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Short-Term Diabetes has no Influence on Mast Cell Infiltration and Reactivity During the Local Shwartzman Reaction

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Abstract: Mast cells numbers and reactivity status based on degranulation was determined in the skin of diabetic rabbits subjected to a local Shwartzman reaction. Diabetes was induced by a single intravenous injection of alloxan. The local Shwartzman reaction was induced seven days after by two inoculations of *Salmonella typhimurium* lipopolysaccharide. Preparatory injection was received in the skin and 24 h later; the provocative injection was administered intravenously. Rabbits were killed at the next day. Skin samples were fixed in Carnoy's and mast cell were identified employing a low pH toluidine blue stain. Results showed that Shwartzman reaction influences dramatically the increment and reactivity of mast cells independently from diabetes. In this model of acute inflammatory response diabetes has no influence on mast cells.

Key words: Local shwartzman reaction, mast cells, short term diabetes, lipopolysaccharide

INTRODUCTION

Diabetes has been considered the most important metabolic disease in human beings (Bonow and Gheorghiade, 2004). Diabetes is related with many complications such as a deficiency during the acute phase of the inflammatory response (Goova *et al*., 2001). This inflammatory deficiency could be linked to other diabetic complications, such as microvascular alterations (Gilbert *et al*., 2000), chemotactic and phagocytic abnormalities in neutrophils (Goova *et al*., 2001) and reduced mast cell infiltration (Diaz *et al*., 2001; de Oliveira Barreto *et al*., 2003).

The local Shwartzman reaction is an allergic-like but non-immune inflammatory process commonly induced in the skin of rabbits by injecting bacterial lipopoly-saccharide. The preparatory inoculation (priming) is administered in the skin and 18-24 h later, animals receive a provocative injection (challenge) intravenously. Microscopically, the reaction is characterized by thrombosis, haemorrhage and necrosis accompanied by a massive neutrophils infiltration at primed sites (Ramírez-Hernández *et al*., 2004). It is well known that this severe inflammatory reaction depends on cytokines, particularly tumour necrosis factor-α (TNF-α) (Ramírez-Romero and Brogden, 2000a; Ramírez-Hernández *et al*., 2004).

Although, mast cells have been traditionally associated with the severe clinical manifestations of anaphylactic reactions, it is now clear that this population of heterogeneous cells intermingle the inflammatory reaction, the tissue restoration and the immune response. Their prominent influence depends on vasoactive compounds, enzymes and an array of cytokines, including TNF-α, which are stored preformed in mast cell granules (Holgate, 2000; Krishnaswamy *et al*., 2001). More recently, it was reported that mast cells have a prominent role during the initial phase of the skin inflammatory response but not during healing (Egozi *et al*., 2003). This was confirmed in a previous work in this laboratory in which mast cells, many of them degranulated, markedly proliferate during earlier stages of the inflammatory response induced in the skin by a local Shwartzman reaction but dramatically decline toward the healing process (Ramírez-Hernández *et al*., 2004).

Taking in account that diabetics suffer from deficiencies during the acute phase of the inflammatory process (Goova *et al*., 2001), we extended our observations with this model of acute inflammatory response employing rabbits with chemically induced diabetes. Based on a work by de Oliveira Barreto (2003) and co-workers in which numbers of mast cells were reduced and their reactivity diminished during an antigen-mediated acute inflammatory response in the skin of

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diabetic rats, we originally supposed that mast cells infiltrating the skin of diabetic rabbits would be less numerous during the acute phase of the local Shwartzman reaction. Furthermore, we also thought that mast cells activation status based on their degranulation would be also diminished in diabetics.

MATERIALS AND METHODS

Animals and treatments: Twenty New Zealand White rabbits of both sexes, weighting 2.2 kg at the beginning of experiment were maintained in conventional conditions under constant veterinary supervision. The research protocol was carried out following the guidelines of the Asociación Mexicana de Especialistas en Animales de Laboratorio. The animals were randomly assigned to 4 groups (n = 5/group). Group 1 was diabetic with Shwartzman. Group 2 was diabetic without Shwartzman. Group 3 was non-diabetic with Shwartzman. Group 4 was control (non-diabetic without Shwartzman).

Induction of diabetes: To induce diabetes animals received a single administration of alloxan (Sigma, St. Louis, Missouri, USA) (150 mg kg^{-1}) intravenously. Controls received saline. A blood sample was taken 48 h after intravenous injection to determine glucose levels (Blood Glucose Meter; Ascensia Elite, Bayer, México, D.F.).

Induction of the local shwartzman reaction: Lipopolysaccharide from *Salmonella typhimurium* (Sigma, St. Louis, Missouri, USA) was administered in 2 sites (0.5 μg per site diluted in 0.1 mL of pyrogen-free saline) of the previously shaved dorsal skin (preparatory inoculation). Subsequently, 24 h later, animals received the *S. typhimurium* lipopolysaccharide (50 μg contained in 1.0 mL of saline) intravenously (provocative injection). Controls were subjected to a similar procedure with saline.

Collection of samples and histological procedures: Animals were killed 24 h after provocative injection with an overdose of sodium pentobarbital. Immediately after euthanasia the two sites of skin were collected to be fixed in Carnoy´s solution for 24 h at 4°C and then rinsed twice in absolute ethanol. All of the samples were routinely processed and sectioned at 4 μm. The sections were stained with a low pH Toluidine Blue (TB) to detect mast cells on a pale background (Ramírez-Romero *et al*., 2000a). In addition, complementary sections were stained with H and E.

Procedure for mast cell quantitation: The two sites obtained from each rabbit were included in the corresponding slide. Mast cells were counted in 20 high-power (×400) fields, equivalent to 3.694 mm^2 and expressed in cells/mm^2 (Ramírez-Romero *et al*., 2000b). The fields were randomly selected following a zigzag pattern of the superficial dermis. A single investigator (A.C. R-A) carried out the counting procedure (mostly by counting the 20 fields in one slide) without knowledge of the treatment assignment of each particular rabbit. When the counts were completed, the values for each rabbit were assigned to the corresponding group. Subsequently, degranulated mast cells were identified as cells with metachromatic granules scattered in their immediate vicinity. The percentage of degranulated cells was established by counting 100 mast cells with highest magnification (×1000). Then, the corresponding absolute value for each animal was calculated from the originally counted cells in the 20 fields. This procedure has been validated previously (Ramírez-Hernández *et al*., 2004).

Statistical procedures: A Student's t-test comparing diabetics versus non-alloxan treated rabbits was carried out. Subsequently, an ANOVA test employing a 2×2 factorial arrangement was utilized to assess the interaction between diabetes and Shwartzman in assumption that the infiltration of mast cells could be decreased in diabetics. The model was $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\ \beta)_{i\ j} + \epsilon_{ijk}$; where μ is the general mean, α_i is the effect of the i-th Shwartzman treatment (1 and 2), β_j is the j-th diabetes treatment (1 and 2), $(\alpha\ \beta)_{i\ j}$ is the interaction between Shwartzman and diabetes and ϵ_{ijk} is the random error associated with each observation, assuming ~ NI (0, $\sigma^2\epsilon$). After this, a Tukey's *w* procedure for multiple comparisons of means was carried out. A similar procedure was employed to assess the degranulation of infiltrating mast cells. The level of significance for all of the procedures was P = 0.05 or lower. The statistical software used was Statistix for Windows (1996, Analytical Software).

RESULTS

Induction of diabetes: Glucose blood levels were markedly higher in animals that received alloxan (474.30±92.05 (mg dL^{-1}; mean±standard deviation) in comparison with animals that received saline (92.70±8.28; $p<0.001$).

Gross lesions and histopathology: Typical haemorrhagic lesions that characterize the local Shwartzman reaction developed in the skin of all of the rabbits in the lipopolysaccharide-treated groups (1 and 3). Microscopically, the skin of the animals had oedema and a marked infiltration of polymorphonuclear cells; also, microthrombi formation and perivascular haemorrhages

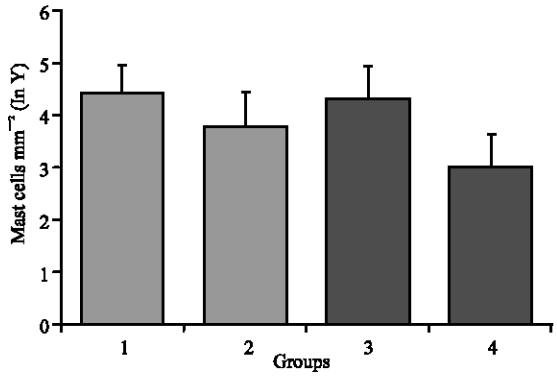

Fig. 1: Mast cell quantification (natural logarithm transformed) for the different groups. Tukey's w test for means comparison showed that groups 1, 3 and 2, corresponding to diabetics with Shwartzman, non-diabetics with Shwartzman and diabetics without Shwartzman, respectively, share similar values ($p>0.05$). Similarly, group 4, controls, share similar values with group 2. However, groups 1 and 3 have different values from group 4 ($p<0.05$)

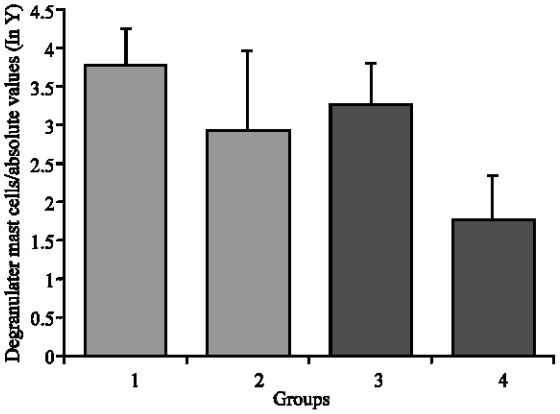

Fig. 2: Absolute values of degranulated mast cells (natural logarithm transformed) for the different groups. Tukey's w test for means comparison showed that groups 1, 3 and 2, corresponding to diabetics with Shwartzman, non-diabetics with Shwartzman and diabetics without Shwartzman, respectively, share similar values ($p>0.05$). Similarly, group 4, controls, share similar values with groups 2 and 3. However, groups 1 and 4 have different values ($p<0.05$)

were prominent. Lesions were more severe in the superficial dermis and often resulted in necrosis of the overlying epidermis. Occasionally, in the most severe lesions, the subjacent hypodermis was also involved. Is relevant to mention that under histopathological examination the skin of animals with Shwartzman, either diabetics (group 1) or non-diabetics (group 3), showed lesions with same severity. Similarly, the skin of diabetics without Shwartzman (group 2) and controls (group 4), appeared without changes.

Quantification of mast cells: Rabbits in the Shwartzman groups had an increase in the number of mast cells. Values obtained were: 92.95±50.25 (cells mm^{-2}; mean±standard deviation) for group 1, diabetic and 86.02±52.43 for group 3, non-diabetic. Non-Shwartzman groups had 52.18±33.01 for group 2, diabetic and 25.60±21.25 for group 4, controls. Degranulated mast cells were numerous in both Shwartzman and diabetics; group 1 had 48.47±23.29, group 2 had 28.71±29.75 and group 3 had 29.49±15.14. Controls had few cells 9.37±6.72.

For valid statistical inference, natural logarithm transformation was required. The ANOVA of the factorial experiment demonstrated that the major contribution for mast cells infiltration was provided by the Shwartzman reaction ($p<0.005$). Diabetes did not contribute ($p>0.05$) and therefore there was no interaction ($p>0.05$). Tukey's test showed that Shwartzman animals with diabetes (group 1) and Shwartzman non-diabetics (group 3) had similar values among them but completely different from controls ($p<0.05$). These results are presented in Fig. 1. Similarly, mast cell degranulation was influenced by Shwartzman ($p<0.025$), but not by diabetes ($p>0.05$), which discard any interaction ($p>0.05$). Tukey's showed that only the diabetic animals with Shwartzman (group1) were different from controls (group 4) ($p<0.05$). All of the results previously mentioned are presented in Fig. 2.

DISCUSSION

Although, the persistence of the inflammatory response is a hallmark in diabetes, the current opinion is consistent with a deficiency in the number of inflammatory cells infiltrating during the earlier phase of inflammation (Goova *et al*., 2001). This condition could be associated with a diminished number as well as a lower reactivity of infiltrating mast cells (de Oliveira Barreto *et al*., 2003). Although, we did not recognize a diminished, but indeed, perceived an augmented trend in the number and reactivity of infiltrating mast cells in diabetics, these values were not statistically different from controls. On the other hand, the number of infiltrating and reactivity of mast cells were markedly influenced by the Shwartzman reaction independently from diabetes.

The influence of diabetes on the numbers of mast cells is contradictory. There are studies in which the numbers of mast cells were increased in the vicinity of

mesenteric arteries (Gilbert *et al*., 2000) and the tongue (Batbayar *et al*., 2003) of diabetic rats. Conversely, Diaz and co-workers (2001) and de Oliveira Barreto *et al*. (2003), encountered that rats with diabetes showed a decreased number of serosal mast cells. In the former cases these mast cell increment were functional because mast cells associated with mesenteric arteries influenced their thickness (Gilbert *et al*., 2000) and mast cells in the tongue showed increased contacts with substance P containing nerve fibers (Batbayar *et al*., 2003). In the same way, decreased numbers of mast cells in serosal surfaces were associated with a diminished hypersensitivity response (de Oliveira Barreto *et al*., 2003). Although our model is different from the experiments referred previously, we think that the Shwartzman reaction presented herein is more compatible with the model reported by de Oliveira Barreto (2003) and co-workers. In fact, it has been proposed a common route dependant of complement for induction of the inflammatory response mediated by both, hypersensitivity and Shwartzman (Ramírez-Romero and Brogden, 2000c). On this respect, our results were discordant with those reported by de Oliveira Barreto *et al*. (2003), because in our assay, we found that the inflammatory response mediated by the Shwartzman reaction influences markedly mast cells, independently of diabetic status, while in the hypersensitivity model previously quoted, diabetes was the major influence.

Mast cells have been traditionally associated with hypersensitivity-mediated inflammatory responses. However, their role during inflammatory responses related with innate defence mechanisms has recently emerged (Holgate, 2000; Krishnaswamy *et al*., 2001). On the latter subject, mast cells possess toll-like receptor 4 and CD14 molecule on their membrane surface (Supajatura *et al*., 2002; Ikeda and Funaba, 2003), which are required for cell activation in presence of bacterial lipopolysaccharides. Apparently, mast cells respond to lipopolysaccharides releasing TNF-α and IL1-β slightly but without degranulation (Supajatura *et al*., 2002; Ikeda and Funaba, 2003). Ramírez-Hernández (2004) co-workers showed that mast cells participate primarily during the earlier phase of the inflammatory response provoked by the Shwartzman reaction and proposed that their degranulation is not massive but piecemeal. In the research presented herein we intended ameliorate as much as possible the influence of lipopolysaccharide on mast cells making instead diabetes ponder able. For this reason the amount of lipopolysaccharide injected in the primed sites was 200 times less than in our previous work (Ramírez-Hernández *et al*., 2004). On the other hand, it has been demonstrated that mast cells of diabetic rats show almost three times more degranulation than controls as soon as seven days after diabetogenic treatment (Kalichman *et al*., 1995). Nonetheless, despite the small amount of lipopolysaccharide employed, the Shwartzman reaction induced here resulted in a strong stimulus that influences mast cells irrespective of diabetes.

CONCLUSION

In conclusion, short-term diabetes did not influence mast cells during the acute phase of the local Shwartzman reaction. This lipopolysaccharide-mediated phenomenon is sufficient, by itself, to markedly increase the number and reactivity of infiltrating mast cells irrespective of diabetic status. It will be of interest to reproduce the experiment herein presented in animals with long-lasting hyperglycemia, expecting a major role for diabetes.

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