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Heterogeneity of Guinea-Pig Homocytotropic Antibodies

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Summary. It is suggested that guinea-pigs produce three different homocytotropic antibodies that can be distinguished by their physicochemical and biological properties. One has the properties of an IgG antibody, is heat and mercaptoethanol resistant and persists in the passively sensitized skin for not more than 72 hours. The other has the properties of an IgE antibody, is heat and mercaptoethanol labile and persists in the passively sensitized skin for at least 20 days. The third antibody is heat stable and mercaptoethanol labile and persists in the passively sensitized skin for more than 72 hours but less than 1 week. This antibody was partially separated from $\gamma 1$ antibody by chromatography on DEAE cellulose and may represent a distinct segment of the $\gamma 1$ antibody population. All three antibodies were able to sensitize guinea-pig mast cells and cause degranulation of these cells on reaction with antigen. Antihistamines produced complete inhibition of PCA reactions induced with all three types of antibodies but were much less efficient in inhibiting PCA reactions induced with $\gamma 1$ antibody obtained from hyperimmune serum.

INTRODUCTION

It is now well established that many species produce at least two different types of homocytotropic antibodies (Bloch, 1968). One has the properties of an IgG antibody, is heat stable and usually unable to persist in the passively sensitized skin for more than 48 hours. The other is a skin sensitizing and heat labile antibody of the IgE class. Until recently the guinea-pig seemed to be exceptional in this regard producing only one homocytotropic antibody of the 7S $\gamma 1$ type. Very recently, however, an IgE-like antibody was found in guinea-pigs infected with *Trichinella spiralis* (Catty, 1969) and in guinea-pigs artificially sensitized with a single intraperitoneal injection of antigen with *Bordetella pertussis* (Mota and Perini, 1970). This antibody was heat labile and mercaptoethanol sensitive. The findings reported in the present paper suggest the presence of a third homocytotropic antibody in this species.

MATERIALS AND METHODS

Animals. Albino guinea-pigs weighing 250–350 g reared at the Instituto Butantan animal farm were used throughout.

Antigens. Five times crystallized ovalbumin (Ov) from Pentex Inc. (Kankakee, Ill.) and dinitrophenylated bovine gamma-globulin (DNP-BGG) containing thirty DNP groups per molecule of protein were used as antigens.

Adjuvants. Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) and a suspension of *Bordetella pertussis* (Laboratory of Bacterial Vaccines, Butantan Institute, São Paulo) containing 2×10^{10} organisms per ml were used.

Antihistamines. Mepyramine maleate was a gift from Merck, Sharpe and Dohme, Rahway, New Jersey. Triprolidine hydrochloride was purchased from Burroughs Wellcome Co. Tuckahoe, N.Y.

Preparation of antisera. Early antisera: guinea-pigs were immunized either with Freund's complete adjuvant or with *B. pertussis* suspension containing 1 mg per ml Ov or DNP-BGG. When Freund's adjuvant was used the animals were injected with 0·25 ml of the antigen mixture in each one of the footpads. When *B. pertussis* was used they were injected intraperitoneally with 1 ml of the micro-organism suspension plus antigen. In both instances the animals were bled by cardiac puncture 10, 17, 20 and 30 days after antigen stimulation. Blood samples were immediately transferred to tubes kept at 4° and centrifuged in a refrigerated centrifuge. Serum samples were used as soon as possible or, when necessary, kept at −20°. All serum manipulations were done in the cold. Hyperimmune antisera: groups of guinea-pigs were injected with Freund's adjuvant plus Ov as stated for early antisera and from the 10th day received additional intradermal injections of 200 μg Ov weekly for 2 months. The animals were bled 10 days after the last antigen injection. Antisera obtained from each group were pooled and the PCA activity of each pool was studied separately.

Chromatography on DEAE cellulose. Chromatography on DEAE cellulose was performed by applying 5 ml antiserum to columns measuring 2·5 cm in diameter and 30 cm in length. Stepwise elution was performed with pH 8·0 phosphate buffers of different molarities. Five-millilitre samples were collected at the rate of 1 ml/minute. The protein concentration of the eluates was determined by spectrophotometry at 2800 Å. When necessary the protein peaks were pooled and the volume of the pool reduced to the original serum volume by positive pressure using an ultrafiltration cell (Amicon Corporation, Cambridge, Mass.).

Preparation of γ1 antibody. Guinea-pig γ1 anti-Ov antibody was obtained from hyperimmune serum by chromatography on DEAE-cellulose according to the method described by Reisfeld and Hyslop (1966). The preparation showed slight contamination with γ2 antibody.

Treatment with heat. To determine the effect of heating on the antibody activity the antisera or the chromatographic fractions were placed for 4 hours in a water bath at 56°.

Treatment with mercaptoethanol. Samples of antisera or their chromatographic fractions were dialysed against 250 ml of 0·1 M 2-mercaptoethanol for 3 hours at room temperature and then dialysed against 500 ml 0·02 M iodoacetamide for 4 hours. Before testing the samples were dialysed against several changes of phosphate-buffered saline pH 7·2 for 18–24 hours at 4°. Control samples were either dialysed against phosphate-buffered saline in place of mercaptoethanol and then treated with iodoacetamide as described above, or simply dialysed against phosphate-buffered saline.

Passive cutaneous anaphylaxis (PCA). Ovary's technique for PCA (1964) was employed for detecting and estimating antibodies. The dorsal region of the animals was previously shaved with an electrical hair clipper, care being taken to avoid irritation of the skin. Several intradermal injections of antiserum or chromatographic fractions were then made on each side of the dorsal skin with a hypodermic needle. After a sensitization period of 4 or more hours the sensitized animals were injected intravenously with 1 ml of 0·25 per

cent solution of Evans' blue in saline (0·85 per cent NaCl) containing 1 mg antigen. Twenty to 30 minutes after antigen injection, the animals were killed, the skin was inverted and the lesion diameter was measured on the inner surface of the skin with a transparent ruler. Antibody was estimated by determining the highest dilution of antiserum which induced a PCA reaction. PCA titres were stated as the reciprocal of the highest serum dilution giving a skin reaction. A minimum of three guinea-pigs was used for each determination.

RESULTS

Effect of heating and mercaptoethanol treatment. Samples of early antisera obtained from guinea-pigs immunized with Ov plus *B. pertussis* or Freund's adjuvant were submitted to heat or mercaptoethanol treatment and their PCA activity compared with that of control samples. As can be seen in Table 1 the results of these experiments suggested the presence of three distinct homocytotropic antibodies in these antisera. One antibody was both heat stable and mercaptoethanol stable, a second antibody was both heat labile and mercaptoethanol labile whereas a third antibody was mercaptoethanol labile but was heat stable. In an additional group of ten guinea-pigs injected with DNP-BGG plus *B. pertussis* and tested with DNP-BSA, two animals produced antisera whose PCA activity was destroyed by heat and mercaptoethanol treatment and eight animals produced antisera whose PCA activity was not destroyed either by heat or mercaptoethanol treatment. It was presumed that the heat and mercaptoethanol resistant activity was due to 7S γ1 antibody, the heat and mercaptoethanol labile activity was due to the reaginic antibody and that the heat stable

TABLE 1

EFFECT OF HEATING (56° 4 HOUR) AND MERCAPTOETHANOL TREATMENT ON PCA ACTIVITY OF GUINEA-PIG EARLY ANTISERA*

Immunization	Antisera		PCA titre		
			Control	Heat treated	Mercaptoethanol treated
B. pertussis +Ov	P_1	HM-labile	12	0	0
	P_2		5	0	0
	P_3		10	0	0
	P_4	M-labile	9	9	0
	P_5		15	15	0
	P_6		12	12	0
	P_7		12	12	0
	P_8	HM-resistant	15	15	15
	P_9		20	20	20
	P_{10}		40	40	40
Freund's +Ov	F_1	HM-resistant	40	40	40
	F_2		80	80	80
	F_3		20	20	20
	F_4		40	40	40
	F_5		15	15	15
	F_6		20	20	20
	F_7	M-labile	15	15	0
	F_8		40	40	0
	F_9		40	40	0
	F_{10}		80	80	0

* Antisera collected 10 days after a single injection of Ov plus adjuvant.

mercaptoethanol labile activity was due to an unknown antibody. For brevity these antibodies will be termed HM-resistant antibody, HM-labile antibody and M-labile antibody.

SENSITIZATION PERIOD AND PERSISTENCE OF SKIN FIXATION OF GUINEA-PIG HOMOCYTOTROPIC ANTIBODIES

Since in other species different homocytotropic antibodies have different biological properties, the sensitization period and skin persistence of guinea-pig early antisera possessing different activities were studied in an attempt to further differentiate the antibodies. Guinea-pigs were prepared for PCA with antisera containing either HM-resistant antibody, HM-labile antibody or M-labile antibody. The animals were then challenged after a sensitization period of either 4 hours or 1, 2, 3, 7, 10 or 20 days. As can be seen in Table 2 the antibodies present in the three types of antisera used seem to have

TABLE 2

SENSITIZATION PERIOD AND SKIN PERSISTENCE OF GUINEA-PIG ANTISERA CONTAINING HM-RESISTANT ANTIBODY, HM-LABILE ANTIBODY AND M-LABILE ANTIBODY

Sensitization period	Antisera and PCA titre		
	HM-resistant	HM-labile	M-labile
4 hours	20	0	10
24 hours	20	10	40
48 hours	0	10	40
7 days	0	UND*	UND
10 days	0	UND	0
20 days	0	UND	0

* UND = undiluted.

different sensitization periods and different ability to persist in the sensitized skin. The HM-resistant antibody has an optimum sensitization period of 4 hours and either disappears from the skin of the recipient animals or produces very diffuse PCA reactions when 48 hours or more are allowed between antibody and antigen injection; the HM-labile antibody has an optimum sensitization period of 24–48 hours and persists in the skin for at least 20 days whereas the M-labile antibody has an optimum sensitization period of 24–48 hours and persisted in the skin for a time much longer than the HM-resistant antibody but shorter than the HM-labile antibody.

Effect of antihistamines. PCA reactions induced with different types of homocytotropic antibodies in rats and mice respond differently to the effects of antihistamines (Mota, 1963, 1964, 1967) and an attempt was made to find out whether the same would occur with guinea-pig homocytotropic antibodies. Guinea-pigs were prepared for PCA with the three different types of guinea-pig early antisera and challenged after the optimum sensitization period for each antiserum. For comparison they were also injected with a $\gamma 1$ antibody preparation obtained from hyperimmune antisera in a dilution adequate to induce PCA lesions of size and intensity comparable to the PCA lesions induced by the early antisera. Five minutes before antigen injection some of the animals in each group were injected intravenously with triprolidine (1 mg/kg) or mepyramine (5 mg/kg). The results of these experiments are shown in Table 3. It was observed that PCA reactions

TABLE 3
EFFECT OF TRIPROLIDINE (1 mg/kg) ON PCA REACTIONS INDUCED WITH GUINEA-PIG HM-RESISTANT ANTISERUM, HM-LABILE ANTISERUM OR M-LABILE ANTISERUM OR γ1 ANTIBODY OBTAINED FROM HYPERIMMUNE ANTISERUM

Antiserum	S.P.	PCA (mm)		
		Dilution	Control	Triprolidine
HM-resistant (early)	4 hour	5	20–18–15	0– 0– 0
		10	17–15–12	0– 0– 0
		20	11–13– 8	0– 0– 0
HM-labile (early)	48 hour	5	16–16–12	0– 0– 0
		10	14–13–10	0– 0– 0
		15	10–10– 7	0– 0– 0
M-labile (early)	24 hour	10	17–20–18	0– 0– 0
		20	14–17–16	0– 0– 0
		40	11– 9–10	0– 0– 0
Hyperimmune γ1 antibody	4 hour	100	19–13–13	11–13–10
		200	17–11–12	8–12–11
		400	10–13–10	6– 7–10

S.P. = sensitization period.

induced with the three different early antisera were completely abolished by pretreatment with triprolidine whereas PCA reactions induced with γ1 antibody from hyperimmune antiserum were only partially abolished by the same treatment. When mepyramine (5 mg/kg) was used the same results were obtained.

Passive sensitization of mast cells. Mesentery mast cells can be passively sensitized *in vitro* by incubation with antibody in adequate conditions. Contact of the sensitized cells with antigen induces histamine release and mast cell degranulation (Humphrey and Mota, 1959; Mota, 1959). Pieces of guinea-pig mesentery were incubated at 37° for 30 minutes or at 4° for 18 hours with HM-resistant antisera, HM-labile antisera and M-labile antisera and transferred to Tyrode's solution containing Ov 1 mg/ml for 15 minutes at 37°. The tissue was subsequently fixed and stained for microscopic observation as previously described (Mota, 1959). Degranulation of the mast cells after contact with antigen was taken as a criterion of passive sensitization. Antigen-induced mast cell degranulation was definitely obtained after incubation of the mesentery with HM-resistant antisera and M-labile antisera but not with HM-labile antisera. However, very slight mast cell degranulation could be obtained with HM-labile antisera when incubation with antisera was performed at 4° for 18 hours. The ability of HM-resistant antisera to passively sensitize the mast cells was not abolished by heat or mercaptoethanol treatment whereas the same treatments suppressed the sensitizing ability of HM-labile antisera. On the other hand mercaptoethanol treatment but not heat treatment abolished the sensitizing ability of M-labile antibody.

FREQUENCY OF THE DIFFERENT HOMOCYTOTROPIC ANTIBODIES IN EARLY ANTISERA

Samples of antisera obtained 10 days after a single injection of Ov plus *B. pertussis* or Freund's adjuvant were submitted to heat or mercaptoethanol treatment and their PCA activity was studied. 300 guinea-pigs were immunized using *B. pertussis* as adjuvant and 64 with Freund's adjuvant. When *B. pertussis* was used 30 per cent of the animals produced HM-labile antibody, 48 per cent produced M-labile antibody and 22 per cent produced

HM-resistant antibody. When Freund's adjuvant was used 60 per cent of the animals produced HM-resistant antibody and 40 per cent produced M-labile antibody. In no instance did we detect an HM-labile antibody in the animals immunized with Freund's adjuvant.

The earliest appearance of both the HM-labile antibody and the M-labile antibody was on the 10th day following sensitization. The HM-labile antibody was still present 20 days after sensitization but had disappeared after 30 days whereas the M-labile antibody was still present at that time. Additional injections of antigen led to disappearance of the HM-labile antibody but had a booster effect on both HM-resistant and M-labile antibody.

Results with hyperimmune antisera. When samples of hyperimmune antisera were submitted to heat or mercaptoethanol treatment no change in their PCA activity was induced by heat whereas treatment with mercaptoethanol produced either no change or a partial reduction varying from 5 to 60 per cent decrease in their PCA activity. Antisera whose activity was substantially reduced by mercaptoethanol treatment were assumed to have both HM-resistant and M-labile antibodies. Antisera whose PCA activity was partially reduced by mercaptoethanol treatment produced better PCA reactions after a sensitization period of 24 hours than after only 4 hours. When a sensitization period of 48 hours was used challenge of the animals produced a diffuse blueing of the entire dorsal skin that prevented the reading of the PCA reactions.

TABLE 4

EFFECT OF MERCAPTOETHANOL TREATMENT ON THE PCA ACTIVITY OF CHROMATOGRAPHIC FRACTIONS OBTAINED FROM GUINEA-PIG HYPERIMMUNE ANTISERUM

Fractions obtained with pH 8·0 PO_4 buffer	PCA titre	
	Control	Mercaptoethanol treated
0·005 M	0	0
0·05 M	40	40
0·07 M	10	10
0·1 M (leading side of the peak)	20	10
0·1 M (back side of the peak)	15	3

Chromatography on DEAE cellulose. Chromatography of antisera containing HM-labile antibody was considered not practical due to the low PCA activity present. Attempts were made to separate the mercaptoethanol labile antibody from hyperimmune antisera whose PCA activity was partially destroyed by mercaptoethanol treatment. Antiserum samples were applied to the column and sequential elution was performed with five pH 8 phosphate buffers: 0·005 M, 0·01 M, 0·05 M, 0·07 M and 0·1 M. The amount of each buffer used was about 1·5 times the void volume of the column. The individual eluates obtained with each buffer were tested for their PCA activity before and after mercaptoethanol treatment and divided according to their resistance to this treatment, into three groups: completely resistant, partially resistant and not resistant. In no case was the PCA activity of these eluates changed by heating. The eluates of each group were pooled, concentrated to the original serum volume and the PCA activity of the resulting pools studied again before and after mercaptoethanol treatment. The results of these experiments (see Table 4) showed that most of the mercaptoethanol resistant antibody was eluted with buffers of lower molarities (0·05 and 0·07) and that most of the mercaptoethanol labile antibody

was detected in the effluent fractions of the back side of the protein peak obtained after application of 0·1 M buffer. However it must be noted that when the eluates whose PCA activity was completely destroyed by mercaptoethanol were pooled and concentrated the PCA activity of the resulting pool was only partially destroyed by this same treatment. This indicates that some mercaptoethanol resistant antibody was present in undetectable amounts in the original eluates but became detectable after pooling and concentrating the eluates. Thus a complete separation of the M-labile antibody from the HM-resistant antibody could not be achieved.

DISCUSSION

Heterogeneity of homocytotropic antibodies has been described in many species. The presence of two types of homocytotropic antibodies has been clearly shown in the rat, mouse and rabbit (Mota, 1963, 1964, 1967; Mota and Peixoto, 1966; Mota, Wong, Sadun and Gore, 1968; Binaghi and Benacerraf, 1964; Lindqvist, 1968; Henson and Cochrane, 1969).

In the guinea-pig two homocytotropic antibodies were already known, the γ1 type described by Ovary, Benacerraf and Bloch (1963) and an IgE type antibody found in guinea-pigs infected with *Trichinella spiralis* (Catty, 1969) and in guinea-pigs sensitized with ovalbumin or DNP-BGG plus *B. pertussis* as adjuvant (Mota and Perini, 1970). More recently the production of reagin-like antibodies has also been observed in guinea-pigs sensitized with picryl chloride in Freund's adjuvant or B-lactoglobulin in aluminium hydroxide (Parish, 1970), in guinea-pig infected with *Ascaris suum* (Dobson, Morseth and Soulsby, 1971) as well as in guinea-pigs injected with repeated minute doses of benzyl-penicilloyl-bovine gamma-globulin (Levine, Chang and Vaz, 1971). The IgE type antibody can be differentiated from the γ1 type antibody by its heat lability and mercaptoethanol susceptibility. The third reagin-like homocytotropic antibody suggested in this paper can be distinguished from γ1 antibody by being skin-sensitizing and from the IgE type antibody by being mercaptoethanol labile but heat stable. Furthermore the M-labile antibody differs from the IgE type antibody by inducing maximal PCA reactions within a shorter sensitization period (24 hours) and also by remaining in the sensitized skin for a shorter time (less than 10 days). In this regard it resembles the 7-day antibody recently described by Parish (1970) in antisera of guinea pigs sensitized with picryl chloride in Freund's adjuvants or B-lactoglobulin in aluminium hydroxide. The sensitivity of this antibody to mercaptoethanol was not described. It seems from Parish's and our own results that antigen and adjuvant are important in preferentially inducing the production of each of these homocytotropic antibodies by sensitized guinea-pigs. In our experiments *B. pertussis* induced a better production of reagin-like antibody than Freund's adjuvant which was very poor in this regard. Under our experimental conditions the reagin-like antibody had disappeared from sera one month after sensitization whereas the M-labile antibody was still present. Indeed hyperimmune guinea-pig antisera though not containing a heat labile antibody still contain an M-labile PCA activity probably due to the presence of the M-labile antibody. Differing from the IgE type antibody, the M-labile antibody attains high levels in sera of guinea-pigs receiving additional injections of antigen. It is possible that under other immunization schedules the IgE type antibody may attain high serum levels and may persist in the sera for longer periods of time as observed by Parish (1970) and Levine and co-workers (1971).

The fact that sensitization of mast cells was only slight when HM-labile antisera was used was probably due to the very low serum content of this antibody and/or its extreme ability *in vitro* (Mota and Perini, 1970). More experiments are necessary on this respect. Anyway, the observation that mast cell sensitization can be obtained with the three types of antisera suggest that all three guinea-pig homocytotropic antibodies act by sensitizing the mast cells and in reacting with antigen causes damage to these cells with consequent activation and release of pharmacologically active substances. This is in contrast to what happens in rats in which PCA induced with different homocytotropic antibodies have different mechanisms not always involving the mast cells (Mota, 1963, 1964; Stechschulte, Austen and Bloch, 1967). The fact that triprolidine, a very specific antihistamine (Becker, Mota and Wong, 1969) was able to inhibit PCA reactions induced with any of the guinea-pig homocytotropic antibodies suggests that histamine is probably the principal mediator in many types of anaphylactic reactions in this species. It must be noted, however, that PCA reactions induced with γ1 antibody preparation obtained from hyperimmune antiserum was only partially abolished by antihistamines although the blue staining and the size of the PCA lesions were comparable to that exhibited by the PCA lesions induced with early antisera. Stechschulte, Austen and Bloch (1967) have reported that reaction of antigen with guinea-pig tissues sensitized with γ1 antibody induces the formation of slow reacting substance (SRS) in addition to histamine release. Thus it is possible that PCA reaction induced with hyperimmune antiserum may in part be due to SRS formation induced by reaction of antigen with γ1 antibody. We can offer no explanation for the discrepancy between the effect of antihistamines on PCA reactions induced with early or late γ1 antibody.

The present evidence suggest that guinea-pigs produce three different homocytotropic antibodies, one being both heat and mercaptoethanol resistant (γ1), other being both heat and mercaptoethanol labile (IgE) and a third one that is heat stable but mercaptoethanol susceptible. This antibody may belong to a different immunoglobulin class. However the fact that chromatography on DEAE cellulose resulted only in a partial separation of the M-labile antibody suggests that it may rather represent a different segment of the γ1 antibody population.

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