

IDENTIFICATION OF IN VIVO IMMUNOSUPPRESSIVE FRACTIONS  
FROM THE RAT SUBMANDIBULAR SALIVARY GLAND

A thesis submitted to the Faculty of Graduate Studies  
in partial fulfilment of the requirements of the degree of  
Master of Science

BY

Mohamed Mahmoud Abdelhaleem

Department of Immunology  
University of Manitoba  
Winnipeg, Manitoba  
February, 1990



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ISBN 0-315-63399-9

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MOHAMED MAHMOUD ABDELHALEM

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ABSTRACT

The presence in the submandibular salivary gland (SMG) of factors with inhibitory effects on the immune system has been suggested by a number of previous reports. We tried to isolate the in vivo immunosuppressive factors from the rat SMG. This has been achieved by testing for in vivo immune activity the SMG fractions obtained in a multi-step purification procedure. The first step was ammonium sulphate precipitation of the SMG homogenate. Gel filtration chromatography of this material resulted in two pools of fractions with significant effect on the in vitro proliferation of murine lymph node Con-A blasts. Of these two pools, only the one with the lower molecular weight (LMW) (between 47 and 14 kD) resulted in prolongation of murine skin allograft survival, suppression of the delayed type hypersensitivity (DTH) response to Picryl Chloride and decrease the number of direct (IgM) plaque forming cells (PFC) against sheep red blood cells (SRBC). Fractionation of the LMW pool through hydrophobic interaction chromatography resulted in three protein peaks designated A, B and C. Of these peaks, only peak A produced significant suppression of the DTH response. Further purification of peak A through anion exchange

- ii -

chromatography produced two fractions with immunosuppressive activity in the DTH response. One fraction had an SDS-PAGE single band of 40 kD, while the other had two bands of 30 and 40 kD.

TABLE OF CONTENTS

|   | Page |
|---|------|
| ABSTRACT .....  | i    |
| TABLE OF CONTENTS .....   | iii  |
| ACKNOWLEDGEMENT .....   | vi   |
| ABBREVIATIONS .....   | vii  |
| LIST OF FIGURES .....   | ix   |
| LIST OF TABLES .....  | xii  |
| REVIEW OF LITERATURE  |      |
| I. Structural freatures of the submandibular<br>salivary gland of rodents ..... | 1    |
| II. SMG as a source of a variety of biologically<br>active factors .....        | 4    |
| II.1. Nerve growth factor .....   | 5    |
| II.2. Epidermal growth factor .....   | 8    |
| III. Regulatory role of the SMG .....   | 10   |
| IV. Relationship between the SMG and the<br>immune system .....                 | 13   |
| IV.1. Effect of injection of the SMG extract<br>on the immune system .....      | 14   |
| IV.2. Effect of extirpation of the SMG<br>on the immune system .....            | 19   |
| IV.3. The rat SMG .....   | 21   |

MATERIALS AND METHODS

|   |    |
|---|----|
| 1. Animals .....  | 23 |
| 2. Buffers .....  | 23 |
| 3. Tissue culture medium .....  | 24 |
| 4. Preparation of the rat SMG crude extract .....                         | 25 |
| 5. Purification of the SMG extract .....                                  | 26 |
| 5.1. Gel filtration chromatography .....                                  | 26 |
| 5.2. Hydrophobic interaction chromatography .....                         | 26 |
| 5.3. Anion exchange chromatography .....                                  | 27 |
| 6. SDS-PAGE .....   | 28 |
| 7. Immunological assays .....   | 29 |
| 7.1. <u>In vitro</u> assays.  |    |
| 7.1.1. Mitogen-induced lymphocyte<br>proliferation (Con A bioassay) ..... | 29 |
| 7.1.2. Mixed lymphocyte reaction .....                                    | 30 |
| 7.1.3. Cell mediated cytotoxicity .....                                   | 31 |
| 7.1.4. Antigen-induced proliferation .....                                | 32 |
| 7.2. <u>In vivo</u> assays.   |    |
| 7.2.1. Skin transplantation .....   | 33 |
| 7.2.2. Delayed type hypersensitivity response                             | 34 |
| 7.2.3. Plaque forming cell response .....                                 | 35 |
| 8. Statistical analysis .....   | 37 |

RESULTS

|   |     |
|---|-----|
| 1. Fractionation of the SMG crude extract<br>through gel filtration chromatography .....                | 38  |
| 2. Heterogeneity of the Sephacryl high and<br>low molecular weight pools .....                          | 38  |
| 3. <u>In vivo</u> immunoreactivity of the high and<br>low molecular weight Sephacryl pools .....        | 43  |
| 3.1. Skin allograft rejection .....   | 43  |
| 3.2. DTH response .....   | 49  |
| 3.3. Direct plaque forming cell response .....  | 62  |
| 4. Fractionation of the low molecular weight pool<br>through hydrophobic interaction chromatography ... | 65  |
| 5. Peak A has the <u>in vivo</u> immunosuppressive activity<br>but it is still heterogeneous .....      | 68  |
| 6. Fractionation of peak A through anion exchange<br>chromatography .....                               | 73  |
| 7. Suppression of the DTH response by both 40k and<br>30-40k fractions .....                            | 83  |
| 8. Summary of the purification steps of the <u>in vivo</u><br>immune activity of the rat SMG .....      | 83  |
| 9. <u>In vitro</u> immune reactivity of the various<br>fractions of the rat SMG .....                   | 88  |
| DISCUSSION .....  | 119 |
| REFERENCES .....  | 128 |

ACKNOWLEDGEMENT

I wish to express my sincere appreciation and gratitude to my supervisor, Dr. Edris Sabbadini, for his invaluable support and guidance. His encouragement and endless patience throughout this project and the writing of this thesis made life easier.

I would like to thank all members of the department of Immunology, University of Manitoba, particularly those located at the Immunology Annex, for their support.

This project was supported by the Medical Research Council of Canada and a Manitoba Health Research Council Studentship.

ABBREVIATIONS

|       |                                     |
|-------|-------------------------------------|
| ATS   | Antimouse thymocyte serum           |
| CD    | Cluster of differentiation          |
| cDNA  | Complementary deoxyribonucleic acid |
| CMC   | Cell mediated cytotoxicity          |
| Con A | Concanavalin A                      |
| DEAE  | Diethylaminoethyl                   |
| DFP   | Diisopropylfluorophosphate          |
| DTH   | Delayed type hypersensitivity       |
| EGF   | Epidermal growth factor             |
| GCT   | Granular convoluted tubules         |
| HBSS  | Hank's balanced salt solution       |
| HMW   | High molecular weight               |
| IL    | Interleukin                         |
| Ig    | Immunoglobulin                      |
| kDa   | Kilodalton                          |
| LMW   | Low molecular weight                |
| L.N.  | Lymph node                          |
| mAb   | Monoclonal antibody                 |
| mg    | Milligram                           |
| MLR   | mixed lymphocyte reaction           |
| mRNA  | Messenger ribonucleic acid          |
| M.W.  | Molecular weight                    |
| NGF   | Nerve growth factor                 |

PBS Phosphate-buffered saline

PFC Plaque forming cell

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel  
electrophoresis

SMG Submandibular salivary gland

SRBC Sheep red blood cells

ug Microgram

LIST OF FIGURES

| Figure  | Page |
|---|------|
| 1 Fractionation of the SMG crude extract through gel filtration chromatography.....                         | 39   |
| 2 Fractionation of the SMG and sublingual glands crude extracts through gel filtration chromatography ..... | 41   |
| 3 SDS-PAGE & silver staining of the crude rat SMG extract and the Sephacryl pools.....                      | 44   |
| 4 Prolongation of the skin allograft survival by treatment with the low molecular weight pool ..            | 47   |
| 5 Rejected skin allograft from the PBS-treated group .....  | 50   |
| 6 Syngeneic skin graft .....  | 52   |
| 7 Intact allograft from the low molecular weight pool-treated group .....                                   | 54   |
| 8 Higher magnification of an intact graft from the low molecular weight pool-treated group ...              | 56   |
| 9 A rejected allograft from the high molecular weight pool-treated group .....                              | 58   |
| 10 Higher magnification of a rejected graft from the high molecular weight pool-treated group ..            | 60   |
| 11 Suppression of the DTH response by treatment with the low molecular weight Sephacryl pool ..             | 63   |
| 12 Suppression of the direct PFC response by the  |      |

|   |    |
|---|----|
| low molecular weight pool .....   | 66 |
| 13 Fractionation of the low molecular weight pool<br>through hydrophobic interaction chromatography.  | 69 |
| 14 Suppression of the DTH response by peak A .....  | 71 |
| 15 SDS-PAGE & silver staining of the low molecular<br>weight pool and the various peak of the<br>Phenyl Sepharose column .....                                | 74 |
| 16 Suppression of the DTH response by peak AII of<br>the DEAE-Sepharose column .....  | 77 |
| 17 Fractionation of peak A through anion-exchange<br>chromatography .....   | 79 |
| 18 SDS-PAGE & silver staining of the fractions of<br>the DEAE-Sepharose column .....  | 81 |
| 19 SDS-PAGE & silver staining of peak AIIa and<br>AIIb of the DEAE-Sepharose column .....   | 84 |
| 20 Suppression of the DTH response by 30-40 K<br>and 40 K fractions .....   | 86 |
| 21 SDS-PAGE & silver staining of the proteins<br>at various steps of purification of the <u>in</u><br><u>vivo</u> immunosuppressive activity of the rat SMG . | 91 |
| 22 Activity of the Sephacryl pools in the Con A<br>bioassay .....   | 94 |
| 23 Activity of the Sephacryl pools in the MLR<br>assay .....  | 96 |

|          |  |     |
|----------|--|-----|
| 24       | Activity of the Sephacryl pools in the antigen-induced proliferation assay ..... | 98  |
| 25       | Activity of the Sephacryl pools in the CMC assay .....                           | 101 |
| 26       | Activity of peaks A, B, and C in the Con A bioassay .....                        | 103 |
| 27       | Activity of 40k and 30-40k fractions in the Con A bioassay .....                 | 105 |
| 28       | Activity of 40k and 30-40k fractions in MLR ...                                  | 107 |
| 29       | Activity of 40k and 30-40k in the antigen-induced proliferation assay .....      | 109 |
| 30,31,32 | Activity of 40k and 30-40k in the CMC assay .....                                | 111 |

LIST OF TABLES

| Table |   | Page |
|-------|---|------|
| 1     | Summary of the purification steps for<br>the <u>in vivo</u> immune activity of the rat<br>SMG ..... | 89   |

REVIEW OF LITERATURE

I. Structural features of the submandibular salivary gland of rodents

Salivary glands consist of specialized collection of cells organized for the purpose of producing and secreting saliva. Saliva is a complex mixture of water, salts, macromolecules, notably mucins and enzymes. Saliva is important in maintaining the integrity of the oral cavity as well as in initiating digestion. However, significant species differences, and sometimes sex differences within the same species, do occur with respect to the gross anatomy, histological architecture, histochemical staining, physiology and biochemistry of the salivary glands (Pinkstaff, 1980).

The submandibular salivary gland (SMG) of rodents is a compound tubulo-acinar organ. The adult male rat SMG consists of five interconnected portions ; acini, intercalated ducts, granular ducts, striated ducts and the main excretory duct. The acini contain both secretory and myoepithelial cells. They are connected to the main conduit system by the intercalated ducts which contain nonsecretory cells. On the other hand, the granular ducts or the granular convoluted tubules (GCT) are lined with cells which are full of secretory granules. These

granules are heterogeneous in size and electron opacity. GCT contain several types of granular cells which are thought to represent different secretory stages of the same cell type. The striated ducts consist of tall columnar cells with extensive infolding of the basal plasma membrane. The excretory duct cells have also basal infoldings. The arrangement of many capillaries around the excretory duct suggests that it is primarily concerned with water transport (Tamarin & Sreebny, 1965).

The structure of the mouse SMG follows the same organization, except that morphological differences do exist between the male and the female glands. The intercalated ducts may contain secretory granules in the glands of the female but not the male. A more notable difference is that the GCT of the male's gland are more extensive with more prominent granules. They constitute around 50% of the gland volume in the male compared to 15 to 20% in the female (Caramia, 1966). Because of these differences, the mouse SMG is said to be sexually dimorphic. In the rat, however, this morphologic sexual dimorphism is not normally evident (Cutler&Chaudry, 1975).

The rodent SMG is not fully developed at birth, but it is rather formed of transitional cellular elements

whose definitive differentiation takes place postnatally. The early stages of development are dominated by the development of acinar cells. The full development of GCT cells from their precursors, the striated duct cells, occupies later stages of development. In mice, GCT cells appear in both sexes at 25 days of age and they do not become sexually dimorphic until 60 days of age when full sexual maturity has been reached (Gresik, 1980). On the other hand, cells of the GCT of the SMG of senescent male mice show structural changes indicative of functional decline. When the GCT cells of 12- and 28-month-old male mice are compared, old cells show changes occurring mainly in secretory granules and lysosomal elements. Secretory granules vary greatly in size with advancement of age. Some GCT cells have very fine apical granules, whereas the number of polysomes and the extent of Golgi apparatus are apparently decreased (Gresik, 1989).

The GCT portion of the SMG attracted a lot of attention because a large variety of factors with striking biological activities has been detected, particularly in the mouse, and they have been specifically located in the this part of the SMG (Barka, 1980).

In adult rodents, GCT are under multihormonal control. The synergistic actions of androgens, thyroxine and the adrenal corticosteroids are necessary for their maintenance. It has been shown that androgens alone are unable to induce GCT cells precociously because of the lack of androgen receptors, while thyroxine alone is able to do so and can act synergistically with androgens in this induction (Gresik, 1980). It has also been shown that hypophysectomy of prepubertal rats results in inhibition of the development of the GCT cells (Kronman, 1963).

## II. SMG as a source of a variety of biologically active factors

The mouse SMG contains a large number of biologically active molecules (reviewed in Barka, 1980). Some of these factors are involved in growth and differentiation such as the nerve growth factor (NGF) and epidermal growth factor (EGF), while other factors have activities related to homeostasis (kallikreins, renin, gastrin, somatostatin), intracellular regulation (esteropeptidases) and digestion (amylase, acid phosphatase).

Direct histochemical studies have shown that the majority of these factors are located in the GCT cells.

Other evidence which support this finding include the presence of higher concentrations in the glands of the male than those of the female, the rise in concentration after androgen treatment and fall following orchietomy and the presence of very low or negligible levels of these substances in prepubertal glands with increase in concentration after puberty in parallel with the cytodifferentiation of the GCT cells (Gresik, 1980).

A detailed discussion of these biologically active factors is beyond the scope to this review and the reader is referred to the review by Barka (Barka, 1980). However, a brief account of two of the most extensively studied of these factors, NGF and EGF, will be presented.

### II.1. NGF

NGF was first isolated from snake venom (Cohen&Levi-Montalcini, 1956). Later, it was also isolated from the male mouse SMG (Cohen, 1960). Although NGF has been also identified in a wide variety of vertebrate tissues, large amounts can be isolated only from these sources which represent true sites of synthesis in vivo (Bradshaw, 1978).

Mouse SMG NGF is isolated from the gland homogenate as a high molecular weight complex containing three polypeptide chains designated, alpha, beta, and gamma.

This complex has a molecular weight of approximately 130,000 dalton and it is referred to as the 7S NGF (Varon et al, 1967). The three subunits of the 7S NGF are held together by noncovalent forces which are stabilized by zinc ions. The 7S NGF has two alpha, two gamma and one beta chain. The beta subunit is the one which possesses the nerve growth promoting activity. The beta subunit is a dimer of two identical polypeptide chains held together by noncovalent bonds. The gamma subunit has been shown to be an arginine-specific esteropeptidase of the serine protease family, while the alpha subunit is an acidic protein of as yet unknown biological function (Bradshaw, 1978).

Immunocytochemical studies have localized NGF in the GCT cells of the adult mice (Schwab et al, 1976). The male mouse SMG contains more NGF than the female's gland (Hendry, 1972). The level of NGF in the SMG of both sexes is androgen-dependent. It is reduced in the male's gland by castration and increased by administration of testosterone to castrated male or female animals (Ishii&Shooter, 1975). Recently, NGF mRNA has been colocalized with the NGF precursor protein in the basal parts of the GCT cells. Conversion of the proNGF to the mature one results in altered localization with a

preferential concentration at the apical pole of the GCT cells (Dicou et al, 1988).

The amino acid (Bradshaw, 1978) as well as the nucleotide sequence of cDNA encoding the murine (Scott et al, 1983) and the human (Ulrich et al, 1983) NGF has been determined. These molecules are highly homologous.

In culture, two types of peripheral neurons respond to NGF by extending neurites, these are the adrenergic neurons of sympathetic ganglia and the mediodorsal sensory neurons of dorsal root ganglia (Levi-Montalcini&Booker, 1960). Injection of anti-NGF antibody into neonatal animals has been shown to prevent the development of their sympathetic nervous system, a procedure called immunosympathectomy (Levi-Montalcini, 1987).

NGF is necessary for the maintenance and function of adult neurons by acting as a trophic agent. NGF has also a neurotropic effect manifested by its ability to direct growing or regenerating axons of sensory and sympathetic fibres along its concentration gradient. Targets of NGF action include central nervous system neurons and cells of nonneuronal origin besides the neural crest derivatives (sympathoadrenal and sensory neurons). The cells of nonneuronal origin which are affected by NGF

include mast cells, mononuclear spleen cells, and possibly thymocytes which have been shown to express specific NGF receptors (Levi-Montalcini, 1987).

NGF binds to specific plasma membrane receptors and the bound NGF is internalized. Then NGF undergoes retrograde axonal transport where it interacts with specific receptors located on the nuclear membrane to directly affect transcriptional events (Greene&Shooter, 1980).

#### II.2. EGF

Injection of extracts of the mouse SMG into newborn animals led to precocious eyelid opening and incisor eruption. The factor responsible for these effects was later isolated and it was shown to have a direct stimulatory effect on epidermal growth and keratinization, so it was given the name "Epidermal Growth Factor" (EGF) (Cohen, 1962).

EGF is a single polypeptide chain of 53 amino acids which has a molecular weight of 6045 Daltons. In the crude homogenate of mouse SMG, EGF is isolated as a component of a high-molecular weight (74,000 Daltons) complex which has two molecules of EGF and two molecules of binding protein. This binding protein has been shown to be an enzyme with arginine esteropeptidase activity

which is similar, but not identical, to the gamma subunit of the 7S NGF (Carpenter&Cohen,1979).

Human EGF has been isolated from urine (Cohen& Carpenter, 1975). The gastric antisecretory hormone urogastrone is identical to human EGF. Urogastrone produces identical biological responses as the mouse SMG EGF (Gregory, 1975). Both molecules compete for the same receptor site on cultured fibroblasts (Hollenberg& Gregory, 1977). EGF has also been isolated from the rat SMG (Moore, 1978).

Like NGF, EGF is located in the GCT cells of mouse SMG which exhibit both sexual dimorphism and sensitivity to androgen level in relation to their content of both factors (Barka, 1980).

EGF has been demonstrated to elicit significant biological responses in intact animals, organ cultures and cell culture systems. These responses include accelerated proliferation and differentiation of skin, corneal, lung, and tracheal epithelia. EGF enhances the proliferation of a wide variety of nontransformed as well as transformed cells (Carpenter&Cohen, 1979). EGF mediates its responses through interaction with a widely distributed receptor of 170,000 Dalton molecular weight. EGF receptor is a transmembrane glycoprotein with

extracellular and intracellular domains separated by a single hydrophobic transmembrane region. Binding of EGF to the extracellular domain leads to activation of the intracellular domain, which has an intrinsic protein tyrosine kinase activity, with subsequent increase in intracellular phosphorylation as well as self-phosphorylation (Gill et al, 1987).

### III. Regulatory role of the SMG

The regulatory role of the GCT of the SMG and their biologically active factors remains an unresolved issue (Barka, 1980). The GCT portion of the gland has been shown to be dependent on the hormonal status of the animal, especially androgen levels. So, before puberty the levels of growth factors, NGF and EGF, are lowest at the time when their potential target cells show maximal sensitivity to them and highest when these cells are least responsive. Moreover, other substances, apparently having nothing to do with growth per se like renin or amylase are also rising simultaneously with the growth factors (Gresik, 1980).

Earlier reports showed that removal of the mouse SMG resulted in an abrupt decrease in the level of serum NGF which returned gradually to the normal level after several weeks suggesting that NGF was synthesized in

tissues other than the SMG (Hendry&Iversen, 1973). In contrast, Murphy et al (1977) reported no decline in serum NGF after sialectomy. However, it has been shown later that significant concentrations of NGF in mouse serum probably do not exist. The mouse serum elicits no biological response although it exhibits marked activity in the NGF radioimmunoassay. It has been suggested that a serum protein may nonspecifically interfere in the immunoassay (Walker, 1982).

The biological significance of the murine SMG NGF is not fully appreciated. The sexually dimorphic male mouse SMG has ten times higher concentrations of NGF in comparison with the female's gland (Barka, 1980). Removal of the male SMG does not lead to any adverse effects on the sympathetic or sensory neurons (Levi-Montalcini, 1987). Moreover, other SMG that are not sexually dimorphic, as in the rat, make no detectable NGF (Bothwell et al, 1979). These results together with the negative attempts to detect the presence of NGF in the circulating blood are against the hypothesis that NGF of SMG origin gains access to the target cells (Levi-Montalcini, 1987).

However, a role of male mouse SMG NGF in fighting has been suggested. Intraspecific fighting,

experimentally induced in adult male mice by six to eight weeks of social isolation, results in massive NGF release into the blood stream. This effect could be prevented by previous removal of the SMG. The released SMG NGF may have a role in the stimulation of the synthesis of adrenal catecholamines. The mechanisms involved in triggering SMG NGF release under these circumstances are not yet understood (Levi-Montalcini, 1987).

Although EGF is synthesized and stored in the GCT cells of murine SMG, removal of the gland does not affect the plasma levels of EGF. Moreover, the androgen-dependent levels of EGF in the mouse SMG do not reflect its levels in plasma and other body fluids. The level of plasma EGF in male mice is not significantly different from that of the female. Also, EGF concentrations in the plasma of immature male mice are not different from adult levels (Carpenter&Cohen, 1979).

A role for the murine SMG as an exocrine organ of growth factors has been suggested. Large amounts of NGF and EGF are secreted into the saliva of adult mice, in forms which are biologically active and chemically similar to the proteins found in salivary gland extracts, following stimulation with either alpha or beta adrenergic agonists. The concentrations of secreted

growth factors in saliva reflect the sexual dimorphism of the gland, male saliva has more of both factors than in the female. Morphological studies have shown that these adrenergic agents induce the GCT cells to secrete their cytoplasmic vesicles into the saliva rather than into the blood (Murphy et al, 1980).

The role of growth factors in murine saliva is not yet known. However, the mammalian kidney has also been shown to be a significant site of EGF synthesis which is secreted in urine. Although the function of this urinary EGF has not been established, a role for EGF in saliva and urine in maintaining the surface integrity of the gastrointestinal and urinary tracts, respectively, has been recently proposed (Fisher et al, 1989).

#### IV. Relationship between the SMG and the immune system

Most of the studies which tried to define the relationship between the SMG and the immune system were done in mice. These studies can be grouped into two categories ; the majority of them dealt with the effect of injection of the mouse SMG homogenate, or purified fractions thereof, on the morphology and/or responsiveness of the immune system, whereas the rest dealt with effects of extirpation of the SMG on the immune system.

IV.1. Effect of injection of SMG extract on the immune system

Takeda et al (1967) showed that injection of the crude SMG extract prepared from male mice for seven days had led to marked atrophy of the lymphoid tissues, particularly the thymus. This effect was not mediated through the adrenals as adrenalectomized animals showed the same degree of atrophy. They suggested that SMG of male mice contained a substance which was responsible for this effect. Later, they tried to purify this factor using ammonium sulphate and ethanol precipitation followed by gel filtration chromatography. They used the effect on the morphology of lymphoid organs as the bioassay to reach the active factor. They identified a pool of five gel filtration fractions which had the effect and was free from irritant, renin-like pressor and depressor substances. However, they showed that these substances may be responsible for part of the inhibitory action of the crude extract (Takeda&Grollman, 1968).

The inhibitory effect on the morphology of lymphoid tissues produced by injection of male mouse SMG extract suggested that immune responses could be suppressed by a similar treatment. Intraperitoneal injection of male mouse SMG extract led to significant prolongation of skin

allograft survival. Although the prolongation in survival was only by a few days, combined treatment of SMG extract and antimouse thymocyte serum (ATS) prolonged the survival time more than in ATS-treated group alone. This proved that SMG extract injection resulted in suppression of this cellular immune response (Kongshavn&Bliss, 1970).

Antibody response to sheep red blood cells (SRBC) was also suppressed by treatment with SMG extract from male mice. SMG extract prepared from female mice did not have any suppressive effect. Moreover, orchietomy of male mice at two weeks of age rendered the extract prepared from their SMG immunologically ineffective. This suggested that the suppressive factor was present only in the male gland and it was androgen-dependent (Kongshavn&Lapp, 1972).

Delayed type hypersensitivity (DTH) response was shown to be suppressed by treatment with SMG extract from male mice. Suppression of the same response was observed in a group of animals injected with EGF and this led the authors to suggest that EGF was responsible, at least partially, for the action of SMG extract in suppressing the DTH response (Roberts et al, 1975). However, Dean et al (1986) showed that EGF had no effect on in vitro two-way mixed lymphocyte reaction (MLR) using mouse spleen

cells, whereas crude SMG extract produced significant suppression. EGF has been shown to be a potent activator of adrenal corticosteroids synthesis and release (Sing-Asa&Water, 1983) an effect which might explain its in vivo suppression of the DTH response.

Efforts have been made to purify and isolate the immunologically active factor (s) from the mouse SMG. Hoffman et al (1967) developed a method to study the in vitro activities of various SMG extract proteins on the morphology of the thymus. They explanted fragments of thymic tissue of newborn mice on thin longitudinal slices of acrylamide gel within which the proteins of crude SMG extract had been separated electrophoretically. They showed that the gel on which the SMG extract was separated had extensive growth of the explanted thymic fragments except over a narrow band where almost all the thymocytes were fragmented and lysed. This band was different from the NGF region (Hoffman&McDougall, 1968). They isolated the responsible factor by gel filtration followed by anion exchange chromatography and reached an electrophoretically homogenous material. The in vitro activity of this factor was dose-related, with lysis and agglutination of thymocytes occurring with higher doses and a characteristic cellular transformation occurring

with lower doses. This transformation was described by the authors as "plasma cell-like" morphology in which the nucleus became eccentric in position with the development of clear areas of low density, the nucleolus became prominent and the cytoplasm increased in volume and became foamy with prominent Golgi system. This factor was also active in vivo. The assay consisted of injecting the factor into both newborn and adult mice for various periods of time followed by sacrificing the animals and studying the effect on their lymphoid organs. Newborn animals injected daily for 4 weeks developed the characteristic runting syndrome. Their thymic cortex showed increased prevalence of "plasma-like" cells as well as pycnotic and moribund small lymphocytes. These activities led the authors to suggest that this factor might be the one that was responsible for the immunosuppressive effects of male mouse SMG extract. They gave it the name "thymotropic factor" (Naughton et al, 1969).

Later characterization of the thymotropic factor showed that it was an esteropeptidase with a molecular weight of approximately 28,500 Dalton. The esterase and peptidase activities were inseparable and were both inhibited by Diisopropyl Fluoro Phosphate (DFP)

suggesting that it was a member of the serine esterase family. DFP also inhibited the thymotropic activity suggesting that the enzymatic activity was essential for the biologic function (Naughton et al, 1972).

The activity of the thymotropic factor was determined on the basis of changes of the morphology of thymocytes. In the studies that led to its isolation there was no report as regards its effect on the immune responses. However, this morphologic effect was selective in the sense that lysis and transformation occurred with explants of new born thymus, while neither of these effects were obtained with explants of femoral lymph nodes either from normal or immunized animals (Hoffman&McDougall, 1968).

Moreover, Koch and Rowe (1976) reported that a similar preparation of bovine glands containing the thymotropic factor had no immunological activity in the antibody response to SRBC. They also showed that some fractions from the mouse SMG depressed that response without producing any thymotropic effect. They showed that purification of male mouse SMG extract by gel filtration produced two fractions which were able to suppress both IgM and IgG antibody responses to SRBC. Because the immunosuppressive effect was transient, it

was called the immunotranquillizer of mouse SMG. The active material produced marked suppression when given as a single dose in the day before immunization. This led the authors to suggest that it interfered with the early phase of the immune response probably through blocking the development of helper T-cells. However, no further purification or characterization of the immunotranquillizer has been published.

From the above mentioned studies a conclusion can be made that the male mouse SMG contain factor (s) which can affect the immune system morphologically and functionally. However, no factor responsible for this effect has been isolated or characterized.

#### IV.2. Effect of extirpation of the SMG on the immune system

Another line of experiments tried to characterize the role played by the mouse SMG in the maintenance and/or regulation of the immune system. Conflicting results were obtained regarding the effect of extirpation of the SMG on the morphology and responsiveness of the immune system.

Takeda and Grollman (1968) reported that extirpation of the SMG from 3 week old males resulted in increase in the size of their thymuses when examined 3 weeks later.

Histologically, the cortex of their thymuses was filled completely with thymocytes in contrast to the Sham-operated animals where it was depleted of these cells. Extirpation of the SMG in the female had no effect, and this led to the conclusion that normally the SMG in the male mouse had an inhibitory effect on the thymus.

Similarly, Hiramatsu et al (1979) showed that normally the weight of thymus and spleen were higher in the ICR strain female mice than in the male. Female animals produced higher DTH response to Picryl Chloride. Also, extirpation of SMG resulted in significant increase in the weight of thymus and spleen as well as enhanced DTH response in males only. The enhanced response was returned to the normal level by injection of crude SMG extract prepared from the male but not from the female or castrated male. However, Roberts et al (1976) reported no change in the DTH response following extirpation of the SMG.

On the other hand, Martinez-Hernandez et al, (1973) reported opposite effects following SMG extirpation of both mice and rats. Thymic as well as splenic weights were reduced. The thymic cortex was thin and contained few thymocytes. Identical results were obtained in male and female animals. Daily injection of SMG extract

equivalent to one gland per dose for 6 days prevented the atrophy of lymphoid organs following SMG extirpation. Moreover, Hatakeyama et al (1980) showed suppression of the antibody response to SRBC in male mice following removal of their SMG in contrast to the enhancement of the DTH response (Hiramatsu et al, 1979). Both of these studies used the same strain of mice which were at a comparable age. Extracts of male SMG were able to bring the affected responses back to the normal level, whereas extracts from the female or castrated male were not effective.

So, extirpation of of the murine SMG can affect, one way or another, the morphology and responsiveness of the immune system. The contradictory results might reflect the complexity of the system and the possible involvement of multiple factors.

#### IV.3. The Rat SMG

Kemp et al, (1985) used the effect on the proliferation of Concanavalin A (Con A)-stimulated murine lymphocytes as an in vitro bioassay to monitor the immunological activity of the rat SMG extract. By using this assay, it has been shown that the rat SMG crude extract has a biphasic effect ; at high concentrations it produces suppression, whereas stimulation is produced at

low concentrations. Separation of the two activities has been accomplished through gel filtration chromatography. Further fractionation of the suppressor fractions has led to partial purification of a material which exhibited suppressor activity in a number of in vitro assays (Kemp et al, 1986). Among these assays was the ability to inhibit the interleukin-1 (IL-1)-induced thymocyte proliferation in a dose-dependent fashion. The competitive nature of this inhibition suggested that inhibition of IL-1 activity might be the mechanism of action of this factor.

## MATERIALS AND METHODS

### 1. Animals

Random bred male hooded rats weighing 200 - 250 grams were obtained from the Central Animal Care Facility of the University of Manitoba. Also obtained from this facility were the following strains of mice : inbred female C57 BL/6J, inbred female DBA/2J, and inbred male BDF1 (hybrids of C57BL/6J and DBA/2J). Inbred female A/J mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were 6 to 8 weeks old at the time of experiments. They were kept in the animal room of the Immunology Annex, Department of Immunology and were allowed food and water ad libitum.

### 2. Buffers

The following buffers were used for various steps of protein purification :

#### a. Phosphate-buffered saline (PBS)

PBS consisted of 0.12 M Sodium Chloride, 0.01 M Disodium Phosphate and 0.003 M Monopotassium Phosphate (Fisher Scientific, Fair Lawn, New Jersey). It had a pH of 7.2. It was used for gel filtration chromatography. The various protein fractions were dialysed against PBS before testing their activities both in vitro and in vivo. PBS was also used as the control in these assays.

b. Phosphate buffer

Phosphate buffer was used for hydrophobic interaction chromatography. It had a composition of 0.01 M Disodium Phosphate and 0.01 M Monopotassium Phosphate and a pH of 7.0. The Phenyl Sepharose column was equilibrated with 30% ammonium sulphate in phosphate buffer. To elute the bound proteins, a linear continuous gradient was established by using equal volumes (70 ml each) of this buffer and 60% Ethylene Glycol in phosphate buffer.

c. Piperazine buffer

Piperazine buffer was used for the anion exchange chromatography (DEAE-Sepharose column). It consisted of 0.025 M Piperazine hexahydrate (Sigma Chemical Co., St. Louis, MO). The pH was brought down to 5.5 by using 1 N HCl. Protein were eluted from this anion exchange column by the use of a linear continuous gradient of increasing salt (NaCl) concentration. This gradient was established by using equal volumes of piperazine-0.0 M NaCl and piperazine-1.5 M NaCl buffers in one extraction and piperzine 0.2 M NaCl and piperazine-0.4 M NaCl in another experiment.

3. Tissue Culture Medium

The medium used for in vitro experiments was RPMI-1640 (GIBCO laboratories, Grand Island, NY) supplemented

with  $5 \times 10^{-5}$  M 2-mercaptoethanol, 100 U/ml penicillin, 100 ug/ml streptomycin and 10 % Fetal Calf Serum. Cultures were done in 96-well U-bottomed plates (Flow Laboratories, Rockville, MD)

#### 4. Preparation of the Rat Crude Submandibular Salivary

##### Gland Extract

Submandibular salivary glands from male hooded rats were dissected free of the sublingual gland, adherent fatty tissues and lymph nodes and were decapsulated. They were immediately frozen using either dry ice or liquid nitrogen and kept at  $-60^{\circ}\text{C}$  until the time of extraction. The crude SMG extract was prepared basically according to a modification of the method described by Takeda and Grollman (1968). The frozen glands were homogenized in phosphate-buffered saline (PBS) using Brinkmann homogenizer (Brinkmann Instruments Co., Switzerland) in an ice bath. The homogenate was then centrifuged at 15,000 r.p.m. at  $4^{\circ}\text{C}$  for 15 minutes to remove the insoluble material. Then, ice-cold saturated ammonium sulphate solution was added to the supernatant to 30 % saturation and was left in an ice bath for 30 minutes. A second centrifugation was done as above and the sediment was discarded. The resulting supernatant from the second centrifugation was made up to 64 %

saturation by adding saturated ammonium sulphate solution, was left for 1 hour and then a third centrifugation was done for 20 minutes. The resulting precipitate was redissolved in a small volume of PBS and dialysed over night against PBS at 4 °C. Then the dialysate was centrifuged at 15,000 r.p.m. for one hour at 4 °C to remove the insoluble material formed during dialysis and the resulting supernatant was referred to as the crude extract of the SMG.

## 5. Purification of the SMG Crude Extract

### 5.1. Gel Filtration Chromatography :

Gel filtration was carried out at room temperature by using Sephacryl S-200 High Resolution gel (Pharmacia Canada Ltd, Dorval, Quebec). The column had a diameter of 2.6 cm and a bed height of 60 cm. The column was equilibrated with PBS pH 7.2 and elution was done with the same buffer in 8 ml fractions at a flow rate of 22 ml/hour/cm<sup>2</sup> or less. The effluents were monitored at 280 nm using LKB detector unit (LKB products, Sweden). Protein concentration of various fractions was determined according to the method of Lowry et al (1951).

### 5.2. Hydrophobic Interaction Chromatography :

Phenyl Sepharose (Sigma, St. Louis, MO) was used for further purification of the low molecular weight pool of

the Sephacryl column. It was packed into a column of 1 cm diameter to a bed height of 29 cm (Biorad Laboratories, Richmond, CA). To the top of Phenyl Sepharose, 1 ml of Sephadex G-25 (Pharmacia, Uppsala, Sweden) was added to a final bed height of 30 cm. The column was equilibrated with 0.01 M phosphate buffer containing 30 % ammonium sulphate, pH 7.0. The sample was dialysed against the same buffer. After sample application, 2 bed volumes of the starting buffer (0.01 phosphate with 30% ammonium sulphate) were run, then a linear continuous gradient was started. The gradient was established using a Pharmacia gradient mixer (GM-1). More than 6 bed volumes of elution buffer was used consisting of equal volumes (70 ml each) of the starting buffer and 0.01 M phosphate buffer containing 60 % ethylene glycol (Fisher Scientific Company, Fair Lawn, New Jersey). The flow rate was adjusted at 0.5 ml per minute. Conductivity of the 4 ml-volume fractions was measured using a conductivity meter (Cole-Parmer Instrument Company, Chicago, Illinois). Then fractions were dialysed against PBS overnight at 4 °C before their protein contents were determined by Lowry's method.

### 5.3. Anion Exchange Chromatography

Anion exchange chromatography was carried out by

using DEAE-Sepharose (Sigma, St.Louis, MO.). The gel was packed into a 1x30 cm column(Biorad) and run the same way as described above for the Phenyl Sepharose column. The bound proteins were eluted by using a continuous linear gradient of increasing salt (NaCl) concentration in piperazine buffer. The specification of the gradient used is mentioned in the results with each extraction. The conductivity of the resulting fractions was determined before they were dialysed against PBS to determine their protein content by Lowry's method.

#### 6. Sodium Dodecyl Sulphate - Polyacrylamide Gel

##### Electrophoresis (SDS-PAGE)

SDS-PAGE was done according to the discontinuous gel method of Laemmli (1970). Electrophoresis was done by using the Mini-Protean II Dual Slab Cell (Biorad Laboratories, Richmond, CA). The 12% resolving gel (0.375 M Tris, 1% SDS, pH 8.8) was deaerated first then polymerized by adding 0.05% Ammonium Persulphate and 0.05% TEMED (Biorad). The top (stacking) gel (0.125 M Tris, 1% SDS, pH 6.8%) was polymerized by adding 0.05% Ammonium Persulphate and 0.1% TEMED and was layered over the stacking gel. The samples were diluted one in four in the SDS-reducing sample buffer. The low molecular weight standards (10,000 to 100,000 , Biorad) were used to

estimate the molecular weight. Electrophoresis was done at room temperature under constant voltage (200 volts) for approximately 60 minutes. Silver staining of the gels was done according to the method of Marshall (1984).

## 7. Immunological Assays

### 7.1. In Vitro Assays

#### 7.1.1. Mitogen-induced Lymphocyte Proliferation (Con A Bioassay)

Lymph node cells from either C57BL/6J or BDF1 mice were obtained from the femoral, brachial, axillary and submandibular lymph nodes. Lymphocytes were extracted and washed twice with tissue culture medium. They were stimulated with Concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO) in U-bottomed tissue culture plates (Flow Laboratories, Rockville, MD). Each culture (in triplicates) consisted of  $0.5 \times 10^5$  cells in 0.18 ml tissue culture medium. Con A was used at a concentration of 1.1 ug/ml. These concentrations of L.N. cells and Con A were chosen because they allowed optimum identification of both the suppressive and enhancing activities of different fractions. 20 ul of various fractions (in PBS) was added to make the total volume of 0.2 ml. The plates were incubated at 37 °C in a humidified atmosphere of 5% Co<sub>2</sub> in air for 72 hours. During the last 4-6 hours, 20 ul

of medium containing 0.5  $\mu$  Ci of tritiated thymidine (ICN Radiochemicals, Irvine, CA) was added to each culture well. The cells were collected at the end of the culture time with a cell harvester (Skatron, Lierbyen, Norway) on glass fiber discs (Titertek, Flow laboratories). The amount of tritiated thymidine incorporated by the cells was measured by liquid scintillation (ScintiLene, Fisher) using a beta counting machine (LKB, Wallac, Finland).

#### 7.1.2. Mixed Lymphocyte Reaction (MLR)

Spleen cells from male BDF<sub>1</sub> mice were used as stimulator cells. They were treated with Mitomycin C (Sigma Chemical Co., St. Louis, MO) at a concentration of 25  $\mu$ g/ $10^7$  cells/ml for 45 minutes at 37 °C with occasional shaking. 100  $\mu$ l of stimulator cell suspension containing  $2 \times 10^5$  splenocytes was added to 100  $\mu$ l of responder cell suspension containing  $10^5$  C57 BL/6J lymph node cells in 96-well U-bottomed tissue culture plates. 20  $\mu$ l of various protein fractions, in PBS, was added to the test cultures, whereas an equal volume of PBS was added to the control cultures. The plates were then incubated for 4 days at 37 °C with 5% CO<sub>2</sub>. Proliferation of the responder cells was measured through the 6-hour Tritiated Thymidine uptake as mentioned above for the Con A bioassay.

### 7.1.3. Cell Mediated Cytotoxicity (CMC)

Mitomycin-treated BDF1 splenocytes were used as stimulator cells and were prepared as described above in the MLR. Their number was kept constant at  $2 \times 10^5$  cells per well. The number of responder cells (C57BL/6J lymph node cells) varied as specified for each experiment. The cultures were done in 96-well U-bottomed tissue culture plates and were incubated at 37 °C with 5% CO<sub>2</sub> for four or five days as specified in the results. At the end of this incubation period, 0.1 ml of the supernatant was carefully withdrawn from each culture to avoid disturbing the pellet. Then, 0.1 ml of tissue culture medium containing  $2 \times 10^4$  <sup>51</sup>Cr-labelled P815 X2 cells (of DBA/2J origin) was added to each culture. <sup>51</sup>Cr-labelling was done at 37°C for 45 minutes by adding 200 uCi of Sodium [<sup>51</sup>Cr] Chromate (Amersham corporation, Arlington Heights, Illinois) to  $8 \times 10^6$  cells in 1 ml with occasional shaking. The labeled cells were introduced into the cultures in such a way to produce enough mixing with the pellet. The plates were then centrifuged 400xg. Then they were incubated for another 4 hours. At the end of this second incubation, 0.1 ml of the supernatant was withdrawn to measure the amount of radioactive chromium released. The percent lysis was calculated from the formula

$(E-S)/(T-S) \times 100$ , where E=cpm in the supernatants of the wells in which target cell lysis was induced with a detergent, S= spontaneous release, i.e. mean cpm in the supernatant of wells in which the target cells has been incubated with tissue culture medium alone. Spontaneous release was around 10%.

#### 7.1.4. Antigen-induced Lymphocyte Proliferation

This assay measures the degree of proliferation of sensitized lymph node cells upon challenge with haptenic syngeneic spleen cells. The assay was done as described before (Kemp et al, 1985). Briefly, A/J mice were immunized by painting their shaved abdomen with 100 ul of 5% Picryl Chloride-Ethanol solution. Six days later, the lymph node cells were cultured in U-bottomed, 96-well tissue culture plates at a concentration of  $2 \times 10^5$  cells in 100 ul per well. The challenge was provided by  $10^5$  Trinitrophenylated spleen cells from normal A/J mice in 100 ul tissue culture medium. Trinitrophenylated spleen cells were prepared by incubating  $10^7$  A/J splenocytes in 1 ml with 25 ug/ml Mitomycin C for 45 minutes at  $37^\circ\text{C}$  with occasional shaking. The cells were then washed in Hank's balanced salt solution (HBSS), and the incubated in 10mM Trinitrobenzene sulphonic acid in HBSS at room temperature with mixing for 30 minutes. The cells were

washed once with HBSS, once with 10mM Glycylglycine in HBSS, and three times with tissue culture medium. The stimulatory cells were incubated with the responder cells for 72 hours, after which the 6-hour Tritiated Thymidine uptake was measured as mentioned above.

## 7.2. In Vivo Assays

### 7.2.1. Skin Transplantation

Female DBA/2J mice (H-2<sup>d</sup>) were used as donors of skin grafts, while female C57BL/6J (H-2<sup>b</sup>) were used as recipients. This combination was chosen to study the effect of SMG-derived fractions on skin transplantation across a strong histocompatibility barrier. Donor mice were sacrificed by ether overdose then full thickness skin grafts were taken from the abdominal surface after shaving and sterilization with 70% ethanol. This was followed by removal of fat and panniculus carnosus by blunt dissection. The clean skin was cut into square pieces 1 cm x 1 cm and was kept in sterile PBS at 4 °C until the graft beds were prepared in the recipient mice. The skin grafts were generally used within one or two hours after preparation.

Recipient mice were anaesthetized by using Nembutal (54 ug per gram body weight) injected intraperitoneally. The lateral chest wall was sterilized with 70% ethanol

after it was shaved. The graft bed was prepared by removing a square area, slightly smaller than the graft size, of the skin down to the fascia avoiding damage to the blood vessels. The grafts were applied, sprayed with Aeroplast Dressing (Parke, Davis & Company, Brockville, Ontario) to keep them in place and bandaged over with a piece of gauze.

The day of transplantation was taken as day 0. The bandage was removed at day 10 and the grafts were inspected daily after bandage removal. Rejection was scored when 50% of the graft became necrotic.

For histological studies, the graft area together with the surrounding skin of the recipient was removed and put in 10% Formaldehyde in PBS. The 6u thick, hematoxylin and eosin-stained sections of the skin grafts were done from paraffin blocks in the Histology Laboratory, Department of Anatomy, University of Manitoba.

#### 7.2.2. Delayed-Type Hypersensitivity (DTH) Response

DTH was carried out basically according to the method of Asherson and Ptak (1968). A/J female mice were used as they showed maximum response in this assay compared to other murine strains (data not shown). They were anaesthetized by using 54 ug Nembutal per gram body

weight. The skin of the abdomen was shaved, then 100 ul of 5 % Picryl Chloride-Ethanol solution was used to paint a confined area of the abdominal skin. The day of sensitization was taken as day 0. At day 6, the thickness of the left ear was measured using a caliper. Then animals were challenged by painting both sides of the left ear with 1 % Picryl Chloride-Olive oil solution. The thickness of the painted ear was measured again 24 hours after the challenge and the increase in thickness was determined by subtracting the second reading from the first one.

### 7.2.3. Plaque Forming Cell (PFC) Response

The liquid monolayer technique of Cunningham and Szenberg (1968) was used with some modifications. Plain glass microscope slides 25 x 75 mm (Canlab, Division of Travenol Canada Inc.) were cleaned after soaking overnight in ether : ethanol (1 : 1) solution. Then, two slides were separated from each other by three parallel strips of 6 mm-wide, double-sided, self-adhesive tape in such a way that two microchambers were formed between the two slides. The two chambers had a volume of about 180 ul. Sheep red blood cells (SRBC) (National Biological Laboratory, Gunton, Man.) were washed three times with PBS before use. Then they were injected into mice at day

0 at a dose of  $8 \times 10^8$  cells per mouse intraperitoneally. To calculate the number of direct (IgM) plaque forming cells, mice were sacrificed at day four. The spleen was removed aseptically and a cell suspension was prepared in RPMI 1640 tissue culture medium supplemented with 10% FCS. After washing twice in that medium, the red blood cells were lysed using Tris-buffered isotonic ammonium chloride. Then the cell count was adjusted so that each 100 ul of medium contained  $10^6$  splenocytes. To this 100 ul cell suspension, 25 ul of guinea pig complement (Cedarlane Laboratories Limited, Hornby, Ontario) diluted one in four was added in a small tube. 50 ul of 12.5% (packed cell volume) of washed SRBC and diluted in tissue culture medium was added to the tube. Then the volume was made up to 200 ul by adding 25 ul of tissue culture medium. The contents of the tube were mixed gently and 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). Then they were incubated for one hour in a humidified atmosphere of air with 5%  $\text{Co}_2$  at 37 °C. The resulting plaques were counted on a dark background with slight magnification.

It should be noted that in the above mentioned in

vivo models, mice were used to test for the biological activity of rat SMG-derived fractions. The species non-specific nature of the activity of these fractions has been suggested from in vitro experiments (Con A and MLR) done previously in the lab. using rat as well as murine lymphoid cells (data not shown).

#### 8. Statistical analysis

The two-sample t test was used to determine the significance of the differences between each mean and the appropriate control (Rosner, 1982)

## RESULTS

### 1. Fractionation of the SMG Crude Extract through Gel Filtration Chromatography

After ammonium sulphate precipitation of the SMG homogenate (between 30% and 64%) the resulting crude SMG extract was further fractionated through gel filtration chromatography. The resulting fractions from the Sephacryl S-200 HR column were tested for their in vitro immunological activity in the Con A bioassay. The pattern shown in Fig.1 was obtained in a typical experiment. The high molecular weight fractions 20 to 25 (M.W. between 96 and 47 kDa) produce significant suppression while the low molecular weight fractions 26 to 30 produced significant stimulation.

When the crude extract of the rat sublingual gland was treated exactly the same and used as a control, no significant effect in the Con A bioassay was present with any of the resulting Sephacryl fractions (Fig. 2).

### 2. Heterogeneity of the Sephacryl high and low molecular weight pools

The immunologically active fractions of the Sephacryl column were pooled. The high molecular weight pool contained fractions 20 to 25 which produced in vitro suppression. The low molecular weight pool contained

Fig. 1 Fractionation of the SMG crude extract through gel filtration chromatography

The Sephacryl fractions were tested for their activity in the Con A bioassay in two dilutions 1 in 6 ( $\blacktriangle$ ) and 1 in 30 ( $\circ$ ). The horizontal lines represent two standard deviations (2SD) above and below the control cultures (20 ul PBS instead of various fractions). Values above or below these lines were considered significant. Also shown in the figure are the protein contents ( $\bullet$ ) of various fractions. The arrows at the top of the figure indicate the positions of the molecular weight standards used to calibrate the column.

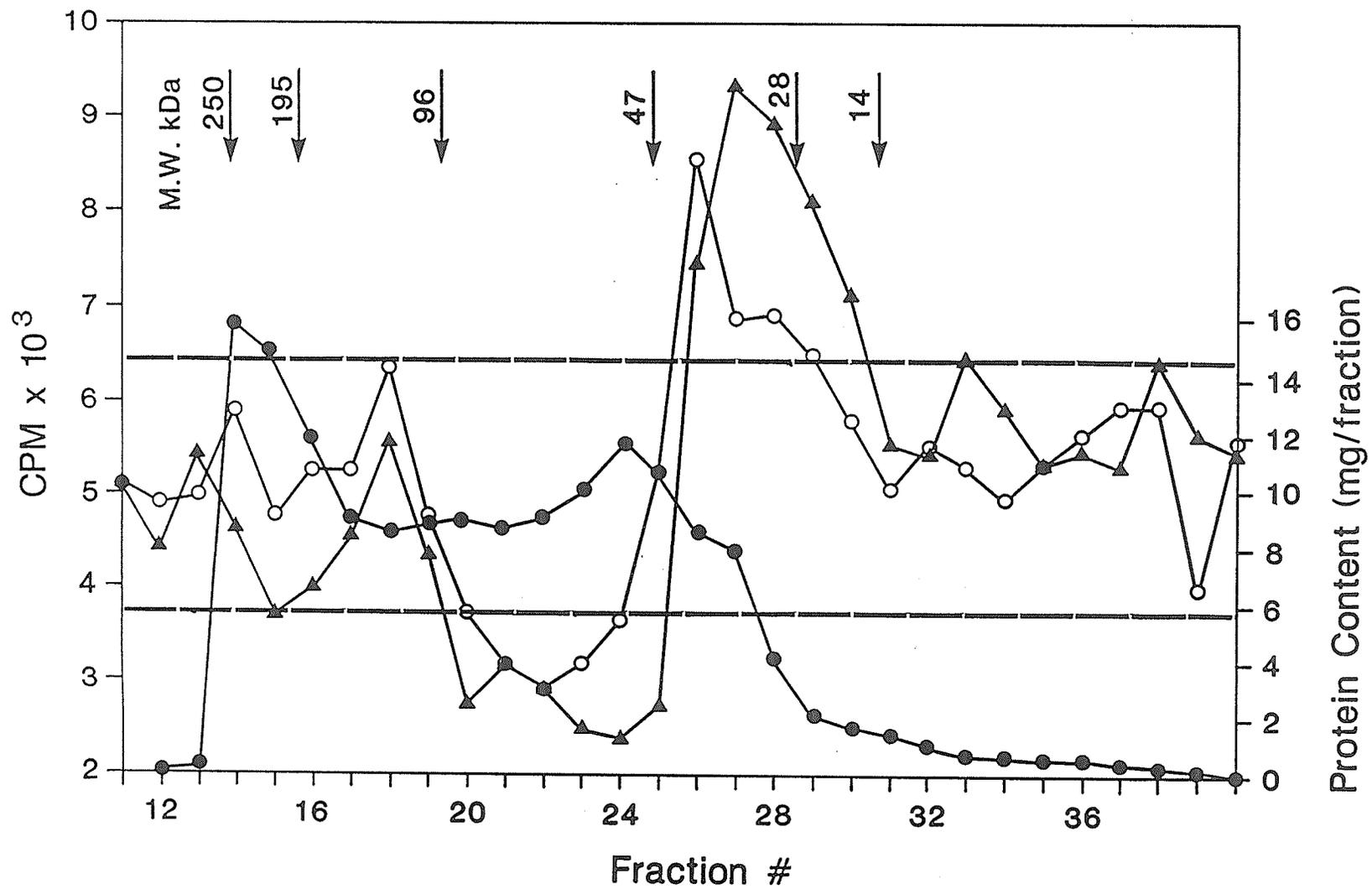
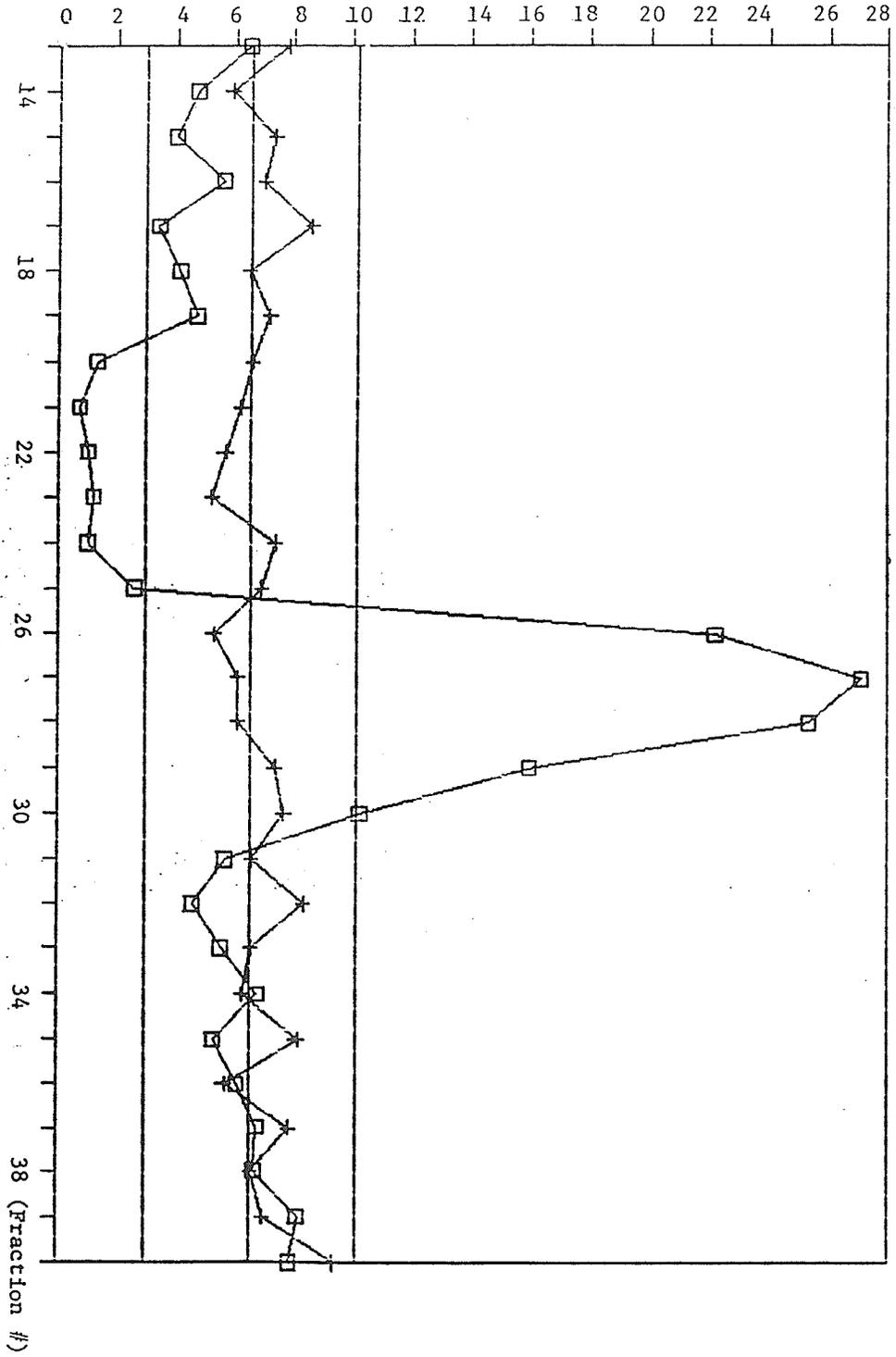


Fig. 2 Fractionation of the submandibular and sublingual gland  
crude extracts through gel filtration chromatography

The activity of the eluted fractions after fractionation of the submandibular (□) and sublingual (+) glands crude extract in the Con A bioassay is shown in the figure. While the submandibular gland fractions exhibit a reproducible pattern of activity in this assay, suppression followed by stimulation, the sublingual gland fractions do not appear to have any effect in this assay. The central horizontal line represents the mean value in the control cultures (PBS instead of various fractions), whereas the other horizontal lines represent two standard deviations from the mean, values above or below these lines were considered significant.

C P M (Thousands)



fractions 26 to 30 which produced in vitro enhancement of the proliferation of Con A-lymphoblasts.

Fig. 3 shows the SDS-PAGE followed by silver staining of both pools as well as the crude SMG extract. Both these pools are quite heterogeneous, each of them has several protein bands.

### 3. In Vivo immune reactivity of the high and low molecular weight Sephacryl pools

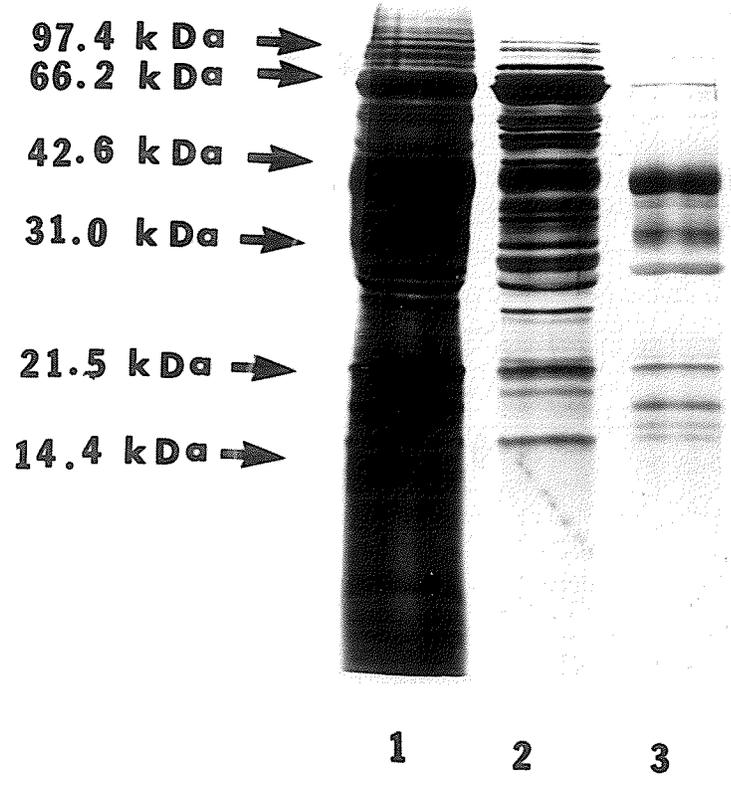
Since the objective of this study was to isolate the factors responsible for the in vivo suppression of the immune response, both the high and low molecular weight pools which showed in vitro immune reactivity were tested in a number of in vivo models.

#### 3.1. Skin allograft rejection

The first in vivo model used was the skin allograft rejection. Grafts were done across a strong histocompatibility barrier using DBA/2J mice (H-2<sup>d</sup>) as donors and C57B/6J (H-2<sup>b</sup>) as recipients. Three groups of skin transplanted animals were used. The control group was injected with PBS, the test groups were injected with either the high or low molecular weight Sephacryl pool. A total of ten injections were given, starting from the day of the operation (day 0) to day 9. Each dose consisted of a material equivalent to half a gland. So each animal

Fig. 3 SDS-PAGE & silver staining of the crude rat SMG extract and the Sephacryl pools

The numerous protein bands present in the crude SMG extract are shown in lane 1. Lanes 2 and 3 represent the high and low molecular weight Sephacryl pools, respectively. The positions of the molecular weight standards are shown on the left side of the figure.

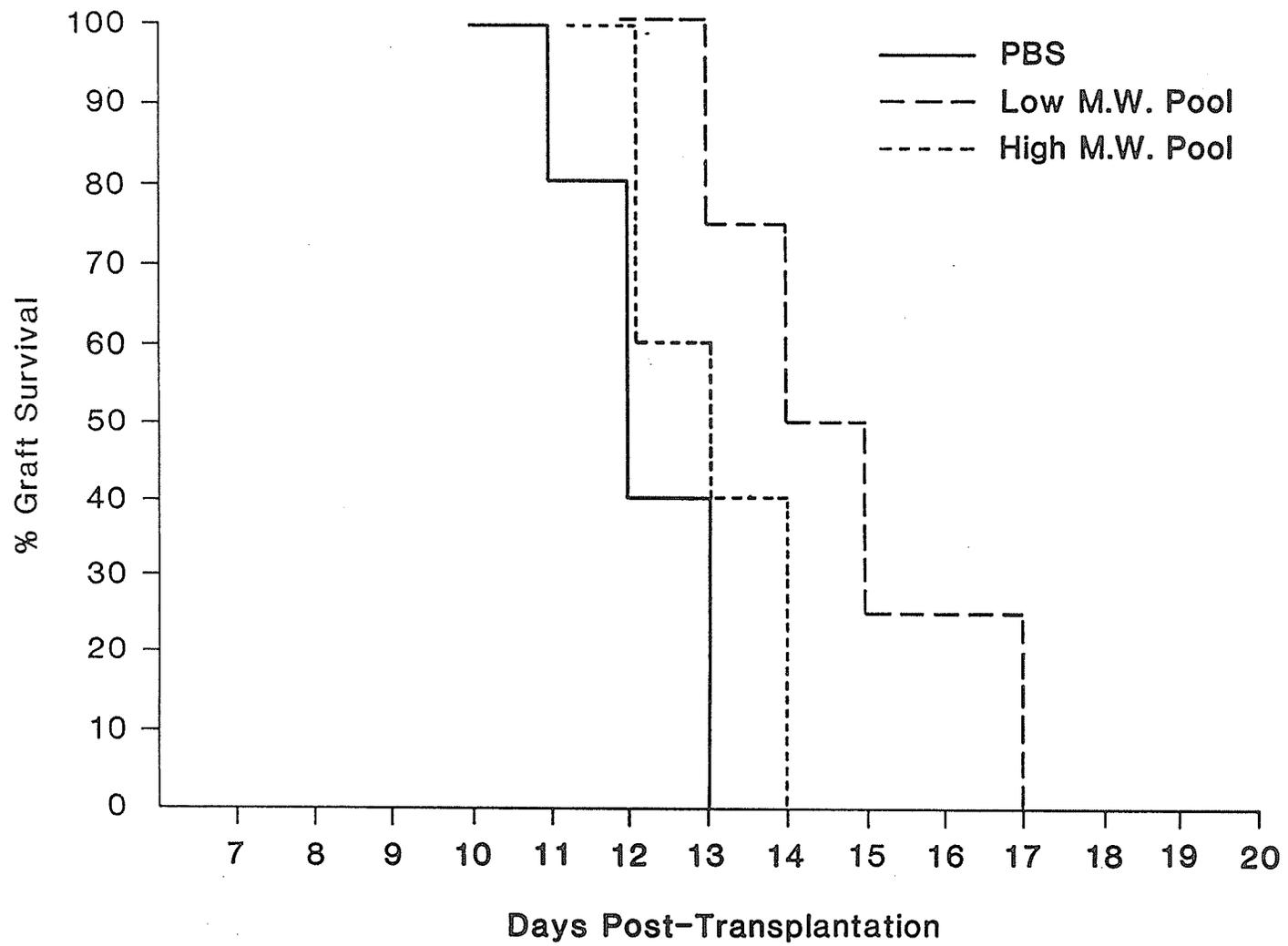


received material from either the high or low molecular weight Sephacryl pool corresponding to a total of 5 submandibular glands. Rejection was scored when 50 % of the graft was already necrotic. Fig.4 shows that the PBS-treated group had a mean graft survival time of  $12.2 \pm 0.37$  days. There was some prolongation in the group injected with the high molecular weight pool ( $13 \pm 0.44$  days) which was not statistically significant. However, significant prolongation of the allograft survival occurred in the group injected with the low molecular weight pool. The mean survival time was  $14.75 \pm 0.76$  ( $0.02 > p > 0.01$ ).

In order to confirm these results histologically, another experiment was carried out. Similar groups of animals were used and they were treated the same way but, instead of allowing the grafts to reach rejection, animals were sacrificed at day 12 post-transplantation. This particular day was chosen because it represented the mean graft survival time in the PBS-treated group in the first experiment. Hematoxylin and eosin-stained sections were made out of the grafts and they were examined microscopically. Five out of six (83.3%) of the grafts in the PBS-treated group showed signs of rejection. The low molecular weight pool-treated group had more (83.3%)

Fig. 4 Prolongation of the skin allograft survival by treatment with the low molecular weight Sephacryl pool

C57BL/6J female mice (H-2<sup>b</sup>) were used as recipients, whereas DBA/2J female mice (H-2<sup>d</sup>) were used as donor of skin grafts. Three groups of animals were used. The control group (5 animals) was treated with PBS. The high molecular weight group (5 animals) and the low molecular weight group (4 animals) were given ten daily injections starting from day 0 (the day of the operation) to day 9, each injection had a material corresponding to half a gland. Rejection was scored when 50% of the graft became necrotic. The control group had a mean graft survival time of  $12.2 \pm 0.37$  days. The high molecular weight pool-treated group had a mean survival time of  $13.0 \pm 0.44$  days which was not significantly different from the control group. However, significant prolongation of the graft survival occurred in the low molecular weight pool-treated group. The mean survival time was  $14.76 \pm 0.76$  days ( $0.02 > p > 0.01$ ).



intact grafts than the high molecular weight pool-treated one (33.3%).

Histological signs of rejection can be seen in one of the PBS-treated grafts, namely loss of the epidermis, increased thickness and marked cellular infiltration of the dermis as seen in Fig.5. Syngeneic grafts (C57BL/6J donor and recipient) were done as a control. Fig.6 shows one of these syngeneic grafts where the epidermis is intact and the dermis is showing a little cellular infiltration but preserved glands and hair follicles. Fig.7 and 8 show two magnifications of an intact graft from the low molecular weight pool-treated group. Similarity to the syngeneic graft can be detected. Both have intact epidermis and preserved glands and hair follicles in the dermis. On the other hand, Fig.9 and 10 show a graft from the high molecular weight treated-group where signs of rejection as in the PBS-treated graft can be seen.

These experiments suggested that the low molecular weight Sephacryl pool of the rat SMG is able to produce prolongation of the survival of the murine skin allograft.

### 3.2. DTH Response

The results of the skin allograft model raised the

Fig. 5 Rejected skin allograft from the PBS-treated group

The picture shows one of the rejected skin allografts from the PBS-treated group. The epidermis is lost, the dermis is thickened with marked mononuclear cellular infiltration.

The section is stained with hematoxylin and eosin and magnified 100 times.

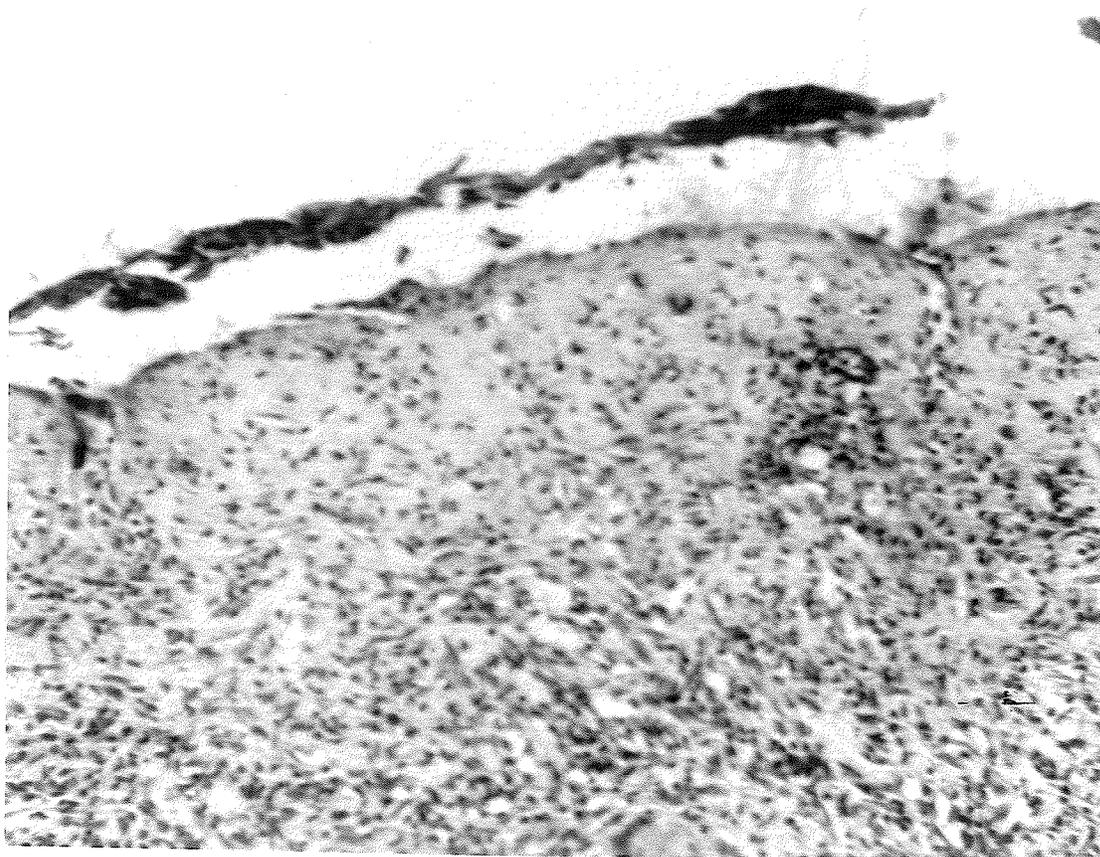


Fig. 6 Syngeneic skin graft

The picture shows a syngeneic skin graft (C57BL/6J donor and recipient) done as a control. The epidermis is preserved and intact. The dermis shows some cellular infiltration but the glands and hair follicles are intact. The section is stained with hematoxylin and eosin and is magnified 100 times.

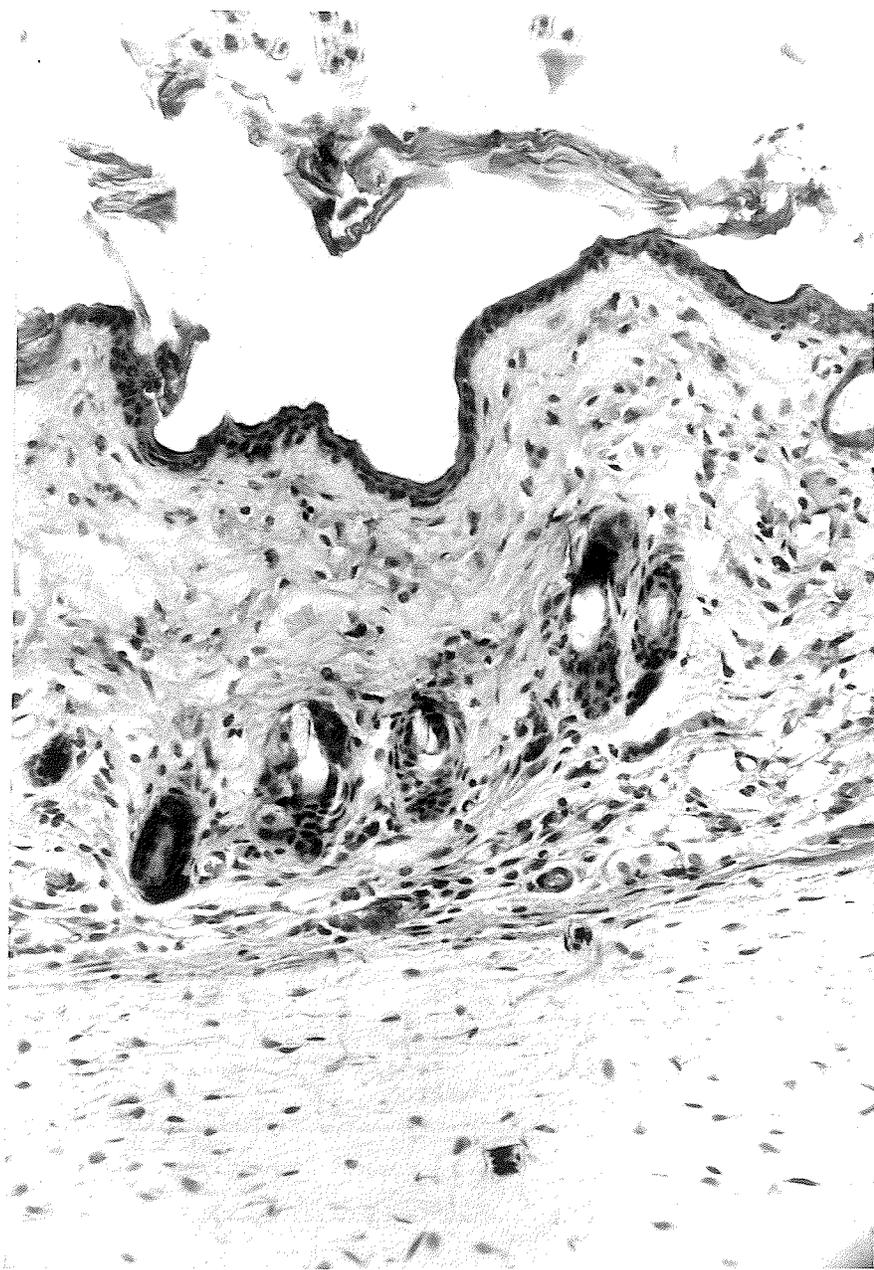
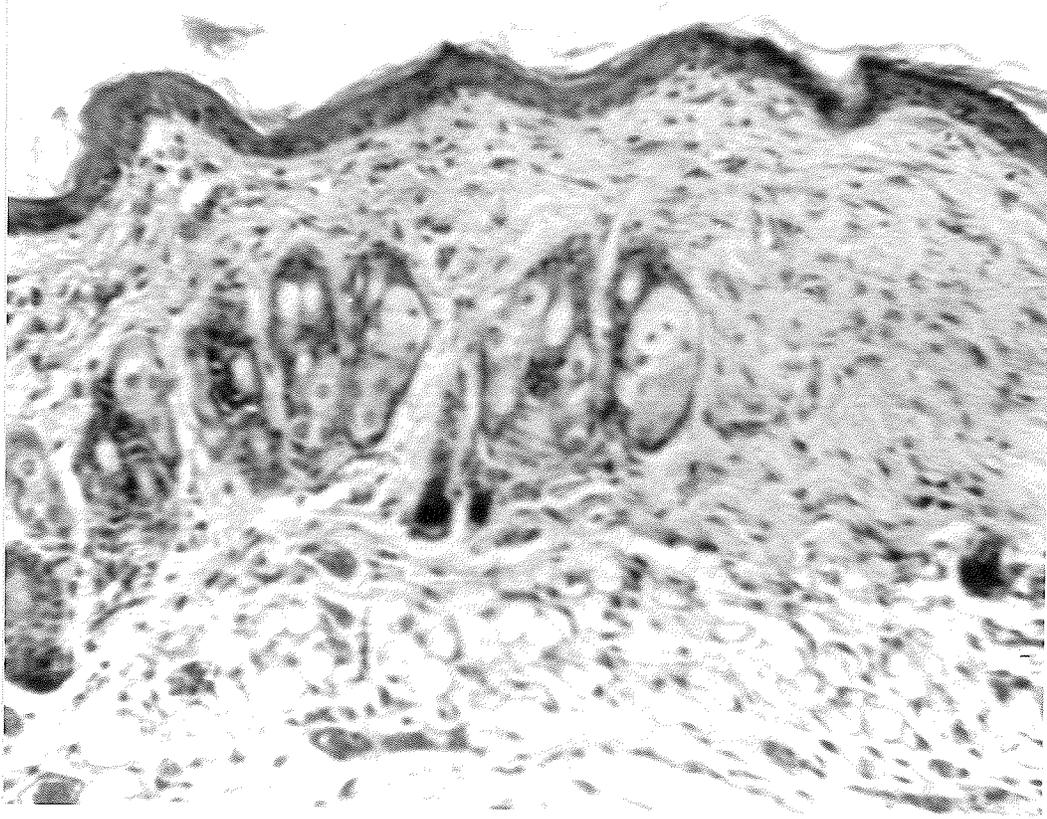


Fig. 7 Intact graft from the low molecular weight pool-treated group

The picture shows a day 12 allogenic graft from the group treated with the low molecular weight Sephacryl pool. The epidermis is intact and as shown in the figure there is a moderate mononuclear cellular infiltration of the dermis, but the dermal glands are intact. Five out of six (83.3%) of the grafts in this group showed a similar picture.

The section is stained with hematoxylin and eosin and is magnified 100 times.



F

Fig. 8 Higher magnification of an intact graft from the low molecular weight pool-treated group

The figure shows a higher magnification (400 times) of the picture shown in Fig. 7. It is intended to show the extent of cellular infiltration of the dermis of an intact graft from the low molecular weight pool-treated group.

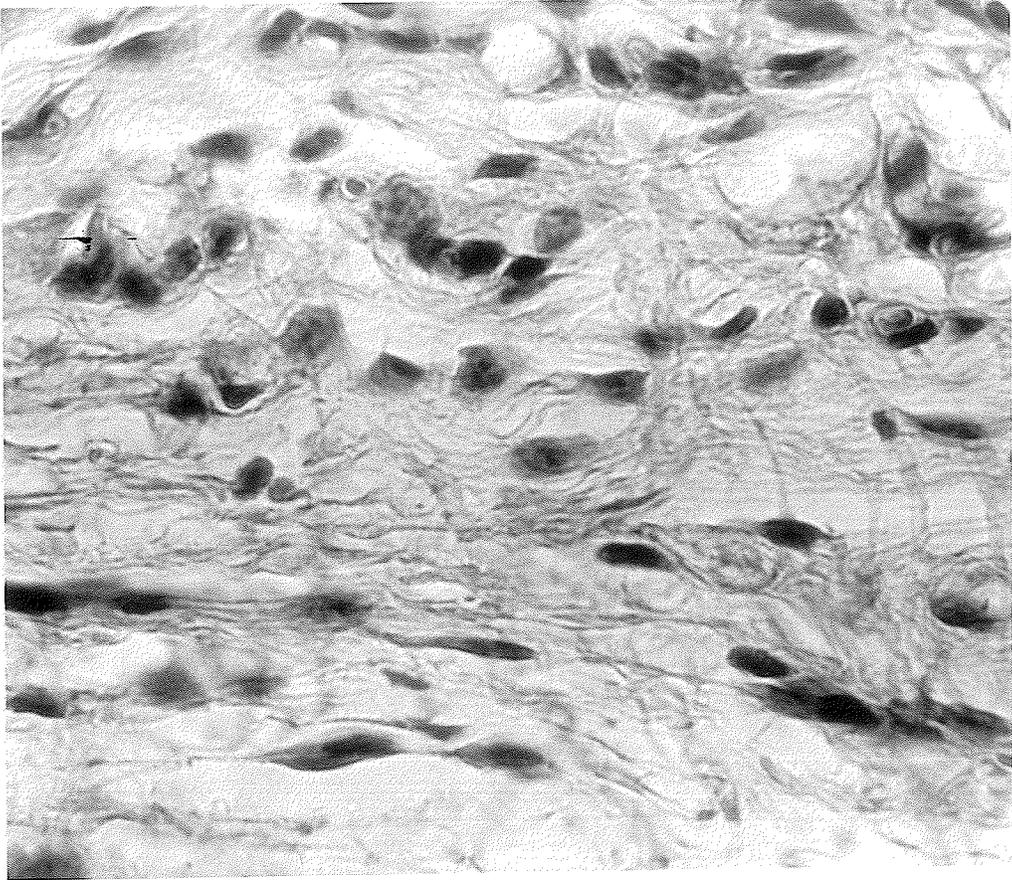


Fig. 9 A rejected graft from the high molecular weight pool-treated group

The picture shows a day-12 allogeneic graft from the high molecular weight pool-treated group. There is a loss of the epidermis and marked cellular infiltration of the dermis indicating rejection. Four out of six grafts in this group showed a similar picture. The section is stained with hematoxylin and eosin and is magnified 100 times.

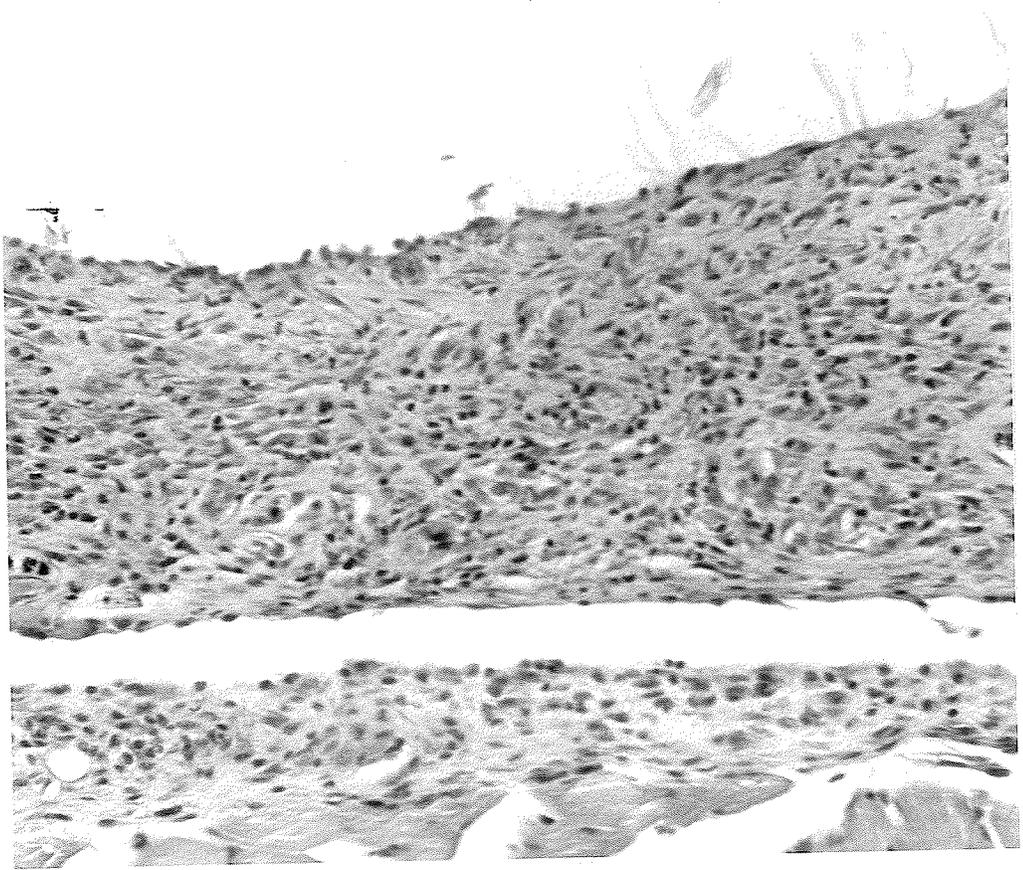
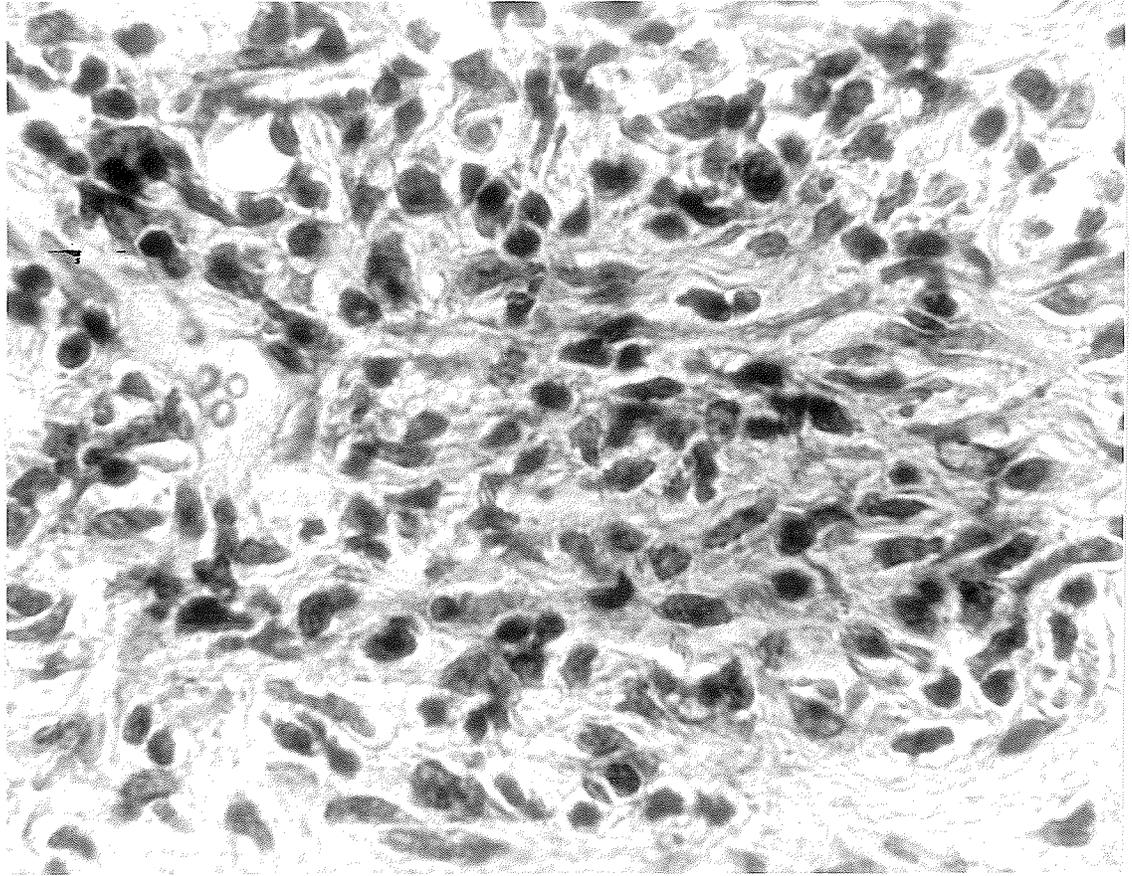


Fig. 10 Higher magnification of the rejected graft from the high molecular weight pool-treated group

This picture is a higher magnification (400 times) of the picture shown in Fig. 9. As shown there is a marked mononuclear cellular infiltration of the dermis of the graft.



question of whether the immunosuppressive activity of the low molecular weight pool was confined to that in vivo model only or it can also suppress other models. This was important particularly because of the apparently contradicting results between the in vitro and in vivo models. To answer this question the activity of the low molecular weight pool in other in vivo models was tested.

The following step was to test the ability of both pools to suppress the DTH response to picryl chloride. Three groups of animals were used, each consisted of five animals. Treatment of the groups with PBS, high or low molecular weight pools was carried out during the effector phase of the response. Two injections were given, one at day 5 and the other at day 6 post-sensitization, each consisted of a material corresponding to one SMG. Fig.11 shows that, consistent with the skin allograft model, significant suppression ( $p < 0.001$ ) occurred only in the low molecular weight pool-treated group.

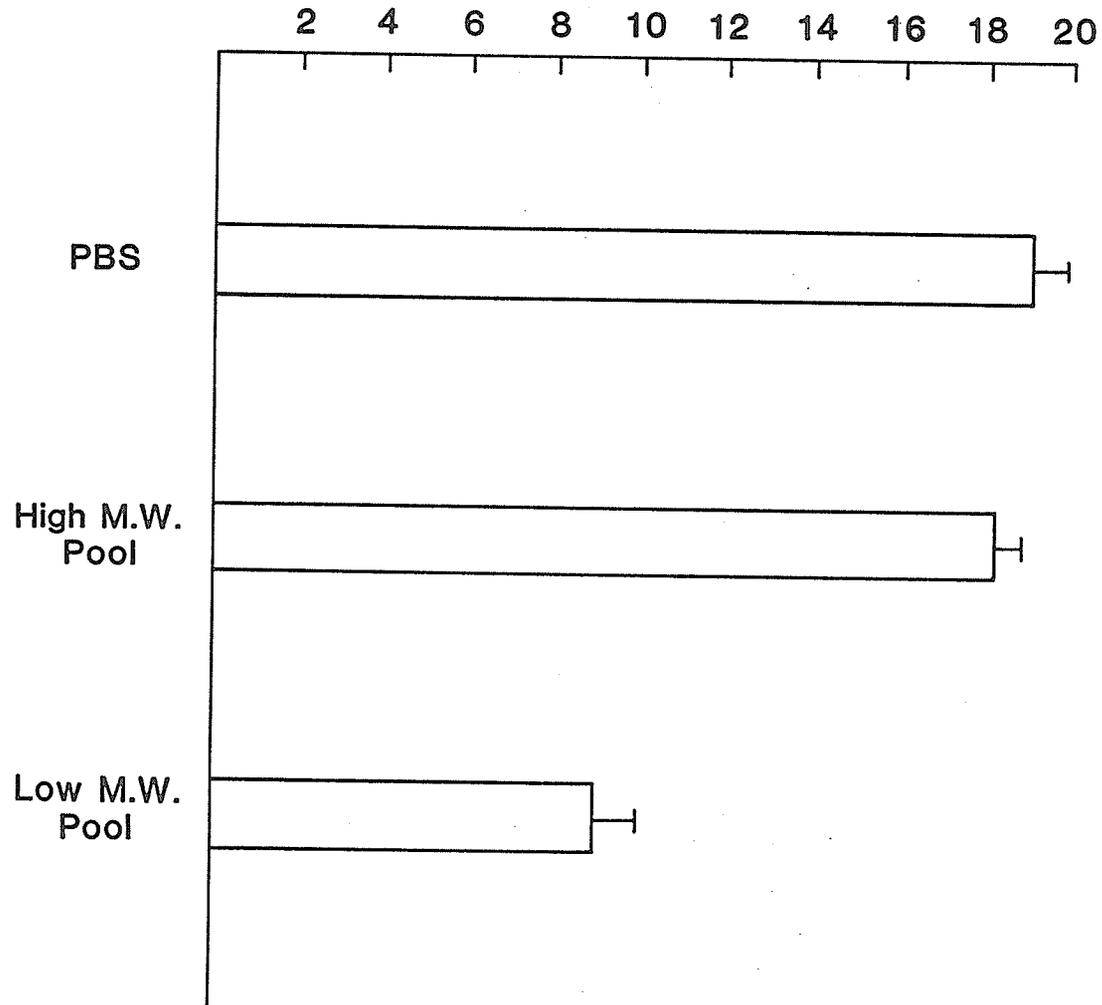
### 3.3. Direct Plaque Forming Cell Response

In order to test whether the low molecular weight pool can also suppress the antibody response to SRBC, direct PFC response was carried out. Female C57BL/6J mice were used in this experiment. Animals were given five

Fig. 11 Suppression of the DTH response by treatment with the low molecular weight Sephacryl pool

Three groups of female A/J mice (five animals each) were used in this experiment. Treatment with either PBS, high or low molecular weight Sephacryl pools was carried out as two daily injections at day 5 and 6 post-sensitization with Picryl Chloride. Each injection contained a material corresponding to one SMG. The mean increase in the thickness of the ear was calculated for each group after challenge. The high molecular weight pool-treated group had a mean of  $18.2 \pm 0.49$  which was not significantly lower than the PBS-treated group ( $19.0 \pm 0.7$ ). The low molecular weight pool-treated group had a mean of  $9.0 \pm 1.0$  which was significantly lower than the control group ( $p < 0.001$ ).

Increase in the Thickness of the Ear ( $\text{mm} \times 10^{-2}$ )  $\pm$  SE



daily injections starting at day -1 (the day before immunization with SRBC) and ending at day 3. Each injection consisted of material from either the high or the low molecular weight pool that was corresponding to one SMG. The control group was injected with PBS. At day 4, animals were sacrificed and the number of plaques produced by  $10^6$  of their splenocytes were counted. Fig.12 shows that significant suppression ( $0.02 > p > 0.01$ ) resulted from treatment with the low molecular weight pool. As in the previous in vivo models, some suppression occurred with the high molecular weight pool which was not statistically significant.

These experiments suggested that the low molecular weight Sephacryl pool of the rat SMG had an immunosuppressive activity in a number of in vivo models. The next step was to try to fractionate this pool to identify the material(s) responsible for this activity.

#### 4. Fractionation of the low molecular weight pool through hydrophobic interaction chromatography

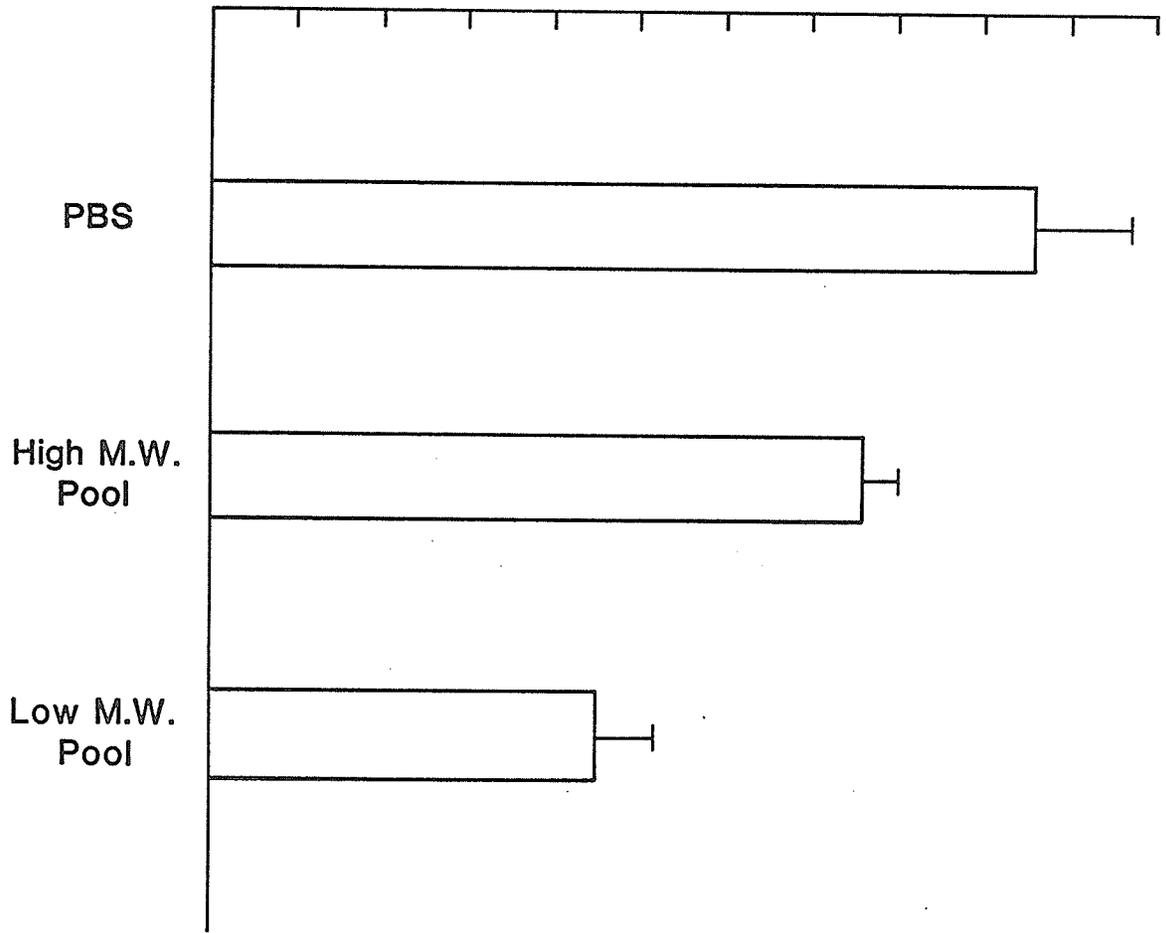
The low molecular weight pool was further fractionated through hydrophobic interaction chromatography by using a Phenyl Sepharose column. Elution of the proteins bound to the column was achieved through a continuous linear gradient of decreasing

Fig. 12 Suppression of the direct PFC response by the low  
molecular weight Sephacryl Pool

Three groups (five animals each) of female C57BL/6J mice were used for this experiment. The number of direct (IgM) plaque forming cells per one million splenocytes was determined for each animal at day 4 post-immunization with SRBC. Significant suppression of this response resulted from treatment with the low molecular weight pool ( $119.6 \pm 10$  direct PFC/ $10^6$  splenocytes compared to  $235 \pm 19.7$  for the PBS-treated group). Treatment with the high molecular weight pool resulted in some suppression which was not statistically significant.

Number of Direct (IgM) Plaque Forming Cells/ $10^6$  Splenocytes

0.25 0.5 0.75 1 1.25 1.5 1.75 2 2.25 2.5 2.75  $\times 10^2$



ammonium sulphate and increasing ethylene glycol concentrations in the phosphate buffer. The main advantage of this method of purification was that the pool could be eluted in three distinct peaks. The fractions forming these peaks were pooled to produce Peak A, B and C (Fig.13). Peak A was eluted first after the gradient was established, followed by Peak B and then Peak C which was eluted towards the end of the gradient. This limited number of peaks was important in order to facilitate testing for in vivo activity. Fig.13 shows also the activity of the various fractions in the Con A bioassay. Most of the resulting fraction had enhancing activity in this assay which was not surprising in light of the enhancing activity of their parent low molecular weight pool.

5. Peak A has the in vivo immunosuppressive activity but it is still heterogenous

The resulting peaks from the Phenyl Sepharose column were then tested for their ability to suppress the DTH immune response. Test groups were injected with material from either the LMW pool, peak A, B, or C corresponding to a total of two SMG in a volume of PBS (0.2 ml) equal to that given to the control group. Fig.14 shows that significant suppression of the DTH immune

Fig. 13 Fractionation of the low molecular weight Sephacryl pool  
through hydrophobic interaction chromatography

The resulting fractions from the Phenyl Sepharose column were tested for in vitro activity in the Con A bioassay using one dilution (1 in 10) (○). The two interrupted horizontal lines represent the two standard deviations from the mean value of the control cultures in this assay. Conductivity (--) in milli MHO as well as the protein concentration (●) were also measured for each fractions. The arrows at the top of the figure define the fractions which were pooled to produce Peaks A, B, and C.

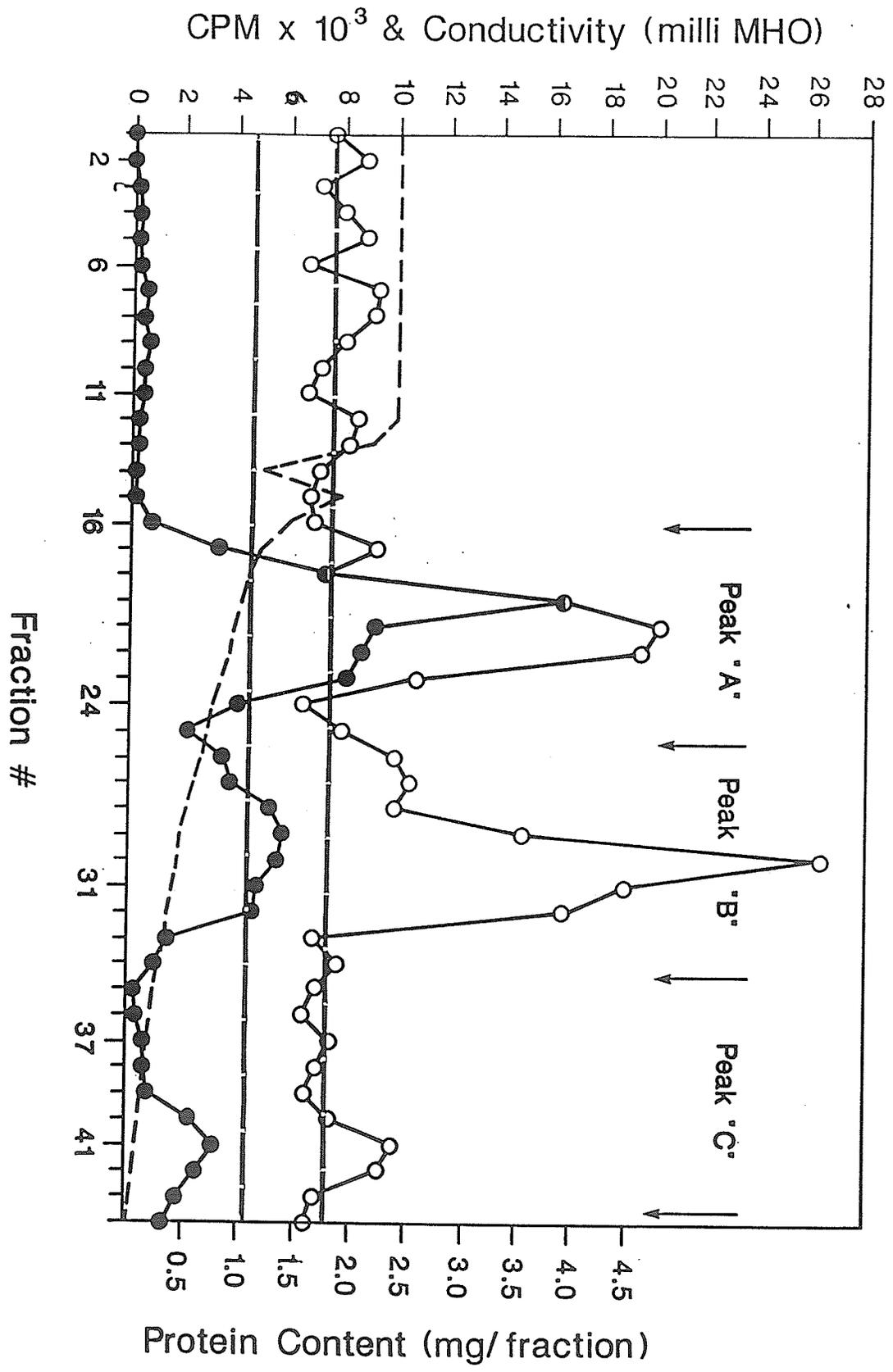
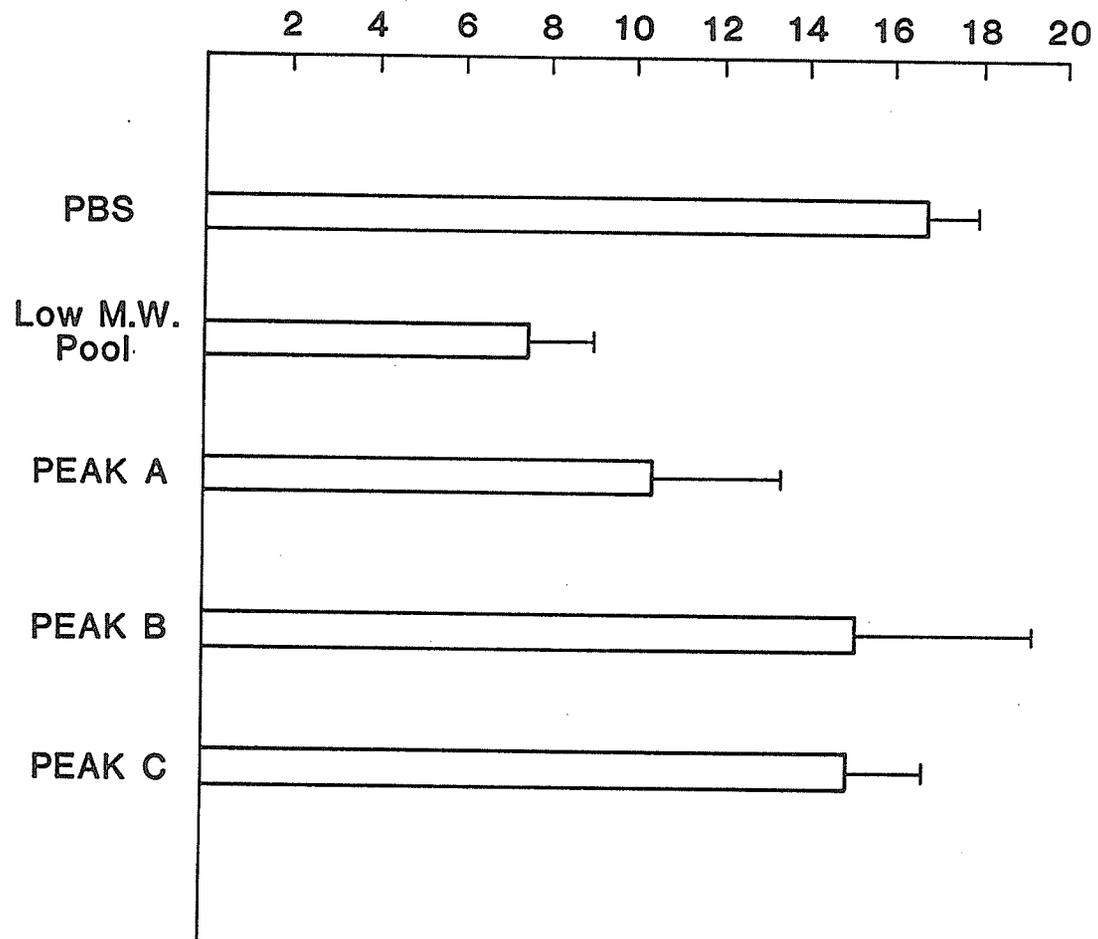


Fig. 14 Suppression of the DTH response by Peak A

The figure shows that suppression of the DTH response occurred in the groups treated with the low molecular weight Sephacryl pool and Peak A. The mean increase in the ear thickness in these groups was  $7.7 \pm 0.76$  and  $10.2 \pm 1.5$ , respectively, which were significantly different from the PBS-treated group ( $16.8 \pm 0.48$ ), the p values were  $p < 0.001$  and  $0.001 < p < 0.01$ , respectively. The values for Peak B and C treated groups ( $14.7 \pm 1.97$  and  $14.3 \pm 0.97$ , respectively) were not statistically different from the control group.

Increase in the Thickness of the Ear ( $\text{mm} \times 10^{-2}$ )  $\pm$  SE



response occurred with both the low molecular weight pool and Peak A ( $p < 0.001$  and  $0.001 < p < 0.01$ , respectively). The finding that the LMW pool is more suppressive than peak A when compared on a per gland basis could be due to loss of some of the active material during fractionation. Alternatively, peak B and/or C may be able to potentiate the suppressive effect of peak A although neither of them suppresses this activity when tested individually.

Fig.15 shows SDS-PAGE of the low molecular weight pool and the various peaks. Peak A is less heterogeneous than the pool but it still contains several bands.

#### 6. Fractionation of Peak A through Anion Exchange

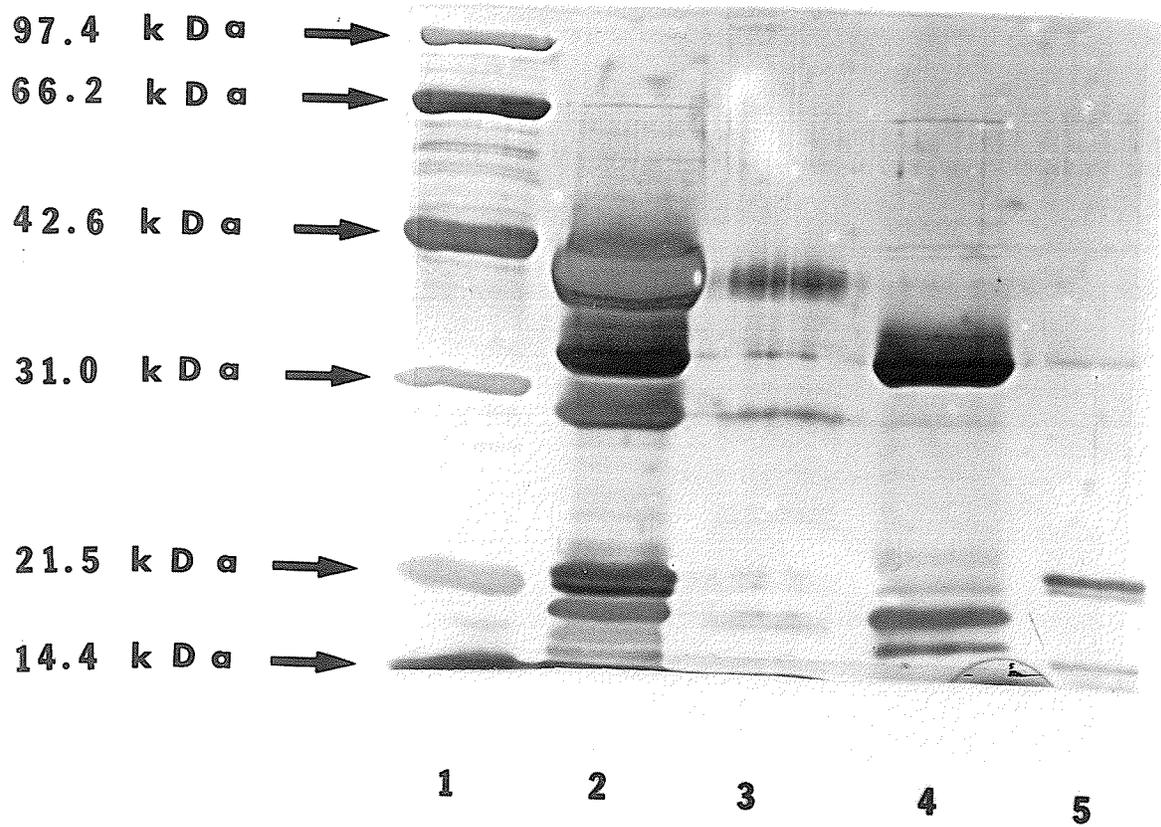
##### Chromatography

Peak A was further fractionated through anion exchange chromatography by using a DEAE-Sepharose column. The linear continuous gradient was established by increasing the sodium chloride concentration from 0 M to 1.5 M in the piperazine buffer. This method of purification resulted in the production of two protein peaks. The first peak was eluted early in the gradient, whereas the second peak was eluted around 0.3 M NaCl concentration.

To determine which of these two peaks ( $A_I$  for the first and  $A_{II}$  for the second) contained the in vivo

Fig. 15 SDS-PAGE & silver staining of the low molecular weight pool and the various peaks of the Phenyl Sepharose column

Lane 1 shows the molecular weight standards. Lane 2 represents the L.M.W. pool before fractionation. Lanes 3, 4 and 5 show the protein bands present in Peaks A, B and C, respectively, that result from fractionation of the pool through the Phenyl Sepharose column.



immunosuppressive activity, another DTH experiment was carried out. As shown in Fig.16, peak A<sub>II</sub> appeared to be the one that was immunosuppressive in that model.

Then, Peak A was fractionated through the DEAE-Sepharose column as mentioned above, but the gradient consisted of increasing NaCl concentration from 0.2 to 0.4 M in the piperazien buffer and more of the eluting buffer was used, about 9 times instead of 6 times the bed volume as in the first fractionation. The reason for these changes was to try to stretch this part of gradient to further separate the components of the second peak (A<sub>II</sub>). Fig.17 shows the resulting fractions from this column. Peak A<sub>I</sub> was eluted before the gradient was started (the proteins did not bind to the column under these conditions). Peak A<sub>II</sub> was eluted in 15 fractions (fractions 14 to 29) and was shown to be composed of two small peaks, A<sub>IIa</sub> and A<sub>IIb</sub>.

Fig.18 shows the SDS-PAGE of fractions 16 to 23 of the DEAE-Sepharose column. The fractions which are part of the peak A<sub>IIa</sub> (16 to 19) contain a single molecular species with a molecular weight around 40,000 Dalton, whereas fractions 20 to 23 (which are part of peak A<sub>IIb</sub>) contain, in addition, a molecule with a molecular weight around 30,000 Dalton. Fractions 15 to 19 were pooled to

Fig. 16 Suppression of the DTH response by Peak AII of the DEAE-  
Sepharose column

Three groups of female A/J mice (five animals each) were used in this experiment. Treatment with either PBS, Peak AI, or Peak AII was carried out as two daily injection at days five and six post-sensitization. Each injection contained a material corresponding to one SMG. Significant suppression of the DTH response occurred only in the group treated with Peak AII,  $10.4 \pm 1.2$  compared to  $15.8 \pm 0.37$  in the control group ( $0.001 < p < 0.01$ ).

Increase in the Thickness of the Ear ( $\text{mm} \times 10^{-2}$ )  $\pm$  SE

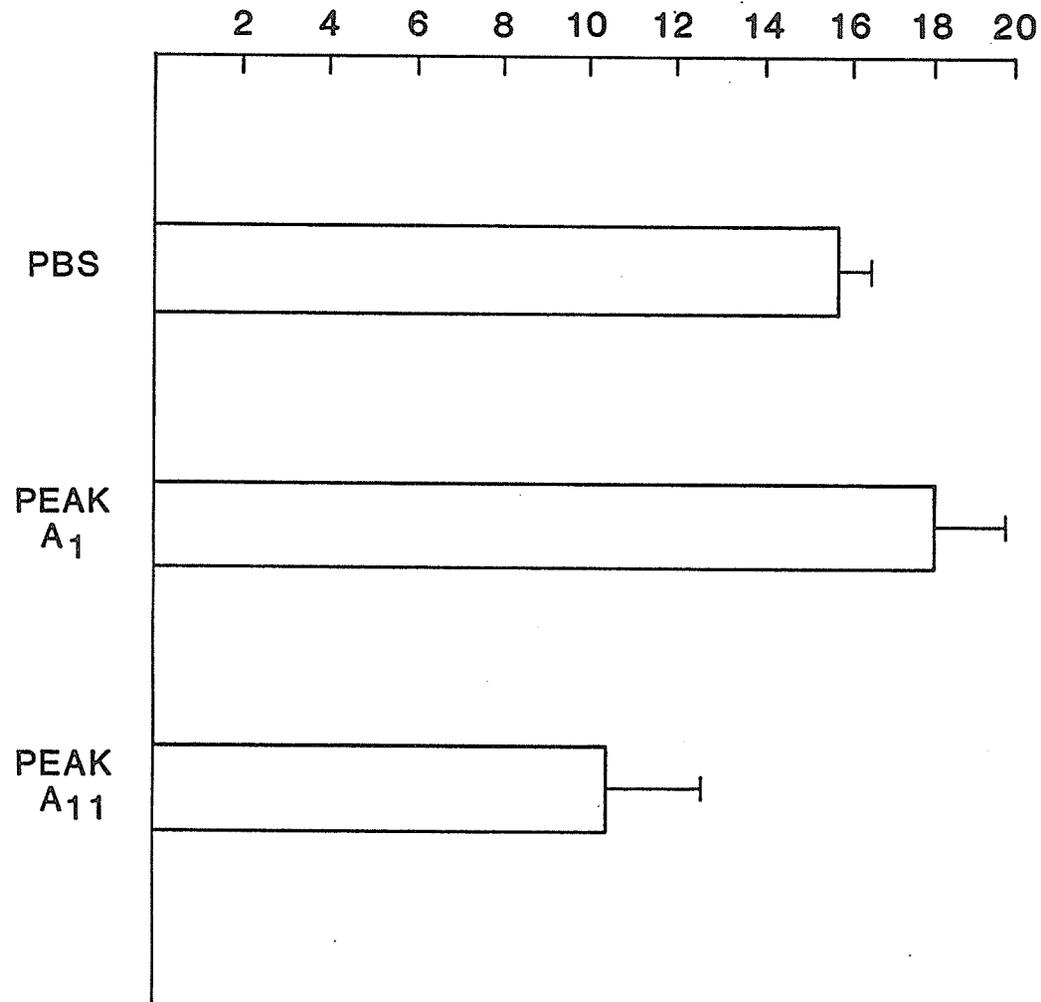


Fig. 17 Fractionation of Peak A through anion-exchange chromatography

The figure shows the protein content (●) and the conductivity (---) of the resulting fractions of the DEAE-Sepharose column. The gradient consisted of equal volumes (100 ml each) of piperazine-0.2 M NaCl and piperazine-0.4 M NaCl buffers. The first peak AI was eluted before the start of the gradient. The second peak AII appeared to consist of two parts, AIIa and AIIb.

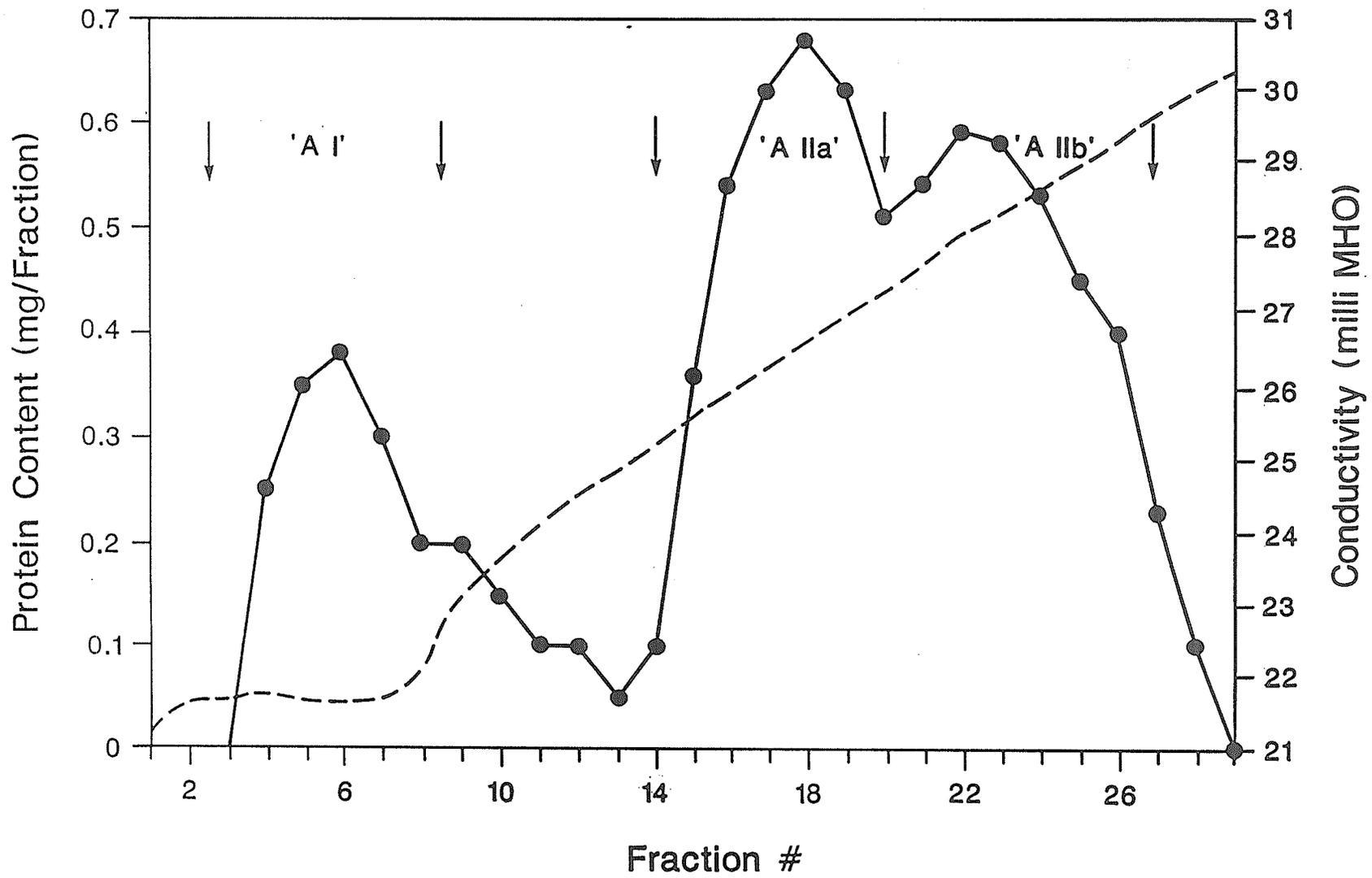
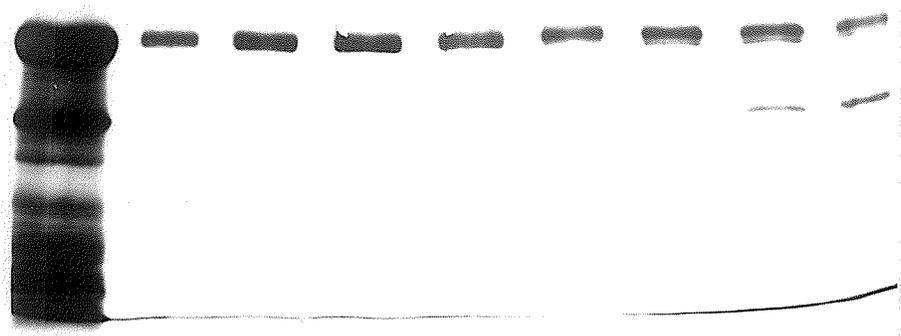


Fig. 18 SDS-PAGE & silver staining of the fractions of the DEAE-  
Sepharose column

The positions of different molecular weight standards are shown at the left side of the figure. Lane 1 represents Peak A before fractionation. Lanes 2 to 5 represent fractions 16 to 19, respectively, which are part of Peak AIIa. Lanes 6 to 9 contain fractions 20 to 23 of Peak AIIb.

97.4 kDa →  
66.2 kDa →  
42.6 kDa →  
31.0 kDa →  
21.5 kDa →  
14.4 kDa →



1 2 3 4 5 6 7 8 9

produce peak A<sub>IIa</sub>, whereas fractions 20 to 27 were pooled to produced peak A<sub>IIb</sub> as shown in Fig.17. Electrophoresis of both pools is shown in Fig.19. Peak A<sub>IIa</sub> has a single band referred to as 40K, whereas peak A<sub>IIb</sub> contains two bands, 30-40K.

#### 7. Suppression of the DTH response by both 40k and 30-40K fractions

Both 40K and 30-40K fractions were isolated from peak A<sub>II</sub> which had an immunosuppressive activity in the DTH response (see Fig.16). In order to determine the immunological activity of these molecules, they were tested for their ability to suppress the DTH response. Three groups (five animals each) of female A/J mice were used. Two daily injections with either PBS (control group), 40K (100ug per injection), or 30-40K (100 ug per injection) were given at day 5 and 6 postsensitization. As shown in Fig.20 both of these fractions produced significant suppression of the increase in ear thickness after challenge in comparison to the control group.

#### 8. Summary of the purification steps of the in vivo immune activity of the rat SMG

The first step of purification was salt precipitation of the rat SMG homogenate by using ammonium sulphate between 30 and 64% concentration to produce the

Fig. 19 SDS-PAGE & silver staining of Peak AIIa and AIIb of the  
DEAE-Sephadex column

The DEAE-Sephadex fractions which form peak AIIa were pooled and as shown in lane 1, they contain a single band with a molecular weight of around 40,000 Daltons (40 K). Lane 2 shows the 30-40 K present in Peak AIIb. The positions of the molecular weight standards are also shown.

97.4 kDa →

66.2 kDa →

42.6 kDa →

31.0 kDa →

21.5 kDa →

14.4 kDa →



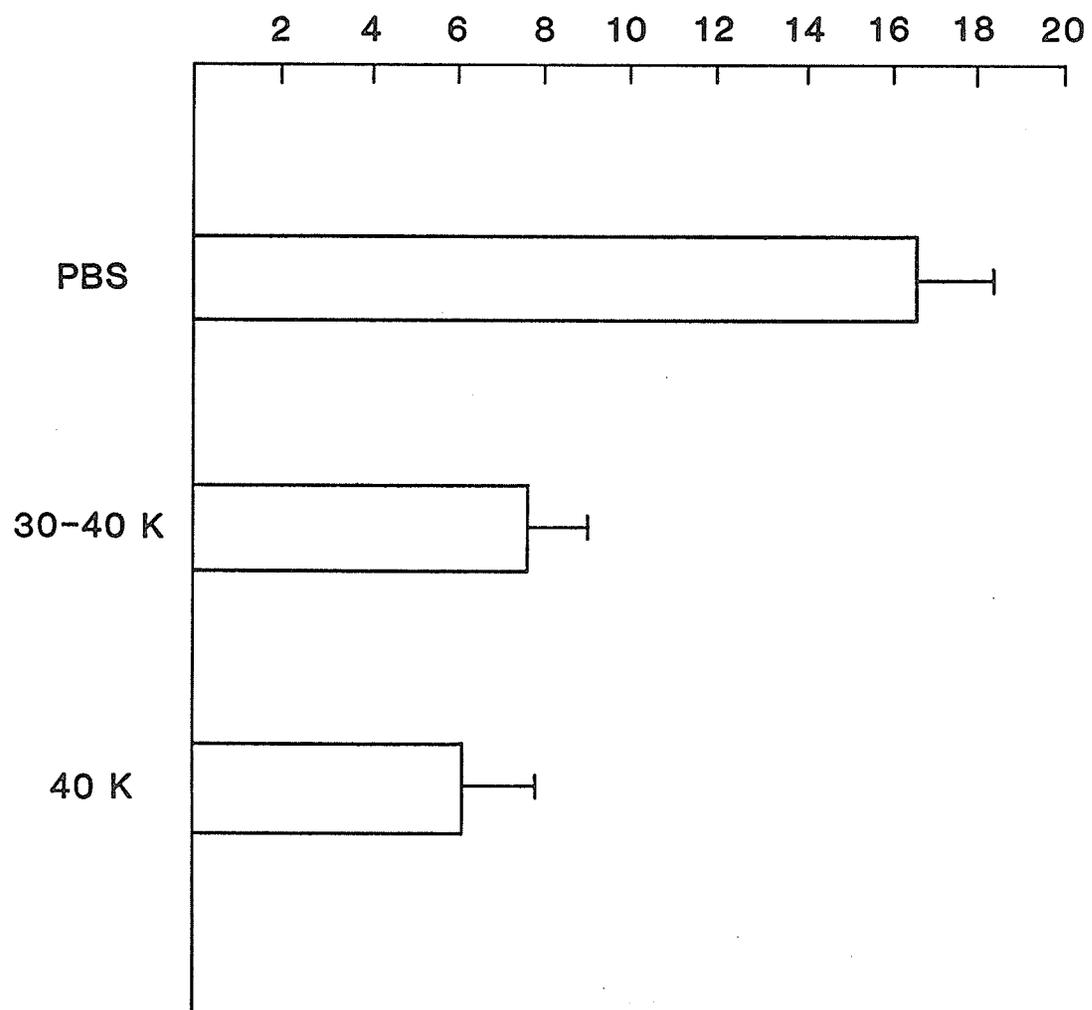
1

2

Fig. 20 Suppression of the DTH response by 30-40 K and 40 K

Both 30-40K and 40K produced significant suppression of the DTH response, the mean increase in the ear thickness after challenge in these groups were  $7.7 \pm 0.43$  and  $6.2 \pm 0.58$ , respectively, compared to  $16.4 \pm 0.6$  in the control group.

Increase in the Thickness of the Ear ( $\text{mm} \times 10^{-2}$ )  $\pm$  SE



crude SMG extract. In a typical experiment, 30 SMG taken from 15 male rats weighed about 5.4 grams. The total amount of protein present in the crude extract after ammonium sulphate precipitation was 235.9 mg. The crude extract was then fractionated through gel filtration chromatography (Fig.1). The amounts of protein in the high and low molecular weight Sephacryl pools were 58 and 32 mg, respectively. The low molecular weight pool was then fractionated through hydrophobic interaction chromatography to produce peak A, B, and C (Fig.13) which had 15.6, 6.5, and 2.6 mg protein, respectively. The in vivo active peak A was further fractionated through anion exchange chromatography (Fig.17). The resulting in vivo active molecules, 40K and 30-40K from this purification step contained 2.29 and 3.76 mg of protein, respectively. The amounts of proteins at different purification steps per one SMG are shown in Table 1. It should be noted that these results represent only one experiment and slightly different figures were obtained from one experiment to another. Fig.21 shows SDS-PAGE of the various in vivo active proteins.

#### 9. In Vitro Immune Activity of the Various Fractions of the Rat SMG

As previously shown (Kemp et al, 1985 and Fig. 1)

Table 1. Summary of the purification steps for the in vivo immune activity of the rat SMG

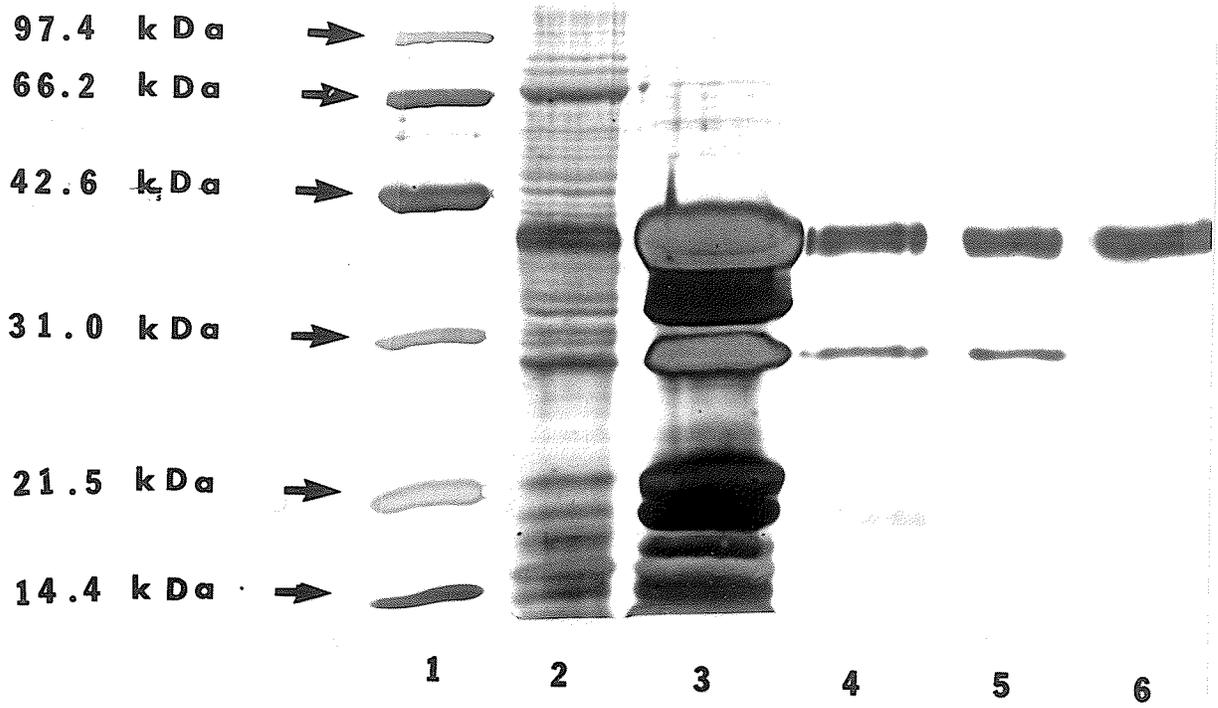
The table shows the different steps used for purification of the in vivo immune activity of the rat SMG. Also shown in the table are the resulting proteins from each step of purification as well as the content of these proteins per one SMG.

| Purification step   | Resulting protein | content per gland |
|---|-------------------|-------------------|
| Frozen gland  | ---               | 0.18 grams*       |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation | Crude extract     | 7.8 mg            |
| Gel Filtration  | H.M.W. Pool       | 1.93 mg           |
|   | L.M.W. Pool       | 1.06 mg           |
| Hydrophobic interaction chromatography of L.M.W. Pool         | Peak A            | 0.52 mg           |
|   | Peak B            | 0.21 mg           |
|   | Peak C            | 0.08 mg           |
| Anion Exchange chromatography of Peak A                       | Peak AI           | 65 ug             |
|   | 30-40 K           | 125 ug            |
|   | 40 K              | 98 ug             |

\* This value represents the weight of the gland

Fig. 21 SDS-PAGE & Silver staining of the proteins at various steps of purification of the in vivo immunosuppressive activity of the rat SMG

The crude SMG extract (lane 2) was fractionated through gel filtration chromatography. The in vivo active low molecular weight pool (lane 3) was further fractionated through hydrophobic interaction chromatography by using the Phenyl Sepharose column. Peak A (lane 4) of this column had the in vivo activity and was further fractionated through anion exchange chromatography. Both 30-40 K and 40 K of the DEAE-Sepharose column (lanes 5 and 6, respectively) were immunosuppressive in the DTH response. Lane 1 contains the molecular weight standards.



The activity of the Sephacryl gel filtration fractions in the Con A bioassay was taken as the measure of the ability of these fractions to affect lymphocyte proliferation in vitro. The fractions which produced significant suppression in this assay were eluted early from the column and were pooled to produce the high molecular weight pool, whereas the fractions of the low molecular weight pool produced significant enhancement. In agreement with the activity of their constituent fractions, the high and low molecular weight Sephacryl pools produced (at three different concentrations) significant suppression and enhancement, respectively, of lymph node-cell proliferation in the Con A bioassay (Fig.22).

In MLR, similar results were obtained (Fig.23). Suppression occurred with the high molecular weight pool, whereas the low molecular weight pool had significant stimulatory effect.

Both pools were also tested for their effect on the mitogenic reaction of sensitized lymph node cell in response to trinitrophenylated syngeneic spleen cells. Fig.24 shows that at high concentrations, both pools were able to suppress that response, whereas at low concentrations significant stimulation occurred only with

Fig. 22 Activity of the Sephacryl pools in the Con A bioassay

The high molecular weight pool produced suppression whereas the low molecular weight pool produced stimulation of the proliferation of the Con A-lymphoblasts. The three concentrations of either pools used in this experiment were 6 (//), 3 (\\), and 1.5 (///) ug per well. These amounts of protein were added in 20 ul of PBS, the control cultures had 20 ul of PBS.

C P M (Thousands)

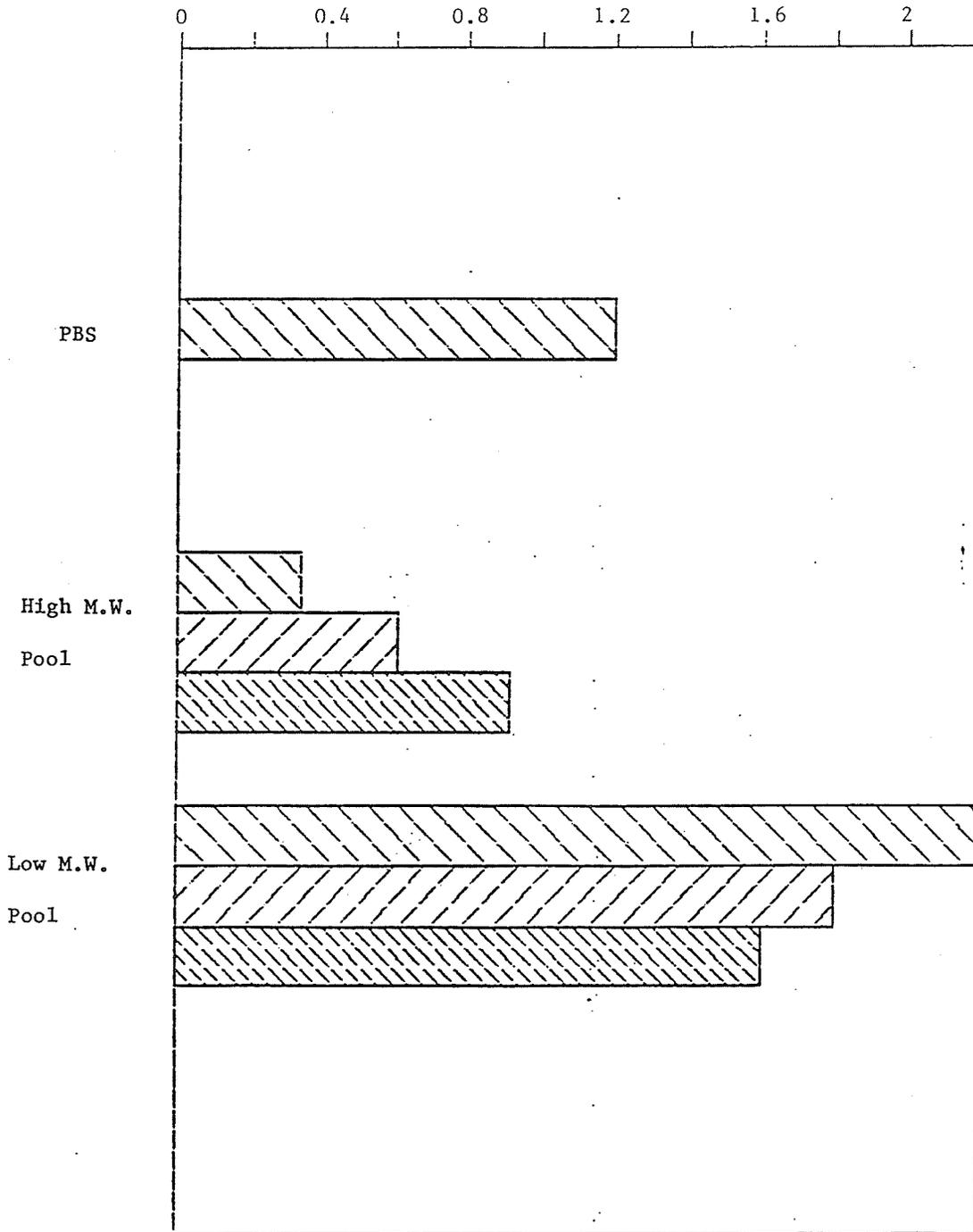


Fig. 23 Activity of the Sephacryl pool in the MLR assay

With three different concentrations, 6 (//), 3 (\\), and 1.5 (///) ug per well, the high molecular weight pool produced significant suppression. Stimulation occurred with the first two concentrations of the low molecular weight pool.

C P M (Thousands)

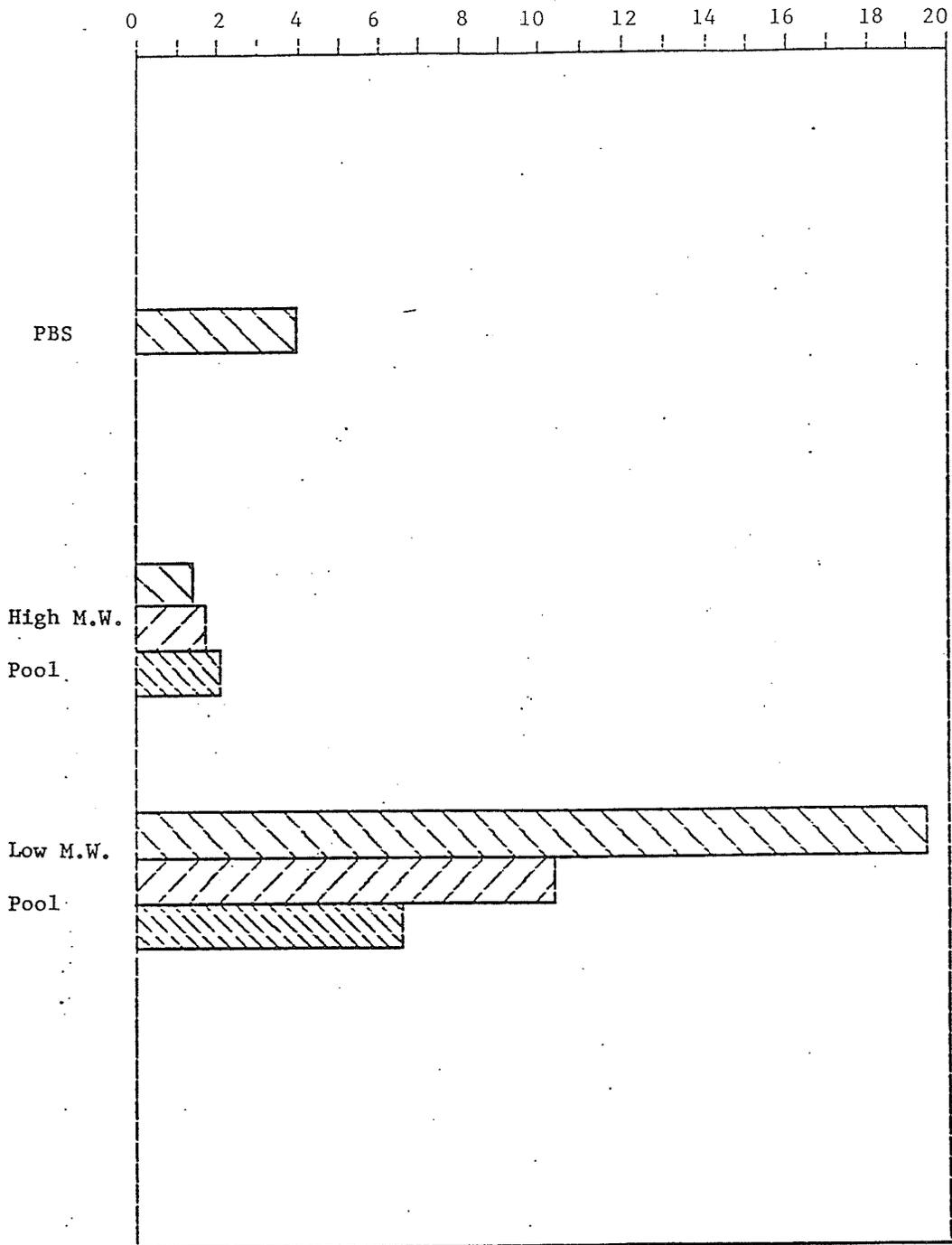
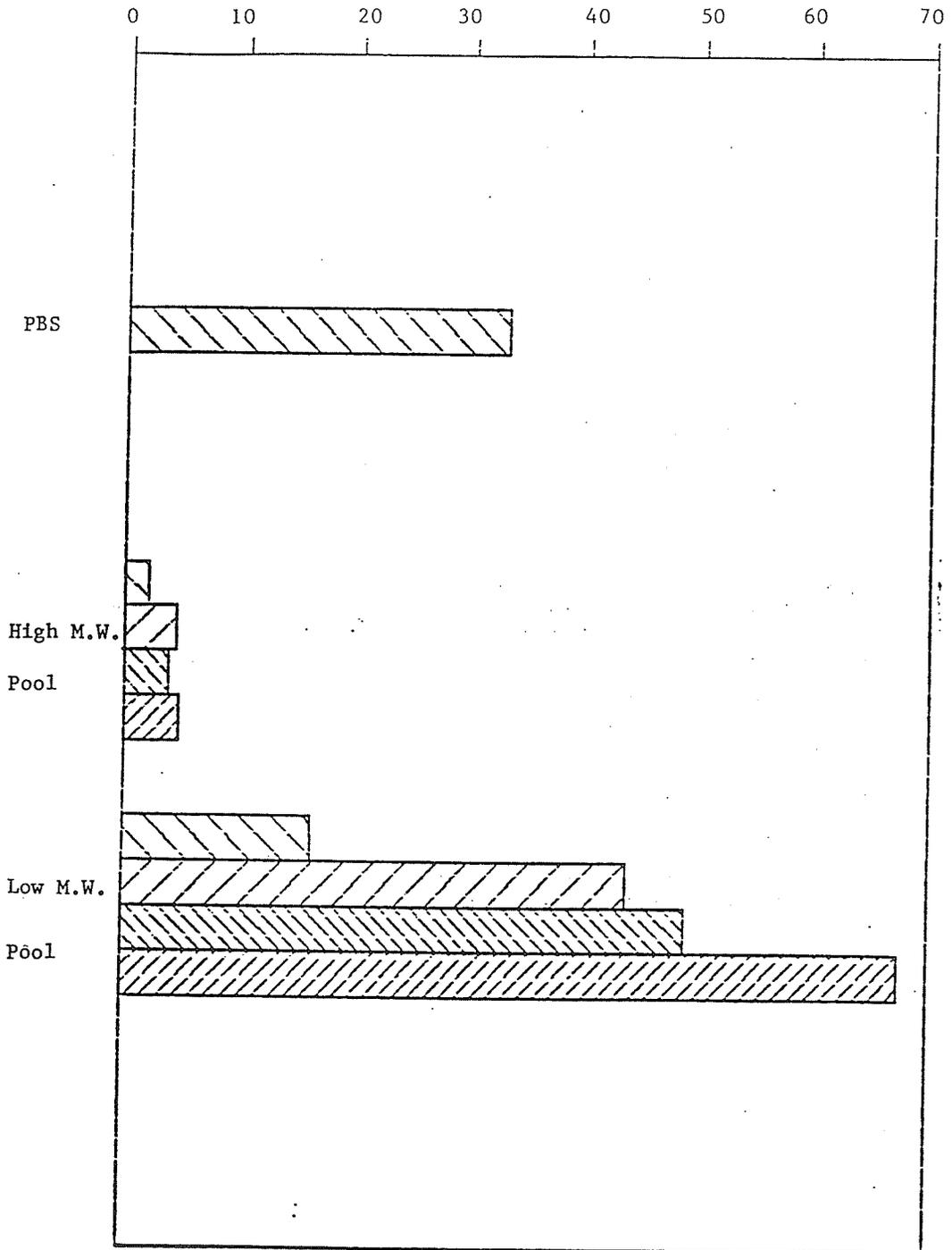


Fig. 24 Activity of the Sephacryl pools in the antigen-induced proliferation assay

The four different dilutions of the high molecular weight pool, 6 (//), 3 (\\), 1.5 (///), and 0.75 (≡) ug per well produced marked suppression of the proliferation of immune lymph node cells three days after challenge with haptenized syngeneic spleen cells. The low molecular weight pool had a mixed effect in this assay. At high concentrations, 6 ug per well, significant suppression of the response occurred, whereas at low concentrations, 0.75 ug per well, significant stimulation occurred.

C P M (Thousands)



the low molecular weight pool.

Both pools were finally tested in the CMC assay. In this model no stimulation of the cytotoxic activity was noted (Fig.25) Both pools were able to suppress the response.

It should be noted that the low molecular weight pool which exhibited in vitro enhancing activity in the ConA and MLR at all concentrations and in the antigen-induced proliferation at low concentrations was immunosuppressive in vivo in all the three models tested (skin transplantation, DTH, direct PFC response).

On the other hand, the finding that the high molecular weight pool was able to suppress all the above mentioned in vitro responses is consistent with the previous results (Kemp et al, 1986) which showed that this pool contained a factor with an IL-1 inhibiting activity.

The Phenyl Sepharose fractions constituting peaks A, B, and C, which resulted from fractionation of the low molecular weight pool through hydrophobic interaction chromatography, were able to enhance the lymphocyte proliferation in the Con A bioassay (see Fig.13). The in vivo active peak A produced a dose-dependent enhancement in this assay. Peak B, however, was more stimulatory

Fig. 25 Activity of the Sephacryl pools in the CMC assay

Four different dilutions, 6 (//), 3 (\\), 1.5 (///), and 0.75 (////) ug per well of both the high and low molecular weight pools were tested for their activity in the CMC assay. Marked suppression of this assay occurred with the all the concentrations of the high molecular weight pool. No stimulation was observed with low molecular weight pool, the first three concentrations produced marked suppression.

PER CENT LYSIS

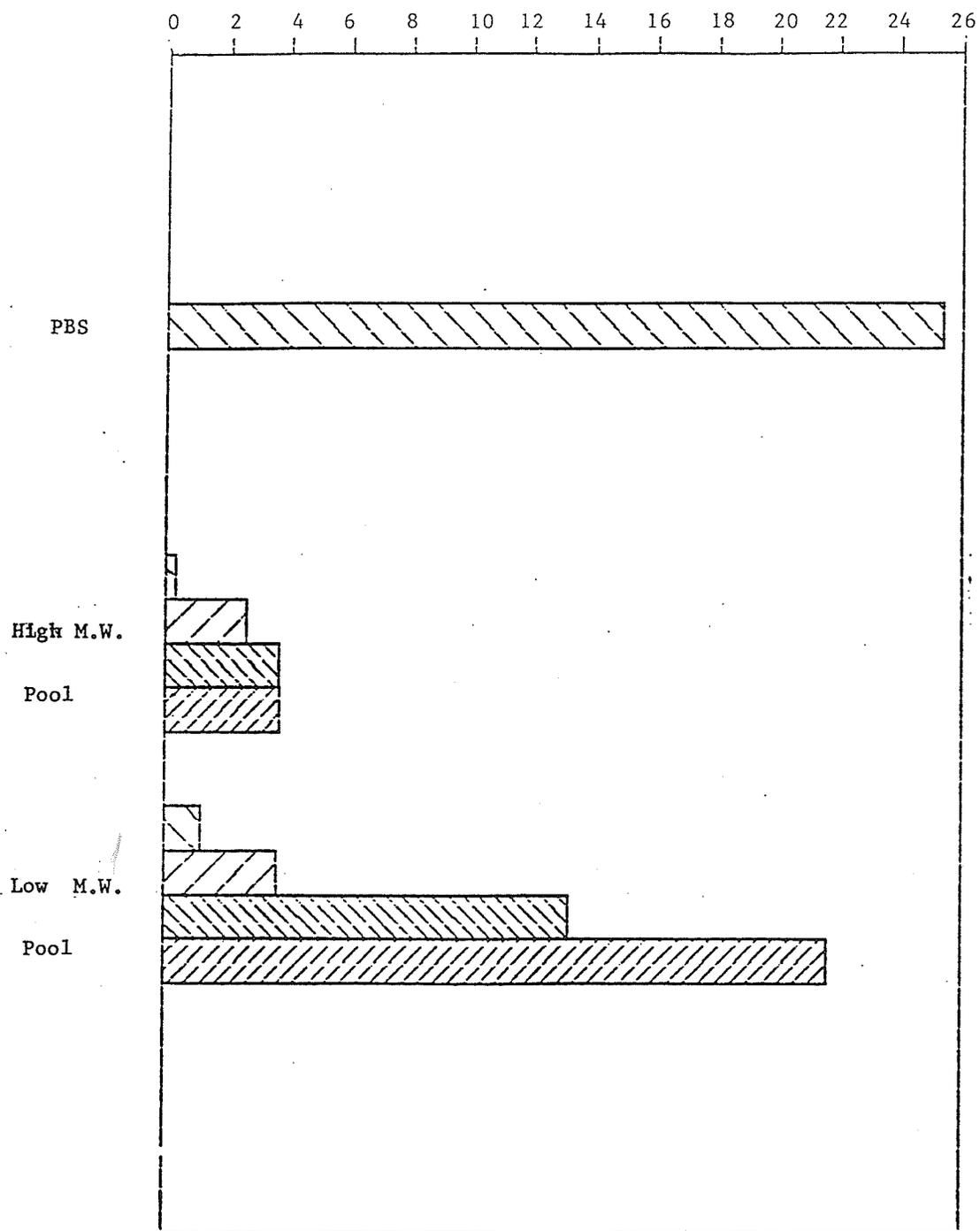


Fig. 26 Activity of Peaks A, B, and C in the Con A bioassay

The protein peaks that result from fractionation of the low molecular weight Sephacryl pool through hydrophobic interaction chromatography were tested for their activity in the Con A bioassay. Four dilutions of the peaks were used, 8 (//), 5 (\\), 2.5 (≡), and 1.25 (≡≡) micrograms in 20 ul of PBS per well. Peak A showed a dose-related stimulation. Peak B produced more stimulation in comparison with Peak A, whereas peak C resulted in lysis of lymphocytes at 5 ug concentration and stimulation at 1.25 ug concentration.

C P M (Thousands)

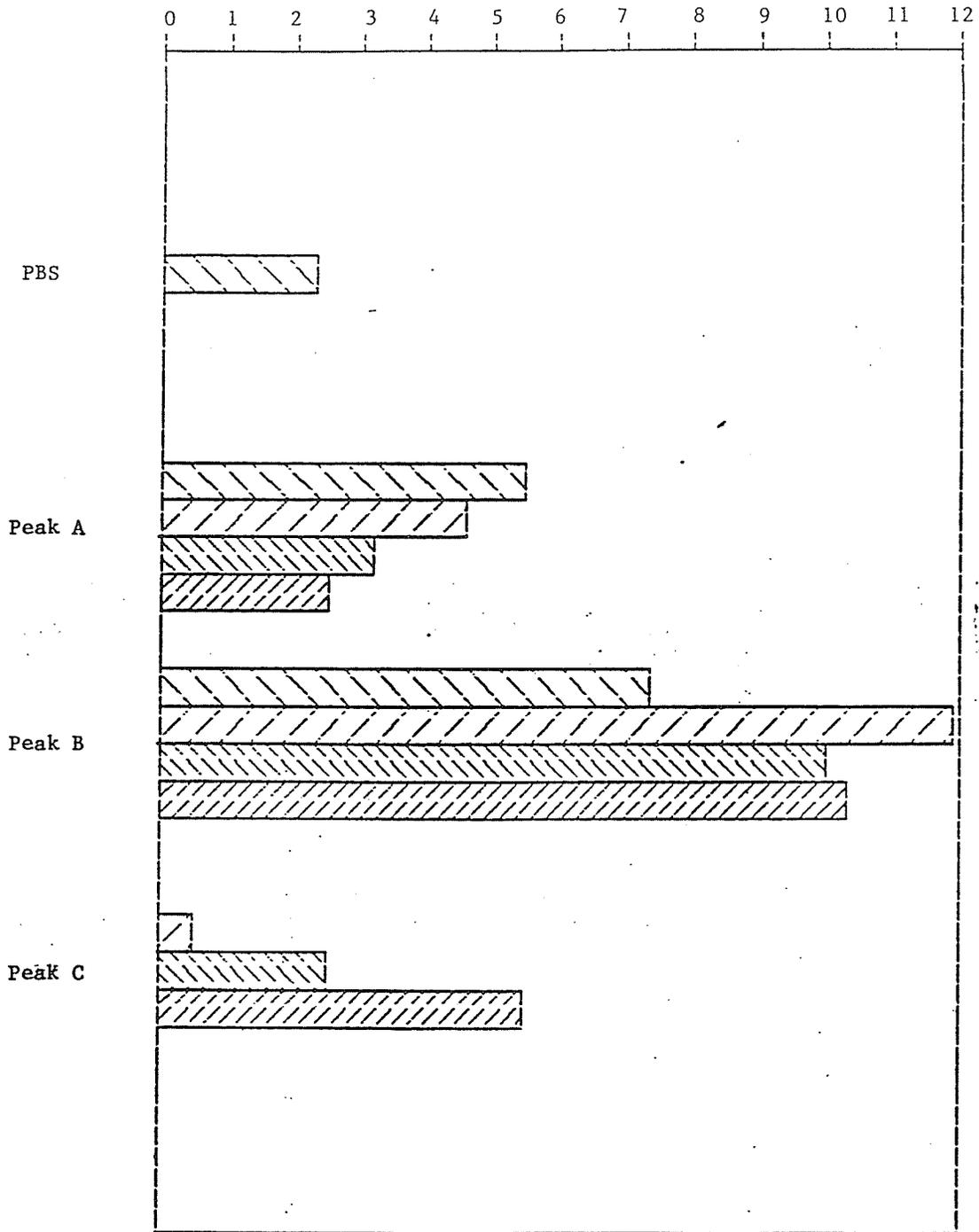


Fig. 27 Activity of 40K and 30-40K fractions in the ConA bioassay

Three different dilutions of each of the 40K and 30-40K molecules were tested in this assay. They were 1.6 (//), 1 (\), and 0.3 (///) ug per well. The results are shown in the figure which illustrates that both fractions have a similar pattern of activity in this assay. Stimulation results only the high concentrations.

C P M (Thousands)

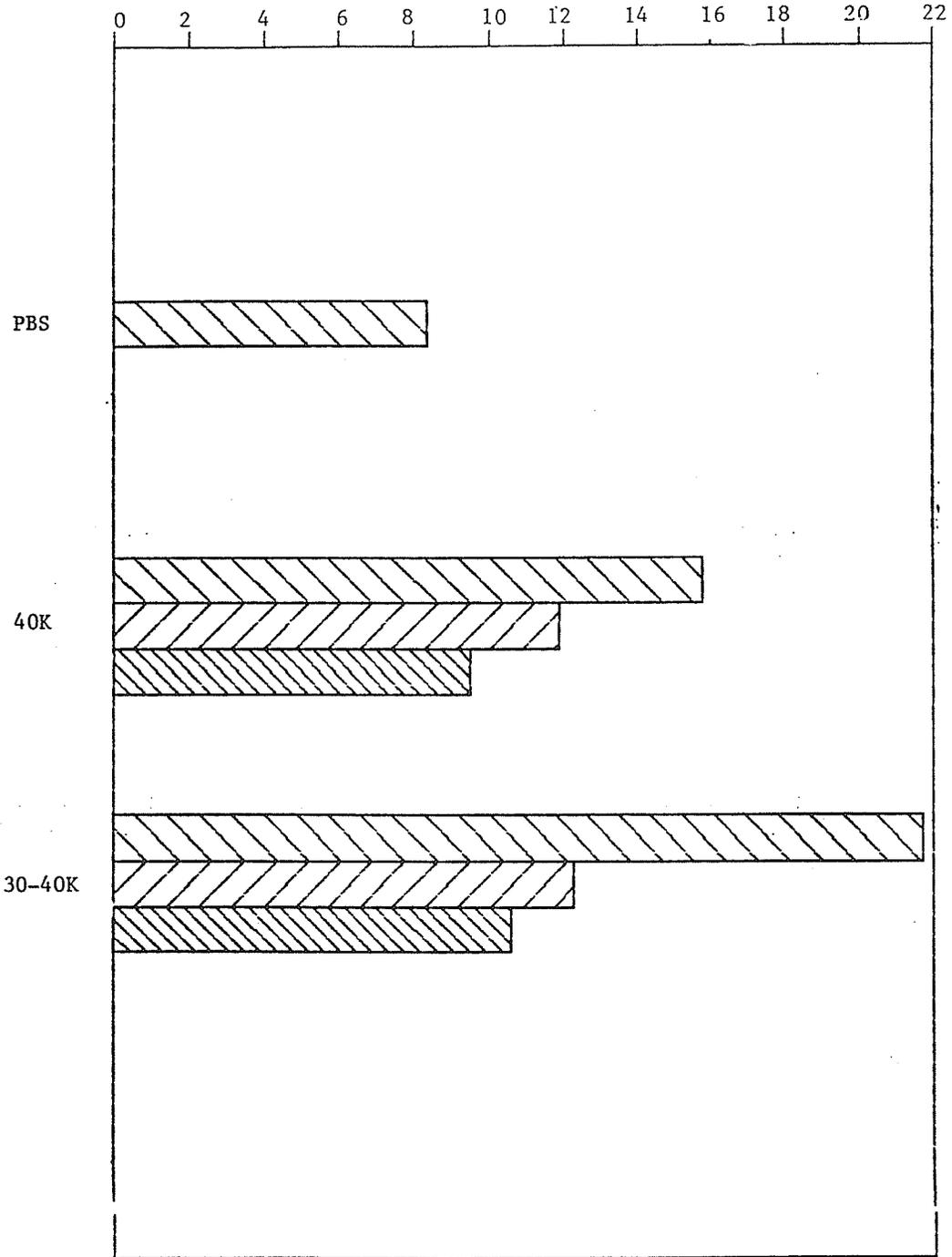


Fig. 28 Activity of 40K and 30-40K fractions in MLR

Four serial dilution, 1.6 (//), 0.8 (\\), 0.4 (///), and 0.2 (\\\\) ug per well, of both fractions were used in this assay. With the highest concentration used, there was a marked stimulation of lymphocyte proliferation with 30-40 K, whereas with 40 K there was only mild enhancement of the response. With the rest of the concentrations used, both 40K and 30-40K had values around the control cultures.

C P M (Thousands)

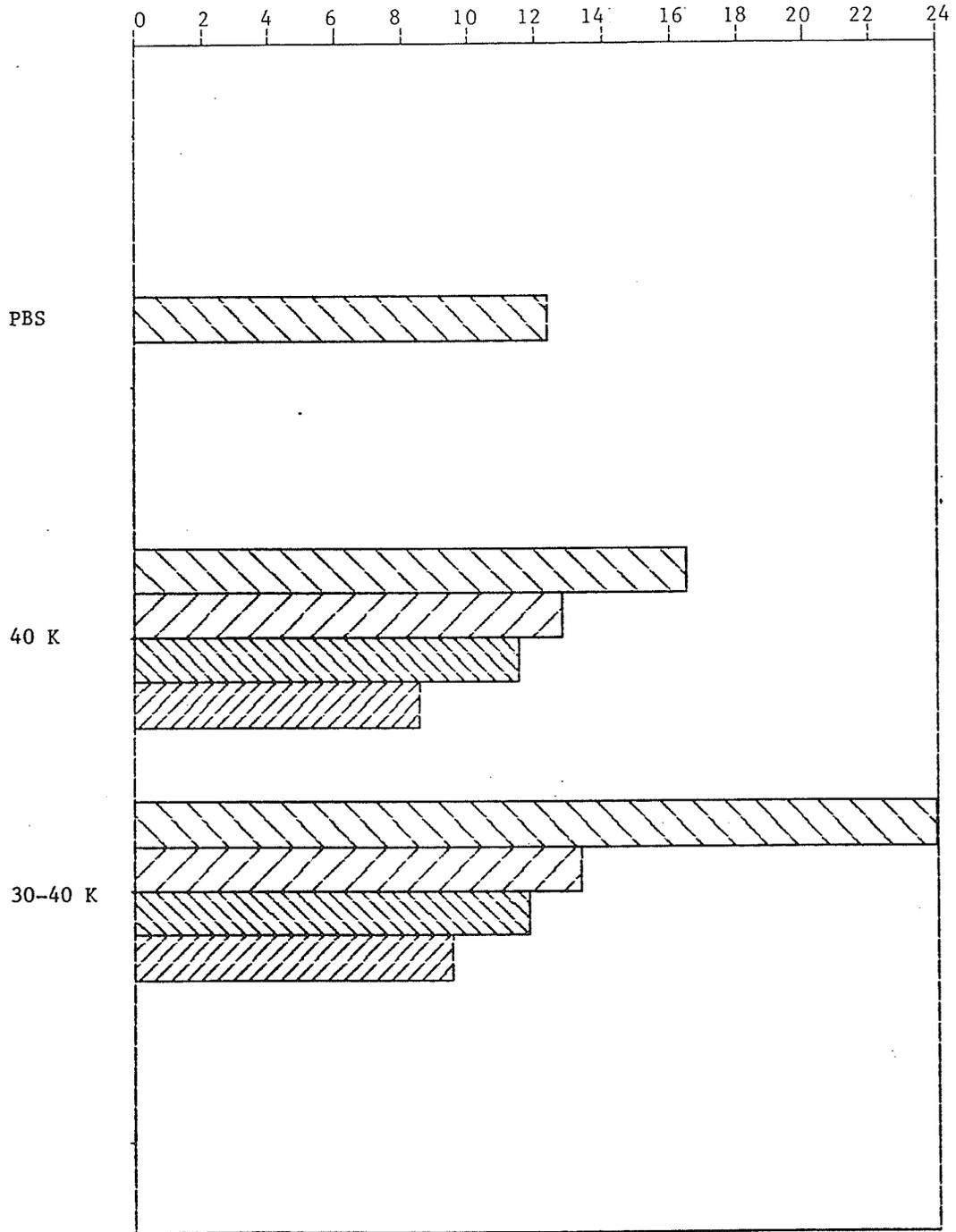


Fig. 29 Activity of 40 K and 30-40 K fractions in the antigen-induced proliferation assay

The figure shows the activity of the 40 K and 30-40 K fractions in the antigen-induced proliferation assay in three different concentrations, 1.6 () , 1 () , and 0.3 () ug per well.

C P M (Thousands)

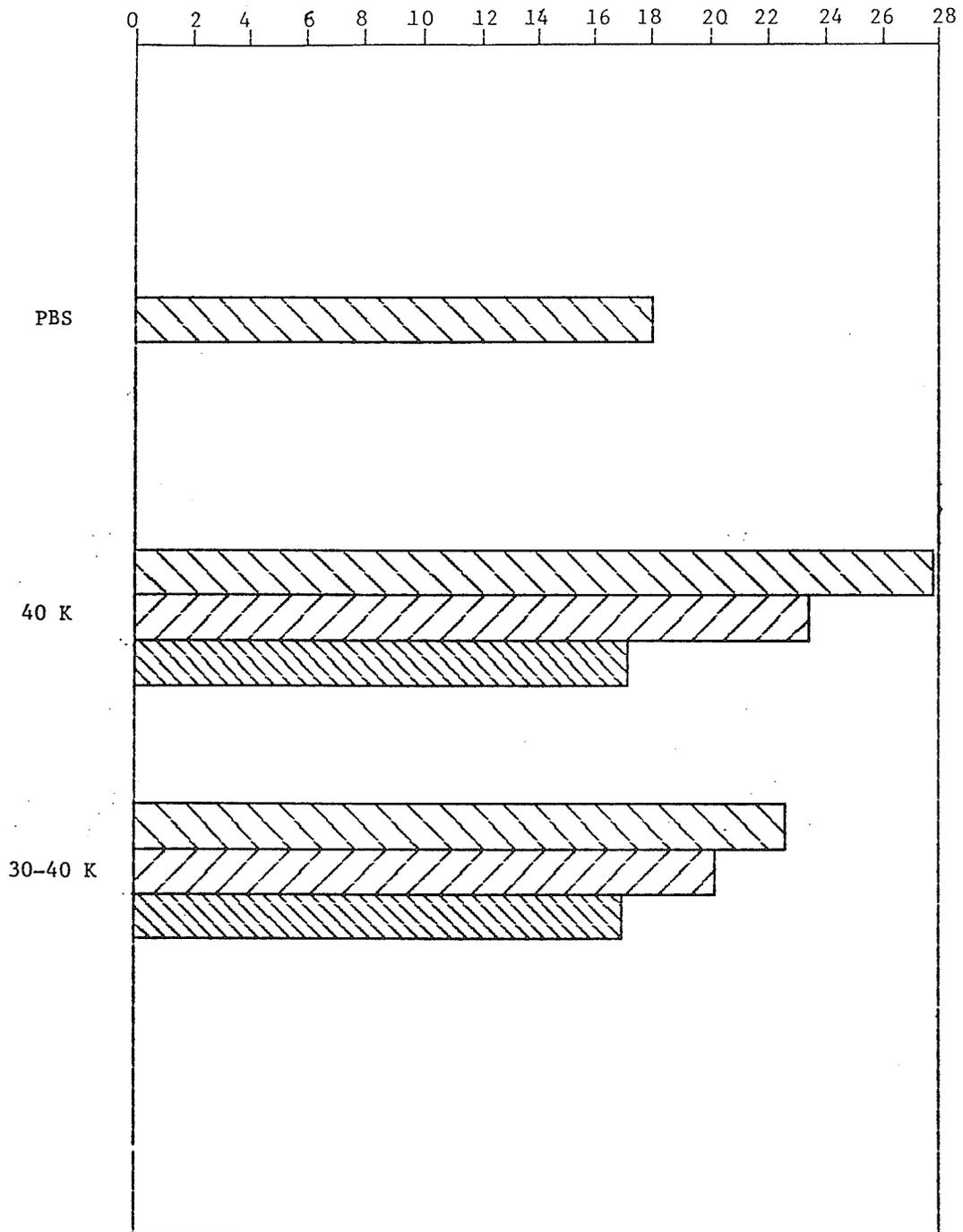


Fig. 30 Activity of 40 K and 30-40 K fractions in CMC assay

Four serial dilution, 1.6 (//), 0.8 (≡), 0.4 (≡≡), and 0.2 (≡≡≡) ug per well of either 40 K or 30-40 K fractions were tested for the activity in the CMC assay. As shown in the figure, stimulation of the cytotoxic activity of the responder cells occurred only with the high concentrations of these fractions.

The cultures were tested for cytotoxic activity against <sup>51</sup>Cr labeled target cells after five days of incubation.

The concentration of both the stimulatory and responder cells was kept constant at  $2 \times 10^5$  and  $1 \times 10^5$ , respectively.

PER CENT LYSIS

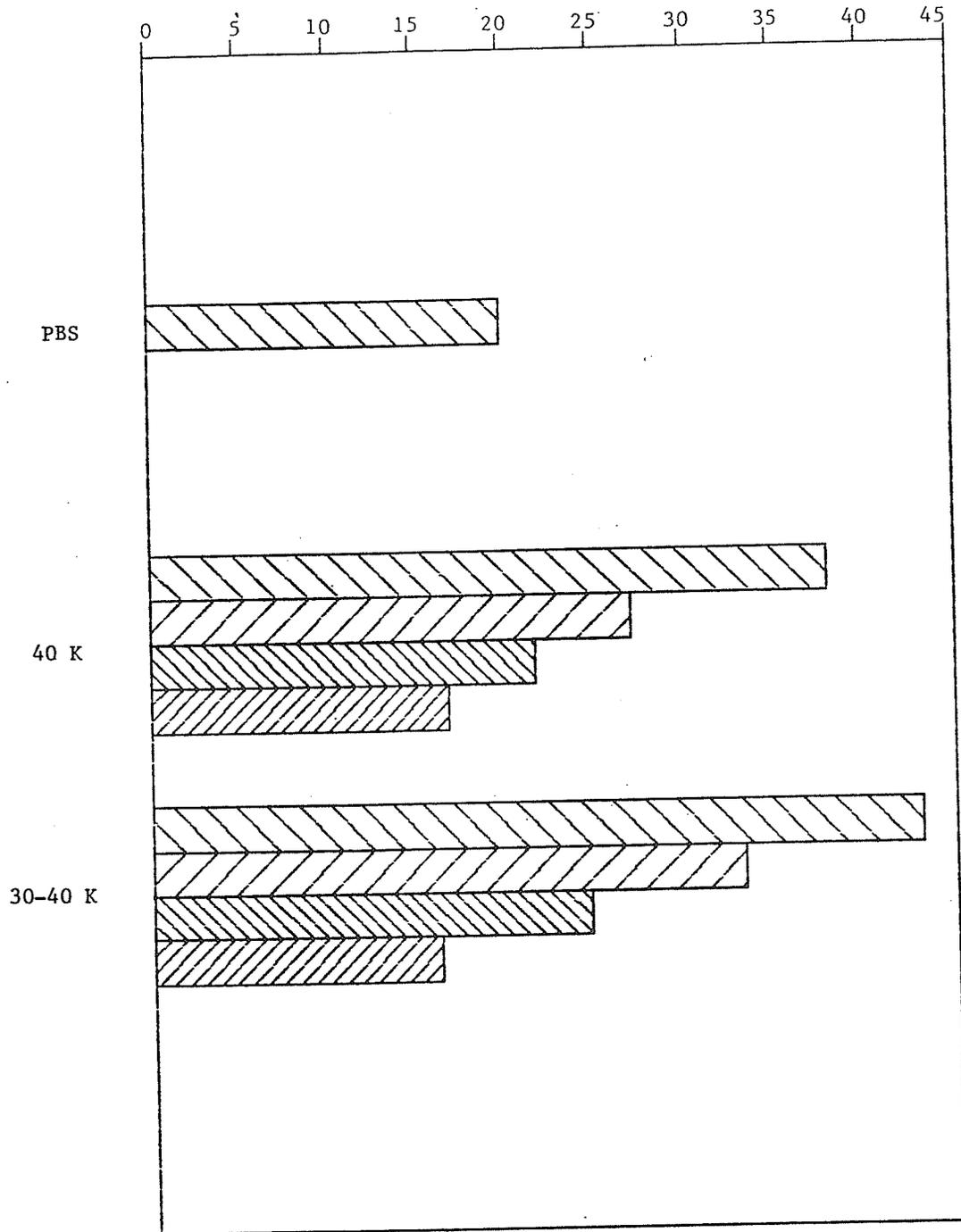
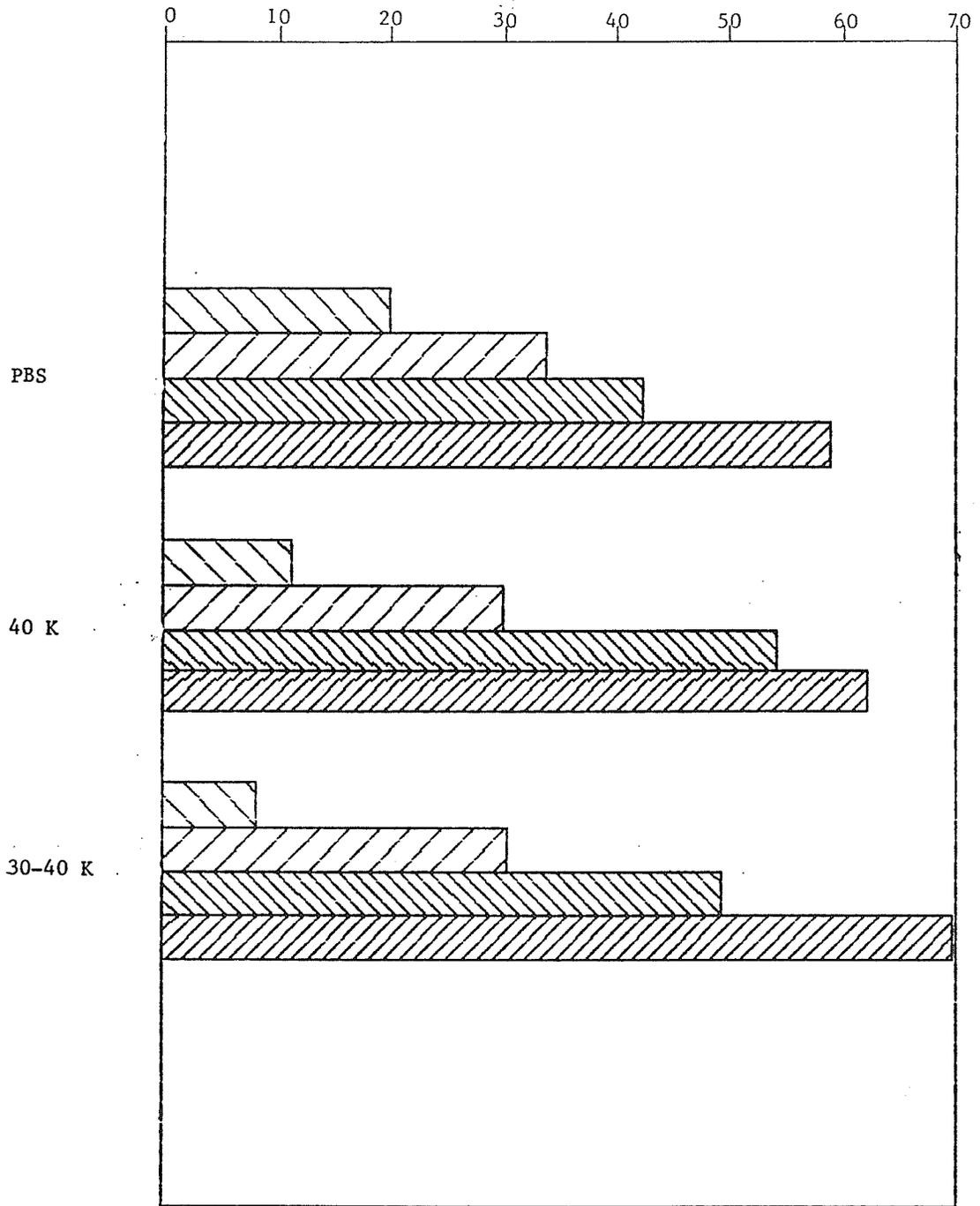


Fig. 31 Activity of 40 K and 30-40 K in the CMC assay

In this assay the concentration which produced enhancement of the cytotoxic activity in the previous assay (Fig. 30), 1.6 ug per well was used, but the concentration of the responder cells varied. Four concentrations were used, 2.5 (///), 5 (\\), 10 (\\), and 20 (\\) x 10<sup>4</sup> responder cells per well. The concentration of stimulatory cells was kept constant at 2 x 10<sup>5</sup> cells per well. The results shown in the figure are after four days of culture.

PER CENT LYSIS



than peak A, whereas peak C led to marked suppression at high concentrations and stimulation at low concentrations. So, the different components of the low molecular weight pool showed different patterns of activity as far as the Con A bioassay was concerned (Fig.26)

The in vivo active 30-40K and 40K were also tested for their in vitro activities in a number of assays. In the Con A bioassay (Fig.27) both molecules had stimulatory effect only in high concentrations. Similarly, in MLR (Fig. 28) and in the antigen-induced proliferation assay (Fig. 29) stimulation occurred only with high concentrations of the 30-40 K fraction and the 40 K fraction, respectively.

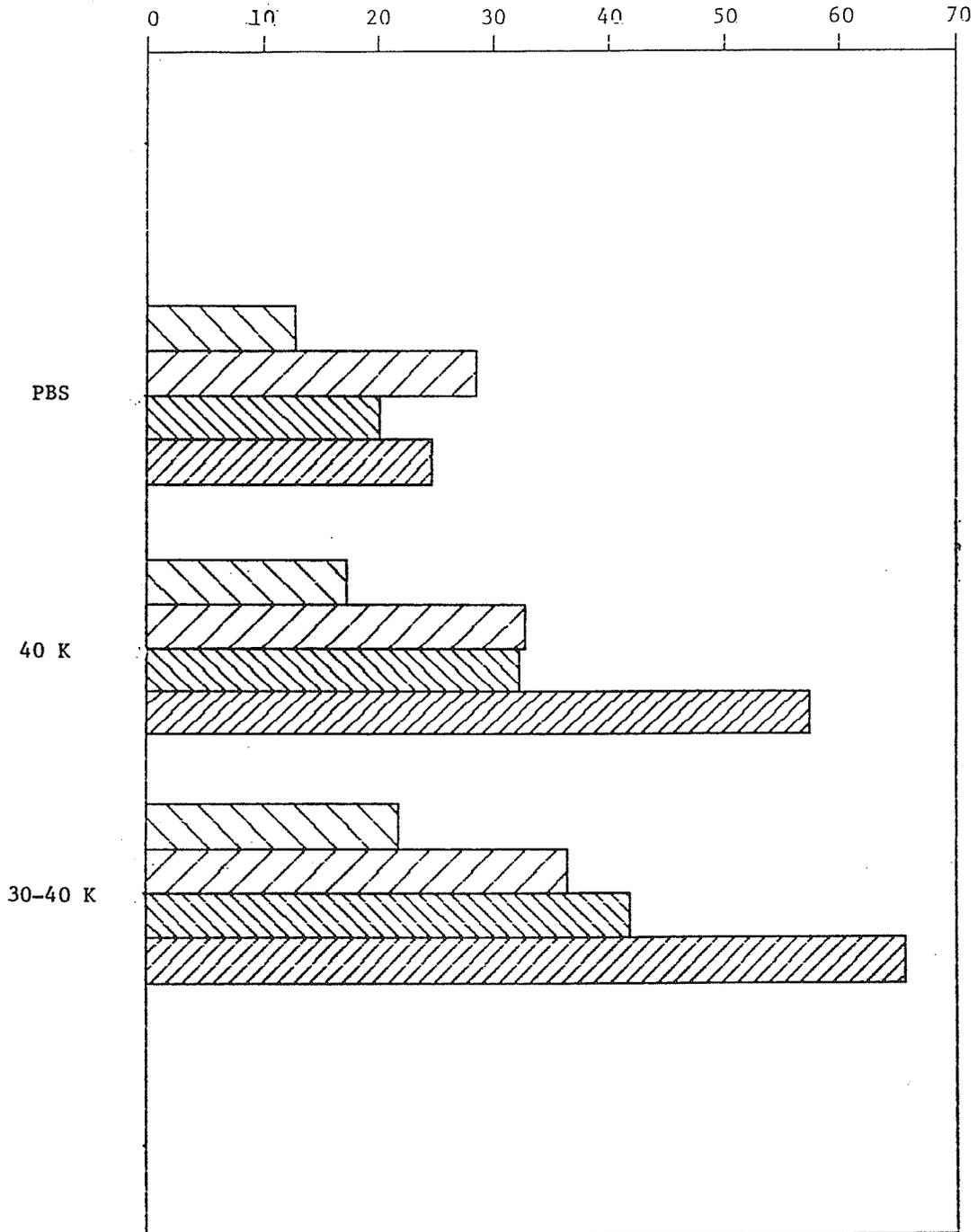
In the CMC assay, both 30-40K and 40K appeared again to have a stimulatory activity at high concentrations when a single responder cell concentration ( $10^5$  cells per well) was used (Fig.30). In an attempt to analyse this activity, the concentrations of both 30-40K and 40K which produced the enhancing effect in this experiment (Fig.30) were tested in an assay which employed different concentrations of the responder cells with the concentration of the stimulatory cells fixed ( $2 \times 10^5$  cells per well). As shown in Fig.31, no significant differences from the control values were observed with either of the

molecules after four days of culture. On the other hand, Fig.32 shows the results of the same experiment after five days of incubation with significant enhancement of the cytotoxic activity occurring only at high concentrations of responder cells.

Fig. 32 Activity of 40 K and 30-40 K fractions in the CMC assay

The figure shows the same cultures used in the previous experiment (Fig. 31), but but after five instead of four days of incubation. As shown in the figure, under these conditions of culture, there is a significant stimulation of the cytotoxic activity when high (  $2 \times 10^5$  ) concentration of the responder cells was used.

PER CENT LYSIS



### DISCUSSION

In this study we have identified 40kD and 30-40kD in vivo immunosuppressive fractions from the rat SMG. These fractions were derived from the low molecular weight pool resulting from fractionation of the SMG crude extract through gel filtration chromatography. This pool showed significant suppression of the in vivo murine models used in this study, namely, skin allograft rejection, PFC response and the DTH response. In addition, the LMW pool suppressed the adjuvant induced arthritis in rats. (Sabbadini, unpublished). In all these models, no significant effect was observed with the high molecular weight pool despite the fact that it showed a strong in vitro immunosuppressive activity. This in vitro activity has been attributed to an IL-1 inhibiting factor Kemp et al (1986). However, the absolute requirement for IL-1 in in vivo immune responses has not been established (Durum&Oppenheim, 1989).

On the other hand, the activity of the low molecular weight pool in the in vitro assays was variable depending on the assay used. In the Con A bioassay and MLR, it produced enhancement of cellular proliferation. In the antigen-induced proliferation assay, suppression was observed at high concentration (6 ug/well) whereas

stimulation occurred at lower concentrations. In the CMC assay, no stimulation was observed at all, and suppression was evident at high concentrations. It should be noted that although these assays are T-cell dependent, the proliferating cells in each of them vary. Con A is a T-cell mitogen which has been reported to favor the induction of suppressor T-cells (Dutton, 1973). MLR and CMC measure the response to alloantigens. In MLR, cellular proliferation in response to lymphokines produced by alloantigen-specific T-cells is detected. In CMC target cell lysis by cytotoxic T-cells depends on the production of lymphokines by alloantigen-specific T-cells as well as the presence of precursors of cytotoxic cells with specificity for alloantigens on the target cell surface. In the antigen-induced proliferation assay, the frequency of specific T-cells in sensitized lymph nodes is usually low and the possibility that unrelated cells also proliferate can not be excluded. The variability of the in vitro activity of the low molecular weight pool can be attributed to the heterogeneity of the material and the possible presence of more than one molecule with different activities acting on the same and/or different targets.

However, the ability of the the low molecular weight pool of the rat SMG to slow down allograft destruction, suppress the increase in ear thickness due to cellular infiltration upon antigenic challenge in the DTH response, and decrease the number of spleen direct plaque forming cells in mice suggested that this pool contained immunosuppressive fractions that can act in vivo to inhibit either directly or indirectly the activity of immunocompetent cells.

We have used the DTH response for further testing of the in vivo activity of various fractions resulting from fractionation of the low molecular weight pool due to its technical simplicity as well as the relatively small amount of material required to effectively suppress the response (two gland-equivalent material per animal compared to five glands in case of either the skin allograft or the PFC response).

DTH is an antigen-specific, T-cell dependent recall reaction. In the induction phase of the contact sensitivity variant of DTH, the reactive chemical binds to skin proteins to create neoantigens which are taken up by antigen presenting cells. These cells migrate into the dermis and enter the lymphatics of the draining lymph node to present antigens to T-cell. Some authors suggest

the presence of at least two antigen presenting cells in the epidermis ; I-A<sup>+</sup> cells that induce CD4<sup>+</sup> T-cells which initiate the DTH response and I-J<sup>+</sup> cells that induce CD8<sup>+</sup> T-cells which suppress the response (Meltzer&Nacy, 1989). The final expression of the DTH response depends on the net result of the effects of these cells. Upon antigenic challenge, sensitized CD4<sup>+</sup> T-cells secrete lymphokines which both recruit and activate macrophages which represent the final effector cells of the DTH response (Meltzer&Nacy, 1989). Recently, two distinct types of murine T-helper cells have been identified by analysis of the pattern of lymphokine secretion by cloned T-helper cells. Thus, TH1 clones secrete interleukin 2, interferon gamma and lymphotoxin, whereas TH2 clones secrete interleukin 4 and 5. Although there is no proof that these T-cell subsets exist in vivo, it has been shown that DTH is mediated by TH1 clones (Mossman&Coffman, 1989). Moreover, it has been shown that these clones lack the receptor of and can not respond to IL-1 (Greenbaum et al, 1988). So, this might explain the lack of activity of the IL-1 inhibitor-containing high molecular weight pool in the DTH response.

On the other hand, the mechanism by which the 40k and 30-40k fractions suppress this response is currently

unknown. Antigen-induced lymphocyte proliferation has been suggested to be an in vitro correlate of the DTH response. However, this correlation is incomplete as the frequency of antigen specific T-cells in the lymph nodes of sensitized animals is very low and the observed result is due partly to proliferation of cells other than those which induce the DTH response (Meltzer&Nacy, 1989). In this assay, only high concentrations (1.6 ug per well) of both fractions induced enhancement of cellular proliferation rather than suppression. The same concentrations also enhanced the proliferation of the L.N. Con A-blasts. Although the phenotypic and functional characterization of the proliferating cells in this assay are yet to be determined, it has been reported repeatedly that Con A induces suppressor T-cells (Dutton, 1973, Peavy&Pierce, 1974, Takakuma&Pierce, 1978). Also, suppressor circuits which include at least three distinct T-cell populations have been shown to be involved in the down regulation of the DTH response (Dorf&Benacerraf, 1984). It is tempting to speculate that injection of SMG-derived fractions induces in vivo imbalance in the regulation of DTH, favoring the proliferation of suppressor cells. The fact that injection of these fractions in the effector phase of the DTH response (day

5 and 6 postsensitization, with the latter being the day of antigenic challenge) produced significant suppression would suggest a mechanism involving the terminal effectors of the response either through specific suppressor effectors or through nonspecific effect. The latter may be mediated through the action of corticosteroids. Determination of the serum corticosteroid levels following treatment with SMG-derived immunosuppressive fractions as well as testing for their ability to suppress the DTH response in adrenalectomized mice can help to resolve this issue.

Previous experiments which showed that the SMG extracts, or fractions thereof, were immunosuppressive in in vivo models similar to those used in this study were done using male mouse SMG. The female gland did not appear to have any significant immunosuppressive effects. It should be noted that species differences do exist between the SMG regarding their content of the biologically active factors. Thus, the rat SMG is not sexually dimorphic and contains no detectable NGF (Bothwell et al, 1979) whereas the male mouse SMG is the richest source of this factor (Bradshaw, 1978). So, direct comparison between our results with those of others can not be made at the present stage. This

comparison must await further characterization of the fractions described here as well as using a similar systematic approach to identify similar murine SMG fractions. However, we used the SMG from male rats and it remains to be determined whether or not these active fractions are found in the female's gland.

Our interest was focused primarily on detecting the fractions that affected the responsiveness of the immune system rather than the morphology of the lymphoid organs. However, we noticed a significant increase in splenic weight together with thymic atrophy in mice with suppressed DTH response due to injections of the 30-40 kD fraction compared to PBS injected animals (data not shown). The splenomegaly might result from hemodynamic effects or from cellular proliferation. Determination of the phenotypic and functional characteristics of these cells will enable a better understanding of the mechanism of immunosuppression.

This study describes four steps to purify the active fractions starting with ammonium sulphate precipitation which is followed by gel filtration, hydrophobic interaction, and anion exchange chromatographies. By using a discontinuous gradient in anion exchange chromatography to fractionate directly the crude extract,

we have been able to isolate the 30-40 kD fraction with greater yield than that described here (data not shown).

The relationship between the 40kD and 30-40kD fractions is currently unknown. Based on the similarity of their activity in the described in vivo and in vitro models, it is possible that the 40kD protein is the same in both fractions and it is the one that is responsible for immunosuppression. Further separation of the 30 and 40 kD proteins followed by physicochemical characterization is required to solve this issue. Production of monoclonal antibodies (mAbs) against the single 40kD fraction will help to define the relationship between the two fractions. If the 40kD protein proves to be the same in both fractions, mAbs can be used to affinity purify the protein. MAbs can also be used in immunohistochemical studies to define the distribution of the immunosuppressive fraction with the SMG and study its presence in other organs.

The physiological significance of the presence of immunosuppressive factors in the rat SMG is a matter of speculation at the present stage. From the work done in our laboratory it appears that the rat SMG contains at least two different immunologically related activities. One with in vitro immunosuppressive activity most likely

due to inhibition of IL-1, and another with in vivo immunosuppressive activity due to as yet undefined mechanism. The rodent SMG is a unique organ in the sense that it contains a variety of biologically active factors with apparently no relation to its primary function of saliva secretion. Attempts to understand why this array of factors does exist or to assign an endocrine function for this organ were largely unsuccessful (see review of literature).

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