Immunology, 1975, **28**, 1033.

Rat Tumour Allografts Evoke Anaphylactic Antibody Responses

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(*Received* 14*th October* 1974; *accepted for publication* 3*rd December* 1974)

Summary. *In vitro* assays of release of histamine from peritoneal mast cells showed that Wistar rats produced anaphylactic antibody in response to a single immunization with an allogeneic sarcoma. The response occurs early after immunization, and no adjuvant is needed. The thermolability of the anaphylactic antibody suggests that it is IgE.

INTRODUCTION

Though the adverse effects of anaphylactic antibody sensitization in the allergic disorders are obvious, a normal biological role for these antibodies remains obscure. Even in parasite infections where very high IgE levels often occur, a primary eradicative role for anaphylactic antibodies is not tenable (Urquhart, Mulligan, Gadie and Jennings, 1965; Kelly, 1973; Kelly and Ogilvie, 1972).

Nonetheless a high incidence of reaginic sensitization in humans following a number of bacterial and fungal infections as well as many other stimuli suggests that such antibodies play some adjunctive role in diverse immune reactions (Flick, 1972).

Reaginic responses detected by passive cutaneous anaphylaxis were reported by Bartholomaeus and Keast (1972) in mice immunized with allogeneic cells. In this study using direct measurement of histamine release from mast cells we found that rats readily and consistently form anaphylactic antibodies after a single immunization with allogeneic tumour cells.

MATERIALS AND METHODS

Animals and tumours

Inbred male Wistar rats aged between 10–17 weeks were used throughout. The tumour used for immunization was a fibrosarcoma (referred to as HSN) which originally had been induced in the Hooded rat with benzpyrene. As a control, a fibrosarcoma was used which had been induced in a Wistar rat with methylcholanthrene (WMC2). Both tumours were maintained by passage in syngeneic hosts.

Preparations of antigens

The procedure for obtaining soluble transplantation antigens involved papain digestion

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(Shimada and Nathenson, 1969). Approximately 300 g of minced HSN or WMC2 tumour tissue was suspended in a 0·25 mol/l sucrose buffer (2 mmol/l $MgCl_2$, 2 mmol/l $CaCl_2$, 1 mmol/l $NaHCO_3$) at pH 7·6, and homogenized with an ultra-turrax in a controlled manner so that the nuclei remained intact. The resulting tumour homogenate was centrifuged (600 ***g*** for 12 minutes) to remove nuclei and debris and the supernatant retained. The pellet was washed twice keeping the supernatants which were pooled and centrifuged (90,000 ***g*** for 2 hours). The pelleted membranes were dispersed in 160 ml of 5 mmol/l Tris–HCl buffer, pH 7·6. To this was added papain 2·5 mg/ml and L-cystine (to 0·05 M) and the whole incubated for 1 hour, at 37°. The reaction was stopped by adding iodoacetamide (to 0·05 M) and NaCl (to 0·05 M). The mixture was centrifuged (90,000 ***g*** for 2 hours) and the supernatant dialysed for 3 days against phosphate-buffered saline (Dulbecco 'A' Oxoid Ltd, London), aliquoted and stored at −20°.

Extracts made with 3 M KCl (as described by Thomson and Alexander, 1973) of both HSN tumour and normal Hooded rat tissue were also used, but these caused higher non-specific histamine release than the extracts prepared with papain.

The alloantigen content of the HSN papain digest preparation was assayed by its ability to inhibit the complement-dependent lytic activity of Wistar anti-Hooded alloantiserum (titration end point of 1:3000). A 1 : 25 dilution of the HSN preparation completely and specifically inhibited the lytic activity of 1 :400 dilution of the antiserum. The control alloantigen from Wistar tumour was assayed by an indirect immunofluorescent sandwich technique because of difficulty in raising a Hooded anti-Wistar alloantiserum with complement-dependent lytic activity. A 1 :16 dilution of the Wistar tumour preparation completely and specifically blocked the affinity for Wistar thymocytes of a 1 :320 dilution of a Hooded anti-Wistar alloantiserum (titration end point of 1 : 1280).

Immunization with tumour

Viable HSN tumour tissue was pressed through a fine wire mesh with a sterile test tube into Medium TC199 (Wellcome). A fine mesh (50 per cent medium, 50 per cent cells) was injected s.c. into the flank and the scruff. Where *Bordetella pertussis* (40×10^9 organisms/ml; Wellcome) was used as adjuvant it was mixed with the tumour inoculum. To get small controlled inocula, cell suspensions were prepared from the mush by slow, short centrifugation removing debris and cell clumps.

Sera

Rats were bled from the heart at varying times after tumour inoculation. Control sera were obtained as required by bleeding out three unimmunized animals of appropriate age and pooling the sera. All sera were held at −20°C and dispersed in small aliquots to avoid the need for repeated freeze-thawing. In all instances phosphate-buffered saline (Dulbecco 'A' pH 7·3; Oxoid) was the basic medium (PBS). Supplementation with Mg^{2+} and Ca^{2+} ions (both to 2 mM) was achieved by adding Mineral Salt Solution (Dulbecco 'B'; Oxoid).

Harvesting and exposure of sensitized mast cells to antigen

Rats were killed with ether and a 12-ml perforated plastic test tube (Henley) inserted through a small lower abdominal incision and 20 ml of ice-cold PBS instilled. After gentle abdominal massage for 90 seconds approximately 17 ml of wash-out fluid was with-

drawn by inserting a 10-ml plastic pipette (Sterilin) into the test tube, whilst suspending the rat by the tail when the PBS pooled in the diaphragmatic area. The cell suspension was held in ice, then spun at 800 ***g*** for 1 minute and re-suspended in PBS supplemented with Ca^{2+} and Mg^{2+} ions (PBS+MSS) and a mast cell count performed after adding 0·03 ml of 0·1 per cent toludine blue (in PBS) to 0·1 ml of cell suspension (at 37°). Between 4 and 10×10^5 mast cells in 0·1 ml were then aliquoted to plastic test tubes (5 ml, Henley) containing 0·9 ml of just PBS−MSS, or PBS−MSS+antigen, at 37°. Antigen dosages are indicated in the tables containing the results. The release phase incubation time was 5 minutes (at 37°), and was terminated by rapid cooling to +4°. All tubes were centrifuged (800 ***g*** for 5 minutes) and the supernatants added to 1 ml of 10% trichloroacetic acid (TCA).

Two millilitres of 5 per cent TCA was added to the pellets which were agitated on a whirlimixer. All tubes were centrifuged again (4500 rev/minute for 10 minutes), on a B.T.L. bench centrifuge and the supernatants assayed for histamine. All assays were performed in duplicate.

An *in vivo* technique for antigen challenge was also used. Antigen dissolved in PBS−MSS (12 ml, 37°, 0·05 ml HSN antigen per millilitre of medium) was injected into the peritoneal cavity and dispersed to all quadrants by posturing and minimal gentle massage. After 5 min 8 ml of ice-cold PBS was instilled and immediately 17 ml of fluid withdrawn. One-millilitre aliquots were taken, rapidly cooled to 4° and centrifuged (800 ***g*** for 5 min). The supernatants and pellets were treated as before.

Attempts were made to trigger mast cells with live HSN cells dislodged mechanically from *in vitro* culture flasks using glass beads. The HSN cells were included in the assay tubes in place of soluble antigen. Alternatively 10^8 spleen cells were used as described in the Results section.

Passive sensitization

In the *in vivo* method 1 ml of immune or control serum (diluted to 3 ml with PBS) was injected intraperitoneally into a normal rat and after 2 hours antigen challenge was performed *in vivo* (vide supra). In the *in vitro* method, mast cells were harvested from three to five normal rats (9 weeks old), pooled and resuspended in 0·9 ml of PBS (not containing Mg^{2+} or Ca^{2+} ions). Mast cells ($2–3 \times 10^5$ in 0·3 ml) were added to 0·3 ml of sensitizing or control serum and incubated at 37° for 1½ hours. Shaking was necessary every 10 minutes to keep the cells suspended. The cells were then washed twice in ice-cold PBS (with very gentle centrifugation; 400 ***g*** for 1½ min). The mast cells were re-suspended at $5–7 \times 10^4$ cells/0·1 ml of PBS+MSS, and added to antigen containing assay tubes as already described.

Histamine assays

Histamine was assayed fluorimetrically by the method of Shore, Burkhalter and Cohn (1959) by conjugation with *o*-phthalaldehyde and measurement of fluorescence using an Aminco fluoromicrophotometer. Results are expressed as percentage histamine release (supernatant histamine) calculated as the fraction of the total cellular histamine(supernatant histamine plus pellet histamine). In all experiments the following controls were carried out: (1) PBS−MSS alone was assayed to give background fluorescence; (2) both sensitized and control mast cells were incubated in the absence of antigen to give the *spontaneous* histamine release during the assay; (3) incubation of control mast cells

with antigen gave non-specific release due to the antigen preparation. All figures in the tables are corrected for background and spontaneous release except for the *in vivo* assays (Table 2) where the spontaneous release is unknown. Unless otherwise stated, spontaneous release was always less than 10 per cent of the total histamine content of the mast cells.

RESULTS

Table 1 shows that allogeneic immunization in conjunction with *Bordetella pertussis* as adjuvant gave rise to mast cells which released histamine on the addition of antigen. Marked sensitization of the mast cells was evident as early as 10 days, and as late as 43 days postimmunization. Specific release of histamine was consistently observed but its magnitude varied with different preparations of the antigen. The specific histamine release was not altered by varying the number of mast cells exposed to antigen *in vitro*.

Table 1 also shows that adjuvant is not necessary to obtain sensitized mast cells following allogeneic immunization. Mast cells from six of the seven rats tested were sensitized to a degree comparable to the adjuvant series.

However, sensitization is not so successful following immunization with a large amount of tumour. Rats that had been immunized with 0·25 ml of tumour mush at four s.c. sites and 0·25 ml intraperitoneally, without adjuvant, failed to become sensitized. Whereas 0·25 ml given s.c. at one site (without adjuvant) or at two sites (with adjuvant) consistently induced sensitized mast cells.

Passive transfer experiments showed clearly that immune serum from rats immunized with allogeneic tumour sensitized non-immune rat mast cells if the procedure outlined in the Materials and Methods section was followed; i.e. the mast cells were washed only twice and that shaking was maintained during the test (see Table 2). Variations from these conditions showed that good transfer occurred only if during sensitization the cells were shaken regularly to enhance cell contact with antibody. Exclusion of Mg^{2+} and Ca^{2+} ions during the sensitization phase seemed to improve sensitization, which is in agreement with findings in the leucocyte system (Levy and Osler, 1966) but differs from those of another group (Evans and Thompson, 1972), who found that the presence of these ions was necessary.

Heat inactivation (56°, 60 min) reduced the serum's sensitizing capacity but did not abolish it. Petillo and Smith (1973) have found that high titre sensitizing sera require a prolonged period of heat inactivation. Five hours at 56° virtually abolished the sensitizing activity of the serum used in the passive transfer experiments (Table 2).

Table 1 also shows that mast cells from a further series of Wistar rats immunized with HSN cells without adjuvant are triggered by the soluble HSN antigen but not by an antigen prepared from the WMC2 tumour. Wistar rats (syngeneic with WMC2 tumour) differ from the Hooded rats (syngeneic with the HSN tumour) at one major histocompatibility locus at least.

Soluble antigen (papain or KCl extracts) were used in all the experiments, but whole cells will also trigger sensitized mast cells. However, to obtain sufficient contact with the mast cells a high concentration of (10^8/ml) allogeneic cells and a long incubation time were used. Such high concentrations of viable cells in suspensions were difficult to achieve with tumour cells and for this reason allogeneic spleen cells were used. Approximately the same specific histamine release was induced by the Hooded rat spleen cells and the soluble antigen from the HSN tumour in Wistar rats immunized with tumour (see Table 1).

TABLE 1

RELEASE OF HISTAMINE FROM MAST CELLS OF WISTAR RATS IMMUNIZED WITH ALLOGENEIC (HSN) SARCOMA*

Time (days) after immunization when mast cells were taken†	Antigen used (quantity in parenthesis)	Percentage histamine release from: Mast cells of immunized rats	Mast cells of control rats
Immunization with adjuvant (*Bordetella pertussis*)*			
9	HSN papain extract number 1 (0·4 ml)	12	3
10	HSN papain extract number 1 (0·4 ml)	37	7
10	HSN papain extract number 1 (0·4 ml)	47	9
12	HSN papain extract number 1 (0·4 ml)	31	2
12	HSN papain extract number 1 (0·4 ml)	28	11
17	HSN papain extract number 1 (0·4 ml)	11	3
17	HSN papain extract number 2 (0·05 ml)	17	6
17	HSN papain extract number 2 (0·1 ml)	38	18
43	HSN KCl extract (0·4 ml) tested against:		
	8×10^4 mast cells**	63	—
	4×10^4 mast cells**	65	20
	2×10^4 mast cells**	68	—
Immunized with tumour only‡			
12	HSN papain extract number 2 (0·05 ml)	31	6
13	HSN papain extract number 2 (0·05 ml)	41	11
13	HSN papain extract number 2 (0·05 ml)	7	3
14	HSN papain extract number 2 (0·05 ml)	15	6
14	HSN papain extract number 2 (0·05 ml)	15	4
17	HSN papain extract number 2 (0·05 ml)	24	6
21	HSN papain extract number 2 (0·05 ml)	55	27¶
21	Hooded spleen cells	25	1
Immunized with multiple doses of tumour§			
17	HSN papain extract number 1 (0·4 ml)	1	10
20	HSN papain extract number 1 (0·4 ml)	13	4
20	HSN papain extract number 2 (0·4 ml)	1·5	4
Specificity of antigen (following immunization with tumour only)‡			
18	HSN papain extract number 2 (0·1 ml)	31	10
	Wistar tumour extract (0·1 ml)	7	0·5
19	HSN papain extract number 2 (0·05 ml)	13	5
	Wistar tumour extract (0·05 ml)	0	0
20	HSN papain extract number 2 (0·05 ml)	21	10
	Wistar tumour extract (0·05 ml)	2	2

* 0·5 ml of HSN tumour mush was mixed with 4×10^{10}/ml *Bordetella pertussis* organisms and injected s.c. into the flanks and scruff (i.e. total for rat 1·5 ml).

† Mast cells taken from an individual rat.

‡ 0·25 ml of HSN tumour mush into scruff.

§ 0·25 ml of HSN tumour mush into 4 s.c. sites and intraperitoneally.

¶ The incubation time of the mast cells with the antigen was 18 minutes and this caused high (i.e. 27 per cent) non-specific release.

** All the mast cells used here were from a single sensitized rat.

TABLE 2

SENSITIZATION OF MAST CELLS BY SERUM FROM WISTAR RATS IMMUNIZED WITH ALLOGENEIC (HSN) SARCOMA

Method of sensitizing mast cells	Serum used	Percentage histamine release from mast cells sensitized with serum
In vivo		
Challenge with antigen *in vivo* also	Immune*	34
	Control†	18·5
In vitro		
Cells shaken, and washed twice	Immune	32
	Immune heated 56° for 1 hour	19
	Control	7
Cells shaken, and washed twice	Immune	33
	Immune heated 56° for 5 hours	14
	Control	11
In vitro		
Cells not shaken, and washed three times	Immune	4·5
	Control	0·5
In vitro		
Cells shaken, and washed three times	Immune	21
	Control	−2
Cells shaken, and washed three times	Immune	8
	Control	1·5

* 0·25 ml of HSN tumour mush injected into scruff without adjuvant and serum taken 13 days later.

† Control serum = pooled normal Wistar serum.

DISCUSSION

The induction of anaphylactic antibody in Wistar rats following immunization with tumour allograft seems to be established in that: (1) mast cells from immunized animals were triggered by soluble HSN antigen and Hooded rat spleen cells to release much greater quantities of histamine than control mast cells; (2) this release appears to be specific because the same mast cells are not triggered by an antigenic extract prepared, in an identical fashion from a fibrosarcoma syngeneic in Wistar rats; (3) the serum from immunized animals will sensitize *in vitro* the mast cells of non-immune rats.

When soluble proteins such as heterologous gamma-globulins, ovalbumin, or hapten–protein conjugates were used for immunization, strong and consistent homocytotropic antibody levels were in general only achieved with the assistance of an adjuvant (Mota, 1958; Mota, 1964; Binaghi and Benacerraf, 1963; Smith, Hwang, Eichelberger and Randall, 1973). This contrasts with this study where the tumour allografts induce readily detectable anaphylactic antibody responses early after primary immunization without the addition of an adjuvant.

Since passive transfer with serum could be demonstrated, circulating anaphylactic antibody must be present. The antibody class involved is probably IgE (Stechschulte, Orange and Austen, 1970; Bazin, Beckers and Querinjean, 1974) because of its thermolability (Bloch and Wilson, 1968). The prolonged period of heating required to inactivate the serum further suggests that the antibody levels achieved were substantial (Petillo and Smith, 1973) but the possibility remains that heat-stable antibody of the IgGa type (Morse, Bloch and Austen, 1968; Morse, Austen and Bloch, 1969) was also present as the serum activity was not completely abolished after 5 hours.

Ability to form reaginic antibody has a strong genetic element (Vaz, Vaz and Levine, 1970; Provoust-Danon, Stiffel, Moreton and Biozzi, 1971) and a reaginic response to an allograft may represent an incidental component of a broad immune reaction to the allograft which happens to have been placed in an animal which is a 'good responder' in terms of IgE production (Provoust-Danon, *et al.*, 1971; Stanworth, 1973). On the other hand, it has been argued that the increased vascular permeability caused by mast cell products may be a useful effect in the control of local bacterial infections (Bloch, 1967). Increased permeability might enhance the access of various elements of the immune effector response to the site of bacterial invasion or in the case of a graft, the site of implantation. The data presented here adds to the growing evidence (Flick, 1972) that a very large variety of stimuli will induce reaginic antibody responses. It may be that normal non-atopic individuals produce minute but, nevertheless, immunologically important quantities of antigen-specific reaginic antibody to these stimuli. The importance of these findings in terms of allograft survival is not clear. It is known that the antihistamine promethazine hydrochloride will depress delayed hypersensitivity and antibody responses (Gusdon, Moore, Myrvik and Holyfield, 1972) and delay parasite rejection (Kelly and Dineen, 1972; Ogilvie and Jones, 1973) but the evidence points to an action at lymphoid cell level. Histidine decarboxylase inhibitors will prolong rat skin allograft survival (Moore and Lawrence, 1969) but the mechanism is uncertain.

ACKNOWLEDGMENTS

The authors are very grateful to Dr D. E. Stanworth and members of his department (Experimental Allergy Group, Department of Experimental Pathology, University of Birmingham) for demonstrating the mast cell and histamine assays; and to Dr C. Dean and Dr L. Gauchi for assaying the tumour membrane preparations for antigen activity. B.C.B. held a New Zealand M.R.C. Training Fellowship in Immunology and this investigation has been supported by grants from the Medical Research Council and the Cancer Research Campaign.

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