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Resident salivary gland macrophages function as accessory cells in antigen-dependent T-cell proliferation

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SUMMARY

The function of salivary gland macrophages in the induction of local immunity in secretory organs was investigated in Fischer 344 rats. Macrophages obtained from dispersed submandibular gland (SMG) cells were characterized and examined for their ability to present antigen to T cells. Populations of SMG-adherent cells contained approximately 80% macrophages, of which 46–62% were I-A$^+$ cells. These numbers were from five to 10-fold greater than the I-A$^+$ cells in macrophage populations from peritoneal exudates (5–11%). SMG macrophages functioned effectively as antigen-presenting cells. Antigen presentation was antigen specific, macrophage dose dependent and inhibitable by monoclonal anti-I-A antibodies. These studies suggest that a functional salivary-gland immune-response pathway exists that can function independently of a gut-associated lymphocyte-homing mechanism.

INTRODUCTION

As proposed in the scheme of the common mucosal immune system, salivary glands are target organs where the clonal progeny of antigen-stimulated IgA-committed B cells, originating in the Peyer's patch follicle, can localize (Craig & Cebra, 1975; Gearhart & Cebra, 1979; Weisz-Carrington *et al.*, 1979; Jackson *et al.*, 1981). However, a mechanism of intestinal stimulation and cell migration for salivary immunity (Michalek *et al.*, 1976; Smith, Taubman & Ebersole, 1979) may not explain completely the findings of antibody responses after local antigen challenge of salivary glands (Emmings, Evans & Genco, 1975; Nair & Schroeder, 1983a). Furthermore, salivary glands contain lymphoid aggregates (Gorlin, 1957; Schroeder, Moreillon & Nair, 1983), in close apposition to the duct system, that appear to be accessible to experimental application of antigen (Nair & Schroeder, 1983b). These observations suggest that the salivary gland-associated lymphoid tissue may play an important role in the initiation of local mucosal immunity to oral antigens. However, it is not known whether antigen recognition and regulation of lymphocyte functions occur in the local secretory microenvironment. Recently, we found that isolated resident mononuclear cells from salivary and lacrimal glands contain T-helper cells and that the macrophage populations contain a high level of I-A$^+$ cells (J. Pappo, J. L. Ebersole and M. A. Taubman, manuscript in preparation). In the present studies, we have investigated the antigen-presenting function of submandibular gland mononuclear phagocytes and their regulatory interactions with T lymphocytes.

Abbreviations: PEC, peritoneal exudate cells; SMG, submandibular gland.

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MATERIALS AND METHODS

Animals

Male Fischer CDF (F-344)/CrlBR strain inbred rats, from 60–90 days old, were obtained from Charles River Breeding Laboratories (Kingston, NY). Animals were maintained on a pelleted diet (Purina Chow, Ralston Purina Co.) and distilled water.

Cell isolation procedures

Submandibular gland (SMG) mononuclear cells were prepared as described elsewhere (J. Pappo, J. L. Ebersole and M. A. Taubman, manuscript in preparation). In brief, the organs were perfused with cold RPMI-1640 medium containing 100 U/ml penicillin, 100 U/ml penicillin, 100 μg/ml streptomycin (Grand Island Biological Company, Grand Island, NY) and 12·5 mM HEPES buffer (Research Organics, Cleveland, OH), sectioned into 1–2 mm^3 fragments and sequentially dispersed with medium containing 120 U/ml of collagenase (Class IV; Worthington Biochemicals, Freehold, NJ). SMG mononuclear cells were obtained in fractions of density 1·044–1·077 gm/ml after centrifugation in discontinuous gradients of Percoll (Pharmacia Fine Chemicals, Piscataway, NJ). Loss of mononuclear cells in other density fractions was $<8\%$.

Cells from peritoneal exudates (PEC) were elicited by i.p. injection of 5 ml sterile 10% proteose peptone (Difco Labora-

tories, Detroit, MI). The cells were harvested 3–4 days later by lavage with cold medium containing 10 U/ml of heparin (Abbott Laboratories, Chicago, IL). Cell viability was determined with ethidium bromide and acridine orange (Ford, 1978) under fluorescence microscopy, and consistently found to be $\geq 90\%$ for SMG cells and $\geq 98\%$ for PEC.

Preparation of antigen-presenting cell populations

SMG mononuclear cell fractions and PEC were resuspended in complete medium supplemented with 2 mM L-glutamine and 10% heat decomplemented fetal bovine serum (Grand Island Biological Company) and incubated for 2 hr at 37° in 5% CO_2 in microculture wells (Costar, Cambridge, MA). Non-adherent cells were rinsed extensively from each well with warm medium. Isolated cells from the SMG were serially plated for 1–2-hr periods to yield a greater number of cells per unit area. The number of macrophages per well was estimated in each experiment in a parallel set of cultures by multiplying the mean number of adherent phagocytic cells in 20 high-power fields by the surface area of the well and dividing this value by the eyepiece micrometer area (0·0575 mm^2). The adherent cells were used as antigen-presenting populations only when the observed value was within 20% of the predicted value.

Characterization of macrophages

Adherent populations were prepared on sterile glass coverslips (Bellco, Vineland, NJ). Phagocytosis was assessed with 1 μm latex particles (Dow Diagnostics, Indianapolis, IN). Non-specific esterase activity (Koski, Poplack & Blaese, 1976) was determined with alpha-naphthyl acetate as substrate. Fc receptor-bearing cells were identified with opsonized sheep red blood cells (Hamburg, Manejias & Rabinovitch, 1978). I-A antigens were detected on adherent phagocytic populations by immunofluorescence with monoclonal antibody OX4 (Reynolds *et al.*, 1981). Adherent phagocytic populations from SMG contained from 77 to 89% non-specific esterase-positive cells and from 64–75% Fc receptor-bearing cells. PEC-adherent phagocytic populations comprised of 96–98% esterase-positive cells and from 96 to 97% Fc receptor-bearing cells.

Preparation of antigen-sensitized T-cell populations

Antigen-stimulated peritoneal exudate T cells were obtained as described elsewhere (Farr, Kiely & Unanue, 1979), with modifications. Briefly, animals were injected i.p. with $2–5 \times 10^5$ live *Listeria monocytogenes* (kindly provided by Dr D. Beller and Dr G. Schreiner, Harvard Medical School, Boston, MA) harvested in log growth phase. Control animals received sterile phosphate-buffered saline (PBS). Seven days after injection, each animal was injected i.p. with 5 ml of 10% proteose peptone and the PEC population harvested by lavage 3 days later. Macrophage depletion was accomplished by double adherence in plastic tissue culture flasks (75 cm^2; Falcon Plastics, Oxnard, CA) for 2 hr, followed by a third cycle of adherence for 20–22 hr. The resulting non-adherent cells were incubated in nylon-wool columns (Julius, Simpson & Herzenberg, 1973) and centrifuged in Ficoll–Hypaque gradients. T-cell populations were functionally devoid of endogenous antigen presentation, as demonstrated by their inability to proliferate significantly in the presence of serial concentrations (from 2×10^7 to 2×10^1) of exogenously administered heat-killed *Listeria*.

Opsonization of L. monocytogenes *and antigen pulsing*

Rabbit anti-*Listeria* serum was prepared (Farr *et al.*, 1979) and its reactivity examined by an enzyme-linked immunosorbent assay (Ebersole *et al.*, 1980). Heat-killed organisms ($3·5–4 \times 10^8$/well) were bound to polystyrene microtitre plates (Flow Laboratories, Hamden, CT) by overnight incubation in 0·25% glutaraldehyde in PBS, pH 7·4, at 4°. Serial dilutions of anti-*Listeria* antiserum or normal rabbit serum were incubated with the antigen, the system developed with goat anti-rabbit IgG (Miles Laboratories, Elkhart, IN) conjugated with alkaline phosphatase, and the extent of reaction determined at 405 nm. Heat-killed *L. monocytogenes* (10^8/ml) were incubated with the optimal dilution of antiserum for 1 hr at 37°, washed and 2×10^7 opsonized organisms dispensed into the appropriate wells containing from 10^2 to 5×10^3 adherence-enriched macrophages. The plates were centrifuged to enhance cell–organism contact, incubated for 1 hr to allow phagocytosis, and the unbound organisms thoroughly rinsed from the wells. The distribution of antigen-binding cells was analysed in macrophage populations stained with Brown and Brenn (Gray, 1954) and evaluated with a Kolmogorov–Smirnov two-sample test. Significant differences in distribution were considered at the $P < 0·05$ level.

Co-culture composition and assay of T-lymphocyte proliferation

Antigen-sensitized T cells (5×10^4) were resuspended in culture medium supplemented with 2 mM L-glutamine and 10% heat-inactivated normal rat serum, dispensed into wells containing varying concentrations of macrophages and cultured for 4, 5 or 6 days. Controls included (i) T cells added to unpulsed macrophages; (ii) macrophages pulsed with antigen; (iii) macrophages alone; (iv) T cells with exogenously administered antigen; (v) T cells alone; and (vi) antigen only. During the last 20 hr of culture, 0·2 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) was added to each well. The cultures were harvested on glass microfibre filters (Whatman Limited, Maidstone, Kent), dried and counted using a xylene-based scintillation fluid (Scintillene; Fisher Scientific Company, Fair Lawn, NJ). The antigen-dependent proliferation of T cells was assayed by incorporation of [^{3}H]thymidine and the incorporation determined from triplicate cultures in a liquid scintillation spectrometer (Beckman Instruments, Palo Alto, CA). The results are expressed as mean c.p.m. $\pm$ SEM or as stimulation indices (SI) (mean experimental c.p.m./control c.p.m.). The control values consisted of the c.p.m. obtained with cultures of sensitized T cells with free antigen.

RESULTS

Antigen dependency of T-cell proliferation

Since the total recovery of SMG-adherent cells was approximately 100–150 times lower than the recovery of adherent cells from PEC, we developed a microculture system in which the antigen-presenting function could be tested with small numbers of antigen-presenting cells and sensitized T cells. The ability of 10^3 macrophages from SMG or PEC to present antigen to 5×10^4 T cells was examined first. Macrophage populations from SMG or PEC were pulsed with antigen or medium and co-cultured with immune T cells or normal T cells. The results illustrated in Fig. 1 show that both SMG and PEC macrophages

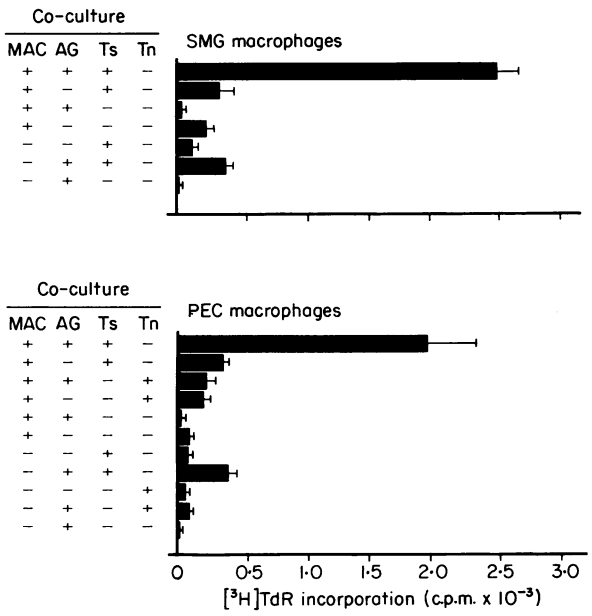

Figure 1. SMG and PEC macrophage populations (MAC) were purified (10^3 per well) and cultured in the presence or absence of antigen (AG) with 5×10^4 sensitized T cells (Ts) or normal T cells (Tn). The bars show the mean c.p.m. and the brackets enclose 1 SEM of triplicate cultures from two separate experiments.

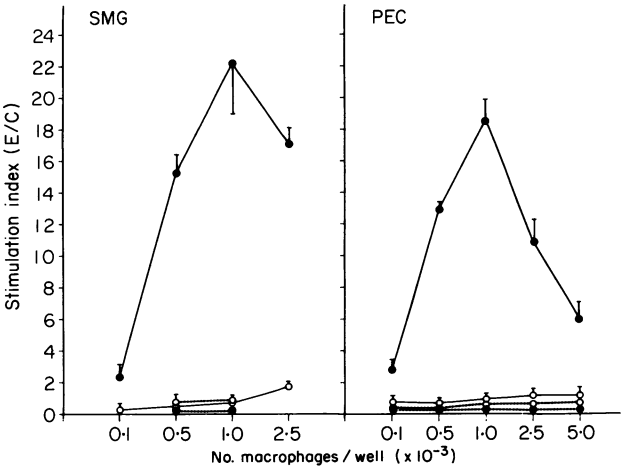

Figure 2. Effect of macrophage titration on the generation of T-cell proliferative responses. SMG or PEC cells were cultured at concentrations determined to yield the indicated numbers of macrophages per well. Increasing numbers of macrophages were pulsed with antigen (●) or medium (○) and cultured with 5×10^4 sensitized T cells (solid line) or without T cells (broken line). Cultures were harvested at Day 5. The points represent the mean stimulation index and the brackets the SEM of triplicate cultures in five separate experiments. The c.p.m. of sensitized T cells were 244 ± 91 and of sensitized T cells with antigen 291 ± 70.

Table 1. Specificity of antigen-presenting function*

Macrophage population	Antigen pulse	[³H]thymidine incorporation
SMG	LM	1245±100
	OVA	118±27
	Medium	94±16
PEC	LM	1165±154
	OVA	255±72
	Medium	113±37

* Listeria-immune T cells were co-cultured with 10^3 SMG or PEC macrophages pulsed with opsonized *L. monocytogenes* (LM), 100 μg/ml ovalbumin (OVA) or culture medium for 1 hr at 37°. Values represent mean C.P.M. ±SEM of triplicate cultures. The C.P.M. of sensitized T cells were 28±7 and of sensitized T cells plus antigen 22±4.

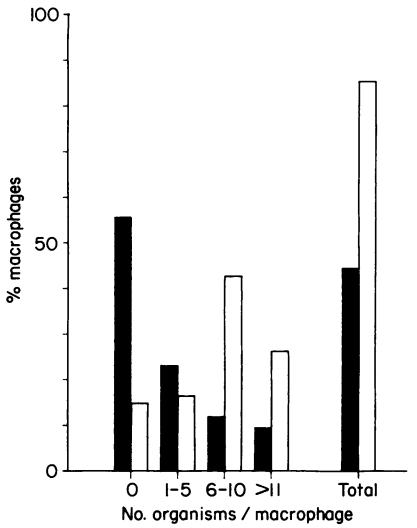

Figure 3. Distribution of antigen uptake in macrophage populations from submandibular gland and peritoneal exudates. Macrophages from SMG (closed bars) and PEC (open bars) were prepared by adherence in flat-bottomed microtitre wells containing glass coverslips. Duplicate preparations were pulsed with opsonized *Listeria* or latex particles for 1 hr at 37°. Adherent cell populations were rinsed, fixed and stained with Brown and Brenn. The proportions of macrophages contained in the adherent monolayers were determined from 100–200 phagocytic cells in the parallel set of latex-pulsed cultures. For the distribution of antigen-binding cells, 50–100 adherent cells from duplicate coverslips were examined for the presence or absence of antigen.

were capable of presenting antigen at this macrophage:T cell ratio. The T-cell proliferative response was antigen dependent, required antigen-bearing macrophages, and specifically sensitized T lymphocytes.

Specificity of antigen-presenting function

Experiments were conducted in which macrophage populations were pulsed with *Listeria* or an irrelevant antigen and subsequently cultured with *Listeria*-sensitized T cells (Table 1). SMG

Table 2. Dependency of I-A$^+$ macrophages for antigen presentation

Co-culture*			
SMG MAC	T cell	Culture† treatment	[^{3}H]thymidine‡ incorporation
+	+	None	1882±155
+	+	IgG	1910±267
+	+	Anti-I-A	1160±216
PEC MAC	T cell		
+	+	None	1441±200
+	+	IgG	1232±158
+	+	Anti-I-A	625±56

* Macrophage populations (10^3 MAC) from SMG or PEC were prepared by adherence, treated as indicated and pulsed with antigen. T cells (5×10^4) were centrifuged onto the adherent cells.

† 5 μg/ml of control IgG (89) or anti-I-A (OX4) monoclonal antibodies for 60 min.

‡ Determined at Day 5 of culture. Values represent mean±SEM of triplicate cultures. The c.p.m. of sensitized T cells with antigen were 41±5.

macrophages supported the proliferation of immune T cells when they were pulsed with the homologous but not with the irrelevant antigen.

Effect of increasing numbers of macrophages on T-cell proliferative responses

We next evaluated the relative antigen-presenting function of equivalent numbers of SMG and PEC macrophages (Fig. 2). T-cell proliferation was related to increases in the number of antigen-bearing macrophages, and achieved maximal levels with 2% macrophages. Greater numbers of macrophages per well inhibited T-cell proliferation. Comparison of antigen-presenting functions indicated that SMG macrophages presented antigen as effectively as PEC macrophages. SMG macrophage populations contained from 46% to 62% I-A$^+$ cells, whereas PEC macrophages harboured from 5% to 11% I-A$^+$ cells.

Antigen uptake in salivary gland and peritoneal macrophages

Macrophage populations from SMG and PEC were examined for content and distribution of antigen-binding/phagocytic cells. The results shown in Fig. 3 demonstrate that SMG macrophages exhibited a significantly lower ($P < 0{\cdot}01$) distribution of antigen-binding/phagocytic cells than PEC macrophage populations.

Requirement of I-A-positive macrophages for T-cell proliferative responses

The ability of I-A-bearing macrophages from SMG or PEC to participate as antigen-presenting cells was examined in a functional inhibition assay with the monoclonal antibody OX4 (anti-I-A). Macrophage populations incubated with anti-I-A monoclonal antibody showed a marked reduction in their capacity to stimulate T-cell proliferation relative to controls (Table 2). Treatment of cultures with monoclonal antibodies did not affect the distribution of antigen-binding cells in the macrophage populations.

DISCUSSION

The experiments described in this report were undertaken to examine the function of salivary gland macrophages in the initiation of T-cell activation. The data support the concept that salivary glands harbour antigen-presenting cells which may function in the generation of local mucosal immunity. SMG macrophages behaved in a similar manner to conventional antigen-presenting cells (PEC macrophages) in relation to their ability to process and present antigen. The ability of SMG macrophages to process a complex multi-determinant bacterial antigen could be abolished by fixation prior to antigen pulsing (unpublished observation) and antigen presentation was macrophage-dose dependent. These observations are consistent with previous reports showing that presentation of the bacterial antigen *Listeria* requires antigen processing and catabolism by phagocytes (Ziegler & Unanue, 1982). Because co-cultures containing purified gland parenchymal cells and antigen were incapable of activating T cells, as measured by T-cell DNA synthesis, it was likely that SMG macrophages were responsible for the antigen-presenting function and T-cell activation. Furthermore, rat SMG epithelial cells did not express I-A antigens, suggesting that they do not normally function as antigen-presenting cells. However, the results reported here do not totally rule out accessory activity by epithelial cells or contaminating B cells, particularly after initial macrophage–antigen interaction. It has been shown that epithelial cells can be induced to express I-A molecules (Cerf-Bensussan *et al.*, 1984) and that fixed B-lymphoma cells present antigen when incubated with processed antigenic fragments (Shimonkevitz *et al.*, 1983). The relative antigen-processing ability of SMG epithelial cells has not been examined. However, results obtained with thyroid Ia expressing epithelial cells suggest that these cells were only capable of presenting viral peptides but not intact virus to T-cell clones (Londei *et al.*, 1984).

The expression of I-A molecules by macrophages has been shown to be under regulatory control mechanisms (Beller & Unanue, 1981; Steeg *et al.*, 1982) and to vary among macrophage populations from different anatomical sites (Cowing, Schwartz & Dickler, 1978). The high levels of I-A-positive macrophages in salivary glands parallel the high proportions of Ia-bearing macrophages in mucosal surfaces (Lipscomb *et al.*, 1981; Lee *et al.*, 1983) continuously subjected to antigenic exposure. These observations suggest that salivary gland macrophages play an important role in the regulation of immune responses to oral antigens. Possibly, the development of local salivary gland immune responses involves interaction between I-A-positive macrophages, resident T-helper cells (J. Pappo, J. L. Ebersole & M. A. Taubman, manuscript in preparation) and oral antigenic molecules which gain access to the salivary gland. Bacteria (Schroeder *et al.*, 1983) and protein antigens (Mazariegos, Tice & Hand, 1984) have been identified in salivary gland ducts, and localized immune responses have been generated by ductal instillation of antigen (Emmings *et al.*, 1975). Although

the mechanisms of translocation from the duct compartment into the salivary gland are not completely understood, there is evidence that duct cells can endocytose luminal protein antigens (Coleman & Hand, 1985).

The activation of antigen-primed T cells by SMG macrophages was comparable to the antigen-presenting function of PEC macrophages. Several possibilities may be proposed to account for the similarity in T-cell activating function, despite observing five to 10-fold differences in the numbers of I-A-expressing cells. One possibility is that the level of I-A antigen expression by macrophage populations is upregulated *in vitro* by lymphokines, and specifically gamma-interferon (Steeg *et al.*, 1982). Rat PEC macrophage populations can display levels of I-A comparable to those expressed by SMG macrophages when incubated with mitogen-stimulated spleen cell supernatants (unpublished observations). Secondly, it has been shown that the concentration of antigen and the number of Ia molecules determine the magnitude of T-cell proliferative responses (Matis *et al.*, 1983). Macrophages derived from SMG contained fewer numbers of antigen-bearing cells and lower antigen concentrations than PEC macrophages. If these antigen concentrations are suboptimal, and the antigen handling and surface recycling capacities of PEC and SMG macrophages are similar, reduced associations between antigen, Ia molecules and T-cell receptors may occur. Since I-region restricted T-cell activation may involve recognition of antigen and Ia molecules on the surface of the antigen-presenting cell (Benacerraf, 1978), a decrease in the concentration of macrophage–associated antigen could result in a reduction in the T-cell triggering capacity.

Finally, the antigen presentation system used here may not have been sensitive to the differences in I-A levels expressed by the small numbers of antigen-presenting cells used. The rapid increment of T-cell proliferation and generation of maximal responses with 10^3 macrophages suggests that this assay required small numbers of I-A-bearing cells for effective T-cell activation.

The T-cell proliferative response was inhibited by monoclonal anti-I-A antibody OX4 . This anti-I-A probe presumably recognized a functional domain of the Ia molecule and diminished the number of ligand–receptor interactions necessary for maximal proliferation. In this regard, it has been shown that anti-Ia monoclonal antibodies shift the antigen-presentation curves to a greater antigen requirement (Matis *et al.*, 1983). However, if the antigen concentration is maintained at a constant level, T-cell proliferation is inhibited. This inhibitory function is dose dependent and roughly proportional to the numbers of I-A-bearing cells in culture (unpublished observations).

The results presented here contribute to our understanding of mucosal immunological mechanisms. Thus, salivary glands may be envisioned not only as structures which accumulate the antigen-activated IgA-committed cell progeny which arise from intestinal antigenic stimulation, but also as organs capable of regulating T-cell proliferation locally. In this regard, while the gut-associated lymphoid tissues supply abundant numbers of IgA B-cell precursors to remote mucosal sites, the distribution of these cells in salivary glands is relatively low when compared with intestinal mucosae (Weisz-Carrington *et al.*, 1979; Jackson *et al.*, 1981). Whether SMG B cells migrate to other mucosal surfaces after local antigenic stimulation is not known at present.

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