

Immune Stimulated Regional Inflammatory Responses Mediating Lung Reactivity in Rats

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Daily sensitization of SPF BNxWi/Fu rats with ovalbumin (OA) in aerosol during 2-week periods with a 4-week interval resulted after 7 weeks in IgE, IgA and IgG antibodies in serum and bronchial fluid. After cultivation of the regional, axillary, brachial and mediastinal (ABM) lymph node cells, IgE antibodies were found in the culture supernatant. Such antibodies were not found in culture supernatants of spleen and inguinal lymph nodes. Regional formation of IgE antibodies was also noted in the ABM lymph node cell culture supernatants after subcutaneous (s.c.) injections of 100 ng OA in the neck region. When the injections were given in the tail-root region, the inguinal but not the ABM lymph nodes produced the IgE antibodies. The s.c. sensitization induced inconsistent and rather low IgG and no IgA antibody responses. The aerosol but not the s.c. sensitization induced accumulations of mononuclear cells and mucous cells in the lungs. Clinically, the rats sensitized s.c. in the neck region reacted to aerosol and intravenous (i.v.) challenge as early as 1 week after sensitization had started, whereas the animals sensitized in the tail-root regions reacted 7 and 8 weeks after repeated sensitization. The animals sensitized by aerosol showed only weak clinical reactivity after i.v. challenge.

Key words: allergy; IgE; mast cells; mucous cells; bronchoconstriction; rats.

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CLINICAL ASPECTS

Various components of the immune system act in concert, which may result in an allergic inflammation. Albeit well-known, the regional occurrence of clinical symptoms has not been easily explained. In the present model using immunization protocols without adjuvant, a regional stimulation of the immune system in the neck region resulted in antibodies of IgE and other isotypes together with locally occurring mediator releasing cells. This resulted in local clinical reactivity early during the immunization which was not found in the animals after sensitization in the tail-root. The clinical reactivity was later manifested systematically. This seems to represent a relevant animal model for allergic reactions in the airways and allows further studies of the mechanisms responsible for allergic inflammatory reactions.

Extrinsic asthma is influenced by various humoral products and cells triggered by immune reactions, e.g. antibodies of IgE and other serotypes, and substances formed by T cells which stimulate mast cells, basophils and mucous cells to mature and differentiate (4).

Studies of parasite infected animals have shown that increased numbers of mast cells and mucous cells appear as a part of T cell-evoked

tissue inflammation. This has been shown to develop in the gut after local stimulation of immune cells with antigen (9, 10). We have recently presented similar results from studies in the lung using simple protein antigens (1, 2) and protein reactive hapten (6). Such local cell accumulations as part of the immune response demonstrate that the events causing an asthmatic reaction are multifactorial. It also em-

phasizes that much of the immune responses developed regionally and that systematically occurring components of immunity are not necessarily relevant for the reactivity in a shock organ (2, 3).

To understand better the importance of local immunity we compared the immune responses developing after regional sensitization of rats in the airways, neck region and tail region. The immunity was reflected as IgE antibody formation, appearance of mast cells in the regional lymph nodes and lung, and mucous cells in the lung. These results were related to clinical reactivity after airway challenge with antigen.

MATERIAL AND METHODS

Animals

Specific pathogen-free hybrid rats of BNxWi/Fu strain of both sexes, 6–12 weeks old, weighing 180–320 g were used (Anticimex, Stockholm, Sweden).

Antigens

Ovalbumin (OA) grade V, and DSA, fraction V (Sigma Chemical Co., St. Louis, Mo) were used.

Immunization

Groups of rats were exposed to antigen Monday through Friday for 1- or 2-week periods with a 4-week interval. The antigens were administered by subcutaneous (s.c.) injections in the neck and tail-root and by aerosol inhalations (1, 12).

For the s.c. immunizations, 0.2 ml OA in sterile saline to a concentration of 0.5 µg/ml was injected. For the aerosol immunizations, OA was dissolved in distilled H₂O to a concentration of 1%, and 5 ml was delivered into a nebulization chamber (56.5 × 56.5 × 32 cm) using a Bird micronebulizer. The chamber was first equilibrated for 5 min with a nebulizer air pressure of 10 psi/in². The immunization then proceeded for 30 min at a nebulizer air pressure of 6 psi/in² and an air flow of 2 l/min through the chamber caused by a pump device, resulting in a

chamber air pressure of 2 cm H₂O below atmospheric pressure. Air was then blown through the chamber for 30 min before opening it after the immunization procedure. Maximum bleeding was performed from the retroorbital plexus under ether anaesthesia, and the animals were then killed using ether.

Test on cell-mediated immunity

The spleen and the axillary, brachial, mediastinal (ABM) and inguinal lymph nodes were immediately removed under aseptic conditions and placed in ice-cold Hank's balanced salt solution (HBSS) (Flow Labs., Svenska AB, Solna, Sweden). Axillary, brachial and mediastinal lymph nodes (ABM) were pooled and single cell suspensions were prepared by perfusion of the specimens. The spleen cell suspensions were separated from red blood cells on Ficoll-Paque (Pharmacia AB, Uppsala, Sweden), washed twice in HBSS and resuspended (2×10^6 /ml) in McCoy's medium, pH 7.4, supplemented with 10% fetal calf serum, 2 mM glutamine, 5×10^{-5} M β -mercaptoethanol, 20 mM HEPES buffer (Flow Labs) and 8 µg/ml gentamicin (Flow Labs.).

Cultures of 2 ml with either inguinal, ABM, spleen cells or a mixture of spleen and ABM cell suspensions were incubated for 21 days in 24-well plates (Flow Labs.) at 37°C in 7% CO₂ and 95% humidity. Cultures were stimulated with 5 µg/ml leucoagglutinin (LA) (Pharmacia AB) or 30 µg/ml OA, or left unstimulated. Half of the culture medium was replaced twice a week with fresh medium containing an appropriate concentration of antigen or mitogen. Culture medium was collected after 4 and 7 days for antibody analysis. Cell preparations were made on days 0 (preculture), 14 and 21 using a Cytospin centrifuge (Shandon Southern Inst., Inc., Pa). The specimens were fixed in 95% ethanol for 15 min and stained with Alcian blue-safranin and analysed for mast cell counts.

Histological examination

After removal of the lymph nodes the bronchi were washed 3 times by infusion of 2 ml HBSS

containing 0.1% EDTA injected into the surgically exposed trachea (Venflon® i.v. cannula, Viggo AB, Helsingborg, Sweden). Cells from bronchial lavage fluid were prepared and stained as already described (1). The fluids were also analysed for antibody content. The lungs were then maximally insufflated with a fixative of 85% methanol, 5% acetic acid and 4% formaldehyde (MAF) in water, removed and kept in MAF for 24 h at +4°C. The specimens were then dehydrated in 99.5% ethanol and embedded in acryl plastic (Sorvall) according to the manufacturer's instruction. The histological examinations were done according to Ahlstedt et al. (1) using a 4-grade scale.

Antibody determination

The IgE antibody levels in all specimens were determined with radioallergosorbent test (RAST) altered according to Hemady et al. for maximum sensitivity (8). Samples from cultures and bronchial lavage were diluted 1:1 with culture medium and serum 1:9 with a phosphate tween-dextran buffer. Aliquots of 100 µl were incubated with OA-conjugated paper discs (5) for 24 h at +18°C under slow shaking. The discs were washed using DA-300 dispenser-aspirator (Pharmacia AB). I¹²⁵-tracer (rabbit anti-rat-IgE antibodies obtained from Dr Torbjörn Karlsson, Biocell, Uppsala) was diluted in culture medium to a concentration of 0.08 ng/ml giving a radioactivity of 35,000–50,000 cpm/200 µl. Aliquots of 200 µl were incubated with the discs as described above. The discs were washed again and the bound radioactivity was measured.

The IgG and IgA antibody levels were determined with the enzyme-linked immunosorbent assay (ELISA) (1), using specific rabbit anti-rat IgG and goat anti-rat IgA sera (Nordic Labs., Tilbury, The Netherlands). The gamma-globulin fractions were conjugated with alkaline phosphatase (Sigma). Optical density was determined at 405 nm after 100 min of enzyme reaction using a Titertek multiscan spectrophotometer (Flow Labs.), as previously described (1). The serum dilution giving the

optical density of 0.25 units above the background of a non-immune control serum at 405 nm was considered the antibody titre.

Respiratory measurements

The animals were anaesthetized with Inactin® (Promota, Hamburg, FRG), 125 mg/kg body weight given as a single intraperitoneal injection, and placed on a heating pad connected to a thermostat to keep the body temperature at 37°C. They were tracheostomized with a polyethylene tube (o.d. 2.4 mm) and polyethylene catheters were inserted into the right common carotid artery and the left external jugular vein for blood pressure recordings and intravenous (i.v.) injections, respectively. The animals were given 0.5 mg/kg i.v. of pancuronium bromide (Pavulon®) for muscle relaxation and were ventilated with a constant volume respirator (Palmer) as previously described (12).

The respiratory frequency was set to 76–80 strokes/min with a tidal volume determined by the formula: $v = mx \cdot 6.26 \times 10^{-3} + 0.727$ (v in ml, m in g). This resulted in a PaCO_2 of 4.7–5.4 kPa. Ventilation was performed with a positive end-expiratory pressure of 2 cm H_2O to avoid spontaneous atelectasis.

Transpulmonary pressure (TPP) was measured by connecting one outlet of a differential pressure transducer (Statham PM 131 TC) to a side arm of the tracheal tube (tracheal pressure), and the other to an indwelling teflon catheter (Braunüle® o.d. 2.0 mm) which was inserted through the 5th or 6th intercostal space (intrapleural pressure). Mean arterial blood pressure (MAP) was measured with an IPM-transducer and both TPP and MAP were continuously recorded on a UV oscillograph recorder (Ultralette, ABEM, Stockholm). Arterial blood samples were collected before and after the airway provocations for analysis of blood gases using an IL automatic blood gas analyser (Instrumentation Labs., Inc., model 613).

The animals were challenged with an antigen aerosol (OA or DSA) of 10 mg/ml in distilled H_2O for 10 min using an Ultrasonic nebulizer model 108 (Gambro-Engström Medical AB,

Stockholm) connected to the respirator. This resulted in a total delivery of 4–5 ml antigen solution to the nebulization chamber. After hyperinflation of 3 strokes and 10 min recovery the animals were challenged intravenously with 5 mg OA in 0.5 ml saline or with serotonin 500 µg/kg.

Statistical analysis

The results from the different groups were analysed using the Wilcoxon rank sum test.

RESULTS

Antibody formation

A local formation of IgE antibodies in the draining lymph nodes but not in other lymph nodes of immunized rats was detectable in the supernatants of cultured cells (Fig. 1). In the supernatants of spleen cell cultures, low levels of IgE antibodies were detectable from aerosol immunized but not from s.c. immunized rats (results not shown).

Aerosol immunization also resulted in the appearance of IgE antibodies in the bronchial lavage fluid, whereas most of the s.c. immunized animals had less antibodies in this compartment. There was no obvious correlation between the IgE antibody levels in the bronchial lavage fluid and the culture supernatants from ABM lymph node cells. The IgE antibody levels were low in serum after the various immunizations.

Considerable levels of IgG and IgA antibodies were noted in serum and bronchial fluid after aerosol immunisation, whereas s.c. immunizations resulted in lower antibody levels detectable in a few animals only (Table 1). In the supernatants of lymph node cell cultures no IgG and IgA antibodies could be detected in any of the animals (not shown).

Histological picture of the lungs

The lungs of control and s.c.-sensitized rats showed scattered accumulations of mononuclear cells associated to bronchioli, vessels and interstitium. In the aerosol-sensitized animals

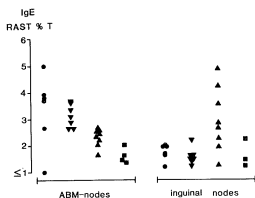


Fig. 1. Levels of IgE antibodies to ovalbumin in non-stimulated supernatants in 4-day cultures of lymph node cell from rats immunized by aerosol (closed circles), s.c. in the tail-root (triangles) or neck (inverted triangles) and in non-immunized controls (squares).

mononuclear cell accumulations were noted predominantly located around bronchioli (Table 2).

Mast cells were seen in the lungs of animals in all groups. They were apparently not influenced by sensitization (Table 2) except the mast cells in the aerosol-exposed animals, which were mostly located adjacent to the bronchioli.

The numbers of mucous cells were significantly higher in the aerosol-sensitized animals than in any of the other groups ($P < 0.01$; Table 2). The mucous cells were preferentially located in connection with accumulations of mononuclear cells in the bronchiolar wall.

Clinical lung reactivity

The non-sensitized animals did not show any increases in TPP (Fig. 2) and MAP after aerosol and i.v. antigen challenge. In sensitized animals clinical reactivity was noted as an increase in TPP and a simultaneous decrease in MAP.

All animals, except one, sensitized s.c. in the neck region (Fig. 2) reacted to aerosol challenge after both one and two sensitization periods. In contrast, none of the animals sensitized in the tail-root region reacted to aerosol after one sensitization period and two of three reacted after two periods. The reactivity to i.v.

challenge appeared before the reactivity to aerosol challenge in both s.c. regimens.

Aerosol-sensitized animals (Fig. 2) reacted only to i.v. challenge, and the increase in TPP was lower and peaked later than in s.c.-sensitized animals.

DISCUSSION

This study demonstrates that the early phase of an immune response resulting in allergic in-

flammation is regional, in regard to formation of IgE antibodies, appearance of mast cells and mucous cells, and clinical reactivity. The IgG and IgA antibodies, however, were possibly more related to systemic immunity, thus supporting a recent study (2). The regional production of IgE antibodies is further supported by a recent study of the immune response to parasitic antigen (3). The development of a systemic IgE antibody response to the simple ovalbumin antigen late during the observation

Table 1

Antibody responses in serum and bronchial fluid in individual rats. The IgG and IgA antibodies were recorded with the ELISA and the results presented as titres. The IgE antibodies were recorded with RAST and the values given as per cent of radioactivity uptake. Titres less than 20 (serum) and less than 2 (bronchial fluid) and uptake less than 2% were considered negative

Immunization rat No.	Serum			Bronchial fluid		
	IgE	IgG	IgA	IgE	IgG	IgA
Aerosol						
1	neg	16943	352	4.3	50	54
2	neg	>20000	>1000	3.8	>100	76
3	neg	17248	685	4.4	54	16
4	neg	11846	515	4.1	52	24
5	neg	12518	376	3.4	>100	77
6	neg	10906	289	3.8	75	91
S.c. neck						
7	neg	neg	neg	3.4	neg	neg
8	2.3	54	neg	3.9	neg	neg
9	neg	neg	neg	3.1	neg	neg
10	3.2	293	neg	3.1	neg	neg
11	2.6	neg	neg	2.6	neg	neg
12	neg	neg	neg	2.1	neg	neg
13	2.9	neg	neg	2.0	neg	neg
14	neg	neg	neg	neg	neg	neg
S.c. tail-root						
15	neg	14713	neg	2.9	16	neg
16	neg	1094	neg	2.7	2	neg
17	neg	5403	145	2.8	6	neg
18	neg	neg	neg	neg	neg	neg
19	2.8	neg	neg	neg	neg	neg
20	2.0	neg	neg	neg	neg	neg
21	2.1	neg	neg	2.3	neg	neg
22	4.2	neg	neg	2.6	neg	neg
Control						
23	neg	neg	neg	neg	neg	neg
24	neg	neg	neg	neg	neg	neg
25	neg	neg	neg	neg	neg	neg
26	neg	neg	neg	neg	neg	neg

Table 2

Histological findings in individual rats sensitized s.c. or by aerosol and in non-sensitized controls. The cell numbers are given for individual animals using a 4-grade scale for inflammatory cells (i.c.) and mast cells (m.c.) and as average number of mucous cells (mu.c.) per mm of bronchioli

S.c. in tail-root (rat Nos. 15-22)			S.c. in neck (rat Nos. 7-11)			Aerosol (rat Nos. 1-6)			Controls (rat Nos. 23-24)		
i.c.	m.c.	mu.c.	i.c.	m.c.	mu.c.	i.c.	m.c.	mu.c.	i.c.	m.c.	mu.c.
+	0.8	0.6	+	1.5	0.5	++	1.2	4.2	-	0.2	0.2
+	1.1	0.0	-	0.7	0.3	++	1.5	5.4	+	1.0	0.0
-	0.8	0.5	-	0.5	0.3	-	1.5	0.6	-	0.7	0.0
-	1.0	0.1	-	1.1	0.0	++	1.2	1.0	-	2.0	0.0
+	0.7	0.3	-	1.0	0.4	++	2.2	6.3			
+	1.0	0.7	-	2.0	0.1	++	0.3	2.0			
-	1.0	0.0	+	2.0	0.2						
-	0.6	0.3	-	0.8	0.0						
Median values											
+/-	0.9	0.3	-	1.1	0.3	++	1.4	3.1*	-	0.9	0.0

* Significantly increased compared with controls and s.c.-sensitized animals.

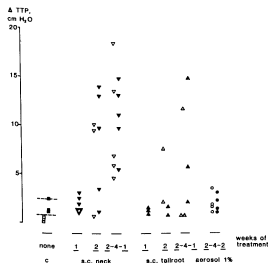


Fig. 2. Increase in transpulmonary pressure (Δ TTP) after challenge with antigen as aerosol (filled symbols) and i.v. (open symbols). Controls: squares; s.c. injection in the tail-root: triangles; s.c. injection in the neck: inverted triangles; aerosol inhalations: circles. The weeks for sensitization are underlined. The upper 95-percentiles of the values for control animals are indicated.

period also corresponds to findings for migrating parasites as shown by Befus et al. (3).

The more accentuated IgG and IgA antibody responses after aerosol sensitization compared with those after s.c. immunization may be related to the presumably higher antigen dose given by aerosol than by the s.c. injections. However, this does not agree with our previous findings of a similar antibody formation occurring in response to the two antigen administration regimens (1, 2, 12). Some of the discrepancy may be because SPF rats were used in this study; these rats have less lung infections than the animals used previously (to be published).

The result of using SPF rather than non-SPF animals used previously was also expressed in the histological picture of the lungs. In the control animals, there was very little inflammation. Consequently, the numbers of mast cells and mucous cells were low. Aerosol sensitization resulted in increased inflammation, reflected as accumulation of mononuclear cells and mucous cells, as we have shown before (1). In contrast to our previous findings, however, the mast cell numbers were only slightly affected by the aero-

sol exposure. This corresponds to our findings in SPF mice, in which irritation or inflammation seem to greatly enhance the immunologically stimulated appearance of mast cells in the lung (6). Mast cells also appear in response to pure irritation, independent of immunization (13).

Despite the presence of antibodies, signs of inflammation and increased numbers of mast cells and mucous cells in the lungs, the aerosol-sensitized animals were only slightly reactive to challenge with antigen. The animals sensitized s.c. in the neck region responded well despite having less antibodies and no inflammation, and normal numbers of mast cells and mucous cells. This agrees with our earlier observations (1, 2, 12). The results are possibly due to increased production of mucus and antibodies of IgG and IgA class protecting the mucous membrane (to be published), in addition to decreased sensitivity to mediators like serotonin (1).

In conclusion, the present study reveals a regional immune response to antigen, including antibodies and inflammatory cells. The clinical reactivity to antigen challenge also varied with the rank of sensitization.

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