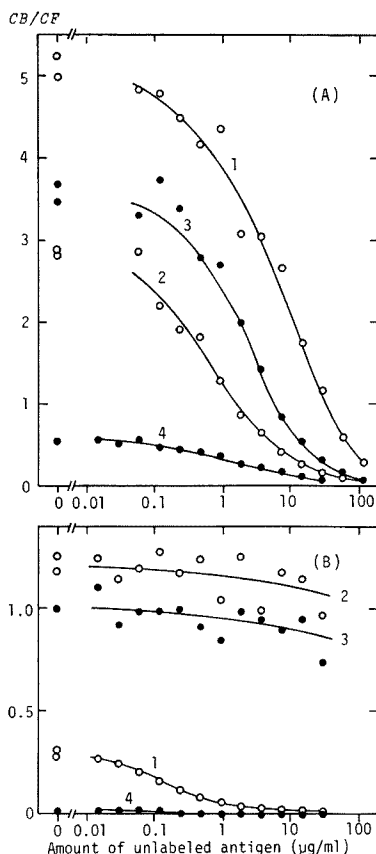


Fig. 2. Inhibition of antibody-binding of a radiolabeled antigen by the other unlabeled antigen. A: Experiments with radiolabeled Gi-rep and unlabeled Ei-M. B: experiments with radiolabeled Ei-M and unlabeled Gi-rep. Curves 1, 2, and 3 are the experiments with anti-Gi-rep, anti-Ei-M, and anti-Gi-2, respectively. Curve 4 is the blank experiment without antiserum but with bovine serum as a carrier.

antibody. In addition, relatively small portions of their reactions with labeled Ei-M appear to be inhibited by a high concentration of Gi-rep (curves 2 and 3 in Fig. 2B), suggesting that both antisera contain not only *type B* antibody but also some *type A* antibody.

On the assumption that, of the values of CB_0/CF_0 ($\approx KB_m$, Eq. 6) for the reactions of anti-Ei-M and anti-Gi-2 with labeled Ei-M, the portion due to *type A* antibody does not exceed

the value for anti-Gi-rep against the same labeled antigen (0.29), the CB_0/CF_0 values observed for anti-Ei-M (1.21) and for anti-Gi-2 (1.04) are large (Figs. 1B and 2B) and, thus, may be considered to depend mainly on the reaction of type *B* antibody. Under such conditions, even when most of the reaction of type *A* antibody was inhibited, the relative decrement of the CB_0/CF_0 value would be very small. This may be the case here.

Accordingly, it is considered that anti-Gi-rep contains only type *A* antibody, while anti-Ei-M and anti-Gi-2 contain the other antibody of type *B* in addition to a relatively small contribution of type *A* to the CB_0/CF_0 value in binding labeled Ei-M.

DISCUSSION

In the present study, two antisera, anti-Gi-rep (L 304) and anti-Ei-M (L 305), were prepared in rabbits against substantially purified antigen preparations, Gi-rep and Ei-M, respectively, and the interaction between the antisera and the antigens were analyzed by radioimmunochemistry. It was found that anti-Gi-rep contained only one type of antibody (type *A*) which reacted with Gi-rep and Ei-M in common (Fig. 1), while anti-Ei-M contained, in addition to type *A*, the other antibody (type *B*) which was strictly specific for Ei-M (Fig. 2B).

Since type *A* antibody is induced by and reacts with either Gi-rep or Ei-M, a certain antigenic determinant (type α) common to the two antigens should be responsible for induction of the antibody. The type *B* antibody is ascribed to another determinant (type β) which is carried by Ei-M. Thus, it is considered that Gi-rep carries only the determinant of type α , while Ei-M carries the two determinants, types α and β .

As anti-Gi-rep contains only type *A* antibody, the values of B_m for Gi-rep and Ei-M may be inversely proportional to the contents of type α determinant in the two antigens. Thus, from the values of B_m of anti-Gi-rep in Table I, the content of type α determinant in Ei-M is estimated to be 7.8 times that in Gi-rep on a weight basis.

As expected in the previous studies (8, 9), anti-Gi-2 (238) serum also contained type *B* antibody in addition to type *A*. However, its value of B_m for Ei-M is much lower than that of anti-Ei-M, indicating that the content of type *B* anti-

body is very much lower than that in anti-Ei-M. Although an apparently high value of CB_0/CF_0 is observed in the reaction of anti-Gi-2 with radio-labeled Ei-M (Figs. 1B and 2B), this value can be ascribed mainly to the higher affinity, K , in the reaction as compared with the case of anti-Ei-M (Table I). In addition, the B_m value of anti-Gi-2 for Gi-rep is more than 65% of that of anti-Gi-rep, indicating that type *A* is the major antibody in anti-Gi-2. Therefore, type *B* antibody in the antiserum may be ascribed to contamination with Ei-M type antigen in the previous preparation, Gi-2, used as the immunogen for the antiserum.

On the other hand, as previously recognized, a certain blocking antibody was induced in asthmatic patients by hyposensitization therapy with any one of the previous antigen preparations, G-2, Gi-2, E-2, and Ei-2. The blocking antibody obviously reacted with those antigens and the presumed allergen in common (2, 4-7). Those clinical observations suggest that the blocking antibody corresponds to the rabbit antibody of type *A* in immunological specificity. If this is the case, the presumed allergen would also carry a determinant similar to type α .

However, it is also supposed that not only rabbit anti-Gi-2 serum but also the sera of the patients treated with the preparation of G-2 or Gi-2 contain a small amount of type *B* antibody in addition to the major antibody of type *A*. Thus, if the type *B* antibody acted as the blocking antibody, the sera of the treated patients would also appear to have common reactivity to the antigens of both Gi-rep and Ei-M types and the presumed allergen. Accordingly, type assignment of the blocking antibody requires further studies in hyposensitization therapy using a preparation of Gi-rep which is free from the antigen of Ei-M type.

The assumption that each antigenic molecule in Ei-M carries the antigenic determinants of both types α and β still remains to be confirmed. In this connection, we have recently observed that anti-Gi-rep immunoglobulin (type *A* antibody alone) immobilized on Protein A-Sepharose CL-4B effectively absorbed the antigenic activity of Ei-M against anti-Ei-M as well as that against anti-Gi-rep (Suzuki, H., Oka, S., Jyo, T., & Katsutani, T., unpublished observation). This observation supports the above assumption regarding the determinants of Ei-M. Details of the study will be

presented elsewhere.

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