THE HAMSTER CHEEK POUCH: AN IMMUNOLOGICALLY PRIVILEGED SITE SUITABLE TO THE STUDY OF GRANULOMATOUS INFECTIONS

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SUMMARY

The hamster cheek pouch is an invagination of oral mucosa, characterized histologically as skin-like. In this paper we describe anatomical, histological and embryological features of the pouch and current on the pouch as an immunologically privileged site since it lacks lymphatic drainage and has few Langerhans cells. We present the review from literature and our observations after inoculation in the pouch of mycobacteriae (BCG, Mycobacterium tuberculosis and Mycobacterium leprae) and a fungus (Paracoccidioides brasiliensis). Lesions in the pouch were granulomatos but smaller and long lasting; even granulomatos, the reaction was inefficient to control the proliferation of agents compared with inoculation in other sites, except for BCG. Appearance of immunity was also delayed or absent and, when it was detected, a sharp decrease in number of agents in pouch lesions was observed. These observations make the pouch an interesting site for the study of the role of immune system in infectious diseases and in granuloma formation.

KEYWORDS: Hamster cheek pouch - development; Inflammatory reactions: Leprosy; Paracoccidioidomycosis: granuloma; Mycobacterium leprae.

INTRODUCTION

Normal and malignant tissues transplanted to histoincompatible hosts are usually rejected. Time and intensity of rejection depend basically on immunogenetic disparity between donor and host. However, there are some anatomical sites in which incompatible grafts survive for longer periods of time, so called immunologically privileged sites. An example and may be the most effective and intensively studied is the hamster cheek pouch (HCP), a food storage place, that has been used in several biomedical experiments.

The HCP is an invagination of the oral mucosa that extends 3-6 cm caudally under the skin shoulders (Fig. 1). It originates as two solid ingrowths of non-differentiated cells from the oral epithelium that reach definitive form only at the second week after birth.

In the foetus, the rudiment of the HCP appears as a bow-shaped area of oral epithelium, caudal to the junction of the anterolateral parts of the maxillary and mandibular arches. At birth, this ingrowth becomes a leaflike structure that grows not only caudally, but also dorsally and ventrally and remains attached to the cheek by a narrow pedicle which eventually becomes the neck of the pouch. At day 5, the central cells of this solid rudiment accumulate large amounts of glycogen before differentiation as stratum spinosum cells; central cluster of cells containing granules of keratohyalin initiates the stratum granulosum. Later, they become keratinized and loose cohesion with the formation of intercellular gaps that coalesce to form a central lumen that opens into the oral cavity. At day 10, connective tissue and groups of striated muscle fibres surround this epithelial growth.

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Although Linderman & Strauli (1968) have described lymphatic vessels in the HCP histologically, several other authors were unable to demonstrate lymphatic routes from HCP. Shepro et al., considered the superficial cervical node as the pouch regional lymph node because of its proximity to the HCP but not by the demonstration of any direct lymphatic drainage. These authors by injecting tritiated Escherichia coli and Tj phage into the HCP reported that, this agents or their antigens came out from the pouch, but did not pass through a regional node before spreading systemically.

Barker & Billingham (1971), injecting the dye Patent Blue V into HCP, also failed to reveal any lymphatic drainage from the pouch. Furthermore, they demonstrated that, when suspensions of viable lymph node cells from specifically sensitized donors were inoculated into the skin of thorax, a hypertrophy of the draining lymph nodes occurred. However, similar cellular suspension inoculated into HCP, did not evoke regional lymphadenopathy, indicating the lack of pathways to the lymph nodes from the pouch.

Goldemberg (1970) has identified ferritin in the ipsilateral submental lymph nodes by histochemical staining, 24 hours after the iron compound had been inoculated into the HCP; nevertheless, the amount of ferritin that reached the lymph nodes was much smaller and the time necessary for accumulation was 10 times longer than showed in the case of extrapouch inoculation. More recently, Bijovsky et al. (1984) referred to the presence of lymphatic vessels in the musculature of the base of the pouch. When the authors inoculated Trypanosoma cruzi into the membranous portion of the pouch, a large number of parasites could be detected until 15 days post-inoculation and the inflammatory infiltrate with a focal distribution was seen. In contrast, when the parasites were inoculated in the footpad, the infiltrate was diffuse and no parasites were detected. Moreover, the dissemination to other tissues occurred 7 days later in animals inoculated into the pouch when compared with those inoculated in the footpad. Since there are indications that the musculature of the base of the pouch presents lymphatic vessel together with the observation of amastigotes in this site, they suggested that the parasites could have reached the lymphatic drainage by spreading through the connective tissue of the pouch and not through direct lymphatic drainage. On the other hand, Sinihorin et al. (1994) injected Indian ink into the subepithelial connective tissue of the pouch and into the superior lips of the hamster. Twenty-four and forty-eight hours later, the animals inoculated into the lips exhibited large quantities of the carbon particles in subcapsular and medullar sinuses of the nodes; in animals inoculated into the pouch, however, no single particle was observed in the regional node. Similarly, when BCG was inoculated into the HCP or lips of the hamsters, the regional lymph nodes of animals injected into the pouch did not increase in weigh, whereas the increase in weigh was observed in the regional lymph nodes of the animals inoculated in the lips.

The HCP as a Immunologically Privileged Site

There is considerable evidence that in HCP, as in other places where grafts survive, the immunological tolerance is a consequence of the absence of lymphatic drainage, since the initial lymphocyte activities, as antigen recognition and interaction with other cells occur in the draining lymph nodes.23
Although not completely conclusive, the available literature supports the hypothesis that the long survival of the grafts in the pouch is related to paucity or lack of lymphatic drainage.

In addition, significantly fewer Langerhans cells (LC) have been observed in HCP. Epidermal LC are regarded as the primary antigen presenting cells in the mammalian skin. Circumstantial evidence showed that LC play an important role in the induction of immunity to cutaneous antigens. Presumably, they take antigens from the epidermal skin compartment where they normally reside and migrate across the dermal-epidermal junction where the T-cell sensitization is initiated either in the dermis or in the regional lymph nodes. BERGSTRESSES et al. (1988) carried studies involving LC in the back, ear and pouch of the hamster. In the pouch, the surface density of LC were reduced 5 to 10 fold. Since LC were absent from the central portion of the cornea, another immunologically privileged site, it is possible that decreased LC surface density may contribute to the immunological tolerance observed in the pouch.

KAPLAN & STREILEIN (1974), proposed that in sites without evident lymphatic routes, antigens may escape through the blood capillaries without passing through lymph nodes; as a consequence, the initial antigen exposure to the host occurs predominantly in the spleen. In this case instead of effective cell-mediated and antibody-mediated immune responses, the response elicited might be suppressive favouring the escape of antigen rather than its destruction. Consequently, the tolerance afforded by HCP could be the result of paucity of residual LC allows antigen to pass through the epithelium without immunological effective presentation plus absence of lymphatic drainage and the spread of antigen through the vascular system leading to a suppressive response.

Because of all these features, the HCP has been used in the study of host-parasite interaction, involving inflammatory reactions supposed to be mediated by the immune response. In addition, it has been used in the investigation of the role of lymphatic route in the spreading of parasites.

THE HCP AS SITE FOR THE STUDY OF GRANULOMATOUS REACTION

The granuloma is the morphological substrate of several diseases as tuberculosis, leprosy and many deep mycoses, suggesting that granulomas play an important role in the host's defense mechanisms. Therefore, it is of interest to study the mechanisms of granuloma formation and the killing capacity of the mononuclear cells in the parasitic granulomas.

ADAMS' referred that the development of a granulomatous lesion can be divided into three steps: 1. Infiltration by young mononuclear cells; 2. Maturation and organization of these cells into a mature granuloma and, 3. further maturation of these cells into epithelioid cells, resulting in the epithelioid granuloma.

The exact mechanism that leads the mature granuloma to transform into epithelioid one is not clear. There are arguments that favour the hypothesis that they are related to delayed type hypersensitivity (DTH) reactions but, inert particles and immune complexes may also evoke this type of reaction. Furthermore, epithelioid granulomas can be induced in athymic or immunosuppressed animals.

Since immunosuppressed or athymic animals may display other metabolic defects that can influence their responses to infection, the HCP is a good alternative for evaluation of the role of immune mechanism in the pathogenesis and evolution of the granuloma in immunological intact animals.

SINHORINI et al. (1994) used the HCP to investigate the influence of lymphatic drainage on the development of Bacillus Calmette-Guerin (BCG)-induced granuloma. They inoculated the bacteria into the pouch and into the footpad, an area rich in lymphatics. Although typical epithelioid granulomas developed in both sites, the footpad granuloma were highly cellular and persistent for longer periods of time, whereas the pouch granulomas were smaller and decreased in volume with time. The inoculation of BCG into the footpad of animals with granuloma in the pouch, reactivated the lesion in the pouch. Furthermore, the PPD test was positive in hamsters inoculated in the footpad, while it was always negative in hamster inoculated in the pouch. Their results provide additional evidence that contact of the bacterial antigens with the regional lymph node is essential to sensitize the animal and that the epithelioid granulomatous response to BCG may develop in the absence of specific DTH. The bacillary load control in the lesions also was not under the influence of DTH, since after 41 days of the inoculation, the number of bacteria either in the footpad or in the pouch was the same. Thus, macrophages, without the interference of immune sensitization were able to control the number and multiplication rate of BCG in the pouch lesion.

ROUXO (personal communication), in a similar study, compared the morphology of granulomas in hamsters inoculated with BCG and H37Rv, a virulent strain of Mycobacterium tuberculosis. The same pattern of evolution was observed with both mycobacteria, but H37Rv disseminated to intern organs and the BCG did not. Like BCG, H37Rv did not reach cervical lymph nodes before they became systemic. The retention of the BCG into the HCP suggests that lymphatics represent an important route of escape and dissemination of attenuated mycobacteria.

We have used the HCP in studies of granulomatous lesions of leprosy and paracoccidioidomycosis (Fig. 2). Leprosy is a chronic infectious disease caused by Mycobacterium leprae (M.leprae) where granulomatous inflammation of the skin and nerves is a prominent manifestation. The lack of in vitro techniques for the evaluation of the role of immune mechanism in the pathogenesis and evolution of the granuloma in immunological intact animals.
FIGURE 2 - A. Check pouch: large epithelioid granuloma containing fungi. 14 days after inoculation of *P. brasiliensis*. HE 250X; B. Check pouch: large epithelioid granuloma containing scarce fungi. 120 days after inoculation of *P. brasiliensis*. HE 250X; C. Footpad of hamster: small granuloma containing scarce fungi. 120 days after inoculation of *P. brasiliensis*. HE 250X; D. Check pouch: small granuloma. 14 days after inoculation of killed *P. brasiliensis*. HE 250X; E. Footpad of hamster: epithelioid granuloma. 14 days after inoculation of *M. leprae*. HE 250X; F. Check pouch: accumulation of vacuolated macrophages. 14 days after inoculation of *M. leprae*. HE 250X.

cultivation of *M. leprae* and the fact that *M. leprae* multiply and produce disease in only very limited number of species represents an important barrier to the progress in leprosy research. The inoculation in the footpad of immunologically intact mice remains the basic tool for assessing the activity of drugs against *M. leprae* and for the study of drug resistance. This animal model, however, has limitations because of the long duration of the experiment and the need of large number of animals, since *M. leprae* growth rate is very slow. Immune-deficient animals are less used in studies of leprosy due to the high cost of the animals and difficulties in their maintenance; furthermore, mortality is high before dissemination of the disease.

Similarly to experiments in which BCG plus *M. tuberculosis* were utilized, we inoculated *M. leprae* into the pouch or into the footpad of hamsters. In the footpad, the mycobacteria evoked focal epithelioid granulomas, with giant cells, lymphocytes and few or no bacilli, whereas in the HCP the *M. leprae* inoculation re-
sulted in a histiocytic reaction, without any epithelioid transformation; the lesion was rich in bacilli and was similar to the nodules of lepromatous leprosy, a multifocal form of disease. This observation confirms the information that, in leprosy, the development of epithelioid granulomas is directly related to the immune response. Furthermore, *M. leprae* grows easily and rapidly in the HCP, a useful feature that permits the study of new antileprosy drugs and drug resistance.

Paracoccidioidomycosis is a deep mycosis caused by *Paracoccidioides brasiliensis* (*P. brasiliensis*). Its clinical manifestations are those of a chronic granulomatous disease, affecting lungs, mononuclear phagocyte system, mucocutaneous areas and other organs. Several experimental models have been used to study the *P. brasiliensis* granuloma, but many of its aspects remain unclear; one of them is the role of immune response in its evolution.

The inoculation of the fungus in HCP resulted in immunological positive reactions only 35 days after inoculation. This permits the study of the lesions in two moments: before and after the development of immunity as tested by the footpad test and ELISA. In the first moment we observed epithelioid granulomas containing viable and proliferating fungi, surrounded by small halo of mononuclear cells. After day 35, when cell mediated immunity was detectable, there was progressive decline in the number of viable fungi. Thus, granulomas elicited by *P. brasiliensis* in the absence of immune response are morphologically similar to those formed in the footpad; however, they were unable to control fungal proliferation, also shown in athymic mice. With time, however, fungi or their antigens escape from the HCP, elicit an immune response and fungi may reach other organs. Our findings point to the importance of lymphatics in the progression of Paracoccidioidomycosis.

We have also utilized the HCP to study the role played by viability of *P. brasiliensis* in the morphology of paracoccidioidomycotic granuloma. We observed that similar to the reported with viable *P. brasiliensis*, killed fungi evoke typical epithelioid granulomas, even in the absence of detectable immune response. However, the granulomas elicited by killed fungi were devoid of giant cells and mononuclear cell halo, suggesting that live proliferating fungi or their products may be involved in those events.

CONCLUSION

Hamsters are docile animals, that can be easily handled and maintained in conventional animal colonies. They are susceptible to many infections and their pouch is an immunologically privileged site that is easy to access for investigation. The HCP is an ideal site for experimental studies of situations in which the role of immunity is supposed to be important, including the participation of the immune system in the development and modulation of granulomatous reactions. The HCP also provide a good system for study of the role of the lymphatic route in the spreading of infections.

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