Canine cutaneous mast cells dispersion and histamine secretory characterization

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Abstract

In view of the high incidence of canine cutaneous atopic disease and the relevance of mast cells to its pathogenesis, it was considered important to isolate firstly cutaneous mast cells from normal dog skin and to assess the histamine secretory activity, as this can be further used as a tool for the study of canine skin mast cell pharmacology in cutaneous atopy. The procedure for canine dermal mast cell dispersion following a skin enzymatic digestion (as for previous human skin mast cell dispersion methods) is described in detail. The number of canine cutaneous mast cells yielded per gram of skin was $2.31 \pm 0.21 \times 10^3$ representing 1.00% of the total cutaneous cells. The total histamine content per mast cell is $4.93 \pm 0.39$ pg. Net histamine release owing to stimulation by calcium ionophore A23187 ($1 \mu M$) and concanavalin A (1 mg ml$^{-1}$) was respectively $32.17 \pm 3.56$% and $20.39 \pm 2.41$% of the total amount per cell. Viability and reactivity to both stimuli of dispersed cutaneous mast cells were similar to the results found in humans. The present study allows further research on the role of mast cells immunopharmacology in allergy by investigation of cells isolated from canine skin in naturally occurring or experimentally induced atopy in the dog to be undertaken.

Abbreviations

AD, atopic dermatitis; FCS, foetal calf serum; HBSS, Hank's balanced salt solution; MC, mast cells; MEM, Minimum Essential Medium; OPT, O-Phthaldialdehyde; TCA, trichloroacetic acid.

Introduction

Like human atopic dermatitis (AD) and allergic asthma, canine AD is thought to be a classic example of an atopic disease (Butler et al., 1983; Willems, 1987). Atopy is generally accepted as being an hereditary allergic condition mediated, at least in part, by a Type I hypersensitivity reaction, the immunological mechanisms of which have been widely studied and recently revised (Hanifin, 1990; Aalberse, 1991).

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Although the exact pathogenesis of canine AD, similarly to that of the human disease, is unknown, the role of mast cells (MC) in mediating Type I hypersensitivity reactions is well recognized (Hanifin, 1990; Aalberse, 1991; Holgate, 1991). Most of the evidence supporting the possible importance of MC in the aetiology of human AD has also been observed in canine AD. In particular Scott (1981) and Nimmo Wilkie et al. (1990) reported an increase in skin MC count as one of the dermal changes observed histologically in biopsy specimens of affected skin from naturally occurring canine AD. Increased cutaneous histamine levels have also been found in skin lesions in both human and canine AD as compared with healthy individuals (Nimmo Wilkie et al., 1990) being the percentage increase over normal versus atopic skin similar for both species. The influence of such difference on the increased MC 'releasability' showed in dog models of allergy (Hirshman et al., 1988; Turner et al., 1989) has to be further confirmed. This enhanced 'releasability' which might indicate a higher sensitivity of the atopic MC versus the non-atopic cells is probably owing to what was called a pre-activated or primed state by Holgate (1991) in relation to asthma. Nimmo Wilkie et al. (1990) sought to improve the diagnostic criteria obtainable from histopathologic and pharmacologic examination of the skin and suggested that both, an increase in MC number and histamine concentration, were valuable findings.

Only a few studies have been made on canine MC, and these indicate that both human and canine cutaneous MC apparently share many constitutive properties. Cutaneous heterogeneity of the MC population in dog skin (typical and atypical MC) distinguished by their staining properties indicates the existence of differences in their morphologic and probably biochemical and functional characteristics (Becker et al., 1985, 1986). As human MC heterogeneity is well documented an effort to further characterize dog MC populations would be of interest.

Previous studies on normal or atopic dog cutaneous MC releasing activity have used skin slices or biopsy specimens. The value of this approach is limited by the presence of diffusional barriers for drugs and immune mediators as skin MC lie deeply within the tissues, being found in the dermis around blood vessels. It also precludes the knowledge of the precise number of MC available for stimulation owing to non-uniformity of their distribution within tissue (Tharp et al., 1983).

The purpose of the present work is to isolate MC from dog skin and characterize the histamine releasing activity. The skin MC dispersion procedure (by enzymatic digestion) is a validated technique for the study of human cutaneous MC pharmacology (Benyon et al., 1987; Lawrence et al., 1987) which has however never been performed previously on normal or atopic canine skin. The present paper describes the procedure for isolation of canine cutaneous MC and the assessment of their histamine secretory characteristics.
Material and methods

Animals

Skin biopsies were obtained from 31 healthy beagle dogs (17 males and 14 females) bred for experimental purposes. The animals were properly vaccinated and kept under close veterinary observation. No evidence of any disease was found.

They were distributed into three groups according to age and sex. Group I included ten males from 2 to 3 years old. Puppies from 5 to 9 months old were divided in Group II of seven males and Group III of 14 females.

Two incisional biopsies were performed on the abdominal craniolateral skin area in 14 dogs. One sample was processed as described later. The second specimen was used to quantify histamine content in whole skin (non-digested skin).

Skin MC dispersion

The dispersion procedure published by Benyon et al. for human foreskin MC was employed in our laboratory with minor modifications (Benyon et al., 1986). A skin incisional biopsy taken from the abdominal craniolateral area of dogs under general anaesthesia (pentobarbitone sodium, 30 mg kg\(^{-1}\) i.v.) was immediately placed in Minimum Essential Medium with 2% Foetal Cal'f Serum (MEM/FCS). Fatty tissue was removed and the remaining tissue weighed. The skin was finely chopped and washed with MEM/FCS by centrifuging at 500 rcf for 10 min. The fragments were further digested for 150 min by incubation with an enzyme mixture in a shaking bath at 37 °C in the presence of penicillin, streptomycin and bovine albumin (1 g of tissue digested per 15 ml of the mixture. The content in 15 ml solution was as follows: collagenase (Type I, Sigma, St. Louis, MO) 34.5 mg, hyaluronidase (Type I-S, Sigma) 18 mg, protease (pronase E-type XIV, Sigma) 12 mg, bovine albumin (fraction V, Sigma) 0.45 g, streptomycin and penicillin (Gibco, Paisley, UK) 1.5 mg and 1500 IU respectively. Dispersed cells were harvested in conic centrifuge tubes after prefiltration at 800 \(\mu\)m and filtration through 150 \(\mu\)m diameter nylon gauze (MAISSA, Barcelona, Spain).

The resultant cell suspension was washed first with MEM/FCS and then with calcium and magnesium-free Hank's buffered salt solution with Hepes, (HBSS-Ca,Mg free) (500 rcf, 10 min). Dispersed cells were pooled and MC number determined by light microscopy using Kimura metachromatic stain (Kimura et al., 1973). Viability was assessed by Trypan Blue dye.
MC stimulation

Cells were resuspended in HBSS-Hepes and distributed at a rate of 15 000 MC per 180 μl aliquot for stimulation.

Concanavalin A (Type IV, Sigma) was used as an immunological stimulus and calcium ionophore A23187 (Sigma) as a non-immunological stimulus at the following concentrations; 1 mg ml$^{-1}$ of Concanavalin A (Con A) and 1 μM of calcium ionophore A23187 (A23187) as the optimal concentration (0.1% dimethyl sulphoxide).

Cells were incubated with the stimulant and histamine release allowed to proceed at 37°C for 20 min. The reaction was stopped in ice and the samples centrifuged at 2630 rcf (4°C) for 20 min. Twenty μl of trichloroacetic acid (TCA) 55% were added to 180 μl of the supernatant. Spontaneous histamine-releasing activity of MC was surveyed by incubating the cells for 20 min at 37°C in a stimulant-free HBSS-Hepes. Pellet histamine content was measured in order to calculate the total amount per MC. The samples were centrifuged once and histamine immediately determined.

Whole skin histamine extraction

Fatty tissue was removed from the biopsy specimen and the skin weighed and chopped into approximately 2 mm pieces. The tissue was placed in 5 ml of HBSS-Hepes and heated at 100°C for 20 min. It was frozen and melted four times before assessing the histamine content.

Histamine determination

Histamine concentration was evaluated by spectrofluorimetry using O-Phthaldialdehyde (OPT from Sigma) derivatization. The classic procedure established by Shore (Shore et al., 1959) was adopted with a few modifications; particularly, the extraction step was omitted (Bergendorf and Uvnäs, 1972). Briefly, 170 μl of 10% TCA were added to a 170 μl aliquot. After centrifuging for 2 min at 1900 rcf, 320 μl of the resulting supernatant was brought to alkaline pH (12.5) by adding 700 μl of 0.3 M NaOH. Histamine was derivatized with 256 μl of OPT (prepared daily at a concentration of 10 mg ml$^{-1}$ in methanol) by 4 min incubation at room temperature. The reaction was stopped with 30 μl of 6 N HCl (pH 2.5). Maximum fluorometric sensitivity was obtained by exciting at 350 nm and reading the emission at 437 nm.

A histamine diphosphate (Sigma) scale ranging from 0.05 to 1 μg ml$^{-1}$ in HBSS-Hepes was prepared at once in order to quantify histamine concentration. The mean correlation coefficient ($r$) for the histamine standard curve regression was 0.997 ± 5 × 10$^{-4}$ SEM ($N=26$).
Analysis

Results are expressed as mean ± SEM indicating sample size (N) between brackets. A Kolmogorov-Smirnov test allowed us to check the normality of every group of data (or parameter, obtained from different dogs). Thereafter a statistic criterium was applied to discard data whose values were too discordant owing to erroneous handling and not natural differences from dog to dog (those values distant ± two times the standard deviation from the mean (7% of the total data) were discarded). Briefly, not all data from each dog were used to calculate means as shown by the differences in the N value from one parameter to another.

The comparison between groups of data was made by Student's t-test at a level of statistical significance of 0.05 (P<0.05).

Results

The mean results differentiated by sex and age with respect to cutaneous cells number and histamine content are shown in Tables 1 and 2. Differences between adult dogs and puppies (Groups I and II) and between males compared with females (Groups II and III) were not statistically significant as to total and MC count. Likewise, the comparative study of total histamine content and release between these groups did not show any significant difference.

Cell viability assessed after manipulating the tissue (washing and digesting) was 94.81 ± 0.55% (N=16).

The mean cutaneous cell number per gram of skin obtained by our proce-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Total cell, mast cell count and histamine content per gram of dog skin in relation to sex and age. (Mean ± SEM)</th>
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<tbody>
<tr>
<td></td>
<td>Total cells g⁻¹ of skin (×10⁶)</td>
</tr>
<tr>
<td>Group I</td>
<td>23.024 ± 1.450</td>
</tr>
<tr>
<td></td>
<td>N=9</td>
</tr>
<tr>
<td>Group II</td>
<td>23.367 ± 2.448</td>
</tr>
<tr>
<td></td>
<td>N=5</td>
</tr>
<tr>
<td>Group III</td>
<td>22.810 ± 0.998</td>
</tr>
<tr>
<td></td>
<td>N=14</td>
</tr>
</tbody>
</table>

¹No available data because of the small skin biopsies obtained from these dogs.
Table 2
Total histamine and histamine release from canine dispersed cutaneous mast cells in relation to age and sex. (Mean ± SEM)

<table>
<thead>
<tr>
<th>Group</th>
<th>Total (pg per mast cell)</th>
<th>Spontaneous (pg per mast cell)</th>
<th>A23187(^1) (pg per mast cell)</th>
<th>Con A(^1) (pg per mast cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>5.57 ± 0.82</td>
<td>0.90 ± 0.18</td>
<td>-(^2)</td>
<td>1.06 ± 0.43</td>
</tr>
<tr>
<td>(♂ 24–36 months of age)</td>
<td>N=9</td>
<td>N=9</td>
<td>N=8</td>
<td></td>
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<tr>
<td>Group II</td>
<td>4.20 ± 0.86</td>
<td>0.84 ± 0.13</td>
<td>1.13 ± 0.33</td>
<td>0.91 ± 0.36</td>
</tr>
<tr>
<td>(♂ 5–6 months of age)</td>
<td>N=5</td>
<td>N=5</td>
<td>N=5</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>4.98 ± 0.61</td>
<td>0.77 ± 0.12</td>
<td>1.25 ± 0.26</td>
<td>0.91 ± 0.20</td>
</tr>
<tr>
<td>(♀ 5–9 months of age)</td>
<td>N=14</td>
<td>N=13</td>
<td>N=11</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Expressed as net histamine by correcting for spontaneous release.

\(^2\)No available data because of the small skin biopsies obtained from these dogs.

Fig. 1. Histamine release by canine cutaneous mast cells expressed as a percentage of the total histamine content. (Results are expressed as net histamine corrected for spontaneous release.) First column, Total; second column, A23187; third column, Con A.

dure was 23.059 ± 0.726 × 10^6 (N=30) and MC count per gram was 2.31 ± 0.21 × 10^5 (N=31). MC purity is thus 1.00%.

Figure 1 summarizes total and released histamine per MC. These results are expressed as a percentage (considering total histamine content as 100%).
Spontaneous histamine represents 16.76 ± 1.26% (N = 29) of MC total histamine (4.93 ± 0.39 pg histamine/MC; N = 31). Net histamine release per cell obtained by A23187 and Con A stimulation was respectively 32.17 ± 3.56% with N = 19 (1.59 pg/MC) and 20.39 ± 2.41% with N = 25 (1.01 pg/MC) of the total histamine contents.

The histamine content per gram of whole skin was 9.00 ± 0.51 μg (N = 14).

**Discussion**

The equivalence in the results between animals of different sex and age allowed us to consider the 31 studied dogs as a homogeneous group for the present work.

The mean of the total yield of cutaneous cells per gram (23.059 ± 0.726 × 10⁶) is close to the value obtained by human foreskin digestion (29.375 × 10⁶) (Benyon et al., 1986). On the other hand, numerical yield of human cutaneous MC (1.41 × 10⁶) is significantly higher than in the dog (2.31 ± 0.21 × 10⁵), leading to lower MC purity in the latter species (Benyon et al., 1986, 1987).

Until now, no data were available on the number of canine cutaneous MC yielded per gram of skin by enzymatic digestion, but a comparison of the number of canine dermal MC number per cubic millimetre against human figures (although the number of MC in human skin has been the subject of conflicting reports) reflects the existence of a lower number in dogs (Becker et al., 1985, 1986), thus supporting our results.

Preliminary studies performed in our laboratory showed that shorter digestion times than 2.5 h would not provide us with the necessary amount of cells to undertake the experiments with the usual skin sample available. Under our conditions, a 1 g sized skin sample is sufficient to perform the series of experiments (spontaneous, total and stimulated cells). As almost the total histamine content is known to be highly specific for MC (Pearce, 1991) it is therefore a reliable indicator of their activity even though MC suspension purity is low.

The histamine content of MC in dog skin (4.93 ± 0.39 pg/MC) is very similar to that found in humans by Benyon et al. (1986); 4.7 ± 0.4 pg/MC and somewhat higher than the results obtained by Lawrence et al. (1987).

Percentage histamine released by A23187 stimulation at the optimal concentration is slightly higher than for human MC; 32.17% against 31% in humans. The immunological stimulation with Con A leads to an elevated net liberation (20.39%) when compared with the Anti-IgE stimulus in humans (15.8%) (Benyon et al., 1986). This leads to significant differences between the two stimuli A23187 and Con A on dog skin MC. Therefore MC reactivity remains satisfactory, as occurs with MC isolated from human skin.

Total whole skin histamine was determined to evaluate the wastage during
the procedure. Most of the skin histamine content is lost (84.92%), as deduced from the real histamine concentration found in whole skin (9.00 ± 0.51 µg g⁻¹). This is probably owing to degranulation and losses of a high number of cells during manipulation. A recent study conducted by Nimmo Wilkie et al. (1990) shows a lower canine cutaneous histamine content per gram of skin in mixed-breed dogs (3.85 ± 2.23 µg g⁻¹), although this is more than double the content found by Turner et al. (1989) in mongrel dogs (1.28 ± 0.28 µg g⁻¹) showing a high variability which is probably dependent on the characteristics of the animal itself and on the technique used. Results of histamine content in human whole skin reported in literature also vary 17.8 ± 2.2 µg g⁻¹ (Benyon et al., 1987) and 5.83 to 8.75 µg g⁻¹ according to age (Lawrence et al., 1987), but are closer to our results.

Besides cell loss, biological enzymatic effects and mechanical handling probably induce degranulation of a proportion of MC which cannot therefore be seen with the Kimura stain and the histamine-releasing activity of which cannot be monitored.

For these reasons, as has been stated previously, after handling the tissue we obtained 15.08%. Lawrence et al. (1987) reported release of between 20 and 30% of the real cutaneous histamine content for human skin. Nevertheless the digestion step and subsequent centrifuging do not significantly alter MC viability, functionality or reactivity, as shown earlier.

As there is sufficient analogy to make comparisons between human and canine AD useful, and considering the high incidence of the latter disease in clinical practice with the consequent availability of biopsy specimens from atopic dogs, it is suggested that canine cutaneous MC might provide an attractive model for the study of the role of these cells in allergies and possibly other MC-mediated diseases.

In conclusion, the present work offers a valuable tool for further studies on canine cutaneous MC immunopharmacological properties in hypersensitivity disorders by comparing control and atopic dogs, either suffering naturally occurring or experimentally induced pruritic dermatitis.

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References


