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Influence of the Medium on the Heat and Acid Denaturation of IgE

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The denaturation of IgE immunoglobulin induced by heating at 56°C or by treatment at low pH is inhibited in the presence of high concentrations of salts or hexoses. Between 50 and 100% of the IgE anaphylactic activity (PCA) of rat and mouse antisera is recovered after heating at 56°C for 1,5 or 5 h, respectively, in 1 M MgSO₄ or 2 M glucose, mannose or fructose. Anaphylactic activity of IgE monoclonal anti-DNP mouse antibody is equally preserved. The specific antigenic determinants of human and rat IgE myeloma proteins are also thermostable in these conditions. The addition of MgSO₄ or glucose protects IgE anaphylactic antibodies against denaturation at pH 2.

It is suggested that IgE denaturation is the consequence of interactions between molecules of immunoglobulin and that such interactions are diminished by steric hindrance in a medium containing high concentrations of ions or hexose molecules.

Key words: *IgE – heat denaturation – acid denaturation – anaphylactic antibody*

1. Introduction

One of the outstanding characteristics of IgE is its lability when heated at 56°C. This treatment, which results in an irreversible loss of functional capacity, is generally used to distinguish IgE from anaphylactic antibodies of the IgG class. Heating affects only the Fc region of the molecule since the capacity of its Fab region to react with the specific antigen is preserved (Ishizaka et al. 1967; Dorring-ton and Bennich, 1973). Recent observations (Prouvost-Danon et al., 1977, 1980) indicate that the denaturation process is greatly dependent on the composition of the medium in which the IgE is heated: when the salt concentration is increased or when the protein content is diminished, the heat stability is increased. The nature of the ionic components is important: NaCl or NaH₂PO₄ are effective in preventing the thermolability while Ca ions have the opposite effect. With regard to the proteins of the serum, the gamma globulin fraction but not the albumin fraction increases the heat denaturation.

We present in this communication further data concerning the influence of the

medium on the denaturation of IgE, showing that high concentrations of salts or hexoses have a protective effect against heat or low pH treatments.

Materials and Methods

Animals, immunizations

Anti-hen egg albumin (EA) sera was produced in BN rats and Swiss mice. Immunizations were done by intraperitoneal injections of 1 μ g EA and 1 mg aluminium hydroxide gel in 1 ml buffered saline, repeated 2 or 3 times at 3-week intervals. The animals were bled one week after the last injection. Anti-*Trichinella* sera were obtained from Wistar rats and Swiss mice 2 months after oral infection with 3000 or 300 muscle larvae, respectively. Wistar rats were used for passive cutaneous anaphylaxis (PCA).

Antigens, antibody determinations

EA crystallized 1 $\times$ was purchased from Serva, G.F.R. *Trichinella* antigen was prepared by sonic disruption of muscle larvae, as described (Perrudet-Badoux and Binaghi, 1974).

Mouse and rat IgE antibodies were evaluated by their capacity to sensitize rat skin. PCA was performed as described by Ovary (1958). Challenge was made after 24–48 h by intravenous injections of 1 ml 0.5% Evans blue containing 5 mg EA, 5 mg DNP₄₈-BSA or 1 mg protein *Trichinella* antigen. Titres were expressed as the inverse of the highest dilution giving a positive reaction. Total precipitating antibodies were measured by quantitative precipitin tests. Sheep anti-human IgE and goat anti-rat IgE were purchased from Nordic Immunology, Holland. Human IgE myeloma protein was a gift of Dr. Ishizaka and rat myeloma protein (IR 2) was a gift of Dr. Bazin. Human IgE determinations were made by the Phadezym-Prist technique (Pharmacia, Sweden) according to the instructions given by the manufacturer.

Monoclonal IgE mouse antibody

The murine hybridoma used was prepared by the method of Liu et al. (1980) by fusion of SP2/0 tumour cells and spleen cells from DNP-*Ascaris*-hyperimmunized CAF₁ mice. Hybridoma ascites fluid was kindly supplied by Dr. A. Prouvost-Danon. The purification of the monoclonal DNP-specific IgE antibodies was performed as described by Liu et al. (1980). The purified IgE antibody was dissolved in buffered saline at a final concentration of 0.6 mg/ml. From this solution, 1 : 50 dilutions were made in normal mouse serum or in buffered saline, and treated as described in Results. After treatment, PCA reactions were performed using DNP₄₈-BSA as challenging antigen.

Heat treatment

Heating was done at 56°C in the presence of various reagents. In general, rat antisera were heated for 1.5 h and mouse antisera for 5 h. These times have been

shown to be sufficient to destroy practically all the anaphylactic activity of the antisera (Binaghi et al., 1964; Prouvost-Danon et al., 1977). MgSO_4 or other salts, 2 M in saline, or D (+) glucose, mannose or fructose 3 M in saline were added to the antisera in the quantity required to attain the final desired concentration. In the case of hexoses the final concentration of the antisera was 1 : 3; in the case of inorganic salts it was 1 : 2. After heating, the samples were extensively dialyzed against saline before testing them.

Acid treatment

Glycine buffer was added to the antisera. The final buffer concentration was 1 M. After 20 min the samples were quickly neutralized at pH 7 with 1 M NaOH and dialyzed against buffered saline.

Results

Heat treatment in the presence of salts

Various rat and mouse antisera were heated at 56°C in the presence of various concentrations of MgSO_4 , MgCl_2 or CaCl_2 . Their anaphylactic activity was measured by PCA before and after the heat treatment, with the results shown in Table I.

It can be seen that MgSO_4 has a protective effect against heat denaturation, which is proportional to its concentration. At 1 M, the maximum concentration tested, complete protection was frequently obtained. In two experiments where mouse antisera were heated for 10 and 24 h, 50% protection was observed with 1 M MgSO_4 . It appeared that MgCl_2 affords less protection and that CaCl_2 does not afford protection at all.

Heat treatment in the presence of hexoses

When heating was performed in a medium containing hexoses, denaturation was prevented, as seen in Table II. No differences were observed between glucose, mannose and fructose. Using 2 M concentrations of the hexoses complete protection was found in many cases, provided that the heating was not continued for very long periods of time. Protection of monoclonal IgE anti-DNP antibody was also obtained in the presence of 2 M glucose, as shown in Table III. In this case the protection was more marked when the monoclonal antibody was diluted in normal mouse serum than when it was diluted in saline.

Experiments were performed to verify whether the protective effect of hexose sugars was equally manifested against loss of antigenic determinants. Two immunochemical methods were employed. In a series of experiments, myeloma IgE protein from rat and man were heated at 56°C for 1,5 and 3 h, respectively, at a concentration of 0.15 mg/ml in buffered saline or in buffered saline containing 2 M glucose. After treatment, the samples were dialyzed against buffered saline and Ouchterlony tests were performed with specific anti-IgE antisera. The results obtained (not shown) confirmed previous observations by Ishizaka et al. (1967) indicating that heating induced denaturation of antigenic determinants of IgE; when

TABLE I
EFFECT OF HEATING AT 56°C IN THE PRESENCE OF SALTS, ON THE IgE ANAPHYLACTIC ACTIVITY OF VARIOUS RAT AND MOUSE ANTISERA

Salt added	PCA titre after heating at 56°C										
	Rat anti-EA ^a					Rat anti- <i>Trich.</i> ^a	Mouse anti-EA ^b				Mouse anti- <i>Trich.</i> ^b
	1	2	3	4	5		1	2	3	4	
None	20	< 20	20	10	< 20	10	< 20	< 15	< 15	< 15	10
MgSO ₄ 0.2 M	20		40	20		20					
0.4 M	40			20		40					
0.6 M	80		80			80					
0.8 M	160		80			160					
1.0 M		160	160	40	80	160	80	120	60 ^{c)}	60 ^{d)}	80
MgCl ₂ 1.0 M		80			40	160					
CaCl ₂ 1.0 M		< 20			< 20						
Control											
non-heated	320	160	160	80	160	320	80	120	120	120	160

Heating for: ^a 1.5 h; ^b 5 h; ^c 10 h; ^d 24 h.

TABLE II

EFFECT OF HEATING AT 56°C. IN THE PRESENCE OF HEXOSES, ON THE IgE ANAPHYLACTIC ACTIVITY OF VARIOUS RAT AND MOUSE ANTISERA

PCA titre after heating at 56°C													
		Rat anti-EA			Rat anit- <i>Trich.</i>				Mouse anti-EA				
Serum number		1	2	3	3	3	3	4	5	6	7	8	
Time of heating (h)		1.5	3	3	3	9	24	1.5	5	5	5	24	
<i>Hexose added</i>													
None		10	< 20	< 20	< 20	< 20	< 20	20	40	< 20	25	< 15	
Glucose	0.25 M			40	20	20							
	0.5 M	20		80	20								
	1.0 M	40		160	160								
	1.6 M			160	160								
	2.0 M	80	160	320	160		80	80	240	80	200	60	
Fructose	2.0 M	80									200		
Mannose	2.0 M	80									200		
Control non-heated		80	160	320	320	320	320	160	480	80	200	120	

TABLE III

EFFECT OF HEATING AT 56°C, IN THE PRESENCE OF GLUCOSE 2 M, ON THE ANAPHYLACTIC ACTIVITY OF A MONOCLONAL IgE ANTI-DNP ANTIBODY

Monoclonal mouse IgE antibody was diluted in normal mouse serum or in buffered saline to a concentration of 0.012 mg/ml, and heated as indicated. After heating, the dilutions to perform PCA were made in buffered saline.

Glucose added	Time of heating (h)	PCA titre after heating at 56° C	
		Diluted in serum	Diluted in saline
None	0	1 600	1 600
	1	400	400
	2	50	50
	4	50	50
2 M	0	1 600	1 600
	1	1 600	800
	2	1 600	400
	4	1 600	100

TABLE IV

TOTAL IgE CONCENTRATION OF VARIOUS HUMAN SERA FRESH, HEATED AT 56°C, AND HEATED AT 56°C IN THE PRESENCE OF 2 M GLUCOSE

Serum	IgE units/ml		
	Non-heated	Heated 3 h at 56°C	Heated 3 h at 56°C in glucose 2 M
1	140	46	118
2	154	56	118
3	1 450	350	1 080
4	1 380	345	990
5	69	15	69

TABLE V

EFFECT OF ACID TREATMENT, IN THE PRESENCE OF MgSO₄ OR GLUCOSE, ON THE IgE ANAPHYLACTIC ACTIVITY OF VARIOUS RAT ANTISERA

Treatment	PCA titre after treatment	
	Rat anti-EA	Rat anti-Trich.
Control, 20 min at pH 7	80 80 160 160 160 80	80
20 min at pH 3	80 80 80	80
20 min at pH 3, 1 M MgSO ₄	80	
20 min at pH 3, 2 M glucose	80 80	
20 min at pH 2	20 20 20 40 20 10	10
20 min at pH 2, 1 M MgSO ₄	40 80 80	40
20 min at pH 2, 2 M glucose	80 80 80	

heating was done in the presence of glucose, no denaturation was apparent. In another series of experiments, the total IgE concentration of various human sera was determined before and after heat treatment in the presence of 2 M glucose, using the Phadezym-Prist technique. The results, expressed in units IgE/ml are reported in Table IV. They indicate that after heating at 56°C for 3 h the IgE is reduced to less than 25% of its initial concentration while more than 70% remains when heating is performed in a glucose medium.

Low pH treatment

In view of the results previously described, it was of interest to verify whether glucose or MgSO_4 could prevent denaturation of IgE during low pH treatment. Different rat sera containing anti-EA or anti-*Trichinella* IgE antibodies were acidified with 1 M glycine buffer to pH 3 or 2, maintained at this pH for 20 min, then neutralized, dialyzed against buffered saline and tested. The results are shown in Table V. Treatment at pH 3 did not affect significantly the anaphylactic activity of the antisera but treatment at pH 2 produced an important loss of activity. In the presence of glucose or MgSO_4 good protection against acid denaturation was obtained.

Discussion

It appears from our results that heat denaturation of IgE can be prevented in the presence of high concentrations of inorganic salts or hexoses. At least 50%, and frequently 100% of the anaphylactic activity of mouse and rat IgE was recovered after heating at 56°C in 1 M MgSO_4 and a similar protection was afforded by 2 M glucose, fructose or mannose. Previous experiments (Prouvost-Danon et al., 1977; 1980) have shown that a significant protection against heat denaturation could be obtained at high dilutions of the antisera (1:50–1:100) and/or by increasing the salt concentration of the diluent, up to 5 M NaCl or 1 M Na_2HPO_4 . Our results confirm these observations and show that a better protection can be obtained using MgSO_4 , a salt giving bivalent ions and consequently higher ionic strength. We have also found that protection is equally well achieved with a high concentration of non-ionic compounds such as hexoses.

One should note that denaturation is not completely abrogated but rather delayed, as can be seen from the results presented in Table II when antisera were heated for many hours.

The protective effect of hexoses at high concentrations was also demonstrated with respect to the antigenicity of the IgE molecule. This protective effect was shown with rat and human IgE myeloma proteins and with normal human IgE.

Another aspect of this study concerned the denaturation induced by acidity. It is known that immunoglobulins in general, and IgE in particular, are rapidly denatured at low pH, the degree of denaturation being proportional to the acidity and to the duration of the treatment. Our results indicate that good protection is achieved when the acid treatment (pH 2) is made in the presence of MgSO_4 or glucose.

In most cases, the degree of denaturation of IgE has been evaluated by measuring the anaphylactic activity of antisera by PCA. The conditions of the test were such that only IgE antibodies were measured. This is particularly true for mouse IgE since IgG₁, the other anaphylactic antibody of the mouse, does not sensitize rat skin (Ovary et al., 1975). In the case of rat anaphylactic antibodies, as tested by PCA in rats, a residual activity, amounting to about 10% of the total, was regularly observed in antisera after heat treatment under conditions at which the IgE was supposed to be denatured. This activity corresponds, in all probability, to the presence of IgG_{2a} heat stable rat anaphylactic antibody (Morse et al., 1971).

The physicochemical basis of the heat denaturation of IgE is unclear. Irreversible unfolding of the molecule, formation of S-S bridges and inter- or intra-protein interactions have been suggested as possible events (Ishizaka et al., 1967; Dorrington and Bennich, 1973). In our experiments the antisera were heated at a dilution of 1 : 2 or 1 : 3, which corresponds to a total protein concentration of about 30 mg/ml. In a solution of 2 M glucose (360 mg/ml) the weight ratio of glucose to protein is therefore 12 and each protein molecule is completely surrounded by glucose molecules. It is conceivable that in these conditions molecular interactions may be considerably impeded. In fact, we have observed that antigen-antibody reactions in solution or in gel are very much retarded in a medium containing 2 M glucose. It can be hypothesized that the heat denaturation of IgE is the result of interactions between IgE and other protein molecules and that the presence of high concentrations of hexoses may reduce the chances of these interactions. The fact that denaturation is increased by the gamma globulin fraction but not by the albumin fraction of normal serum (Prouvost-Danon et al., 1977) as well as the fact that denaturation takes place when pure IgE (myeloma proteins or monoclonal antibody) is heated, suggests that IgE-IgE interactions play a role in the heat denaturation process.

In the case where the protection is obtained by high salt concentrations, with MgSO₄ or with other salts, a similar mechanism may be operative but in that case the supposed interactions should be hindered by the ionic clouds formed around the polar groups of the protein molecule. Another possibility could be that the heat protection determined both by hexoses and by salts is due to specific interaction of these reagents with some particular protein residues, but this possibility is difficult to sustain from a chemical point of view. On the contrary, the denaturing action of Ca²⁺ ions could well be due to the formation of protein-protein bonds by this ion.

Apart from the implications that they may have concerning the physicochemical properties of the IgE molecule, the present results are of practical interest for the measurement of the IgE activity and for devising technical procedures for the preservation and purification of IgE antibodies.

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