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Identification of immunosuppressive fractions from the rat submandibular salivary gland

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SUMMARY

It had previously been suggested that the submandibular gland (SMG) of mice and rats may contain *in vivo* immunosuppressive factors(s). To identify such factor(s), we used a multi-step purification procedure of rat SMG extracts. Gel filtration chromatography of the SMG crude extract resulted in two pools of fractions with significant effects on lymphocyte reactivity in the *in vitro* concanavalin A (Con A) bioassay. Of these two pools, only the one with lower molecular weight resulted in the prolongation of murine skin allograft survival, the suppression of the delayed-type hypersensitivity (DTH) response to picryl chloride and the decrease in number of direct (IgM) plaque-forming cells against sheep red blood cells. Fractionation of this low molecular weight (LMW) pool through hydrophobic interaction chromatography resulted in three protein fractions designated A, B and C. Of these fractions only fraction A produced significant suppression of the DTH response. Further purification of fraction A with anion exchange chromatography produced two fractions with immunosuppressive activity in the DTH response. One fraction demonstrated on SDS–PAGE a single component of 40,000 MW, while the other had two components of 30,000 and 40,000 MW respectively.

INTRODUCTION

The submandibular salivary gland (SMG) of several animal species contains a large number of biologically active factors some of which affect the functions of the immune system. Many of the biologically active factors have been shown to be localized in the granular convoluted tubule portion of the gland and to be regulated by several hormones.[1,2] The physiological role of these factors largely remains a matter of speculation.[1-5]

Previous studies done to define a relationship between the SMG and the immune system were carried out largely by using mice. The majority of these studies investigated the effects of injections of murine SMG homogenates, or fractions thereof, on the morphology and/or responsiveness of the immune system. Thus, atrophy of the lymphoid tissues,[6] prolongation of skin allograft survival,[7] suppression of the antibody response to sheep red blood cells (SRBC),[8] and of the delayed hypersensitivity response[9] were reported after injection of the SMG homogenates from male mice. A number of factors referred to as the thymotropic factor[10,11] and the immunotranquillizer of the mouse SMG[12] were reported to be responsible for some of these effects.

Previous studies done to define a relationship between the SMG and the immune system were carried out largely by using graphy into two major pools depending on their molecular size and their effects on the proliferation of concanavalin A (Con A)-stimulated murine lymphocytes. The high molecular weight (HMW) pool produced suppression whereas the low molecular weight (LMW) pool produced stimulation of such a proliferative activity.[13] Moreover, an *in vitro* immunosuppressive factor was partially purified from the HMW pool and was shown to inhibit the activity of interleukin-1 (IL-1).[14] Here we show that this HMW pool lacks *in vivo* immunosuppressive activity and report on the identification of *in vivo* immunosuppressive fractions derived from the LMW pool.

Abbreviations: Con A, concanavalin A; DTH, delayed-type hypersensitivity; HMW, high molecular weight; LMW, low molecular weight; PBS, phosphate-buffered saline; PFC, plaque-forming cell.

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MATERIALS AND METHODS

Animals

Random bred male hooded rats (200–250 g) and female mice of the inbred strains C57BL/6J and DBA/2J were obtained from the Central Animal Care Facility of University of Manitoba, Canada. Female A/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were 6–8 weeks old at the time of experiments.

Preparation and purification of the rat crude SMG extract
As described previously in more detail,[15] the crude SMG extract was prepared from male hooded rats by homogenization of the SMG and ammonium sulphate precipitation (between 30 and 64% saturation) of such homogenate. Gel filtration chromatography of this crude extract was done at room temperature by using Sephacryl S-200 high resolution gel (Pharmacia Canada Ltd, Dorval, Quebec, Canada) equilibrated with phosphate-buffered saline (PBS, 0·12 M sodium chloride, 0·01 M disodium phosphate and 0·003 M monopotassium phosphate) in a 2·6 × 60 cm column. Elution was done with PBS in 8-ml fractions at a flow rate of 22 ml/hr/cm^2.

Two fractions of the eluate demonstrated significant effects on lymphocyte proliferation in the Con A mitogen assay. One fraction with molecular weight between 47,000 and 96,000 is referred to here as the HMW fraction or pool. The other one with molecular weight between 14,000 and 47,000 is referred to as the LMW fraction or pool.

The LMW pool of the Sephacryl column was further fractionated by hydrophobic interaction chromatography. Phenyl Sepharose (Sigma Chemical Co., St Louis, MO) was packed into a 1 × 30 cm column, with 1 ml of Sephadex G-25 (Pharmacia) added to the top. The column was equilibrated with 0·01 M phosphate buffer (pH 7·0) containing 30% ammonium sulphate. After sample application, two bed volumes of this buffer were run, then a linear continuous gradient was started by using a Pharmacia gradient mixer (GM-1). The gradient consisted of equal volumes (70 ml each) of the equilibrating buffer (0·01 M phosphate/30% ammonium sulphate) and 0·01 M phosphate buffer with 60% ethylene glycol. The gradient was monitored by measuring the conductivity of the 4-ml fractions by using a conductivity meter (Cole-Parmer Instrument Co., Chicago, IL).

The final fractionation was done by using 1 × 30 cm DEAE-Sepharose anion exchange chromatography column (Sigma) equilibrated with 0·025 M Piperazine buffer, pH 5·5. The bound proteins were eluted in 4-ml fractions by using a continuous linear gradient of increasing sodium chloride concentration in the Piperazine buffer.

The protein concentration of the various fractions was measured by Lowry's method.[16]

SDS–polyacrylamide gel electrophoresis
SDS–PAGE was performed according to Laemmli[17] by using a 12% resolving gel (0·375 M Tris, 1%SDS, pH 8·8) and 4% stacking gel (0·125 M Tris, 1% SDS, pH 6·8) in a Mini-Protean II dual slab cell (BioRad Lab., Richmond, CA). Samples were diluted one in four in the SDS-reducing sample buffer. Silver staining of the gels was done according to the method of Marshall.[18]

Mitogen-induced lymphocyte proliferation assay (Con A) bioassay.
The Con A assay was carried out as described previously.[13] Briefly, lymph node cells from either C57BL/6J or BDF1 mice were stimulated with 1·1 μg/ml Con A (Sigma) in RPMI-1640 medium supplemented with 5 × 10^{-5} 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% foetal calf serum. Each culture (in triplicate) consisted of 0·5 × 10^5 cells in 0·18 ml tissue culture medium to which 20μl of various SMG fractions (in PBS) were added to a final volume of 0·2 ml. Control cultures received 20μl of PBS. The plates were incubated at 37° in a humidified atmosphere of 5% CO_2 in air for 72 hr. During the last 4–6 hr, 20 μl of medium containing 0·5 μCi of tritiated thymidine (ICN Radiochemicals, Irvine, CA) was added to each culture well. The cells were then collected with a cell harvester (Skatron, Lienbyen, Norway). Radioactivity incorporated by the cells was measured in a liquid scintillation counter.

Skin transplantation
Full thickness skin grafts were taken from the abdominal surface of donor mice (DBA2/J, H-2^d) after shaving and sterilization with 70% ethanol. After the removal of the fat and panniculus carnosus, the skin was cut in 1 × 1 cm pieces and kept in sterile PBS until the graft beds were prepared. Recipient mice (C57BL/6J, H-2^b) were anaesthesized (i.p. injection of Nembutal, 54 μg/g body weight) before removing a piece of the skin slightly smaller than the graft from the lateral wall of the chest. The graft was then applied, sprayed with aeroplast dressing (Park, Davis & Co., Brockville, Ontario, Canada) and bandaged over a piece of gauze. The day of transplantation was taken as Day 0 and the bandage was removed on Day 10, after which the grafts were inspected daily. For histological studies, the graft was removed on Day 12, after killing the animal, together with the surrounding recipient's skin and fixed in 10% formaldehyde in PBS. The 6 μm thick, haematoxylin and eosin-stained sections were prepared from paraffin blocks in the Histology Lab., Dept. of Anatomy, University of Manitoba, Canada.

Delayed-type hypersensitivity (DTH)
The contact sensitivity variant of the DTH response was studied in A/J mice according to the method of Asherson and Ptak.[19] Animals were immunized by the application of 0·1 ml of 5% solution of picryl chloride in ethanol to the clipped skin of the abdomen. The challenge was done by the application of 1% picryl chloride in olive oil on both sides of one ear at Day 6 post-sensitization. The thickness of the ear was measured with calipers immediately before challenge and 24 hr after challenge. The increase in ear thickness was determined by subtracting the first reading from the second one.

Plaque-forming cell response
The liquid monolayer technique of Cunningham and Szenberg[20] was used with some modifications. The two microchambers were prepared by separating two clean 25 × 75 mm plain glass slides with three parallel strips of self-adhesive tape. The two chambers had a volume of about 180 μl. SRBC (National Biological Lab., Gunton, Manitoba, Canada) were washed three times in PBS before being used to immunize C57BL/6J i.p. at a dose of 8 × 10^8 cells per mouse. Mice were killed on Day 4, their spleens were removed aseptically and a spleen cell suspension was prepared in RPMI-1640 medium supplemented with 10% foetal calf serum. After lysing the red cells with a Tris-buffered isotonic ammonium chloride solution, the cell count was adjusted so that each 100 μl of medium contained 10^6 splenocytes. To this, 100 μl cell suspension, 25 μl of diluted guinea-pig complement (Cedarlane Lab. Ltd, Hornby, Ontario, Canada), 50 μl of 12·5% (packed cell volume) of washed SRBC, and 25 μl of tissue culture medium were added in a small tube to a final volume of 200 μl. The contents of the tube were mixed

gently, then withdrawn immediately and used to fill the two microchambers. The chambers were then sealed by painting the edges with a molten mixture of paraffin wax and petroleum jelly (7:3 w/w). They were then incubated for 1 hr in a humidified atmosphere of air with 5% CO_2 at 37°. The resulting plaques were counted on a dark background with low magnification.

RESULTS

After ammonium sulphate precipitation of the male rat SMG homogenate (between 30 and 64% saturation), the resulting crude SMG extract was fractionated by gel filtration chromatography. The resulting fractions were tested for their ability to influence the proliferation of lymph node Con A blasts. As shown in Fig. 1, and in agreement with an earlier study,[13] two pools of fractions capable of modifying the Con A response could be identified. The HMW pool (fractions 20 to 25, 96,000–47,000 MW) and the LMW pool (fractions 26 to 30, 47,000–14,000MW) produced significant suppression and stimulation, respectively. When the crude extract of the rat sublingual gland was treated exactly in the same way and used as a control, no significant effect in the Con A bioassay was detected in any of the resulting fractions (data not shown). SDS–PAGE of both SMG pools showed several protein bands (data not shown).

As reported earlier, the HMW pool showed *in vitro* immunosuppressive activity in a number of assays, an activity which was attributed to a factor with an IL-1 inhibitory effect.[14] We tested the *in vivo* activity of both pools in the skin allograft, direct plaque-forming cell response and the DTH models. As shown in Table 1 and Figs 2 and 3, and contrary to what one might have expected in view of their *in vitro* suppressive activity, the HMW pool did not have a significant effect in these models. The LMW pool, on the other hand, produced significant suppression in all models. Moreover, the LMW pool produced significant suppression of the adjuvant-induced arthritis in rats (data not shown).

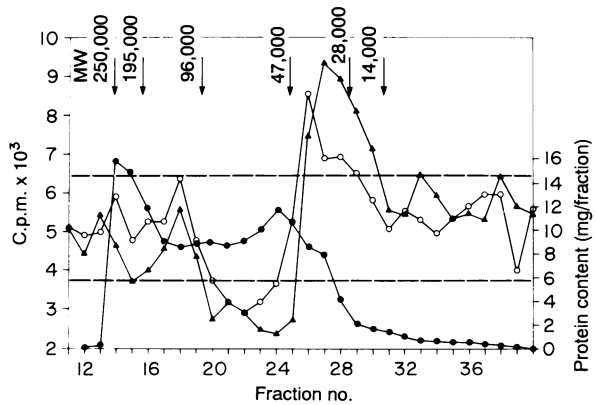

Figure 1. Fractionation of the SMG crude extract by sephacryl gel filtration chromatography. The figure shows the total protein content (●) of the 8-ml fractions. These fractions were tested for their activity in the Con A bioassay in two dilutions 1:6 (▲) and 1:30 (○). The broken horizontal lines represent two standard deviations above and below the mean c.p.m. values of the control cultures (PBS instead of various SMG fractions). Values above or below these lines were considered significant. The arrows at the top of the figure indicate the positions of the MW standards used to calibrate the column.

In order to identify the factor(s) responsible for these *in vivo* immunosuppressive effects, we fractionated the LMW pool by hydrophobic interaction chromatography in a phenyl sepharose column. As shown in Fig. 4, three protein peaks were observed in the elution profile. They were designated peaks A, B and C, respectively. Figure 4 also shows the activity of the various fractions in the Con A bioassay. Most of the resulting fractions

Table 1. Effects of the HMW and LMW pools of the rat SMG in three *in vivo* immunological assays

Exp. model	Treatment*	Groups†	Results ± SD‡	Significance§
Skin transplantation (DBA/2J, donor, C57Bl/6J, recipient)	10 daily doses Days 0 to 9	PBS (5)	12·2 ± 0·37	—
		HMW (5)	13·0 ± 0·44	NS
		LMW (4)	14·7 ± 0·76	$0·02 > P > 0·01$
Direct PFC response (C57Bl/6J mice)	5 daily doses Days 1 to 3	PBS (5)	237·0 ± 19·7	—
		HMW (5)	193·0 ± 12·0	NS
		LMW (5)	119·6 ± 10·0	$0·02 > P > 0·01$
DTH (A/J mice)	2 daily doses Days 5 and 6	PBS (5)	19·0 ± 0·70	—
		HMW (5)	18·2 ± 0·49	NS
		LMW (5)	9·0 ± 1·00	$P < 0·001$

* The animals received the subcutaneous injection of 200 μl of PBS or of PBS containing either the HMW or the LMW fractions. The doses of HMW or LMW fractions corresponded to one-half of a SMG in the case of skin transplantation (0·965 mg or 0·53 mg/dose, respectively) and to one full gland in the other models (1·93 mg or 1·06 mg/dose, respectively).

† Numbers in parentheses indicate animals per group.

‡ Skin transplantation results are expressed as mean survival time; PFC response is expressed as the number of IgM anti-SRBC PFC per one million splenocytes; DTH results are expressed as increases in ear thickness in 1/100 mm units 24 hr after challenge.

§ Significance was determined by the two-sample *t*-test, using the PBS-treated group as a control; NS, not significant.

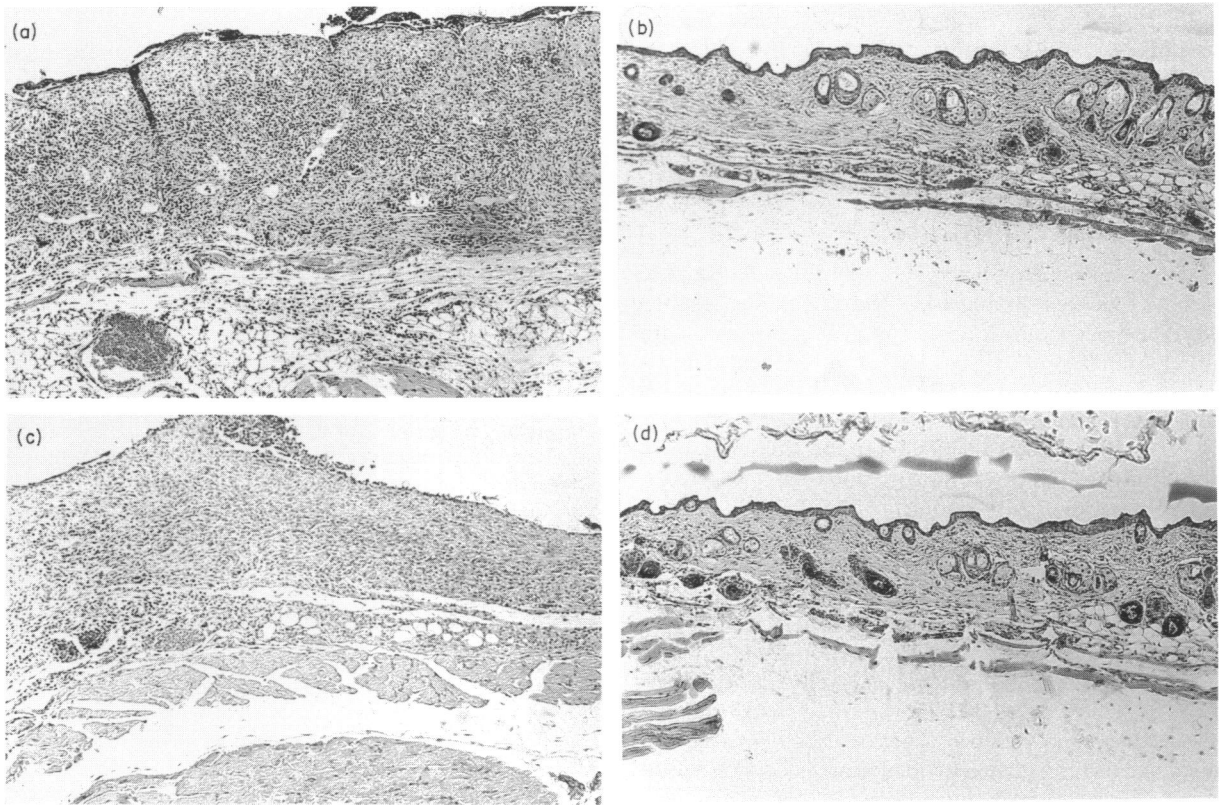

Figure 2. Histological sections of skin grafts. The animals were grafted on Day 0 and killed on Day 12. Five out of six of the skin allografts in the PBS-treated group showed advanced rejection (a). Syngeneic grafts were well preserved (b). In the HMW pool-treated group four out of six of the grafts showed signs of rejection including loss of the epidermis and marked mononuclear cellular infiltrate of the dermis (c). In the LMW-treated group five out of six of the grafts were intact (d). The sections were stained with haematoxylin and eosin and magnified 100 times.

had enhancing activity in this *in vitro* model. When we tested the fractions belonging to these three peaks in the *in vivo* DTH model (see Fig. 3), significant suppression was observed with peak A ($P < 0{\cdot}01$). SDS–PAGE demonstrated less heterogeneity in the *in vivo* active peak A than in the LMW pool (data not shown).

Further fractionation of the immunosuppressive peak A was obtained with anion exchange chromatography. By using a gradient of increasing sodium chloride concentration from 0 to 1·5 M in the piperazine buffer, two major peaks were eluted. The material that did not bind to the gel was eluted early in the gradient (peak AI). This was followed by a second peak (AII). Upon testing the activity of both peaks in the DTH model, peak AII was found to contain the *in vivo* immunosuppressive activity (see Fig. 3c). Using a slightly modified gradient so that the concentration of sodium chloride was 0·2 M at the start and 0·4 M at the end a partial separation of peak AII into two subfractions (peaks AIIa and AIIb) was obtained (Fig. 5). SDS–PAGE followed by silver staining of the gels detected the presence of a single band of approximately 40,000 MW in the fractions of peak AIIa, whereas two bands of 40,000 and 30,000 MW, respectively, were detected in the fractions of peak AIIb (Fig. 6). Both the 40,000 MW and the 40,000 and 30,000 MW fractions showed immunosuppressive activity in the DTH response (Fig. 3d).

DISCUSSION

We demonstrate here that two fractions from the rat SMG, one containing an electrophoretically homogeneous 40,000 MW protein and the other containing a similar 40,000 MW protein and a 30,000 MW one, suppress the DTH reaction in mice. This phenomenon could be attributed to an immunosuppressive or to an anti-inflammatory mechanism. Moreover, less purified fractions also suppressed the rejection of allografts and the plaque-forming cell [PFC) response in mice, the latter model suggesting a true immunosuppressive mechanism. Although the experimental evidence presented here would be compatible with more than one active factor being contained in the LMW pool, the similarity of the models suggests that all these effects may have been induced by a single factor with a molecular size of 40,000 MW which, in this case, would have to be considered immunosuppressive.

The experiments reported here were suggested by our observation that the rat SMG contains a factor with an IL-1 inhibiting activity which produces *in vitro* immunosuppressive effects.[14] When we tested the hypothesis that this factor was the one responsible for the previously reported *in vivo* immunosuppressive activity of the SMG,[6-9] our results (Table 1 and Figs 2 and 3) showed this was not the case. Since the absolute requirement for a stimulatory role of IL-1 in *in vivo* immune

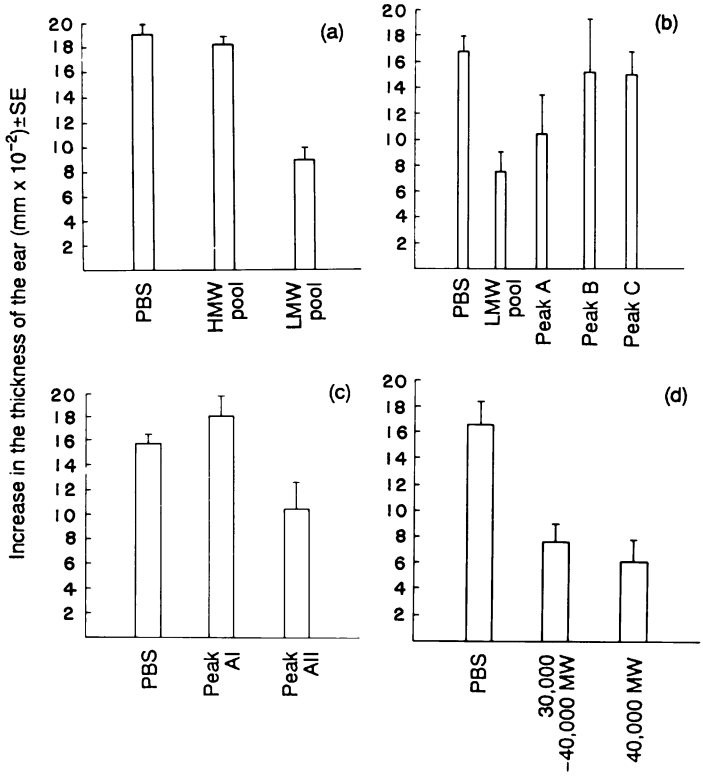

Figure 3. Activity of the rat SMG-derived fractions in the DTH response. A/J mice received two subcutaneous doses, one on Day 5 and the other on Day 6 after sensitization with picryl chloride. Each consisted of a volume of 200 μl of either PBS (the control group) or various SMG fractions in the amount given below which is equivalent to one SMG. (a) Treatment with the LMW pool (1·06 mg/dose) resulted in significant suppression ($P < 0{\cdot}001$), whereas the HMW pool (1·93 mg/dose) had no effect. (b) Significant suppression occurred with the LMW pool and with peak A obtained by hydrophobic interaction chromatography (0·52 mg/dose) ($P < 0{\cdot}001$, $0{\cdot}001 > P > 0{\cdot}01$, respectively) but not with either peak B or C (0·21 and 0·08 mg/dose, respectively). (c) Significant suppression was seen with peak AII (250 μg/dose, $P > 0{\cdot}001$) but not with fraction AI (65 μg/dose). (d) Both the 40,000 MW and the 40,000 and 30,000 MW fractions (peak AIIa and AIIb of Fig. 5, respectively) effectively suppressed the DTH response (98 and 125 μg/dose, respectively).

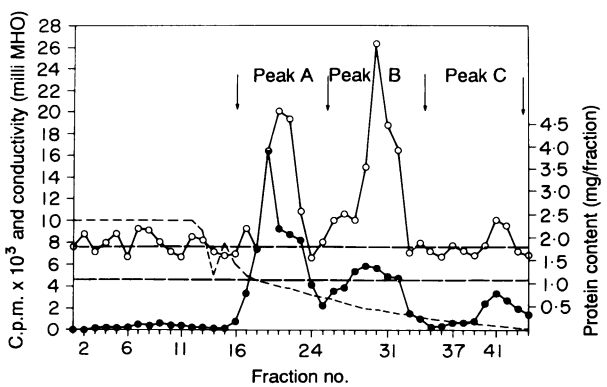

Figure 4. Fractionation of the LMW pool through hydrophobic interaction chromatography. Elution of the protein was achieved by using a continuous linear gradient of decreasing ammonium sulphate (from 30 to 0%) and increasing ethylene glycol (from 0 to 60%) concentration. Monitoring of the gradient was done by measuring the conductivity (- - -) of the resulting 4-ml fractions. Also shown in the figure is the protein content (●) as well as the activity of each fraction (diluted 1:10) in the Con A bioassay (○).

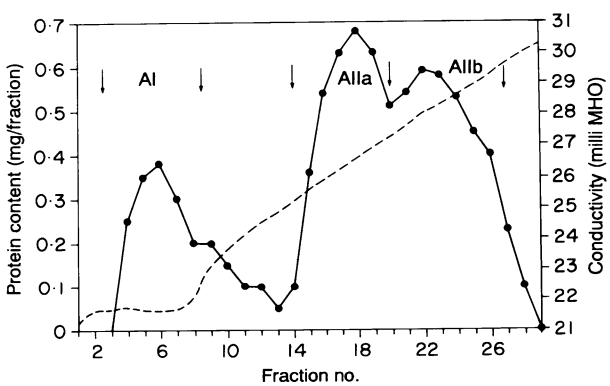

Figure 5. Fractionation of peak A through anion exchange chromatography. A linear continuous gradient of increasing sodium chloride concentration (from 0·2 to 0·4 M) was used. The protein content (●) and the conductivity (- - -) of each fraction was measured. The arrows at the top of the figure indicate the cuts used to obtain the various fractions for the experiments in Fig. 3.

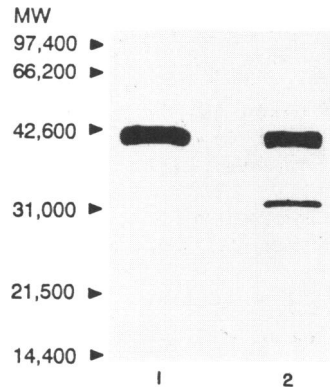

Figure 6. SDS–PAGE and silver staining of peak AIIa and AIIb of the DEAE-sepharose column. The pooled DEAE-sepharose fractions 15 to 19 (Fig. 5) forming peak AIIa (lane 1) presented a single protein band of approximately 40,000 MW. Lane 2 shows fractions 20 to 26 (peak AIIb of Fig. 5) containing two proteins of 40,000 and 30,000 MW. The arrows show positions of the molecular weight markers.

responses has not been proven,[21] it is not totally surprising that the HMW pool, which was shown to contain an IL-1 inhibitory factor, did not suppress *in vivo* DTH. This result is also in agreement with the major role attributed to Th1 cells in DTH, [22] since these cells appear to lack the receptors for, and the ability to respond to , IL-1.[23]

On the other hand, we found *in vivo* immunosuppression to be induced by the LMW pool which is associated with fractions capable of stimulating *in vitro* lymphocyte proliferation. Further purification of this pool showed that the suppressive activity in the DTH model was contained in the 40,000 MW and the 40,000 and 30,000 MW fractions. Even with these more purified fractions, an *in vitro* stimulatory activity in the Con A assay was associated to the *in vivo* immunosuppressive activity. As a possible explanation for this association of two apparently opposite effects, it may be suggested that some T-cell subsets may have been stimulated by the biologically active factor while others may have been suppressed. The increased proliferative activity of the former subsets would be detected in the Con A assay, while the suppression of the latter would be expressed in the *in vivo* immunological assays employed by us. Alternatively, the cells that are stimulated by the factor may have suppressive effects on Th cells, thus explaining the suppression of both DTH and PFC reactions.

The relationship between the 40,000 MW and the 30,000 and 40,000 MW fractions is currently unknown. However, on the basis of the similarity of molecular weight, and of the immunological cross-reactivity of the 40,000 MW proteins contained in the two adjacent fractions (M. Abdelhaleem and E. Sabbadini, unpublished data), it is likely that the 40,000 MW protein may be the same in both fractions and is the one that is responsible for *in vivo* immunosuppression. Further separation of the 40,000 and 30,000 MW proteins is required to confirm this possibility.

The SMG contains several biologically active factors that may have been responsible for the phenomena described here. For example, nerve growth factor (NGF) was shown to possess anti-inflammatory properties.[24,25] The biologically active β chain of NGF has a molecular weight of about 13,000[26] and is frequently observed as a dimer of 26,000 MW. Thus, the 40,000 MW we observed for the putative active factor in our experiments would indicate either that NGF was not responsible for the phenomena described here, or that an unusual complex of three β chains was involved.

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REFERENCES

1. Barka T. (1980) Biologically active polypeptides in submandibular glands. *J. Histochem. Cytochem.* **28,** 836.
2. Gresik E.W. (1980) Postnatal developmental changes in submandibular glands of rats and mice. *J. Histochem. Cytochem.* **28,** 860.
3. Levi-Montalcini R. (1987) The nerve growth factor 35 years later, *Science,* **237,** 1145.
4. Murphy R.A., Watson A.Y., Metz J. & Frossman W.G. (1980) The mouse submandibular gland: an exocrine organ for growth factors. *J. Histochem. Cytochem.* **28,** 890.
5. Gill G.N., Bertics P.J. & Santon J.B. (1987) Epidermal growth factor and its receptor. *Mol. Cell. Endocrinol.* **51,** 169.
6. Takeda T., Yamasaki Y., Yamabe H., Suzuki Y., Haebera H., Irino T. & Grollman A. (1967) Atrophy of the lymphoid tissues of mice induced by extracts of the submandibular gland. *Proc. Soc. exp. Biol. Med.* **126,** 212.
7. Kongshavn P.A.L. & Bliss J.Q. (1970) Effect of mouse submandibular gland extracts on survival of H-2 incompatible skin allografts. *Immunology,* **19,** 363.
8. Kongshavn P.A.L. & Lapp W.S. (1972) Immunosuppressive effect of male mouse submandibular gland extracts on plaque-forming cells in mice: abolition by orchiectomy. *Immunology,* **22,** 227.
9. Roberts M.L., Freston J.A. & Reade P.C. (1976) Suppression of immune responsiveness by a submandibular gland factor. *Immunology,* **30,** 811.
10. Naughton M.A., Koch J., Hoffman H., Bender V., Hagopian H. & Hamilton E. (1969) Isolation and activity of a thymocyte-transforming factor from the mouse submaxillary gland. *Exp. Cell Res.* **57,** 95.
11. Naughton M.A., Geczy C., Bender V., Hoffman H. & Hamilton E. (1972) Esteropeptidase and thymotropic activity of a protein isolated from the mouse submaxillary gland. *Biochem. biophys. Acta,* **263,** 106.
12. Koch J.H. & Rowe J. (1976) Immunosuppressive activity of submaxillary gland extracts of the mouse. I. Effect on antibody formation in response to sheep red blood cells. *Eur. J. Immunol.* **6,** 583.
13. Kemp A., Mellow L. & Sabbadini E. (1985) Suppression and enhancement of *in vitro* lymphocyte reactivity by factors in rat submandibular gland extract. *Immunology,* **56,** 261.
14. Kemp A., Mellow L. & Sabbadini E. (1986) Inhibition of interleukin 1 activity by a factor in submandibular glands of rats. *J. Immunol.* **137,** 2245.
15. Takeda T. & Grollman A. (1968) Inhibitory action of submaxillary gland of thymus and lymphoid tissues of the mouse. *Am. J. Physiol.* **215,** 1337.
16. Lowry O.M., Rosenbrough J., Farr A.L. & Randall R.J. (1951) Protein measurement with the Folin Phenol reagent. *J. biol. Chem.* **193** 265.
17. Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227,** 680.
18. Marshall T. (1984) Detection of a protein in polyacrylamide gels using an improved silver stain. *Anal. Biochem.* **136,** 340.
19. Asherson G.L. & Ptak W. (1986) Contact and delayed hypersensitivity in the mouse. I. Active sensitization and passive transfer. *Immunology,* **15,** 405.

20. CUNNINGHAM A.J & SZENBERG A. (1968) Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology*, **14,** 599.
21. DURUM S.K. & OPPENHEIM M. (1989) Macrophage derived mediators. In *Fundamental Immunology*, edn 2 ed. W. E. Paul, p.642. Raven Press, New York.
22. MOSSMAN T.R. & COFFMAN R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Ann. Rev. Immunol.* **7,** 145.
23. GREENBAUM L.A. HOROWITZ J.B., WOODS A., PASQUALINI T. & REICH E.-P. (1988) Autocrine growth of $CD4^+$ T-cells: differential effects of IL-1 on helper and inflammatory T-cells. *J. Immunol.* **140,** 1555.
24. BANKS B.E.C., VERNON C.A. & WARNER J.A. (1984) Nerve growth factor has anti-inflammatory activity in the rat hindpaw edema test. *Neurosci. Lett.* **47,** 41.
25. AMICO-ROXAS M., CARUSO A., LEONE M.G., SCIFO R., VANELLA A. & SCAPAGNINI U. (1989) Nerve growth factor inhibits some acute experimental inflammations. *Arch. Int. Pharmacodyn. Ther.* **299,** 285..
26. GREENE L.A. & SHOOTER E.M. (1980) The nerve growth factor: biochemistry, synthesis, and mechanism of action. *Ann. Rev. Neurosci.* **3,** 353.