

EFFECT OF BENZODIAZEPINE ON ANTIGEN-INDUCED EOSINOPHIL
INFILTRATION INTO GUINEA PIG CONJUNCTIVA

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The effect of benzodiazepine (BZP) on experimental allergic conjunctivitis was studied. Male Hartley guinea pigs were sensitized with ovalbumin (OVA) and then homologous anti-OVA serum was injected intravenously into guinea pigs for passive sensitization. BZP was administered at a dose of 5 mg/kg by intraperitoneal injection (i.p.) according to the following schedule respectively; a single, repeated twice a day for 3 days, repeated twice a day for 3 days plus a single and repeated twice a day for 7 days. OVA challenge was performed to the conjunctiva, 20 minutes later, conjunctival edema during the early phase response (EPR) was observed and again at 6 hours later, both eosinophil infiltration into the conjunctiva and the platelet activating factor (PAF) serum level during the late phase response (LPR) were examined. BZP did not inhibit the development of conjunctival edema during the EPR. A single dose of BZP did not inhibit, but repeated doses of BZP for 3 or 7 days significantly suppressed eosinophil infiltration during the LPR. And after repeated doses of BZP for 7 days, all PAF serum levels during the LPR were under the lower detection limit of the assay. These results suggest that BZP has an inhibitory effect on antigen-induced eosinophil infiltration into the conjunctiva during the LPR.

Key words: benzodiazepine — eosinophil infiltration — experimental allergic conjunctivitis — late phase response — platelet activating factor

INTRODUCTION

It has been clarified that neurotransmitters such as substance P (SP) and calcitonin gene-related peptide (CGRP), which are known to cause neurogenic inflammation, play important roles in allergic reactions¹⁾²⁾. On the other hand, benzodiazepine (BZP) is known to suppress various types of neurotransmission through the central-type benzodiazepine receptor (CBR) by conjugating with γ -aminobutylic acid (GABA), an inhibitory neurotransmitter. In addition to this central-type receptor, peripheral-type ben-

zodiazepine receptors (PBRs) have been identified both in the periphery and the central nervous system³⁾, and have also been identified in mast cells⁴⁾.

In humans, immediate allergic reactions in the conjunctiva develop rapidly after antigen challenge and subside within a few hours. A clinical ocular late phase response (LPR), occurring 6-10 hours after the antigen challenge and characterized by recurrence of irritating eye symptoms, has been described⁵⁾. In passively sensitized guinea pigs, the early phase response (EPR), i.e., hyperemia and edema in the conjunctiva is observed about 20 minutes after the antigen challenge. The peak of eosinophil infiltration into the conjunctiva was observed 6 hours after the antigen challenge⁶⁾.

We have found that capsaicin, a pungent agent of red pepper, induces eosinophil infiltration into guinea pig conjunctiva via the release of endogenous SP and that endogenous SP is involved in increasing antigen-induced eosino-

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Abbreviations: BZP benzodiazepine; CBR central-type benzodiazepine receptor; CGRP calcitonin gene-related peptide; EPR early phase response; GABA γ -aminobutylic acid; LPR late phase response; MBP major basic protein; OVA ovalbumin; PAF plate activating factor; PBR peripheral-type benzodiazepine receptor; PCA passive cutaneous anaphylaxis

phil infiltration (unpublished data). Eosinophils have been highlighted as the effector cells in allergic inflammatory disease because of their granule proteins⁷. Platelet activating factor (PAF) is the most potent known chemotactic factor for eosinophils. Hence, we examined the effects of BZP on eosinophil infiltration and on the PAF serum level during the LPR in guinea pigs.

MATERIALS AND METHODS

Passively sensitized guinea pigs preparation

As shown in Fig. 1, male Hartley guinea pigs (300–350 g) were sensitized by intraperitoneal injection (i.p.) of 1 μ g ovalbumin (OVA) and 5 mg aluminum gel (10 mg/ml) mixture once a month three times or more. One week after the last sensitization, blood was collected from the auricle artery and analyzed using the 48-hour PCA (passive cutaneous anaphylaxis) reaction according to Ovary's method⁸. When the PCA titer was 640-fold or higher, as much as possible

of the animal's blood was collected after being anesthetized with pentobarbital, and its serum was isolated. Sera obtained from each guinea pig were combined, mixed and then stored at -80°C . A 2 ml aliquot of the homologous anti-OVA serum thus prepared was intravenously injected into the guinea pig for passive sensitization. Forty eight hours later, a 10 μ l drop of ovalbumin (30 mg/ml physiological saline) was instilled onto the conjunctival cul-de-sac (OVA challenge) to provoke an immediate allergic reaction.

Benzodiazepine administration procedure

BZP (Diazepam, Takeda Chemical Industries, Tokyo, Japan) was suspended in 1% methyl cellulose (Sigma, St. Louis, USA) at a 10 mg/ml concentration, and administered according to the following schedule. Since no significant difference was observed between the no-administration group and the vehicle group administered 1% methyl cellulose twice daily for 7 days at the same volume as the 5 mg/kg BZP group did, the vehicle group was used for

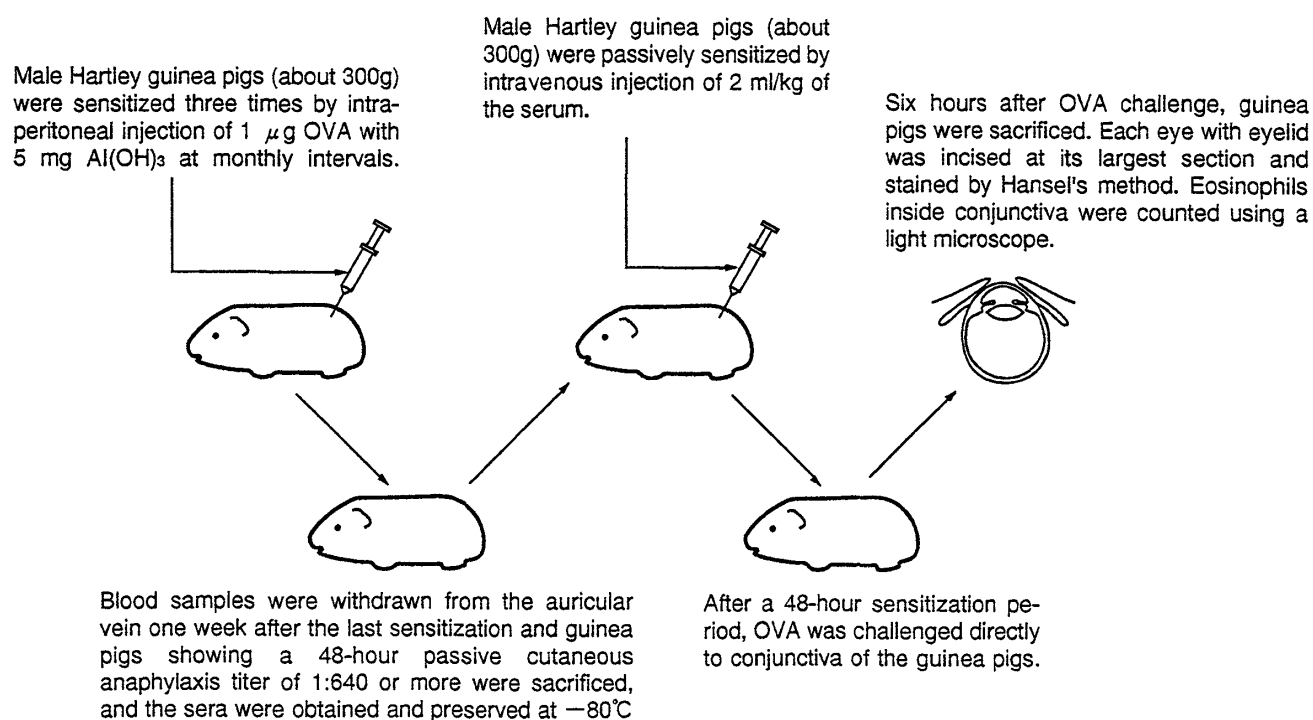


Fig. 1. Schematic illustration of the experimental design for studying the influence of allergen-induced anaphylaxis on conjunctival histological findings.

the control group.

- Group-1: 5 mg/kg BZP i.p. 30 minutes before OVA challenge.
- Group-2: 5 mg/kg BZP i.p. twice daily for 3 days (the last dose was injected 3 hours before OVA challenge).
- Group-3: the same treatment as for group-2, plus a single 5 mg/kg BZP i.p. 30 minutes before OVA challenge.
- Group-4: 5 mg/kg BZP i.p. twice daily for 7 days (the last dose was injected 3 hours before OVA challenge).

Macroscopical changes of the conjunctiva during the EPR

Twenty minutes after OVA challenge, conjunctival edema was observed and was graded into three levels, (–) (±) (+), in each group. The designation (+) indicated an obvious edema detectable without lid eversion, (±) indicated an edema detectable after lid eversion and (–) indicated no edema detectable.

Histological examination during the LPR

Six hours after OVA challenge, guinea pigs were sacrificed using overdosed pentobarbital sodium (100 mg/kg, i.p.) and the orbital contents together with the eyelids, were collected, fixed in 10% formalin and embedded in paraffin. Each eye was incised at its largest section and microtomed into a 2.5 μ m thick section. After staining by Hansel's method, eosinophils were counted under a light microscope ($\times 400$). At this largest section of the eye, the length of the conjunctival epithelial regions of male Hartley guinea pigs weighing 300 g and 350 g were 13.195 ± 0.040 mm and 13.188 ± 0.034 mm respectively (n=6, each). Since it was assumed that the total conjunctival area at the largest section was constant, the area for the eosinophil count was limited to inside the total conjunctiva, from the epithelial region to the muscular layer and the tarsus. Eosinophils in blood vessels and lymph nodes were not counted. The conjunctiva of each animal was examined to assess morphological changes.

PAF serum level determination

Blood of the animals in the control group and group-4 (repeated BZP i.p. for 7 days) was collected at the time the eyelids and orbital contents were resected 6 hours after OVA challenge. The collected blood was extracted using ethyl alcohol, and PAF serum level was measured using radioimmunoassay.

Statistical analysis

The data was analyzed statistically using Student's t-test. Significant differences were determined at the $p < 0.05$ level.

RESULTS

Macroscopical changes of the eyes during the EPR

As shown in Table 1, in the passively sensitized guinea pigs, no differences were observed in conjunctival edema among the groups at 20 minutes after OVA challenge. In all groups, obvious conjunctival edema was detected. It seemed that DZP did not suppress the EPR.

Conjunctival histological findings during the LPR

As shown in Fig. 2, in the no-BZP-administration group (the control group), local eosinophil infiltration into the conjunctiva was

Table 1 Conjunctival edema 20 minutes after OVA challenge

Guinea pigs	eyes	Conjunctival edema		
		(+)	(±)	(–)
Control	8	0	1	7
Group-1	6	0	0	6
Group-2	6	0	0	6
Group-3	6	0	1	5
Group-4	6	0	1	5

The designation (+) indicates an obvious edema detectable without lid eversion, (±) indicates an edema detectable after lid eversion and (–) indicates no edema detectable.

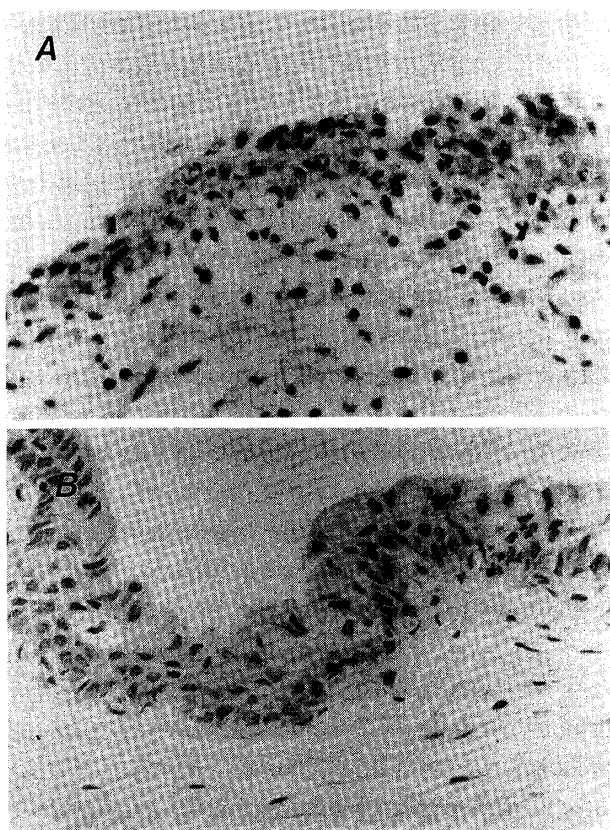


Fig. 2. Conjunctival histological findings 6 hours after OVA challenge.

A: In the no-BZP-administration group, local eosinophil infiltration was remarkable. The epithelium sloughed and the tissue became loose also. ($\times 100$)

B: In the BZP-administration group, the tissue was tighter and less epithelium sloughing was observed. ($\times 100$)

remarkable. Moreover, the epithelium sloughed and the tissue became loose also. In the BZP-administration group, the tissue was tighter and less epithelium sloughing was observed.

Eosinophil counts the LPR (mean $\pm$ S.D.)

Control group: 537 ± 106 cells ($n=8$)

Group-1: 509 ± 102 cells ($n=6$)

Group-2: 386 ± 140 cells ($n=6$)

Group-3: 276 ± 55 cells ($n=6$)

Group-4: 260 ± 72 cells ($n=6$)

As shown in Fig. 3, no significant difference was observed between the control group and group-1. However, the eosinophil count was significantly suppressed in group-2 ($p < 0.05$),

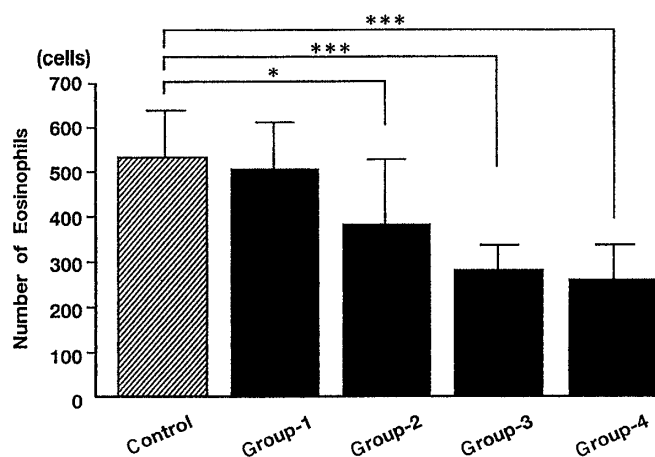


Fig. 3. The effect of benzodiazepine (5 mg/kg i. p.) on conjunctival eosinophil infiltration during the late phase response in guinea pigs. (control: repeated vehicle i.p. for 7 days, group-1: a single BZP i.p., group-2: repeated BZP i.p. for 3 days, group-3: repeated BZP i.p. for 3 days plus a single BZP i.p., group-4: repeated BZP i.p. for 7 days.)

Columns and bars represent the mean $\pm$ S.D., control; $n=8$, others $n=6$. * $p < 0.05$, *** $p < 0.001$; significantly different by the Student's t-test.

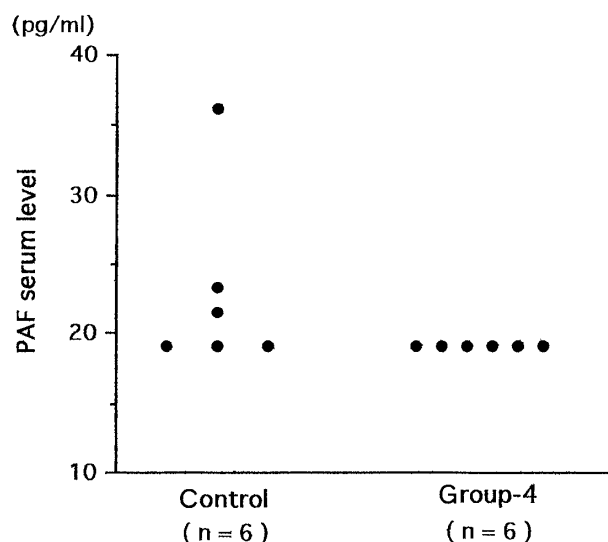


Fig. 4 PAF serum level 6 hours after OVA challenge. (control: repeated vehicle i.p. for 7 days, group-4: repeated BZP i.p. for 7 days.) The lower detection limit of the assay was 20 pg/ml and values lower than the limit are arbitrarily expressed as 19 pg/ml. ($n=6$)

group-3 ($p < 0.001$) and group-4 ($p < 0.001$).

PAF serum level during the LPR

As shown in Fig. 4, every PAF serum level

collected in group-4 (repeated BZP i.p. for 7 days) six hours after OVA challenge was under the lower detection limit of the assay. In the control group, the increase of PAF serum level was observed in 3 out of 6 guinea pigs. The lower detection limit of the assay was 20 pg/ml and values lower than this were arbitrarily expressed as 19 pg/ml.

DISCUSSION

In this BZP administration study, the IgE mediated immediate type reaction was not suppressed, but eosinophil infiltration was significantly suppressed during LPR after repeated BZP administration, and at the same time, each PAF serum level in group-4 was under the lower detection limit of the assay. These results indicate that the release of preformed mediators derived from mast cells, mainly such as histamine, have not been suppressed, but the newly generated mediators, such as PAF, have been suppressed. BZP has been widely used in clinical practice because of its sedative and anxiolytic effects. The mechanism of its action is the promotion of the inhibitory effect of GABA through CBR by GABA-BZP receptor complex agonistic action, and it suppresses the release of various neurotransmitters. In contrast with the CBR, the PBRs are distributed in several glandular and secretory tissues, such as adrenal glands, salivary glands, testes, kidneys, nasal and lingual epithelium³⁾, and have also been identified in mast cells⁴⁾ and the spinal cord⁹⁾. PBRs are found in mostly abundance in the mitochondrial outer membrane¹⁰⁾. The inhibitory effect of various compounds on mitochondrial respiration is well correlated with their affinity for PBRs^{11)–14)}. It has been postulated that PBRs might be linked to cyclo-oxygenase and inhibit arachidonic acid cyclo-oxygenation¹⁵⁾ and that there might be some common regulatory mechanisms for the biosynthesis of PAF and arachidonic acid metabolites¹⁶⁾. On the other hand, PBRs are coupled to calcium channels and the blockade of

calcium influx is involved in PBRs functions¹¹⁾. Immunological stimulation of human mast cells leads to the exocytosis of preformed granule-associated products and the generation of newly synthesized eicosanoids. The suppression of eosinophil infiltration and low PAF serum level during the LPR after repeated BZP administration indicate that the newly generated mediators, such as PAF through IgE dependent activation after allergen challenge, might be suppressed through PBRs by BZP and that occupation of PBRs by BZP might require repeated administration.

In the periphery, sensory nerve endings are distributed in the vicinity of mast cells. When neuropeptides, such as SP and CGRP, are released from peripheral sensory nerve terminals, various physiological activities, such as mucous secretion¹⁷⁾, vasodilatation¹⁾, increase in vascular permeability¹⁸⁾, degranulation of mast cells¹⁹⁾, and taxis of eosinophils²⁰⁾ are induced. In the conjunctiva, sensory nerves branching from the trigeminal nerve are distributed densely. According to Luhtala et al.²¹⁾²²⁾, 10–20% of gangliocytes in the trigeminal ganglion are SP-positive, and about 40% are CGRP-positive. The role of GABA as an inhibitory neurotransmitter in the central nervous system has been recently complemented by the identification of GABA receptors on the primary afferent neurons²³⁾ of the peripheral nervous system. GABA receptors have been classified into GABA-A (bicuculline-sensitive) and GABA-B (bicuculline-insensitive) subtypes²⁴⁾. The GABA-A receptor and the GABA-B receptor are controlling the CGRP and SP secretion, respectively, in an inhibitory fashion *in vitro*²⁵⁾²⁶⁾. On the other hand, SP and CGRP immunoreactivity co-exist in capsaicin-sensitive primary sensory neurons and are simultaneously from rat spinal cord sections by capsaicin or potassium²⁷⁾. CGRP is potently inhibit SP endopeptidase isolated from human cerebrospinal fluid and potentiates the SP action²⁸⁾. *In vivo*, the GABA-A receptor forming the GABA-BZP receptor complex may suppress CGRP release in the

peripheray. Because of these effects, neurogenic inflammation may be suppressed, then the eosinophil count and PAF serum level during the LPR may decrease. However, further studies are needed to better understand the mechanism of BZP action.

Eosinophils are commonly encountered in the conjunctiva of ocular allergies, and are thought to play pathophysiologic roles in allergic inflammation. The activation of eosinophils and subsequent degranulation result in the release of major basic protein (MBP) and other eosinophil granule proteins, which induce histamine release from mast cells²⁹⁾³⁰⁾. Further more, MBP's cytotoxic³¹⁾ and neutrophil activating³²⁾ properties are important factors for generating allergic inflammation.

In conclusion, the results suggest that BZP suppresses the antigen-induced eosinophil infiltration into the guinea pig conjunctiva during the LPR.

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モルモット結膜における抗原誘発好酸球浸潤への ベンゾジアゼピンの効果

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ベンゾジアゼピン (BZP) をモルモットに投与し、実験的眼結膜アレルギーへの影響を調べた。同種抗 ovalbumin (OVA) 血清で受身感作したモルモットに、OVA を点眼投与 (OVA challenge) して結膜局所に I 型アレルギー反応を起こさせた。OVA challenge 20分後 early phase response (EPR) の結膜浮腫を観察し、同 6 時間後、late phase response (LPR) の結膜好酸球を測定し、同時に血清 PAF 値を測定した。結果、1) BZP 投与は EPR の結膜浮腫の肉眼的所見に影響を与えなかった。2) BZP 1 回投与は LPR の結膜好酸球浸潤に影響を与えなかった。3) BZP 3～7 日連続投与は LPR の結膜好酸球浸潤を有意に抑制した。4) BZP 7 日間連続投与で、LPR の血清 PAF 値は、すべて測定限界値以下であった。以上の結果より BZP はモルモットにおける LPR の結膜好酸球浸潤に対して抑制的効果を持つことが示唆された。