

Binding of Antigen to Surface Ig during *in vitro* Hyposensitization of Isolated Rat Mast Cells

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The mechanism of *in vitro* hyposensitization was examined in isolated rat mast cells. Surface distribution of immunoglobulin was examined by immunofluorescence technique. Hyposensitization could be ascribed neither to changes in the distribution of surface immunoglobulins nor to capping or shedding phenomena. The distribution of cell-bound antigen and the number of antigen-positive cells (patch-bearing cells) was not changed by hyposensitization. The binding of antigen to surface Ig was quantitated by ^{125} I-labelled antigen. In rats sensitized to two different antigens, hyposensitization of the mast cells with one of these inhibited the subsequent histamine release induced by either of the two antigens, whereas no changes were observed in the antigen binding capacity.

The present investigation indicates that the mechanism of *in vitro* hyposensitization is the result of events secondary to the initial binding of antigen to the mast cell surface immunoglobulin.

Key words: antigen; hyposensitization; mast cells; surface immunoglobulin.

Accepted for publication 8 September 1978

The mechanism of specific hyposensitization therapy in immediate type allergic diseases is still unsettled. It is well known that hyposensitization results in an increased titer of blocking antibodies (1, 5, 7), but the correlation between this titer and clinical improvement is controversial. This points to an involvement of factors other than blocking antibodies which are so far unknown (3, 8). Hyposensitization of rat mast cells and human leukocytes has been demonstrated *in vitro* by preincubation of the cells with specific antigen in suboptimal concentrations (4, 5). The reduction of the histamine release was caused by changes in the target cell but could not be ascribed to blocking antibodies (4). The mechanism might be induction of capping phenomena which in human

basophils is followed by a reduction in histamine release (2). It might also be the result of a successive binding of surface immunoglobulin with antigen, or the reaction between antigen and cell-fixed antibody may alter the ability of free, fixed antibody molecules to react as a result of steric hindrance (4). The present study was undertaken to examine the importance of these mechanisms for induction of *in vitro* hyposensitization of isolated mast cells.

MATERIAL AND METHODS

Antigens

Bovine serum albumin (BSA) was obtained from Behringwerke, Germany. Only one line

was obtained by crossed immunoelectrophoresis of BSA against rabbit anti-bovine serum. To obtain partial purification of bovine IgG and IgM, complement inactivated bovine serum was dialysed against buffered salt solution, pH 7.0 (see below) and 3 ml was applied on a 2.5×100 cm Sephadex G-200 column (Pharmacia, Sweden). IgG and IgM were eluted from the column by Tris-HCl containing 0.02 M sodium azide (pH 7.35, ionic strength 0.154) by a flow rate of 10 ml/h. The fractions were identified in u.v.-absorption light and they were dialysed against buffered salt solution (pH 7.0). The protein concentrations of the IgG and IgM enriched fractions were 0.5 and 1.1 mg/ml, respectively. In the following these fractions are designated IgG and IgM, respectively.

Sensitized rats

Adult male albino rats, weight 200–300 g, were sensitized by a subcutaneous injection of bovine serum, bovine serum albumin (BSA), bovine IgG or IgM. The rats were used 3 weeks after the sensitization. Bovine serum was complement inactivated by heating to 56°C for 30 min. 1.0 ml of the serum was given mixed with 1.0 ml (10^{10} bacilli) pertussis vaccine as adjuvant. BSA was given as a mixture of 625 μg BSA (Behringwerke, Germany), 75 μl $\text{Al}(\text{OH})_3$ (aluminium hydroxide gel, Alhydrogel, Superfoss Export Comp. A/S, Denmark), 125 μl 0.9% NaCl and 0.8 ml pertussis vaccine. IgG and IgM were given as 1.0 ml fractions (see above) mixed with 1.0 ml pertussis vaccine.

Cell suspension

Mast cells from the rat peritoneal and thoracic cavities were isolated according to the method of Thon & Uvnäs (10). If not otherwise stated, the pure mast cells were finally suspended in buffered salt solution in a concentration of 2,500 to 7,500 cells/ml. The salt solution was buffered to pH 7.0 with

10% Sørensen phosphate buffer and contained 0.131 M NaCl, 2.4 mM KCl, 1.0 mM CaCl_2 , 4.7 mM Na_2HPO_4 , 2.0 mM KH_2PO_4 , 5 mM glucose, and 1 mg/ml gelatine. For the radiolabelled antigen binding studies cells were suspended in buffered salt solution without CaCl_2 and glucose to avoid mast cell degranulation.

Hyposensitization

200 μl mast cell suspension was equilibrated at 37°C for 5 min. The cells were hyposensitized by preincubation at 37°C with specific antigen in suboptimal concentrations. The preincubation consisted of four 5-min periods with increasing antigen concentrations. BSA: 5×10^{-5} , 5×10^{-4} , 1.5×10^{-3} , and 6.5×10^{-3} mg/ml. IgG: 2.5×10^{-4} , 8×10^{-4} , 6×10^{-3} , and 1.6×10^{-2} mg/ml. IgM: 2×10^{-3} , 7×10^{-3} , 1.7×10^{-2} , and 3.7×10^{-2} mg/ml. All in final concentrations. Anaphylactic challenge was performed by addition of antigen up to final concentrations of 1.7×10^{-2} , 6.6×10^{-2} , and 1.4×10^{-1} for BSA, IgG and IgM, respectively. The cells were then incubated at 37°C for 10 min. The histamine released was quantitated according to Skov (7). All values were corrected for the spontaneous histamine release amounting to 1–4%. The inhibition of histamine release resulting from the preincubation was calculated as shown in the footnote of Table 1.

Statistics

In the Tables each figure represents mean value $\pm$ s.d. of eight experiments run in duplicates.

Fluorescein conjugates

Fluorochrome conjugated goat anti-rat immunoglobulin (GARa/FITC) batch 7-1073 (Nordic Pharmaceuticals and Diagnostics, Holland) was used. The antiserum was tested for specificity according to Skov (6).

The isolated IgG fraction from rabbit anti-BSA serum (Dakopatts, Denmark) was used

Table 1

Effect of preincubation of mast cells with antigen on histamine release and percentage of patch-bearing cells

Preincubation with BSA ^a (for 5 min unless otherwise stated)				Incubation with BSA ^a	Total histamine release (%)	RABSA/FITC
Without BSA for 20 min (control)				1.7×10^{-2}	24.7 ± 6	80 ± 11
5×10^{-5}	5×10^{-4}	1.5×10^{-3}	6.5×10^{-3}	0	8.2 ± 4.9	44 ± 12
5×10^{-5}	5×10^{-4}	1.5×10^{-3}	6.5×10^{-3}	1.7×10^{-2}	10.8 ± 5.6^b	82 ± 10
	6.5×10^{-5}	for 20 min		0	8.3 ± 3.0	41 ± 7
	6.5×10^{-5}	for 20 min		1.7×10^{-2}	18.8 ± 6.9^c	85 ± 2

^a Final concentration mg/ml^b % inhibition of histamine release $\frac{24.7-10.8}{24.7} \times 100 = 56\%$.^c % inhibition of histamine release $\frac{24.7-18.8}{24.7} \times 100 = 24\%$

and showed a monospecific reaction to BSA as visualized by immunoelectrophoresis (9). It was labelled with fluorescein isothiocyanate according to Wiik (11). Non-specific binding of this conjugate to mast cells was not seen. The fluorescein/protein ratio as estimated by OD 495/280 nm was 0.5. In the following this conjugate is designated RABSA/FITC.

Immunofluorescence technique

Before and after exposure to antigen, mast cell pellets were suspended in a droplet of GARA/FITC or RABSA/FITC, both diluted 1:10 with buffered salt solution. After incubation for 1 h at 4°C the cells were washed three times and immediately thereafter examined for surface fluorescence as judged by the presence of patch-bearing cells. Identical fluorescence patch formation was seen after GARA/FITC and RABSA/FITC (9).

All preparations were read in a Leitz Orthoplan microscope according to Skov (9). Each sample was counted twice, and 100 mast cells were counted each time. The number of patch-bearing cells was expressed as a percentage of the cells counted.

Radio-iodination of antigens

Bovine IgM and IgG fractions were labelled with I^{125} using chloramine-T. 100 µg of the protein was labelled with 5 mCi I^{125} . Free I^{125}

was separated from labelled antigen by Sephadex G-25 medium gel filtration (Pharmacia, Sweden). Approximately 80% of I^{125} was associated with antigen.

Assay for I^{125} -antigen bound to surface Ig on mast cells

Iodinated bovine IgM and IgG were used in the same final concentrations as used for the allergic histamine release. Samples of 2×10^5 mast cells/400 µl were incubated with I^{125} -antigens at 37°C for 10 min. All concentrations were in duplicate, and each experiment included sensitized as well as non-sensitized cells. Each sample was washed three times in 10 ml of buffered salt solution, the cell pellet was resuspended in 500 µl of buffered salt solution and 400 µl of the cell suspension was transferred to a counting tube with an Oxford automatic pipette. The pipette tip was left in the counting tube and the samples were placed in an automatic gamma-counter (Selektronik, Denmark).

Specific binding of iodinated antigen to sensitized mast cells was estimated as the difference between antigen bound to sensitized and non-sensitized cells. Variation in duplicate tubes was less than 5%. Approximately 1% of the total amount of labelled antigen was bound to sensitized mast cells. The amount of antigen bound to non-sensitized cells constituted only $15 \pm 4\%$

($n=6$) of that bound to sensitized cells. Bound antigen was estimated from a standard curve, in which known amounts of iodinated antigen were plotted against counts per minute. Specific binding was expressed as molecules of antigen per mast cell, which was calculated according to the formula:

$$\frac{\text{X g of antigen/mast cell}}{\text{MW}} \times 6 \times 10^{23}$$

in which $\text{MW}_{\text{IgG}} = 160,000$ and $\text{MW}_{\text{IgM}} = 800,000$.

RESULTS

Histamine release

Fig. 1 shows the release of histamine in mast cell suspensions from rats sensitized to bovine serum. Challenge of non-hyposensitized cells with bovine IgG in the range of 0.0003 to 0.3 mg/ml caused a gradual increase in histamine liberation up to 60%, whereas higher concentrations of IgG (0.3–1 mg/ml) resulted in a reduced histamine release. When hyposensitized cells were challenged, the maximal release of histamine was still obtained by 0.3 mg/ml IgG but the release was reduced by approximately 30%. Thus, the inhibition in histamine release by hyposensitization could not be overcome by challenge with higher

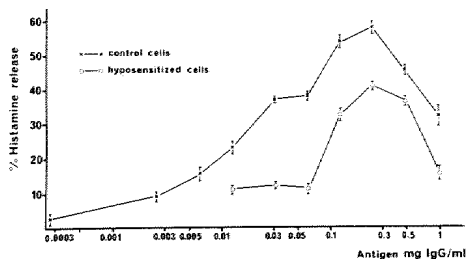


Fig. 1. Histamine release from hyposensitized and non-hyposensitized mast cells as a function of the antigen concentration. Each point represents the mean of triplicates $\pm$ s.d.

concentrations of antigen. The hyposensitization procedure caused in itself a histamine release of 11%.

Patch-bearing cells

Table 1 shows the relationship between BSA-induced histamine release and the percentage of cells showing fluorescent patches (Fig. 2) by the use of RABSA/FITC. The experiments include hyposensitized as well as non-hyposensitized cells. During preincubation (hyposensitization) some histamine was released. Preincubation of the cells by a stepwise increase in BSA concentration up to 6.5×10^{-3} inhibited the subsequent histamine release by 56%, whereas a single concentration of 6.5×10^{-3} caused only a 24% inhibition. Fluorescent patches on 40% of the cells were seen after either stepwise preincubation or one preincubation with 6.5×10^{-3} mg/ml BSA. After allergic challenge with optimal antigen concentration (1.7×10^{-2} mg/ml BSA) preincubated cells as well as control cells (non-hyposensitized cells) showed 80% patch-bearing cells.

Capping and shedding

No capping phenomena were observed after antigen-specific hyposensitization of BSA-sensitized cells using GARa/FITC as con-

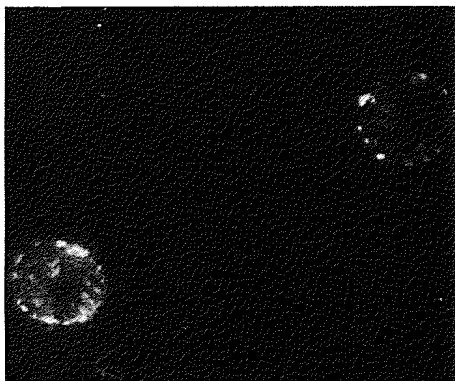


Fig. 2. Fluorescent patch formation on the mast cell surface.

Table 2

Percent patch-bearing cells before and after hyposensitization

Exp. No.	Inhibition of histamine release (%)	GARA/FITC	
		Before hyposensitization	After hyposensitization
1	52	100%	100%
2	58	85%	90%
3	78	90%	90%
4	79	95%	95%

jugate. The distribution of immunoglobulins on the cell surface was identical before and after hyposensitization (Fig. 2) and no change in the number of patch-bearing cells was observed (Table 2). This indicates that no shedding of immunoglobulins took place from the cell surface by hyposensitization.

Histamine release and number of antigen molecules bound to surface Ig

Mast cells sensitized to bovine serum were hyposensitized with radiolabelled bovine IgG or IgM and thereafter challenged for allergic histamine release. Histamine release was measured, and by counting the radioactivity, the number of antigen molecules bound to cell-fixed immunoglobulins was estimated. Tables 3 and 4 show the relationship between histamine release and the number of antigen molecules bound per cell.

Specific hyposensitization of the cells by preincubation and subsequent challenge with the same antigen resulted in a reduced histamine release. Reduced histamine release was also obtained by "crossed hyposensitization", i.e. preincubation with IgG and allergic challenge with IgM or vice versa. In both specific and "crossed hyposensitization" a stepwise increase of the antigen concentration caused a 50–60% reduction in histamine release in contrast to a reduction of 20–35% by non-stepwise (one dose) preincubation. However, specific and "crossed hyposensitization" with four increasing concentrations of antigen, or with the final concentration, resulted in quantitatively identical antigen binding.

After subsequent allergic challenge with optimal antigen concentration a higher binding was obtained, which was quantitatively identical whether the cells were hyposensitized or not hyposensitized by specific or "crossed hyposensitization" or by stepwise or non-stepwise hyposensitization.

The great difference in antigenic size between IgG and IgM did not influence the ability to induce hyposensitization in the mast cell.

Allergic cross reactivity

No allergic cross reactivity was observed between IgG and IgM, since IgG-sensitized cells responded only to IgG with allergic histamine release and hyposensitization and since these responses were obtained only by IgM in IgM-sensitized cells.

DISCUSSION

In vitro hyposensitization of pure mast cells was examined to obtain information about possible changes in the target cell which might explain cellular events responsible for the mechanism of specific hyposensitization therapy. Immunofluorescence technique and binding of radiolabelled antigens were used to study changes in the immunological events of the cell surface. The hyposensitization was evaluated by the reduction in allergic histamine release (4).

Surface distribution of immunoglobulins was examined by direct immunofluorescence technique. No changes were seen before and after hyposensitization in the surface dis-

Table 3

Binding of IgG molecules per mast cell after hyposensitization and after allergic histamine release

Preincubation	Incubation	Histamine release (%)	IgG molecules/cell
Without antigen for 20 min (control)	IgG ^d	39.7 ± 11	1.9 × 10 ⁵ ± 0.4 × 10 ⁵
IgG (four doses) ^a	0	10.6 ± 6	0.9 × 10 ⁵ ± 0.6 × 10 ⁵
IgG (four doses) ^a	IgG ^d	16.0 ± 8 ^b	2.3 × 10 ⁵ ± 0.6 × 10 ⁵
IgM (four doses) ^a	IgG ^d	21.2 ± 4 ^b	2.0 × 10 ⁵ ± 0.2 × 10 ⁵
IgG (one dose) ^b	0	28.5 ± 5	0.9 × 10 ⁵ ± 0.6 × 10 ⁵
IgG (one dose) ^b	IgG ^d	32.9 ± 9 ^b	1.6 × 10 ⁵ ± 0.6 × 10 ⁵
IgM (one dose) ^c	IgG ^d	26.1 ± 10 ^c	1.9 × 10 ⁵ ± 0.2 × 10 ⁵

^a See Method.^b 1.6 × 10⁻² mg/ml for 20 min.^c 3.7 × 10⁻² mg/ml for 20 min.^d 6.6 × 10⁻² mg/ml.Inhibition of histamine release in per cent: ^b60; ^c47; ^b18; ^c34.

tribution of Ig (Fig. 2), indicating that redistribution is not involved in the inhibition of histamine release by hyposensitization. Furthermore, it was obvious that neither capping nor shedding of detectable amounts of immunoglobulin took place on hyposensitized cells (Table 2). In an earlier study (9) it was shown that allergic challenge did not cause redistribution, capping or shedding in sensitized mast cells. The results strongly indicate that all these phenomena

do not occur after stimulation with antigen, either by weak stimulation (hyposensitization) or by optimal stimulation (allergic challenge).

The influence of hyposensitization on the distribution of antigen bound to surface Ig was evaluated by indirect immunofluorescence technique. Hyposensitization did not change the patchy distribution (Fig. 2) or the number of patch-bearing cells (Table 1).

Table 4

Binding of IgM molecules per mast cell after hyposensitization and after allergic histamine release

Preincubation	Incubation	Histamine release (%)	IgM molecules/cell
Without antigen for 20 min (control)	IgM ^d	38.4 ± 10	2.2 × 10 ⁴ ± 0.4 × 10 ⁴
IgM four doses) ^a	0	14.0 ± 3	1.2 × 10 ⁴ ± 0.4 × 10 ⁴
IgM (four doses) ^a	IgM ^d	20.1 ± 5 ^b	2.1 × 10 ⁴ ± 1.2 × 10 ⁴
IgG (four doses) ^a	IgM ^d	15.9 ± 9 ^b	2.5 × 10 ⁴ ± 0.9 × 10 ⁴
IgM (one dose) ^b	0	21.1 ± 9	1.0 × 10 ⁴ ± 0.5 × 10 ⁴
IgM (one dose) ^b	IgM ^d	25.1 ± 4 ^b	2.3 × 10 ⁴ ± 0.9 × 10 ⁴
IgG (one doses) ^c	IgM ^d	30.1 ± 7 ^c	2.6 × 10 ⁴ ± 0.6 × 10 ⁴

^a See Method^b 3.7 × 10⁻² mg/ml for 20 min^c 1.6 × 10⁻² mg/ml for 20 min^d 1.4 × 10⁻¹ mg/mlInhibition of histamine release in per cent: ^b48; ^c59; ^b35; ^c22

A change in the amount of cell-bound antigen by hyposensitization was examined by quantitation of I^{125} -labelled antigen according to Skov et al. (9). The results indicate that in rats sensitized to two different antigens, which do not cross react, preincubation of the mast cells with one of these inhibited subsequent histamine release induced by either of the two antigens, whereas no changes in cell-bound antigen were observed. This means that specific and "crossed hyposensitization" inhibit the histamine release without changing the antigen binding. Therefore, the hyposensitization does not seem to reduce the amount of free Ig on the cell surface. This also seems to be in accordance with the finding of a reduction in the histamine release curve but not of a parallel shift towards higher antigen concentrations (Fig. 1).

The mechanism of the hyposensitization cannot be ascribed to changes in the distribution of Ig on the cell surface or to a changed amount of antigen bound to the surface Ig. Therefore, capping and shedding phenomena as well as successive binding of surface immunoglobulin with antigen or steric hindrance can be excluded.

The mechanism might well be the result of events secondary to the initial antigen-antibody reaction, such as modifications in the cell membrane not detectable by the techniques employed in the present study, or exhaustion or modification of biochemical mechanisms involved in the allergic histamine release.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs. Kirsten Bak Sørensen for skilful technical assistance.

This study was supported by the Danish Medical Research Council (Grant No. 512-10238) and by the Merchant of Odense Johann and Hanne Weimann née Seedorff legacy.

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