MANIPULATION OF BODY FAT BY PASSIVE AND ACTIVE IMMUNISATION AGAINST THE ADIPOCYTE

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by

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SUMMARY

The aim of the work described in this thesis was to devise a technique for the reduction of body fat by passive or active immunisation against the adipocyte. Passive immunisation of rats with an antiserum, raised in sheep, against rat adipocyte plasma membranes (A/S 83) caused a 50% reduction in parametrial adipocyte number which persisted, at least until 24 weeks after treatment. This was accompanied by a reduction in parametrial adipocyte size which persisted for 8 weeks but had recovered by 24 weeks, resulting in a 50% reduction in parametrial adipose mass at 24 weeks. Subcutaneous and peri-renal adipose depots were, initially, less affected and had recovered their total mass by 24 weeks after treatment. Differential effects on different adipose depots were found to be due largely to the site of injection of the antiserum. Body composition analysis showed that total body fat was reduced by 30% 8 weeks after treatment and lost fat was replaced by protein and water. Side effects of A/S 83 were limited to a reduction in food intake on the first day of treatment, which gradually recovered to reach normal levels by 4 days and transient proteinurea with fluctuations in body weight about 2 weeks after treatment in some rats. The initial effects of A/S 83 on adipose tissue and food intake were dependent on the presence of circulating complement. A doubling of serum free fatty acids and triglycerides occurred 6-24 h after treatment, then returned to normal levels by 48 h after treatment and was probably evidence of adipocyte destruction. Treatment with A/S 83 did not affect the ability of rats to undergo a normal pregnancy and lactation.

More recent bleeds of the same sheep did not reproduce the in vivo effects of earlier bleeds of A/S 83 and so 2 additional antisera were raised against rat adipocyte plasma membranes. These new antisera, while reproducing the effects of A/S 83 on adipose tissue, had the additional side effects of anaesthetic-like effects immediately after administration and the production of gross liver abnormalities. The presence or absence of side effects could not be correlated with strength of binding to nervous tissue or hepatocyte plasma membranes, as determined by ELISA and Western blotting.

An attempt was made to prepare rat adipocyte specific antigens by affinity purification of anti-(adipocyte plasma membrane) antibodies, adsorption of those antibodies with non-adipose tissues and the use of the adipocyte specific antibodies to affinity purify antigens from solubilized adipocyte plasma membranes. Six polypeptides, with molecular weights of 53-96 kD, were considerably enriched in the adipocyte specific antigen preparation. Antisera raised against the adipocyte specific antigens showed some binding to non-adipose tissues and antisera raised against hepatocyte and erythrocyte plasma membranes showed some binding to adipocyte specific antigens, which cast doubt on the true adipocyte specificity of some of the components of the adipocyte specific antigen preparation. However, the anti-(adipocyte specific antigen) antiserum showed considerably more adipocyte specificity than antisera raised against whole adipocyte plasma membranes, had fat-reducing properties in vivo and did not cause liver abnormalities. Adsorption of anti-(adipocyte plasma membrane) antisera with liver homogenate provided an antiserum that retained its effects on adipose tissue but no longer caused liver

abnormalities. It is, therefore, likely that adipocyte specific antisera can have fat-reducing properties, but both the anti-(adipocyte specific antigen) antiserum and the adsorbed antiserum reproduced the anaesthetic-like effects of anti-(adipocyte plasma membrane) antisera and caused a reduction in food intake on the first day of treatment. Antisera raised against hepatocyte and erythrocyte plasma membranes had no effects on adipose tissue.

Attempts were made to reproduce the effects of passive immunisation by means of active immunisation against the adipocyte, as this might prove a more practical approach for the treatment of large species. Rats were immunised with rat adipocyte plasma membranes or adipocyte specific antigens conjugated to BSA, or with BSA alone in complete Freunds adjuvant. Despite the absence of a convincing demonstration of circulating anti-(adipocyte plasma membrane) antibodies, adipocyte plasma membrane- and adipocyte specific antigen-treated rats showed a 40-50% reduction in adipocyte numbers and a compensatory increase in adipocyte size. In only 1 out of 3 groups of rats did active immunisation induce a reduction in adipose mass and, in this group, body weight was also reduced.

Antisera raised against rat, sheep, pig and chicken adipocyte plasma membranes showed some binding to adipocyte plasma membranes of other species but the binding was less than 5% of that to homologous adipocyte plasma membranes. Sheep and chicken adipocyte specific antigens were prepared, in addition to rat adipocyte specific antigens and the 3 preparations included some polypeptides of similar electrophoretic mobilities. Western blotting of anti-(adipocyte plasma membrane) antisera against adipocyte specific antigens of the 3 species showed some cross-reactivity, particularly between proteins

of molecular weights of 72 and 96 kD, but the binding was always weaker than that to the homologous protein of the same molecular weight, suggesting that the adipocyte specific antigen preparations of the different species did not contain antigenically identical proteins.

Active immunisation of lambs with sheep adipocyte plasma membranes, conjugated to rabbit serum albumin, in Fruends adjuvant did not result in any reduction in body weight or adiposity, compared with rabbit serum albumin-immunised controls. In a second experiment, lambs were immunised, initially, with sheep adipocyte plasma membranes, followed by sheep adipocyte specific antigens, conjugated to rabbit serum albumin and were compared with untreated controls. Despite the absence of a convincing demonstraton of circulating anti-(adipocyte plasma membrane) antibodies, immunised lambs showed significantly reduced body weights and fat depot weights, compared with untreated controls. Although fat pad weight was still reduced in the treated group when expressed as a percentage of body weight, this difference was not significant.

ABBREVIATIONS

Ach-R	–	Acetylcholine receptor
ADCC	–	Antibody dependent cell-mediated cytotoxicity
APM	–	Adipocyte plasma membrane
A/S	–	Antiserum
ASA	–	Adipocyte specific antigens
BAT	–	Brown adipose tissue
BM	–	Brain membranes
BSA	–	Bovine serum albumin
CAM	–	Chronic autoimmune hepatitis
CFA	–	Complete Freunds adjuvant
EAE	–	Experimental autoimmune encephalomyelitis
ELISA	–	Enzyme-linked immunoassay
EPM	–	Erythrocyte plasma membrane
FCE	–	Food conversion efficiency
HPM	–	Hepatocyte plasma membrane
KPM	–	Kidney plasma membrane
LDH	–	Lactate dehydrogenase
MBP	–	Myelin basic protein
MG	–	Myasthenia gravis
MS	–	Multiple sclerosis
RSA	–	Rabbit serum albumin
SDS PAGE	–	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLE	–	Systemic lupus erythematosus

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CHAPTER 1

I N T R O D U C T I O N

INTRODUCTION

Antisera raised against adipocyte plasma membranes have been shown to be cytotoxic to isolated adipocytes in the presence of complement (Pillion and Czech, 1978a; Flint, Coggrave, Futter, Gardner and Clarke, 1986). This suggests that such an approach might prove useful in the reduction of adiposity in vivo.

The reduction of fat deposition is of considerable commercial interest. Coronary heart disease is the major cause of death in adult men in developed countries, where dietary fat, most of which is of animal origin, constitutes 40% of total caloric intake. The possible associations between dietary fat intake, serum cholesterol, artherosclerosis and coronary heart disease have led to a demand for the reduction of the fat content of animal products (Brisson, 1986).

Vast amounts of fat are deposited by all commercially important meat-producing animals. There is, therefore, considerable interest in techniques that increase the efficiency of meat production by repartitioning energy from fat into protein deposition.

A technique for the reduction of fat deposition would also be of interest to the dairy industry. The nutrient requirements for milk production are met, in some species, by both increased food consumption and lipid mobilisation (Vernon and Flint, 1984). During pregnancy and lactation co-ordinated changes occur in the numbers of insulin receptors in adipose tissue and the mammary gland. These changes favour lipid deposition in adipose tissue during pregnancy, while restricting lipogenesis in adipose tissue and favouring lipogenesis in the mammary gland during lactation (Flint, Sinnet-Smith, Clegg and Vernon, 1979). In late lactation, as milk

production declines, lipid deposition in adipose tissue occurs. In rats the decline in milk production is also accompanied by an increase in the number of insulin receptors per adipocyte but no change in the number of insulin receptors of the mammary gland. The decline in milk production can be delayed by the substitution of younger offspring for the original litter and fat deposition in adipose tissue is prevented. This raises the question of whether competition between adipose and mammary tissue for common precursors contributes to the decline in milk yield in late lactation (Flint, 1986). A reduction in fat deposition in dairy cows might, therefore, lead to increases in milk yield during the later stages of lactation.

In addition to commercial applications, an immunological means of reducing body fat could be used as a research tool for the investigation of the control of adipose tissue development. The health problems associated with obesity have led to a growing interest in factors that control adipocyte hypertrophy and hyperplasia. Recent work has suggested that adipocyte number is not 'fixed' at an early age but that, given appropriate stimuli, adipose tissue of adult animals may proliferate (Faust and Miller, 1981). A means whereby adipocyte number or size could be reduced at different stages of maturity might yield valuable information about the role cellularity plays in the control of adipose tissue development and obesity. An important development in this field was the recognition of adipocyte precursors (Van and Roncari, 1977). These cells, isolated from adult rats, can develop in vitro and in vivo into 'mature adipocytes' (Roncari and Van, 1978). Antisera raised against adipocyte plasma membranes have been used to monitor precursor cell differentiation in vitro (Cryer, 1985a).

An immunological technique for the reduction of body fat would, therefore, have many practical applications but the immune response must first be shown to cause long-term reduction of adiposity, to be devoid of adverse side effects and to be tissue specific.

1.1 ADIPOSE TISSUE DEVELOPMENT

1.1.1 A comparison with non-adipose tissues

At birth rats and pigs contain about 1% fat while ruminants are born with 2-4% fat and humans with 16% fat (Leat and Cox, 1984). Poultry hatch with very little adipose tissue as the embryo develops as an isolated system with no constant supply of maternal glucose and hence rates of lipogenesis are very low (Hood, 1984).

Adipose tissue is a 'late-developing' tissue, its growth being exceeded by that of bone and muscle in early life. During later stages of growth the rate of protein accretion declines and the deposition of fat exceeds that of lean (Bergen, 1974). Beyond a certain body weight, fat gain becomes a large and constant fraction of body-weight gain. In sheep, pigs and cattle fat deposition exceeds that of muscle beyond body weights of 30, 50 and 350 kg respectively (Searle, Graham and O'Callaghan, 1972). In poultry rates of fat deposition increase rapidly on hatching but decline after 2-3 weeks. In the female fat synthesis increases at sexual maturity, in readiness for egg production. In laying hens both protein and fat deposition increase up to 32 weeks, thereafter fat deposition increases but protein deposition declines (Vogt and Harnisch, 1983). Rapid accumulation of fat normally occurs, in human development, during 2 periods, perinatally, during the last part of pregnancy and

the first few months of infancy, and during the adolescent period up to adulthood (Hirsch and Batchelor, 1976, Scott, 1981).

1.1.2 The cellular characteristics of adipose tissue development

The assessment of the relative contributions of increases in cell size and number to increases in mass of adipose tissue has been hindered by difficulties in measuring the size and number of adipocytes (Hirsch and Gallian, 1968; Sjöström, Björntorp and Varana, 1971). Microscopic measurements of histological sections may lead to underestimation of cell size due to shrinkage of the tissue. Microscopic and automatic measurements on cells liberated by collagenase digestion suffer from the problems of breakage of large cells and loss of small cells (De Martinis, 1985). Determination of DNA content of the intact tissue is an inaccurate estimation of adipose cellularity since supporting cells may contribute up to 50% of the total amount of DNA, even in mature tissue. Based on the use of these techniques, the accepted concepts were that the adult adipocyte number was 'fixed' very early in life and the full complement of mature adipocytes was reached by sexual maturity. Subsequent increases in adipose mass could only occur by increases in adipocyte size.

However, the techniques used were unable to distinguish between the formation of new cells, lipid-filling of preformed cells and dedifferentiation of mature adipocytes. To overcome this problem Greenwood and Hirsch (1974) measured the synthesis of new adipose cells by *in vivo* tritiated thymidine incorporation and proposed that the development of adipose tissue in the rat was divided into 3 periods. The first was a highly proliferative period to 4 weeks

after birth, followed by a predominantly lipid-filling period with some proliferation until puberty (60-80 days) and a period exclusively of lipid-filling during maturity. Roncari and Van (1978) isolated adipocyte precursors from adult rats that replicated and differentiated completely into adipocytes, both in culture and after re-implantation into rats. These authors proposed that, while the majority of proliferation occurs early in life, a slow continuous rate of adipocyte precursor replication and maturation is balanced by dedifferentiation of mature adipocytes in the adult.

Pre-adipocytes have been isolated from adult bovine, ovine, avian and human adipose tissue and have been shown to be capable of replicating and differentiating into mature adipocytes in culture, indicating that, like the rat, there is capacity for the production of new adipocytes in the adult in these species (Van, Bayliss and Roncari, 1976; Plaas and Cryer, 1980; Broad and Ham, 1983; Hood, 1986). Growth of extramuscular adipose tissue depots occurs through both hypertrophy and hyperplasia in poultry, sheep, pigs and cattle up to 14 weeks, 12 months, 5-6 months and 14 months respectively (Hood, 1977; Hood, 1982). The cellular development of adipose tissue in man is not fully understood. The major period of proliferation may range from the thirtieth week of gestation to 9-12 months of age, although adipocyte numbers continue to increase after this age, to reach adult values at about 15 years (Hirsch and Batchelor, 1976). Kirtland and Gurr (1979), however, suggested that the major period of adipocyte synthesis is completed by birth.

The times, quoted above, at which hyperplasia is diminished are approximate. The precise characteristics of the development of adipose tissue depend upon the breed or strain, the sex, the

nutritional status and the adipose depot of the animal. Apparent cell number increases in the rat cease earlier in the epididymal than the peri-renal and subcutaneous depots (Bertrand and Masoro, 1978). Peri-renal adipose tissue is also an early developing tissue in meat species, followed by intermuscular depots, and the subcutaneous depot (Leat and Cox, 1984). In the later stages of fattening fat is deposited intramuscularly, giving meat a marbled appearance. The distribution of fat also varies between species. The ratio of subcutaneous to intermuscular fat varies from 0:6 for cattle to 1:2 in sheep and 2:4 in pigs.

It would appear, therefore, that while the majority of proliferation in adipose tissue occurs early in life, there is capacity for the production of new adipocytes in the adult. Hyperplastic obesity can be of adult onset and is not easily treated by dieting, which leads to an abnormally low fat cell size and no reduction in adipocyte number (Miller, Faust, Goldberger and Hirsch, 1983). A very low adipocyte size is frequently associated with a reduced metabolic rate, a reduced level of thyroid hormone production and mental problems. Therefore, a method of reducing adipocyte numbers rather than adipocyte size by an immunological approach may be of interest in the treatment of certain forms of human obesity.

1.2 THE CONTROL AND MANIPULATION OF ADIPOSE TISSUE DEVELOPMENT

In the preceding section 'normal' adipose tissue development has been described, in terms of the contributions made by increases in adipocyte number and size. Within a species and within individuals there is an enormous capacity for variation in total adipose mass,

whether due to hypertrophy or hyperplasia. Studies of naturally-occurring and experimentally-induced variation have indicated that the body composition of an animal is a result of a combination of genetic, physiological and nutritional factors. The manipulation of these factors has yielded information about the control of adipose tissue development and possible means of regulating the body composition of commercial species.

Obese strains of laboratory animals have been developed and different breeds of birds, pigs, cattle and sheep have widely differing propensities to fatten. This indicates the importance of a genetic component in the control of adipose tissue development and has led to attempts to select for reduced fat deposition.

Physiological factors affecting degree of adiposity include sex, external environment and maturity (Moran, 1986; Webster, 1986). Intact males tend to be leaner than castrate males or females. Low temperatures tend to reduce growth rate and increase fat deposition. The manipulation of photoperiod can influence body composition. Extending the day length tends to lead to an increased ratio of protein to fat deposition. The fall in day length at the beginning of autumn tends to lead to a shift towards fat deposition. Maturity is the major non-nutritive determinant of body composition, as described in the previous section. The effects of physiological factors on adiposity are primarily mediated by hormones and so hormone treatment has been used in meat species in an attempt to repartition energy from protein into fat deposition.

The effects of nutritional manipulations on fat deposition depend, in part, on the age, strain, species and sex of the animal

(Combs, 1976). Manipulation of total energy intake and diet composition of laboratory animals and meat species have resulted in alterations in growth rate and body composition. These factors are discussed in greater detail below.

1.2.1 Genetic selection for reduced fat deposition

Fat deposition is likely to be subject to little natural selective pressure because, when a new ecological opportunity is exploited, population numbers rapidly reach an equilibrium with the amount of food necessary for maintenance, growth and reproduction only. Therefore, fat deposition is likely to be heritable. In contrast, fat secretion in milk for the nurture of the young is likely to have been subject to natural selection pressure resulting in the arrival at a stable equilibrium of fat content that would best support the growth and survival of the offspring. Fat secretion, therefore, is likely to have very low heritability (Willham, 1976). Domestication has resulted in food consumption exceeding the requirements for maintenance, growth and reproduction, resulting in the ability to deposit more fat to be expressed.

Vast differences exist between breeds in the rate at which they mature and fatten. Within breeds estimations of the heritability of fat deposition have been made by comparing relatives and by measuring the response to selection for increased or decreased fat deposition. Such studies suggest that fat deposition has an average heritability of about 50% (Willham, 1976). Selection for reduced fat deposition in pigs, for instance, resulted in a 25% reduction in backfat from 1926-1956 (Croft, 1958). Selection for either milk or meat production in cattle has led to differences in fat deposition within

the species. Dairy cattle breeds tend to have more abdominal fat as it has a greater blood supply which can be mobilized more rapidly to meet the demands of lactation, while beef breeds have more subcutaneous fat (Webster, 1986).

Genetic selection has several disadvantages as a method for reducing fat deposition in animals. The effect that can be achieved is limited to the available genetic variation and is a long-term method that is not able to adapt to rapid changes in requirements. Carcass composition must either be measured on the live animal or siblings must be used, reducing the selective pressure. In addition, undesirable traits have been produced by selection for reduced fat deposition, such as Porcine Stress Syndrome and double-muscling in cattle (Willham, 1976).

1.2.2 Repartitioning agents

Growth hormone, while stimulating growth in young animals, is lipolytic. The daily secretion of growth hormone is positively correlated with lean tissue growth and negatively correlated with carcass fat in cattle (Bauman, Eisemann and Currie, 1982). Similar trends have been found in lambs and pigs. The administration of growth hormone to pigs, sheep and cattle has been shown to increase weight gain, food conversion efficiency and protein deposition and decrease fat deposition (Hart and Johnson, 1986). Recombinant bovine growth hormone has similar effects (Muir, Wien, Duquette, Rickes and Cordes, 1983). Such studies have shown considerable variation depending on species, breed and mode of administration. The administration of androgens or oestrogens to cattle can increase growth rate, food conversion efficiency and decrease fat deposition

(Roche and Quirke, 1986). Female cattle and bulls respond best to androgens and oestrogens respectively. Similar responses have been obtained with sheep but not pigs.

There has been growing interest in recent years in pharmacological repartitioning agents, such as clenbuterol and cimeratol. These β-agonists have been demonstrated to increase lean tissue accretion and limit fat deposition in rats, broilers, cattle, sheep and pigs (Dalrymple, Baker, and Rickes, 1984; Hanrahan, Quirke, Bomann and Roche, 1986). Their precise mode of action is unknown but they bind specifically to β-adrenergic receptors on the cell membrane, effecting their responses through the stimulation of cAMP production within the cell. β-agonists inhibit protein degradation in muscle, while stimulating lipolysis in adipose tissue and β oxidation and possibly protein synthesis in muscle (Dalrymple et al., 1984).

The approaches for the improvement of animal performance, described above, suffer from several serious limitations, the most important of which is the current EEC ban on the use of steroid growth promoters in animal production. Hormones and β-agonists are also expensive and frequent administrations are necessary to achieve the desired effects on growth promotion and carcass composition. These limitations, together with the possibility of adverse effects on animal health and behaviour, have led to the search for alternative strategies to improve animal performance. Several immunological approaches have yielded promising results, including active immunisation against somatostatin, which inhibits growth hormone secretion, resulting in increased growth rates and food conversion efficiencies in lambs (Spencer, Garssen and Bergström,

1983). Anti-idiotypic antibodies, raised against growth hormone antibodies, have been shown to resemble growth hormone, both structurally and functionally (Morrison, 1986). The use of anti-idiotypic antibodies as hormone images may prove useful for a number of hormones having growth-promoting effects (Flint, 1987). Passive or active immunisation against the adipocyte would share the advantages of these methods of giving the option of treatment or no treatment, depending on market trends, and of not leaving harmful residues in the edible tissues. In addition, this approach may not require continuous or frequent administration of antiserum or immunogen and, unlike the methods described above, might reduce the number of adipocytes, rather than alter metabolism.

1.2.3 Nutritional manipulations

The effects of nutritonal manipulations on adipose tissue development partly depend on the age of the animal. Maternal undernutrition of rats and man can cause a permanent increase in adiposity of the offspring (Ravelli, Stein and Sussex, 1976; Jones and Friedman, 1982). Food restriction of swine in early pregnancy resulted in a reduction in adiposity and total body weight (Pond, Mersmann and Jong-Iseng, 1985). Food restriction during the final part of pregnancy may cause a permanent reduction in body weight and adipocyte number, possibly because proliferation of foetal adipose tissue has begun (Winnick, 1981).

In the rat underfeeding pre-weaning causes a permanent reduction in adipocyte number while underfeedng post-weaning causes a reduction in cell size only (Knittle and Hirsch, 1968; Stern and Greenwood, 1974). Even severe long-term food deprivation in the

adult does not cause loss of adipocytes (Miller, Faust, Goldberger and Hirsch, 1983). Reduced adipocyte size, produced as a result of underfeeding, is recovered, in the adult, upon refeeding. Overfeeding pre-weaning may cause a permanent increase in cell number (Knittle and Hirsch, 1968). In the adult rat the feeding of a high fat diet induces hyperphagia and leads, initially, to an increase in adipocyte size. The reaching of a 'critical' cell size appears to trigger an increase in adipocyte number (Peckham, Entenman and Carrol, 1962; Klyde and Hirsch, 1979; Faust and Miller, 1981). Upon returning to a normal diet, adipocyte size returns to a normal level but adipocyte number remains elevated, resulting in a permanently elevated fat mass.

Evidence for a relationship between adipocyte size and food intake has come from studies on the obese Zucker rat, which is hyperphagic until 20 weeks of age. This has been correlated with an increase in adipocyte size which ceases at 20 weeks and is followed by cycles of cellular proliferation followed by increases in adipocyte size, coupled with cycles of hyperphagia (Vasselli, 1985). A mechanism was proposed whereby the reaching of a critical cell size has a depressive effect on food intake. The nature of the signal linking adipocyte size with food intake is unknown. Parabiosis experiments between obese and normal rats have suggested that blood-borne factors are involved with the regulation of food intake (Harris and Martin, 1984). Liebel (1977) proposed a biological radar system whereby the intensity of a humoral signal, such as insulin or the ratio of insulin and glucose, to the CNS varied according to the adipose cell surface area. Adipsin is a recently discovered candidate for an adipocyte-derived signal that might exert effects on

food intake (Flier, Cook, Usher and Spiegelman, 1987). Adipsin is a serine protease homologue that is synthesized and secreted by adipocytes and is found in the circulation. Adipsin secretion is decreased during continuous infusion of glucose, which results in an increased adipose mass, and is also decreased in genetically obese rodents. However, little change in adipsin secretion is found in rats that become obese as a result of high-fat feeding.

A number of studies have attempted to increase the growth rates of meat-producing animals by increasing dietary energy intake. However, an over-consumption of dietary energy tends to lead to increased fat deposition (Combs, 1976). The feeding of diets with a high protein to energy ratio tends to minimise carcass fat. Unless the breed, cross or strain has the genetic potential for a high lean to fat ratio, most dietary manipulations have little effect on carcass fat.

1.2.4 Lipectomy

Clearly, surgical removal of adipose tissue is not a technique to be used in meat species but studies in rats have yielded information on the regulation of adipose tissue development. In general, lipectomy of internal fat depots in the adult rat is not followed by regeneration of lost tissue or compensatory hypertrophy of other depots unless the animal is genetically predisposed towards obesity or is fed a high fat diet (Faust, Johnson and Hirsch, 1976). Lipectomy of internal depots early in life can result in some regeneration, (Faust, Johnson and Hirsch, 1977) and subcutaneous adipose tissue shows some regenerative capacity (Bailey and Anderson, 1980). Faust et al. (1977) found that lipectomised animals fed a

high fat diet had a lower food intake than sham-operated controls, providing further evidence that body fat stability in the adult is achieved by the regulation of adipocyte size and that such regulation may operate by influencing food intake.

Surgical removal of adipose tissue in humans has proved successful only for removing small amounts of fat in areas refractory to dieting.

1.3 CHARACTERISATION OF THE ADIPOCYTE PLASMA MEMBRANE AND ANTI-(ADIPOCYTE PLASMA MEMBRANE) ANTIBODIES

A number of groups have attempted to identify and characterise the components of the adipocyte plasma membrane by gel electrophoresis with the ultimate aim of studying hormone-plasma membrane interactions. Lee, Tume, Cryer and Cryer (1986) have attempted to characterise the adipocyte plasma membrane using adipocyte specific antisera, in order to identify 'differentiation' antigens that can be used to identify differentiation of adipocyte precursors. Pillion, Grantham and Czech (1979) and Flint et al. (1986) have raised antisera to adipocyte membranes and studied their effects on isolated adipocyte metabolism.

1.3.1. Characterisation of the adipocyte plasma membrane by gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of rat adipocyte plasma membranes has identified between 13 and 20 major peptide components with molecular weights ranging from 15 kD to 178 kD (Czech and Lynn, 1973 a & b; Trosper and Levy, 1974; Avruch, Leone and

Martin, 1976; Kawai and Spiro, 1980 and Tume, Lee and Cryer, 1985). These groups have also identified 2 prominent glycopeptides with molecular weights of 88-108 kD and 77-81 kD. Czech and Lynn (1973a) found that the 2 glycoproteins represented about 50% of the total membrane protein and constituted almost all of the protein on the exterior of the adipocyte surface, as determined by iodination of intact cells.

Two reports comparing the polypeptide composition of adipocyte plasma membranes of rats with those of other species, have yielded conflicting results. Both groups found differences in polypeptide and glycoprotein composition between plasma membranes of different species. However, while Kawai and Spiro (1980) concluded that rabbit, rat and bovine plasma membranes were alike in containing a major glycoprotein component with a molecular weight of 74-79 kD, Tume et al. (1985) reported that mouse, ox, chick, rabbit and rat adipocyte plasma membranes differed in the number and electrophoretic mobility of all major glycopeptides. These authors also found differences in the polypeptide composition of adipocyte plasma membranes from rats of different strains.

1.3.2 Characterisation of the adipocyte plasma membrane using anti-(adipocyte plasma membrane) antisera

Thompson and Abraham (1979) raised antisera to mouse mammary adipose cells that could be used to distinguish mammary adipose cells from mammary epithelial cells and fibroblasts. Lee et al. (1986) raised antisera to mouse, rat, bovine and chicken adipocyte plasma membrane antigens. Each antiserum showed some reactivity towards erythrocyte, liver and heart membranes but this could be removed by

absorption without affecting reactivity against adipocytes (Cryer, 1985; Plaas, Woodhead and Cryer, 1981). The antisera showed low levels of reactivity against adipocytes from different species with the exception of anti-mouse and anti-(rat adipocyte plasma membrane) antisera, which showed 30% cross-reactivity with rat and mouse adipocyte membranes respectively.

Lee et al. (1986) have attempted to identify adipocyte differentiation-related antigens by reacting solubilized plasma membranes with adipocyte specific antisera and analysing the resulting immunoprecipitates by gel electrophoresis. The components immunoprecipitated were distinguishable when antisera and plasma membranes from different species were used. Three components were isolated from rat adipocyte plasma membranes with molecular weights of 124, 90 and 60 kD, one component was obtained from bovine adipocyte plasma membranes with a molecular weight of 87.5 kD and 3 components were prepared from chicken adipocyte plasma membranes, with molecular weights of 37, 47 and 57 kD. These antigens were not identified on freshly confluent adipocyte precursors and their appearance could be used as a marker of adipocyte differentiation before the development of enzymic markers of differentiation.

1.3.3 In vitro effects of anti-(adipocyte plasma membrane) antisera on isolated adipocytes

Pillion and Czech (1978b) raised antisera, in rabbits, to rat adipocyte intrinsic plasma membrane proteins, prepared by the treatment of plasma membranes with dimethylmaleic anhydride. This fraction consisted of 2 major glycoproteins of molecular weights of 94 kD and 78 kD and has been shown to exhibit D-glucose transport

activity when incorporated into liposomes (Shanahan and Czech, 1977). The anti-(adipocyte plasma membrane) antiserum caused cytolysis of isolated adipocytes. This effect could be abolished by heat inactivation of the antiserum to destroy complement. The heat-inactivated antiserum stimulated glucose uptake and carbon dioxide production from glucose by isolated adipocytes, comparable to effects seen with maximal doses of insulin, while catecholamine stimulated lipolysis was reduced (Pillion and Czech, 1978a). Similar effects have been produced by antibodies raised against rat insulin receptors and by human autoantibodies to the insulin receptor isolated from the sera of patients suffering from insulin-resistant diabetes (Jacobs, Chang and Cuatrecasas, 1978; Kahn, Baird, Flier and Jarrett, 1977).

Insulin binding to the adipocyte plasma membrane was not affected by the anti-(adipocyte plasma membrane) antiserum of Pillion and Czech (1978a) which led to the suggestion that the antiserum reacted directly with the hexose transport system. Studies with fab fragments of the anti-(membrane) antibodies showed that multivalency was required for activity (Pillion, Grantham and Czech, 1979). The authors proposed a mechanism of action for the antibodies involving aggregation of membrane components, similar to the model proposed for the mechanism of action of anti-(insulin receptor) antibodies (Kahn, Kasuga, King and Grunfeld, 1982). Immunofluorescence studies with anti-adipocyte plasma membrane) antibodies showed capping on the cell surface (Pillion and Campbell, 1984). There was no correlation between the extent of capping and antibody concentration or between capping and glucose oxidation, but there was a clear relationship between glucose oxidation and antibody concentration. Adipocytes

fixed in paraformaldehyde before exposure to anti-(plasma membrane) antibodies showed a similar number of caps to unfixed cells. These studies suggest that adipocytes have areas on their cell surfaces where antigenic components are clustered into groups. Anti-(membrane) antibodies do not appear to mimic insulin action in isolated adipocytes by causing aggregation of antigenic sites on the cell surface.

The work of Pillion and Czech has been supported by that of Flint et al. (1986), where an antiserum was raised against whole rat adipocyte plasma membranes in sheep. The antiserum was shown to have insulin-like effects on adipocytes at low concentrations, stimulating glucose transport and glucose incorporation into lipid, while, at higher concentrations, an inhibitory effect on glucose incorporation was observed. This effect was found to be due to complement-mediated cytotoxicity since, under such conditions, large amounts of the intracellular enzyme, lactate dehydrogenase, were released and the effect could be abolished by heat-inactivation of the antiserum. This antiserum appeared to interact directly with the glucose transport system by a mechanism not involving the insulin receptor since antibodies were able to stimulate glucose transport in cells from which the insulin receptors had been removed by trypsinisation and the antiserum was unable to immunoprecipitate solubilized insulin receptors.

The adipocyte plasma membrane, therefore, has a number of antigenic components some of which are tissue and species specific. The fact that antisera raised against adipocyte plasma membranes are cytotoxic to isolated adipocytes in vitro suggests that such antisera might have fat-reducing properties in vivo.

1.4 MECHANISMS OF IMMUNE TISSUE DAMAGE

The immune system serves as a defence against viral, bacterial, fungal and parasitic infections. Under normal circumstances an individual does not mount a destructive immune response against its own tissues but virally-infected or tumour cells can elicit a cytotoxic immune response against host cells. Both passive and active immunisation have been used to augment the effective immune response against infectious agents and tumour cells. Passive immunisation involves the administration of preformed antibody in the hope that it will activate the recipient's antibody-dependent immune effector mechanisms. By active immunisation antigen-specific antibodies and/or antigen specific T cell-mediated immune responses to the injected antigen may be induced. The cytotoxic mechanisms used by an organism in defence against infection or in response to immunisation vary according to the nature of the infection or immunogen, and several factors are usually involved.

1.4.1 Complement-mediated cytolysis

Complement consists of a complex series of proteins the primary functions of which are chemotaxis and activation of polymorphonuclear leucocytes, and macrophages, cytolysis of target cells and opsonisation. Complement may be activated via the classical pathway, in which case the primary stimulus is binding of antibody to antigen, or via the alternative pathway, which occurs independently of antibody and is often initiated by carbohydrate components of microbial cell walls.

1.4.1.1 Complement activation

The biochemistry of complement has been reviewed by Porter and Reid (1978). The sequential activation of the complement cascade by the classical pathway is shown in Fig. 1.1. Binding of certain subclasses of IgG and IgM to the C1q component of the C1 complement protein complex activates C1r and C1s in turn. C1s cleaves C4 into a small peptide C4a and a larger peptide C4b. C1s is highly proteolytic for C2 when complexed to C4b. A small C2a fragment is lost and the larger C2b fragment joins with C4b to form the C3 convertase $\overline{C4bC2b}$. The C3 convertase activates C3 by splitting off the anaphylatoxin C3a, revealing a nascent thio-ester-reactive binding site on the larger fragment C3b. C3b cleaves C5 into C5b and the anaphylatoxin C5a. C5b, when fixed to biological membranes, is followed by the sequential addition of C6, C7, C8 and C9 to form the membrane attack complex.

The chemistry of the alternative pathway of complement has been reviewed by Müller-Eberhard and Schreiber (1980). The C3 convertase of the alternative pathway is formed by an association between C3b and factor B (Fig. 1.2). C3b is thought to be continuously formed in small amounts. Factor H competes with factor B for binding of C3b and which is preferably bound depends on the nature of the substrate to which C3b is attached. Certain polysaccharides on microbial cell walls favour the uptake of factor B onto C3b. $\overline{C3bB}$ is susceptible to enzymatic cleavage by factor D. The small Ba fragment is lost and $\overline{C3bBb}$ forms the C3 convertase, thus forming a positive feedback loop. $\overline{C3bBb}$ has a half life of approximately 5 minutes unless it is bound to properdin, forming $\overline{C3bBbP}$. This complex has a half life of 30 minutes. When factor H is bound to C3b, factor I cleaves C3b into C3c and C3d which are inactive.

Fig. 1.1. Complement activation via the classical pathway

The binding of immune complexes to C1q causes the activation of C1r and C1s in turn. C1s cleaves C4 into C4a and C4b. When C2 is bound to C4b, C1s cleaves C2 into C2a and C2b. C2b joins with C4b to form the C3 convertase $\overline{C4bC2b}$. The C3 convertase cleaves C3 into the anaphylatoxin, C3a and C3b. C3b cleaves C5 into C5b and the anaphylatoxin, C5a. C5b, when fixed to biological membranes, is followed by the sequential addition of C6, C7, C8 and C9 to form the membrane attack complex.

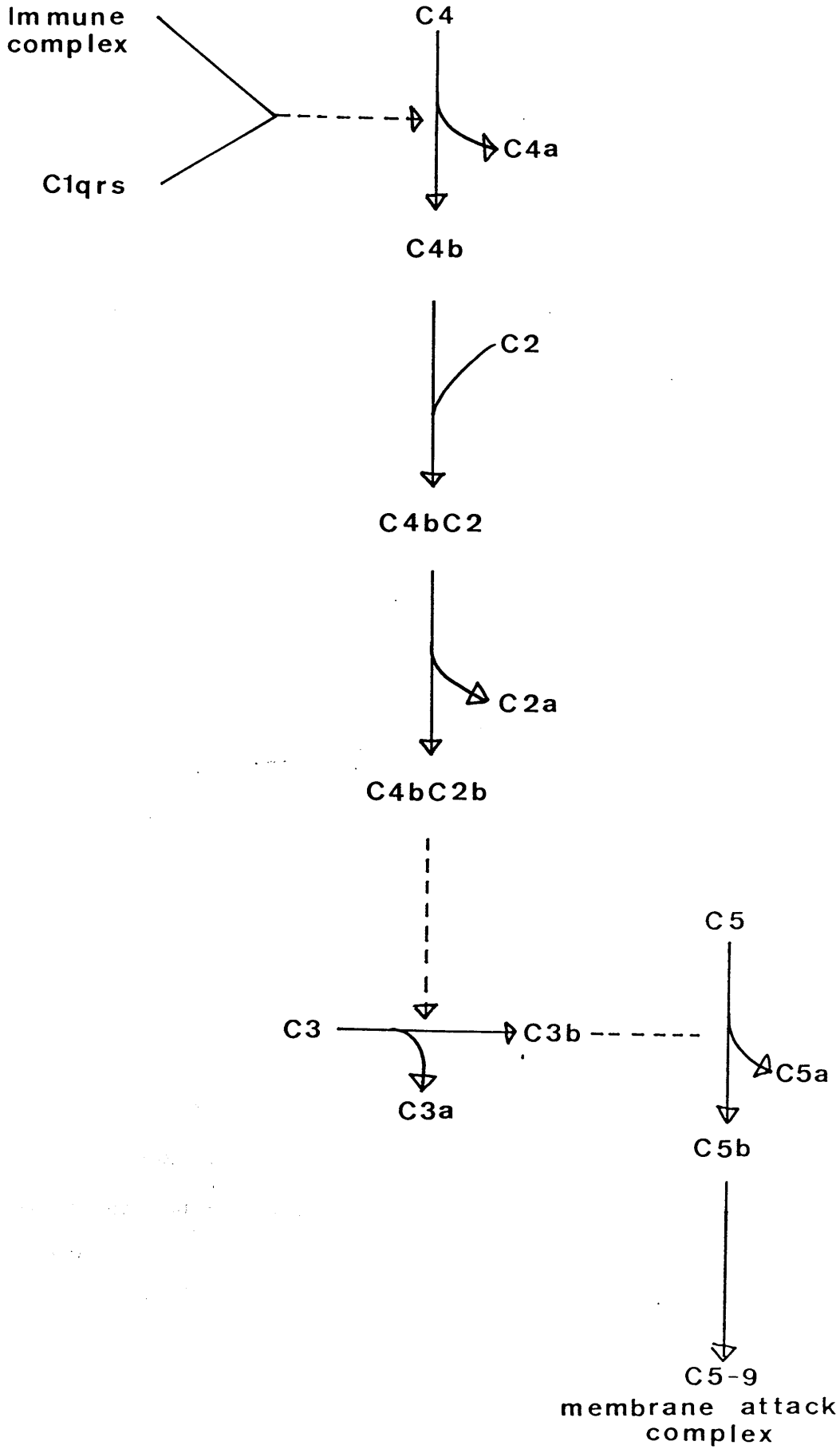
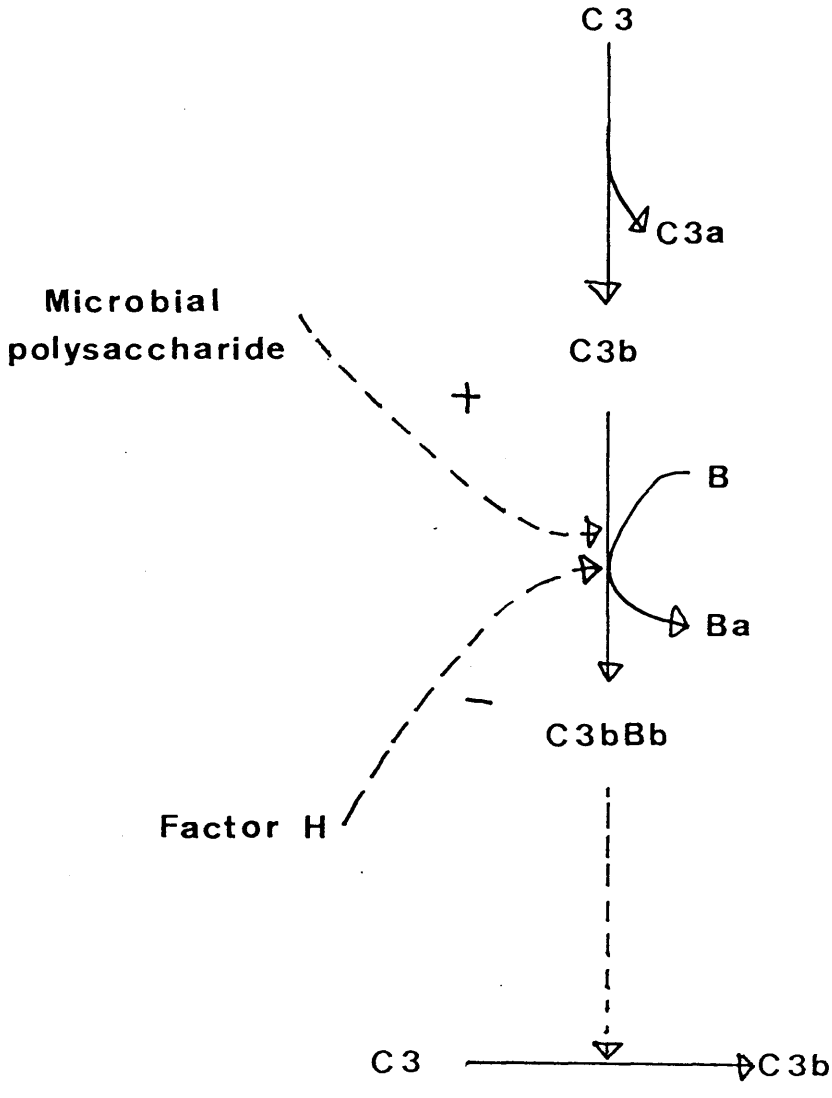

Immune complex
C1qrs
C4
C4a
C4b
C2
C4bC2
C2a
C4bC2b
C3
C3b
C3a
C5
C5a
C5b
C5-9
membrane attack complex

Fig. 1.2 Complement activation via the alternative pathway



The C3 convertase is formed by an association between C3b and factor B. Factor H competes with factor B for binding of C3b but certain polysaccharides on microbial cell walls favour the uptake of factor B onto C3B.

Animals can be depleted of complement by activation of the alternative pathway with cobra venom factor (Cochrane, Müller-Eberhard and Aikin, 1970). A complex is formed between cobra C3b and host Bb which is resistant to factors H and I and has a half life of 37 hours. This results in massive conversion of C3 to C3b, discharging the feedback loop to exhaustion.

1.4.1.2 Cytolysis

Much of the available information on complement-induced lysis comes from studies on the destruction of erythrocytes, although lysis of nucleated cells appears to proceed similarly (Humphrey and Dourmashkin, 1969). Ultrastructural studies have suggested that C9 mediates the fusion of two C5-9 complexes to form a tubule traversing the membrane, which is highly amphiphilic, 1700 kD molecular weight, 15 nm long and 10 nm in diameter (Podach, Essex, Biesecker and Müller-Eberhard, 1980). Such a complex would allow the free exchange of electrolytes and water across the plasma membrane. The net influx of sodium ions and water would lead to cytolysis.

Different classes and subclasses of antibody vary in their ability to activate complement. For the lysis of sheep red blood cells by anti-Forssman antibody, the relative effectiveness of IgM:IgG molecules has been calculated as 800:1 (Humphrey and Dourmashkin, 1969).

One of the commonest examples of complement-mediated cytolysis is the transfusion reaction caused by the transfusion of blood into a recipient who has antibodies to the transferred cells. Haemolytic disease can also occur in newborn infants where the mother has circulating antibodies to blood group antigens on the infant's red

blood cells, the most commonly involved antigen being the Rhesus D antigen (Wintrobe, 1956).

In vitro studies have demonstrated a relative inefficiency of complement to lyse cells from homologous species (Shin, Hausch, Hu and Nicholson-Weller, 1986). A 'decay-accelerating factor' (DAF) has been isolated from erythrocyte membranes which is believed to be involved in the regulation of C3/C5 deposition on the surface of homologous erythrocytes (Nicholson-Weller, Burge and Austen, 1981). Patients with Paroxysmal Nocturnal Haemoglobinuria produce red blood cells that are deficient in DAF and are abnormally sensitive to complement-mediated lysis (Nicholson-Weller, March, Rosenfield and Austen, 1983). The existence of an additional homologous restriction factor that regulates C9 insertion has also been demonstrated (Shin et al., 1986).

Antibody has also been shown to cause complement-mediated cytolysis of virally infected human cell lines. Here the alternative pathway is required to amplify the triggering of the lytic sequence by the classical pathway, which is not, by itself, sufficient to cause damage (Sissons and Oldstone, 1980).

Antibodies have been detected in the sera of patients with Burkitt's lymphoma, malignant myeloma and sarcomas which show complement-mediated cytotoxicity to tumour cells in vitro. Their in vivo significance, however, is not clear (Herberman, 1977).

There are, therefore, several examples of cytotoxicity caused by the formation of the complement membrane attack complex. However, in some responses to infections and in some auto-immune conditions, where complement is activated, tissue damage may occur as a result of cell-mediated reactions.

1.4.2 Cell-mediated cytolysis

1.4.2.1 Polymorphonuclear leukocytes and macrophages

Neutrophils and macrophages possess Fc receptors and so may bind to Fc-coated cells and be two of the cell types responsible for antibody-dependent cell-mediated cytotoxicity. C5a, generated by complement activation, is chemotactic for and activates polymorphonuclear leukocytes. Neutrophils are phagocytic, but if unable to phagocytose their target, their lysosomal contents may be released, damaging the underlying tissue. The main role of complement in immune complex-induced kidney injury has long been thought to be C5a attraction of neutrophils to the site of immune deposits, and the subsequent release of toxic products of neutrophil activation adjacent to the glomerular basement membrane (Couser, 1985; Ulich, Bannister and Wilson, 1987). Neutrophils have also been found to exert toxic effects on tumour cells. The administration of a streptococcal preparation to patients with malignant ascites increased the numbers of neutrophils with cytotoxic activity against tumour cells, probably due to C5a generated via the alternative pathway (Fujimura and Torisu, 1987).

Antigen-activated T and B lymphocytes release lymphokines that may activate macrophages in an antigen non-specific manner. T cell lymphokines are the most well defined but have been identified by their activity and so it is not always clear whether an activity is due to one factor or several or whether there is an overlap between lymphokines. Lymphokines that modulate the action of macrophages include migration inhibition factor, macrophage activation factor and the interferons (Rocklin, Bendtzen and Greineder, 1980). Lymphokines

activate macrophages by inducing the formation of more Fc and C3 receptors and by stimulating the production of various enzymes and other factors, including toxic oxygen metabolites (Nathan, Karnovsky and David, 1971; Klimetzek and Sorg, 1977). T cell activated macrophages have been shown to have enhanced phagocytic activity (Nathan et al., 1971), tumoricidal capacity (Piessons, Churchill and David, 1975) and bacteriocidal activity (Godal, Rees and Lamvik, 1971).

1.4.2.2 K cells

The cells involved in antibody-dependent cell-mediated cytotoxicity (ADCC) are poorly defined. Classical K cells are lymphoid cells that do not consistently carry the surface markers of either T or B cells but possess Fc receptors (van Boxel, Stobo, Paul and Green, 1972). ADCC has been demonstrated in vitro, using antibodies to tumour or MHC-associated antigenic determinants, to haptens conjugated to cell membranes, or to membranes of nucleated erythrocytes (Sanderson, Clark and Taylor, 1975; Korec, Herberman, Dean and Canon, 1980). The in vivo significance of ADCC is open to question but K cells may kill virus-infected cells by binding to specific antibody via surface Fc receptors (Kohl and Loo, 1982). The antibody requirements for ADCC appear to be different to those required for complement-mediated cytotoxicity. All classes of immunoglobulins are capable of mediating ADCC although IgG predominates. A very much smaller number of antibodies is required to coat the cell than for complement-mediated cytotoxicity and a single effector cell appears to be able to cause lysis (Perlmann, Perlmann and Wigzell, 1972).

1.4.2.3 Natural killer cells

Natural killer cells are a heterogeneous population of cells, both with respect to surface markers and the target cells that they recognise. They have Fc receptors but killing is not achieved through Fc receptor binding and is not antigen-specific.

1.4.2.4 Cytotoxic T lymphocytes

Cytotoxic T lymphocytes (CTLs) recognise antigen in association with self MHC products, predominantly class I MHC products on the surface of antigen-presenting cells (Marrack and Kappler, 1986). A single cytotoxic T lymphocyte is able to lyse an antigen-bearing target cell (Kranz, Pasternack and Eisen, 1987). The precise mechanism whereby cytolysis is achieved is not clear but, in the process, a novel serine esterase and a C9-like protein, perforin, are released from the cytolytic granules of the CTL which has led to the suggestion that CTLs and complement have similar cytolytic mechanisms. However, the resistance of some CTLs to lysis by other CTLs but not to lysis by antibody-activated complement suggests that there are differences between the two cytolytic mechanisms (Kranz et al., 1987).

One of the major physiological functions of CTLs is the elimination of virally-infected cells, where the CTL recognises foreign viral antigens on the host cell surface in conjunction with self MHC class I antigens (Zinkernagal and Doherty, 1974). Some cancer patients exhibit peripheral blood cytotoxicity against autologous tumour cells. Here, the CTL recognises neoantigens on the tumour cell in conjunction with MHC I antigens (Henney, 1977). CTL may also play a role in graft rejection. One theory is that T helper

cells recognise foreign MHC II antigens on the graft cell and stimulate host CTLs to destroy the graft cell. The CTLs may recognise the graft via foreign class I MHC antigens (Roitt, 1986).

1.4.3 Phagocytosis

C3b coating of target cells and particles is probably the major biological effect of complement. Neutrophils, eosinophils, monocytes and macrophages bear C3b receptors. C3b coating facilitates adherence of foreign particles to phagocytic cells and facilitates the ingestion of foreign particles by macrophages.

1.4.4 Cytolysis enhanced by immunotoxins

The use of antibodies to target toxins and drugs to cells has received a lot of attention in recent years, particularly as agents of tumour destruction (Vitetta, Krolick, Miyama-Inaba, Cushley and Uhr, 1983). Plant and bacterial toxins show structural homology. Ricin, one of the best characterised, is composed of a polypeptide B chain, which is a galactose-specific lectin and hence binds to virtually all eukaryotic cells, and an A chain which displays enzymatic activity (Vitetta and Uhr, 1985). Entry of toxins into the cell is achieved by receptor-mediated endocytosis and, after cleaving of the disulphide bonds linking the A and B chains, the A chain may be translocated into the cytoplasm. The entry of a single ricin A chain into the cytoplasm is sufficient for cell killing. The A chain catalytically inactivates ribosomal subunits by binding at or near the Elongation Factor 2 binding site and thereby inhibits protein synthesis.

Most immunotoxins are composed of antibodies conjugated to ricin A chains only, since the B chain confers excessive non-specific toxicity. Immunotoxins have been assessed for their ability to facilitate bone marrow transplantation for the treatment of cancer (Krolick, Uhr and Vitetta, 1982). In a mouse model, bone marrow from tumour-bearing animals was treated with a specific anti-tumour A chain toxin. After supra-lethal irradiation mice were reconstituted with immunotoxin-treated bone marrow and 75% of such treated animals remained disease-free. The prevention of graft versus host disease in allogeneic bone transplants by eliminating T lymphocytes with immunotoxins has been successful in mice and man (Vitetta and Uhr, 1985).

Potential problems for the use of immunotoxins in vivo include non-specific cytotoxicity of immunotoxins, particularly to the reticulo-endothelial system, specific cytotoxicity to non-target cells that bear cross-reacting antigens with target cells and the host immune response to the immunotoxin. Successful treatment of tumour-bearing mice with immunotoxins has been reported, where 95% of the tumour burden had already been removed by other methods (Krolick, Uhr, Slavin and Vitetta, 1982).

The most important requirement for the use of immunotoxins in vivo is probably for specific antibodies to antigens that show no cross-reactivity with other tissues. The work of Lee et al. (1986) suggests that such antigens may exist on adipose tissue.

1.5 SELF TOLERANCE AND AUTOIMMUNITY

At the beginning of the century Ehrlich proposed that the body does not have lymphocyte receptors directed against self molecules, a phenomenon he termed "horror autotoxicus" (Ehrlich and Morgenroth, 1900). This was based on the fact that he failed to induce animals to generate antibodies to their own tissues. The idea that all self-reactive lymphocytes were deleted early in life persisted for many years until several lines of evidence demonstrated the existence of self-reactive lymphocytes in normal adult individuals. Immune responses have been induced in experimental animals to many self tissues including the liver (Watanabe, Kawakami, Kawamoto, Ikemoto, Masuda, Takzaki, Nakanishi, Kajiyama and Takeno, 1987), kidney (Germuth, Senterfit and Pollock, 1967; Vnanue and Dixon, 1967), the CNS (Källen and Nilsson, 1986) and the thyroid (Clagett and Weigle, 1974) by immunising with either autologous or heterologous antigens in complete Freunds Adjuvant. B and T lymphocytes have been identified in normal individuals binding many self antigens, including thyroglobulin (Roberts, Whittingham and Mackay, 1973), DNA (Pisetsky, Caster and Steinberg, 1983), various common serum proteins (Guilbert, Dighiero and Avrameas, 1982), antigens of the CNS (Silberg and Swanborg, 1986) and immunoglobulin (Defranco, Raveche, Asorfsky and Paul, 1982). It is now generally accepted that self-reactive lymphocytes and self-antigens must be able to make contact in normal individuals although, under normal circumstances, controlling mechanisms must exist which prevent the triggering of an autoimmune reaction.

1.5.1 Immunoregulation

The immune response to any antigen is under complex regulatory control mediated by antibody and cellular circuits of helper and suppressor cell interactions and lymphocyte products (Roitt, 1986). An outline of a minimal model for immunoregulation is given here in order to facilitate discussion of the control of autoimmunity.

Before a B lymphocyte can respond to an antigen, the antigen must be processed and presented by an antigen-presenting cell, frequently a macrophage. Most B lymphocytes also require T cell help. T helper cells recognise different antigenic determinants to those recognised by B cells in conjunction with MHC class II antigens on the antigen-presenting cell. Both B and T cells are subject to T cell suppression. The nature of interactions between lymphocytes are not clearly defined. Soluble antigen-specific and MHC restricted factors are produced by T cells *in vitro* but their *in vivo* significance is not clear.

The variable region of an antibody molecule, the idiotype, may act as an antigenic determinant, inducing the formation of anti-idiotypic antibodies. Such antibodies may recognise the antigen-combining site of the idiotypic antibody and, hence, will compete with the antigen for binding of the idiotypic antibody. In the early 1970s Jerne (1974) proposed a network hypothesis, whereby idiotypes and anti-idiotypes function in the maintenance of immunological homeostasis by forming a network with multitudinous connections. He proposed that any antibody made in response to an antigen will induce the formation of an anti-idiotypic antibody which will regulate production of the idiotypic antibody. The anti-idiotypic antibody will, in turn, induce the formation of an anti-anti-idiotypic

antibody and so on. T and B cells also bear idiotypes. Jerne's hypothesis envisages idiotype-bearing cells interacting with anti-idiotype bearing cells to suppress or stimulate them.

In vitro studies have suggested that circuits of helper and suppressor cells may exist, possibly including inducer, transducer and contrasuppressor cells (Flood, Chue and Green, 1986). The balance betwen stimulation and suppression by T helper cells, T suppressor cells and idiotype-anti-idiotype interactions determines whether a state of immunity or tolerance is induced by an antigen.

1.5.2 Mechanisms of self tolerance

Theories of how immune responses against self tissues are avoided in the normal individual centre around lack of T cell help, T cell suppression and suppression by the idiotype network. Since T cells bear idiotypes and anti-idiotypes, these theories are not mutually exclusive.

Autoimmune responses to some self antigens may be induced by immunising animals with heterologous antigen or antigens modified by linkage to a drug or hapten (Allison, Denman and Barnes, 1971). Clagett and Weigle (1974) found that immunisation with heterologous thryoglobulin induced an autoimmune response against self thyroglobulin as long as T cells were present. This work suggested that some autoantigens are recognised by B cells but not T helper cells. If T helper cells are presented with foreign antigenic determinants on a heterologous antigen or a hapten linked to a self determinant, they may provide T cell help to self-reactive B cells.

There is substantial evidence for an important role of T cell suppression in the prevention of autoimmunity. Romball and Wiegle

(1987) transferred autoimmunity to thyroglobulin from autoimmune mice into irradiated syngeneic mice but could not transfer autoimmunity into normal mice. They suggested that autoimmunity could be transferred into irradiated mice due to abrogation of suppressor function. Autoimmune diseases, such as thyroiditis and gastritis, can be induced in some strains of mice by thymectomy at a critical stage, without further treatment (Taguchi and Nishizuka, 1987). This led to the proposal that autoantigen-reactive suppressor cells are continuously generated by stimulation from self antigens and inhibit the activation of potentially autoreactive cells. Neonatal thymectomy increases the frequency of autoimmune disease because, at this stage, suppressor T cells have not been peripheralized while cytotoxic T cells have. Support was given to this hypothesis by the prevention of autoimmune disease in thymectomised animals given injections of spleen cells from syngeneic adults 24 hours after thymectomy.

Theories of how the idiotype network might be involved in the prevention of autoimmunity are controversial. The evidence is mostly indirect and is derived from studies where autoimmunity has been induced in experimental animals by manipulation of the idiotype network. Mice immunised with insulin produced anti-(insulin) antibodies which stimulated the production of anti-idiotypic antibodies which behaved like antigen images, binding to the insulin receptor and mimicking the action of the hormone (Shechter, Maron, Elias and Cohen, 1982). Cross-reacting idiotypes are of widespread occurrence on human and animal autoantibodies, suggesting that they may have arisen as the result of a perturbation of the idiotype network (Zanetti, 1986). Bigazzi (1986) found that established

autoimmune responses in a rat model of autoimmune thyroiditis could be inhibited by the administration of the corresponding anti-idiotypic antibodies. Immunisation against the idiotype resulted in more vigorous and protracted autoimmune responses, possibly through the production of anti-anti-idiotypic antibodies. Anti-idiotypic antibodies binding to anti-acetylcholine receptor and anti-thyroglobulin antibodies have been identified in serum of patients suffering from Myasthenia gravis and Hashimoto's Thyroiditis respectively (Dwyer, Bradley, Oh and Kearney, 1984; Sikorska, 1986). In some cases the presence of anti-idiotypes is associated with a recession of the disease. It is, therefore, possible to induce autoimmune responses by deliberate perturbation of the idiotype network and there is evidence of disturbances of the network in established autoimmunity.

1.5.3 Autoimmune diseases

In the normal individual a variety of complex mechanisms maintain a delicate immunological balance, preventing pathological autoimmune responses. This balance can be disturbed experimentally, but can also breakdown naturally. A number of diseases are now recognised that are associated with the production of autoantibodies and autoreactive T cells. Autoimmune diseases range from the highly organ-specific, such as Hashimoto's thyroiditis, where antibodies to thyroglobulin are produced, to non-organ specific, such as Systemic Lupus Erythematosus, where antibodies to a wide range of tissues are produced.

1.5.3.1 The pathogenesis of autoimmune disease

In some autoimmune diseases, while autoantibodies are present, they may not be responsible for the pathogenesis of the disease. Cell-mediated immune responses may be more important or the autoimmunity may be a consequence rather than a cause of the disease.

The most convincing evidence for the cause of disease by autoimmune processes is the production of lesions characteristic of the disease by deliberate induction of autoimmunity. Immunisation of animals against thyroglobulin produces infiltration of the thyroid gland with mononuclear cells and lesions resembling those seen in Hashimoto's thyroiditis (Clagett and Weigle, 1974). Immunisation against myelin basic protein induces lymphocyte infiltration of nervous tissue and demyelination, resembling lesions seen in Multiple Sclerosis (Kâllén and Nilsson, 1986). While in these models autoantibodies against thyroglobulin and myelin basic protein are present, they may not be responsible for the production of the lesions characteristic of the diseases. Antigen-specific autoreactive T cells, rather than antibody, also appear to be responsible for the induction of Experimental Allergic Encephalomyelitis and Experimental Autoimmune Synovitis, which is induced by intra-articular immunisation against IgG (Goldberg, Lance and David, 1974).

In other models of autoimmune diseases, a role for antibody in the pathogenesis of the disease has been indicated. In the Obese Strain chicken, in which thyroid autoantibodies and thyroiditis occur spontaneously, the severity of the thyroiditis is greatly diminished by removing the site of B cell synthesis, the bursa of Fabricius, soon after hatching (Wick, Boyd, Hala, de Canalho, Kofler, Muller and

Cole, 1981). Removal of the thymus at birth increases the severity of the disease, indicating a role for T cell suppression controlling the normal outcome of the disease. Transient thyroiditis occurs in guinea pigs upon passive immunisation with anti-(guinea pig thyroglobulin) antiserum (Kåreson, 1970). Symptoms of Myasthenia Gravis can be reproduced by passive immunisation with anti-(acetylcholine receptor) antibodies (Newson-Davies, 1981). The mechanism of action of antibodies in the production of autoimmune lesions is not clear. Complement-mediated membrane attack, blocking of receptors and induction of an increased rate of receptor loss from the post-synaptic membrane by anti-(acetylcholine receptor) antibodies may contribute towards the pathogenesis of Myasthenia Gravis. Antibody-dependent cell-mediated cytotoxicity may play a role in Type I insulin independent diabetes (Pozilli, DiMario and Andreani, 1982).

It is likely that, in many autoimmune diseases, a combination of T cell-mediated and antibody-mediated responses is responsible for the pathogenesis of the disease.

1.5.3.2 The aetiology of autoimmune diseases

The mechanisms by which autoimmune diseases arise are not clear. There is evidence of suppressor cell defects in patients with SLE, Graves disease, insulin dependent diabetes mellitis and autoimmune chronic active hepatitis (Topliss, How, Lewis, Row and Kolpé, 1983; Vento, Hegarty, Bottazzo, Macchia, Williams and Eddlestone, 1984).

Thyroid cells can be induced to express class II MHC molecules upon stimulation with phytohaemmaglutinin. Lymphokines, such as

λ-interferon and interleukin I, released during viral infections, can also induce class II MHC antigens on cells on which they are not normally expressed (Cooke, Rayner and Lydyard, 1986). Such inappropriate expression of MHC antigens may lead to activation of helper T cells reactive against self antigens expressed on that tissue and induce the production of autoantibodies or cytotoxic T cells (Zanetti, 1986).

Hansen (1986) suggested a means whereby viral infections might disturb the idiotype network and induce autoimmunity. Antibodies raised in the normal immune response to the virus might recognise the part of the virus that binds to the host cell surface receptor. Anti-idiotypic antibodies might then bind to the cell surface receptor and would, therefore, be autoantibodies. Any infectious agent that can cause a disturbance in the idiotype network may lead to autoimmune disease.

It is likely that multiple factors are responsible for the production of autoimmunity. Several genetic loci, particularly those of the MHC, appear to be associated with susceptibility to autoimmune diseases. In addition, there is an age-dependent increase in autoantibody production, which is possibly due to the relaxation of hormonal control of the regulation of the idiotype network (Hansen, 1986).

1.6 IMMUNOLOGICAL ADJUVANTS

In order to raise an antiserum against a heterologous or homologous antigen, the immunogen is frequently administered in the presence of an adjuvant. An adjuvant can be defined as an agent that

acts non-specifically to augment an immune response to a specific antigen. An enormous number of compounds have been found to modulate the immune response which vary in the type of immune response they stimulate and their mode of action.

1.6.1 Bacterial adjuvants

Freunds complete adjuvant, an emulsion of mineral oil, Arlacel A, and mycobacterium, is one of the most potent adjuvants known for stimulating both humoral and cellular immunity (Warren, Vogel and Chedid, 1986). Water in oil emulsions form a depot from which antigen is slowly released. This prolongs the time of interaction between the antigen and antigen-presenting cells. The mycobacterial component attracts lymphocytes to the site of injection where they accumulate and release lymphokines. This leads to the aggregation and proliferation of macrophages. Granulomas, thus formed, serve to expose antigen-presenting cells and lymphocytes to high concentrations of antigen. The toxicity of Freunds complete adjuvant (CFA), mainly because it contains non-metabolizable mineral oil and because the mycobacterial elements elicit granulomatous reactions, has led to attempts to purify the adjuvant-active components. The smallest structure that will replace the mycobacterium in CFA is muramyl-dipeptide (MDP). MDP enhances T helper function and is mitogenic for T and B lymphocytes. MDP is also chemotactic for and activates macrophages. MDP, administered in saline, stimulates humoral immune responses and, in mineral oil, stimulates both humoral and cell-mediated immunity (Audibert, Chedid, Lefrancier and Choay, 1976). Derivatives of MDP have now been isolated that are devoid of pyrogenicity but retain adjuvant activity (Chedid, Parant, Audibert, Riveau, Lederer, Choay and Lefrancier, 1982).

Lipopolysaccharide (LPS), a component of bacterial cell walls, is another potent stimulator of both humoral and cell-mediated immunity (Warren et al., 1986). It appears to act directly on macrophages, stimulating the production and release of lymphokines and increasing expression of Ia molecules, thus improving the efficiency of antigen presentaton. LPS is also a polyclonal B cell mitogen and, hence, may stimulate antibody responses by bypassing the requirement for T cell help. LPS is highly toxic but the lipid A portion of the molecule, while retaining adjuvanticity, is less toxic. Relatively non-toxic synthetic analogues of lipid A have now been produced.

Whole cell Bordetella pertussis vaccine has 2 adjuvant components, LPS and pertussis toxin. Pertussis toxin is a protein exotoxin that has been linked to the harmful effects and long-lasting immunity to whooping cough. Pertussis toxin potentiates cell-mediated immunity through its ability to alter the recirculation of T lymphocytes (Allison, 1979).

Bacterial adjuvants, though widely used in experimental animals, are toxic and are most effective when administered in water-in-oil emulsions which are non-metabolizable and induce granuloma formation, rendering their use unacceptable in humans. Consequently, efforts have been made to develop non-bacterial, metabolizable immunological adjuvants.

1.6.2 Non-bacterial adjuvants

Aluminium compounds have been used since the 1920s as adjuvants. They appear to act by slowing the excretion of antigen and by the attraction of immunocompetent cells

to the area of injection. They may also direct antigens to T-cell-containing areas of the lymph node. However, aluminium compounds have little effect on cell-mediated immunity and are not as potent as CFA in stimulating antibody responses (Warren et al., 1986).

Studies using synthetic polymer adjuvants show that the induction of antibody responses can be dissociated from the induction of inflammation and granuloma formation. Large insoluble polymers with hydrophilic moieties flanking hydrophobic moieties stimulate chemotaxis, complement activation and antibody formation. Smaller polymers induce inflammation rather than adjuvant effects while polymers with hydrophobic moieties flanking hydrophilic moieties induce granuloma formation (Warren et al., 1986; Kohm, Niemi, Albert, Murphy, Langer and Fox, 1986). Such polymers are thought to act by directing antigens to highly efficient antigen presenting cells in the lymph nodes. They are in an early stage of development and their in vivo metabolism and toxicity need to be further evaluated.

Liposomes have been shown to confer adjuvant activity (Heath, Edwards and Ryman, 1976). They can elicit both humoral and cellular immunity, the adjuventicity being determined by their charge, composition and method of preparation. They appear to act as a depot for antigen, prolonging its release. The presentation of antigen in a hydrophobic environment may cause increased uptake of antigen by antigen presenting cells. Liposomes themselves may act as antigen presenting cells if MHC class II molecules are present on their surface. The potency of liposomes as immunological adjuvants may be increased by the inclusion of LPS, lipid A or MDP within the vesicles (Alving and Richards, 1983).

Despite the development of apparently non-toxic vehicles showing immunostimulatory effects, none have been shown to have consistent effects equivalent to those of CFA and further characterisation of their toxicity and effects on the immune system is required.

1.7 A SUMMARY OF THE AIMS AND POSSIBLE PROBLEMS OF PASSIVE AND ACTIVE IMMUNISATION AGAINST THE ADIPOCYTE

The cost of excess fat deposition in farm animals and the demand for leaner meat have led to a search for methods of reducing fat deposition and increasing protein deposition. Existing approaches include genetic selection for reduced fat deposition, nutritional manipulations and the administration of hormonal repartitioning agents. Cost, lack of flexibility, possible adverse effects on animals and consumers and the EEC ban on the use of steroid hormones to improve animal performance, are likely to limit the practical use of many of these approaches. The immunological reduction of body fat using passive or active immunisation against adipocytes is an attractive approach as harmful residues would not be left in the edible tissues, the option to treat or not to treat could be used, according to demand, and the repeated administration of antiserum or immunogen may not be necessary.

Initial attempts to reduce body fat by an immunological approach were made by passive immunisation with antisera raised against whole adipocyte plasma membranes. Passive immunisation has a number of advantages when compared with active immunisation. High titre antisera against heterologous adipocyte plasma membranes could

be easily raised and have been shown to be cytotoxic to isolated adipocytes in vitro. With active immunisation it is necessary to overcome the individual's immunoregulatory mechanisms that normally prevent pathological immune response against self tissues. Self-reactive lymphocytes are present in normal individuals and, given the appropriate immunisation protocols, can be induced to mount autoimmune responses resulting in tissue lesions resembling those seen in autoimmune diseases. Since autoimmune responses have been induced against a wide variety of tissues by immunisation with homologous or heterologous self antigens in the presence of adjuvants, it is likely that an autoimmune response against adipose tissue could be induced. However, while passive immunisation involves the administration of antisera of defined specificity and titre, the results of active immunisation are inevitably subject to the variability of the individual's own immune response. Passive immunisation also allows the conjugation of immunopotentiators to antibody to enhance efficacy, as has been attempted in tumour immunotherapy.

For passive immunisation against the adipocyte to be a useful approach for the commercial reduction of fat deposition, any effects on body fat must be long-lasting. Recent evidence suggests that, while the majority of proliferation in adipose tissue occurs early in life, there is capacity for new adipocyte production in the adult. Regeneration of lost tissue is a potential limitation of the use of passive immunisation for the reduction of body fat since, if heterologous antiserum were used, repeated treatments would not be effective unless animals were tolerized to the antibody administered. Active immunisation against the adipocyte may have the advantage of

inducing a longer-lasting immune response which may not allow regeneration of lost tissue. Active immunisation also requires the administration of minute amounts of antigen and so may prove a more practical approach for the treatment of farm animals on a commercial scale.

Theoretically, active immunisation allows the recruitment of a wider range of mediators of immune tissue damage than passive immunisation. The injection of preformed antibody allows the activation of complement which may result in complement-induced cytolysis or the attraction and activation of phagocytic cells. In addition to these mechanisms, active immunisation, using an appropriate adjuvant, may induce the formation of antigen-specific cytotoxic T cells, which may be responsible for the pathogenesis of several autoimmune diseases.

In order to determine whether either passive or active immunisation against the adipocyte could cause a reduction in body fat deposition, initial experiments were performed using immunisation against whole adipocyte plasma membranes. After the demonstration of initial effects, efforts were made to increase the adipocyte specificity of antisera by the preparation of adipocyte specific antigens as immunogens for both passive and active immunisation.

The majority of the work described in this thesis was performed using the rat. An antiserum against adipocyte cell membranes was already available and so the effects of passive immunisation in the rat could be characterised as far as possible before attempts were made to extend the approach to other species. Preliminary experiments in sheep, pigs and chickens were aimed at raising antisera against whole adipocyte plasma membranes of these species

and comparing any adipocyte specific components with those of the rat. The main aim of experments involving active immunisation in rats and sheep was to devise an immunisation protocol that induced an immune response able to cause a reduction in fat deposition. It was not possible to fully characterise the nature of that immune response or to develop adjuvants suitable for use in commercial species.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 CHEMICALS

All chemicals were obtained from Sigma Chemical Co., Poole, Dorset, Boehringer Corp. Ltd., Lewes, East Sussex or BDH Chemicals Ltd., Poole, Dorset, unless otherwise stated.

2.2 ANIMALS

2.2.1 Rats

Female and male Wistar rats and female Sprague-Dawley rats from A. Tuck and Son (Rayleigh, Essex) were given a Labsure (Poole, Dorset) irradiated diet composed of 57% carbohydrate, 19% crude protein, 2.4% crude oil and 3.6% crude fibre and water ad libitum, unless otherwise stated. Rats were maintained on a 12 h light-dark cycle (light phase from 08.00-20.00 h).

2.2.2 Sheep and goats

Sheep used as antiserum donors at the Hannah Research Institute were either Clun or Finn x Dorset Horn cross-bred ewes, whilst those provided by S. Kestin from the Institute of Food Research (Bristol Laboratory) were either Welsh Mountain, Dorset Down crosses or Scottish half breeds. The goat used as an antiserum donor at the Hannah Research Institute was a British Saanen goat. Experiments involving the active immunisation of lambs were performed in collaboration with Dr. S. Rhind at Hill Farming Research

Organisation (Penicuik, Midlothian) where the animals were kept. Twin Scottish Blackface lambs were housed with their dams and fed a complete ration ad libitum until weaning, at about 16 weeks of age. Thereafter the lambs were fed the same complete diet ad libitum.

2.3 PLASMA MEMBRANE PREPARATION

2.3.1 Adipocyte plasma membrane preparation

Rat adipose tissue was obtained from female Wistar rats at the Hannah Research Institute. Pig and sheep adipose tissue was obtained from Landrace and Scottish Blackface crosses respectively from the local abattoir. Fresh chicken adipose tissue was obtained from Ross F1 hybrid chickens at the West of Scotland Agricultural College (Ayr, Strathclyde). Frozen chicken adipose tissue from Ross F_1 Hybrids was obtained from S. Kestin, Institute of Food Research (Bristol Laboratory).

Membranes for immunisation and for screening antisera were prepared from isolated adipocytes (unless otherwise stated), in order to obtain a preparation as free from contaminating connective tissue and red and white blood cells as possible. Membranes for antigen purification were prepared by homogenisation of whole tissue, in order to obtain large quantities of membrane protein.

2.3.1.1 Preparation of adipocyte plasma membranes from isolated adipocytes

Approximately 5 g of fresh adipose tissue was chopped with scissors and incubated in 10 ml of Krebs Ringer Phosphate containing 3% BSA (essentially fatty acid free), 0.1% collagenase (Type II, Sigma Chemical Co., Poole, Dorset) and 0.02% dispase (Grade II, Boehringer

Mannheim, Lewes, East Sussex) at 37° C for pig, rat and chicken tissue and 42° C for sheep tissue, for 45 minutes, under constant agitation. The suspension was filtered through a nylon sieve to remove undigested material and washed 3 times by flotation in Krebs Ringer Phosphate, containing 1% BSA. Adipocytes were suspended in at least twice their volume of extraction medium, containing 0.25 M sucrose, 0.05 M Tris-HCl pH 7.4, 2 mM EGTA and 2 mM PMSF. After vortexing for 60 seconds, adipocytes were centrifuged at 2,500 g, at 37° C for rat, pig and chicken cells and 42° C for sheep cells, for 5 minutes. The infranatant and pellet were collected onto ice and the remaining unbroken cells were resuspended in extraction medium and vortexed and centrifuged once more. The pooled pellets and infranatants were centrifuged at 40,000 g at 4° C for 30 minutes. The pellet was resuspended in 0.05 M Tris-HCl pH 7.4, containing 2 mM EGTA and 40% w/w sucrose, and homogenised using a ground glass homogeniser. A similar solution, but containing 32% w/w sucrose, was layered onto the 40% sucrose solution. 0.05 M Tris-HCl, containing 2 mM EGTA, was layered onto the 32% sucrose solution and the discontinuous gradient was centrifuged at 75,000 g at 4° C for 1 h. The material lying on the top of the 32% sucrose layer (plasma membranes) was removed, diluted at least 10-fold with 0.05 M Tris-HCl, containing 2 mM EGTA, and centrifuged at 75,000 g for 1 hour. The pellet was resuspended and homogenised in 0.05 M Tris-HCl, containing 2 mM EGTA, snap frozen in liquid nitrogen and stored at -20° C.

2.3.1.2 Preparation of adipocyte plasma membranes by homogenisation of whole adipose tissue

One part of adipose tissue was homogenised in at least 2 parts of extraction medium, containing 10 mM phosphate pH 7.4, 0.25 M

sucrose, 1 mg/ml bacitracin and 2 mM PMSF, at 37° C for rat, pig and chicken tissue and 42° C for sheep, in a Waring blender for 30 seconds. The homogenate was spun at 2,500 g for 5 minutes at 22° C for rat, pig and chicken tissue and 42° C for sheep tissue. The pellet and infranatant were centrifuged at 50,000 g for 1 h. The resulting pellet was purified as described in the previous section but using 10 mM phosphate in place of 0.05 M Tris-HCl.

2.3.2 Hepatocyte, erythrocyte, kidney and brain plasma membrane preparation

Liver, kidney or brain tissue was homogenised in extraction buffer as described for adipose tissue in Section 2.3.1.2, except that the temperature was maintained as close to 4° C as possible. Erythrocytes were washed 3 times with ice cold Krebs Ringer Phosphate by centrifugation at 1,000 g for 5 minutes. The cell pellet was suspended in 0.87% ammonium chloride for 5 minutes and homogenised in a Waring blender for 60 seconds. All homogenates, where plasma membranes were to be used for affinity purification of antigens, were purified as described for adipose tissue in Section 2.3.1.2. Plasma membranes for screening of antisera by ELISA were purified by centrifugation at 40,000 g for 30 minutes. The pellet was discarded and the supernatant was centrifuged at 75,000 g for 1 h at 4° C. The resulting pellet (microsomal fraction) was homogenised in 10 mM phosphate, 2 mM EGTA, snap frozen in liquid nitrogen and stored at -20° C.

2.4 PREPARATION OF ANTISERA FROM SHEEP AND GOATS

200 μg of antigen protein in 1 part of aqueous phase was emulsified in 2 parts of Freunds complete adjuvant for the first immunisation. Subsequent immunisations were given in incomplete Freunds adjuvant. A total of 2 ml of emulsion were injected subcutaneously at 3 different sites. Sheep were bled from the jugular vein 10-18 days after the second and subsequent boosts. The blood was allowed to clot for 16 h at 4° C and the serum was decanted and spun at 2,500 g for 15 minutes. The clear serum was then snap frozen in liquid nitrogen and stored at -20° C.

2.5 PREPARATION OF IgG FRACTION FROM SERUM

90% (w/v) saturated ammonium sulphate solution was added to serum to a final concentration of ammonium sulphate of 40%. The suspension was incubated for 30 minutes with stirring and then centrifuged at 2,500 g for 30 minutes at 4° C. The pellet was resuspended in 10 mM phosphate pH 7.4, and dialysed for 16 h at 4° C against 10 mM phosphate. The IgG fraction was snap frozen in liquid nitrogen and stored at -20° C.

2.6 ENZYME-LINKED IMMUNOASSAY (ELISA)

2.6.1 Coating of antigen to microtitre plates

Insoluble antigens were sonicated using a Kontes micro-ultrasonic cell disrupter (Burkard Scientific, Uxbridge, Middlesex) on setting 2 for 60 seconds before coating to plates.

Antigens available in the absence of detergent were coated to plates by adding 100 μl of protein (10 μg/ml) in phosphate buffered saline (PBS) pH 7.4 and incubating for 16 h at 4° C. Plates were washed 3 times with PBS-Tween (containing 0.05% Tween 20, Sigma Chemical Co., Poole, Dorset) using a Titertek microplate washer 120 (Flow Laboratories, Rickmansworth, Herts.) and incubated for 2 h at room temperature in PBS Tween to block non-specific binding sites on the plate.

Purified antigens, prepared in the presence of Triton X-100, could not be adsorbed onto the plate by the method described above because of the presence of detergent, so plates were first coated with 0.0005% (w/v) poly-l-lysine in PBS for 30 minutes at room temperature. Plates were then aspirated but not washed. Antigen was diluted to a concentration of 2.5 μg/ml which had previously been shown to be the concentration that resulted in maximum binding to the plate. 100 μl of antigen solution was added to each well and incubated for 16 h at 4° C. 100 μl of 0.25% glutaraldehyde in PBS was then added to each well and incubated at 22° C for 15 minutes. Plates were washed 3 times with PBS Tween and incubated for 1 h in 100 mM glycine in PBS (to block any remaining glutaraldehyde), containing 10% normal serum of the species in which the enzyme-conjugated second antibody was raised (to reduce non-specific binding of the conjugate) or 1 mg/ml casein, to block non-specific binding sites on the plate as described in Results. If the plate was not to be used immediately, wells were filled with PBS Tween and stored at -20° C.

2.6.2 Antibody binding assay

100 μl of test serum or non-immune serum, diluted in PBS Tween, was added to each well and incubated for 2 h at room temperature. Plates were washed 4 times with PBS Tween and 100 μl alkaline phosphatase-conjugated second antibody raised against immunoglobulin of the species of the first antibody (Sigma Chemical Co, Poole, Dorset), diluted 1:600 in PBS Tween, was added to each well. Plates were incubated for 2 h at 22° C, were then washed 4 times with PBS Tween and 200 μl of 0.1% 4-nitrophenyl phosphate (Boehringer Mannheim, Lewes, East Sussex) in 0.1 M glycine, pH 10.4, containing 1 mM zinc chloride and 1 mM magnesium chloride, was added to each well. Plates were incubated in the dark at 22° C until wells with the greatest colour development had an absorbance of approximately 1.2 at 405 nm. The reaction was stopped by the addition of 50 μl of 0.5 M sodium hydroxide and the absorbance at 405 nm was read using a Titertek Multiskan (Flow Laboratories, Rickmansworth, Herts.).

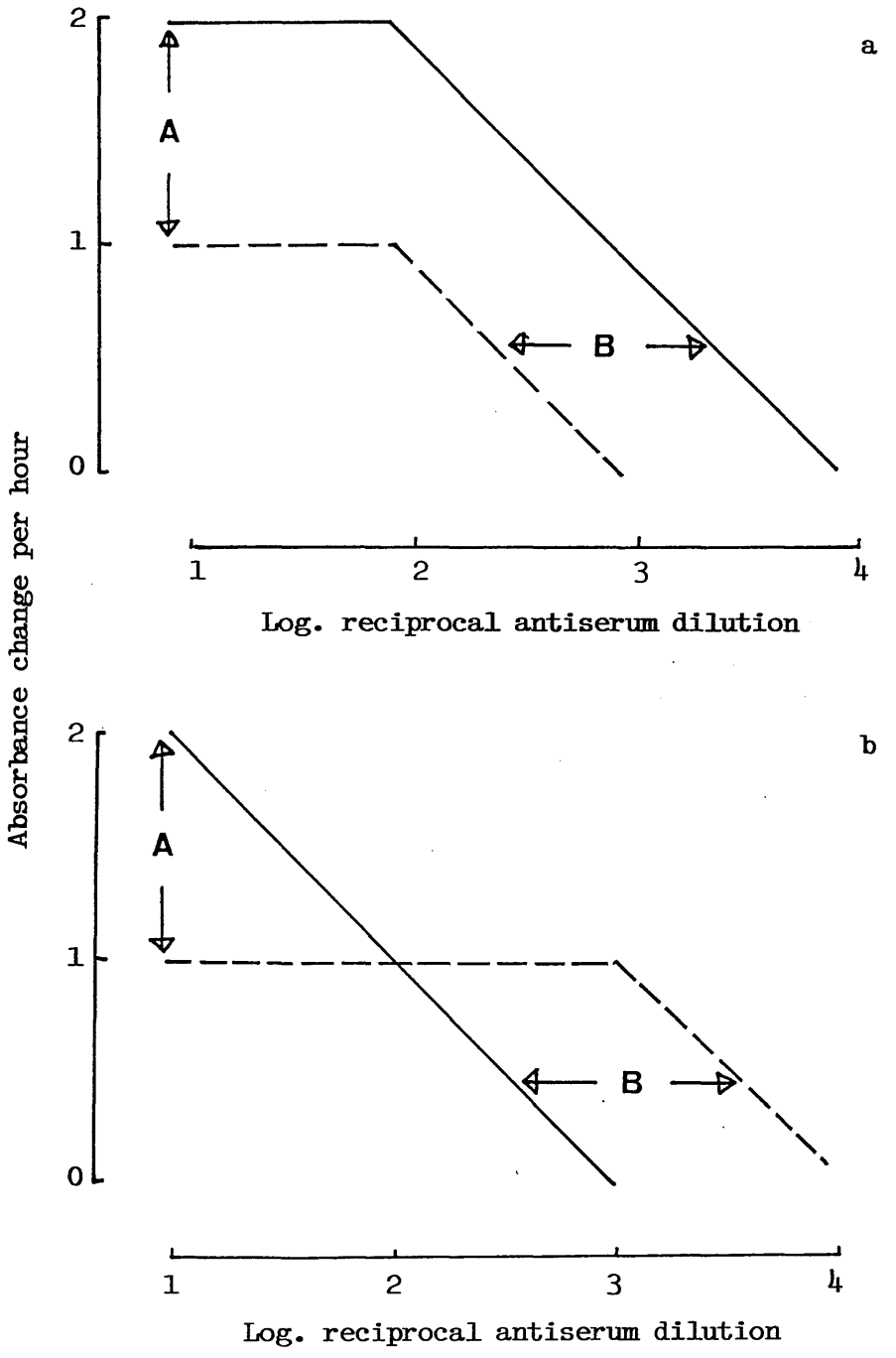
2.6.3 Quantitation of results from ELISA

Unless otherwise stated, serial dilutions of antisera were made with the aim of covering the range of antiserum concentrations from a negative result to a plateau, where antigen had become limiting. In practice this was not always achieved as the range of antiserum concentrations occasionally exceeded 4 $\log_{10}$ dilutions. Equivalent dilutions of non-immune serum were always tested in parallel and the absorbances subtracted from those achieved with antisera so that the dose response curves for antisera represented specific binding only.

Difficulties in quantitation of results arose because dose-response curves for antisera were not always parallel and cross-overs, presumably due to differences in affinity, were observed. In an attempt to overcome this problem, one antiserum or pool of antisera was selected as a standard and, for each test antiserum, 2 values relative to the standard were determined, relative antibody binding and relative antigen abundance. Fig. 2.1a represents the ideal situation where the curves are parallel. The relative antigen abundance (A) measures the relative abundance of the particular epitopes within the complex antigenic mixture on the ELISA plate to which the antisera bind. Thus, in this example, the test antiserum recognises 50% of the epitopes recognised by the standard. The relative antibody binding (B) measures the relative amounts of antisera required to produce the same absorbance at a point where the curves are as parallel as possible. Thus, in this example, the test antiserum shows 10% of the binding to the antigen of the standard when antigen is in excess. This may be due to a lower affinity, or lower titre or a combination of both. Fig. 2.1b represents a situation where the test antiserum has a lower antibody titre or affinity but recognises a larger number of epitopes than the reference antiserum. Thus, while the relative antibody binding (A) is 10%, the relative antigen abundance (B) is $\geqslant$200%.

Despite the problem of lack of parallellism of curves, quantitation of results in this way has advantages over titration methods, where the highest dilution that produces a positive result is determined. In addition to giving an indication of the relative numbers of epitopes bound by an antiserum, relative binding is measured at the linear part of the sigmoidal curve where sensitivity

Fig. 2.1. The quantitation of results from ELISA



The above is a schematic diagram showing how relative antigen abundance (A) and relative antibody binding (B) are calculated. The standards are represented by unbroken lines and the test samples are represented by broken lines.

is maximal. This overcomes the problems of distinction between positive and negative values at the flattening part of the curve.

In active immunisation experiments, where differences between groups of antisera were investigated, dose-response curves with standard errors of absorbances are presented. The statistical significance of differences in absorbance at each antiserum dilution can then be determined. Absorbances for pooled normal sera (non-specific binding) were subtracted from antiserum values.

2.7 CYTOTOXICITY ASSAYS

2.7.1 Cytotoxicity against erythrocytes

Erythrocytes were washed as described in 2.3.2 and resuspended in Krebs Ringer Phosphate containing 1% BSA and 0.1% glucose. This buffer was also used for the dilution of antisera and complement. Antisera and non-immune sera were heat-inactivated at 56° C for 30 minutes to inactivate complement. Guinea pig complement was obtained from Wellcome diagnostics (Dartford, Kent), while rat and sheep complement were prepared by allowing blood to clot for 3 h at 4° C and spinning at 11,000 g for 5 minutes at 4° C. If not to be used immediately, the serum was frozen in liquid nitrogen and stored at -20° C. In the standard assay procedure 50 μl of erythrocytes (2.5% haematocrit), 50 μl of 10% complement and 50 μl of serial dilutions of non-immune serum or antiserum were incubated in v-shaped microtitre plates for 1 h at 37° C. Wells were also included that contained erythrocytes and 100 μl of 2% Triton X-100 in distilled water to obtain a measure of total lysis. Additional wells contained erythrocytes, complement and buffer to obtain a measure of

spontaneous lysis. In typical assays spontaneous lysis and lysis in the presence of non-immune serum represented less than 10% of total lysis. After 1 h plates were centrifuged at 1,000 g for 5 minutes. 75 μl of supernatant was removed into a flat-bottomed well microtitre plate and the absorbances were read at 405 nm (optimal for haemoglobin). Specific cytotoxicity was measured by subtraction of spontaneous lysis from both lysis in the presence of antiserum and total lysis. Corrected lysis in the presence of antiserum was divided by corrected total lysis to obtain specific cytotoxicity as a percentage of the maximum.

$$\frac{\text{Lysis by antiserum - lysis by non-immune serum}}{\text{Lysis by Triton X-100 - lysis by non immune serum}} \times 100$$

2.7.2 Cytotoxicity against adipocytes

Isolated adipocytes were prepared as described in Section 2.3.1.1 but the tissue was allowed to digest for only 30 minutes in order to maximise the viability of the isolated cells. The assay was performed in the same way as for erythrocyte cytotoxicity except that 50 μl of approximately 50% packed adipocytes (approximately 5×10^4 cells) were used. After a 1 h incubation at 37° C the assay was stopped by layering both cells and medium onto dinonylpthalate oil in 400 μl microfuge tubes. Tubes were spun for 2 minutes at 11,000 g to separate the medium from the cells. The tubes were cut and the medium was assayed for lactate dehydrogenase (LDH) content. Wells containing cells, complement and buffer were stopped before incubation at 37° C to assess initial LDH content. LDH was assayed by incubating 40 μl of medium with 200 μl of 0.25M triethanolammonium chloride pH 7.4, containing 0.05% NADH until the absorbance at 340 nm

was constant. 25 μl of 10 mM sodium pyruvate was added and the rate of change in absorbance at 340 nm was measured. LDH in the medium at time 0 varied from 15-30% of total release. Spontaneous release and release in the presence of non-immune serum varied from 25-50% of total release. Specific cytotoxicity (expressed as a percentage of total release) was measured as described in the previous section for erythrocytes.

2.8 ANALYSES OF ADIPOSE TISSUE

2.8.1 Determination of adipocyte size and number

Adipocyte size and number were determined by the method of Vernon (1977), except that after digestion cells were spun at 200 g for 30 seconds and measured without washing, in order to minimise the loss of small cells. The diameter of 100 cells for each tissue was measured at a magnification of X145 using a haemocytometer placed under a projecting microscope and, from this, the mean adipocyte volume was calculated.

The dry weight of the cells or tissue was determined and, assuming a specific gravity of 0.91 for lipid, the cell number could be calculated using the following formula:

$$\text{Cell number} = \frac{\text{Dry weight of tissue (g)} \times 0.91 \times 10^9}{\text{Mean cell volume (pl)}}$$

2.8.2 U-^{14}C-glucose incorporation into total lipid in isolated adipocytes

Isolated adipocytes were prepared as described in 2.3.1.1. The medium used for diluting cells, hormone and glucose was Krebs Ringer

Phosphate containing 1% BSA and 0.01% glucose. 100 μl of adipocytes were incubated with increasing concentrations of insulin from 0 to 100 ng/ml and 0.025 μCi of U-^{14}C-glucose (Amersham International, Amersham, Bucks.) in flat-bottomed wells of 96-well plates, with a total incubation volume of 300 μl. Blank wells were included that contained no U-^{14}C-glucose during incubation but to which U-^{14}C-glucose was added immediately before stopping. After incubation for 2 h at 37° C, cells and medium were removed into toluene scintillation fluid (Packard Instruments, Caversham, Berks.). The incorporation of U-^{14}C-glucose into lipid was measured using a Packard (Tri-Carb 2405, Packard Instruments, Caversham, Berks.). U-^{14}C-glucose was added to emulsifier scintillant 299 (Packard Instruments, Caversham, Berks.) to measure total counts. The number of adipocytes per well was determined as described in Section 2.8.1 so that results could be expressed as nmol U-^{14}C-glucose incorporated by 10^6 cells. (Moody, Stan, Stan and Ghiamm, 1974)

2.8.3 Glycerol release from isolated adipocytes

The assay was performed as for glucose incorporation into lipid but U-^{14}C-glucose was excluded and the insulin was replaced by noradrenalin concentrations of 0-200 ng/ml. After incubation for 2 h at 37° C, medium was removed from beneath the adipocytes and assayed for glycerol.

Glycerol was assayed by incubating 50 μl of sample with 200 μl of assay buffer containing 0.25 M triethanolammonium chloride pH 7.5, 0.6% (w/v) Triton X-100, 2.5% NAD, 6.25% ATP, 12.5 mM $MgCl_2$, 0.5% α glycerophosphate dehydrogenase, 0.25% diaphorase and 4% MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetazolium bromide,

thiazolyl blue). When absorbance at 600 nm had become constant, 50 μl of 2% glycerokinase was added and the change in absorbance at 600 nm with time was measured. The standard used was 1 mM glycerol.

2.9 PREPARATION OF ADIPOCYTE SPECIFIC ANTIGENS

An outline of the method used for the preparation of adipocyte specific antigens is shown in Fig. 2.2. The γ-globulin fraction of an antiserum raised against whole adipocyte plasma membranes was passed down an affinity column bearing solubilized adipocyte plasma membranes. Unbound material, which contained irrelevant antibodies, was washed through the column and the bound antibodies were eluted by washing with glycine buffer at pH 2.5. The glycine wash was then passed down an affinity column bearing hepatocyte, kidney and erythrocyte plasma membranes to remove anti-(adipocyte plasma membrane) antibodies that cross-reacted with these tissues. The unbound material, containing adipocyte specific antibodies, was used to prepare a third affinity column. Solubilized adipocyte plasma membranes were passed down the third column. Unbound material, which contained antigens that cross-reacted with other tissues or to which antibodies had not been raised, was washed through the column and bound material, which contained adipocyte specific antigens, was eluted with glycine buffer at pH 2.5.

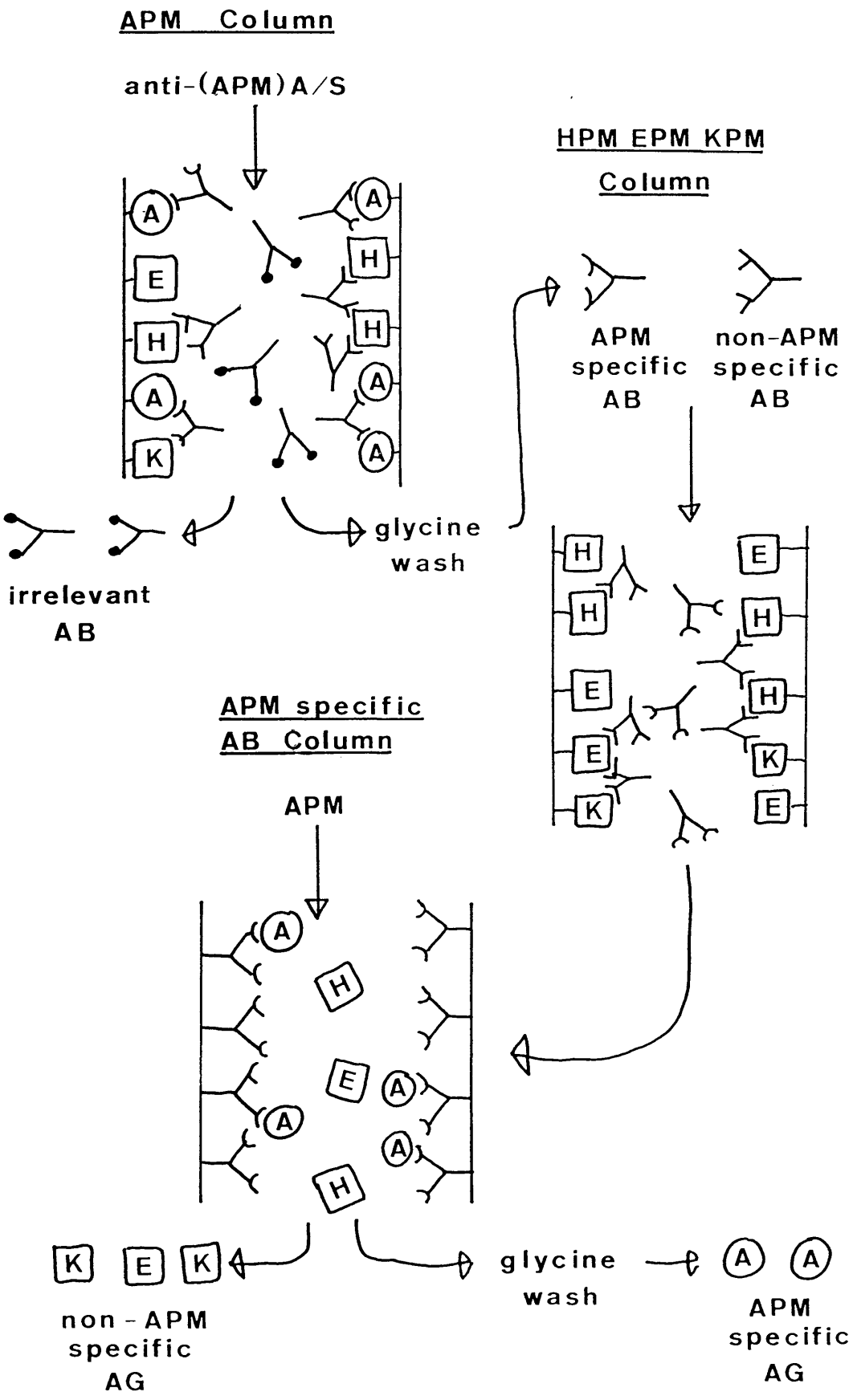
2.9.1 Preparation and use of affinity columns

Plasma membranes were prepared by homogenisation of whole adipose tissue and purification, as described in Section 2.3.1.2. Membrane pellets were resuspended in 0.1 M sodium

Fig. 2.2. An outline of the method

for the preparation of adipocyte specific antigens

The anti-(APM) antiserum was passed down an affinity column of whole APM which bound antibodies recognising adipocyte specific antigens (A) and antigens shared with non-adipose tissues (H,E,K). Bound antibody was eluted with glycine buffer pH 2.5 and passed down a column of HPM, EPM and KPM which bound antibodies recognising antigens shared between adipocytes and hepatocytes (H), erythrocytes (E) and kidney (K). Unbound antibodies were used to prepare a 3rd affinity column down which were passed solubilised APM. This column bound antigens that were not present on HPM, EPM and KPM.


APM Column
anti-(APM)A/S
A
E
H
K
irrelevant AB
glycine wash
HPM EPM KPM Column
APM specific AB
non-APM specific AB
APM specific AB Column
APM
non - APM specific AG
glycine wash
APM specific AG

bicarbonate buffer pH 8.0 and sonicated as described in Section 2.6.1.

Purified Triton X-100 (Pierce) was then added to a final concentration of 2% and the suspension was sonicated as above. After stirring for 1 h, the membrane solution was centrifuged at 75,000 g for 1 h at 4°C to remove insoluble material.

Freeze-dried activated CH-sepharose 4B (Pharmacia Fine Chemicals, Milton Keynes, Bucks) was swollen for 15 minutes in 1 mM ice-cold HCl and washed on a sintered glass filter with the same solution, using 200 ml per g of freeze-dried powder. The plasma membranes were incubated overnight at 4° C with the swollen gel in an end-over-end mixer. Two parts of membrane solution to 1 part of gel were used. The protein concentration found to give the most efficient binding to the column was 5 mg/ml of gel which resulted in a binding efficiency of 50-70%. 10-15 ml plasma membrane columns and 5-10 ml specific antibody columns were prepared, depending upon the amount of ligand available.

After coupling, excess ligand was washed away with coupling buffer and any remaining active groups were blocked by treatment with 1 M ethanolamine pH 9.0, containing 0.1% Triton X-100, for 1 h at 22° C. The column was then washed alternately with borate buffer, containing 0.1 M disodium tetraborate pH 8.0, 0.5 M NaCl and 0.1% Triton X-100, and glycine buffer, containing 0.1 M glycine pH 2.5, and 0.1% Triton X-100. The protein concentration of all washes was measured by Bradford assay so that the amount of protein bound to the column could be determined.

Antibody was coupled to CH-sepharose 4B in the same way as used for solubilised plasma membranes, except that detergent was absent.

Where columns of less than 5 ml of swollen gel were prepared, 0.8 x 4 cm Bio-Rad Poly-Prep chromatography columns (Bio-Rad, Watford, Herts) and flow rates of 0.5 ml/minute for all washes and elutions were used. Where columns of more than 5 ml of gel were prepared, 60 ml plastic syringes with a 0.25 μm filter and flow rates of 1.5 ml/minute for all washes and elutions were used. When not in use, affinity columns were stored in borate buffer, containing 0.02% thiomersal, and 0.1% Triton X-100 where appropriate.

When antisera were to be passed down plasma membrane columns, γ-globulin preparations containing 0.1% Triton X-100 were used. Where plasma membranes were to be passed down antibody columns, membranes were dissolved in borate buffer containing 2% Triton X-100 as described above. Approximately 500 μl of anti-(APM) γ globulin (100 mg/ml) was added per ml of APM column. Approximately 500 μl of anti-(APM) antibody (2 mg/ml), purified from the APM column, was added per ml of HPM, EPM and KPM column. Approximately 500 μl of solubilized APM (2 mg/ml) was added per ml of specific antibody column. Antibody or membrane solutions were run into affinity columns and incubated for 4-16 h at 4° C. Unbound material was washed from the column using 10 column volumes of borate buffer containing 0.1% Triton X-100. Fractions of appropriate volume were collected and assayed for protein to ensure that all unbound material had been eluted. The column was then incubated in glycine buffer, containing 0.1% Triton X-100 at pH 2.5 for 20 minutes. The column was then washed with 10 column volumes of the same glycine buffer. Fractions collected were neutralised immediately with 2 M Tris and assayed for protein. Fractions of borate and glycine washes that contained the bulk of the protein were frozen in liquid nitrogen and stored at -20° C.

The performance of membrane affinity columns was monitored by measuring antibody activity in the borate and glycine washes. The borate and glycine washes from adipocyte plasma membrane columns were adjusted to the same protein concentration and tested, by ELISA, against adipocyte plasma membranes. The borate and glycine washes from the hepatocyte, kidney and erythrocyte plasma membrane columns were tested, by ELISA, against adipocyte, hepatocyte, kidney and erythrocyte membranes. For the preparation of chicken and rat antigens, sheep antisera raised against whole chicken and rat adipocyte plasma membranes respectively were used. For the preparation of sheep antigens, a goat antiserum, raised against whole sheep adipocyte plasma membranes, was used. The antigens prepared were characterised by SDS polyacrylamide gel electrophoresis and Western blotting.

2.10 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS polyacrylamide gel electrophoresis was performed using a Bio-Rad Mini Protean II electrophoresis system (Bio-Rad, Watford, Herts), using the buffer systems of Laemmli (1970). Samples for electrophoresis were boiled in 62.5 mM Tris-HCl pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 0.001% bromophenol blue and 10% glycerol. The 12% running gel was composed of 375 mM Tris-HCl pH 6.8, containing 12% acrylamide, 0.07% methylene-bis-acrylamide, 0.1% SDS, 0.034% tetramethylethylenediamine (Temed) and 0.01% ammonium persulphate. The stacking gel was composed of 125 mM Tris pH 6.8, containing 5% acrylamide, 0.15% methylene-bis-acrylamide, 0.1% SDS, 0.05% Temed and 002% ammonium persulphate. The upper running buffer

% are w/v except for 2-mercaptoethanol, glycerol and Temed which are v/v

was composed of 25 mM Tris-HCl pH 8.3, containing 192 mM glycine and 0.1% SDS. The lower running buffer had the same composition, except that glycine was excluded. Gels were run for 45-75 minutes at 150 mV. Gels to be stained for protein were fixed in 45% (v/v) methanol and 9% acetic acid for 15 minutes. They were stained for 30 minutes in the same fixative containing 0.1% (w/v) Coomassie blue. Gels were destained in 7% (w/v) acetic acid, 5% (w/v) methanol.

2.11 WESTERN BLOTTING

Transfer of proteins onto nitrocellulose was performed using a semi-dry electroblotter (Sartorius, Belmont, Surrey). The graphite plates of the electroblotter were rinsed with distilled water. Filter paper, nitrocellulose membrane and dialysis membranes were trimmed to the size of the gel. Two layers of filter paper soaked in anode buffer no. 1 (300 mM Tris-HCl pH 10.4, containing 20% methanol) were placed on the anodic graphite plate. A 'trans-unit' was then assembled, composed of 1 layer of filter paper soaked in anode buffer no. 2 (25 mM Tris-HCl pH 10.4, containing 20% methanol), on top of which was placed a nitrocellulose membrane soaked in distilled water. Next was placed the polyacrylamide gel soaked in anode buffer no. 2. An additional layer of filter paper soaked in cathode buffer (25 mM Tris-HCl pH 9.3, containing 40 mM 6-amino-n-hexanoic acid and 20% methanol) was placed on the gel. Finally, a dialysis membrane, soaked in distilled water was placed on top of the trans-unit. Several trans-units could be stacked on top of each other. The stack was covered with 2 layers of filter paper soaked in cathode buffer Electroblotting was carried out for 1 h at 0.8 mA/cm^2 of gel. After

electroblotting the gel was stained with Coomassie blue, as described in Section 2.10, to ensure that all the protein had transferred onto the nitrocellulose membrane. The nitrocellulose was washed twice in transblot saline (50 mM Tris-HCl pH 10.3, containing 150 mM NaCl). Free binding sites were blocked with transblot saline containing 0.5% Tween 20 for 1½ h at 22° C. The nitrocellulose was then washed 5 times with transblot saline and incubated with the first antibody, which was raised in sheep, diluted 250-500 times in transblot saline, containing 0.5% Tween, for 16 h at room temperature. After washing 5 times with transblot saline, a hundred-fold dilution of donkey anti-(sheep IgG) serum in transblot saline containing 0.5% Tween was added. After incubation at 37° C for 1½ h, the nitrocellulose was washed 5 times with transblot saline. A hundred-fold dilution of peroxidase-anti-peroxidase complex, raised in sheep, was then added and incubated for 1½ h at room temperature. The membrane was washed 5 times with transblot saline and twice with 50 mM Tris-HCl pH 7.6. The membrane was then incubated in 50 mM Tris-HCl pH 7.6, containing 0.05 (w/v) 4-chloro-naphthol and 0.011% H_2O_2, until colour development was considered optimal. The reaction was stopped by rinsing in distilled water and membranes were dried between filter paper and stored in the dark.

2.12 SERUM ASSAYS

2.12.1 Complement

Rat serum complement levels were measured by assessing the ability of the test sera to support complement-mediated lysis of sensitized chicken red blood cells. 50 μl of 0.5% sheep anti-chicken

erythrocyte) antiserum was incubated with 50μl of chicken erythrocytes (2.5% haematocrit) and 50 μl of 1-10% test rat serum to serve as a source of complement. Dose-response curves were performed for normal and test sera and the relative concentration of serum required to support the same degree of lysis as the control was determined.

2.12.2 Glucose

Serum glucose was measured using a glucose analyser, Analox GM-6 (Analox Instruments, London) according to the manufacturer's instructions.

2.12.3 Insulin

Rat serum insulin was measured by radioimmunoassay. The buffer used for all dilutions was 50 mM Na_2HPO_4 pH 7.4, containing 0.15 M NaCl, 15 mM NaN_3, 0.5% BSA (RIA grade, fraction v, Sigma Chemical Co., Poole, Dorest). 25 μl of test serum was incubated with 100μl of 0.01% anti-(insulin) serum (raised to bovine insulin in guinea pigs by Dr. D. J. Flint) at 4° C for 20 h in a total volume of 300μl. Approximately 20,000 cpm (0.1 ng) ^{125}I-labelled insulin was then added in a volume of 100μl and the incubations were continued at 4° C. Porcine insulin (Sigma Chemical Co., Poole, Dorset) was iodinated using the lactoperoxidase method (see Morrison & Bayse, 1970). After 20 h 300 μl of second antibody, containing 12μl of anti-(guinea-pig) precipitating serum, 150 μl 16% polyethylene glycol, 14 mg EDTA, 1 μl normal guinea-pig serum and 150 μl of buffer. The samples were incubated at 22° C for 5 h and then centrifuged at 2,500 g for 30 minutes. The supernatant was removed

and the pellet was counted in a gamma counter (Rackgamma 11, LKB Instruments Ltd., Selsdon, Surrey). The insulin content of the pellet was determined using a standard curve which was obtained by performing the assay as described above but with a range of concentrations of porcine insulin (0-10 ng/ml) replacing the serum sample.

2.12.4 Triglycerides and free fatty acids

Serum triglycerides and non-esterified free fatty acids were measured using Wako kits (Alpha Laboratories Ltd., Eastleigh, Hampshire) according to the manufacturer's instructions.

2.12.5 Urea

Serum urea nitrogen was measured using kit no. 640 (Sigma Chemical Co., Poole, Dorset), according to the manufacturer's instructions.

2.13 PROTEIN ASSAY

Protein was assayed using the Bio-Rad protein assay reagent. 200 μl of 20% (v/v) Bio-Rad reagent in distilled water was added to 50 μl of protein solution and the absorbance was measured at 600 nm after a 15 minute incubation at 22° C. When membrane or antibody protein solutions were measured, sheep IgG was used as a standard. When protein concentrations of urine were to be measured BSA was used as a standard. Where the concentrations of detergent-solubilized proteins were measured a maximum of 50 μl of 0.02% Triton could be used without interfering with colour development.

2.14 BODY COMPOSITION ANALYSIS

2.14.1 Neutron activation analysis

Neutron activation analysis of live rats was performed according to the method of Preston, Reeds, East and Holmes (1985) at the Scottish Universities Research and Reactor Centre, East Kilbride. This provided a measure of body nitrogen, phosphorus, chlorine, sodium, calcium, potassium and oxygen. The body nitrogen value was multiplied by 6.25 to obtain an estimation of total body protein.

(Reeves, Schouten and Dalrymple, 1974)

2.14.2 Estimation of total body water by tritiated water dilution

Rats were injected i.p. with 20 μCi of tritiated water (Amersham International, Amersham, Bucks.). Rats were bled from the tail under ether anaesthesia at 1, 2, 3 and 6 days after injection. Serum protein was precipitated by the addition of an equal volume of 6% perchloric acid. After 10 minutes, 300 μl aliquots of protein-free serum were added to emulsifier scintillant and counted on a Packard counter (Tricarb 240S, Packard Instruments, Caversham, Berks.). A blank was included containing equal volumes of 6% perchloric acid and distilled water. By plotting the logarithm of counts per minute per ml (cpm/ml) against time, the cpm/ml at time 0 could be calculated by extrapolation. The dilution factor, i.e. total body water, could be determined from the cpm/ml of the original injected solution.

2.14.3 Rat carcass analysis

After dissection of the major fat depots and removal of the stomach and intestines, rats were freeze-dried in a centrifugal

freeze drier, Model 30 T12/796 (Edwards High Vacuum Ltd., Sussex), for 5-7 days until the weight of the dried carcass no longer changed. Dry weights of the dissected depots were determined by heating at 70° C for 2-4 days. 0.4 times the wet weight of the stomach and intestines had previously been shown to approximate to the wet weight of the stomach and intestinal wall, 30% of which was assumed to be dry matter. Subtraction of the dry weight of the carcass, gut wall and adipose depots from the wet weight of the same provided an estimation of their water content.

The lipid content of the carcass was determined by ether extraction in a Soxhlet apparatus at Western General Hospital, Edinburgh by Mr. D. Shirling.

Total body nitrogen was determined by the Kjeldahl method and was multiplied by 6.25 to obtain total body protein. Body minerals were estimated by difference.

2.14.4 Lamb carcass analysis

Carcass dissection of lambs was performed at the Macaulay Land Use Research Institute (Penicuik, Midothian) according to the Meat and Livestock Commission's standard.

CHAPTER 3

PASSIVE IMMUNISATION OF RATS WITH AN ANTI-(ADIPOCYTE PLASMA MEMBRANE) ANTISERUM (A/S 83)

PASSIVE IMMUNISATION OF RATS WITH AN ANTI-(ADIPOCYTE PLASMA MEMBRANE) ANTISERUM (A/S 83)

3.1 INTRODUCTION

The administration of an anti-(adipocyte plasma membrane) antiserum (A/S 83) to 6-week old rats caused a 50% reduction in mass of parametrial adipose tissue 8 days after treatment (Flint et al., 1986). The inital aim of the work described in this chapter was to determine whether lost adipose tissue could be regenerated. This was difficult to predict since studies of the effects of surgical removal of adipose tissue have reported no regeneration (Kral, 1976), partial regeneration (Bailey and Anderson, 1980), total regeneration of lost tissue (Taylor and McBean-Hopkins, 1971) or compensatory hypertrophy of remaining depots (Larson and Anderson, 1978; Bailey and Anderson, 1980). Treatment with A/S 83 could, therefore, result in the recovery of lost adipose tissue mass. Alternatively a permanent reduction in mass of adipose tissue and, consequently, a decrease in total body mass, could occur. The final possibility was that energy released from adipose tissue and possibly excess energy intake could be used by other tissues, including possible 'repartitioning' into protein deposition, resulting in improved protein:fat ratios in the carcass. In order to investigate these possibilities, body mass, food intake and body composition of A/S 83-treated rats were examined.

Before the development of antibiotics, diseases, such as diptheria, were treated by the administration of large quantities of antiserum, usually raised in the horse. Serum sickness sometimes occurred, caused by the patient's immune response to the injected

foreign protein. Since A/S 83 was raised in a sheep, the rat immune response to sheep γ globulin was examined and kidney function was monitored.

Intraperitoneal injection of A/S 83 caused a greater reduction in mass of parametrial than of peri-renal or subcutaneous adipose tissue. This was despite the absence of differences in specificity of A/S 83 for adipocyte plasma membranes of different depots (Flint et al., 1986). The importance of the site of injection on the relative effects of A/S 83 on different adipose depots was, therefore, determined. The effects of different durations of treatment with A/S 83 were also examined.

The administration of A/S 83, but not non-immune sheep γ globulin, resulted in a reduction in food intake that was most dramatic during the 24 h after the first treatment (Flint et al., 1986). A role for serum glucose, free fatty acids, triglycerides and insulin in the control of food intake has been suggested (Harris and Martin, 1984). The short-term effects of A/S 83 on these metabolites and hormones were, therefore, investigated.

While the work of Flint et al. (1986) demonstrated complement-mediated cytotoxicity of A/S 83 against adipocytes in vitro, the in vivo importance of complement in mediating the effects of A/S 83 was not demonstrated. Therefore the in vivo effects of A/S 83 administration on circulating complement levels and the effect of complement-depletion on A/S 83-mediated effects were investigated in vivo.

Gross abnormalities of parametrial adipose tissue were observed 8 days after treatment with A/S 83 (Flint et al., 1986). In order to determine whether this resulted in damage to the underlying uteri or

ovaries, the ability of A/S 83-treated animals to mate and undergo a normal pregnancy was examined.

3.2 AN ASSESSMENT OF THE LONG-TERM EFFECTS OF PASSIVE IMMUNISATION OF RATS WITH A/S 83

3.2.1 Experimental

Female Wistar rats, weighing 120-140 g, were injected intraperitoneally, between 09.00 h and 11.00 h on 4 consecutive days, with the equivalent of 2 ml of A/S 83 γ globulin. These rats were killed 1, 3, 8 and 24 weeks after the first injection. An equivalent group of rats were pair fed with the treated group for 5 days after the first injection. These rats were killed at 1, 3 and 8 weeks after treatment. A second group of control rats were injected intraperitoneally, on 4 consecutive days, with the equivalent of 2 ml of non-immune sheep γ globulin. These animals were pair fed with the treated group for 4 days after the first injection and were killed 24 weeks after the first injection. Non-immune sheep γ globulin had previously been shown to have no effects on body weight gain, food intake or adiposity, but was included in the second group of controls in order to study the rat immune response to sheep γ globulin.

Blood samples were taken from the tail, under ether anaesthesia, at 1, 2, 3, 5, 10 and 24 weeks after treatment. Serum glucose, non-esterified free fatty acids, triglycerides, insulin and urea and urine protein concentrations were measured as described in sections 2.12 and 2.13. Anti-(sheep γ globulin) responses were measured by ELISA. Body weight gain and food intakes were determined daily, for the first 3 weeks after treatment and weekly thereafter.

Five weeks after treatment 6 A/S 83-treated rats and 6 untreated controls were subjected to neutron activation analysis to obtain estimations of total body protein and minerals, according to the method of Preston et al (1984). Body water was measured by tritiated water dilution (see Section 2.14.2) enabling body fat to be calculated by difference.

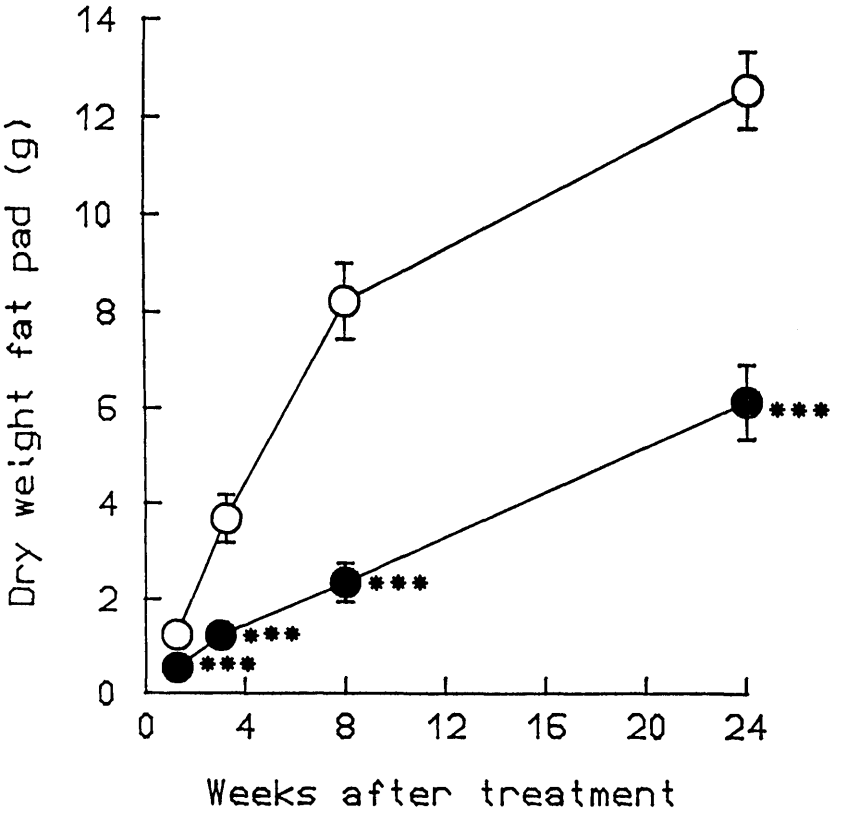
Rats were killed 1, 3, 8 and 24 weeks after treatment by cervical dislocation and the dry weights, mean adipocyte volumes and adipocyte numbers of inguinal subcutaneous, parametrial and peri-renal fat depots were determined. The wet weights of the liver, kidneys, spleen, gut and adrenals were also determined. Carcass analysis was performed on rats killed 8 weeks after treatment, as described in Section 2.14.3.

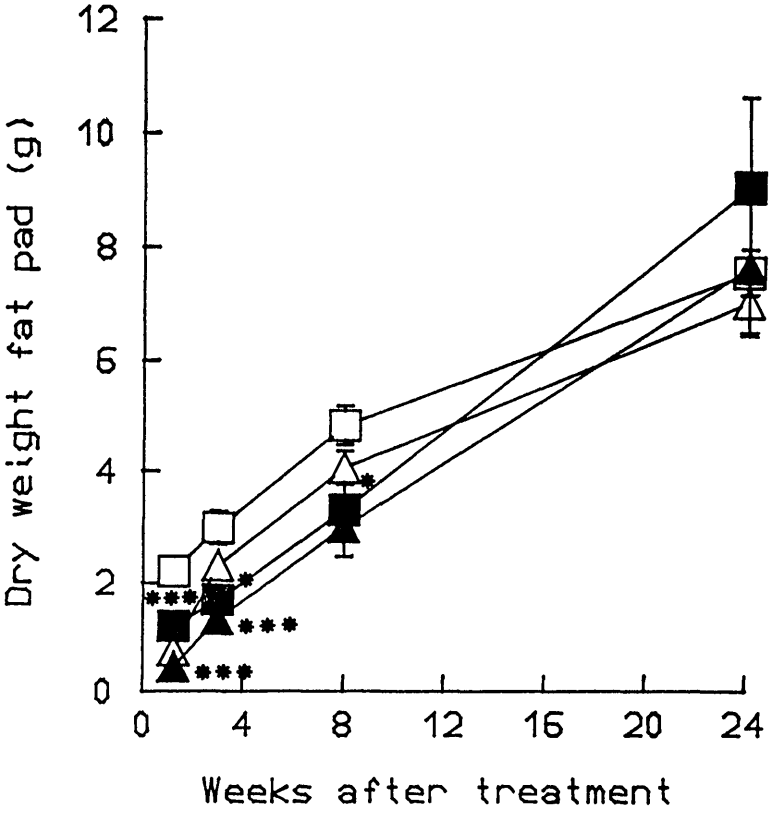
3.2.2 Results

The dry weight of the parametrial fat pad was significantly reduced in A/S 83-treated rats, compared with controls, 1,3, 8 and 24 weeks after treatment (Fig. 3.1). This reduction was due to reduced adipocyte numbers and adipocyte volume 3 and 8 weeks after treatment and to reduced adipocyte numbers alone 24 weeks after treatment (Fig.s 3.2, 3.3). The dry weight of the parametrial fat pad showed a 71% and 49% reduction in treated animals after 8 and 24 weeks, respectively. In contrast, the dry weights of peri-renal and subcutaneous fat pads 8 weeks after treatment showed reductions of only 32 and 26% respectively (Fig. 3.1). This was due, primarily, to a reduction in mean adipocyte volume, although this was not significant. Twenty four weeks after treatment the dry weights, mean adipocyte volumes and adipocyte numbers of peri-renal and subcutaneous adipose depots had completely recovered.

Fig. 3.1. The effects of A/S 83 on the dry weight of fat pads 1, 3, 8 and 24 weeks after treatment

Rats were injected intraperitoneally with the equivalent of 2 ml of A/S 83 γ globulin for 4 consecutive days (filled symbols). Control rats were pairfed with the treated group for 5 days and then allowed to feed ad libitum (open symbols). Dry weights of the parametrial (circles), peri-renal (triangles) and inguinal subcutaneous (squares) fat pads were determined. Results are mean ± SEM of 3-8 observations. *P<0.01, ***P<0.001, compared with controls (Student's unpaired t test).


Dry weight fat pad (g)
0
2
4
6
8
10
12
14
0
4
8
12
16
20
24
Weeks after treatment
***
***
***


Dry weight fat pad (g)
0
2
4
6
8
10
12
0
4
8
12
16
20
24
Weeks after treatment
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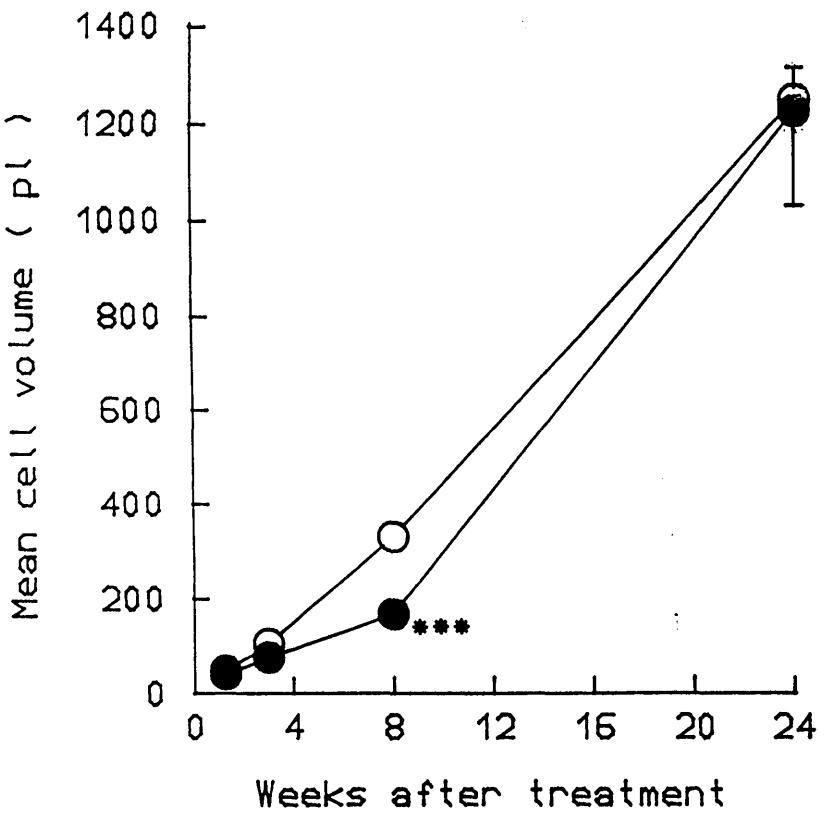
***

Fig. 3.2. The effects of A/S 83 on mean adipocyte volume 1, 3, 8 and 24 weeks after treatment

Treatment and symbols as for Fig. 3.1. Mean adipocyte volumes were determined by collagenase digestion of the tissue and the measurement of the diameters of 100 adipocytes. Results are mean ± SEM of 3-8 observations. ***P<0.001, compared with controls (Student's unpaired t test).


Mean cell volume (pl)
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1200
1000
800
600
400
200
0
0
4
8
12
16
20
24
Weeks after treatment
***

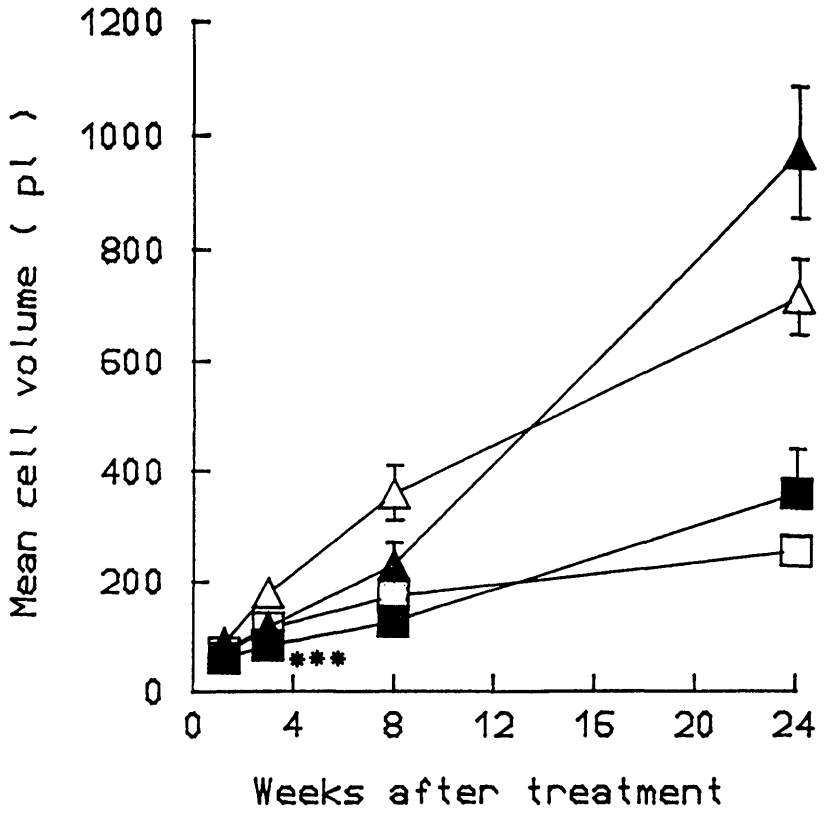
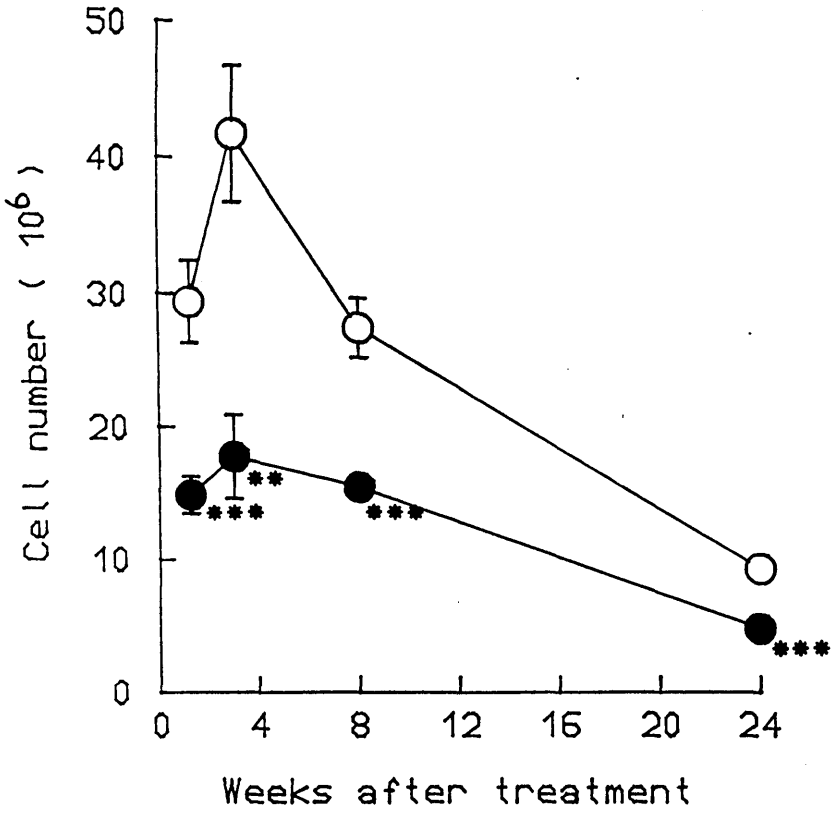

Mean cell volume (pl)
1200
1000
800
600
400
200
0
0
4
8
12
16
20
24
Weeks after treatment
***

Fig. 3.3. The effects of A/S 83 on adipocyte numbers 1, 3, 8 and 24 weeks after treatment

Treatment and symbols as for Fig. 3.1. Adipocyte numbers were calculated from the dry fat pad weights and mean adipocyte volumes. Results are mean $\pm$ SEM of 3-8 observations. $^{*}P<0.01$, $^{**}P<0.05$, $^{***}P<0.001$, compared with controls (Student's unpaired t test).


Cell number (10^6)
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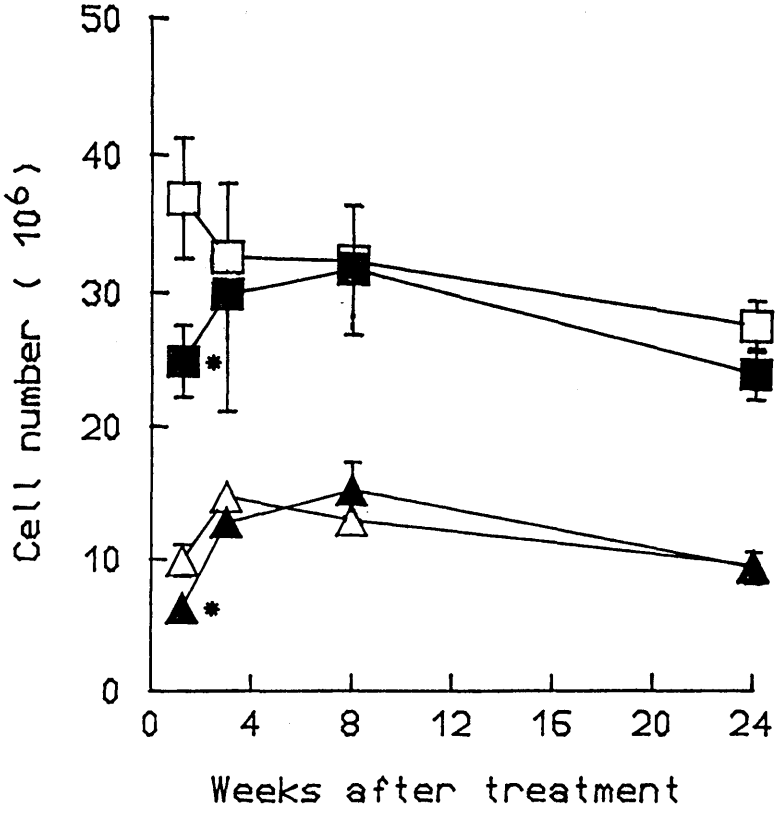
40
30
20
10
0
0
4
8
12
16
20
24
Weeks after treatment
*
**
***


Cell number (10^6)
50
40
30
20
10
0
0
4
8
12
16
20
24
Weeks after treatment
*

Between 1 and 8 weeks the parametrial adipose depots of A/S-treated rats accumulated about 33% of the lipid accumulated by controls while, between 8 and 24 weeks, the same depot in A/S-treated rats accumulated 86% of that of controls (Table 3.1). Lipid accumulation in peri-renal and subcutaneous fat pads was slightly reduced in A/S-treated rats between 1 and 8 weeks but was nearly twice that of controls between 8 and 24 weeks after treatment (Table 3.1).

Adipocytes isolated from parametrial adipose tissue 3 and 8 weeks after treatment showed no significant differences in basal and insulin-stimulated glucose incorporation into lipid or in basal or noradrenalin-stimulated glycerol release (Figs 3.4, 3.5).

The relative antibody binding of A/S 83 to hepatocyte, kidney and erythrocyte plasma membranes were, respectively, 32, 25 and 7% of that to adipocyte plasma membranes. Relative antigen abundances against hepatocyte, kidney and erythrocyte plasma membranes were, respectively, 100, 66 and 37% of that against adipocyte plasma membranes (Fig. 3.6). Western blotting of A/S 83 against adipocyte and hepatocyte plasma membranes showed that A/S 83 bound to at least 19 major polypeptides of the adipocyte plasma membrane and at least 7 of the hepatocyte plasma membrane (Fig. 3.7). No significant differences in the wet weights of liver, kidney or spleen were observed between control and A/S 83-treated rats 1, 3, 8 and 24 weeks after treatment, except the spleen weight, which was elevated in A/S-treated rats after 1 week (Table 3.2).

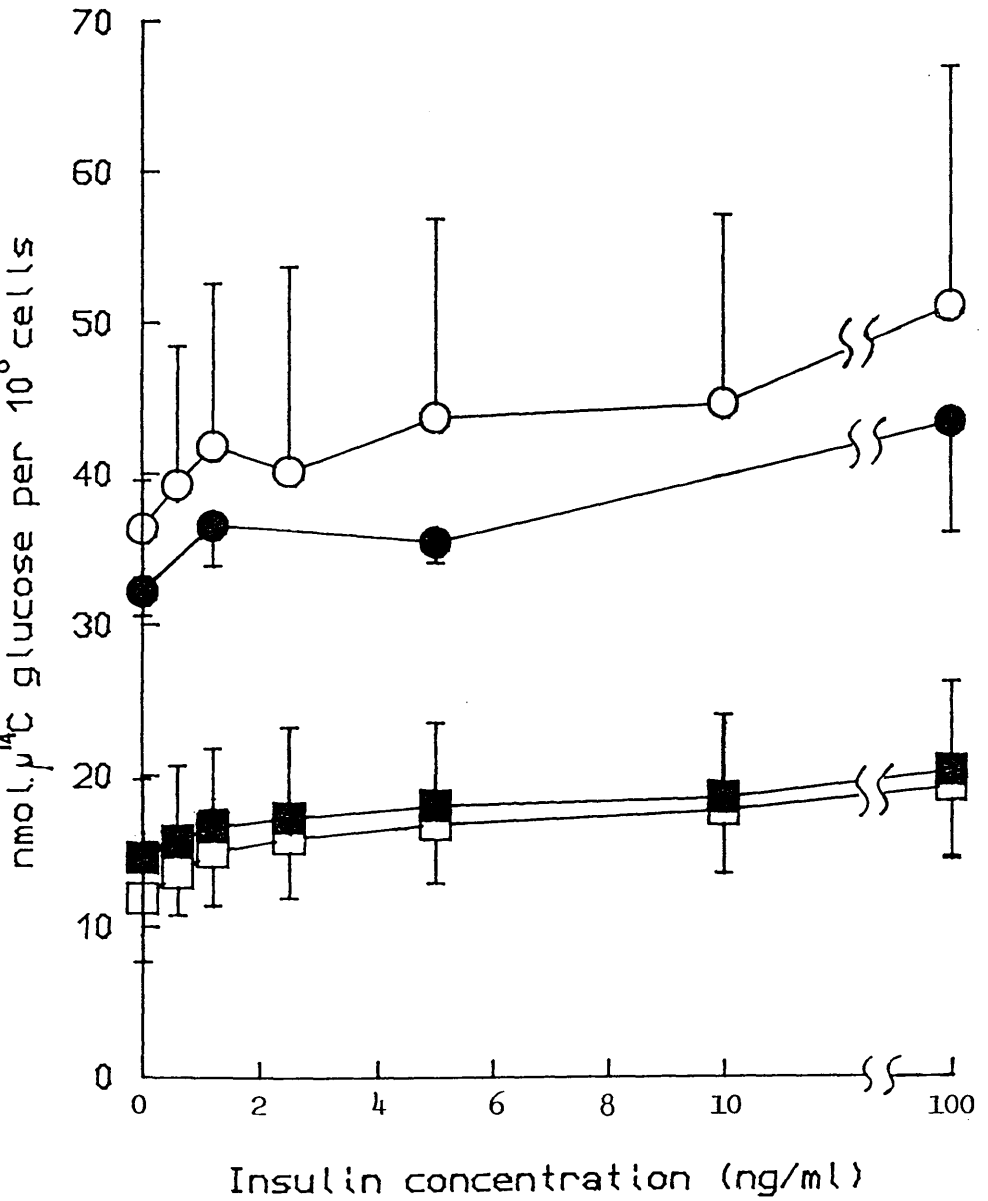
A/S-treated rats showed an 8-10 g drop in body weight, accompanied by a 75% fall in food intake, on the first day of treatment (Fig. 3.8). The rats showed no signs of distress. Some

Table 3.1. The effects of A/S 83 on lipid accumulation in adipose depots between 1 and 24 weeks after treatment

Treatment	Time after treatment (weeks)	Lipid accumulation (g/depot)			
		Parametrial	Peri-renal	Inguinal subcutaneous	Total dissected
Control	1- 8	5.4	3.3	2.6	11.4
	8-24	4.4	2.9	2.8	10.1
A/S 83	1- 8	1.8	2.5	2.1	6.5
	8-24	3.8	4.6	5.7	14.1

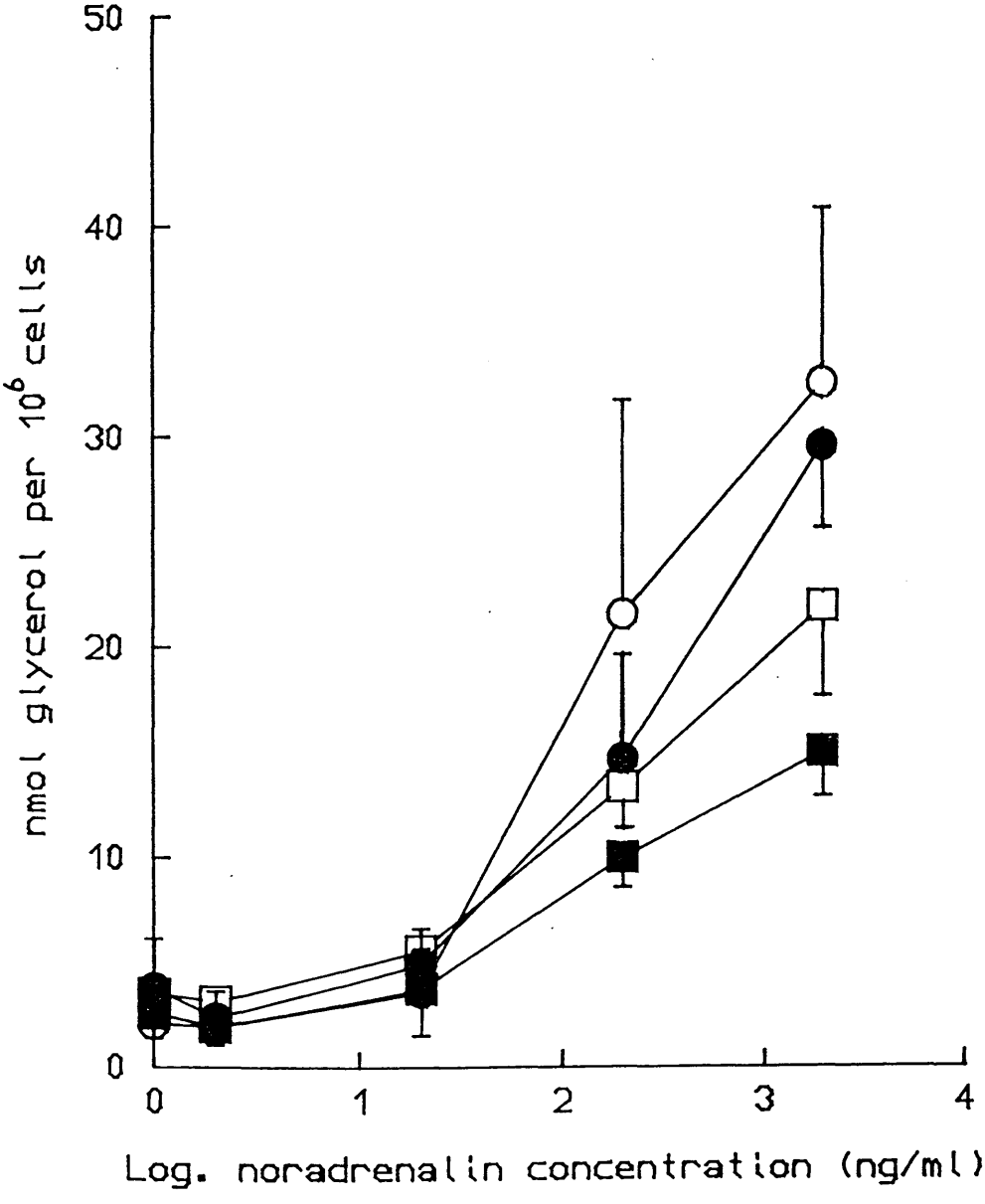
Rats were injected intraperitoneally with the equivalent of 2 ml of A/S 83 γ globulin for 4 consecutive days. Control rats were pairfed with the treated group for 5 days and then allowed to feed ad libitum until the animals were killed at 1, 8 or 24 weeks after the first treatment. Lipid accumulation was calculated by subtracting the dry weights of fat pads of animals killed at the first time point from those of animals killed at the second time point. Results are 3-9 observations.

Fig. 3.4. The stimulation of glucose incorporation into lipid by insulin in parametrial adipocytes isolated from A/S 83-treated and control rats



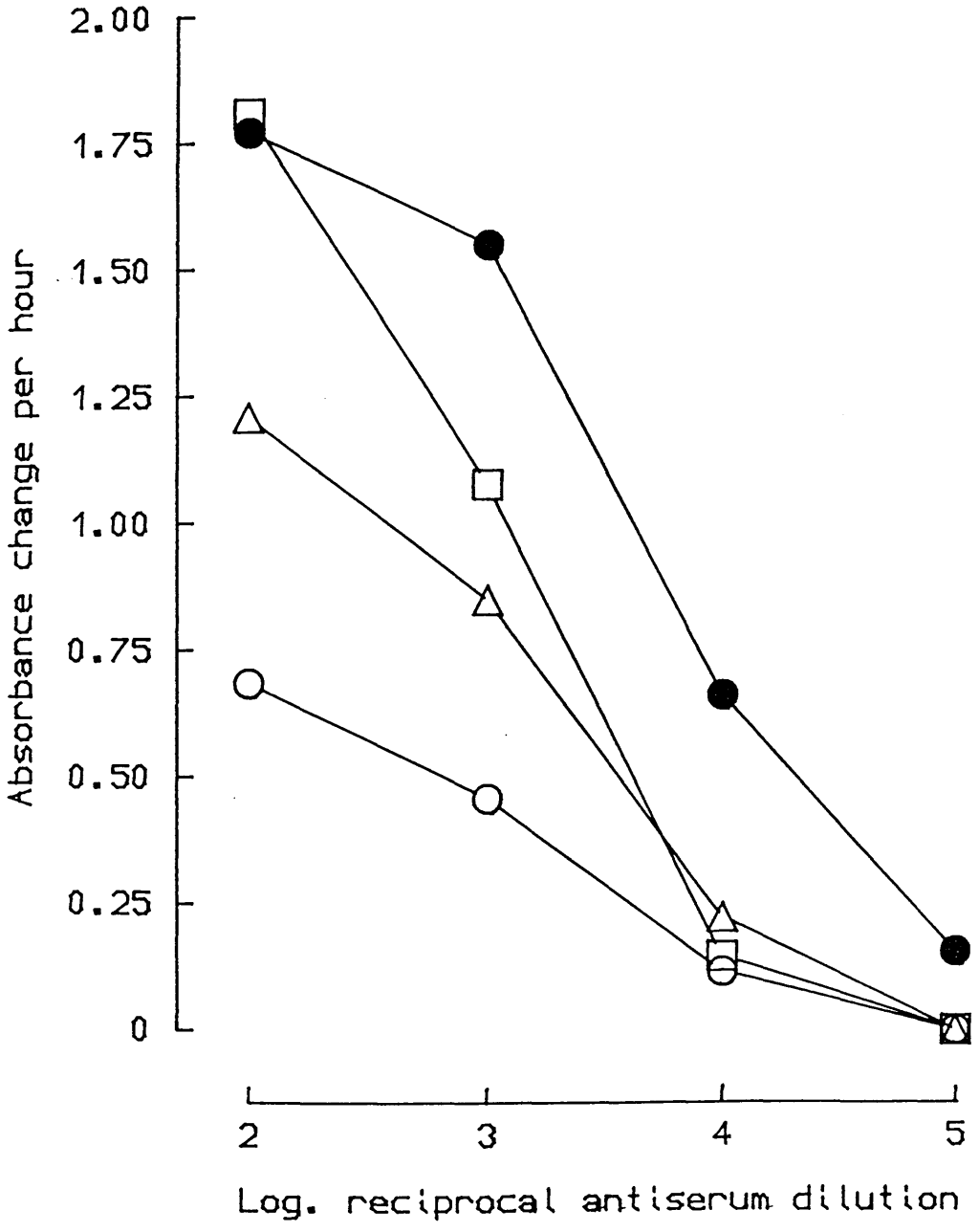
Adipocytes isolated from A/S 83-treated (filled symbols) and control (open symbols) rats 3 weeks (circles) and 8 weeks (squares) after treatment were incubated in Krebs Ringer phosphate pH 7.4, containing 1% BSA, 0.1 % glucose, μ-^{14}C-glucose and insulin for 2 h at 37° C. Results are mean ± SEM of 3-5 observations.

Fig. 3.5. The stimulation of glycerol release by noradrenalin in parametrial adipocytes isolated from A/S 83-treated and control rats



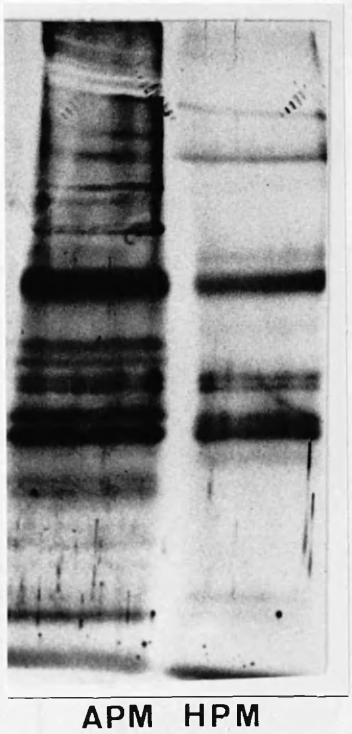
Symbols as for Fig. 3.4. Adipocytes were incubated in Krebs Ringer phosphate pH 7.4, containing 1% BSA, 0.1% glucose and noradrenalin for 2 h at 37° C. Results are mean ± SEM of 3-5 observations.

Fig. 3.6. The binding of A/S 83 to adipocyte, hepatocyte, erythrocyte and kidney plasma membranes as determined by ELISA



Binding of A/S 83 to rat adipocyte (filled circles), hepatocyte (open squares), erythrocyte (open circles) and kidney (open triangles) plasma membranes was measured by ELISA. Samples were assayed in duplicate and results have been corrected for non-specific binding, using values obtained with non-immune sheep γ globulin.

Fig. 3.7. The binding of A/S 83 to adipocyte and hepatocyte plasma membranes as determined by Western blotting



Adipocyte plasma membranes (APM) and hepatocyte plasma membranes (HPM) were separated by SDS PAGE and transferred to nitrocellulose and binding of A/S 83 to transferred proteins was detected, as described in Section 2.11.

Table 3.2. Liver, kidney and spleen weights 1, 3, 8 and 24 weeks after treatment with A/S 83

Weeks after first treatment	Wet weight (g)					
	Liver		Kidneys		Spleen	
	Control	A/S	Control	A/S	Control	A/S
1	8.7 ±0.3	8.8 ±0.2	1.60 ±0.37	1.48 ±0.05	0.78 ±0.18	1.29* ±0.10
3	10.6 ±0.6	11.2 ±1.1	1.83 ±0.09	2.30 ±0.24	0.65 ±0.05	0.66 ±0.07
8	10.3 ±0.4	11.3 ±1.2	1.92 ±0.10	2.34 ±0.30	0.61 ±0.04	0.72 ±0.04
24	13.1 ±1.0	15.8 ±1.8	2.23 ±0.15	2.64 ±0.16	0.74 ±0.05	0.84 ±0.04

Rats were injected intraperitoneally with the equivalent of 2 ml of A/S 83 γ globulin for 4 consecutive days. Control rats were pairfed with the treated group for 5 days and then allowed to feed ad libitum. Results are means ± SEM for 3-9 observations. *$P<0.01$, compared with controls (Student's unpaired t test).

rats showed slightly abnormal postures for the first 2 h after treatment but showed no signs of pain in the area of injection. Food intake and body weight gain returned to normal levels by the fourth day of treatment. During the first 2 weeks after treatment the A/S 83-treated rats that were killed 24 weeks after treatment showed 2% more weight gain but 7% less food intake than controls (Table 3.3). A/S-treated rats gained 23% more weight and ate 12% more food than controls between 2 and 6 weeks after treatment. These differences resulted in a 9% increase in food conversion efficiency (FCE), calculated as food intake divided by body weight gain, in A/S-treated rats, compared with controls, during the first 6 weeks after treatment. From weeks 6 to 20, FCEs of the 2 groups were equal because A/S-treated rats gained 7% more weight but ate 7% more food than controls. The differences in body weight gain were not statistically significiant. Food intake was measured on groups of rats, rather than individuals, and so statistical significance of differences in food intake and efficiency could not be determined.

Control rats, pairfed with A/S 83-treated rats, showed a voluntary reduction in water intake of the same magnitude as the enforced reduction in food intake. A/S 83-treated rats, however, showed no reduction in water intake (Fig. 3.8). Subsequently, there was no difference in water intake between control and treated rats (results not shown).

Neutron activation analysis 5 weeks after treatment showed a 38% reduction in total body fat in A/S-treated rats, compared with controls, which was balanced by an increase in total body water and protein (Table 3.4). Carcass analysis after 8 weeks showed a 29% reduction in body fat and increases in body water and body protein (Table 3.4).

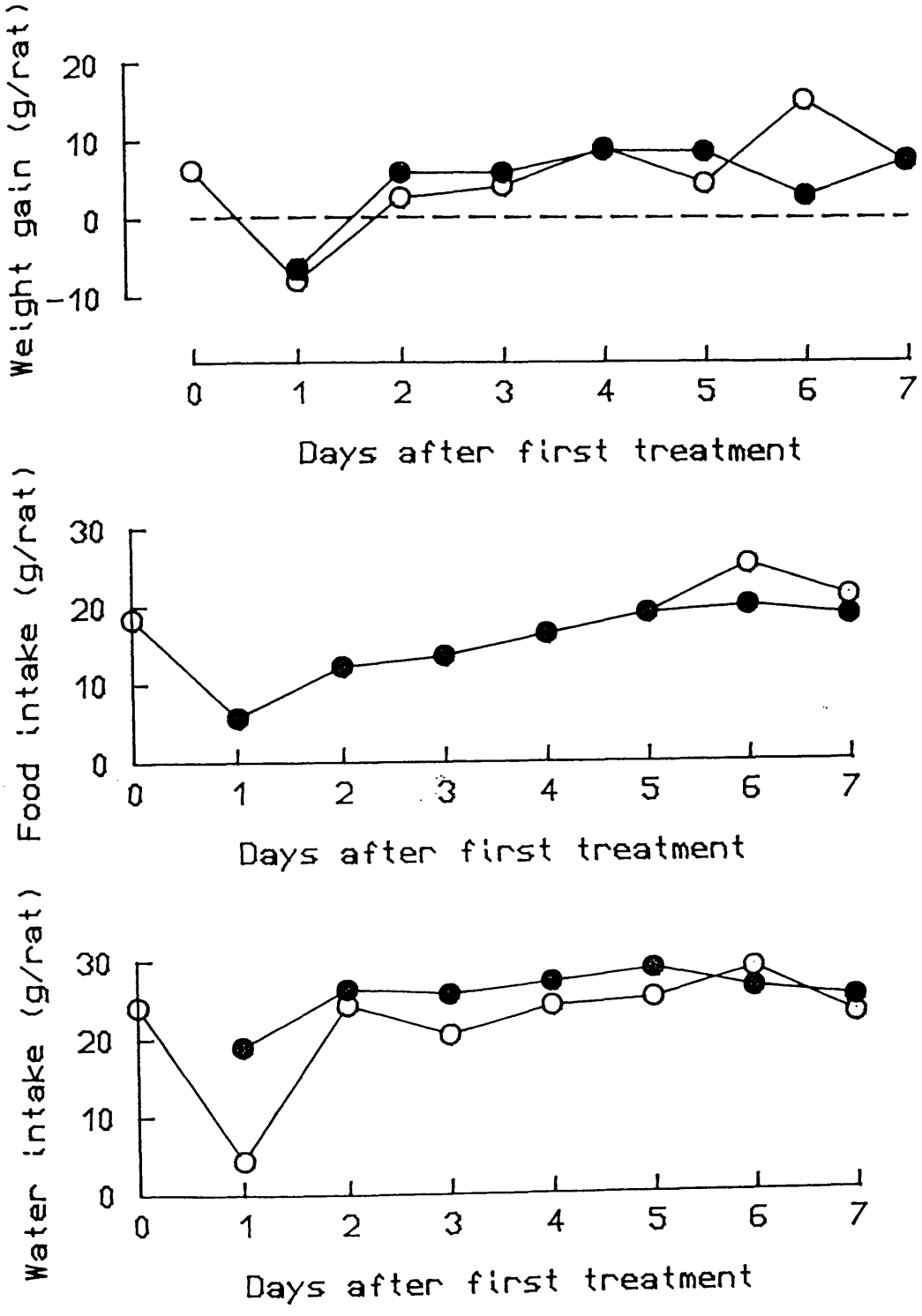
Table 3.3. The long-term effects of A/S on body weight gain food intake and food conversion efficiency

	Treatment	Number of observations	Weeks after treatment 0–2	2–6	6–20
Body weight gain (g/rat)	NS	8	67 ±3	68 ±6	72 ±5
	A/S 83	5	69 ±5	84 ±4	78 ±12
Food intake (g/rat)	N/S	8	301	646	1997
	A/S 83	5	279	724	2133
$\frac{\text{Intake}}{\text{Gain}}$	N/S	8	4.5	9.5	27.6
	A/S 83	5	4.1	8.7	25.5

Rats were injected intraperitoneally with the equivalent of either 2 ml of A/S 83 γ globulin or 2 ml of non-immune sheep γ globulin (N/S) for 4 consecutive days. N/S-treated rats were pairfed with the A/S-treated group for the first 5 days and then allowed to feed ad libitum.

Fig. 3.8. The short-term effects of A/S 83

on body weight gain, food intake and water intake



Rats received the equivalent of 2 ml of A/S 83 γ globulin intraperitoneally for 4 consecutive days (filled circles). Control rats were pairfed with the treated group for 5 days and were then allowed to feed ad libitum (open circles). All rats were allowed free access to water throughout. Body weight gains are means ± SEM of 10 observations.

Table 3.4. Body composition studies of A/S-treated and control rats 5 and 8 weeks after treatment

Treatment	Number of observations	Time after treatment	Water (g/kg)	Protein (g/kg)	Minerals (g/kg)	Fat (g/kg)
Control	6	5	674 ±6	183 ±2	34 ±2	109 ±7
A/S 83	6	5	710** ±9	188 ±3	34 ±2	68*** ±7
Control	6	8	574 ±12	199 ±3	46 ±2	182 ±12
A/S 83	4	8	614** ±11	209 ±2	49 ±2	129*** ±13

Rats were injected intraperitoneally with the equivalent of 2 ml of A/S 83 γ globulin for 4 consecutive days. Control rats were pairfed with the treated group for the first 5 days and then allowed to feed ad libitum. Body composition was measured by neutron activation analysis and tritiated water dilution at 5 weeks and by carcass analysis at 8 weeks, all as described in Section 2.14. Results are means ± SEM. **P<0.005, ***P<001, compared with controls (Student's unpaired t test).

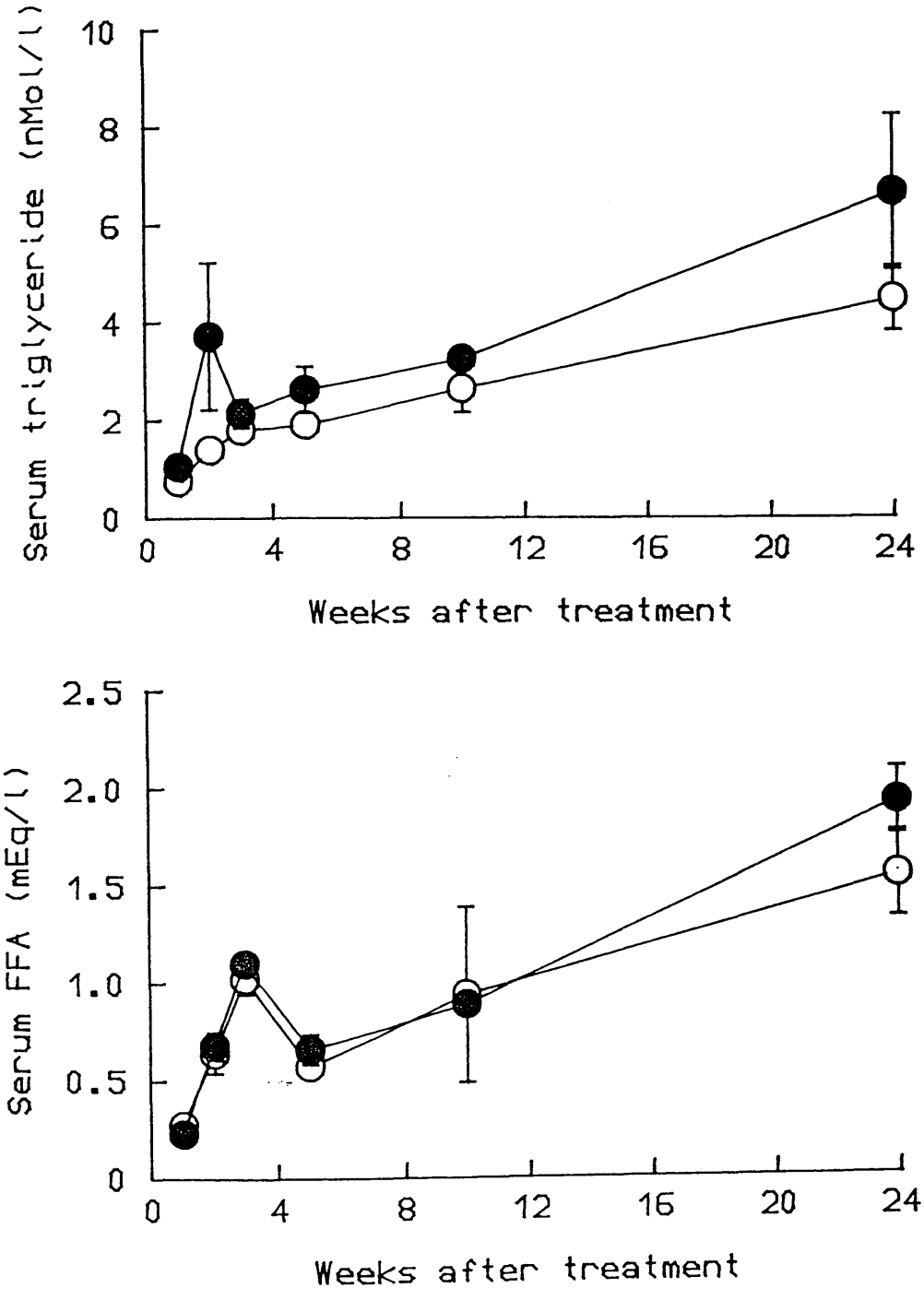
No significant differences between serum glucose, triglyceride, non-esterified free fatty acids or insulin were found between control and A/S 83-treated rats (Fig. 3.9, 3.10). A/S 83-treated rats showed a greater anti-(sheep IgG) response than non-immune sheep γ globulin-treated rats (Fig. 3.11). The response in both groups was first measurable 2 weeks after treatment and reached a peak 5 weeks after treatment, before gradually declining. Some A/S 83- and non-immune sheep γ globulin treated rats showed a transient proteinurea and fluctuations in body weight gain between 1 and 3 weeks after treatment (Fig. 3.12).

3.2.3 Discussion

3.2.3.1 The effects of A/S 83 on parametrial adipocyte number

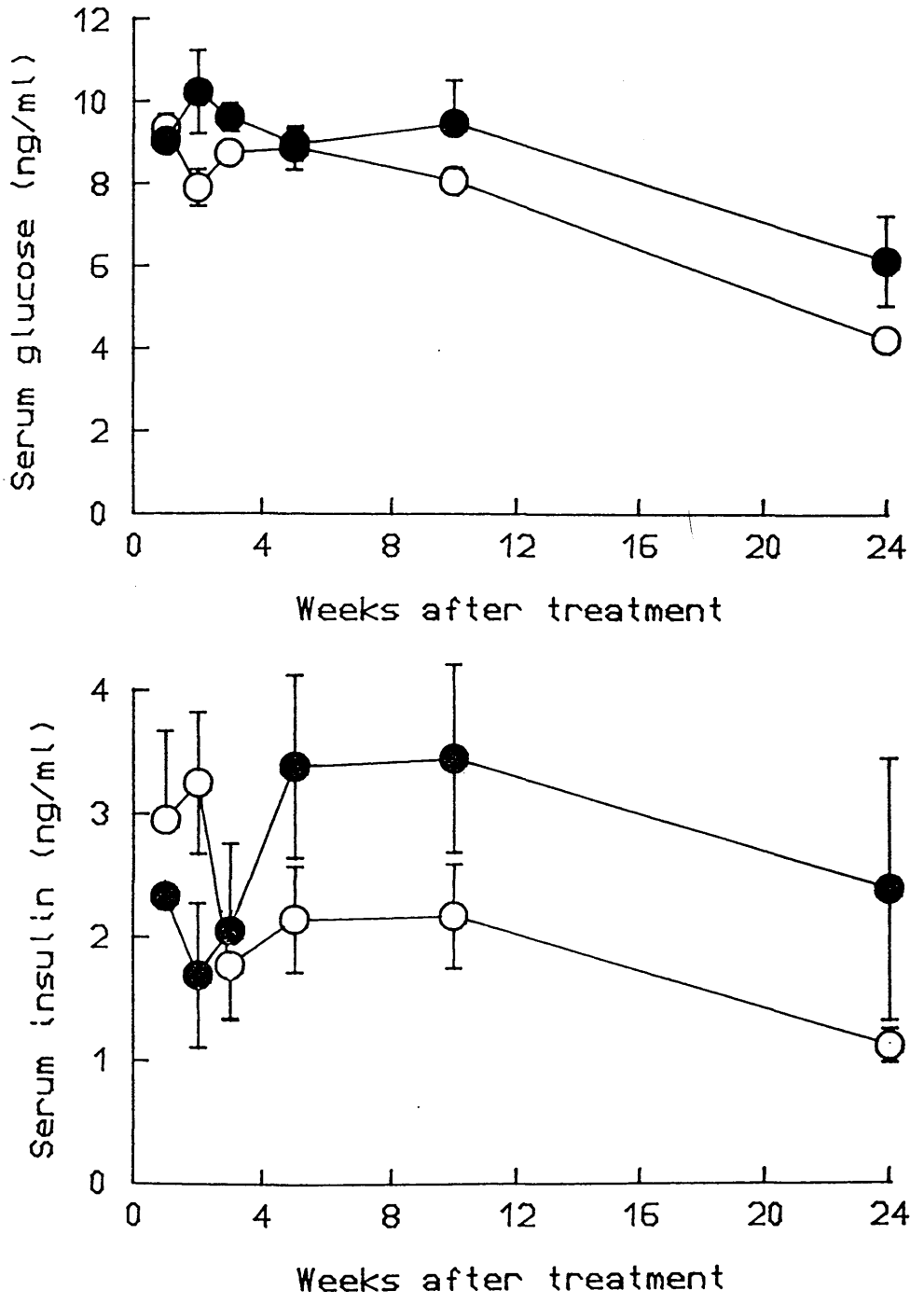
The most dramatic effects of A/S 83 were observed on parametrial adipose tissue. This depot was reduced by 75% at 3 and 8 weeks after treatment and by 50% at 24 weeks after treatment (Fig. 3.1). At 3 and 8 weeks this was due to reduced adipocyte numbers only (Figs. 3.2, 3.3). Apparent adipocyte numbers of both treated and control rats at 24 weeks were less than those at 8 weeks. Since adipocyte numbers cannot be reduced in normal adult rats, even after severe food restriction (Miller et al., 1983), cell numbers were probably underestimated at 24 weeks, due to overestimation of adipocyte volume. The presence of very large adipocytes in rats killed after 24 weeks may have obscured a proportion of the very small adipocytes, leading to an inaccurate estimation of mean adipocyte volume. However, parametrial adipose tissue of treated animals had half the number of adipocytes, compared with controls, at all time points measured, indicating that the relative adipocyte

Fig. 3.9. The effects of A/S 83 on serum triglycerides and non-esterified free fatty acids



Rats received the equivalent of 2 ml of A/S 83 γ globulin (filled circles) or 2 ml of non-immune sheep γ globulin (open circles) for 4 consecutive days. Blood samples were taken from the tail under ether anaesthesia. Serum triglycerides and free fatty acids were measured as described in Section 2.12. Results are means ± SEM of 5-8 observations.

Fig. 3.10. The effects of A/S 83 on serum glucose and insulin concentrations



Treatments and symbols as for Fig. 3.9. Serum glucose and insulin were measured as described in Section 2.12. Results are means ± SEM of 5-8 observations.

Fig. 3.11. The anti-(sheep IgG) response of A/S 83-treated and non-immune sheep γ globulin-treated rats

Treatments and symbols as for Fig. 3.9. The anti-(sheep IgG) response was measured by ELISA and urinary protein was measured as described in Section 2.13. Results are means of 5-8 observations.

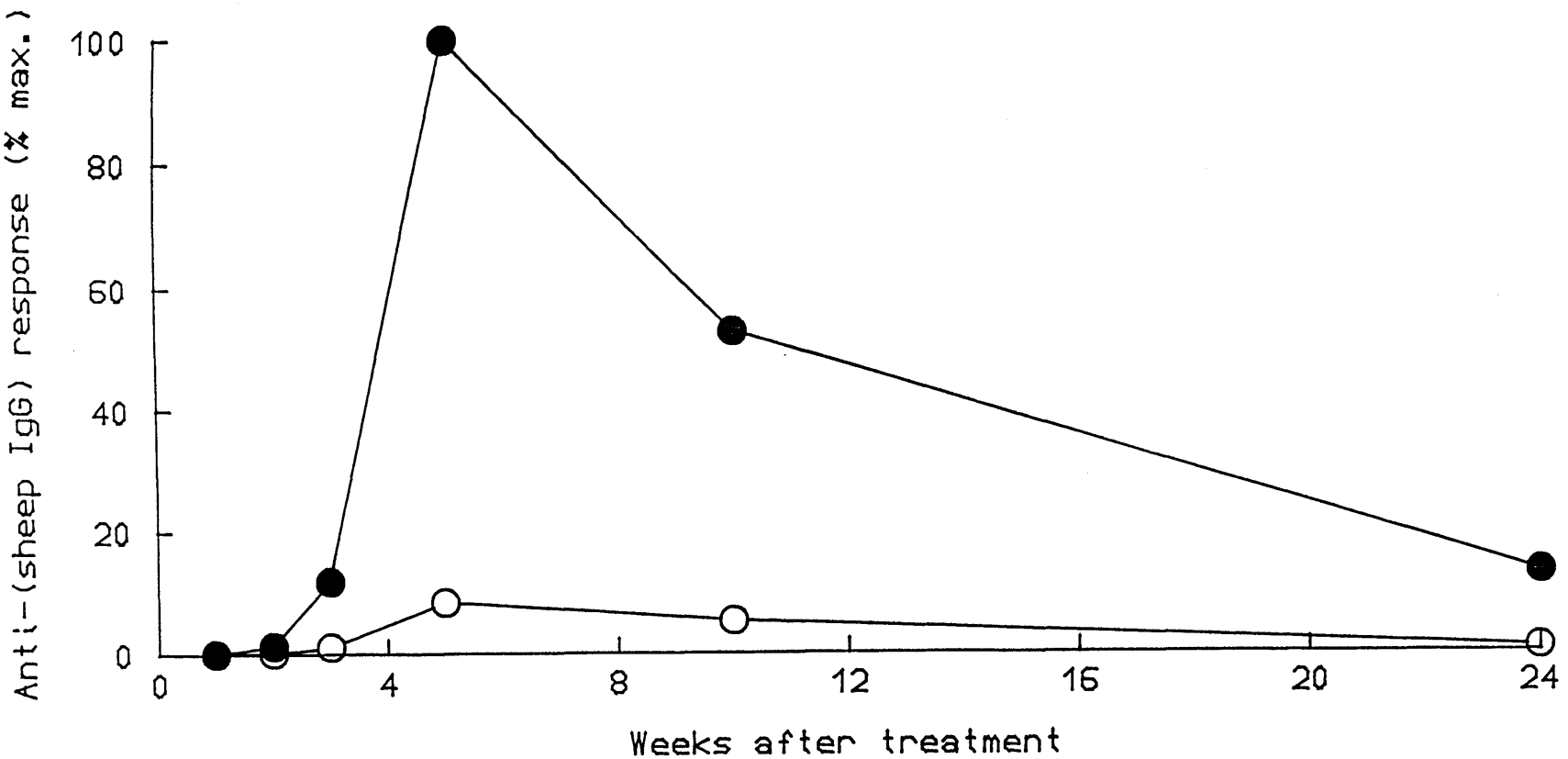
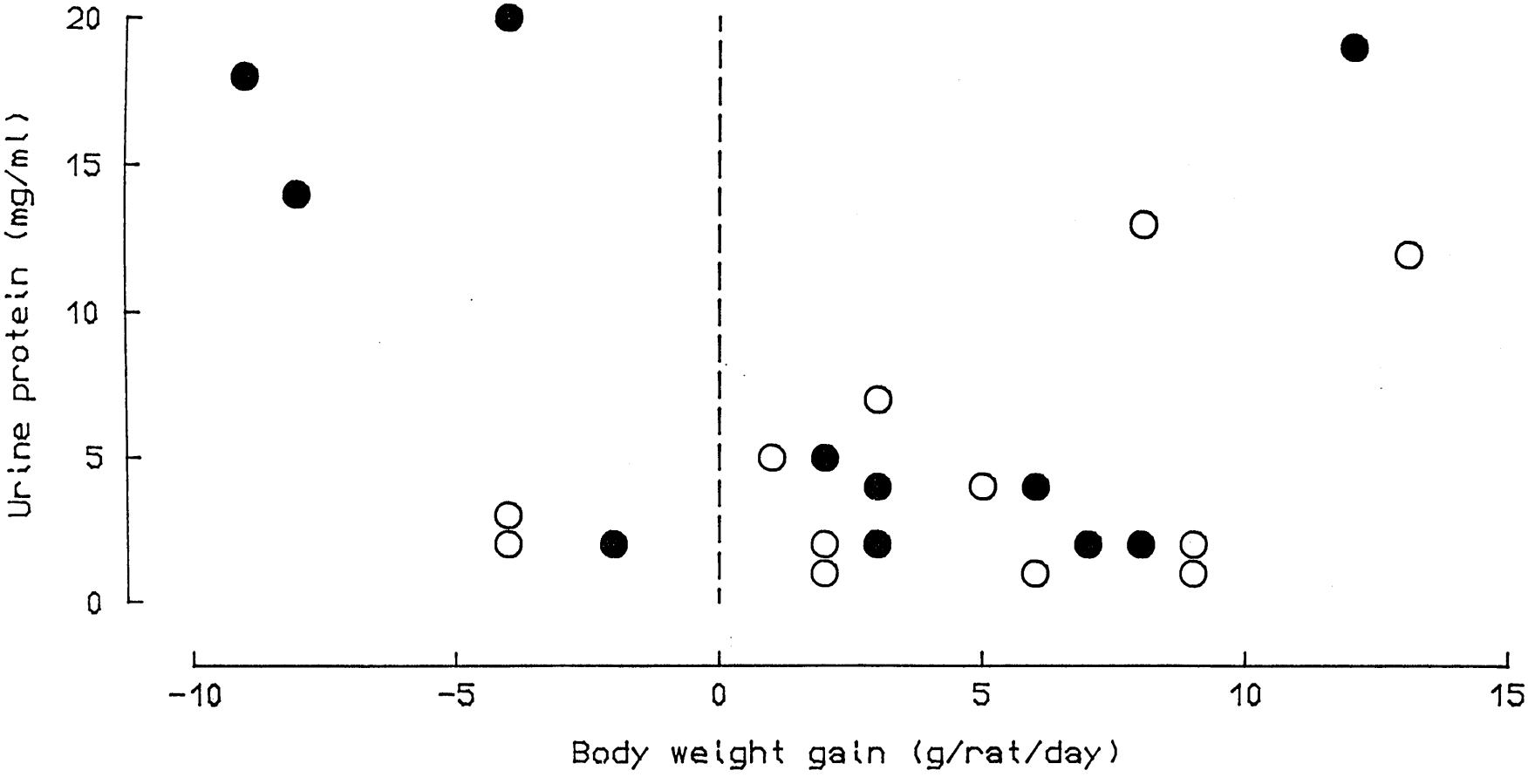

Anti-(sheep IgG) response (% max.)
100
80
60
40
20
0
0
4
8
12
16
20
24
Weeks after treatment

Fig. 3.12. The effects of A/S 83 on body weight gain and urinary protein 1-3 weeks after treatment

Treatments and symbols as for Fig. 3.9. Urinary protein was measured between 1 and 3 weeks after treatment and has been plotted against the weight gain during the previous 24 h.


Urine protein (mg/ml)
20
15
10
5
0
-10
-5
0
5
10
15
Body weight gain (g/rat/day)

numbers are probably valid and that lost parametrial adipocytes were not replaced in A/S-treated rats.

Studies of the regenerative capacity of adipose tissue in rats have been performed exclusively on males and so no information is available on the effects of lipectomy on parametrial adipose tissue. Schemmel (1971), Faust et al. (1976), Kral (1976) and Bailey and Anderson (1980) found no recovery of adipocyte numbers of epididymal adipose tissue following lipectomy. Since the degree of adiposity is more variable in the female than the male, particularly during pregnancy and lactation, it is possible that adipose tissue regeneration may occur more readily in the female than the male.

Pre-adipocytes have been isolated from adult rats, capable of proliferating and differentiating into 'mature adipocytes' in vitro and in vivo (Roncari and Van, 1978). There is, therefore, capacity for the production of new adipocytes in the adult rat. The work of Faust, Johnson, Stern and Hirsch (1978) showed that prolonged feeding of rats with a high-fat diet initially caused an increase in adipocyte size, which was followed by increases in adipocyte number. The authors suggested that the reaching of a 'critical cell size' acts as a trigger for the production of new adipocytes. In A/S 83-treated rats increases in parametrial adipocyte size were inhibited, at least until 8 weeks after treatment. One explanation for the long-term reduction of adipocyte numbers caused by A/S 83 is, therefore, that a 'critical adipocyte size' was not attained and so proliferation was not triggered.

An alternative explanation for the permanent reduction in adipocyte numbers of A/S 83-treated rats is that the pre-adipocyte population was reduced. Preliminary experiments suggested that A/S

83 was cytotoxic to cells isolated from the stromal vascular fraction of adult rat adipose tissue (results not shown). However, the precise nature of the target cells used in these experiments was not determined, as time did not allow the setting up of a culture system capable of supporting adipocyte precursor proliferation and differentiation. Antisera, raised in rabbits, to rat adipocyte plasma membranes showed low level binding to freshly isolated adipocyte precursors but a sharp increase in binding was observed upon the reaching of confluence (Cryer, Gray and Woodhead, 1984). The authors suggested that the low but significant binding to preconfluent precursors might indicate that these cells were already partially determined and derived from pluripotent stem cells, only present during embryogenesis. It is likely, therefore, that mature adipocytes bear surface antigens specific to the mature cell and antigens that are also present on adipocyte precursors in adult adipose tissue. It is possible that adipocyte precursor numbers, in addition to mature adipocyte numbers, were reduced in A/S 83-treated rats, thus reducing the regenerative potential of the tissue.

3.2.3.2 The effects of A/S 83 on parametrial adipocyte volume

The reduction in dry weight of parametrial adipose tissue 24 weeks after treatment was 50%, compared with 75% at 8 weeks (Fig. 3.1). This partial recovery of lost adipose mass was due to a complete recovery of mean adipocyte volume. Table 3.1 shows the amounts of lipid accumulated by each adipose depot during different times after treatment. The measurements at different time points were made on different animals so statistical significance cannot be determined. However, the results indicate that between 1 and 8 weeks the parametrial depot of A/S-treated rats accumulated about 33% of

the lipid accumulated by that of control rats. Between 8 and 24 weeks the parametrial depot of A/S-treated rats accumulated 86% of the lipid accumulated by that of control rats. Since A/S-treated rats had 50% fewer parametrial adipocytes throughout the experiment, lipid accumulation per adipocyte must have been reduced in treated animals betwen 1 and 8 weeks and increased between 8 and 24 weeks, compared with controls.

The causes of the reduction in the ability of parametrial adipocytes to accumulate lipid for about 8 weeks, followed by recovery, is not clear. The massive infiltration of adipose tissue by lymphocytes and polymorphonuclear leucocytes seen one week after treatment (Flint et al., 1986) may cause damage to supporting tissues, reducing the blood and/or nervous supply to adipocytes. A reduction in supply of nutrients, hormones or other stimuli might lead to a reduction in the rate of lipid accumulation by the tissue. Recovery after 8 weeks may have been the result of regeneration of supporting tissues.

Alternatively, adipocytes of A/S-treated rats, while apparently intact, may have suffered damage, causing a reduction in their intrinsic ability to accumulate lipid. This may have been due, for example, to a reduction in uptake of glucose or fatty acids. Such a reduction could operate through the modification of hormone sensitivity, membrane transporters or lipoprotein lipase (LPL) activity. LPL activity was not measured in A/S-treated animals. Adipocytes isolated from parametrial adipose tissue of A/S-treated and control rats showed no differences in basal or insulin-stimulated glucose incorporation into lipid or in basal or noradrenalin-stimulated glycerol release (Figs. 3.4, 3.5). These

results suggest that, in addition to there being no differences in nutrient uptake, there were no direct effects on lipogenesis or lipolysis in adipocytes. However, the adipocytes of A/S-treated animals were smaller than those of controls and smaller adipocytes show higher rates of glucose incorporation and are more insulin-sensitive than larger adipocytes (Salans, Knittle and Hirsch, 1968). Differences between adipocytes of A/S-treated and control rats may, therefore, have been obscured by differences in cell size. The in vitro studies were performed on cells isolated from whole adipose tissue by collagenase digestion. Cells suffering from A/S-induced membrane damage may have been less able to survive the effects of collagenase digestion than undamaged cells.

The recovery of mean adipocyte volume of A/S-treated rats by 24 weeks may have been the result of a slow rate of turnover of adipocytes, suggested to occur in the adult rat (Roncari and Van, 1978). In this way, damaged adipocytes may have been gradually, randomly or preferentially lost from the population to be replaced by newly-formed cells.

3.2.3.3 Differential effects of A/S 83 on parametrial, peri-renal and subcutaneous adipose depots

In contrast to the long-term reduction of parametrial adipocyte numbers caused by A/S 83, adipocyte numbers of peri-renal and inguinal adipose depots, while showing a 30% reduction one week after treatment, approached normal levels by 3 weeks (Fig. 3.3). Mean adipocyte volumes of both depots were still reduced 8 weeks after treatment, but not significantly so (Fig. 3.2). Between 1 and 8 weeks after treatment lipid accumulation in subcutaneous and peri-renal adipose depots of A/S-treated rats was 75-80% of controls

(Table 3.1). During this period, there was a gradual recovery of lost adipocyte numbers in both depots and so any inhibition of lipid accumulation in remaining adipocytes in these depots was probably very short-lived.

There was no difference in the specificity of A/S 83 for adipocyte plasma membranes prepared from parametrial, peri-renal or inguinal subcutaneous adipose depots (Flint et al., 1986). Differences in the pattern of development of adipose depots and their capacity for regeneration in rats have been reported although this work has been performed predominantly with males. Bertrand and Masoro (1978) measured adipocyte number increases in rat peri-renal adipose tissue up to 18 months of age, while increases in cell number of epididymal adipose tissue ceased before 6 months of age. Subcutaneous adipose tissue has a greater capacity for regeneration after lipectomy than internal fat depots (Larson and Anderson, 1978; Faust, 1979). Differential effects of A/S 83 on different adipose depots may, therefore, have been due to differences in the ability of different depots to regenerate or the result of the site of injection.

Twenty four weeks after treatment fat pad weights and adipocyte volumes of peri-renal and subcutaneous adipocytes were larger in A/S-treated rats than in controls. These results, though not significant, suggest that a limited amount of compensatory hypertrophy of other depots, to compensate for the loss of parametrial adipose tissue, had occurred. A number of studies have been undertaken to test the theory, proposed by Kennedy (1966), that total body fat mass is maintained, in the adult, at a constant level through the control of food intake. Such studies have yielded

conflicting results. Bailey and Anderson (1980) reported that lipectomy of epididymal fat depots resulted in compensatory hypertrophy of peri-renal, omental and subcutaneous depots until total fat mass was the same as controls. Similarly, Larson and Anderson (1978) reported that surgical removal of inguinal and epididymal adipose tissue resulted in enlargement of other depots such that total body fat was not reduced 13 weeks after surgery. Internal fat depots of lipectomised animals responded by increases in cell number. In contrast, Kral (1976) found that the removal of various adipose depots did not result in compensatory growth of remaining depots. He suggested that body fat stability in the adult is achieved by regulation of adipocyte size, rather than total adipose mass. He proposed that, after a period of food restriction, missing fat is replaced because adipocyte size is reduced, but, after lipectomy, missing fat is not replaced because adipocyte size is unchanged and only adipocyte numbers are reduced. A/S 83-treated rats showed some compensatory hypertrophy of peri-renal and inguinal adipose depots, which may have been stimulated by the reduced total body fat mass or by the reduced adipocyte size of the parametrial depot. However, compensation was not total, possibly because the remaining reduction in adiposity was due to reduced adipocyte numbers, rather than size.

3.2.3.4 Cross-reaction of A/S 83 with non-adipose tissues

There were no significant differences in wet weights of kidneys, spleen or liver between A/S-treated and control rats 1, 3, 8 and 24 weeks after treatment, with the exception of the spleen weight which was elevated in A/S-treated rats 1 week after treatment (Table 3.2). The elevated spleen weight may have been due to an immune

response against the immunising antiserum or to the recruitment of lymphoid cells to the site of antiserum deposition. A/S 83 appeared, at least in gross terms, to be tissue-specific in vivo, despite clear cross-reactions with kidney, hepatocyte and erythrocyte plasma membranes in vitro (Fig. 3.6). Discrepancies between apparent in vitro and in vivo specificity of A/S 83 may be due to differences in affinity of antibodies for different tissues. Cross-reactivity of antibodies may be based on the binding of structurally different determinants by the same antibody, known as polyfunctional antibodies. The covalent bonds responsible for binding of antibody to antigen are critically dependent on the distance between reacting groups. The higher the degree of complementarity between the antigenic determinant and the antibody combining site, the higher is the affinity of the antibody for the antigen. Hence, an antibody raised against an adipocyte plasma membrane determinant would be expected to have high complementarity with and so high affinity for that antigenic determinant. If the same antibody bound to a different determinant on, for instance, hepatocyte plasma membranes, the degree of complementarity and hence the affinity of the antibody for the liver antigen would be less than that for the adipocyte antigen. Low affinity antibodies tend to bind antigen more effectively at low temperatures. Therefore ELISAs, performed at 22° C, may measure low affinity antibodies that are not of physiological importance in vivo.

In addition to antibodies binding to structurally unrelated determinants, cross-reactivity may involve the existence of common epitopes on different antigens, in which case there would be no difference in the affinity of antibodies for the antigen.

A/S 83 is a polyclonal antiserum composed of antibodies raised against numerous antigens. The overall specificity of the antiserum is, therefore, the result of a combination of the affinity and titre of a range of different antibodies raised against a variety of different antigenic determinants. Western blotting showed that A/S 83 binds to at least 7 major polypeptides of hepatocyte plasma membranes compared with at least 19 polypeptides of adipocyte plasma membranes (Fig. 3.7), indicating that the antiserum recognises some adipocyte plasma membrane antigens that are not shared with hepatocyte plasma membranes.

The site of injection may confer extra specificity on the antiserum *in vivo*. As only tissue weight rather than function was measured, direct effects of A/S 83 on tissues other than adipose cannot be discounted but the most dramatic effects certainly occurred in adipose.

3.2.3.5 The effects of A/S 83 on food intake, body composition and food conversion efficiency

The administration of A/S 83 caused an 8-10 g drop in body weight, accompanied by a 75% decrease in food intake on the first day of treatment (Fig. 3.8). Slightly abnormal postures were sometimes adopted for about 2 h after treatment. Non-immune sheep γ globulin treatment caused none of these effects. It is possible that the antiserum cross-reacted with a component of the nervous system, affecting muscle co-ordination, near the site of injection. Cross-reactivity of A/S 83 with nervous tissue was investigated and is described in Chapter 4. Control rats, pairfed with A/S 83-treated rats, showed an equal fall in body weight on the first day of treatment. Therefore, the reduction in body weight, seen in

A/S-treated rats, can be entirely attributed to a reduction in food intake and this reduction in food intake could be due to the rise in serum metabolites, such as triglycerides, released from adipose tissue.

The fate of lipids, released from adipose tissue in A/S-treated rats, is unclear. Body composition analysis at 5 and 8 weeks showed that protein deposition was increased in A/S-treated animals (Table 3.4). Eight weeks after treatment in A/S-treated rats, a reduction of about 50 g/kg of fat was balanced by increases of 10 and 40 g/kg of protein and water respectively. While fat is deposited without or with the partial replacement of water, protein deposition is accompanied by the deposition of water. Muscle has a protein:water ratio of 1:3-4 (van Es, 1977) so the ratio of protein increase to water increase, described above, is as would be expected.

The increase in protein deposition at 5½ and 8 weeks could not be entirely due to the incorporation into protein of substrates released from adipose tissue as a result of immune attack. Between 1 and 8 weeks A/S 83-treated rats showed an elevated food intake and reduced fat deposition. There must, therefore, have been a repartitioning of energy intake from fat into protein deposition. Alterations in nutrient partitioning in A/S-treated rats could occur by a modification of hormone levels, by direct differential effects on adipose tissue, muscle and liver or by direct effects on adipose tissue which indirectly affect nutrient uptake by muscle or the liver.

A/S 83 may cross-react in vivo with any of the numerous endocrine organs which influence growth and carcass composition, such as the pituitary, pancreas and adrenal. The only hormone measured

was insulin which was reduced at 2 weeks and elevated at 5, 10 and 24 weeks in A/S-treated rats, although these differences were not significant. Insulin concentrations are generally correlated with degree of fatness, as the anabolic effects of insulin tend to be greater on fat than on muscle. Elevated serum insulin concentrations may have contributed towards the elevated fat accumulation, seen in peri-renal and subcutaneous depots between 8 and 24 weeks (Table 3.1). Growth hormone has anabolic effects on muscle, probably mediated by somatomedins, and is lipolytic. The administration of exogenous growth hormone frequently results in the repartitioning of nutrients from fat to protein deposition (Hart and Johnsson, 1986). Elevated growth hormone levels are frequently correlated with decreased insulin levels, so it would be of interest to measure growth hormone levels 2 weeks after treatment with A/S 83 when insulin levels, in some rats, were reduced. The pulsatile release of growth hormone and its response to stress make meaningful measurements of growth hormone levels difficult to obtain, without the use of chronically cannulated animals. The repartitioning effects of the synthetic β agonists, clenbuterol and cimeratol, are believed to involve their known β adrenergic stimulation of lipolysis in adipocytes, coupled with their known limiting effects on muscle protein degradation (Dalrymple et al., 1984). Direct effects of A/S 83 on muscle have not been investigated, so stimulatory effects of A/S 83 on nutrient uptake and protein deposition by muscle cannot be excluded.

Alternatively, repartioning of nutrients in A/S 83-treated rats may occur simply as a result of competition between adipose tissue and muscle for common precursors, such as glucose. Reduced uptake of

nutrients by adipose tissue may have led to compensatory increases in uptake by other tissues.

Table 3.3 shows the effects of A/S 83 on body weight gain, food intake and food conversion efficiency (FCE). FCE is expressed as food intake divided by weight gain, making no adjustment for maintenance requirements. As A/S 83-treated rats were heavier than controls, any differences in efficiency would be accentuated by subtracting maintenance requirements from food intakes, as larger animals have greater maintenance requirements. FCE was increased in A/S-treated rats, particularly between 2 and 7 weeks. During this period A/S-treated animals deposited more protein and less fat than controls. It should be noted, however, that measurements of FCE and body composition were made on different animals and the animals whose body composition was measured showed no increase in body weight gain (results not shown). A number of studies have attempted to estimate the energy cost of fat and protein deposition in rats (McCracken and Weatherup, 1973). Such studies have yielded varied results, partly because both maintenance requirements and energy partitioning are influenced by maturity and so are not independent variables. However, more recent experiments have been designed to overcome this problem by using fat and lean Zucker rats, between which energy partitioning into fat and protein varies independently of maturity (Pullar and Webster, 1974, 1977). Such work has indicated that the amount of energy required to deposit 1 KJ of fat and protein are 1.36 and 2.25 KJ, respectively. The energetic efficiency of net protein synthesis is, therefore, lower than that of net fat synthesis. Assuming energy contents of 39 and 24 KJ per gram of protein and fat respectively, the energy requirements of the deposition of 1 g of

protein and 1 g of fat become almost identical (about 53 KJ). As the deposition of 1 g of protein is accompanied by the deposition of 2-3 g of water, a shift from fat deposition to protein deposition results in a reduction in the amount of energy required for a given amount of weight gain. The increase in FCE in A/S-treated rats could, therefore, have been due to decreased fat deposition and increased protein deposition. Body composition was not measured at 24 weeks, but fat deposition, in the depots measured, was greater between 8 and 24 weeks in A/S-treated than in control rats. A repartitioning of nutrients, favouring fat rather than protein deposition, may account for the reduction in the difference in FCE between A/S-treated and control rats between 7 and 24 weeks.

3.2.3.6 The rat immune response to injected sheep immunoglobulin

The administration of 2 ml of A/S 83 to a rat resulted in the injection of about 100 mg of foreign protein, mainly γ globulin. The rat immune response to sheep IgG was, therefore, investigated. Fig. 3.11 shows that A/S 83-treated rats produced a much greater immune response to sheep IgG than non-immune sheep IgG-treated rats. This was probably because, in A/S-treated rats, where the antibodies had a target, antigen-presenting cells were attracted to the site of antibody deposition, possibly by complement activation. This would lead to improved antigen presentation and, thereby, antibody production. Immune clearance of xenogeneic serum is normally rapid, but A/S 83 bound to tissues may have provided an antigenic stimulus for a longer period than non-immune sheep IgG. A suitably assay for the detection of sheep IgG in rat serum was not available.

Body weight gain of both A/S 83- and non-immune sheep IgG-treated rats fluctuated at about 2 weeks after treatment. This

was accompanied by transient proteinurea which tended to be more severe in A/S-treated rats (Fig. 3.12). Proteinurea is indicative of abnormal kidney function, which may be caused by antibodies directed against renal antigens or by the deposition of circulating immune complexes in the glomerulus.

A/S 83 binds to renal antigens (Fig. 3.6) so antibodies may have localized in the kidneys of A/S-treated rats, in the absence of immune complex formation. Passive nephrotoxic nephritis has been induced, in rats, by the injection of a foreign antiserum raised against rat renal antigens (Unanue and Dixon, 1967). The progress of the disease occurs in 2 phases. The first is a heterologous phase, characterised by the reaction of nephrotoxic antibodies with glomerular antigen. If sufficient amounts of nephrotoxic antibodies are administered, this phase is immediate and proteinurea can be detected within a few hours. In the few instances where it was possible to obtain urine samples a few hours after treatment with A/S 83, proteinurea was not detected and there were no changes in serum urea concentrations during the first week after treatment (results not shown). The second phase of the disease is the autologous phase, which occurs after a week, and results from the immunological response of the host to heterologous immunoglobulin. As nephritis progresses, rats develop elevated blood urea nitrogen levels, decreased urea and creatinine clearance, persistent urinary abnormalities, weight loss and hyperlipidaemia. In addition to transient elevated urine protein concentrations and weight fluctuations, 1 out of 6 A/S-treated rats showed a doubling in serum urea concentations and 3 out of 6 A/S-treated rats showed hyperlipidaemia 2 weeks after treatment. These had all returned to

normal levels by 3 weeks after treatment. It is also possible that the hyperlipidaemia, seen at 2 weeks after treatment with A/S 83, was the result of host anti-(sheep IgG) antibodies binding to anti-(adipocyte) antibodies, already coating adipocytes, resulting in further lysis of adipocytes.

No circulating immune complexes could be measured in A/S 83- or non-immune sheep IgG-treated rats, although a lack of positive controls makes this inconclusive (results not shown). It is possible that the rats may have suffered from mild serum sickness about 2 weeks after treatment, such as that commonly studied by immunisation of rabbits with BSA (Dixon and Cochrane, 1970). In this model, after a week, antibodies to BSA appear in the circulation. Complexes formed in antigen excess are small and persist in the circulation. Complex deposition along the glomerular basement membrane occurs and symptoms of glomerulonephritis, such as proteinurea and weight loss, appear. As antigen is removed from the circulation and antibody is in excess, complexes become larger. Large immune complexes are more rapidly removed from the circulation because of multiple binding of antibody to Fc receptors of phagocytic cells of the mononuclear phagocyte system (Wener and Mannik, 1986).

Circulating immune complexes in A/S 83-treated rats would be composed of either A/S 83 bound to adipocyte plasma membrane antigens or A/S 83 bound to rat anti-(sheep IgG) antibodies. Complexes of the first type would be likely to be formed in the greatest quantity within the first few days after treatment. The appearance of nephritis about 2 weeks after treatment and the presence of nephritis in some non-immune sheep IgG-treated rats implicates a rat immune response as the cause. Since there was a considerable rat immune

response against sheep IgG, it is not possible to be certain whether anti-(kidney) antibodies or circulating immune complexes were responsible for the transient nephritis seen in A/S 83-treated rats.

3.2.4 Conclusions

1. The administration of A/S 83 to 120-140 g female rats caused a 50% reduction in parametrial adipocyte numbers, which persisted at least until 24 weeks after treatment. The absence of recovery of lost adipocyte numbers may have been because the pre-adipocyte population was damaged in some way. Alternatively, since the ability of parametrial adipocytes to accumulate lipid was reduced for the first 8 weeks after treatment, the remaining adipocytes may not have attained the 'critical size' necessary for the triggering of new adipocyte production.
2. The ability of parametrial adipocytes to accumulate lipid was reduced for the first 8 weeks after treatment and had recovered by 24 weeks after treatment. Damage to supporting tissues may have resulted in a reduced blood supply or innervation of parametrial adipose tissue during the first 8 weeks or, remaining adipocytes, while apparently intact, may have been damaged and were gradually replaced by newly-formed adipocytes after 8 weeks.
3. A/S 83 had a greater effect on parametrial than on subcutaneous or peri-renal adipose tissue, possibly because of the site of injection of A/S 83 or because of intrinsic differences in different adipose depots.

4. While A/S 83 showed cross-reactivity with non-adipose tissues in vitro, there was no gross anatomical evidence of cross-reactivity with non-adipose tissues in vivo.

5. A/S 83 caused a 75% reduction in food intake on the first day of treatment, possibly because of an increase in serum metabolites, such as free fatty acids and triglycerides, released from adipose tissue. Food intake recovered by the fourth day of treatment.

6. Body composition analysis at 5½ and 8 weeks after treatment indicated that lost fat was replaced by protein and there was repartitioning of food intake from fat into protein deposition. This may be due either to direct effects of A/S 83 on muscle or liver, to effects on hormone levels or to effects on adipose tissue, thus reducing competition between adipose tissue and muscle for common precursors.

7. A/S 83 caused the transient development of nephritis about 2 weeks after treatment, which was manifested by fluctuations in body weight gain and proteinurea. The nephritis was either due to immune attack of the kidney by A/S 83 or due to the deposition of circulating immune complexes, formed as the result of the immune response to sheep IgG, in the kidney.

3.3 AN ASSESSMENT OF THE EFFECTS OF THE SITE OF INJECTION AND DURATION OF TREATMENT WITH A/S 83

3.3.1 Experimental

Female Wistar rats, weighing 120-140 g, were injected intraperitoneally with the equivalent of 2 ml of A/S 83 γ globulin,

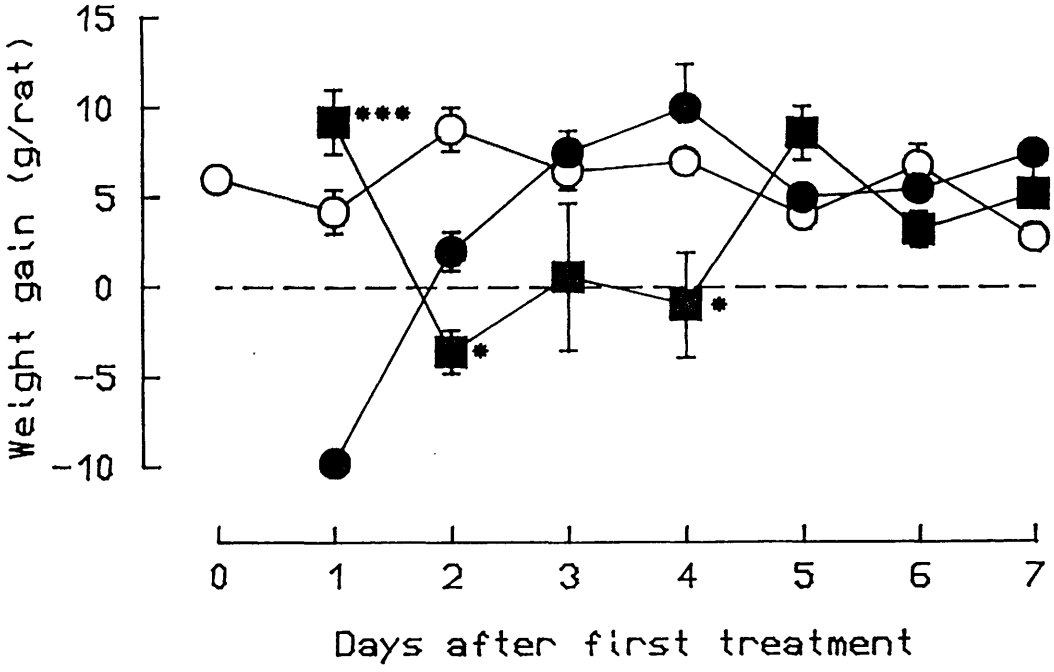
between 9.00 h and 11.00 h, for 0, 1, 2 and 4 days. An additional group of rats were injected subcutaneously with the equivalent of 2 ml of A/S 83 on 4 consecutive days. Food intakes and body weights were recorded daily for the first 2 weeks and every 3 days thereafter. Rats were killed upon reaching a weight of approximately 250 g, which was about 5½ weeks after treatment. The dry weights of the parametrial, peri-renal, omental, interscapular subcutaneous white adipose tissue and the wet weight of the interscapular brown adipose depots were determined.

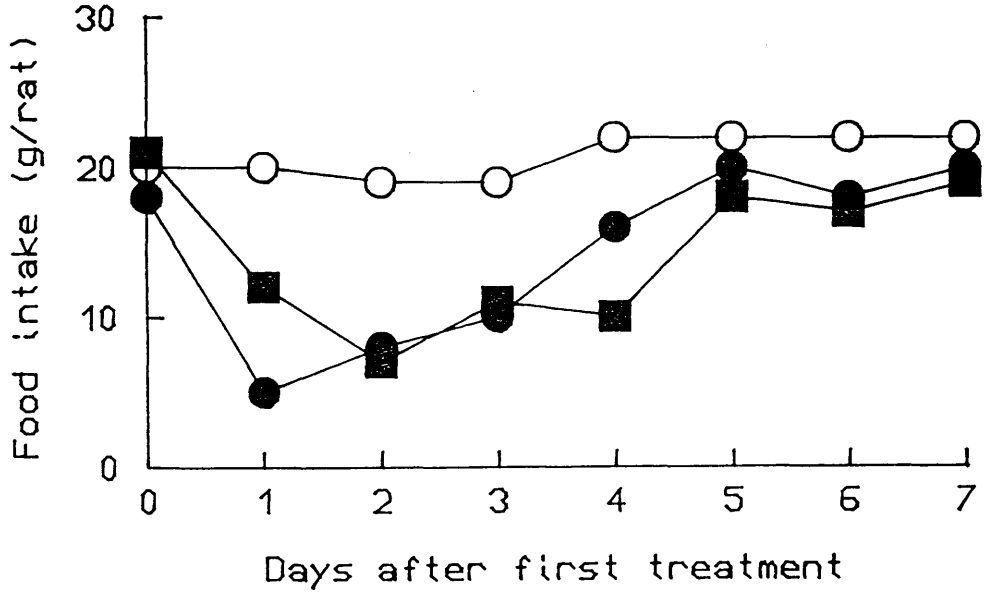
3.3.2 Results

While intraperitoneal injection of A/S 83 caused a drop in body weight of 10 g during the first day after treatment, the injection of A/S 83 subcutaneously caused no drop in body weight gain on the first day (Fig. 3.13). Body weight gain in intraperitoneally injected rats partially recovered on the second day and was normal by the third day after treatment. Subcutaneously injected rats showed a reduction in body weight of 4 g on the second day of treatment. In this group of rats, weight gain fluctuated around 0 on the third and fourth day after treatment before achieving slightly greater than normal levels 5 days after treatment.

There was a 75% reduction of food intake in intraperitoneally injected rats during the first day of treatment, compared with a 40% reduction in subcutaneously injected rats (Fig. 3.13). Food intakes were similar in intraperitoneally and subcutaneously injected rats after the first day of treatment and there were no differences in weight gain and food intake between controls and intraperitoneally and subcutaneously injected animals during the final 4½ weeks of the experiment.

Fig. 3.13. The effects of the site of injection of A/S 83 on body weight gain and food intake





Rats were injected intraperitoneally or subcutaneously with the equivalent of 2 ml of A/S 83 γ globulin for 4 consecutive days. Control rats were allowed to feed ad libitum. Results are means ± SEM of 4-6 observations. *P<0.01, ***P<0.001, compared with intraperitoneally injected rats (Student's unpaired t test).

Table 3.5 shows the effects of different sites of injection on adipose tissue 5½ weeks after treatment. The dry weight of subcutaneous adipose tisue was significantly less in subcutaneously than in intraperitoneally injected rats, whilst the dry weight of parametrial and omental adipose tissue was significantly less in intraperitoneally than in subcutaneously injected animals. The wet weight of interscapular brown adipose tissue was significantly greater in intraperitoneally injected rats than in controls.

There were no significant differences in body weight gain or food intake between rats injected with 1 ml, 2 x 1 ml or 4 x 1 ml of A/S 83 (Fig 3.14). There were no signficiant differences in adipose tissue weight between rats given 1 ml, 2 x 1 ml or 4 x 1 ml of A/S 83 (Table 3.6).

3.3.3 Discussion

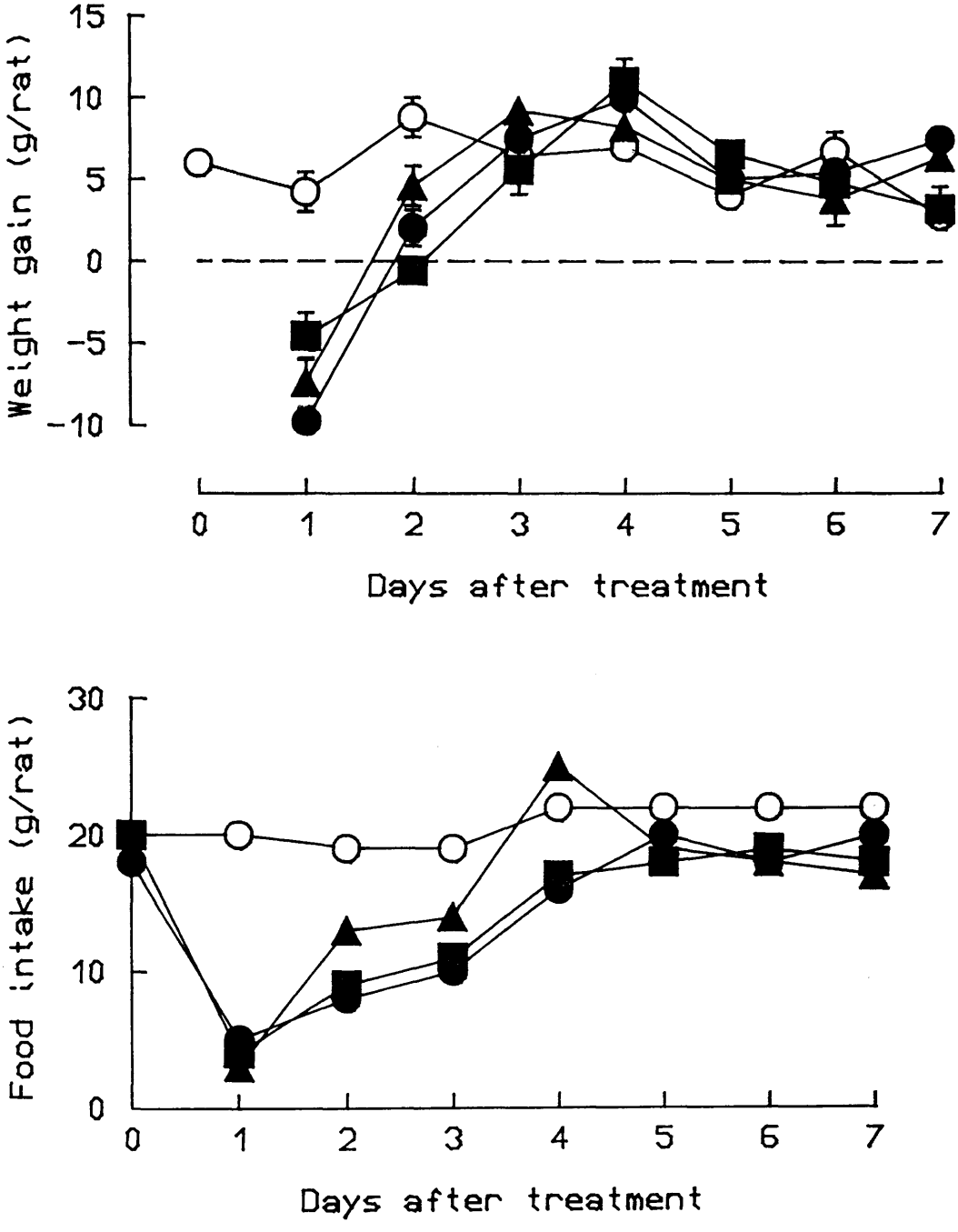
The reduction in food intake and body weight induced by A/S 83 was delayed, but not prevented, in subcutaneously, compared with intraperitoneally, injected rats (Fig. 3.13). Since an antiserum injected subcutaneously would enter the circulation more slowly than if injected intraperitoneally, the reduction in food intake may have resulted from the interaction of circulating antibodies with cells or proteins of the circulation, or with a non-adipose target tissue reached via the circulation. The slower release of antiserum from subcutaneous sites may have permitted a more controlled effect with less dramatic adverse reactions. As parametrial adipose tissue was most affected by intraperitoneal injection of A/S 83 and subcutaneous adipose tissue was most affected by subcutaenous injection (Table 3.5), the antiserum probably does not need to enter the general

Table 3.5. The effects of the site of injection of A/S 83 on fat pad weight 5½ weeks after treatment

Treatment	Number of observations	Wet weight interscapular brown fat (g)	Dry weights fat pad (g)				
			Interscapular subcutaneous	Inguinal subcutaneous	Parametrial	Peri-renal	Omental
Control	4	0.25 ±0.05	1.89 ±0.39	5.07 ±0.57	7.90 ±1.20	3.18 ±0.33	3.80 ±0.49
A/S 83 (intraperitoneal)	6	0.35 ±0.02	1.33 ±0.20	4.08 ±0.18	2.04*** ±0.18	2.48 ±0.25	1.72*** ±0.13
A/S 83 (subcutaneous)	5	0.28 ±0.04	0.82	1.50***§§§ ±0.21	3.67***§§§ ±0.23	2.09 ±0.40	3.08§§ ±0.31

Rats were injected intraperitoneally or subcutaneously with the equivalent of 2 ml of A/S 83 γ globulin for 4 consecutive days. Control rats were untreated. Results are means ± SEM. ***P<0.001, compared with controls (Student's unpaired t test). §§P<0.005, §§§P<0.001, compared with intraperitoneal injection (Student's unpaired t test).

Fig. 3.14. The effects of the duration of treatment with A/S 83 on body weight gain and food intake



Rats were injected intraperitoneally with the equivalent of 2 ml of A/S 83 γ globulin for 1, 2 or 4 consecutive days. Control rats were allowed to feed ad libitum. Results are means ± SEM of 3-6 observations.

Table 3.6. The effects of the duration of treatment with A/S 83 on fat pad weight 5½ weeks after treatment

Treatment	Number of observations	Wet weight interscapular brown fat (g)	Dry weights fat pad (g)				
			Interscapular subcutaneous	Inguinal subcutaneous	Parametrial	Peri-renal	Omental
Control	4	0.25 ±0.05	1.89 ±0.39	5.07 ±0.57	7.90 ±1.20	3.18 ±0.33	3.80 ± 0.49
A/S 83 (1 x 2 ml)	5	0.28 ±0.03	1.08 ±0.11	2.85* ±0.35	2.18*** ±0.13	2.04** ±0.10	2.26* ±0.23
A/S 83 (2 x 2 ml)	3	0.35 ±0.05	1.36 ±0.39	3.38 ±1.11	3.08 ±0.91	2.57 ±0.79	2.12 ±0.37
A/S 83 (4 x 2 ml)	6	0.35 ±0.02	1.33 ±0.20	4.08 ±0.18	2.04*** ±0.18	2.45 ±0.25	1.72** ±0.13

Rats were injected intraperitoneally with the equivalent of 2 ml of A/S 83 γ globulin for 1, 2 or 4 consecutive days. Control rats were untreated. Results are means ± SEM. *P<0.01, **P<0.005, ***P<0.001, compared with controls (Student's unpaired t test).

circulation before interaction with target adipocytes. The reduction of food intake may have resulted from the release of lipid and lipid breakdown products into the circulation, as a result of immune attack of adipose tissue. As parametrial adipose tissue is more highly vascularised than subcutaneous adipose tissue, products of parametrial adipocyte lysis may have been released more rapidly into the circulation than those of subcutaneous adipocytes.

In the experiments described above all rats treated with A/S 83 showed an increase in brown adipose tissue weight, although only in rats given 4 intraperitoneal injections of A/S 83 was this difference significant. It is not known whether the increase in mass of the tissue was due to hyperplasia or to enlargement of existing brown adipocytes. Hyperplasia in brown adipose tissue occurs, in some rodents, upon prolonged exposure to a cafeteria diet, where, despite marked hyperphagia, the animal gains little weight, due to an increased rate of non-shivering thermogenesis (Rothwell and Stock, 1979). However, since the A/S 83-treated rats gained slightly more weight and showed a slightly greater food conversion efficiency than controls, it is unlikely that there was a significant increase in non-shivering thermogenesis in A/S-treated animals. A rise in the weight of brown adipose tissue was more likely to have been due to an increase in triglyceride storage in the tissue.

3.3.4 Conclusions

1. Differential effects of A/S 83 on different adipose depots were due, largely, to the site of injection.
2. The same effects could be achieved on adipose tissue and food intake by a single treatment with A/S 83 as with the same treatment on 4 consecutive days.

3. Treatment with A/S 83 induced an increase in brown adipose tissue mass which was more likely to have been the result of increased triglyceride storage in the tissue than to increased non-shivering thermogenesis.

3.4 AN INVESTIGATION OF THE SHORT-TERM EFFECTS OF A/S 83

3.4.1 Experimental

130-160 g female Wistar rats were anaesthetised at 09.30 a.m. by intraperitoneal injection of 40 mg/kg body weight of 10 mg/ml pentobarbital in pyrogen-free saline. Once anaesthetised, rats were wrapped in cotton wool to maintain body temperature. 0.5 ml blood samples were taken, by cardiac puncture, 20 minutes after onset of anaesthesia. Rats were then injected with either the equivalent of 2 ml of A/S 83 or non-immune sheep γ globulin or were uninjected. Blood samples were taken, as described above, at 3, 6, 12 and 24 hours after administration of γ globulin. Rats were allowed to recover after anaesthesia and 2 sets of animals were used so that rats were not bled more than 3 times in 24 hours. Blood samples were obtained 2 and 4 days after treatment, using ether anaesthesia. At 6 days rats were killed by cervical dislocation and blood samples taken from the jugular vein. Wet and dry weights of parametrial adipose tissue were determined.

All serum samples were allowed to clot at 4° C for 3 h, centrifuged at 11,000 g for 5 mins at 4° C, snap frozen and stored at -20° C. Serum glucose, triglyceride, free fatty acids, urea, complement and insulin concentrations were measured as described in Chapter 2. Serum A/S 83 concentations were measured by ELISA.

3.4.2 Results

A/S 83-treated rats showed a doubling of serum non-esterified free fatty acids 6, 12 and 24 hours after treatment, compared with non-immune sheep γ globulin-treated controls (Fig. 3.15). There was also a doubling of triglyceride levels 6 and 12 hours after A/S treatment. There was no difference in either free fatty acid or triglyceride levels by 48 hours after treatment. Serum glucose and insulin levels were not significantly affected, although 24 hours after treatment insulin levels tended to be reduced in A/S-treated rats (Fig. 3.16). The administration of both A/S 83 and normal sheep γ globulin caused a 50% reduction in circulating complement levels but this had returned to nearly normal levels in non-immune sheep γ globulin-treated rats by 12 hours (Fig. 3.15). Complement levels of A/S-treated rats had fallen to about 20% of normal levels by 12 hours, returning to near normal levels by 24 hours. A/S 83 was detectable in the serum by 3 hours after treatment and had reached maximum levels (0.23 μl/ml) by 6 hours after treatment (Fig. 3.16). This level was maintained until 24 hours after treatment, when it declined and was undetectable by 6 days.

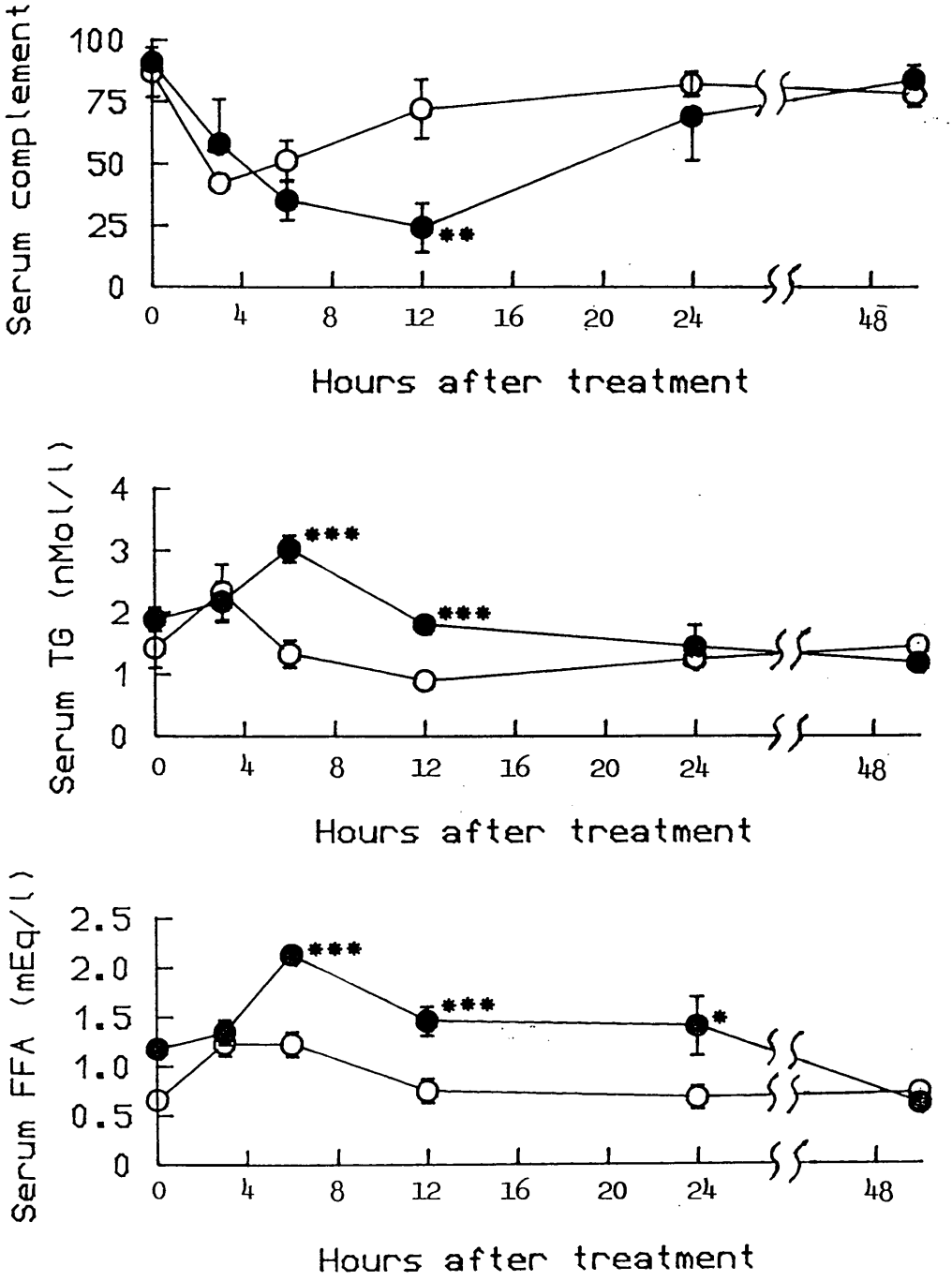
The dry weights and percentage dry weights of parametrial adipose tissue of A/S 83-treated rats showed similar reductions, compared with controls, as had been seen with A/S 83-treated rats in previous experiments (results not shown).

3.4.3 Discussion

Pentobarbital anaesthesia was used in these experiments so that rats could be anaesthetised 20 minutes before blood sampling to allow any stress-induced release of metabolites or hormones to cease before

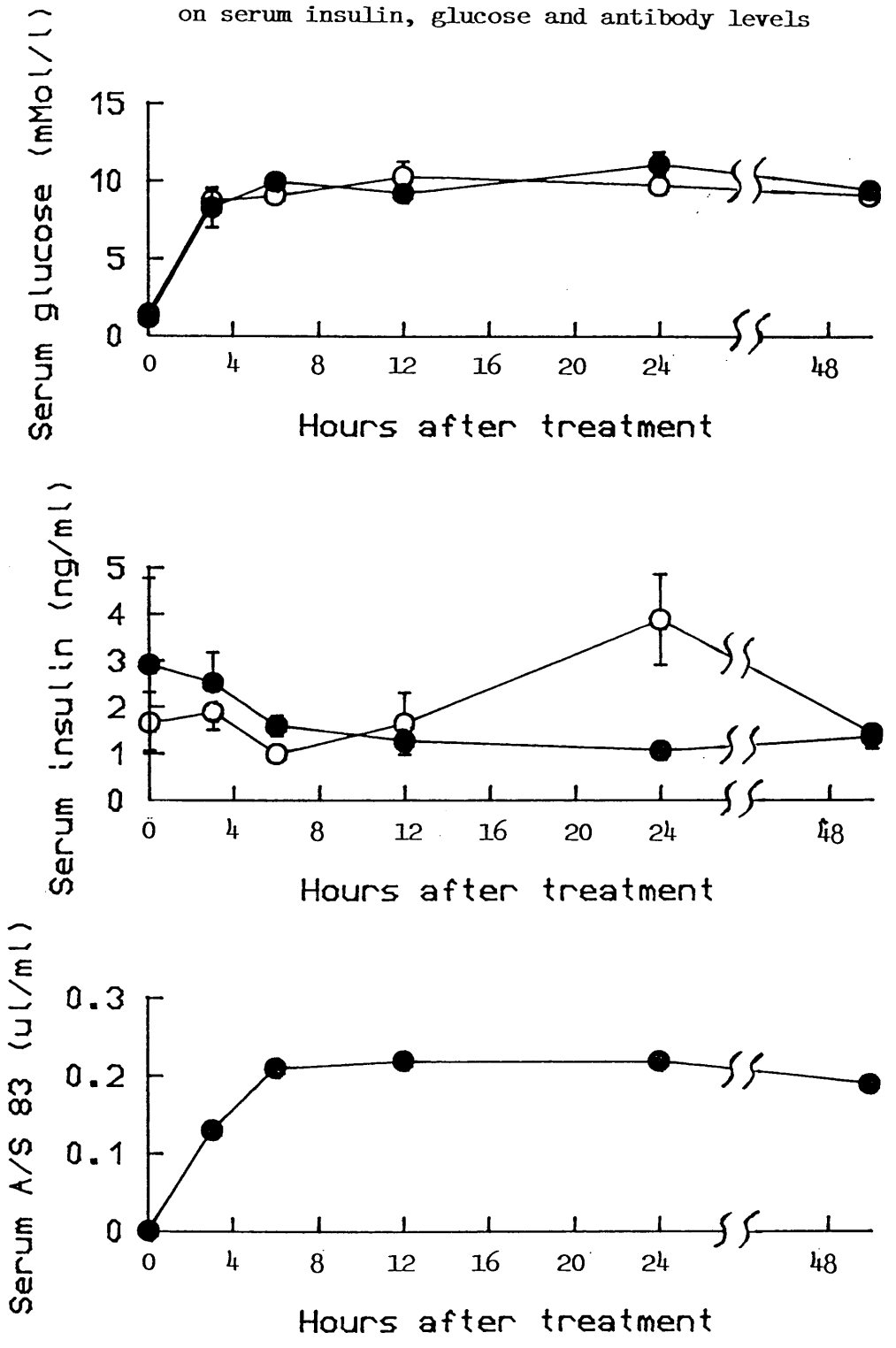
Fig. 3.15. The short-term effects of A/S 83

on serum complement, triglyceride and free fatty acid levels



Rats were injected intraperitoneally with the equivalent of 2 ml of A/S 83 (filled circles) or non-immune sheep γ globulin (open circles) at time 0. Blood samples were taken by cardiac puncture under pentobarbital anaesthesia. Serum complement, triglycerides and free fatty acids were measured, as described in Section 2.12. Results are means ± SEM of 3-8 observations. *P<0.01, **P<0.005, ***P<0.001, compared with controls (Student's unpaired t-test).

Fig. 3.16. The short-term effects of A/S 83 on serum insulin, glucose and antibody levels



Treatments and symbols as for Fig. 3.15. Serum antibody was measured by ELISA and serum insulin and glucose were measured as described in Section 2.12. Results are means $\pm$ SEM of 3-8 observations.

sampling. The increase in serum free fatty acids and triglycerides, seen at 6-24 hours after treatment with A/S 83 (Fig. 3.15) was probably the result of adipocyte lysis. Serum glucose (Mayer, 1953), free fatty acids and insulin (Liebel, 1977) have all been implicated in the control of food intake and so the rise in serum free fatty acids may have contributed towards the reduction in food intake seen during the first 24 hours after treatment (Fig. 3.8). The reduction in insulin levels seen in some A/S-treated rats 24 hours after treatment (Fig. 3.16) may have been the result of cross-reaction of A/S 83 with pancreatic tissue, temporarily affecting insulin secretion, but is more likely to have been the result of a reduction in food intake in A/S-treated rats. Since the rats in this experiment were anaesthetised for long periods, meaningful measurements of food intake could not be obtained.

The reduction in circulating complement levels, seen in both non-immune sheep γ globulin- and A/S 83-treated rats (Fig. 3.15) may have been due to the presence of complement fixing aggregates in both preparations. The decline of complement levels to 20% of normal 12 hours after treatment with A/S 83, while those of non-immune sheep γ globulin-treated rats had returned to normal, suggests that the binding of A/S 83 to target tissues in vivo resulted in complement activation, but does not necessarily demonstrate the importance of complement in mediating the fat-reducing effects of the antiserum.

The maximum level of circulating anti-(adipocyte plasma membrane) antibodies measured after treatment with A/S 83 was a more than 1,000-fold dilution of the anti-(adipocyte plasma membrane) activity of A/S 83. As 1 ml of A/S 83 was injected and the blood volume of the rats would have been no more than 10 ml, a considerable

proportion of the antibodies must have been either cleared from the circulation very rapidly, bound to the tissues or circulating in the form of immune complexes and so not detectable by the assay used.

3.4.4 Conclusions

1. Treatment with A/S 83 resulted in a doubling of serum free fatty acids and triglycerides 6 and 12 hours after treatment which returned to normal levels by 48 hours. This was probably the result of the release of the contents of lysed adipocytes into the circulation and may have contributed towards the reduction in food intake seen on the first day of treatment with A/S 83.
2. The doubling of serum free fatty acids and triglycerides 12 hours after treatment was accompanied by a reduction in circulating complement to 20% of the normal level, showing the ability of A/S 83 to activate rat complement in vivo.
3. A/S 83 reached maximum levels in the serum by 6 hours after treatment, when the anti-(adipocyte plasma membrane) activity was more than 1,000-fold diluted, compared with A/S 83.

3.5 DETERMINATION OF THE IMPORTANCE OF COMPLEMENT IN MEDIATING THE IN VIVO EFFECTS OF A/S 83

3.5.1 Experimental

The dose of cobra venom factor (CVF) required to deplete rats of complement for at least 5 days was determined by injecting 120-140 g female Wistar rats, intraperitoneally, with 10 or 30 μg of CVF in 200 μl of PBS, 3 times, at 8-hour intervals. One rat that had

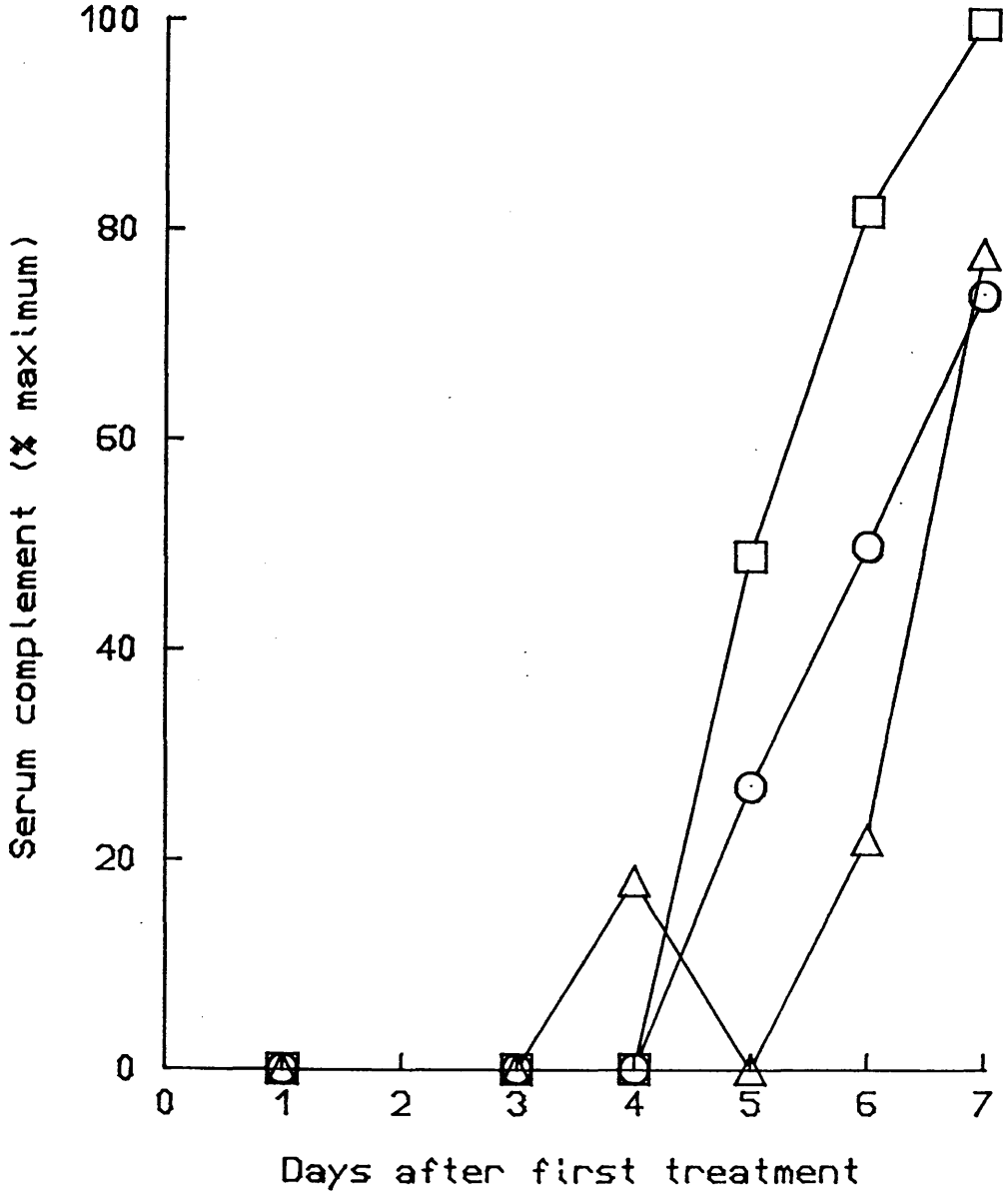
received 3 x 10 μg of CVF on day 0 was given a second 3 x 10 μg dose on day 4. Rats were bled daily, from the tail, under ether anaesthesia. Serum was prepared and stored as described in Section 3.4.1. Complement was assayed as described in Section 2.12.1. The effects of CVF on body weight and food intake were determined.

To assess the role of complement in mediating the in vivo effects of A/S 83, 120-140 g female Wistar rats were injected with 3 x 10 μg of CVF on day 0, as described above. Rats received the equivalent of 2 ml of A/S 83 γ globulin intraperitoneally on days 1, 2, 3 and 4. On day 4 they also received a second 3 x 10 μg dose of CVF. A second group of rats received the same treatment, but with no CVF, and a third group were pairfed with the second group. Body weights and food intakes were measured daily. Rats were killed 6 days after the first treatment with A/S 83. Wet and dry weights of parametrial, peri-renal and inguinal subcutaneous adipose depots were determined. These depots were also scored by visual appearance on a scale of 0-3 for lymphocytic infiltration, as shown by a nodular appearance. Mean adipocyte volumes and adipocyte numbers of parametrial adipose tissue were also determined.

3.5.2 Results

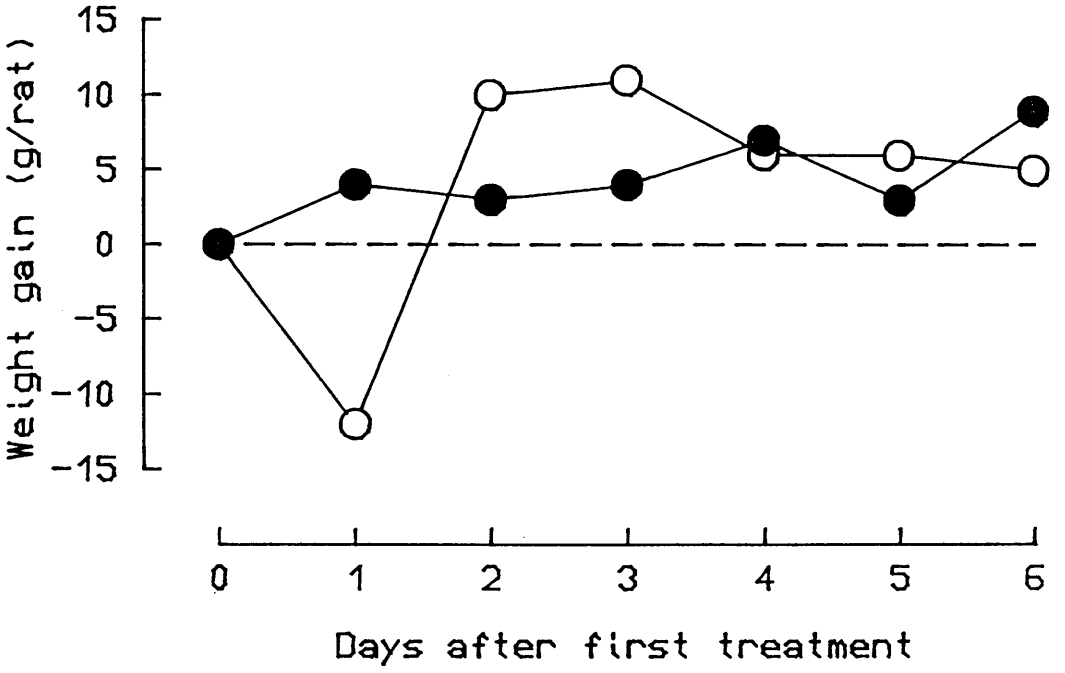
Treatment of rats with 3 x 10 μg of CVF on days 0 and 4 caused decomplementation for 5 days (Fig. 3.17). Treatment with CVF alone had no effect on food intake or body weight. CVF treatment prevented the A/S 83-induced reduction in food intake and weight gain (Fig. 3.18). Six days after treatment with A/S 83, lymphocytic infiltration was clearly visible in parametrial, peri-renal and omental adipose tissue, while that of A/S 83-treated rats, that had

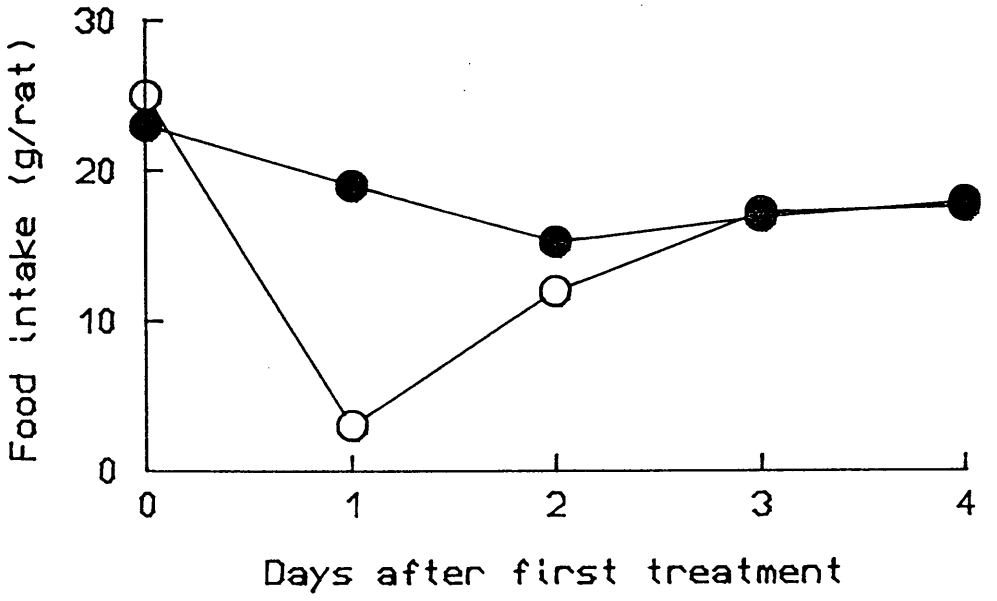
Fig. 3.17. The effects of Cobra Venom Factor on serum complement levels



Rats received either 30 µg (open squares), 90 µg (open circles) or 30 µg on day 0 and 30 µg on day 4 (open triangles) of CVF in 3 intraperitoneal injections separated by 8 h. Rats were bled from the tail and serum complement was measured as described in Section 2.12. Results are single observations.

Fig. 3.18. Body weight gain and food intake of CVF and A/S 83-treated rats





Rats received 30 µg of CVF on day 0 and day 4 (filled circles). CVF-treated and control rats (open circles) were injected with the equivalent of 2 ml of A/S 83 γ globulin intraperitoneally on days 1 to 4. Results are means ± SEM of 3 observations.

also received CVF, showed no lymphocytic infiltration (Table 3.7). Dry weights of parametrial, peri-renal, omental and subcutaneous adipose tissue were reduced in A/S 83-treated animals, but not in rats also given CVF. The percentage dry weights of these depots, used as an indication of lymphocytic infiltration, were slightly reduced in rats that had received A/S 83 and CVF but were reduced to a much greater extent in animals given A/S 83 alone (Table 3.7). Parametrial adipocyte numbers were not significantly reduced in rats given A/S 83 and CVF, while those of rats given A/S 83 alone were significantly reduced by 60%.

3.5.3 Discussion

As treatment with CVF alone had no effect on food intake or body weight gain, the A/S 83-induced reduction in food intake could not be due to complement activation and the resulting liberation of anaphylatoxins.

Lymphocytic infiltration of adipose tissue and immediate reductions in adipocyte number were shown to be, at least in part, dependent on the presence of circulating complement. It is not clear, however, whether the role of complement in mediating reductions in adipocyte number was the attraction of polymorphonuclear leucocytes and lymphocytic cells, which then participated in antibody-dependent cell-mediated cytotoxicity, or the direct lysis of adipocytes through the formation of the membrane attack complex. The lowest A/S concentration, where *in vitro* cytotoxicity against adipocytes was measurable, was 4 μl/ml, while the highest measured concentration of A/S 83 *in vivo* in the circulation was 0.23 μl/ml (Fig. 3.16). However, the portion of A/S

Table 3.7. The effects of cobra venom factor and A/S 83 on adipose tissue 1 week after treatment

Treatment	Number of observations	Body weight (g)	Parametrial fat pad				
			Dry weight (g)	% dry weight	Mean adipocyte volume (pl)	Adipocyte number ($x10^6$)	Infiltration score (0–3)
Control	3	166 ±6	0.56 ±0.12	0.73 ±0.03	45 ±15	12.2 ±1.8	0
A/S 83	3	159 ±6	0.32 ±0.04	0.47 ±0.04	65 ±4	4.5*** ±0.4	2.3
A/S 83 + CVF	3	164 ±9	0.64 ±0.11	0.61 ±0.04	58 ±10	8.5[§] ±0.9	0

Rats received 30 μg of CVF on day 0 and day 4. A/S 83-treated rats were injected intraperitoneally with the equivalent of 2 ml of A/S 83 γ globulin for 4 consecutive days (days 1-4). Control rats were pairfed with rats receiving A/S 83 alone. Results are means ± SEM. [§]$P<0.01$, compared with A/S 83 treatment alone (Student's unpaired t test). ***$P<0.001$, compared with controls (Student's unpaired t test).

83 of physiological significance may have been bound to the tissues, rather than in the circulation. *In vitro* assays cannot completely represent the *in vivo* situation, so a lower concentration of antiserum may be adequate for *in vivo* cytotoxicity. Maximum measurable antibody levels *in vivo* persisted for at least 18 h, while *in vitro* cytotoxicity assays were performed over a period of 1 h, to minimise non-specific LDH release. Prolonged exposure to low antibody concentrations may have been as cytotoxic as short exposure to high concentrations. Alternatively, the main role of complement in mediating the *in vivo* effects of A/S 83 may have been the attraction of phagocytic cells to the site of antibody deposition, as has been suggested for the effects of anti-(kidney) antibodies in the induction of passive nephrotoxic nephritis (Ulich, Bannister and Wilson, 1987). It would be of interest to determine whether the infiltration of adipose tissue with phagocytic cells occurred before or after the rise in serum free fatty acids and triglycerides seen in A/S 83-treated rats. If the latter, the role of the cellular infiltrate may be the phagocytosis of the products of the breakdown of adipose tissue, rather than attack of the intact tissue.

Complement was not shown in these experiments to be necessary for the A/S 83-induced reduction in adipocyte numbers, seen at 3, 8 and 24 weeks. Clearly, long-term experiments investigating the *in vivo* effects of complement are necessary, although CVF is highly immunogenic, rendering it impossible to decomplement rats for more than 6 days. Antibody may still be present after this time point and so complement-mediated adipocyte damage may only be delayed, rather than prevented.

3.5.4 Conclusions

1. The cellular infiltration of adipose tissue and the immediate reduction in adipocyte numbers, induced by A/S 83, was clearly dependent on the presence of complement.

3.6 EFFECTS OF A/S 83 ON PREGNANCY AND LACTATION

3.6.1 Experimental

Female Wistar rats, weighing 120-140 g, were given a single intra-peritoneal injection of the equivalent of 2 ml of A/S 83 γ globulin. Control rats were pairfed with the treated group for 4 days. Three weeks after treatment, treated and control rats were mated with normal males. Body weights and food intake were monitored throughout pregnancy and lactation. Two treated and 2 control rats were killed on day 20 of pregnancy. Dry weights of parametrial, omental and peri-renal adipose depots and a combination of mammary and inguinal subcutaneous adipose tissue were determined. Litters were adjusted to 8 pups at birth and pup weights were monitored during lactation. Pups were removed from dams on day 21 of lactation. A/S-treated and control rats were killed 3 weeks after day 21 of lactation, which was 12 weeks after A/S treatment. Virgin A/S 83-treated and control rats were also killed 12 weeks after treatment. Dry weights of parametrial, peri-renal, omental and inguinal subcutaneous adipose tissue were determined.

3.6.2 Results

Treatment with A/S 83 3 weeks before mating had no effect on the ability of rats to mate or on body weight gain during pregnancy.

There were no differences in foetal numbers and weights, mammary weights and adipose tissue weights between the 2 controls and 2 A/S-treated rats killed on day 20 of pregnancy (Table 3.8). There were no significant effects of treatment with A/S 83 on the body weight of the dam or on weight gain of the litter (Fig. 3.19).

Twelve weeks after treatment, the dry weights of parametrial adipose tissue of A/S 83-treated rats, whether they were virgin or had undergone a cycle of pregnancy and lactation, were almost identical (Table 3.9). Parametrial adipose mass of A/S 83-treated rats, whether virgin or ex-lactating, was less than that of virgin controls and of controls that had undergone a cycle of pregnancy and lactation, although this only achieved statistical significance when compared with virgin controls. Control lactating rats showed a slightly lower parametrial adipose mass than virgin controls and both groups of rats that were ex-lactating showed more variability in adipose mass than virgin rats. A/S-treated rats, whether virgin or ex-lactating, showed no significant changes in subcutaneous, peri-renal and omental adipose mass, compared with controls.

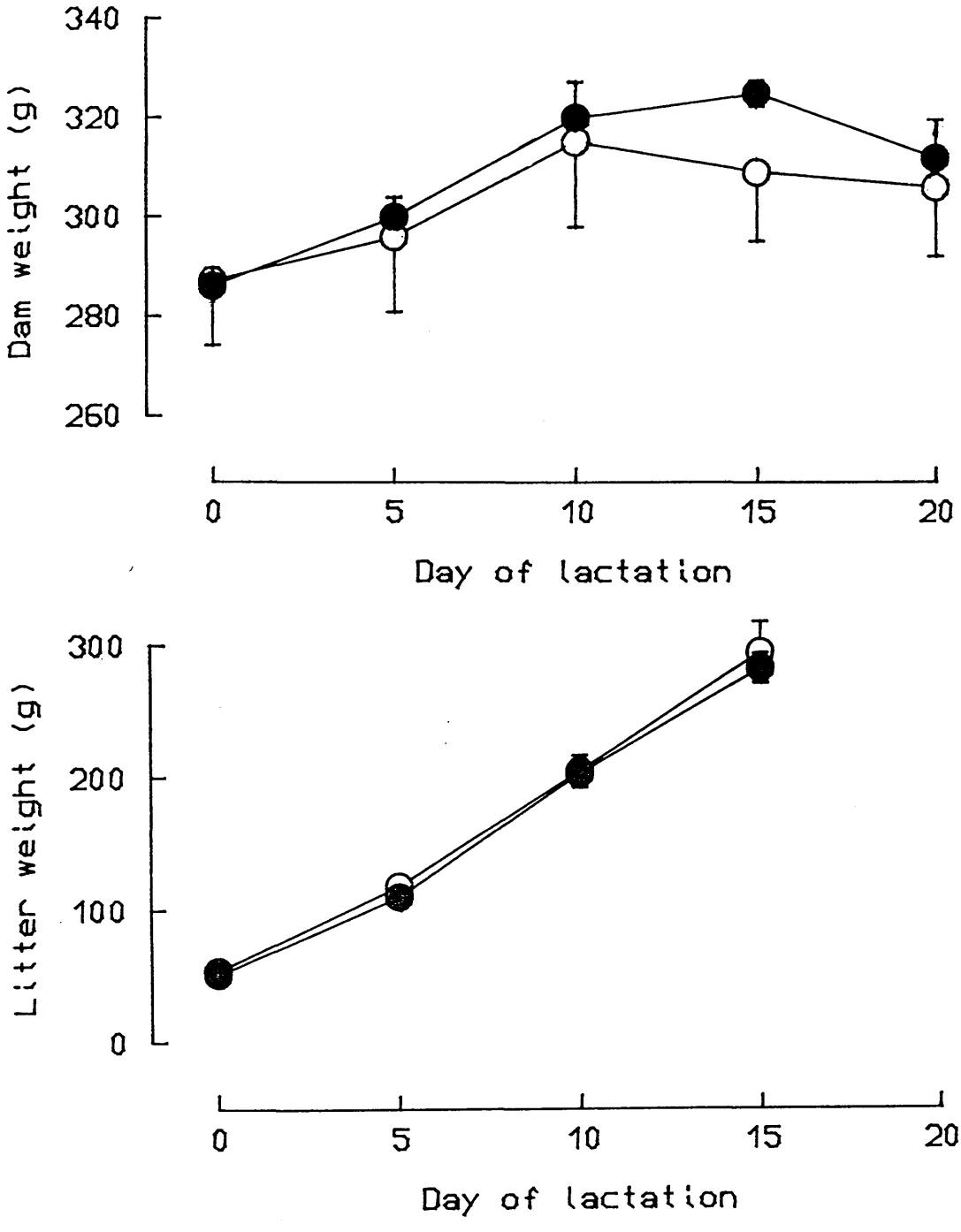
3.6.3 Discussion

Since A/S 83-treated rats showed no abnormalities in their ability to mate or in their foetal numbers or weights, the massive cellular infiltration of parametrial adipose tissue, induced by A/S 83, cannot have caused lasting damage to the uterus or ovaries.

The A/S 83-treated rats killed at the end of pregnancy had the same amount of parametrial adipose tissue as control rats, implying that pregnancy induced recovery of lost adipose mass. However, A/S 83-treated rats, killed after a cycle of pregnancy and lactation, had

Fig. 3.19. The effects of A/S 83

on dam and litter weight gain during lactation



Rats received 1 ml of A/S 83 γ globulin intraperitoneally (filled circles). Control rats were pairfed with treated rats for 4 days and then allowed to feed ad libitum (open circles). Rats were mated 3 weeks after treatment. Results are means ± SEM of 6 observations.

Table 3.8. The effects of A/S 83 on foetal weight and mammary and adipose tissue weight

Treatment	Number of observations	Mean foetal weight	Wet weight mammary (g)	Dry weight fat pad		
				Parametrial	Peri-renal	Omental
Control	2	6.9	6.4	3.96	3.50	3.00
A/S 83	2	6.2	7.8	5.60	2.88	3.00

Rats were injected intraperitoneally with 1 ml of A/S 83 γ globulin. Control rats were pairfed with A/S-treated rats for 4 days and then allowed to feed ad libitum. Rats were mated 3 weeks after treatment and killed on day 20 of pregnancy.

Table 3.9. The effects of one cycle of pregnancy and lactation on fat pad weights of A/S-treated and control rats

Treatment		Number of observations	Body weight (g)	Dry weight fat pad (g)				
				Interscapular subcutaneous	Inguinal subcutaneous	Parametrial	Peri-renal	Omental
Control	Virgin	6	269 ±13	1.71 ±0.29	3.22 ±0.32	7.28 ±0.62	3.73 ±0.59	3.71 ±0.52
A/S 83	Virgin	5	274 ±8	1.61 ±0.34	4.04 ±0.57	4.97* ±0.44	4.09 ±0.47	4.54 ±0.53
Control	Ex-lactator	6	282 ±11	1.27 ±0.15	2.55 ±0.29	6.59 ±0.77	3.18 ±0.31	3.11 ±0.30
A/S 83	Ex-lactator	6	283 ±2	1.49 ±0.06	2.96 ±0.17	4.94* ±0.82	3.27 ±0.38	3.64 ±0.27

Rats were injected intraperitoneally with 1 ml of A/S γ globulin. Control rats were pairfed with A/S-treated rats for 4 days and then allowed to feed ad libitum. Rats were mated 3 weeks after treatment and litters were removed on day 21 of lactation. Rats were killed and the fat pads weighed 2 weeks after litter removal. Results are means ± SEM. *$P<0.01$, compared with virgin controls (Student's unpaired t test).

less parametrial adipose tissue than controls (Table 3.9), implying that pregnancy did not induce recovery of lost adipose mass. This discrepancy may be partly the result of the very small numbers of rats killed at the end of pregnancy. Both sets of results imply that the reduction in fat deposition, seen in parametrial adipose tissue of A/S 83-treated rats between 1 and 8 weeks, was due to a defect in a proportion of the total adipocyte population and the remaining adipocytes were capable of accumulating large quantities of lipid, given the stimulus of pregnancy. However, for unknown reasons, the reduction in adipose mass in virgin A/S 83-treated rats was not as great as had been observed in previous experiments. The degree of A/S 83-induced reduction of adipose tissue mass was probably not sufficient to affect any possible competition between adipose and mammary tissue during lactation.

3.6.4 Conclusions

1. The results of this experiment suggested that treatment with A/S 83 did not affect the rat's ability to mate and undergo a normal pregnancy and lactation. However, the numbers of animals used were low and A/S 83 did not, in this experiment, have as great an effect on adipose tissue as had been seen in previous experiments.

CHAPTER 4

IN VITRO AND IN VIVO CHARACTERISATION OF ANTISERA RAISED AGAINST RAT ADIPOCYTE, HEPATOCYTE AND ERYTHROCYTE PLASMA MEMBRANES AND ADIPOCYTE SPECIFIC ANTIGENS

IN VITRO AND IN VIVO CHARACTERISATION OF ANTISERA RAISED AGAINST RAT ADIPOCYTE, HEPATOCYTE AND ERYTHROCYTE PLASMA MEMBRANES AND ADIPOCYTE SPECIFIC ANTIGENS

4.1 INTRODUCTION

In this chapter the sheep anti-(adipocyte plasma membrane) antiserum (A/S 83), used in the experiments described in Chapter 3, is compared with antisera from a more recent bleed from the same sheep and a number of other sheep antisera against rat adipocyte plasma membranes, as well as hepatocyte and erythrocyte plasma membranes.

Attempts were made to examine the role of antibodies that cross-react with non-adipose tissues in mediating the *in vitro* and *in vivo* effects of anti-(adipocyte plasma membrane) antisera. It would, clearly, be desirable to increase the specificity of the antisera as much as possible, without removing the effects on adipose tissue. Whilst A/S 83 caused no gross abnormalities of non-adipose tissue *in vivo*, the function of non-adipose tissues, with the exception of the kidney, was not monitored at a biochemical level. A/S 83 showed considerable cross-reactivity with other tissues *in vitro* (Fig. 3.6). The possibility of effects on non-adipose tissues *in vivo* cannot, therefore, be excluded. In addition, a reduction in the cross reactivity of A/S 83 with kidney tissue may reduce the transient nephritis seen in A/S 83-treated rats. It is also possible that the reduction in food intake during the first day after A/S 83 treatment, in some way, related to cross-reaction with other tissues.

In order to determine whether cross-reacting antibodies were necessary for the fat-reducing properties of anti-(adipocyte plasma membrane) antisera, the antisera were adsorbed with a liver homogenate to render the antisera more specific for adipose tissue. In addition, an antiserum was raised against adipocyte specific antigens prepared by the method described in Section 2.9. Antisera were also raised against erythrocyte and hepatocyte plasma membranes, in order to determine whether adipocyte specific antibodies were necessary for the *in vivo* reduction of adiposity or whether the apparently adipocyte specific effects of A/S 83 were due to the site of injection or to the nature of the tissue itself.

All of the antisera described above were characterised by ELISA, *in vitro* cytotoxicity assays, Western blotting and short-term *in vivo* experiments. Discrepancies in apparent cytotoxicity and tissue specificity between *in vitro* and *in vivo* assays resulted in efforts to design *in vitro* assays that could effectively predict the likely effects of an antiserum *in vivo*.

The characteristics of adipose tissue development and the response to lipectomy and repartitioning agents partly depend on sex and genetic composition (Willham, 1976; Hood, 1977; Bern, Novakofski and Bechtel, 1985; Moran, 1986). Due to the lack of *in vivo* effects of recent bleeds of A/S 83, it was not possible to compare the effects of A/S 83 on male and female rats, or on rats of different strains. It was possible, however, to compare the binding of this antiserum to plasma membranes from male and female Wistar and female Sprague-Dawley rats, using the small quantities of early A/S 83 available.

4.2 A COMPARISON OF THE IN VITRO AND IN VIVO EFFECTS OF EARLY AND LATE BLEEDS OF A/S 83

4.2.1 Experimental

A/S 83 was raised according to the method described in Section 2.4. Both early and late bleeds were obtained from the same sheep, 10-14 days after a booster immunisation of adipocyte plasma membranes in incomplete Fruends adjuvant. The late bleed of A/S 83 was obtained about 18 months after the early bleed. Binding of the 2 antisera to adipocyte, hepatocyte, erythrocyte and kidney plasma membranes by ELISA, cytotoxicity against adipocytes and erythrocytes and Western blotting against adipocyte and hepatocyte plasma membranes and adipocyte specific antigens were all performed according to the methods described in Chapter 2.

4.2.1.1 In vivo effects of early and late A/S 83 in female rats

Female Wistar rats, weighing 120-130 g, were injected intraperitoneally, between 09.00 h and 11.00 h on 3 consecutive days, with either 2 ml of early A/S 83, 2 ml of late A/S 83, 1 ml of late A/S 83, a mixture of 1 ml of early and 1 ml of late A/S 83 or were untreated. Body weights and food intakes were recorded. Rats were killed 6 days after the first treatment. Wet and dry weights of parametrial, peri-renal, omental and inguinal subcutaneous adipose depots were determined. These depots were also scored by visual appearance on a scale of 0-3 for lymphocytic infiltration, as shown by the nodular appearance of the tissue.

4.2.2 Results

The antibody binding of the late bleed of A/S 83 to adipocyte plasma membranes (APM), hepatocyte plasma membranes (HPM) and kidney

plasma membranes (KPM) were reduced by about 50%, compared with early bleeds, while the binding to erythrocyte plasma membranes (EPM) was reduced by 90% in the late bleed (Table 4.1). Relative antigen abundances against these antigens were also reduced but not to the same extent as the antibody binding. The 2 bleeds of A/S 83 showed similar levels of in vitro cytotoxicity against adipocytes and erythrocytes (Fig. 4.1). Western blotting showed that the late bleed of A/S 83 apparently recognised only 15 APM polypeptides, compared with 21 polypeptides recognised by early A/S 83, but this may have been due only to a lower titre of late A/S 83 (Fig. 4.2). Late A/S 83 bound more strongly to a polypeptide of a molecular weight of about 85 kD of the adipocyte specific antigen preparation (ASA) than early A/S 83. Late A/S 83 bound to only 5 HPM polypeptides, compared with 12 recognised by early A/S 83, although, again, this may have been due to differences in titre of the 2 bleeds.

The administration of late A/S 83 caused only 30% of the fall in body weight and food intake caused by an equivalent amount of A/S 83 (Table 4.2). Late A/S 83 caused less lymphocytic infiltration of adipose depots than early A/S 83. A mixture of early and late A/S 83 caused similar drops in food intake and body weight and more lymphocytic infiltration than A/S 83 alone.

While the results described above were obtained using a single bleed of late A/S 83 and pooled bleeds of early A/S 83, subsequent experiments showed that several bleeds of late A/S 83, taken after the same and after a subsequent booster immunisation, gave similar results (results not shown).

Table 4.1. Antibody binding to adipocyte, hepatocyte, kidney and erythrocyte plasma membranes by early and late bleeds of A/S 83

Plasma membrane antigen	Antibody activity as % of anti-(adipocyte plasma membrane activity) of early A/S 83			
	Early A/S 83		Late A/S 83	
	Ab binding	Ag abundance	Ab binding	Ag abundance
Adipocyte	100	100	54 (54)	81 (81)
Hepatocyte	32	99	11 (33)	79 (80)
Kidney	25	66	12 (48)	45 (68)
Erythrocyte	7	37	1 (9)	24 (64)

Early and late A/S 83 were antisera obtained from the same sheep 10-14 days after a booster immunisation with adipocyte plasma membranes but late A/S 83 was obtained more than 18 months after early A/S 83. Relative antibody binding (Ab binding) and relative antigen abundance (Ag abundance) were measured by ELISA and calculated as described in Section 2.6.3. Figures in parentheses represent the % of the antibody activity of early A/S 83 against that particular antigen.

Fig. 4.1. *In vitro* cytotoxicity against adipocytes and erythrocytes of early and late bleeds of A/S 83

Heat-inactivated antisera were incubated with isolated adipocytes (a) or erythrocytes (b) for 1 h at 37° C. Adipocytes were separated from the medium by spinning through oil and the medium was assayed for LDH, as described in Section 2.7.2. Erythrocytes were separated from the medium by centrifugation and the medium was assayed for haemoglobin by measuring absorbance at 405 nm. LDH or haemoglobin release in the presence of non-immune serum was subtracted from that in the presence of antiserum and expressed as a percentage of total LDH release (determined in the presence of 2% Triton X-100). Results are mean ± SEM of 3-5 observations. The cytotoxicity of an early bleed of A/S 83 (filled circles) was compared with a bleed taken from the same sheep more than 18 months later (open circles).

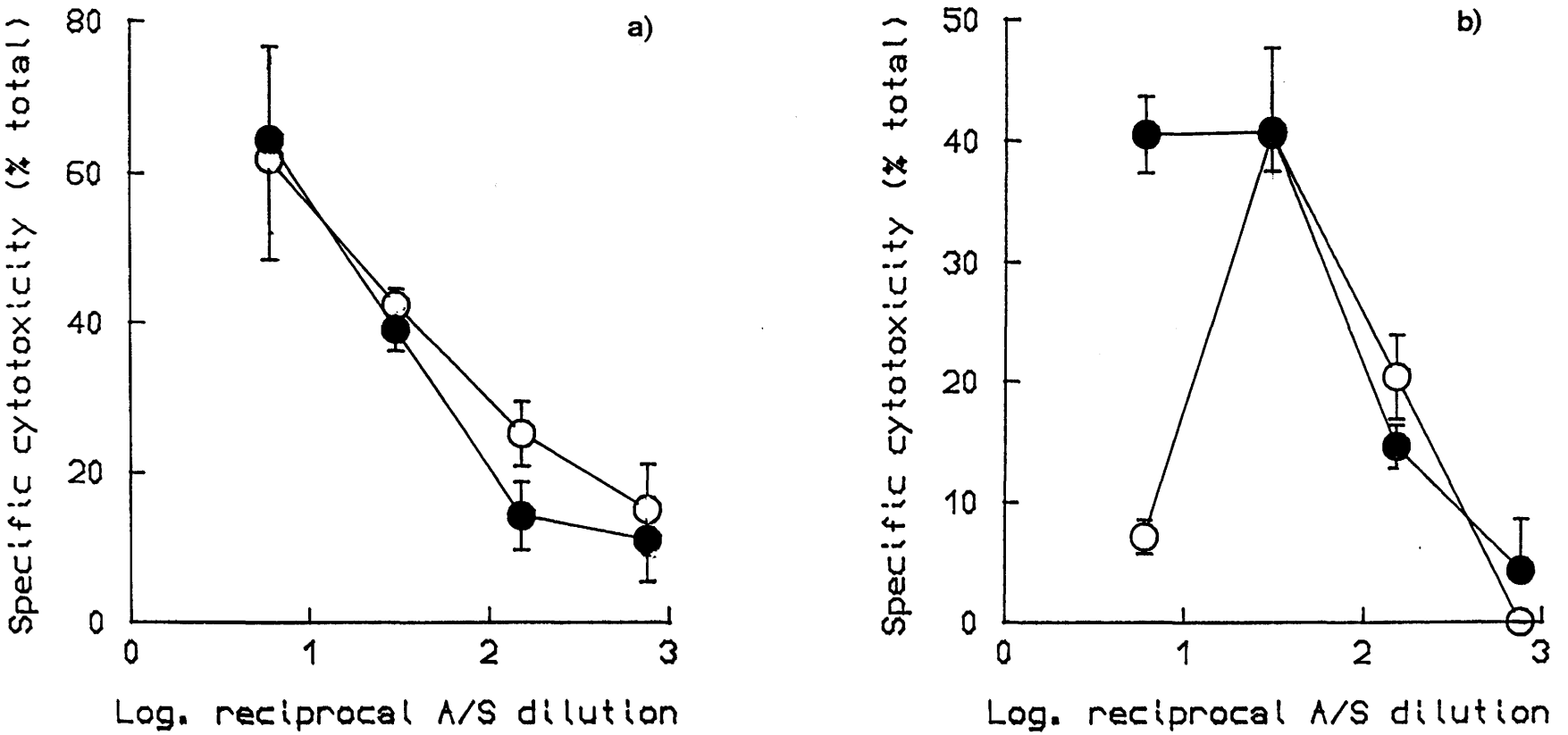
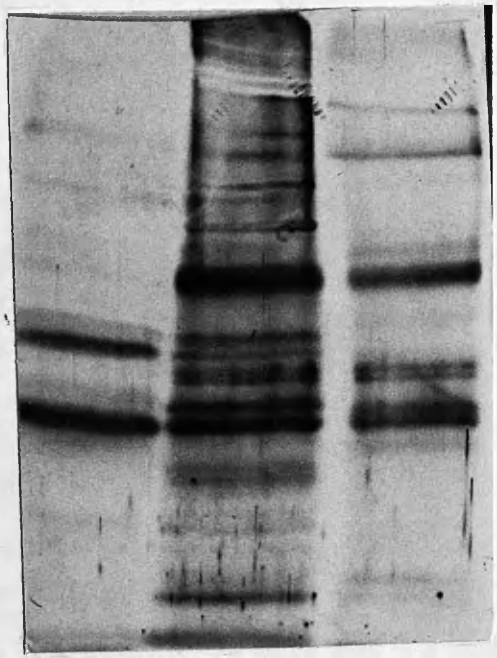

a)
Specific cytotoxicity (% total)
80
60
40
20
0
0
1
2
3
Log. reciprocal A/S dilution
b)
Specific cytotoxicity (% total)
50
40
30
20
10
0
0
1
2
3
Log. reciprocal A/S dilution

Fig. 4.2. Antibody binding
to adipocyte and hepatocyte plasma membranes
and adipocyte specific antigens
by early and late bleeds of A/S 83,
as determined by Western blotting

Adipocyte specific antigens (ASA), adipocyte plasma membranes (APM) and hepatocyte plasma membranes (HPM) were separated by SDS PAGE and transferred to nitrocellulose and binding of early and late bleeds of A/S 83 to transferred proteins was detected, as described in Section 2.11. Proteins mentioned in the text have been labelled by P, followed by the approximate molecular weight in kD.

Early A/S 83



Late A/S 83

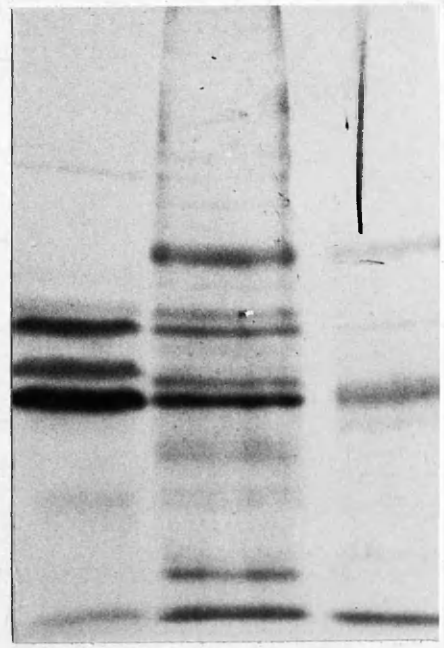


Table 4.2. *In vivo* effects of early and late bleeds of A/S 83

Treatment	Number of observations	Body weight change (1st day) (g)	Food intake (1st day) (g)	Infiltration score of fat pad		
				Parametrial	Omental	Peri-renal
None	4	+3.1	20.6	0	0	0
Early A/S 83 (2 ml)	3	-10.3	5.3	2	0.7	1
Late A/S 83 (2 ml)	3	-2.9	16.5	1	0.7	0.7
Early A/S 83 (1 ml)	3	-6.0	8.2	0.7	0	0.3
Early and late A/S 83 (1 ml of each)	3	-7.7	3.7	2.7	2	2

Female Wistar rats were injected intraperitoneally with the treatments indicated on 3 consecutive days. Rats were sacrificed 6 days after the first treatment and infiltration of adipose tissue was scored on a scale of 0-3 by visual appearance of yellow nodules.

4.2.3 Discussion

The reduction in the in vivo effects of late bleeds of A/S 83, compared with earlier bleeds, may be due, either to differences in the immunogen used to boost the sheep prior to bleeding, or to differences in the response of the sheep to the same immunogen.

The bleeds tested were separated by at least 18 months and the APM were prepared up to a month before immunisation. Outbreeding of the Wistar rat colony used to provide the adipocytes may have resulted in differences in the expression of adipocyte surface antigens, particularly highly polymorphic proteins, such as those encoded by the Major Histocompatibility Complex (MHC). However, the differences between the 2 bleeds of A/S 83 were detected using rats likely to be of similar genetic composition to those used to provide the immunogen for boosting the sheep prior to obtaining the late bleed. The differences between the 2 bleeds, therefore, were not likely to be entirely due to differences in the immunogen. Western blotting of the 2 different bleeds against ASA showed that late A/S 83 bound more strongly to an 85 kD protein than early A/S 83 (Fig. 4.2) suggesting that this protein may have been expressed at a higher frequency on APM used to boost the late bleed, compared with those used for early immunisations (Fig. 4.2).

Studies of mitogen responsiveness and T lymphocyte-dependent and T lymphocyte-independent immune responses in humans, rats and mice have shown that B cell responses decline with age (Makinodan and Kay, 1980). A decline in secondary immune responses with age has been demonstrated, characterised by decreases in antibody avidity and selective losses of IgG-producing cells (Goidl, Innes and Webster, 1976). This decline was thought to be due to specific suppression or

defective T cell help (Segre and Segre, 1976; Krogsrud and Perkins, 1977). An increase in the production of suppressive anti-idiotypic antibodies in aged animals has also been demonstrated (Goidl, Thorbecke, Webster, and Siskund, 1980; Szewezyuk and Campbell, 1981). Since the treatment of rats with a mixture of early and late A/S 83 induced an anti-fat response of a greater magnitude than early A/S 83 alone, suppressive anti-idiotypic antibodies were unlikely to be responsible for the reduced in vitro and in vivo effects of late A/S 83 (Table 4.2).

Late A/S 83 was as cytotoxic in vitro against erythrocytes as early A/S 83, implying that the antiserum had not lost the ability to fix guinea pig complement (Table 4.1). However, when rat serum was used as a source of complement, neither early nor late A/S 83 showed measurable cytotoxicity (results not shown). Different classes or subclasses of immunoglobulin differ in their ability to fix complement (Borsos and Rapp, 1965). It is possible, therefore, that an immunoglobulin class or subclass switch occurred, with time and repeated immunisation of sheep 83, such that later bleeds of the antiserum were composed, predominantly, of a subclass of IgG unable to fix rat complement. Time did not permit this possibility to be examined.

4.2.4 Conclusions

1. Recent bleeds of A/S 83 did not reproduce the in vivo effects on food intake, body weight gain and adiposity of early bleeds. This did not appear to be due to the presence of suppressive antibodies in recent bleeds.

2. Recent bleeds of A/S 83 had a lower titre or affinity of anti-(APM) antibodies than early bleeds, as shown by ELISA and Western blotting, possibly due to an age-dependent decline in the function of the immune system of the sheep used as antiserum donor.

4.3 A COMPARISON OF THE IN VITRO AND IN VIVO EFFECTS OF ANTISERA RAISED AGAINST ADIPOCYTE, HEPATOCYTE AND ERYTHROCYTE PLASMA MEMBRANES AND ADIPOCYTE SPECIFIC ANTIGENS

4.3.1 Experimental

The following antisera were raised, in sheep, by S. Kestin at the Institute of Food Research (Bristol Laboratory), according to the method described in Section 2.4:

Antiserum	Immunising antigen
A/S 164	Adipocyte plasma membranes (APM)
A/S 283	Adipocyte plasma membranes (APM)
A/S 361	Adipocyte specific antigens (ASA)
A/S 479	Hepatocyte plasma membranes (HPM)
A/S 217	Erythrocyte plasma membranes (EPM)

The results of immunisation of a sheep at the Hannah Research Institute with ASA suggested that this detergent solubilized preparation was not very immunogenic. Consequently, for the raising of A/S 361, 250 μg of ASA was conjugated to 250 μg of rabbit serum albumin (RSA) by co-incubating the 2 proteins with 50 μl of 0.5% glutaraldehyde (EM grade, Sigma, Poole, Dorset) for 30 minutes at 22° C. Three immunisations of the conjugated ASA, followed by 2 immunisations with ASA alone, were performed.

Antibody binding of all the antisera described above was measured, by ELISA, to the following antigens: adipocyte plasma membranes (APM), hepatocyte plasma membranes (HPM), kidney plasma membranes (KPM), erythrocyte plasma membranes (EPM), brain membranes (BM), adipocyte specific antigens (ASA), myelin basic protein (from rabbit brain, Sigma Chemical Co., Poole, Dorset) and sphingomyelin (from bovine brain, Sigma Chemical Co., Poole, Dorset). Coating of plates with sphingomyelin was performed by dissolving the lipid in a solution of 10% chloroform in methanol. 100 μl of 10 μg/ml sphingomyelin was added to each well and the solvent allowed to evaporate at 37° C. Binding of A/S 83 to interscapular brown adipocyte plasma membranes, prepared by the same method as white adipocyte plasma membranes, was also measured.

ASA were characterised by SDS PAGE, using carbonic anhydrase, ovalbumin, BSA, phosphorylase b, β galactosidase and myosin (Sigma Chemical Co., Poole, Dorset) as molecular weight markers. APM, prepared by digestion of adipose tissue in the presence of BSA and by homogenisation of the tissue in the absence of BSA, and HPM were also characterised by SDS PAGE. Western blotting of all the antisera against APM, ASA and HPM was performed. Antibody binding of all the antisera to BSA, rat albumin and APM prepared in the presence and absence of BSA was compared, by ELISA.

Cytotoxicity against erythrocytes and adipocytes of all the antisera was measured, according to the method described in Section 2.7. In addition, cytotoxicity against erythrocytes in the presence of guinea-pig, sheep and rat complement was compared. The effects of coating ELISA plates with antigen using poly-L-lysine (see Section 2.6.1) and the effects of temperature on antibody binding to EPM were also examined, as described in the Results Section.

Each of the antisera described above were tested in vivo by injecting female Wistar rats, weighing 120-140 g, intraperitoneally with 1 or 2 ml of antiserum at 09.00 h to 11.00 h, for 4 consecutive days. Some antisera showed anaesthetic-like effects in some animals and, where this persisted, the treatment was discontinued and the affected animal killed. The remaining rats were killed 6 days after treatment and examined, as described in the previous Section.

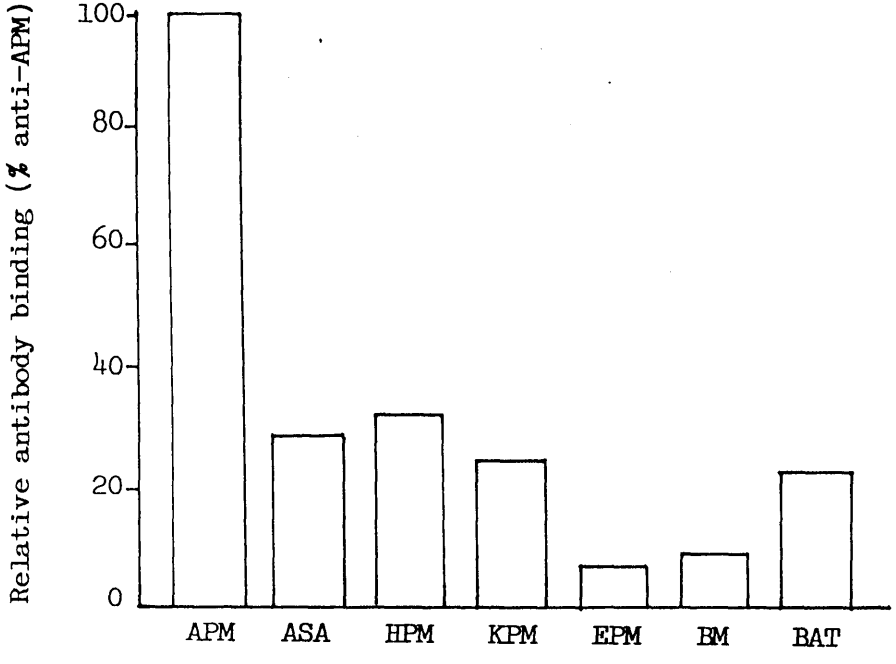
4.3.2 Results

The antibody binding of A/S 83 to ASA, BAT, HPM and KPM was between 20 and 30% of the anti-(APM) binding, assuming that all the antigens bound equally well to micro-titre plates (Fig. 4.3). The antibody binding to EPM and BM was less than 10% of the anti-(APM) binding. Relative antigen abundances of A/S 83 against HPM and BAT were over 90% of that against APM, while antigen abundances against ASA, KPM and BM were between 55 and 70% of that against APM. The antigen abundance against EPM was 37% of that against APM. Little or no antibody against myelin basic protein or sphingomyelin could be detected, although there were no positive controls for these antigens (results not shown).

Antibody binding and antigen abundances of the other antisera tested were, for the most part, less than those of A/S 83 against all the antigens tested (Fig. 4.4 and 4.5). However, there were some exceptions. A/S 164 and 283 (anti-(APM)) showed twice the binding to APM of A/S 83. A/S 164 and A/S 361 (anti-(ASA)) showed about twice the binding to ASA of A/S 83. A/S 479 (anti-(HPM)) and A/S 217 (anti-(EPM)) showed about 8 times and 3 times, respectively, the binding to HPM of A/S 83. A/S 283 (anti-(APM)) and A/S 479

Fig. 4.3. The tissue specificity of A/S 83, as determined by ELISA

Antibody activity of A/S 83 was measured, by ELISA, against adipocyte plasma membranes (APM), adipocyte specific antigens (ASA), hepatocyte plasma membranes (HPM), kidney plasma membranes (KPM), erythrocyte plasma membranes (EPM), brain membranes (BM) and brown adipose tissue membranes (BAT). Relative antibody binding and relative antigen abundance were determined as described in Section 2.6.3.


Relative antibody binding (% anti-APM)
100
80
60
40
20
0
APM
ASA
HPM
KPM
EPM
BM
BAT

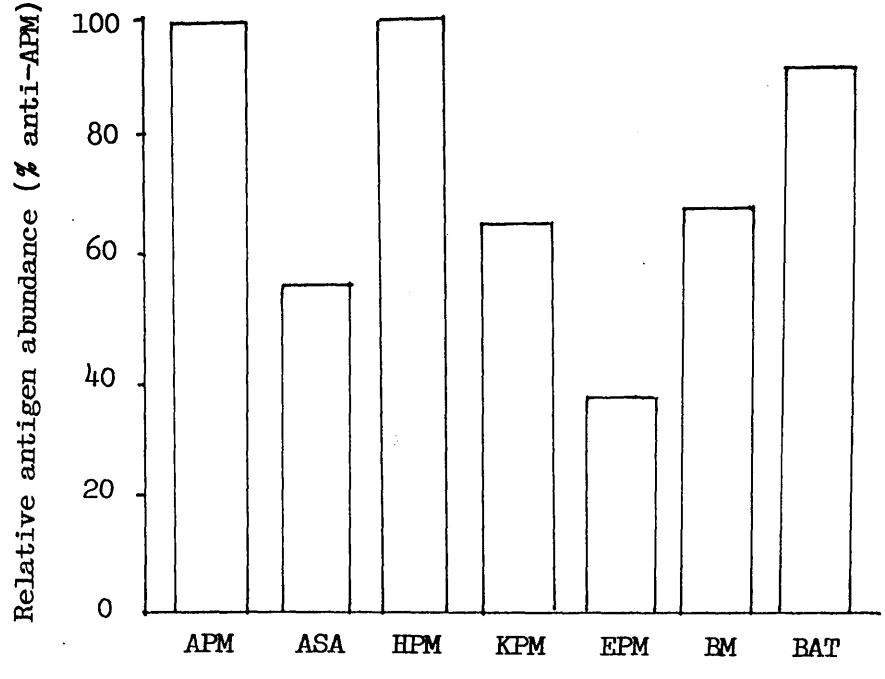

Relative antigen abundance (% anti-APM)
100
80
60
40
20
0
APM
ASA
HPM
KPM
EPM
BM
BAT

Fig. 4.4. The tissue specificity of antisera raised against adipocyte plasma membranes and adipocyte specific antigens as determined by ELISA

Abbreviations are as for Fig. 4.3. Unbroken lines represent relative antibody binding and broken lines represent relative antigen abundance.

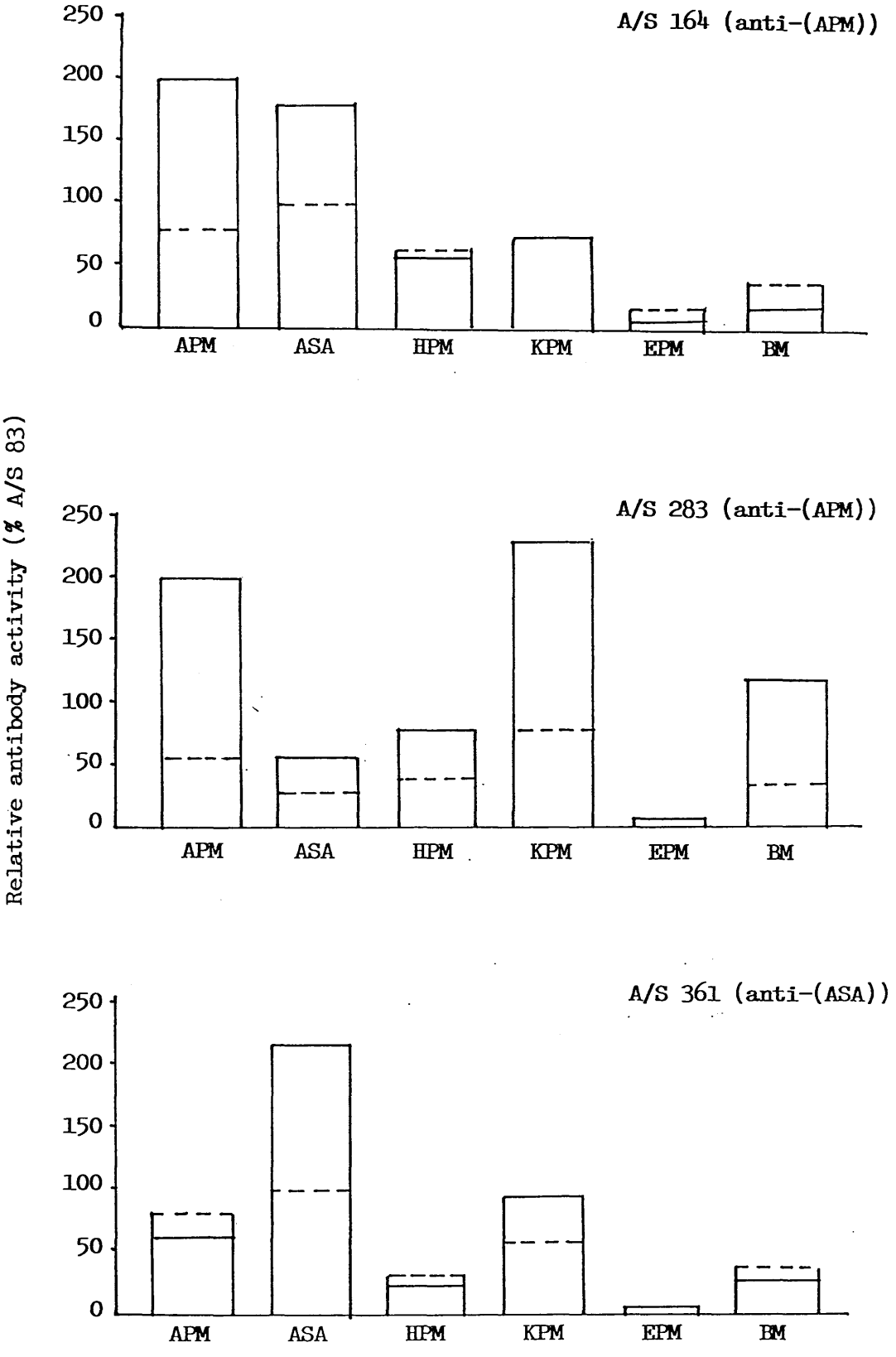
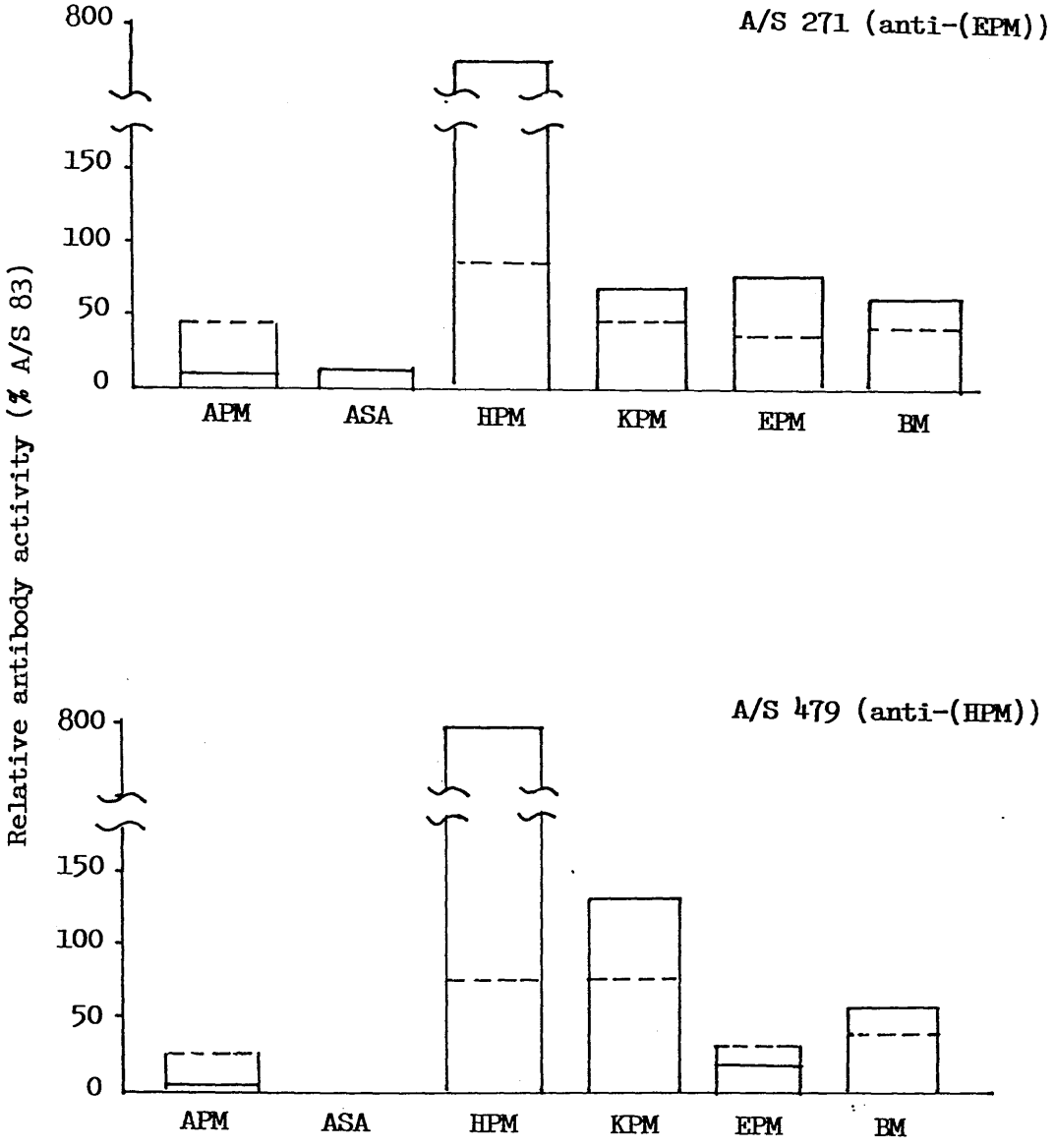

A/S 164 (anti-(APM))
A/S 283 (anti-(APM))
A/S 361 (anti-(ASA))
Relative antibody activity (% A/S 83)
250
200
150
100
50
0
APM
ASA
HPM
KPM
EPM
BM

Fig. 4.5. The tissue specificity of antisera raised against erythrocyte and hepatocyte plasma membranes, as determined by ELISA

Abbreviations and methods as for Fig. 4.3.


A/S 271 (anti-(EPM))
800
150
100
50
0
APM
ASA
HPM
KPM
EPM
BM
Relative antibody activity (% A/S 83)
A/S 479 (anti-(HPM))
800
150
100
50
0
APM
ASA
HPM
KPM
EPM
BM

(anti-(HPM)) showed 2.3 and 1.6 times the anti-(KPM) binding of A/S 83. The greatest reductions in antibody binding, compared with A/S 83, included those of A/S 479 (anti-(HPM)) and A/S 217 (anti-(EPM)) to APM and ASA. A/S 479 showed no binding to ASA and only 5% of the binding of A/S 83 to APM, while A/S 217 showed about 15% of the binding of A/S 83 to APM and ASA. A/S 164 and 283 (anti-(APM)) and A/S 361 (anti-(ASA)) showed less than 7% of the binding to EPM of A/S 83. No antigen abundance of any antiserum against any antigen tested was greater than that of A/S 83.

An outline of the preparation of the ASA, using 3 affinity columns, was given in Chapter 2 (see Fig. 2.2). Table 4.3 summarizes the performance of the affinity columns used to prepare the rat ASA. Only 3% of the IgG fraction of A/S 83 was bound by the APM column, resulting in the bound fraction being enriched in anti-(APM) activity by a factor of 3.4, compared with the fraction added to the column. 33% of this enriched fraction was bound by the HPM, KPM, and EPM column and the fraction that did not bind to this column contained less than 2% of the anti-(HPM) activity of the fraction added to the column. Binding to KPM and EPM was similarly reduced in the unbound fraction (results not shown). This apparently adipocyte specific fraction was used to prepare a third affinity column which bound approximately 5% of the total solubilized APM protein added.

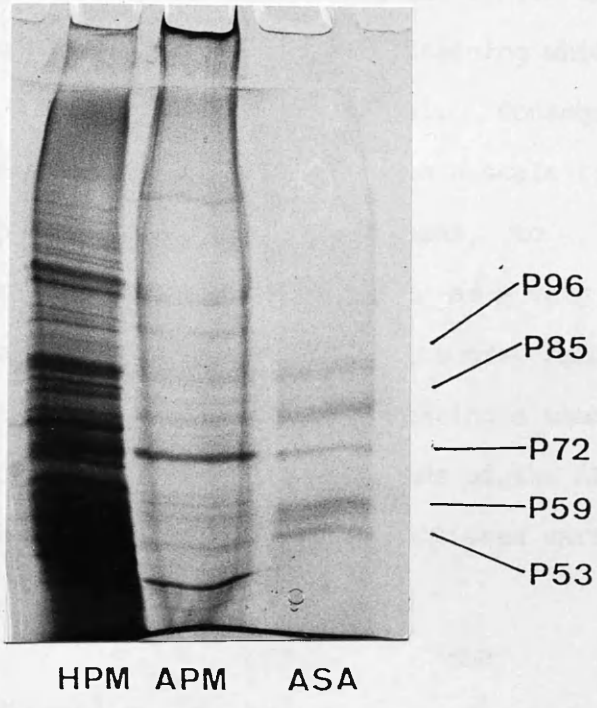
SDS PAGE of HPM, APM and ASA showed that APM consisted of at least 20 major polypeptides of molecular weights varying from 35 to 200 kD (Fig. 4.6). The ASA preparation was greatly enriched in 6 major polypeptides of apparent molecular weights of approximately 53, 59 (a doublet), 72, 85 and 96 kD, while also including very small

Table 4.3. The performance of affinity columns used for the preparation of adipocyte specific antigens

ADIPOCYTE PLASMA MEMBRANE COLUMN		
IgG binding capacity	mg.antibody bound per mg membrane protein on column	0.49
	mg.antibody bound per mg.added IgG	0.031
Purification factor (fold)	Anti-(APM) activity of bound IgG vs. initial IgG	3.4
NON-ADIPOCYTE PLASMA MEMBRANE COLUMN		
IgG binding capacity	mg.antibody bound per mg membrane protein on column	0.064
	mg antibody bound per mg.added IgG	0.33
Purification factor (fold)	anti-(HPM) activity of unbound IgG vs. added IgG	<0.02
ADIPOCYTE SPECIFIC ANTIBODY COLUMN		
Membrane binding capacity	mg. antigen bound per mg IgG on column	0.028
	mg antigen bound per mg.added membrane protein	0.050

The affinity columns were used to prepare adipocyte specific antigens according to the method summarised in Fig. 2.2. IgG binding capacity of affinity columns was measured where that amount of antibody that gave maximum binding to the column was added. Purification factors were calculated by comparing the relative antibody binding to the column ligand per mg. of antibody in added, bound and unbound fractions, by ELISA.

Fig. 4.6. Polyacrylamide gel electrophoresis of adipocyte and hepatocyte plasma membranes and adipocyte specific antigens



Adipocyte specific antigens (ASA), adipocyte plasma membranes (APM) and hepatocyte plasma membranes (HPM) were separated by SDS PAGE and stained with Coomassie blue, as described in Section 2.10.

amounts of 4 further polypeptides. The HPM consisted of more than 35 major polypeptides of a similar molecular weight range to that of APM.

Difficulties were experienced in the interpretation of Western blots, as the same dilution of each antiserum was used for detection of protein bands, resulting in widely differing intensities of colour development of individual blots. This problem could not be overcome by varying the time for which the blot was incubated in the substrate responsible for colour development, as increasing the length of this incubation resulted in higher levels of background staining while not greatly increasing the intensity of the signal. Consequently recognition of protein bands has been classified on a scale ranging from +++, where bands appear black in the photographs, to −, where bands are either not visible or are just visible as a very faint discoloration of the photograph. Dark, mid- and light grey bands are represented by ++, + and ± respectively, ± representing a band that was only very weakly recognised. The number of bands of the APM and HPM recognised, at least weakly, by the different antisera varied as follows (see Figs. 4.7, 4.8):

	APM	HPM
A/S 83 (anti-(APM))	21	9
A/S 164 (anti-(APM))	11	8
A/S 283 (anti-(APM))	8	5
A/S 361 (anti-(ASA))	9	3
A/S 217 (anti-(EPM))	2	5
A/S 479 (anti-(HPM))	4	12

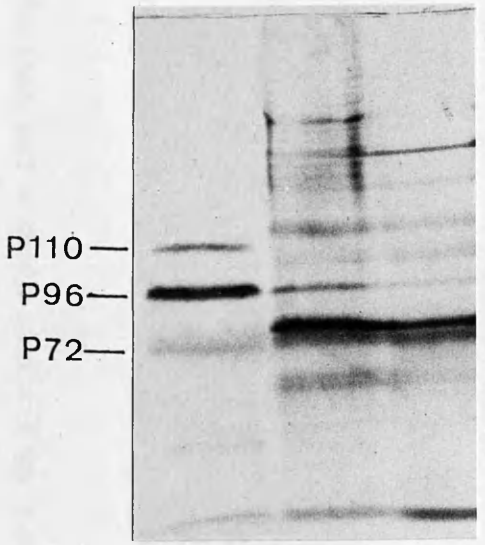
No bands of the APM were clearly recognised by any antiserum that were not also recognised by A/S 83. Only A/S 479 and A/S 217

Fig. 4.7. Antibody binding to adipocyte and hepatocyte plasma membranes and adipocyte-specific antigens by antisera raised against adipocyte plasma membranes, as determined by Western blotting.

Abbreviations and methods as for Fig. 4.2.

A/S 83

A/S 164



A/S 283

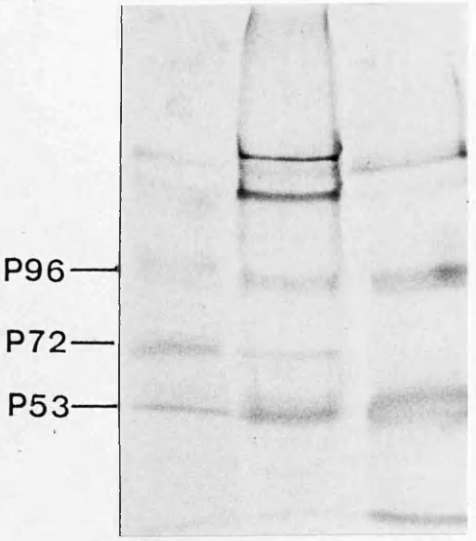
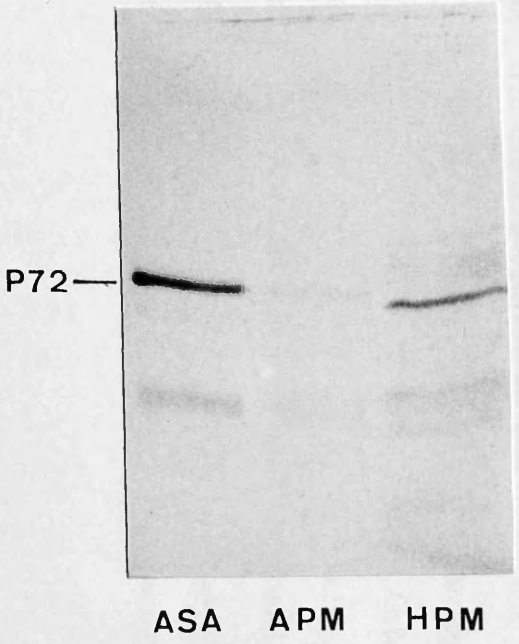


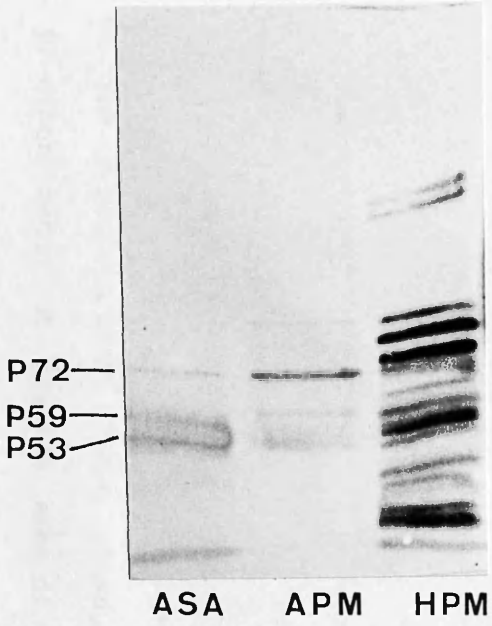
Fig. 4.8. Antibody binding to adipocyte
and hepatocyte plasma membranes
and adipocyte-specific antigens
by antisera raised against hepatocyte and erythrocyte plasma membranes
and adipocyte specific antigens,
as determined by Western blotting

Abbreviations and methods as for Fig. 4.2

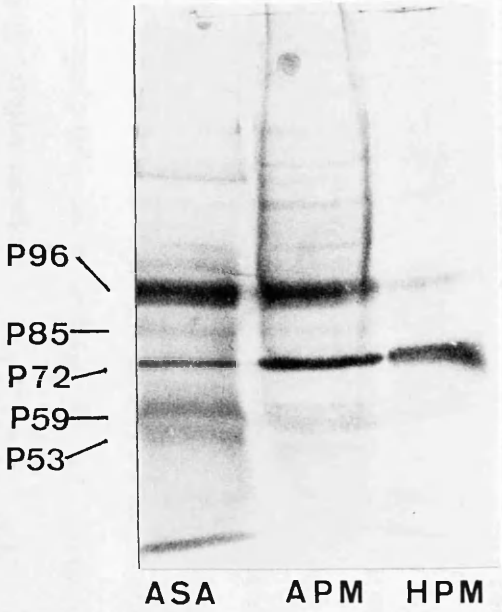
A/S 217 (anti-EPM)



A/S 479 (anti-HPM)



A/S 361 (anti-ASA)



clearly recognised HPM protein bands that were not also recognised by A/S 83.

There was also variation in the binding of different antisera to ASA, both in the total number of bands recognised and in which bands were recognised. In the following table, proteins have been given the letter P, followed by their molecular weight in kD and recognition of each band has been analysed using the scale described above. P59 was not recognisable as a doublet on any Western blots and so is referred to as a single band.

	COMPONENTS OF THE ASA				
	P96	P85	P72	P59	P53
A/S 83 (anti-(APM))	+++	±	+++	-	-
A/S 164 (anti-(APM))	+++	-	+	-	-
A/S 283 (anti-(APM))	±	-	+	-	+
A/S 361 (anti-(ASA))	+++	++	+++	++	++
A/S 217 (anti-(EPM))	-	-	+++	-	-
A/S 479 (anti-(HPM))	-	-	±	+	+

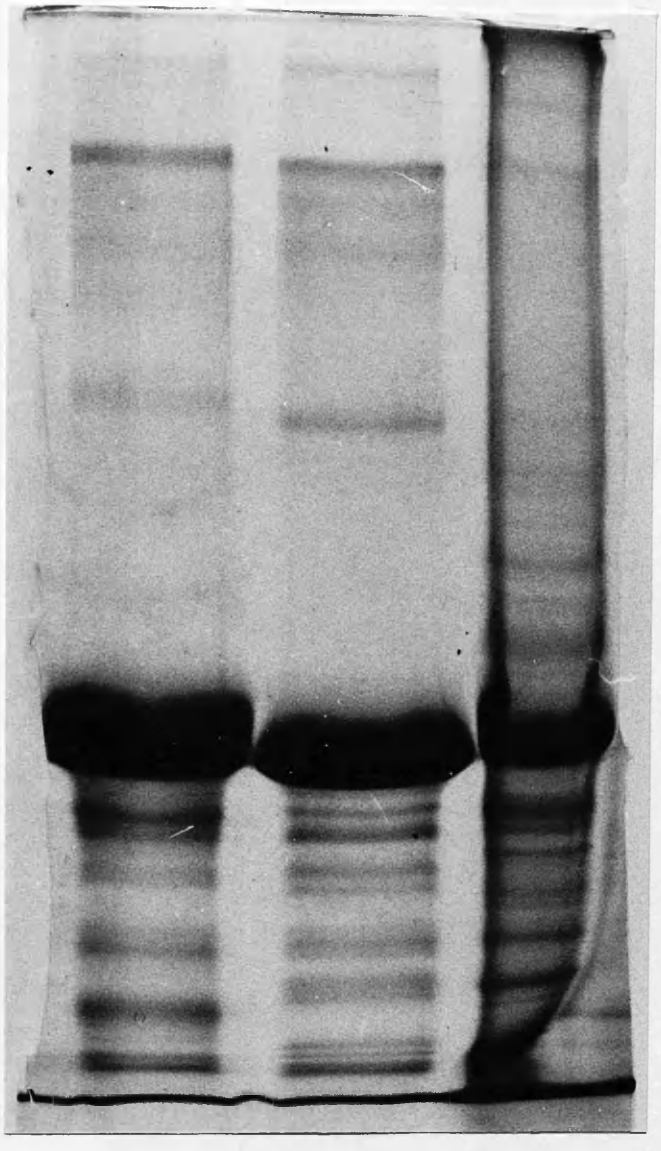
In addition to the 5 protein bands shown above, A/S 361 recognised at least 5 other bands of the ASA, some of which were visible as very minor components of the ASA by SDS PAGE. A/S 217 also bound to an ASA protein not clearly recognised by any of the other antisera and not clearly visible by SDS PAGE of the ASA. Similarly A/S 164 bound to an ASA protein with an approximate molecular weight of 110 kD, not visible by SDS PAGE. Unfortunately pre-immune serum was not available for any of the antisera tested, but pooled non-immune sheep serum showed no binding to APM, HPM or ASA when the antigens were added directly to nitrocellulose and then treated in the same way as the Western blots described above.

SDS PAGE of APM (prepared after digestion of whole adipose tissue in the presence of BSA) showed that a major proportion of the APM preparation consisted of a protein with electrophoretic mobility identical to that of BSA (Fig. 4.9). None of the antisera tested showed a significant anti-(BSA) response, while A/S 361 (anti-(ASA)) showed a small response to rat albumin (Table 4.4). There was little difference between the binding to APM, prepared in the presence or in the absence of BSA, of any of the antisera, with the exception of A/S 217 (anti-(EPM)) which showed 5 times the antibody binding to APM, prepared in the absence of BSA, compared with APM prepared in the presence of BSA.

Relative cytotoxicity of different antisera was determined by comparing the dilutions of antisera required to lyse a given number of target cells, as shown by the amount of haemoglobin or LDH released for erythrocytes and adipocytes respectively. A/S 83, 283 and 164 (anti-(APM)) and A/S 361 (anti-(ASA)) showed similar levels of cytotoxicity against adipocytes, while A/S 479 (anti-(HPM)) and A/S 217 (anti-(EPM)) showed about 40% and 15% of the relative cytotoxicity respectively, of A/S 83 (Fig. 4.10). Total cytolysis was not achieved by any of the antisera tested, even at antiserum concentrations of 15%

A/S 283, 164 and 361 showed more than 10 times the relative cytotoxicity of A/S 83, A/S 479 and A/S 217 against erythrocytes in the presence of guinea-pig complement (Fig. 4.11). While A/S 283, 164 and 361 achieved total lysis of erythrocytes at 3% antiserum, A/S 83 appeared capable of lysing only 40% of the total erythrocyte population since both 3% and 15% antiserum lysed only 40% of the erythrocytes. There was little difference in the cytotoxicity of A/S

Fig. 4.9. Polyacrylamide gel electrophoresis of adipocyte plasma membranes prepared in adipocytes isolated in the presence of BSA



Adipocyte plasma membranes were prepared from adipocytes isolated from adipose tissue by collagenase digestion in the presence of BSA, separated by SDS PAGE and stained with Coomassie blue, as described in Section 2.10. BSA and rat serum albumin (RSA) are included for comparison.

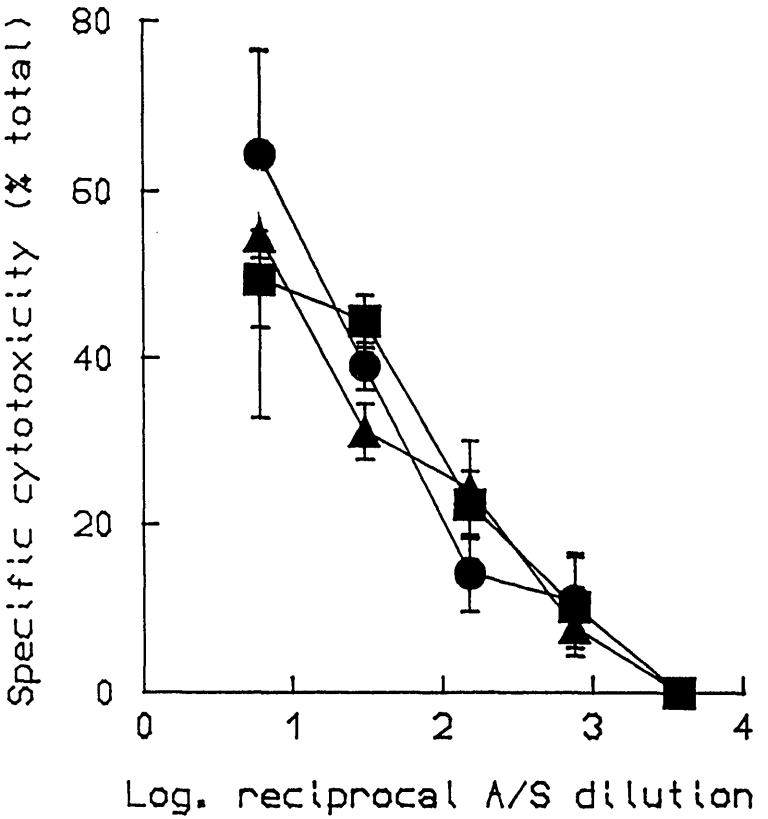
Table 4.4. The contribution of anti-(albumin) responses to apparent anti-(adipocyte plasma membrane) responses

	Relative antibody binding as % of antibody binding to adipocyte plasma membranes (prepared in the presence of BSA) of A/S 83			
	Adipocyte plasma membranes (+ BSA)	Adipocyte plasma membranes (-BSA)	BSA	Rat serum albumin
A/S 83 [anti-adipocyte plasma membrane)]	100	89 (89)	1 (1)	1 (1)
A/S 361 [anti-(adipocyte specific antigen)]	63	63 (100)	1 (1)	3 (4)
A/S 479 [anti-(hepatocyte plasma membrane)]	5	3 (50)	0 (0)	1 (23)
A/S 271 [anti-(erythrocyte plasma membrane)]	13	65 (500)	0 (0)	4 (<1)

Adipocyte plasma membranes (+ BSA) were prepared from adipocytes isolated by collagenase digestion of adipose tissue in the presence of BSA. Adipocyte plasma membranes (- BSA) were prepared by homogenisation of whole adipose tissue in the absence of BSA. Antibody binding was measured by ELISA. Figures in parentheses represent antibody binding as a % of the binding to adipocyte plasma membranes (+ BSA) by each antiserum.

Fig. 4.10. In vitro cytotoxicity against adipocytes of antisera raised against adipocyte, hepatocyte and erythrocyte plasma membranes and adipocyte-specific antigens

Cytotoxicity assays were performed as for Fig. 4.1. Three anti-(adipocyte plasma membrane) antisera, A/S 83 (filled circles) A/S 164 (filled triangles) and A/S 283 (filled squares) are shown in (a). A/S 83 (filled circles), an anti-(adipocyte-specific antigen) antiserum, A/S 361 (open circles), an anti-(hepatocyte plasma membrane) antiserum, A/S 479 (open squares) and an anti-(erythrocyte plasma membrane) antiserum, A/S 271 (open triangles) are shown in (b).


Specific cytotoxicity (% total)
80
60
40
20
0
0
1
2
3
4
Log. reciprocal A/S dilution

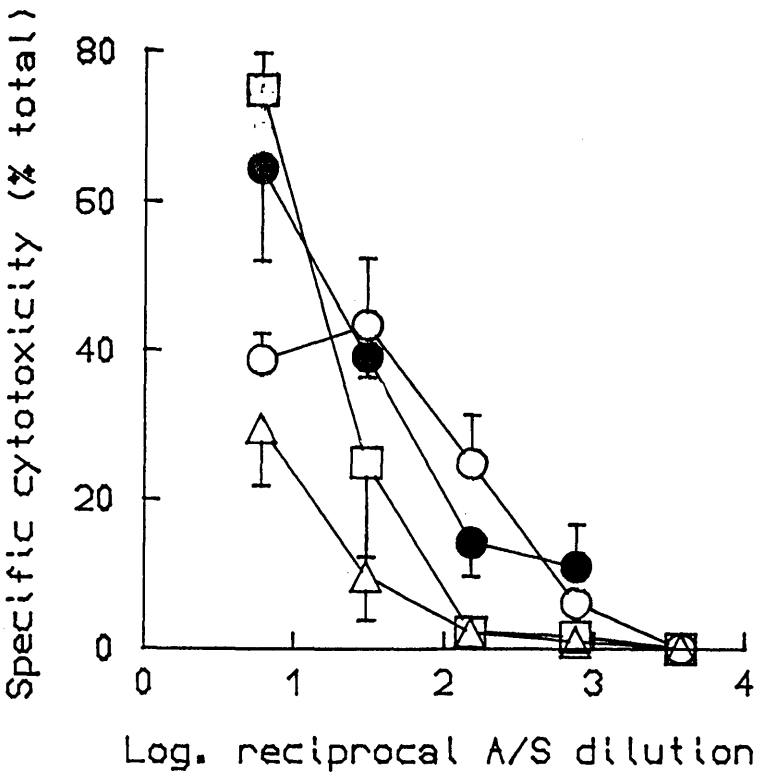
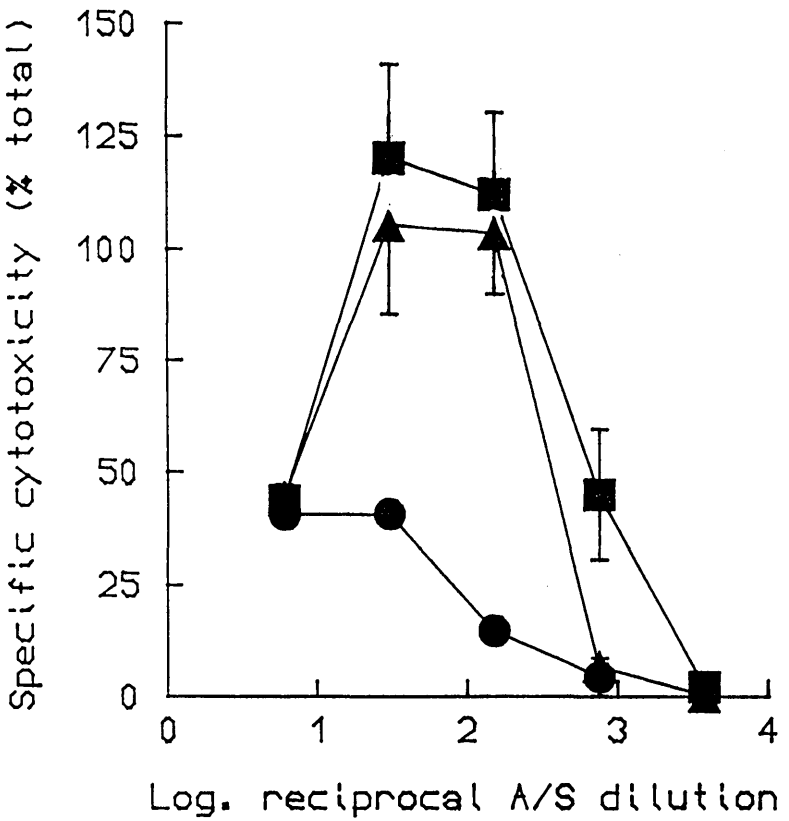
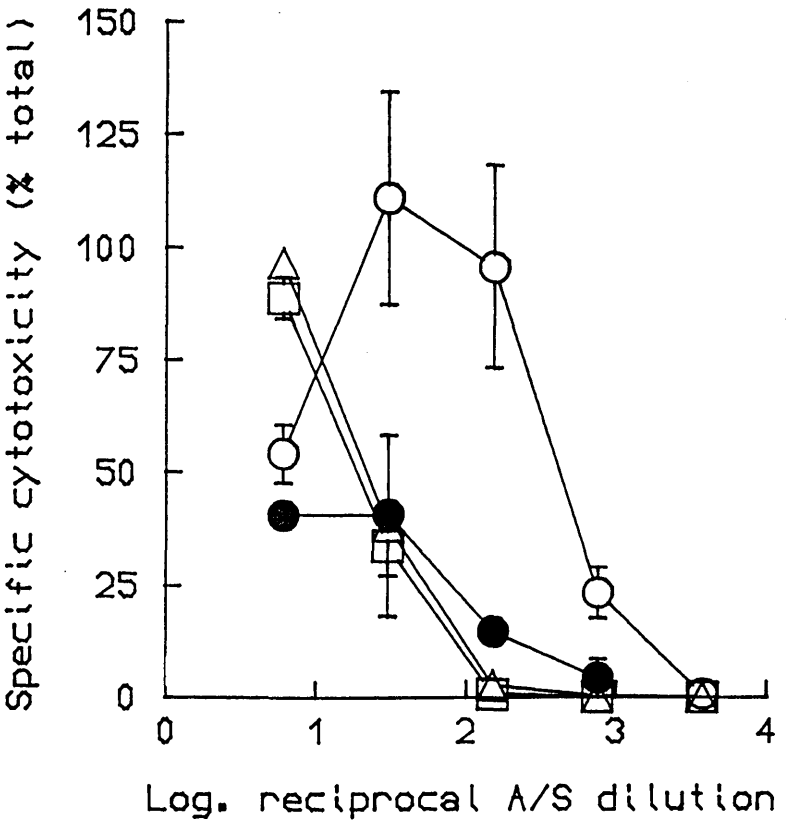

Specific cytotoxicity (% total)
80
60
40
20
0
0
1
2
3
4
Log. reciprocal A/S dilution

Fig. 4.11. *In vitro* cytotoxicity against erythrocytes of antisera raised against adipocyte, hepatocyte and erythrocyte plasma membranes and adipocyte-specific antigens

Cytotoxicity assays were performed as for Fig. 4.1. Symbols as for Fig. 4.10.


Specific cytotoxicity (% total)
150
125
100
75
50
25
0
0
1
2
3
4
Log. reciprocal A/S dilution


Specific cytotoxicity (% total)
150
125
100
75
50
25
0
0
1
2
3
4
Log. reciprocal A/S dilution

217 (anti-(EPM)) against erythrocytes, in the presence of guinea-pig, sheep or rat complement (Fig. 4.12). The cytotoxicity of all the other antisera tested, against erythrocytes, was considerably reduced in the presence of rat complement, compared with guinea-pig or sheep complement. In the presence of sheep complement A/S 83, at low concentations, showed greater cytotoxicity against erythrocytes than A/S 283, 164 or 361 but, at high concentrations, A/S 83 was able to lyse only 40-50% of erythrocytes, while A/S 283, 164 and 361 lysed 75% of the same number of erythrocytes at the same antiserum concentration.

The only antiserum of those described above able to haemagglutinate erythrocytes was A/S 217 (anti-(EPM)) (results not shown). Relative differences in antibody binding to EPM of all the antisera tested was the same, whether the ELISA was performed at 37° C or 22° C (Fig. 4.13). Coating EPM to ELISA plates using poly-l-lysine increased the apparent binding of A/S 164 and 361, compared with A/S 83, but A/S 83 still showed 10 times the binding to EPM of A/S 164 and 361 (Fig. 4.13).

The in vivo administration of 1 ml of A/S 164, 283 or 361 caused anaesthetic-like effects and the adoption of strange postures, indicative of hindlimb weakness, in some rats. These symptoms were evident within 2-5 minutes of injection and lasted for 30 minutes to a few hours after injection. The rats did not appear to be in pain and showed no signs of sensitivity in the area of the injection. When stimulated, by handling, all symptoms disappeared, thus mimicking the outward signs of onset of anaesthetic. The dose of antiserum for these antisera was not increased to 2 ml because of these effects but 2 ml of A/S 83 and 3 ml of A/S 217 and A/S 479 caused only slight anaesthetic-like effects.

Fig. 4.12. A comparison of the ability of complement from different species to support the cytotoxicity against erythrocytes of antisera raised against adipocyte and erythrocyte plasma membranes and adipocyte specific antigens

Cytotoxicity assays were performed as for Fig. 4.1 in the presence of guinea-pig complement (a), sheep complement (b) or rat complement (c). Symbols are as for Fig. 4.10.

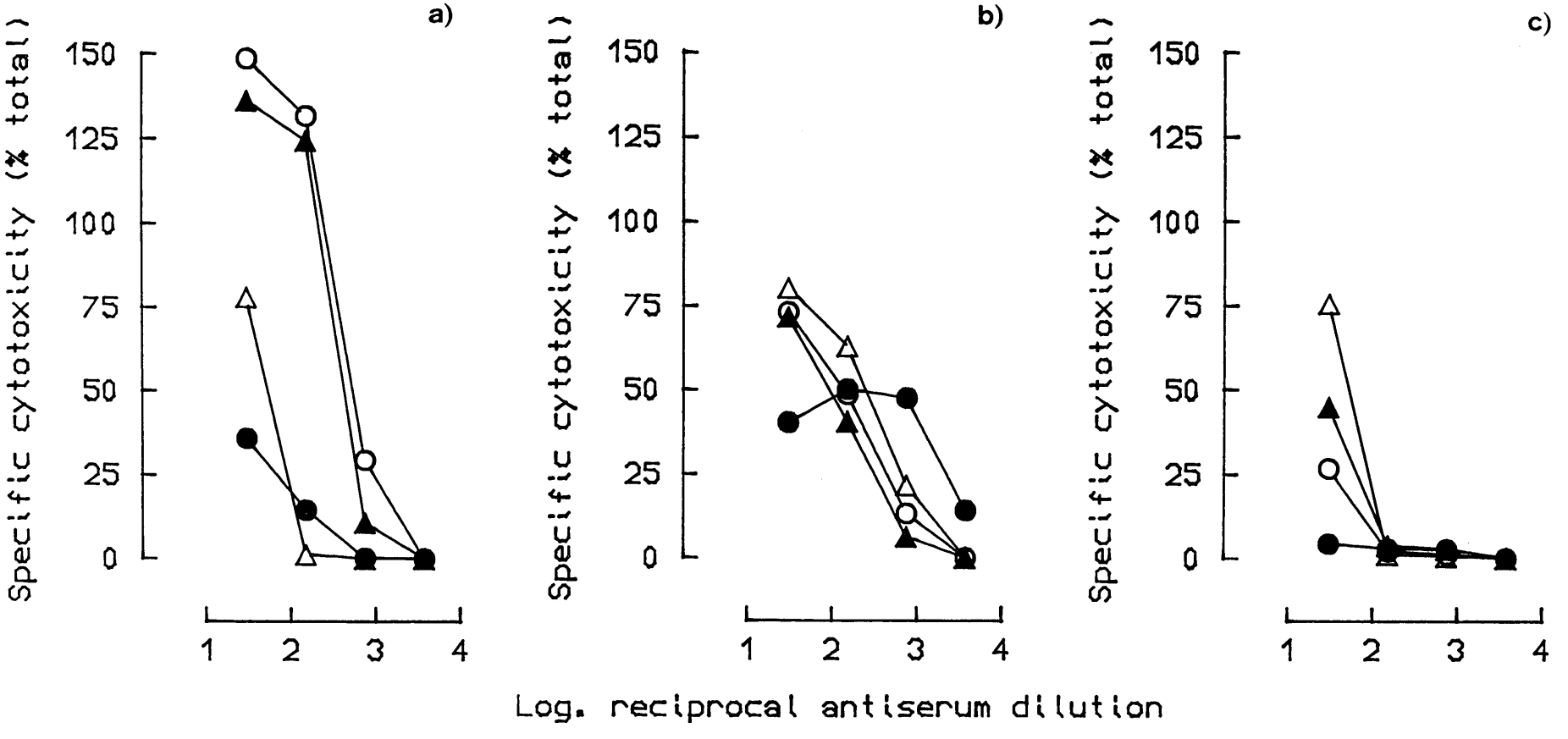
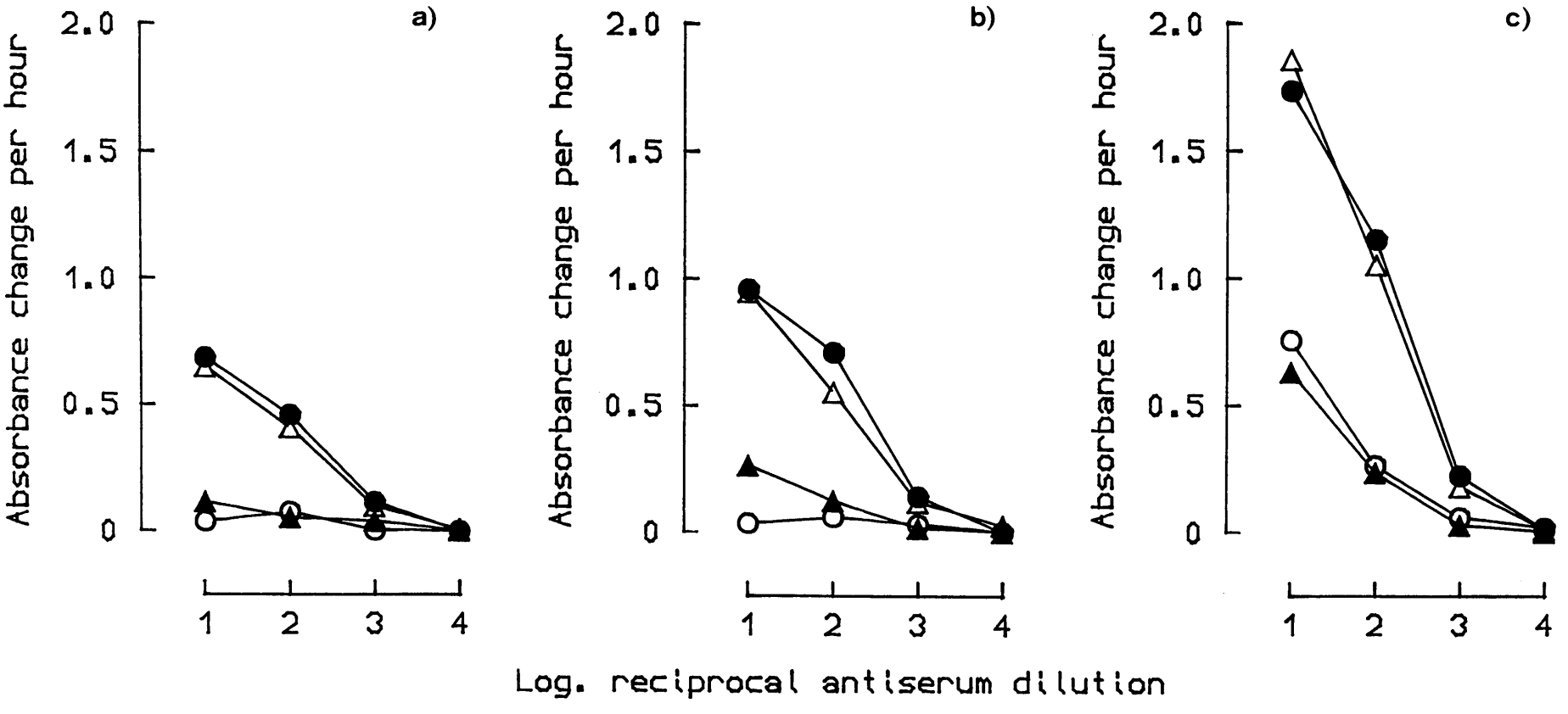

a)
b)
c)
Specific cytotoxicity (% total)
150
125
100
75
50
25
0
1
2
3
4
Log. reciprocal antiserum dilution

Fig. 4.13. The effects of temperature and method of coating antigen to ELISA plates on apparent anti-(erythrocyte plasma membrane) binding of different antisera, as determined by ELISA

Erythrocyte plasma membranes were adsorbed onto uncoated ELISA plates (a and b) or poly-l-lysine coated plates (c), as described in Section 2.6.1. The ELISA was performed at 22° C (a and c) or at 37° C (b). Symbols are as for Fig. 4.10.


a)
b)
c)
Absorbance change per hour
2.0
1.5
1.0
0.5
0
1
2
3
4
Log. reciprocal antiserum dilution

Administration of all the antisera caused a drop in food intake and body weight gain which was most severe in A/S 83-treated rats and least severe in rats treated with A/S 217 and A/S 479 (Table 4.5). All the anti-(APM) antisera caused a significant reduction in the dry weight of parametrial adipose tissue of about 50% and significant reductions in the % dry weight of the tissue (Table 4.5). Visual evidence of lymphocytic infiltration of parametrial adipose tissue was greater in A/S 283- and 83-treated rats than in A/S 164-treated rats. The dry weight of parametrial adipose tissue of A/S 361-treated rats was not significantly reduced but the % dry weight of the tissue was significantly reduced and lymphocytic infiltration of the tissue could be seen in most animals, although this was not as great as in A/S 83- and A/S 283-treated rats. No effects on adipose tissue could be seen following treatment with A/S 217 or A/S 479.

While A/S 83, A/S 361, A/S 217 and A/S 479 caused no gross abnormalities to the liver, A/S 164 and A/S 283 caused abnormalities of the liver, characterised by fusion of liver lobes, a rounded appearance of the liver lobe margins and, in some cases, an abnormally pale colour. Histology, performed at the Veterinary Pathology Laboratory of the West of Scotland College of Agriculture, reported an unidentified cellular infiltration of the tissue. Recent experiments in this laboratory have shown that the administration of 6 ml of A/S 479 (anti-(HPM)) caused gross liver abnormalities, but had no effect on adipose tissue (results not shown).

Table 4.5. In vivo effects of antisera raised against adipocyte, hepatocyte and erythrocyte plasma membranes and adipocyte specific antigens

Female Wistar rats were injected intraperitoneally with 1, 2 or 3 ml of antiserum for 3 or 4 consecutive days, as indicated. Rats were sacrificed 6 days after the first treatment and infiltration of adipose tissue and liver abnormalities were scored on a scale of 0-3, as indicated by yellow nodules and disrupted liver lobes respectively. ***$P<0.001$, *$P<0.01$ compared with controls (Student's unpaired t test)

Treatment	Number of observations	Body weight change (1st day) (g)	Food intake (1st day) (g)	Parametrial adipose tissue			Liver abnormalities score
				Dry weight (g)	% dry weight	Infiltration score	
None	8	6.0 ±1.5	20.4	0.61 ±0.10	75.3 ±1.4	0 ±0	0 ±0
A/S 83 (4 x 2 ml)	3	-11.7*** ±2.9	3.0	0.32 ±0.04	47.3*** ±4.3	2.3 ±0.7	0 ±0
A/S 164 (3 x 1 ml)	3	0.1* ±0.9	9.5	0.28 ±0.03	60.2*** ±0.8	1.0 ±0.6	2.3 ±0.7
A/S 283 (3 x 1 ml)	3	-5.2*** ±2.3	6.4	0.27 ±0.04	50.1*** ±5.6	2.3 ±0.7	2.3 ±0.7
A/S 361 (3 x 1 ml)	7	-5.8*** ±0.9	6.6	0.51 ±0.06	62.0*** ±2.3	0.7 ±0.4	0 ±0
A/S 479 (3 x 3 ml)	2	-1.9 ±1.9	11.7	0.58	70.0	0	0
A/S 271 (3 x 3 ml)	3	-1.5 ±0.5	11.5	0.78 ±0.08	73.7 ±0.3	0 ±0	0 ±0

4.3.3 Discussion

4.3.3.1 A comparison of 3 antisera raised against adipocyte plasma membranes

Following the discovery that recent bleeds of sheep 83 did not reproduce the *in vivo* effects of earlier bleeds, new antisera were raised against APM, in the hope of providing an antiserum that had *in vivo* effects that mimicked those of early bleeds of A/S 83. The results of ELISA suggested that A/S 164 and 283 showed twice the binding to APM of A/S 83, but did not recognise as many antigenic determinants as A/S 83 (Fig. 4.4). Western blotting suggested that A/S 83 bound more strongly to APM than A/S 164 and 283, as well as binding to more APM proteins (Fig. 4.7). These conflicting results emphasize the caution required in the interpretation of the results of ELISA and Western blotting. Both techniques measure a combination of titre and affinity but, since the binding of antisera to antigen was performed under different conditions (for 2 hours in ELISA, compared with 16 hours in Western blotting) the relative influences of titre and affinity on the final result may vary in the 2 techniques. A/S 83 may consist of a higher proportion of low affinity antibodies than A/S 164 or 283, and such antibodies may be able to bind more effectively given the longer incubation period in Western blotting. The technique of Western blotting relies on the retention of antigenicity of proteins after denaturation and running on SDS polyacrylamide gels under reducing conditions. The results described above show that some proteins did retain antigenicity under such conditions, but it is not known how many antigenic determinants of APM, HPM or ASA were lost in this process. Binding to proteins resistant to solubilization by SDS would not be detected by Western

blotting. While, in ELISA, the native proteins are immobilized on the solid phase support, different proteins may vary in efficiency of adsorption to PVC wells and some adherent proteins may be orientated in such a way as to render some epitopes inaccessible to antibody. Quantitation of the results of ELISA and Western blotting also presents difficulties. The quantitation of the results of ELISA was discussed in detail in Section 2.6.3. It should be noted that, for many antisera, dose-response curves obtained by ELISA did not plateau at high antiserum concentrations and so the values shown for antigen abundance give only an indication of the relative number of epitopes recognised by different antisera. Quantitation of the results of Western blotting had to be performed by eye as densitometrical scanning of the blots was not possible.

Despite the possible limitations of ELISA and Western blotting, it was clear that all 3 anti-(APM) antisera bound to components of the adipocyte plasma membrane. This conclusion was supported by the demonstration of *in vitro* cytotoxicity against isolated adipocytes (Fig. 4.10) and *in vivo* fat-reducing properties of all 3 antisera (Table 4.5). A/S 83, 283 and 164 showed similar levels of *in vitro* cytotoxicity and each caused a reduction in parametrial fat pad weight of a similar magnitude 6 days after treatment. A/S 164 induced a lesser degree of lymphocytic infiltration of the tissue than did A/S 83 or 283. This, perhaps, suggests that lymphocytic infiltration was not the only or the direct cause of the reduction in fat pad mass, but more observations are required before such a conclusion can be drawn. However, 2 undesirable side effects of A/S 164 and 283 led to a reluctance to pursue further studies with these antisera. While mimicking the effects of A/S 83 on adipose tissue,

A/S 164 and 283 caused anaesthetic-like effects immediately after treatment, seen only to a much lesser extent in A/S 83-treated rats, as well as gross abnormalities of the liver, never seen in A/S 83-treated rats. Non-immune sheep serum, from a sheep of the same breed and from the same establishment as those from which A/S 164 and 283 were obtained, did not have these effects. The cause was unlikely, therefore, to have been the presence of toxins or microbial infection in A/S 164 or 283 and is more likely to have been the presence of antibodies directed against antigens not recognised, or recognised only weakly, by A/S 83. Recent experiments in this laboratory have shown the anaesthetic-like effects to be dependent on the presence of complement. Complement activation results in the release of the anaphylatoxins, C3a and C5a and the anaesthetic-like effects shared some of the properties of anaphylactic shock. However, the administration of cobra venom factor, which induces massive complement activation, did not have anaesthetic-like effects.

The speed of onset of the A/S 164- and 283-induced anaesthetic-like effects suggests that interference with synaptic transmission, in a way similar to that caused by general anaesthetics, might have occurred. As adipose tissue is innervated, it is possible that the APM preparation, used for raising of the antisera, could have been contaminated with nervous tissue. Antibodies within these antisera may, therefore, have been directed against components of APM that cross-react with nervous tissue or against antigens specific for nervous tissue. One of the most extensively studied autoimmune diseases associated with the nervous system is Myasthenia gravis (MG), characterised by circulating antibodies to the acetylcholine receptor (Ach-R). The injection of antibodies to the Ach-R into

experimental animals induces symptoms, such as muscle fatigue, similar to those seen in patients with MG. The mechanism of action of the antibodies in unclear, but blocking of the Ach-R, complement-mediated lysis of the post-synaptic membranes and cross-linking of Ach-R, increasing the rate of receptor internalization, have all been proposed (Newson-Davis, 1981). It is possible that A/S 164 or 283 bound to the Ach-R or some other component of the post-synaptic membrane and, by one of these mechanisms, interfered with nervous stimulation of muscle. It is, clearly, of importance to examine the binding of these antisera to the muscle cell surface. Acetylcholinesterase, found on the post-synaptic membrane, is a major component of erythrocyte membranes. Since A/S 164 and 283 were highly cytotoxic against eythrocytes, this enzyme might be a useful starting point in the examination of binding to muscle membrane protein.

Antibodies directed against components of the central nervous system can also occur. Multiple sclerosis (MS), a demyelinating disease, is characterised, in most cases, by anti-(myelin) antibodies. Experimental autoimmune encephalomyelitis (EAE), proposed as a model of MS, is induced by immunisation against Myelin basic protein (MBP). In its acute form, animals suffer weight loss, develop hindleg weaknesses and ascending paralysis (Kallen and Nilsson, 1986). Although EAE is characterised by antibodies to MBP, the pathogenesis of the disease appears to be T-cell rather than antibody-mediated (Silberg and Swanborg, 1986) and so, despite the similarities of some of the symptoms to the immediate effects of A/S 283 and 164, it is not a good model for the effects of passive immunisation. No significant responses of A/S 164 or 283 against

sphingomyelin or MBP could be measured by ELISA. Both antisera recognised antigens of brain tissue, although antibody binding to brain membranes of A/S 164 and 283 was less than that of A/S 83 (Fig. 4.4). Under normal circumstances, antibodies cannot cross the blood-brain barrier. However, circulating immune complexes can deposit in the choroid plexus in the same way as in the glomerulus (Lampert and Oldstone, 1973). Theoretically immune complex deposition in the choroid plexus could activate complement, causing damage to the blood-brain barrier and leakage of anti-(brain) antibodies into the brain parenchyma. There is some evidence of increases in albumin and IgG levels in the cerebrospinal fluid of animals with immune complex disease (Harbeck, Hoffman, Hoffman and Shucard, 1979). It is difficult to envisage entrance of antibodies into the bloodstream quickly enough to mediate the anaesthetic-like effects through immunological attack of the brain. However, antibodies may bind to that part of the central nervous system directly responsible for innervation of the lower limbs.

The liver damage seen in A/S 283- and 164-treated rats shares some characteristics with that seen in patients suffering from autoimmune chronic active hepatitis (CAH). This disease is characterised by infiltration of the liver with mononuclear cells, which starts in the portal tract and spreads into the liver parenchyma, disrupting the limiting plate of periportal hepatocytes. The parenchyma tends to be subdivided by fibrous septa accompanying the inflammation and this, together with the appearance of regenerating hepatocytes, leads to the development of cirrhosis (Vento and Eddleston, 1987). Histological examination of the livers of A/S 164-treated rats showed a cellular infiltration of the tissue,

although the nature of these cells was not clear. The edges of the liver lobes were disrupted and the gross appearance of the liver was reminiscent of that of regenerating livers following partial hepatectomy. Several lines of evidence suggest that antibodies directed against surface membrane antigens may contribute towards the liver damage seen in CAH. The first is the demonstration of liver-directed autoantibodies in the serum of CAH patients. Incubation of the serum from these patients with peripheral blood lymphocytes of normal subjects resulted in cytotoxicity against hepatocytes, which has lead to the suggestion that ADCC may contribute towards liver damage in CAH (Vento and Eddleston, 1987). In addition, the immunisation of experimental animals with crude liver proteins can induce the production of autoantibodies against the liver and inflammatory effects similar to those seen in CAH (Watanabe et al., 1987). The precise nature of the target antigens on the hepatocyte surface for damaging immune reactions has not been identified. A/S 164 and 283 are unlikely to bind to liver specific antigens. The 2 antigen pareparations that have received most attention in studies of liver-associated autoimmune diseses are liver specific protein and liver membrane antigen and both include organ-specific and non-organ-specific determinants (Frazer and Mackay, 1984). According to ELISA, the binding of A/S 283 and 164 to HPM was less than that of A/S 83, which was devoid of apparent anti-(liver) activity in vivo. The possibility that A/S 283 and 164 might bind to one or more liver membrane proteins, not recognised by A/S 83, was investigated by Western blotting (Fig. 4.7). No HPM polypeptide band, not bound by A/S 83, was clearly recognised by A/S 283 or 164 but, because of the limitations of this technique described above, this does not prove

conclusively that such a protein does not exist. Unfortunately, preliminary efforts to obtain a viable hepatocyte population suitable for in vitro cytotoxicity assays were unsuccessful.

It is possible that the liver abnormalities seen in A/S 164- and 283-treated rats resulted, not from an anti-(liver) component of the antiserum, but from non-specific effects of the presence of circulating immune complexes. A major portion of the reticulo-endothelial system responsible for the uptake of circulating immune complexes resides in the Fc and C3b receptor-bearing Kupffer cells of the liver (Shogh, Blomhoff, Eskild and Berg, 1985). A/S 164 and 283 showed a higher in vitro level of cytotoxicity against erythrocytes than A/S 83 (Fig. 4.10). If antibody coating or antibody-mediated cytolysis of erythrocytes occurred in vivo upon injection of A/S 164 or 283, such complexes would, in the presence of complement, be rapidly taken up by the liver (Veerhuis, Krol, van Es and Daha, 1986). The gross abnormalities seen in the liver may, therefore, be the result of gross overloading of the hepatic reticulo-endothelial system. CAH is characterised by hyper-gammaglobulinaemia and, in some cases, circulating immune complexes, but a direct causal link with the pathogenesis of the disease has not been demonstrated. Recent experiments in this laboratory demonstrated the in vivo effects of A/S 164 to be dependent upon the presence of complement but since complement increases the rate of hepatic uptake of particulate immune complexes (Veerhuis et al., 1986), this does not resolve the question of whether anti-(liver) antibodies or non-specific clearance of antibodies and immune complexes was responsible for the liver damage.

4.3.3.2 The characterisation of antisera raised against hepatocyte and erythrocyte plasma membranes and adipocyte specific antigens

The characterisation of antisera raised against HPM, EPM and ASA, both *in vitro* and *in vivo*, has yielded information on the true adipocyte specificity of the ASA, the possible causes of the liver abnormalities seen in A/S 283- and 164-treated rats and the role of adipocyte specificity in the fat-reducing properties of antisera *in vivo*.

The performance of the affinity columns used for the preparation of the ASA is summarised in Table 4.3. Only 3% of the IgG fraction of A/S 83 was bound by the APM column, which was less than would be expected for a high titre antiserum. Some antigenic components of the APM may have been resistant to Triton X-100 solubilization or may have had reduced antigenicity after solubilizaton or binding to the affinity column. It is, therefore, possible that antibodies binding to adipocyte specific antigens were lost at this stage in the ASA preparation. The bound fraction contained only 3.4 times as much anti-(APM) activity as the original IgG fraction (per mg of protein) which further suggests that a significant proportion of the anti-(APM) activity of A/S 83 was not bound by the column and that there may have been non-specific adsorption of protein by the column. Further purification of anti-(APM) fraction on a column bearing non-adipocyte plasma membranes resulted in the isolation of antibodies from which more than 95% of the cross-reactivity with HPM, KPM and EPM had been removed. It was, therefore, hoped that this antibody fraction could be used for the purification of adipocyte specific antigens (ASA).

The first indication that these antigens were not totally adipocyte specific came from ELISA of an antiserum raised against EPM, which showed that this antiserum recognised components of the ASA. When cross-reactivity with non-adipose tissues of an antiserum raised against ASA was examined by ELISA, it was found that, while the antiserum (A/S 361) was considerably more adipocyte specific than antisera raised against whole APM, antibody binding to HPM, EPM, KPM and BM was nevertheless detectable (Fig. 4.4). In order to determine which of the proteins of the ASA, if any, were adipocyte specific, binding of antisera raised against APM, ASA, HPM and EPM to the different components of the ASA was examined by Western blotting (Figs. 4.7, 4.8).

A/S 83 (anti-(APM)) did not, apparently, recognise the 2 lowest molecular weight components of the ASA, P59 and P53. This was, perhaps, surprising as the antibodies used to isolate the ASA were purified from A/S 83. It is possible that the antigenicity of P59 and P53 had not been retained after denaturation and transfer onto nitrocellulose. If this were the case, however, other antisera might also not be expected to bind to these polypeptides, unless some epitopes on the protein were destroyed and others were not. A/S 361 (anti-(ASA)) bound to all the major components of the ASA, including P59 and P53. A/S 361 may include antibodies of a higher titre or affinity against P59 and P53 than A/S 83. Immunoblotting at a single antiserum dilution does not allow determination of whether or not a protein is recognised, but only gives an indication of the strength of the response to particular proteins. If the ASA did contain proteins, not recognised by A/S 83, they may have been the result of non-specific adsorption of proteins to the affinity columns used in

the ASA preparation or leeching of antibody from the Sepharose affinity-support. Passage of solubilized APM down an affinity column bearing non-immune sheep IgG would give an indication of the potential for non-specific adsorption to the column. Time and the scarcity of APM material did not permit this important control experiment to be performed. In addition, since the purified ASA was of a greatly different composition to the APM material from which it was purified, it was assumed that non-specific adsorption was not a major problem. It is, however, possible that some APM proteins were adsorbed preferentially to sepharose or sheep IgG. A rabbit antiserum, raised against sheep IgG, recognised the ASA, when measured by ELISA, providing strong evidence for the contamination of the ASA with sheep IgG (results not shown). SDS PAGE of immunoglobulins, under reducing conditions, breaks intra-chain but not inter-chain disulphide bonds so, if sheep IgG constituted a significant proportion of the ASA, a polypeptide of a molecular weight of 150 kD would be visible after SDS PAGE of ASA. Such a protein was not clearly visible by SDS PAGE, but Western blotting of A/S 361 against ASA did reveal a protein with a molecular weight of approximately 150 kD, suggesting that, in terms of percentage protein, sheep IgG constituted a very small part of the total ASA. However, it is not known whether the intact sheep IgG molecule or only part of it became detached from the affinity column following repeated washings in buffers of extreme pH. Unfortunately time did not allow Western blotting of an anti-(sheep IgG) antiserum against the ASA to be performed, which might have shown whether P59 or P53 were components of the sheep IgG molecule. An alternative explanation for the apparent lack of recognition of some components

of ASA by A/S 83 is that incomplete solubilisaton of APM may have resulted in the co-purification of non-specific antigens (such as P59 or P53) with adipocyte specific antigens.

A/S 479 (anti-(HPM)) recognised P59 and P53, suggesting that these polypeptides were non-adipocyte specific components of the ASA, rather than sheep IgG, resulting either from non-specific adsorption to the affinity column or from incomplete solubilisation of APM from which ASA were prepared. Since the recognition of these proteins by A/S 479 was weak, they may only be minor components of HPM and, as such, may still be 'useful' antigens of a 'relatively specific' nature. It should also be noted that different antisera that bound to proteins of similar electrophoretic mobility may not have recognised the same protein. Two-dimensional gel electrophoresis would seem an appropriate way to further this examination.

P72 was apparently recognised by anti-(APM), anti-(ASA), anti-(HPM) and anti-(EPM) antisera. Rat albumin has a molecular weight of 68 kD, raising the possibility that P72 was albumin. Fig. 4.8 shows that APM (prepared following digestion of adipose tissue in the presence of BSA) was heavily contaminated with BSA. This material was used to raise A/S 83, which was then used to purify the ASA. The ASA, however, were prepared exclusively from homogenised whole adipose tissue, in the absence of BSA. Contamination of the ASA with albumin would, therefore, have had to have been due to contamination of APM with rat albumin. Table 4.4 shows that antibody binding of A/S 83 to both BSA and rat albumin were very poor. Antibody components of A/S 83 that cross-reacted with liver (a tissue likely to have a higher albumin content than adipose tissue) were removed prior to preparation of the ASA. Any contamination of the

ASA with albumin, therefore, is likely to have been due to non-specific adsorption on to the column. A/S 361 (anti-(ASA)) showed slightly higher binding to rat albumin than did A/S 83, but the ASA were conjugated to rabbit serum albumin for immunisation of sheep 361 and rabbit and rat albumin are likely to show antigenic cross-reactivity. Several of the antisera tested recognised a protein with a molecular weight of approximately 72 kD of HPM providing a further indication that this protein is not adipocyte specific. However, A/S 283 bound to P72 of APM and ASA, but did not apparently recognise a protein with a similar molecular weight of HPM. The liver polypeptide, therefore, may not have been the same protein as the P72 of APM and ASA.

Several groups have identified 2 prominant glycoproteins with molecular weights of 74-84 and 88-100 kD by SDS PAGE of rat APM (Czech and Lynn, 1973; Avruch, 1976; Kawai and Spiro, 1977; Tume et al., 1985). It is possible that P85 and P96 were these 2 glycoproteins. Czech and Lynn (1973) suggested that these proteins constituted a large proportion of the protein on the exterior of the adipocyte surface, and so might be expected to be recognised strongly by antisera raised against APM.

P96 was strongly bound by anti-(APM) and anti-(ASA) antisera, but was apparently not recognised by anti-(EPM) and anti-(HPM) antisera and so may be a true adipocyte specific antigen. P85 was recognised weakly by A/S 83 and more strongly by A/S 361 (anti-(ASA)), but again was not apparently recognised by anti-(EPM) or anti-(HPM) antisera. The weak recognition of P85 by A/S 83 and the absence of recognition by other antisera may be because the protein had reduced antigenicity as a result of the procedure of

Western blotting and so it is not possible to be entirely confident of the adipocyte specificity of this protein from these results.

Lee et al. (1986) have identified 3 adipocyte specific 'differentiation' antigens on the rat APM, with molecular weights of 124, 92 and 59 kD. P85 and P59 may be the same polypeptides as Lee's 92 and 59 kD polypeptides. There are several possible causes of the differences between the ASA described here and those of Lee et al. (1986). Both preparations were isolated using antisera raised against whole APM. However, the antigens used for immunisation were not identical. Al-jafari, Lee, Tume and Cryer (1986) and Thompson and Abraham (1979) found it necessary to condition isolated adipocytes for at least 3 h after enzymatic digestion of the tissue to allow restoration of surface antigens. In this laboratory, APM were prepared from freshly isolated cells, since antisera raised against this material showed strong anti-(adipocyte) activity, both in vitro and in vivo (Flint et al., 1985). These differences may relate to the amount of tryptic activity in the collagenase used for tissue digestion. The APM used here were isolated from female Wistar rats, while those of Lee were isolated from Hooded rats. Tume et al. (1985) have demonstrated differences in the polypeptide composition of APM of rats of different strains.

While it was obviously disappointing to find evidence of cross-reacting antigens in the ASA preparation, A/S 361, raised against ASA, was considerably more adipose tissue specific than antisera raised against whole APM. According to ELISA, while retaining 63% of the binding to APM, binding to HPM, EPM and brain membranes was reduced by more than 75% compared with A/S 83 (Fig.

4.4). The only exception measured was the anti-(KPM) response, which was similar to that induced by immunisation with whole APM. These results suggest that, while the ASA were not 100% adipocyte specific, the majority of cross-reacting antigens had been excluded or reduced to levels such that their immunogenicity was reduced.

In the previous Section, liver abnormalities caused by the administration of 2 antisera raised against whole APM, A/S 283 and 164, were described. It was proposed that these abnormalities might result, either from antibodies directed against components of the liver, or from overloading of the hepatic reticulo-endothelial system responsible for the clearance of immune complexes. The failure to demonstrate higher anti-(HPM) activity in A/S 283 and 164, compared with A/S 83 by in vitro assays provided evidence against the first possibility. However, in vitro assays have inevitable limitations and so it was hoped that an investigation of the in vivo effects on the liver of antisera raised exclusively against liver components and an antiserum devoid of reactivity against liver might help to resolve the cause of the effects of A/S 283 and 164 on the liver. If anti-(liver) antibodies were responsible, an antiserum raised against liver should induce similar or more severe liver abnormalities than A/S 283 and 164 and an antiserum raised against adipocyte specific antigens only should not cause liver abnormalities. These 2 conditions were satisfied, in that A/S 479 (anti-(HPM)), when administered at a sufficiently high dose, reproduced the effects of A/S 283 and 164 on the liver and A/S 361 had no apparent effect on the liver. However, A/S 479 cross-reacted with and was cytotoxic against erythrocytes in vitro. The anti-(liver) effects of the antiserum may have been due to overloading of the hepatic

reticulo-endothelical system with circulating immune complexes composed of antibody-coated erythrocytes or of antibody and another target cell or protein, rather than due to anti-(liver) antibodies. More A/S 479 than A/S 283 and 164 was required to produce the same effect on the liver, although A/S 479 apparently bound more strongly and to more HPM proteins than A/S 283 and 164, as suggested by ELISA and Western blotting (Figs. 4.5, 4.8). This, coupled with the fact that A/S 361 did show some anti-(HPM) activity *in vitro* but no anti-(liver) effects *in vivo*, suggests that anti-(HPM) antibodies may not have been the only factor involved in the induction of liver abnormalities in A/S 283- and 164-treated rats. It should also be noted that A/S 479 probably bound to liver-specific proteins that would not be recognised by A/S 283 or A/S 164. Consequently, despite the apparent similarities in the gross appearance of livers from A/S 479-, 283- and 164-treated rats, different mechanisms may have been responsible for the abnormalities.

The presence of liver abnormalities in the absence of an effect on adipose tissue in A/S 479-treated rats and the absence of liver abnormalities in the presence of an effect on adipose tissue in A/S 361- and 83-treated rats strongly suggests that liver abnormalities were not a consequence of the release of lipid induced by immunological attack of adipose tissue.

While A/S 361 had no apparent effect *in vivo* on the liver, increasing the adipocyte specificity of the antiserum did not remove the immediate anaesthetic-like effects or the decline in food intake and body weight gain on the first day of treatment, although both effects were of a reduced severity, compared to those seen with some antisera raised against whole APM. A decline in food intake was

observed in all rats treated with antisera that had fat-reducing properties and so may be an inevitable consequence of fat mobilization, possibly mediated by a rise in serum free fatty acids or triglycerides (see Fig. 3.15). A/S 83 induced only very mild anaesthetic-like effects and this antiserum had very potent fat-reducing properties, implying that the anaesthetic-like effects are not an inevitable consequence of treatment with a fat-reducing antiserum. As antibodies used to isolate the ASA had only been preadsorbed against HPM, EPM and KPM, the antigens to which antibodies responsible for the anaesthetic-like effects bind, may not be present on these tissues. It is, clearly, of great importance that this antigen or antigens should be identified.

Despite the demonstration of cytotoxicity against adipocytes *in vitro* of a similar magnitude to that seen with A/S 83, A/S 361 showed variable effects on adipose tissue *in vivo* (Table 4.5). In most rats treatment with A/S 361 induced lymphocytic infiltration of adipose tissue and a reduction in parametrial fat pad weight, but the effects were not as great or as consistent as those caused by treatment with antisera raised against whole APM. It is possible that, since A/S 361 bound to a restricted number of antigens on the adipocyte surface, a reduced number of antibodies bound to adipose tissue *in vivo*, resulting in reduced complement activation and, hence, reduced cytotoxicity or reduced production of chemotactic factors for phagocytic cells, compared with A/S 83, 164 and 283.

Antisera raised against HPM and EPM had no effects on adipose tissue *in vivo*, suggesting that adipocyte specific components are an essential part of an antiserum with *in vivo* fat-reducing properties. However, the absence of an *in vivo* effect of any antiserum on a

particular tissue may simply be because the dose administered was not sufficiently high.

4.3.3.3 Discrepancies between ELISA and in vitro cytotoxicity assays

Some of the limitations of ELISA and Western blotting were discussed in 4.3.3.1. In this section emphasis is placed on the limitations of both ELISA and in vitro cytotoxicity assays by comparing antibody binding to EPM with cytotoxicity against erythrocytes by different antisera.

Two anti-(APM) antisera, A/S 283 and A/S 164, showed equivalent levels of cytotoxicity against erythrocytes at antiserum dilutions of nearly 100-fold greater than A/S 83 (Fig. 4.11). However, the relative antibody binding and antigen abundances of A/S 283 and 164 to EPM were nearly 100-fold less than those of A/S 83, as measured by ELISA (Fig. 4.13). An antiserum raised against erythrocytes showed intermediate cytotoxicity and antibody binding between A/S 283 and 164 and A/S 83.

All ELISAs were performed using an anti-(goat IgG) second antibody. IgM is a far more effective activator of complement than IgG (see Section 1.4.1) and so cytotoxicity of A/S 283 and 164 may have been due to IgM antibodies which were not detected by ELISA. Time did not permit this possibility to be examined. ELISAs were performed at 22° C, while in vitro cytotoxicity assays were performed at 37° C. Low affinity antibodies tend to bind more effectively at low temperatures and so ELISA may measure low affinity antibodies that are not cytotoxic. This possibility was tested by comparing the results of ELISAs where antibody binding was performed at room temperature with those performed at 37° C (Fig. 4.13). While absorbances were higher in assays performed at 37° C than at room temperature, relative differences between antisera remained constant.

The protein adsorbed to the ELISA plate may not have been a representative sample of the total EPM protein. It is possible that proteins, to which binding is important for mediating cytotoxicity, did not adsorb effectively to PVC wells. Poly-l-lysine was, therefore, used to coat ELISA plates in an attempt to adsorb more EPM protein onto the plate (Fig. 4.13). While the relative differences between antisera were reduced under these conditions, A/S 83 still apparently bound more strongly to EPM than did A/S 283 or 164.

A/S 283 and 164 may have been particularly good activators of guinea-pig complement. To test this possibility, sheep, rat and guinea-pig complement were compared in cytotoxicity assays, with erythrocytes as the target cell (Fig. 4.12). In the presence of sheep complement, at low antiserum concentrations, A/S 83 showed greater cytotoxicity against erythrocytes than A/S 283 or 164, but at high concentrations, A/S 83 was able to lyse only 40-50% of erythrocytes, while A/S 283 and 164 could achieve total lysis. This implies either that there are 2 populations of erythrocytes bearing different antigenic determinants, or that high doses of A/S 83 are anti-complementary. In the presence of rat complement, the cytotoxicity of all the antisera measured was so much reduced that relative differences between antisera were difficult to determine. Any differences in the relative cytotoxicity in the presence of rat and guinea-pig complement may be due to differences in the antibody binding to rat erythrocyte homologous restriction factor, which interferes with the formation of the C5-9 membrane attack complex (Shin et al., 1986). Binding to the restriction factor may mask it and render the erythrocyte more susceptible to lysis by homologous complement. Such an effect would preclude the necessity for antibody binding and cytotoxicity to be closely related.

In vitro assays of antibody binding to and cytotoxicity against erythrocytes were the best characterised in this study, as erythrocytes were easy to obtain and could be stored for several days. There were no clear discrepancies between antibody binding to APM and cytotoxicity against adipocytes. It would, clearly, be of interest to examine more closely antibody binding to HPM and cytotoxicity against hepatocytes and compare the relative effects of different antisera on liver components in vitro and in vivo.

4.3.3.4 Conclusions

1. Three different antisera, raised in sheep, against APM were cytotoxic to adipocytes in vitro and had fat-reducing properties in vivo, despite apparent differences in the titre or affinity of anti-(APM) antibodies, as determined by ELISA and Western blotting.
2. Two out of 3 anti-(APM) antisera caused gross liver abnormalities in vivo. The presence or absence of an anti-(liver) effect in vivo could not be correlated with differences in titre or affinity of anti-(HPM) antibodies or differences in binding to particular HPM antigens by ELISA or Western blotting.
3. Anti-(APM) antisera with anti-liver effects in vivo showed greater in vitro cytotoxicity against erythrocytes than the anti-(APM) antiserum that had no apparent effect on the liver in vivo. This raised the possibility that liver abnormalities might be caused by overloading of the hepatic reticulo-endothelial system with immune complexes, formed as a result of reaction of antibodies with non-hepatic antigens, such as erythrocytes.

4. Two out of 3 anti-(APM) antisera, in addition to causing liver abnormalities, had transient anaesthetic-like effects *in vivo* whose speed of onset (within 5 minutes of administration) suggested that antibodies bound rapidly to a component of nervous tissue, in some way interfering with synaptic transmission.

5. An anti-(HPM) antiserum, when given in a sufficiently high dose, caused liver abnormalities *in vivo* but had no effect on adipose tissue, suggesting that the fat-reducing properties of anti-(APM) antisera were due to a specific anti-fat component of the antisera.

6. Polyacrylamide gel electrophoresis showed that the ASA preparations were composed of 6 major polypeptides with approximate molecular weights of 53 (a doublet), 59, 72, 85 and 96 kD and 6 minor polypeptides.

7. Antisera raised aainst EPM and HPM recognised components of the ASA, and an antiserum raised against ASA recognised components of EPM and HPM, suggesting that not all the components of the ASA were truly adipocyte specific. This could be due to inadequate adsorption of anti-(APM) antibodies with non-adipose tissues, before their use to immuno-isolate the ASA or non-specific adsorption by immuno-adsorbants. Despite some cross-reactivity with other tissues, the anti-(ASA) antiserum was more adipocyte-specific than anti-(APM) antisera, as shown by ELISA and Western blotting.

8. The anti-(ASA) antiserum retained, at least partly, the effects of anti-(APM) antisera on adipose tissue, food intake and body weight gain and had immediate anaesthetic-like effects but did

not cause liver abnormalities. This suggests that a reduction in food intake on the first day of treatment may be an inevitable consequence of massive lipid mobilization from adipose tissue and that the antigens responsible for the production of anaesthetic-like properties of antisera remain in the ASA preparation.

4.4 THE EFFECTS OF ADSORPTION OF ANTISERA RAISED AGAINST ADIPOCYTE PLASMA MEMBRANES WITH LIVER

4.4.1 Experimental

Whole female Wistar rat livers were homogenised in an equal volume of PBS, using a Waring blender on high speed for 1 minute. The homogenate was washed 3 times with PBS by centrifugation at 2400 g for 20 minutes. Equal volumes of packed liver homogenate were incubated with A/S 83 or A/S 164 for 16 h at 4° C in an end-over-end mixer. The adsorbed antiserum was centrifuged at 50,000 g for 1 h to remove the liver homogenate.

Binding of the adsorbed antisera to all the antigens tested in the previous Section was measured by ELISA and Western blotting. Cytotoxicity against erythrocytes was measured but the LDH content of the adsorbed antisera was too high to allow measurement of cytotoxicity against adipocytes.

The effects of adsorption against liver on the in vivo effects of A/S 164 were examined by injecting female Wistar rats, weighing 120-140 g, intraperitoneally at 10 h with 1 ml of unadsorbed, 1 ml of adsorbed or 3 ml of adsorbed antiserum for 4 consecutive days. The rats were killed 6 days after the first treatment and examined as described in Section 4.2.1.

4.4.2 Results

While adsorption of A/S 83 and A/S 164 with liver homogenate removed approximately 60% of the antibody binding to APM and ASA, the adsorption removed 85-99% of the binding to non-adipocyte plasma membranes (Tables 4.6 and 4.7). Both adsorbed antisera were more than 10 times more APM specific with respect to HPM and approximately 10 times more APM specific with respect to KPM than the antisera before adsorption, as shown by relative antibody binding. Binding to EPM and brain membranes by the adsorbed antisera was less than or equal to 1% of that by the antisera before adsorption.

While adsorption of A/S 83 and A/S 164 with liver homogenate removed approximately 20% of the antigen abundance against APM, the adsorption removed about 50% of the antigen abundance against ASA. A/S 83 after adsorption was 2-5 times more APM specific with respect to non-adipocyte plasma membranes than A/S 83 before adsorption and A/S 164 after adsorption was about 10 times more APM specific with respect to HPM and brain membranes and 1-3 times more APM specific with respect to KPM and EPM than A/S 164 before adsorption, as shown by relative antigen abundances.

Adsorption of A/S 83 and A/S 164 with liver homogenate removed at least 85% of the *in vitro* cytotoxicity against erythrocytes (Fig. 4.14).

Western blotting showed that the adsorbed antisera clearly recognised only 1 HPM polypeptide, with a molecular weight of approximately 65 kD, compared with at least 9 and 8 recognised by A/S 83 and A/S 164, respectively, before adsorption (Fig. 4.15). Both adsorbed antisera bound strongly to APM polypeptides with

Table 4.6 The effects of adsorption of A/S 83 with liver homogenate on tissue specificity, as determined by ELISA

A/S 83 was adsorbed with packed liver homogenate (1 vol. of serum:1 vol. of packed liver) for 16 h at 4° C. Relative antibody binding and antigen abundances were calculated as described in Section 2.6.3. Relative APM specificity was calculated by dividing antibody activity against APM by the antibody activity against the non-APM antigen.

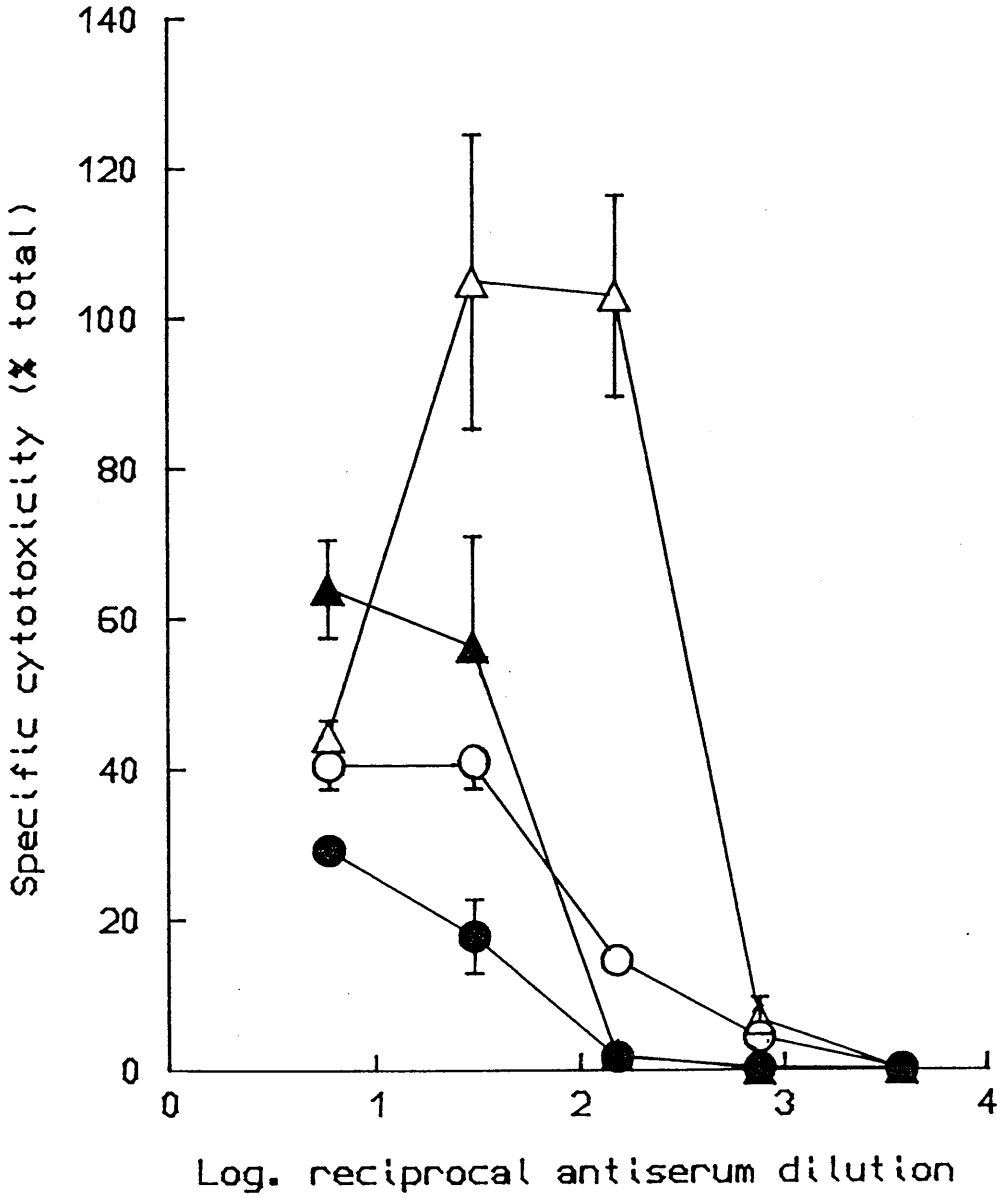
Antigen	Relative antibody binding					Relative antigen abundance				
	Unadsorbed (% of anti-APM)	Adsorbed (% of unadsorbed)	Adsorbed (% of anti-(APM) of unadsorbed	Relative APM specificity		Unadsorbed	Adsorbed (% of unadsorbed)	Adsorbed (% of anti-(APM) of unadsorbed)	Relative APM specificity	
				Unadsorbed	Adsorbed				Unadsorbed	Adsorbed
Adipocyte plasma membrane	100	37	37			100	72	72		
Adipocyte specific antigens	29	41	12	3.4	3.1	56	50	28	1.8	1.4
Hepatocyte plasma membrane	32	3	1	3.1	37.0	100	22	22	1.0	3.3
Kidney plasma membrane	25	3	1	4.0	37.0	66	32	21	1.5	3.4
Erythrocyte plasma membrane	7	1	<1	4.3	>37.0	37	14	5	2.7	14.4
Brain membrane	9	12	1	11.1	37.0	68	19	13	1.5	3.8

Table 4.7. The effects of adsorption of A/S 164 with liver homogenate on tissue specificity, as determined by ELISA

A/S 164 was treated as described for A/S 83 in Table 4.6.

Antigen	Relative antibody binding					Relative antigen abundaance				
	Unadsorbed (% of A/S 83)	Adsorbed (% of unadsorbed A/S 164)	Adsorbed (% of anti-(APM) of A/S 83)	Relative APM specificity		Unadsorbed (% of A/S 83)	Adsorbed (% of unadsorbed A/S 164)	Adsorbed (% of anti-(APM) of A/S 83)	Relative APM specificity	
				Unadsorbed	Adsorbed				Unadsorbed	Adsorbed
Adipocyte plasma membrane	200	40	80			74	88	65		
Adipocyte specific antigens	180	37	66	1.1	1.2	100	41	41	0.74	1.6
Hepatcyte plasma membrane	58	5	1	3.4	80.0	63	9	6	1.2	10.8
Kidney plasma membrane	74	15	3	2.7	26.7	72	30	22	1.0	3.0
Erythrocyte plasma membrane	7	14	0	28.6	∞	17	54	9	4.4	7.2
Brain membrane	18	3	0	11.1	∞	34	8	3	9.3	21.7

Fig. 4.14. The effects of adsorption of A/S 83 and A/S 164 with liver homogenate on <u>in vitro</u> cytotoxicity against erythrocytes

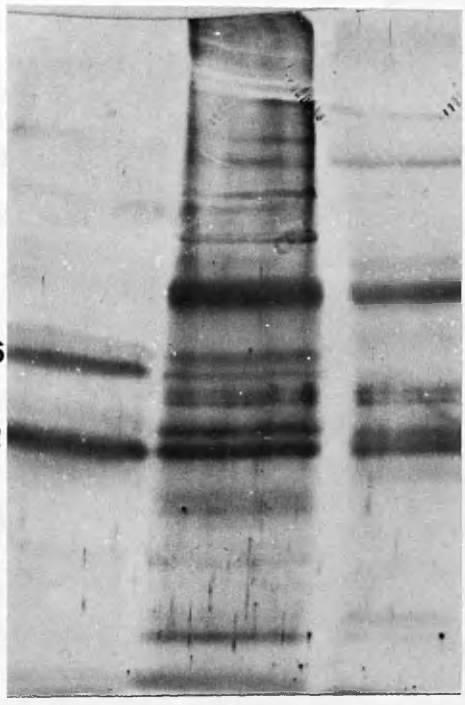


Cytotoxicity against erythrocytes of A/S 83 (open circles), A/S 83 adsorbed with liver homogenate (filled circles), A/S 164 (open triangles) and A/S 164 adsorbed with liver homogenate (filled triangles) was measured as for Fig. 4.1. Results are means ± SEM of 3-5 observations.

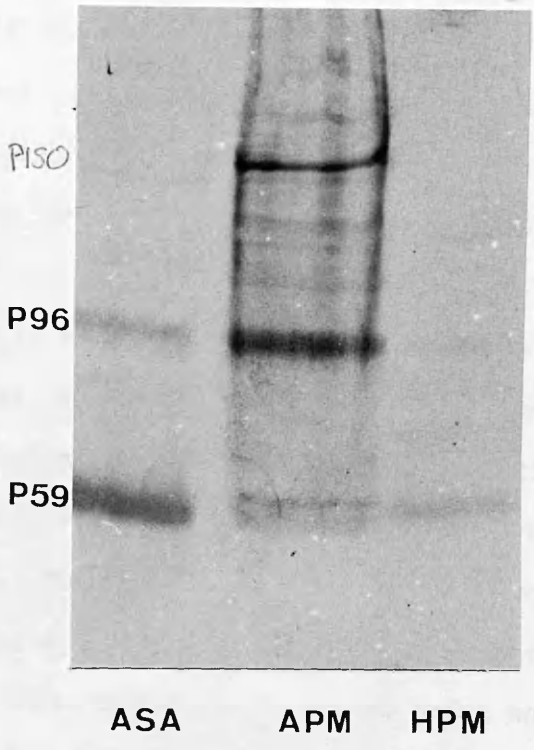
Fig. 4.15. The effects of adsorption of A/S 83 and A/S 164 with liver homogenate on antibody binding to adipocyte and hepatocyte plasma membranes and adipocyte specific antigens, as determined by Western blotting

Abbreviations and methods as for Fig. 4.2

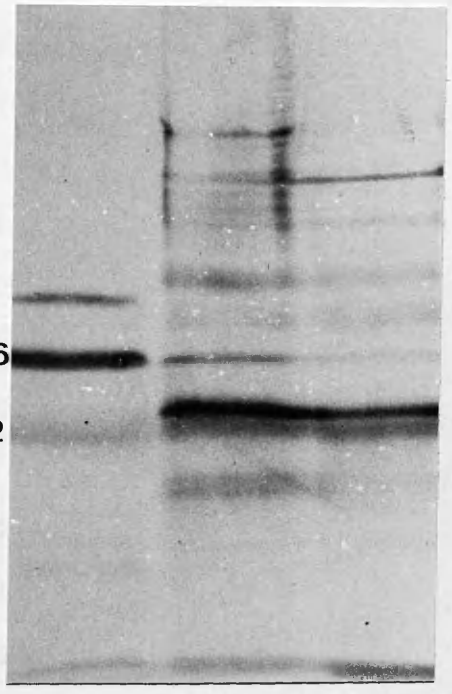
A/S 83



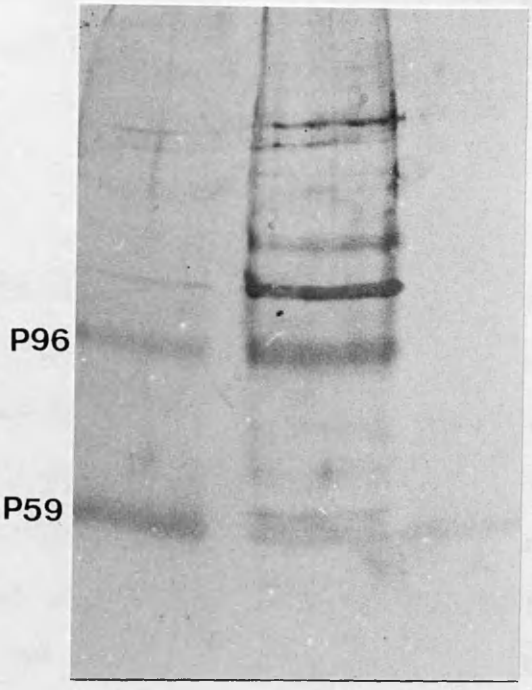
Adsorbed A/S 83



A/S 164



Adsorbed A/S 164



molecular weights of approximately 59, 96 and 150 kD and recognised at least 8 other APM polypeptides. Clear binding to APM polypeptides within a molecular weight range of 60-90 kD was not visible by the adsorbed antisera, while a number of APM polypeptides within this range were recognised before adsorption. P59 and P96 of the ASA were clearly recognised by the adsorbed antisera but binding to P53, P72 and P85 was not clearly visible.

The treatment of rats with 3 ml of A/S 164 adsorbed with liver homogenate caused a slightly greater reduction in the dry weight and percentage dry weight of parametrial adipose tissue and had a much reduced effect on the liver, compared with treatment with 1 ml of A/S 164 before adsorption (Table 4.8). Treatment with 1 ml of adsorbed A/S 164 caused some reduction in dry weight and percentage dry weight of parametrial adipose tissue but these effects were not as great as those observed using 1 ml of A/S 164 before adsorption. No liver abnormalities were observed in rats treated with 1 ml of adsorbed A/S 164. Adsorption with liver homogenate had little effect on the inhibitory effects of A/S 164 on food intake and body weight gain on the first day of treatment.

4.4.3 Discussion

The removal by adsorption with liver homogenate of 60% of the antibody binding to APM of A/S 83 and A/S 164, while removing more than 85% of the binding to non-adipocyte plasma membranes, suggests that 60% of the antibody activity of these anti-(APM) sera was directed against cross-reacting antigens. This may be higher than the true figure, however, because of non-specific adsorption of antibody by the liver. Liver was used for the adsorption because it

Table 4.8. The effects of adsorption of A/S 164 with liver homogenate on the in vivo effects of the antiserum

Treatment	Number of observations	Body weight change (1st day) (g)	Food intake (1st day) (g)	Parametrial adipose tissue		Liver abnormalities score
				Dry weight	% dry weight	
None	3	10.8 ±1.2	22.2	0.97 ±0.22	81 ±1.8	0 ± 0
A/S 164 (3 x 1 ml)	3	1.8*** ±1.0	10.0	0.67 ±0.05	68*** ±1.6	3 ± 0
Ads A/S 164 (3 x 1 ml)	3	1.4*** ±2.7	14.0	0.74 ±0.08	74* ±1.4	0 ± 0
Ads A/S 164 (3 x 3 ml)	3	-9.0*** ±1.0	3.2	0.53 ±0.13	66* ±4.1	1.8 ± 0.3

Female Wistar rats were injected intraperitoneally with 1 or 3 ml of antiserum for 3 consecutive days, as indicated. Rats were sacrificed 6 days after the first treatment and liver abnormalities were scored on a scale of 0-3, as indicated by disrupted liver lobes. *P<0.01, ***P<0.001, compared with controls (Student's unpaired t test).

was a tissue that could be obtained in relatively large quanitites but was, otherwise, perhaps not the ideal tissue for adsorption because of the presence of Fc receptors on Kupffer cells. ELISA and Western blotting showed that, while the majority of the anti-(HPM) activity of A/S 83 and A/S 164 was removed by adsorption with liver homogenate, some binding to HPM was retained, particularly to a polypeptide with a molecular weight of about 60 kD. The antisera were adsorbed with a liver homogenate, harvested by low speed centrifugation, while the antisera were screened against HPM, harvested only by high speed centrifugation. It is possible that the 60 kD HPM protein was barely visible when Western blotting was performed using the antisera before adsorption, suggesting that antibodies directed against this protein are very minor components of the antisera.

ELISA suggested that adsorption with liver homogenate removed some of the antibody activity of A/S 83 and A/S 164 against ASA. Western blotting suggested this was due to a reduction in the binding of both P72 and P96 but that to P72 suffered the greatest reduction. This casts further doubt on the true adipocyte specificity of these antigens but it is possible that non-specific adsorption by the liver homogenate may have resulted in the reduction in binding to ASA. The binding of the adsorbed antisera to P59 of the ASA, which was not clearly recognised before adsorption, was surprising but may have been the result of a longer incubation of the blots in the peroxidase substrate.

The majority of the <u>in vivo</u> anti-(liver) effects of A/S 164 were removed by adsorption with liver homogenate and, by increasing the dose of adsorbed A/S 164, it was possible to induce an effect on

adipose tissue of a magnitude at least as great as that induced by A/S 164 before adsorption. This indicates that antibodies that cross-react with the liver are not an essential component of an antiserum with fat-reducing properties and that the liver abnormalities were not a consequence of fat reduction. These results support those obtained using A/S 361, raised against ASA (see Section 4.3.2). The adsorbed antiserum, like A/S 361, retained the immediate anaesthetic-like effects and the effects on food intake and body weight gain, seen using A/S 164 before adsorption. Therefore, despite the greater apparent specificity of adsorbed A/S 164 than A/S 361, the antigens responsible for anaesthetic-like effects were still recognised by the adsorbed antiserum.

4.4.4 Conclusions

1. Adsorption of anti-(APM) antisera with liver homogenate rendered the antisera considerably more adipocyte specific than the anti-(ASA) antiserum, as indicated by ELISA and Western blotting. This suggests that the anti-(APM) antisera do contain adipocyte specific components.
2. The anti-(APM) antiserum, after adsorption, retained the effects on adipose tissue, food intake and body weight gain of the unadsorbed antiserum and had anaesthetic-like effects but had no effects on the liver, thus supporting the results of the *in vivo* administration of an anti-(ASA) antiserum. This suggests that adipocyte-specific antisera can have fat-reducing properties *in vivo*.

4.5 ANTIBODY BINDING TO PLASMA MEMBRANES FROM MALE AND FEMALE WISTAR AND FEMALE SPRAGUE-DAWLEY RATS BY ANTISERA RAISED AGAINST ADIPOCYTE PLASMA MEMBRANES AND ADIPOCYTE SPECIFIC ANTIGENS

4.5.1 Experimental

The binding of A/S 83 (anti-(APM)), A/S 361 (anti-(ASA)) and A/S 83 adsorbed with liver homogenate to APM and HPM prepared from female Wistar, male Wistar and female Sprague-Dawley rats was compared, by ELISA.

4.5.2 Results

A/S 83, A/S 361 and A/S 83 adsorbed with liver homogenate showed reduced binding to male compared with female Wistar APM and HPM (Tables 4.9, 4.10). Adsorption of A/S 83 with female Wistar liver homogenate removed more antibody binding to male than to female Wistar APM but removed the same amount of antibody binding to male and female Wistar HPM. A/S 361 (raised against ASA isolated from female Wistar APM) showed less binding to male Wistar APM than A/S 83, (raised against whole female Wistar APM).

Adsorption of A/S 83 with female Wistar liver homogenate removed more antibody binding to female Sprague-Dawley APM than to female Wistar APM but removed less antibody binding to female Sprague-Dawley HPM than to female Wistar HPM. A/S 361 showed less binding to female Sprague-Dawley APM than to female Wistar APM.

The relative antigen abundances showed similar trends to the relative antibody binding but the relative differences between different antisera and antigens were reduced.

Table 4.9. Antibody binding to adipocyte plasma membranes of male and female Wistar and female Sprague-Dawley rats by antisera raised against adipocyte plasma membranes and adipocyte specific antigens

Antigen	Antibody activity as % anti-(female Wistar plasma membrane) activity					
	A/S 83 [anti-(APM)]		A/S 83 (adsorbed with female Wistar liver)		A/S 361 [anti-(ASA)]	
	Antibody binding	Antigen abundance	Antibody binding	Antigen abundance	Antibody binding	Antigen abundance
Female Wistar APM	100	100	100	100	100	100
Male Wistar APM	60	98	25	100	19	88
Female Sprague-Dawley APM	84	109	63	112	38	80

Antibody activity was measured by ELISA and antibody binding and antigen abundance were calculated as described in Section 2.6.3.

Table 4.10. Antibody binding to hepatocyte plasma membranes of male and female Wistar and female Sprague-Dawley rats by antisera raised against adipocyte plasma membranes and adipocyte specific antigens

Antigen	Antibody activity as % of anti-(female Wistar plasma membrane) activity					
	A/S 83 [anti-[APM])		A/S 83 (adsorbed with female Wistar liver)		A/S 361 [anti-(ASA)]	
	Antibody binding	Antigen abundance	Antibody binding	Antigen abundance	Antibody binding	Antigen abundance
Female Wistar HPM	100	100	100	100	100	100
Male Wistar HPM	21	65	22	50	47	80
Female Sprague-Dawley HPM	47	56	150	49	35	68

Methods as for Table 4.9

4.5.3 Discussion

Antibody binding of A/S 83 to APM prepared from male Wistar and female Sprague-Dawley rats was reduced, compared with binding to female Wistar APM. This suggests that there are differences in the antigenic composition of the APM prepared from the 3 different sources. There was little difference in the relative antigen abundances of A/S 83 against female Wistar or Sprague-Dawley or male Wistar APM. It is possible, therefore, that minor differences in the expression of antigens on adipocytes from the 3 different sources, due to slight alterations in polypeptide composition or orientation on the cell surface, resulted in differences in antibody affinity for the APM antigens.

Studies of the effects of adsorption with female Wistar liver homogenate and A/S 361 (raised against female Wistar ASA) provide an indication of whether the differences in antigenic composition of APM from rats of different strains and sex concern adipocyte specific or cross-reacting antigens. In the ensuing discussion, 'adipocyte specific' refers to antigens present on female Wistar adipocyte but not on female Wistar hepatocyte plasma membranes. Female Sprague-Dawley or male Wistar APM may bear adipocyte-specific or cross-reacting antigens not present on female Wistar APM but these antigens would not be detected by the antisera described above, which were all raised against female Wistar antigens. Adsorption of A/S 83 with female Wistar liver homogenate removed more binding to Sprague-Dawley and male Wistar APM than to female Wistar APM, but removed the same amount or less binding to male Wistar and Sprague-Dawley HPM. This suggests that A/S 83, before adsorption, bound to a similar number of cross-reacting antigens with a similar

affinity on APM from all 3 sources but bound to a smaller number of adipocyte specific antigens, or to the same number of adipocyte specific antigens but with a lower affinity on male Wistar and Sprague-Dawley APM than female Wistar APM. Thus, adsorption with female Wistar liver would remove a large proportion of the male Wistar and Sprague-Dawley APM binding, since most of this binding was to cross-reacting antigens. If the proportion of binding of A/S 83 to cross-reacting antigens on the APM from the 3 different sources was the same, then adsorption with a tissue containing those cross-reacting antigens would be expected to have a similar effect on binding to HPM of the 3 different sources. It is clearly impossible to draw firm conclusions about the antigenic composition of plasma membranes from different sources from the study of a single antiserum, especially as the method of study was ELISA which has all the limitations discussed in Section 4.3.3.1. However, the suggestion that male Wistar and Sprague-Dawley APM bear fewer adipocyte specific antigens than female Wistar APM is supported by the results of binding of A/S 361 (anti-(ASA)) to APM of the 3 different sources. A/S 361 showed less binding to APM from male Wistar and Sprague-Dawley APM than to female Wistar APM. However, in view of the questionable adipocyte specificity of A/S 361, these results should be interpreted with caution.

4.5.4 Conclusions

1. Antisera raised against APM or ASA (isolated from female Wistar rats) showed reduced binding to APM from male Wistar or female Sprague-Dawley rats. These results suggest that there are both differences and similarities between the antigenic compositon of APM from rats of different strain and sex.

2. Adsorption of anti-(Wistar APM) antisera with female Wistar liver homogenate removed more of the anti-male Wistar and female Sprague-Dawley HPM than APM activity. The differences between APM of different strain and sex may reside, therefore, predominantly in the adipocyte specific antigens. This hypothesis is supported by the fact that the anti-(female Wistar ASA) antiserum bound less well to APM from male Wistar or female Sprague-Dawley than female Wistar rats.

CHAPTER 5

ACTIVE IMMUNISATION AGAINST THE ADIPOCYTE PLASMA MEMBRANE IN RATS

ACTIVE IMMUNISATION AGAINST THE ADIPOCYTE PLASMA MEMBRANE IN RATS

5.1 INTRODUCTION

The induction of autoantibody production directed against a variety of 'self tissues' has been achieved, in experimental animals, by immunisation with homologous or heterologous antigens, usually in Freunds adjuvant (Germuth et al., 1967; Källen and Nilsson, 1986; Watanabe et al., 1987). The aim of the work described in this chapter was to extend this approach to the induction of autoantibodies directed against adipose tissue.

Initial experiments involved immunisation of rats with rat APM conjugated to BSA, in the hope that BSA would stimulate T helper lymphocytes to provide the necessary helper signals to stimulate APM reactive B cells to secrete autoantibodies. The conjugate was emulsified in complete Freunds adjuvant since this is still the most potent stimulator of both B and T cell immune responses known.

Subsequent experiments involved attempts to augment this immune response by the use of Pertussis vaccine and 2'-deoxyguanosine. Pertussis vaccine and toxin have been shown to enhance the induction of experimental autoimmune encephalomyelitis and autoimmune uveoretinitis in rats, when administered in conjunction with complete Freunds adjuvant and encephalogenic protein and retinal specific antigen respectively (McAllister et al., 1974; Kallen and Nilsson, 1986). Deoxyguanosine is an inhibitor of ribonucleotide reductase and, therefore, inhibits DNA synthesis (Bril, van den Akker, Molendijk, Bianchi and Benner, 1984). 2'-deoxyguanosine prevents an antigen dose-dependent suppression of antibody responses at

supra-optimal concentrations (Dosh, Mausour, Cohen, Shore and Gelfand, 1980). These authors found that T helper cells and B lymphocytes were more than 1000-fold more resistant to 2'-deoxyguanosine than T suppressor cells and suggested that suppressor cells are low in 5' nucleotidase and high in deoxyguanosine kinase activity, resulting in the accumulation of intracellular deoxyguanosine triphosphate. As T cell suppression is thought to play a crucial role in the prevention of autoimmunity, it was hoped that the administration of 2'-deoxyguanosine in conjunction with autoantigen might augment the autoimmune response by inhibiting T suppressor function.

Attempts were also made to induce an autoimmune response to adipose tissue by manipulation of the idiotype network. Bigazzi (1986) found that the immunisation of rats, suffering from autoimmune thyroiditis (due to the presence of anti-(thyroglobulin) antibodies) with anti-(thyroglobulin) antibodies increased the severity of the disease, possibly through the production of anti-anti-idiotypic antibodies. It was hoped, therefore, that immunisation of rats with an affinity purified anti-(APM) antibody would induce the formation of anti-idiotypic antibodies, bearing an internal image of APM antigens (Fig. 5.1). Such a disturbance of the idiotype network might provoke the formation of anti-anti-idiotypic antibodies, recognising APM antigens.

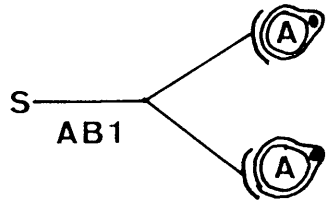
5.2 EXPERIMENTAL

5.2.1 Experiment 1

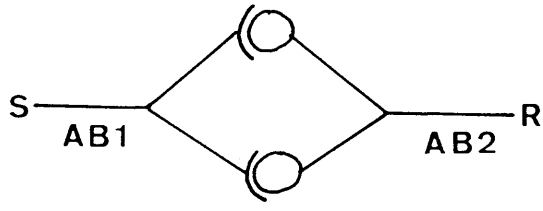
In this experiment different members of the same litters were placed in different treatment groups so that littermates could be

Fig. 5.1. The induction of anti-(adipocyte plasma membrane) antibodies by manipulation of the idiotype network

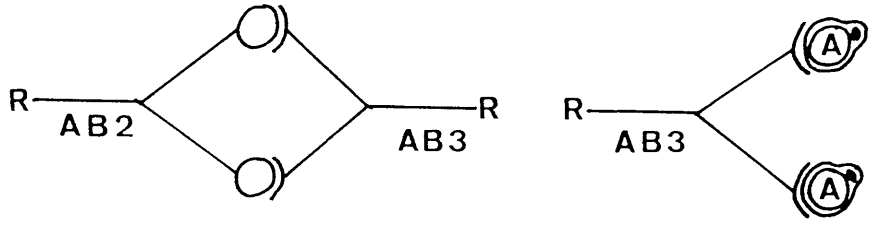
1. Rats are immunised with sheep anti-(rat APM) antibodies



2. Rats respond by the production of anti-idiotypic antibodies that bear internal images of APM antigens



3. Anti-idiotypic antibodies induce the formation of anti-anti-idiotypic antibodies that recognise APM antigens



The above is a diagrammatic representation of the proposed means whereby anti-(APM) antibodies might be induced by manipulation of the idiotype network. S and R indicate sheep and rat antibodies (AB) respectively, while A represents adipocyte.

compared. Four-week-old female Wistar rats, weighing 40-50 g, were immunised subcutaneously at 2 sites, with 100 μg of rat APM conjugated to 100 μg of BSA emulsified in 200 μl of complete Freunds adjuvant. Conjugation was performed by incubating 100 μg of APM with 100 μg of BSA and 20 μl of glutaraldehyde (0.5%) in 100 μl of PBS for 30 minutes at 22° C. Rats were boosted with APM conjugated to BSA in incomplete Freunds adjuvant at 2 and 4 weeks after the first immunisation and with unconjugated APM in incomplete Freunds adjuvant at 6 and 10 weeks. Control rats were given the same treatment, with the exclusion of APM, at 0, 2 and 4 weeks and received no subsequent boosts. Body weights were recorded daily. Blood samples were taken from the tail, under ether anaesthesia, at 6, 8, 10 and 16 weeks after the first treatment and assayed for anti-(APM) antibodies by ELISA. Rats were killed 16 weeks after the first treatment and the dry weights of the inguinal subcutaneous, interscapular subcutaneous, parametrial, peri-renal and omental adipose depots were determined.

5.2.2 Experiment 2

Experiment 2 was carried out in the same way as Experiment 1 except that littermates were not used and a group of control rats were included that received no immunisations. Adipocyte numbers and mean adipocyte volumes of parametrial and inguinal subcutaneous adipose tissue were determined.

5.2.3 Experiment 3

Experiment 3 included a repeat of Experiment 2. Additional groups of rats received either APM conjugated to BSA plus 0.5 ml of

Pertussis vaccine (Wellcome, London) (given subcutaneously at the same time as the first immunisation), or APM conjugated to BSA and 4 mg of 2'-deoxyguanosine (Sigma Chemical co., Poole, Dorset) (given intraperitoneally at the same time as the first immunisation and for the 6 days following each immunisation). Another group of rats received 50 μg of ASA conjugated to BSA. The final group of rats received either 100 μg of affinity-purified anti-(APM) antibody, from the same pool that was used to prepare the ASA. In all groups, antigens were emulsified in complete Freunds adjuvant for the first immunisation, whilst subsequent immunisations on weeks 2, 4, 6 and 10 were given in incomplete Freunds adjuvant. All immunisations were given at 2 subcutaneous sites and, where initially antigens were conjugated to BSA, the final 2 immunisations were given in the absence of BSA.

Body weights and food intakes were measured weekly. Blood samples were taken as for Experiment 1 at 2, 4, 6, 8, 10, 12 and 16 weeks after the first treatment and assayed, by ELISA, for antibody binding to APM, HPM, KPM, EPM and ASA. Delayed type hypersensitivity tests were performed on 2 rats that had received APM conjugated to BSA and Pertussis vaccine 2 weeks after the fourth immunisation. A small area of the skin was shaved and 10 μl of BSA (1 mg/ml) and 10 μl of APM (1 mg/ml) were injected just under the skin at different sites. At 24 and 48 h the injection sites were examined for swelling.

Rats were killed 16 weeks after the first immunisation and the dry weights, mean adipocyte volumes and adipocyte numbers of the parametrial, peri-renal and inguinal subcutaneous adipose depots were determined.

Binding of sera from all actively immunised rats to APM, prepared from adipocytes isolated in the presence of BSA, and to APM, prepared by homogenisation of whole tissue in the absence of BSA, was compared by ELISA. In addition, the ability of pre-incubation with BSA to reduce the binding capacity of sera to APM, prepared in the presence of BSA, was examined. 100 μl of test serum (4%) was incubated with either 100 μl of PBS or 100 μl of BSA (2 mg/ml) (Fraction V, Sigma Chemical Co., Poole, Dorset) or casein (Technical, from Bovine milk, Sigma Chemical Co., Poole, Dorset) for 2 h at 22° C. Casein was included in order to determine the effects of pre-incubation with a non-specific protein. After pre-incubation, the binding capacity of the sera to APM, prepared in the presence of BSA, and to BSA alone, was examined by ELISA.

The ability of pre-incubation with sheep γ globulin (prepared by ammonium sulphate precipitation of whole serum) to reduce binding to ASA was examined for all groups of actively immunised rats. Pre-incubations were performed as described in the previous Section. ELISA plates, coated with ASA, were blocked with casein. Therefore, after pre-incubation, binding of sera to casein alone, as well as to ASA and sheep γ globulin, was tested.

5.3 RESULTS

5.3.1 Apparent anti-(adipocyte plasma membrane) titres in actively immunised rats

In Experiment 1 serum from rats immunised with APM conjugated to BSA or with BSA alone (controls) bound to APM coated onto ELISA plates. There were no significant differences in the absorbances

achieved between serum from the 2 groups of rats, when apparent anti-(APM) responses were measured by ELISA (Fig. 5.2). The APM used for this screening were prepared from adipocytes isolated in the presence of BSA and which were probably contaminated with BSA (see Fig. 4.9). However, the use of APM prepared from adipocytes isolated in the presence of gelatin gave similar results (results not shown). The rats of Experiment 2 showed similar antibody responses to those achieved in Experiment 1 and the apparent anti-(APM) titres were relatively constant between 6 and 16 weeks after the first immunisation in both groups of rats (Fig. 5.3). Apparent anti-(APM) titres in APM- and BSA-immunised rats from Experiment 3 reached a peak of about 5 times those achieved in Experiments 1 and 2 6 weeks after the first immunisation, before falling to levels similar to those observed in Experiments 1 and 2 (Fig. 5.3)

Treatment of rats with Pertussis vaccine and 2'-deoxyguanosine did not augment apparent anti-(APM) titres (Fig. 5.4). Immunisation with anti-(ASA) antibody induced an apparent anti-(APM) response that was more than 100-fold less than that induced by APM conjugated to BSA, although there was still a positive apparent anti-(APM) response suggesting perturbation of the idiotypic network (Fig. 5.1).

Immunisation of rats with Freunds adjuvant alone or Freunds adjuvant and glutaraldehyde did not induce an apparent anti-(APM) response. However, immunisation with BSA in Freunds adjuvant, in the absence of glutaraldehyde, induced similar apparent anti-(APM) responses to those achieved by immunisation with BSA and glutaraldehyde in Freunds adjuvant (Fig. 5.4).

Absorbances achieved by sera from actively immunised rats of Experiment 3, when tested by ELISA against APM, prepared by

Fig. 5.2. The apparent antibody response to adipocyte plasma membranes in actively immunised rats

Rats were immunised with either BSA (open circles) or APM conjugated to BSA (closed circles) in CFA. Antibody binding to APM (prepared in the presence of BSA) was measured 6 weeks after the first immunisation, by ELISA. The results of experiments 1, 2 and 3 are shown in (a), (b) and (c) respectively. Results are means $\pm$ SEM of 5-8 observations.

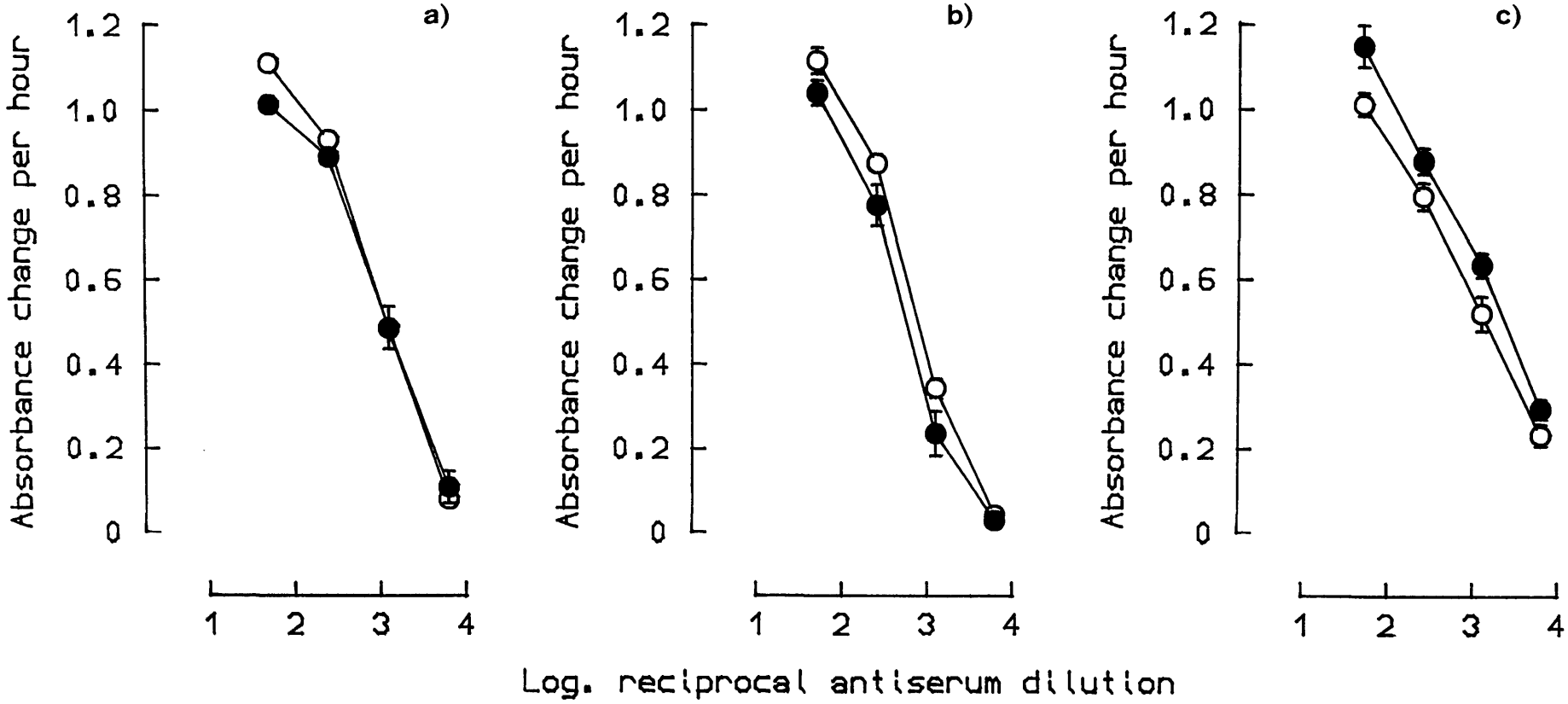

a)
b)
c)
Absorbance change per hour
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
Log. reciprocal antiserum dilution

Fig. 5.3. The change in the apparent antibody response to adipocyte plasma membranes in actively immunised rats, with time

Rats were immunised with either BSA (open symbols) or APM conjugated to BSA (closed symbols) in CFA. Antibody binding to APM (prepared in the presence of BSA) was measured by ELISA. The results of experiments 1 and 2 were pooled (squares) and compared with those of experiment 3 (circles).

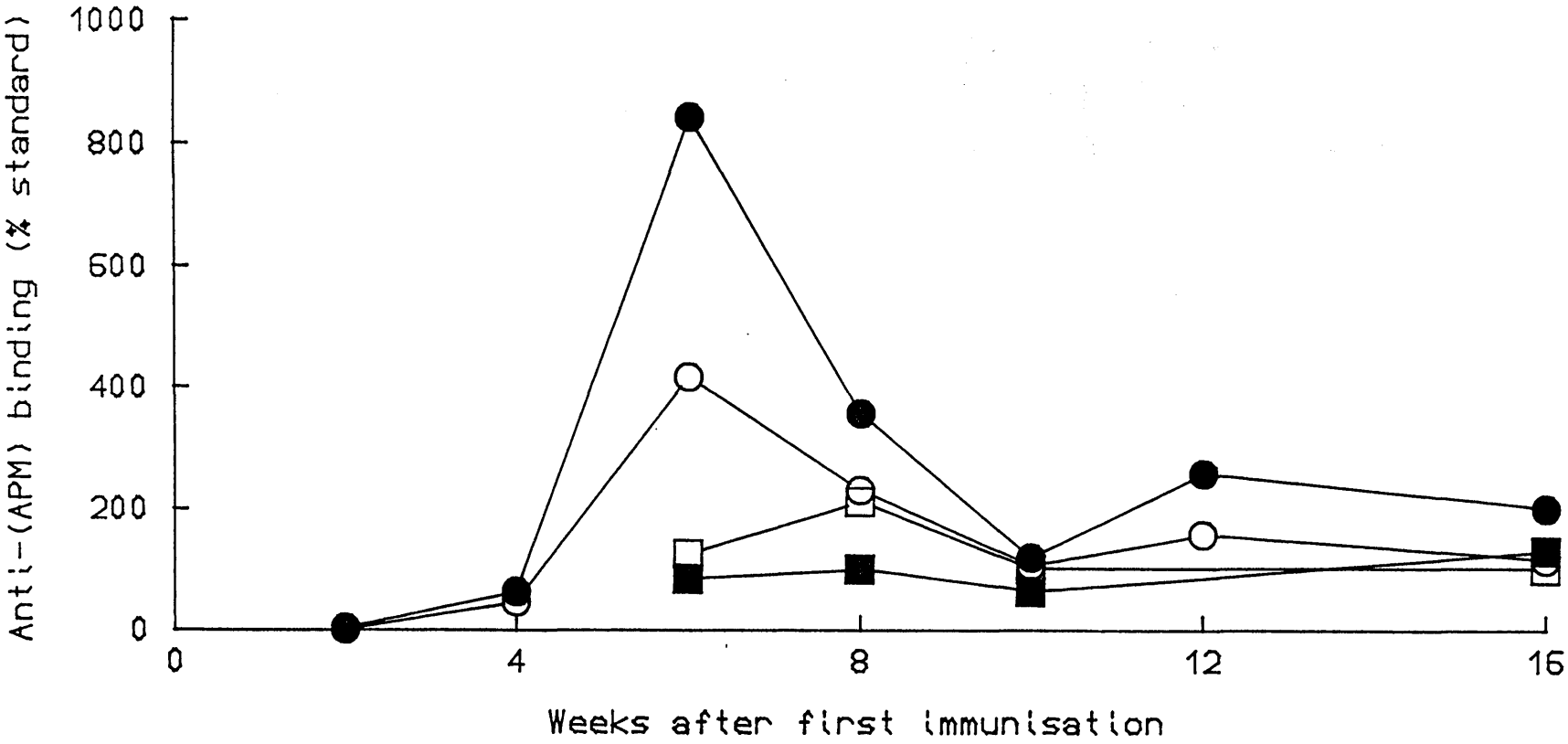
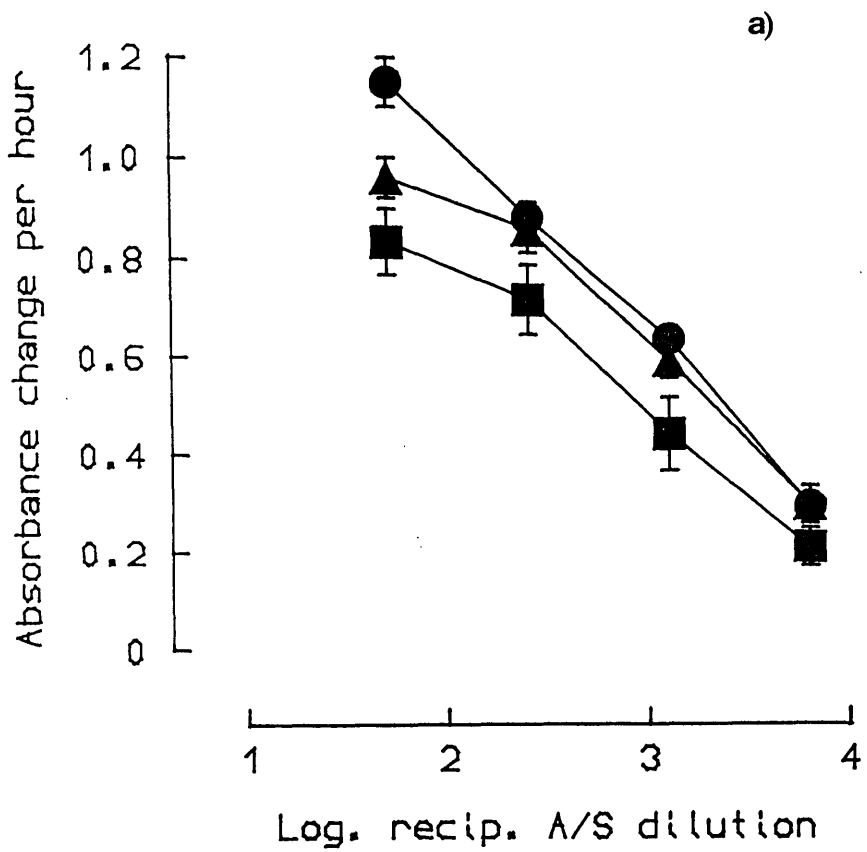
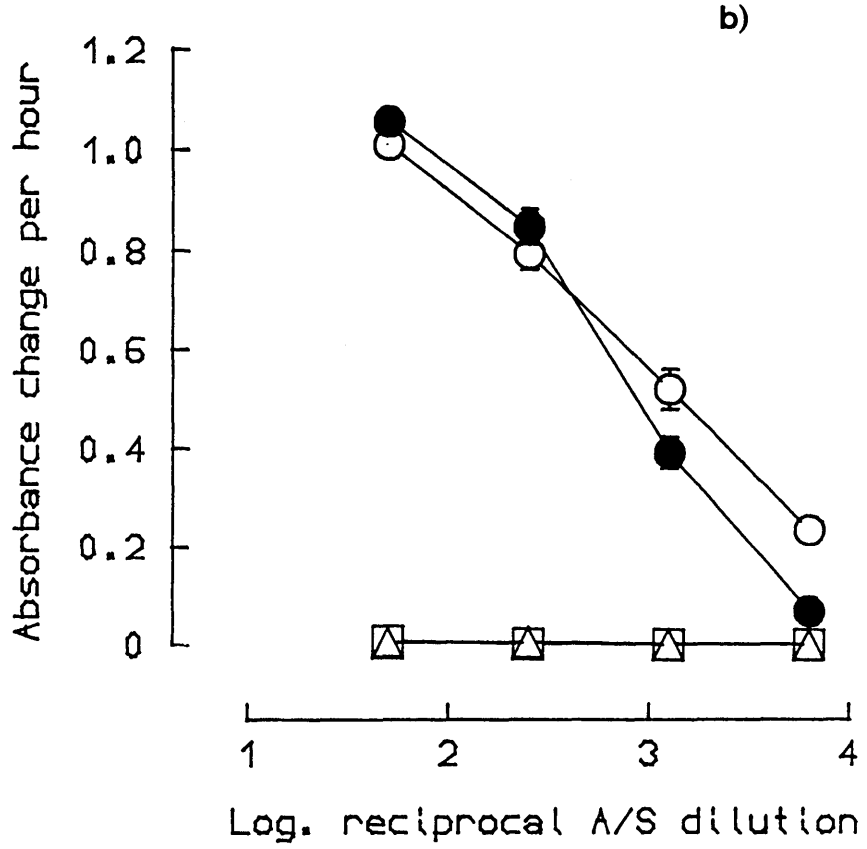

Anti-(APM) binding (% standard)
1000
800
600
400
200
0
0
4
8
12
16
Weeks after first immunisation

Fig. 5.4. The effects of different treatments and immunogens on the apparent antibody response to adipocyte plasma membranes

In (a) rats were immunised with either APM conjugated to BSA and Pertussis vaccine (filled squares), APM conjugated to BSA and 2'-deoxyguanosine (filled triangles) or APM conjugated to BSA only (filled circles). In (b) rats were immunised with either CFA (open squares), CFA and glutaraldehyde (open triangles), CFA and BSA (open circles) or CFA, BSA and glutaraldehyde (filled circles). Antibody binding to APM (prepared in the presence of BSA) was measured by ELISA, 6 weeks after the first immunisation. Results in (a) are means $\pm$ SEM of 8 observations while results in (b) are means $\pm$ SEM of 3 observations.


a)
Absorbance change per hour
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
Log. recip. A/S dilution


b)
Absorbance change per hour
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
Log. reciprocal A/S dilution

homogenisation of adipose tissue in the absence of BSA, were not significantly above those achieved by sera from untreated rats (Table 5.1). Binding to BSA alone by sera from the same actively immunised rats was only about 10% of that to APM prepared in the presence of BSA, as determined by ELISA.

Pre-incubation with BSA of serum from rats immunised with BSA, APM or ASA (both conjugated to BSA) or anti-(ASA) antibody reduced the binding to APM, prepared in the presence of BSA, and to BSA alone to a level such that the absorbances achieved in ELISA were not significantly greater than those of serum from untreated rats (Table 5.2). Pre-incubation with casein caused some reduction of binding to APM but the absorbances achieved were still significantly greater than those achieved using untreated rat serum (Table 5.2).

Pre-incubation with sheep γ globulin of serum from rats immunised with BSA, APM, ASA or anti-(ASA) antibody reduced the binding to ASA and sheep γ globulin to a level such that the absorbances achieved in ELISA were not significantly greater than those of untreated rat serum (Table 5.3). Again pre-incubation with casein caused some reduction of binding to ASA and sheep γ globulin but the absorbances achieved were still significantly greater than those achieved with untreated rat serum (Table 5.3).

5.3.2 The tissue specificity of antibody responses of actively immunised rats

Antibody binding to APM (prepared in the presence of BSA) of serum from rats immunised with APM or BSA alone was more than 100-fold greater than that of serum from rats immunised with anti-(APM) antibody (Fig. 5.5). However, antibody binding to HPM of

Table 5.1. The role of anti-(BSA) responses in apparent anti-(adipocyte plasma membrane) responses of actively immunised rats

Antigen	Relative binding of serum from rats immunised with APM in presence of BSA as % of anti-(APM in presence of BSA) response
BSA	10
APM in absence of BSA	0
APM in presence of BSA	100

Antibody binding to BSA, APM prepared by homogenisation of adipose tissue in the absence of BSA and APM prepared from cells isolated by collagenase digestion of adipose tissue in the presence of BSA in serum of rats from Experiment 3, immunised with APM conjugated to BSA, was measured by ELISA.

Table 5.2. The effects of preincubation with BSA and casein on anti-(BSA) and apparent anti-(adipocyte plasma membrane) antibodies in sera from actively immunised rats

Sera from rats immunised with BSA, APM conjugated to BSA, ASA conjugated to BSA or anti-(APM) antibody were preincubated with either PBS, casein or BSA before measuring antibody binding (AB binding) and antigen abundance (AG abund.) against BSA and APM prepared in the presence of BSA, by ELISA.

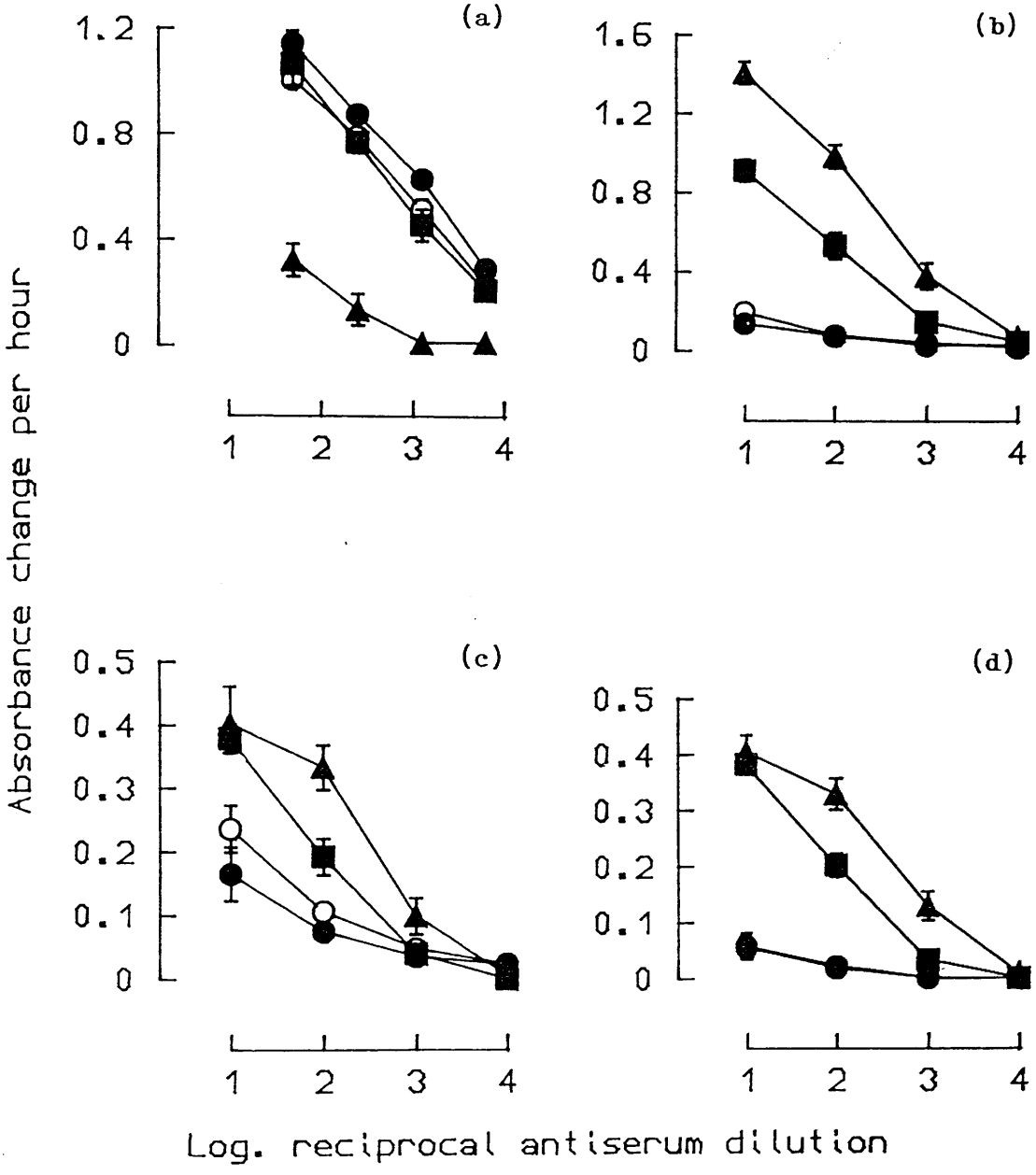
Pre-incubation	Antibody activity against BSA						Antibody activity against APM					
	None (% of that induced by APM-BSA)		Casein (% of that of the same A/S after no preincubation)		BSA (% of that of the same A/S after no preincubation)		None (% of that induced by APM-BSA)		Casein (% of that of the same A/S after no preincubation)		BSA (% of that of the same A/S after no preincubation)	
Treatment	AB binding	AG abund.	AB binding	AG abund.	AB binding	AG abund.	AB binding	AG abund.	AB binding	AG abund.	AB binding	AG abund.
BSA	398	109	13	160	0	0	316	114	13	121	0	3
APM-BSA	100	100	40	153	0	0	100	100	20	95	0	3
ASA-BSA	251	153	13	115	0	0	158	108	13	94	0	13
Anti-(APM) AB	25	63	0	7	0	0	13	69	6	12	0	17

Table 5.3. The effects of preincubation with sheep IgG and casein on anti-(sheep IgG) and apparent anti-(adipocyte specific antigen) antibodies

Sera from rats immunised with BSA, APM conjugated to BSA, ASA conjugated to BSA or anti-(APM) antibody were preincubated with either PBS, casein or sheep IgG before measuring antibody binding (AB binding) and antigen abundance (AG abund.) against sheep IgG and ASA, by ELISA.

Pre-incubation	Antibody activity against sheep IgG						Antibody activity against ASA					
	None (% of that induced by ASA-BSA)		Casein (% of that of the same A/S after no preincubation		Sheep IgG (% of that of the same A/S after no preincubation		None (% of that induced by ASA-BSA)		Casein (% of that of the same A/S after no preincubation		Sheep IgG (% of that of the same A/S after no preincubation	
Treatment	AB binding	AG abund.	AB binding	AG abund.	AB binding	AG abund.	AB binding	AG abund.	AB binding	AG abund.	AB binding	AG abund.
BSA	0	5	0	50	0	114	2	16	0	0	0	89
APM-BSA	0	9	0	165	0	45	0	6	0	29	0	157
ASA-BSA	100	100	63	102	0	16	100	100	25	86	2	16
Anti-(APM) AB	158	104	63	106	0	5	13	55	40	105	2	18

Fig. 5.5. The tissue specificity of antibody responses of actively immunised rats



Antibody binding of sera from rats immunised with BSA (open circles), APM conjugated to BSA (filled circles), ASA conjugated to BSA (filled squares) and anti-(APM) antibodies (filled triangles) to APM (prepared in the presence of BSA) (a), HPM (b), KPM (c) and EPM (d) was measured by ELISA. Results are mean ± SEM of 8 observations.

serum from rats immunised with APM or BSA was more than 100-fold less than that of serum from rats immunised with ASA or anti-(ASA) antibody. Antibody binding to KPM and EPM was also greater by serum from rats immunised with ASA or anti-(ASA) antibody than by serum from rats immunised with APM or BSA (Fig. 5.5).

5.3.3 The effects of active immunisation on adipose tissue

Immunisation of rats with APM conjugated to BSA caused a significant reduction in mass of peri-renal, omental and inguinal subcutaneous adipose tissue, compared with BSA-immunised controls in Experiment 1 (Table 5.4). While the mass of virtually all measured adipose depots was also reduced in APM compared with BSA-immunised rats in Experiments 2 and 3, these reductions were not significant. In Experiment 2, totally untreated controls, in general, showed lower fat pad weights than APM- or BSA-immunised rats. Mean adipocyte volumes of parametrial and subcutaneous depots of rats immunised with APM, in Experiments 2 and 3, were elevated compared with those of BSA- and untreated controls but these differences were not significant (Table 5.5). Adipocyte numbers of these depots were reduced in APM-treated rats and these differences were significant in Experiment 3.

Rats immunised with APM and also treated with 2'-deoxyguanosine or Pertussis vaccine showed similar fat pad weights, mean adipocyte volumes and adipocyte numbers as those immunised with APM alone in Experiment 3 (Table 5.6). Rats immunised with anti-(ASA) antibody showed elevated weights and mean adipocyte volumes of internal fat depots, compared with controls, but these differences were not significant (Table 5.6). Rats immunised with ASA also showed

Table 5.4. The effects of immunisation with BSA and adipocyte plasma membranes on fat pad weight

Treatment	Number of observations	Dry weight fat pad (g)											
		Parametrial			Peri-renal			Omental			Inguinal subcutaneous		
		Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3
None	4–8		5.38 ±0.66	8.55 ±0.81		2.51 ±0.50	4.24 ±0.56		2.73 ±0.56	5.30 ±0.65		3.53 ±0.52	5.67 ±0.74
BSA	4–8	12.17 ±1.29	7.34 ±1.07	8.21 ±0.72	6.07 ±0.82	3.31 ±0.20	4.10 ±0.51	6.89 ±0.92	3.45 ±0.34	5.34 ±0.64	6.50 ±0.99	3.64 ±0.28	4.90 ±0.62
APM + BSA	4–8	9.60 ±0.76	6.77 ±0.61	7.74 ±0.62	4.78* ±0.65	2.86 ±0.34	4.11 ±0.52	4.74* ±0.70	3.03 ±0.29	4.47 ±0.69	4.74* ±0.39	3.37 ±0.15	3.99 ±0.31

Rats were immunised with either BSA or APM conjugated to BSA or were untreated. The dry weights of fat pads were determined 16 weeks after the first immunisation. *$P<0.01$, compared with BSA-immunised controls (Student's paired t test, pairing littermates).

Table 5.5. The effects of immunisation with BSA and adipocyte plasma membranes on adipocyte volume and number

Treatment	Number of observations	Dry weight fat pad (g)				Mean adipocyte volume (pl)				Adipocyte numbers ($x10^6$)			
		Parametrial		Subcutaneous		Parametrial		Subcutaneous		Parametrial		Subcutaneous	
		Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3	Expt. 2	Expt. 3
None	4–8	5.38 ±0.60	8.55 ±0.81	3.53 ±0.52	5.67 ±0.74	329 ±38	646 ±77	114 ±6	177 ±30	14.8 ±3.5	12.7 ±1.8	29.1 ±5.5	31.9 ±5.0
BSA	4–8	7.34 ±1.07	8.21 ±0.72	3.64 ±0.28	4.90 ±0.62	264 ±50	684 ±101	112 ±25	159 ±20	14.6 ±3.7	11.6 ±1.2	35.7 ±9.1	28.6 ±2.7
APM + BSA	4–8	6.77 ±0.61	7.74 ±0.62	3.37 ±0.15	3.99 ±0.31	391 ±28	913 ±101	114 ±10	267 ±37	8.0 ±0.6	7.9* ±0.6	27.2 ±1.8	15.6* ±3.0

Rats were treated as for Table 5.4. *$P<0.01$, compared with BSA-immunised controls (Student's unpaired t-test).

Table 5.6. The effects of immunisation with adipocyte plasma membranes and Pertussis vaccine and 2'-deoxyguanosine, adipocyte specific antigens and anti-(adipocyte plasma membrane) antibody on fat pad weight, adipocyte volume and adipocyte numbers

Rats were immunised with either APM or ASA conjugated to BSA or anti-(APM) antibody or were untreated. Some APM-immunised rats also received Pertussis vaccine or 2' deoxyguanosine as described in Section 5.2. The dry weights of fat pads, mean adipocyte volumes and adipocyte numbers were determined 16 weeks after the first immunisation. *$P<0.01$, **$P<0.005$, compared with controls (Student's unpaired t test).

Treatment	Number of observations	Dry weight fat pad (g)			Mean adipocyte vlume (pl)			Adipocyte numbers ($x10^6$)		
		Para-metrial	Peri-renal	Sub-cutaneous	Para-metrial	Peri-renal	Sub-cutaneous	Para-metrial	Peri-renal	Sub-cutaneous
None	8	8.55 ±0.81	4.24 ±0.56	5.67 ±0.74	646 ±77	457 ±61	177 ±30	12.7 ±1.8	8.8 ±0.7	31.9 ±5.0
APM - BSA	8	7.74 ±0.62	4.11 ±0.52	3.99 ±0.31	913 ±101	736* ±84	267 ±37	7.9 ±0.6	5.2 ±0.5	15.6 ±3.0
APM - BSA + Pertussis	8	7.42 ±0.99	3.34 ±0.38	4.14 ±0.42	701 ±89	573 ±64	233 ±189	9.6 ±0.6	5.5** ±0.6	16.2* ±1.0
APM - BSA + 2'-dGuo	8	8.83 ±1.07	4.08 ±0.56	4.96 ±0.73	923 ±185	726* ±86	244 ±43	9.5 ±1.0	5.2* ±0.4	20.9 ±4.3
ASA - BSA	8	11.08 ±1.36	4.97 ±0.42	5.76 ±0.90	1062* ±143	677** ±26	234 ±20	9.6 ±0.4	6.7 ±0.6	22.6 ±2.7
Anti-(APM) antibody	8	11.17 ±.1.58	5.27 ±0.35	5.45 ±0.78	823 ±139	543 ±68	180 ±42	13.1 ±1.6	9.3 ±0.9	30.9 ±3.4

elevated fat pad weights and significantly elevated mean adipocyte volumes and reduced adipocyte numbers of internal fat depots, compared with controls.

5.3.4 Effects of active immunisation on body weight gain, food intake and food conversion efficiency

In Experiment 1, rats immunised with APM showed a reduced body weight gain compared with BSA-immunised controls, which became significant by 10 weeks after the first treatment (Fig. 5.6). In Experiment 2, rats immunised with APM and totally untreated controls showed a reduced body weight gain compared with BSA-immunised controls between 5 and 6 weeks after the first treatment. There were no significant differences in body weight gain between any of the treatment groups of Experiment 3.

In Experiment 3 food intakes were measured, allowing food conversion efficiency, as defined by food intake divided by body weight gain, to be determined (Table 5.7). In general, APM-treated rats had a slightly lower food intake and a similar body weight gain compared with untreated controls, resulting in a slightly increased food conversion efficiency. BSA-treated rats had a similar food intake to controls but gained slightly more weight which resulted in a similar food conversion efficiency to APM-treated rats. There was little difference between the food intakes or food conversion efficiencies of rats immunised with anti-(ASA) antibody, ASA, or APM with 2'-deoxyguanosine or Pertussis vaccine when compared with untreated controls.

Fig. 5.6. Body weight gain in actively immunised rats

Rats were immunised with either BSA (open circles), APM conjugated to BSA (filled circles) or were untreated (filled squares). The results of experiments 1, 2 and 3 are shown in (a), (b) and (c) respectively. *$P<0.01$ compared with BSA-immunised controls (Student's paired t test). *$P<0.01$ compared with BSA-immunised controls (Student's unpaired t test).

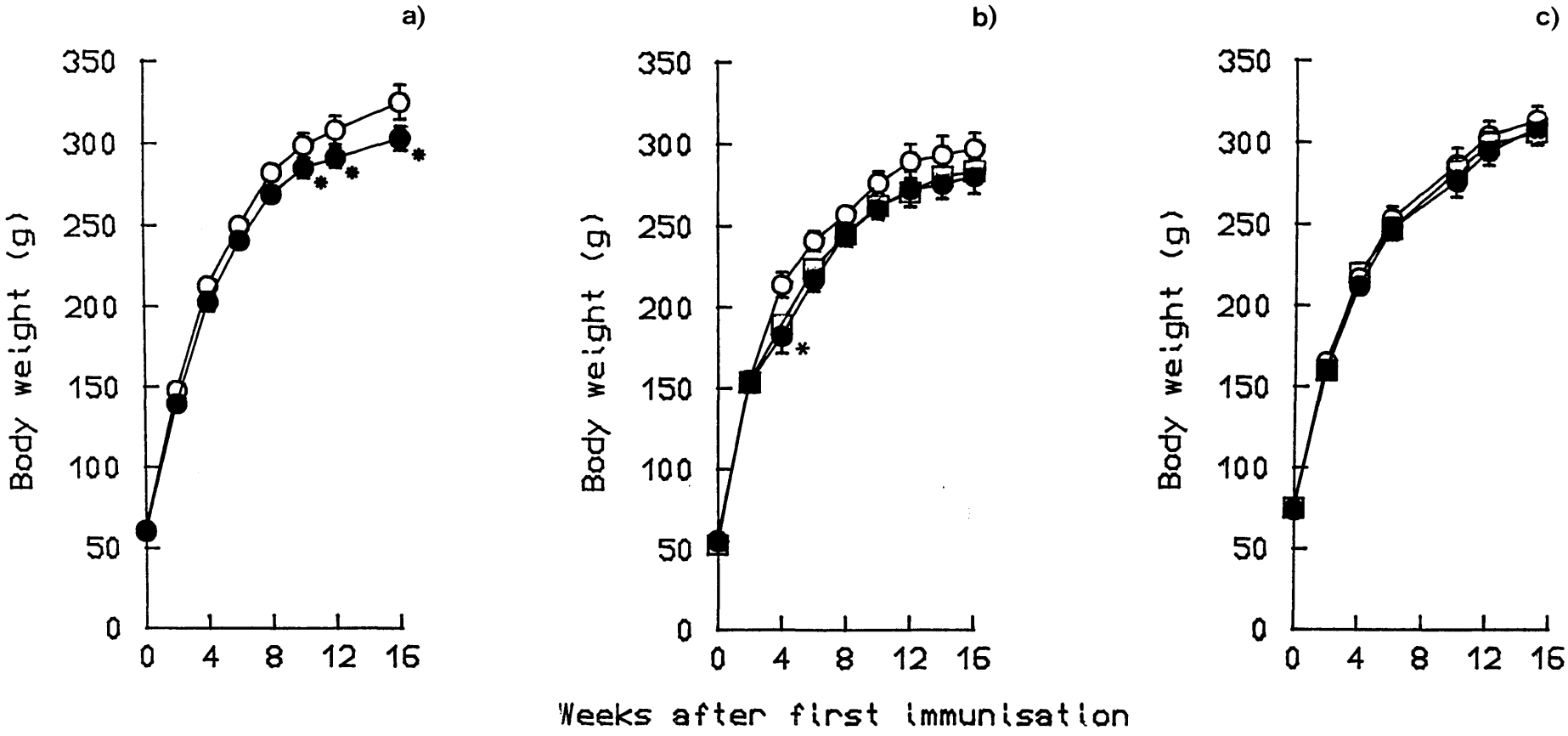

a)
b)
c)
Body weight (g)
350
300
250
200
150
100
50
0
0
4
8
12
16
Weeks after first immunisation

Table 5.7. The effects of active immunisation on body weight gain, food intake and food conversion efficiency

	Treatment	Number of observations	Weeks after first immunisation					
			0–3	4–6	7–9	10–12	13–15	0–16
Weight gain (g/rat/21 days)	None	8	111	61	26	25	8	234
	BSA	8	115	63	25	26	10	245
	BSA – APM	8	111	61	23	24	14	240
Food intake (g/rat/21 days)	None	8	379	424	417	434	428	2345
	BSA	8	386	424	414	427	423	2360
	BSA – APM	8	365	403	413	407	423	2279
$\frac{\text{Intake}}{\text{Gain}}$	None	8	3.4	6.9	16.0	17.4	53.6	10.0
	BSA	8	3.4	6.7	16.5	16.4	42.6	9.6
	BSA – APM	8	3.3	6.6	18.0	17.0	30.2	9.5

Rats were treated as for Table 5.4. The results were obtained from Experiment 3.

5.4 DISCUSSION

5.4.1 Apparent antibody responses to adipocyte plasma membranes in actively immunised rats

The detection of apparent anti-(APM) antibodies in rats immunised with BSA alone immediately raised the question of contamination of the APM preparation with BSA. In order to test this, the same sera were tested, by ELISA, against APM, prepared from adipocytes isolated in the presence of gelatin, rather than BSA. As the sera bound to APM, prepared in the absence of BSA (results not shown), it was assumed that immunisation with BSA induced a genuine anti-(APM) response. Such a response could be induced by the injection of a powerful non-specific stimulator of the immune system, Freunds adjuvant, into an adipose depot, or by BSA acting as a carrier *in situ*, stimulating T helper cells to provide 'help' for autoreactive B cells. In subsequent experiments, a totally untreated group was always included.

In the third active immunisation experiment, the cause of the apparent anti-(APM) antibodies in BSA-immunised rats was examined in more detail and found to be due to immunisation with BSA in Fruends adjuvant, rather than Freunds adjuvant or glutaraldehyde alone (Fig. 5.4). This, coupled with the discovery of contamination of APM with BSA, as indicated by SDS PAGE (see 4.9), prompted a re-examination of the role of anti-(BSA) responses in apparent anti-(APM) responses.

Rats immunised with BSA, APM or ASA showed little or no binding to APM prepared from homogenised whole adipose tissue (Table 5.1). Binding to APM, prepared in the presence of BSA, by the same sera could be completely removed by pre-incubation of the sera with BSA

(Table 5.2). These results indicate that little or no genuine anti-(APM) responses were induced in these rats. Gelatin is formed by hydrolysis of collagen. Therefore, apparent anti-(APM) responses, measured against APM prepared in the presence of gelatin, may have represented anti-(collagen) responses or responses to its degradation products. Measurement of antibody binding to BSA of APM-treated rats by ELISA did not show as much binding as would be expected if all the apparent anti-(APM) response measured against APM, prepared in the presence of BSA, were due to anti-(BSA) antibodies (Table 5.1). Since BSA is a soluble protein, it may not have adsorbed as efficiently to PVC ELISA plates as the insoluble APM. Time did not permit the testing of this possibility using poly-L-lysine to promote the adsorption of BSA to the plates. Alternatively, the rats may have responded to neo-antigens on the APM, formed by collagenase digestion of adipose tissue or to epitopes formed by the combination of BSA with APM.

5.4.2 The tissue specificity of antibody responses in actively immunised rats

Antisera, raised in rats by immunisation with whole APM conjugated to BSA, showed very little cross-reaction with non-adipose tissues while antisera, raised by immunisation with ASA conjugated to BSA, showed considerable cross-reaction with HPM, KPM and EPM (Fig. 5.5). This is not easy to explain since, while the ASA preparation probably contained non-adipocyte specific antigens, this would not explain why immunisation with ASA would induce a less adipocyte specific response than immunisation with whole APM. It is possible that a non-adipocyte specific antigen(s) was co-purified with a

specific antigen due to incomplete solubilization of the APM used to prepare the ASA. If the non-specific antigen were present at a very low frequency on the APM but was concentrated in the ASA preparation, a greater non-specific immune response might be elicited by immunisation with ASA than by immunistion with APM. The ASA preparation was clearly contaminated with sheep IgG. Since pre-incubation with sheep γ globulin removed virtually all the binding to ASA of serum from rats immunised with ASA, sheep Ig G was the major immunogenic component of the ASA preparation in rats (Table 5.3). Anti-(sheep IgG) antibodies are likely to cross-react with rat IgG. If the HPM, KPM and EPM preparations showed greater contamination with rat IgG (present in serum) than the APM preparation, this could account for the apparent cross-reaction of antisera, raised gainst ASA, with these tissues. There is no evidence, however, to suggest that this is the case. Alternatively, sheep IgG and rat HPM, KPM and EPM, may share common epitopes, although this, again, seems unlikely. However, the fact that rats immunised with sheep anti-(ASA) antibodies alone showed even more binding to HPM, KPM and EPM than those immunised with ASA does suggest that the cross-reaction with non-adipose organs relates, in some way, to the anti-(sheep IgG) response.

5.4.3 Effects of active immunisation on adiposity, body weight gain and food intake

In Experiment 1, APM-immunised rats showed significant reductions in the dry weight of adipose depots compared with BSA-immunised controls (Table 5.4). The reduction in adiposity may have been antibody-mediated, despite the absence of differences in

apparent anti-(APM) antibody responses between APM and BSA-immunised rats (Fig. 5.2) and despite the absence of a convincing demonstration of true anti-(APM) antibodies in either group of rats. Only circulating antibodies were measured and anti-(APM) antibodies may have been sequestered in adipose tissue of APM-treated rats. Alternatively, the reduction in adiposity, seen in APM-immunised rats, may have been due to antibody-independent cell-mediated autoimmune reactions. In experimental autoimmune encephalomyelitis (McAllister et al., 1974) and experimental autoimmune synovitis (Goldberg et al., 1974), induced by the injection of autoantigens in Fruends adjuvant, T-cell mediated immune responses have been shown to play a greater part in the pathogenesis of the disease, despite the presence of autoantibodies. Delayed type hypersensitivity tests on 2 actively immunised rats showed some response to BSA but not to APM but alternative measures of in vivo T cell activation should be employed before conclusions can be drawn about the role of T cell responses in mediating the effects of active immunisation on adipose tissue.

In Experiment 2, the dry weights of none of the adipose depots measured in APM-immunised rats were reduced 16 weeks after the first treatment, compared with BSA-immunised controls (Table 5.4). However, adipocyte numbers of parametrial and subcutaneous adipose depots were reduced in APM-immunised rats, but this reduction was compensated for by an increase in adipocyte size (Table 5.5). The rats in Experiment 2 did not reach the body weight of those in Experiment 1 (Fig. 5.6). It is possible, therefore, that the maximum capacity for fat storage was reached in the APM-immunised rats of Experiment 1, while the rats of Experiment 2 did not reach a sufficiently high body weight to

reach their maximum capacity for fat storage. A reaching of the maximum capacity for fat storage must result in either a reduction in food intake, a repartitioning of nutrients into protein deposition or the formation of new adipocytes. Food intake was not measured in the first experiment but there was a reduction in body weight gain in APM-treated rats (Fig. 5.6). Therefore, a mechanism similar to that proposed by Faust et al (1977) to explain the reduction in food intake in lipectomised rats, fed a high fat diet, may have been operating. They found that rats whose adipocyte numbers had been reduced by lipectomy had a normal food intake, when fed a low fat diet, but the feeding of a prolonged high fat diet resulted in a reduction in food intake, compared with sham-operated controls. It was suggested that the fat storage capacity of lipectomised rats had been reduced such that, while there were sufficient numbers of adipocytes to accommodate fat deposition when fed a low fat diet, the feeding of a high fat diet caused an accelerated attainment of a maximum adipocyte size and, hence, a reduction in fat storage capacity. A feedback mechanism, whereby elevated adipocyte size in some way elicited a signal that caused a reduction in food intake, was proposed.

In Experiment 3, adipocyte numbers of parametrial and subcutaneous adipose tissue were significantly reduced in APM-treated rats, compared with BSA-treated and untreated controls (Table 5.5). There was, again, a compensatory increase in adipocyte size in APM-treated rats resulting in a slight, but not significant, reduction in adipose tissue mass. These animals had reached body weights similar to those attained by the APM-treated rats of Experiment 1 but there was no evidence of a reduction in body weight

gain. The reason for the difference in response to active immunisation in Experiments 1 and 3 is not clear. It is possible that, since the experiments were separated by about 18 months and fresh immunogens were prepared for each experiment, the APM used for immunisation differed in antigenic composition. Alternatively, genetic variation, induced by outbreeding of the Wistar rat colony, may have resulted in a different response to the same immunogen. The importance of a genetic component in the response to active immunisation was shown in Experiment 1 where littermates were used. While there was considerable overlap between APM- and BSA-treated rats, in terms of body weight gain and adiposity, when littermates were compared, all APM-treated rats showed reduced body weight gain and adiposity compared with their BSA-treated littermates.

Rats immunised with anti-(ASA) antibody were heavier and had a greater adipose tissue mass and mean adipocyte volume than BSA-treated or untreated controls and showed no reduction in adipocyte numbers (Table 5.6). None of these differences, however, were significant. A/S 83, at low doses, stimulated glucose incorporation into lipid (Flint et al., 1986). It is possible that the anti-(APM) response induced in antibody-immunised rats was of too low a titre or affinity to be cytotoxic but had stimulatory effects on lipid deposition. It would be of interest to examine the effects of sera from actively immunised rats on glucose incorporation into lipid in isolated adipocytes. The majority of the apparent anti-(APM) response of antibody-immunised rats could be removed by pre-incubation of the sera with BSA (Table 5.2). This was surprising as these rats were not deliberately immunised with BSA. The sheep anti-(ASA) antibodies, used for immunisation, may have been

contaminated with ovine albumin and induced the production of antibodies that cross-react with BSA. Alternatively, some of the anti-(APM) antibodies may have been directed against rat albumin (a possible contaminant of the rat APM) and activation of the idiotype network may have resulted in the production of anti-(rat albumin) antibodies that cross-react with BSA.

5.5 CONCLUSIONS

1. Despite the absence of a convincing demonstration of circulating anti-(APM) antibodies, rats immunised with APM conjugated to BSA in Freunds adjuvant showed significantly reduced adipocyte numbers, compared with BSA-immunised controls. This reduction may have been due to anti-(APM) antibodies sequestered in adipose tissue, or to T-cell mediated immune responses.
2. In Experiment 1, the APM-immunised rats showed reduced fat pad weights and body weights, suggesting that a critical adipocyte size had been reached, resulting in a reduction in food intake, fat deposition and body weight gain. However, in 2 out of 3 experiments, loss of adipocyte numbers was compensated for by enlargement of remaining adipocytes, so that total dissected fat mass was the same as in control rats, suggesting that total fat mass rather than adipocyte size is regulated.
3. The differences in the response of different groups of rats to the same treatment emphasizes the influence of variation in individual's immune response on the results of active immunisation, which may limit the use of this approach commercially.

4. Immunisation of rats with ASA apparently induced antibodies showing a higher degree of cross-reactivity with non-adipose tissues than did immunisation with APM. As the ASA was contaminated with sheep IgG and immunisation with affinity-purified sheep anti-(ASA) antibodies also induced an antibody response showing a high degree of cross-reactivity with non-adipose tissues, this cross-reactivity may have related, in some way, to an anti-(sheep IgG) response.

CHAPTER 6

PRELIMINARY ATTEMPTS TO EXTEND THE USE OF PASSIVE AND ACTIVE IMMUNISATION AGAINST THE ADIPOCYTE TO SPECIES OTHER THAN THE RAT

PRELIMINARY ATTEMPTS TO EXTEND THE USE OF PASSIVE AND ACTIVE IMMUNISATION AGAINST THE ADIPOCYTE TO SPECIES OTHER THAN THE RAT

6.1 INTRODUCTION

Passive immunisation of meat-producing animals, on a large scale, with antisera raised against adipocyte plasma membranes would probably only be practical for the treatment of avian species. For the treatment of larger species or humans, active immunisation or passive immunisation with monoclonal antibodies linked to a cytotoxic agent may be required. However, the most convincing demonstration of a reduction in adiposity, in the rat, was achieved by passive immunisation with polyclonal antisera raised against whole APM. Therefore, in order to determine whether a reduction in body fat in species other than the rat was possible, by an immunological approach, antisera were raised against chicken, pig and sheep APM. The work described in this Chapter involves the *in vitro* characterisation of these antisera and their use to prepare adipocyte specific antigens. The antisera and ASA were compared with antisera raised against rat APM and rat ASA with 3 main aims. The first was to determine whether high titre antisera could be generated against APM of other species, the second was to determine whether, like rat APM, the APM of other species were composed of both adipocyte specific and non-adipocyte specific antigenic components. The final aim was to investigate the species cross-reactivity between APM antigens and hence the possibility of using the same antiserum to treat animals of different species.

The majority of the work described in this thesis describes efforts to characterise the effects of passive and active immunisation against the adipocyte in the rat, as it was hoped that the rat would prove a useful model for the effects of similar approaches in other species. In order to give some indication as to whether this was the case, active immunisation experiments were performed with lambs, based on those performed in the rat. Preliminary investigations at the Hannah Research Institute suggested that conjugation of rabbit serum albumin to sheep APM provided an immunogen that stimulated the production of anti-(sheep APM) antibodies of a similar titre to those induced by a similar approach in the rat. Rabbit, rather than bovine albumin, was chosen as it was thought to show less antigenic similarity to ovine albumin. The first experiment was set up, in collaboration with Dr. S. Rhind at Hill Farming Research Organisation (Penicuik, Midlothian), using the approach described above. When sheep ASA became available, a second experiment was performed using this more highly purified immunogen.

6.2 A COMPARISON OF ANTISERA RAISED AGAINST ADIPOCYTE PLASMA MEMBRANES OF DIFFERENT SPECIES

6.2.1 Experimental

The following antisera were raised against whole APM, either at the Hannah Research Institute or by S. Kestin at the Institute of Food Research (Bristol Laboratory), according to the method described in 2.4, with the exception of the goat anti-(sheep APM) antiserum. Since it was likely that sheep and goat APM were antigenically similar, the sheep APM were conjugated to rabbit serum albumin, by

the method described in Section 5.2.1, for the first 3 immunisations but were not conjugated for subsequent boosts.

ANTISERUM	SPECIES OF ANTISERUM DONOR	SPECIES OF IMMUNOGEN
A/S 83	Sheep	Rat
A/S 642	Sheep	Chicken
A/S 816	Sheep	Pig
A/S Perky	Goat	Sheep

The antisera were adsorbed with liver homogenate of the same species as that from which the immunogen was prepared, according to the method described in Section 4.4.1. Binding of adsorbed and unadsorbed antisera to APM (prepared in the absence of BSA) and HPM of rats, chickens, pigs and sheep was measured by ELISA.

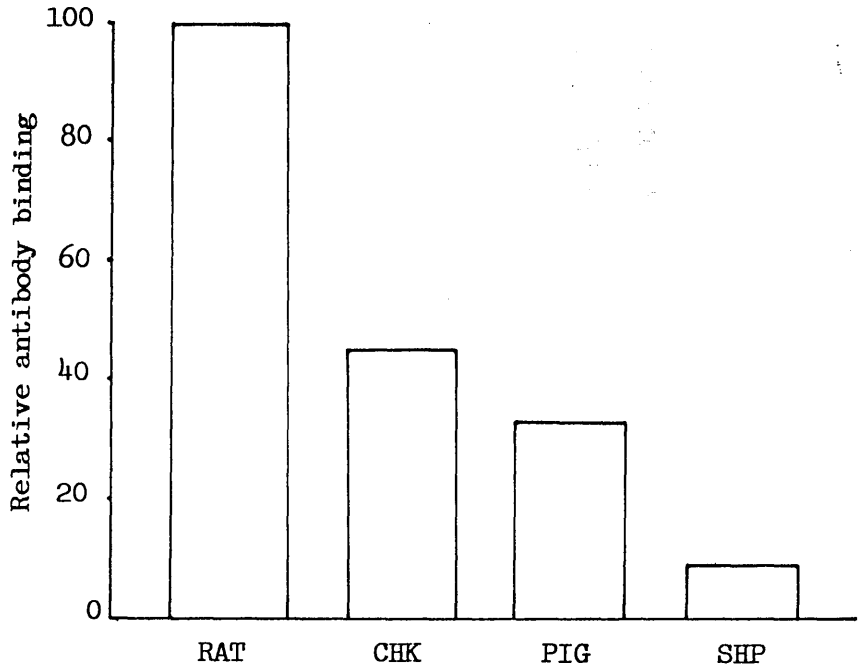
6.2.2 Results

Antisera raised against chicken, pig and sheep APM showed 45, 33 and 9% of the relative antibody binding to homologous APM, compared with A/S 83 (anti-(rat APM)) and about 70% of the relative antigen abundance against homologous APM, compared with A/S 83, as measured by ELISA (Fig. 6.1).

Antisera raised against chicken, pig and sheep APM showed less cross-reactivity with homologous HPM than A/S 83 but, after adsorption with homologous liver homogenate, A/S 83 showed slightly less cross-reactivity with HPM than the other adsorbed antisera (Table 6.1). Adsorption with liver homogenate removed about 60% of the antibody binding to homologous APM of anti-(rat APM), anti-(sheep APM) and anti-(pig APM) antisera and about 75% of that of the anti-(chicken APM) antiserum.

Fig. 6.1. Antibody binding to homologous adipocyte plasma membranes of antisera raised against adipocyte plasma membranes of different species, as determined by ELISA

Antibody binding to APM of the same species as those against which the antiserum was raised, by antisera raised against rat APM (RAT), chicken APM (CHK), pig APM (PIG) and sheep APM (SHP), was measured by ELISA. The APM used for screening were prepared in the absence of BSA. Relative antibody (AB) binding and antigen (AG) abundances were determined, as described in Section 2.6.3.


Relative antibody binding
100
80
60
40
20
0
RAT
CHK
PIG
SHP

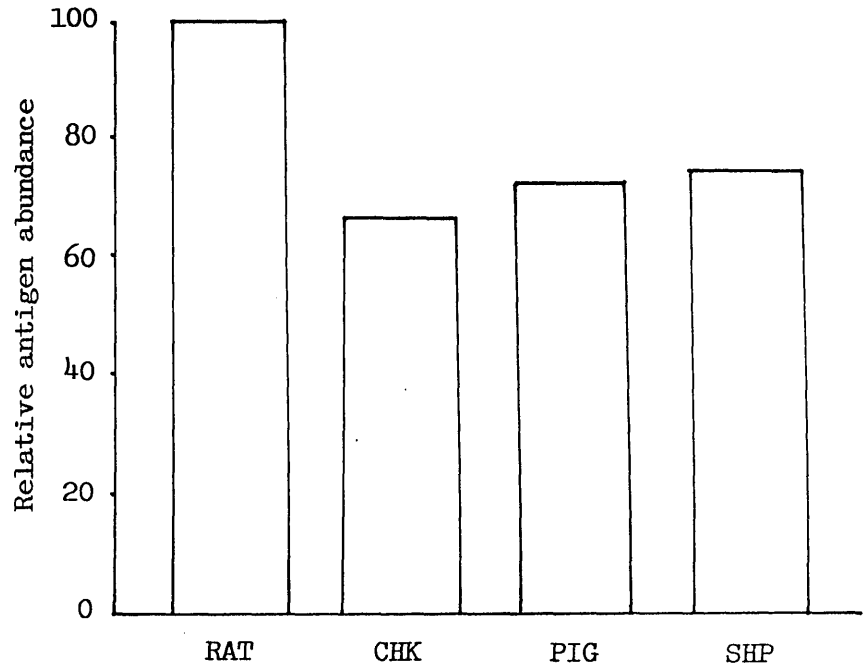

Relative antigen abundance
100
80
60
40
20
0
RAT
CHK
PIG
SHP

Table 6.1. The effects of adsorption with liver homogenate on the specificity of antisera raised against adipocyte plasma membranes of different species, as determined by ELISA

Unadsorbed antisera were adsorbed with packed liver homogenate (1 vol. of serum : 1 vol. of packed liver) for 16 h at 4° C. Relative antibody binding and antigen abundances were calculated as described in Section 2.6.3. Relative APM specificity was calculated by dividing binding to APM by binding to HPM.

	Antiserum	Binding to APM		Binding to HPM			Relative APM specificity	
		Unadsorbed	Adsorbed (% of unadsorbed)	Unadsorbed (% of anti-(APM))	Adsorbed (% of unadsorbed)	Adsorbed (% of anti-(APM) of unadsorbed)	Unadsorbed	Adsorbed
Antibody binding	Anti-(rat APM)	100	37	32	3	1	3.1	37.0
	Anti-(chicken APM)	100	26	14	13	2	7.1	13.0
	Anti-(pig APM)	100	38	10	25	3	10.0	12.7
	Anti-(sheep APM)	100	40	7	34	2	14.3	20.0
Antigen abundance	Anti-(rat APM)	100	71	100	22	22	1.0	3.3
	Anti-(chicken APM)	100	90	74	58	43	1.4	2.1
	Anti-(pig APM)	100	100	54	18	10	1.9	10.0
	Anti-(sheep APM)	100	85	78	77	60	1.3	1.4

6.2.3 Discussion

The antibody activity against homologous APM of antisera, raised against chicken, pig and sheep APM, were not as high as those of A/S 83. However, the relative antibody binding and antigen abundances of antisera, raised against rat, chicken and pig APM, were all of the same order of magnitude. The antisera described here were obtained from the best responders of only 2 or 3 animals and so it is not possible to draw firm conclusions from this data about the antigenicity of APM of different species. Antigenic similarities between sheep and goat APM may have contributed towards the rather poor response of a goat to sheep APM.

A/S 83 showed more than twice as much binding to homologous HPM as did antisera raised against sheep, pig and chicken APM. However, adsorption of A/S 83 with homologous liver homogenate removed a greater proportion of the anti-(HPM) binding than did adsorption of the antisera raised against APM of other species. After adsorption A/S 83 showed greater relative adipocyte specificity, as defined by the ratio of APM to HPM binding, than the adsorbed anti-(pig, sheep and chicken APM) antisera. Adsorption with liver homogenate removed approximately 60% of the antibody binding to rat, pig and sheep APM, while removing about 75% of that of the anti-(chicken APM) antiserum, suggesting that chicken APM may bear a particularly high proportion of non-adipocyte specific antigens. The anti-(chicken APM) antiserum did not appear to have high anti-(HPM) activity before adsorption but anti-(HPM) activity was measured against purified HPM, while the antisera were adsorbed against a crude liver homogenate. The crude homogenate may have been contaminated with other tissues, particularly erythrocytes and connective tissue, so the antisera may

also have been adsorbed with non-hepatic tissues. Again, since single antisera were tested, it is not possible to draw conclusions about the relative proportions of antigens that cross-react with liver of the APM of different species.

6.2.4 Conclusions

1. High titre antisera can be generated against APM of chicken and pig APM, in addition to rat APM.
2. Antisera raised against rat, chicken, pig and sheep APM all cross-react with liver but the majority of this cross-reactivity can be removed by adsorption with liver homogenate of the species against which the antiserum was raised.

6.3 A COMPARISON OF ADIPOCYTE SPECIFIC ANTIGENS OF DIFFERENT SPECIES

6.3.1 Experimental

Rat, chicken and sheep ASA were prepared using the anti-(rat APM), anti-(chicken APM) and anti-(sheep APM) antisera described above, by the method described in Section 2.9. The antigens were characterised by SDS PAGE as described in Section 2.4.

6.3.2 Results

Table 6.2 summarizes the performance of affinity columns used to prepare the chicken, sheep and rat ASA. The maximum amount of anti-(APM) antibody that could be bound by the APM columns was greatest for the anti-(rat APM) antiserum, 3% of which could be bound

Table 6.2. The performance of affinity columns used for the preparation of adipocyte specific antigens of different species

The affinity columns were used to prepare adipocyte specific antigens, according to the method summarized in Fig. 2.2. IgG binding capacity of affinity columns was measured where that amount of antibody that gave maximum binding to the column was added. Purification factors were calculated by comparing the relative antibody binding to the column ligand per mg of antibody in added, bound and unbound fractions, by ELISA.

Species		Chicken	Sheep	Rat
ADIPOCYTE PLASMA MEMBRANE COLUMN				
IgG binding capacity	mg antibody bound per mg membrane protein on column	0.27	0.38	0.49
	mg antibody bound per mg added IgG	0.27	0.015	0.031
Purification factor (fold)	Anti-(APM) activity of bound IgG vs. initial IgG	5.0	4.4	3.4
NON-ADIPOCYTE PLASMA MEMBRANE COLUMN				
IgG binding capacity	mg antibody bound per mg membrane protein on column	0.030	0.027	0.064
	mg antibody bound per mg added IgG	0.20	0.23	0.33
Purification factor (fold)	Anti-(HPM) activity of unbound IgG vs. added IgG	<0.02	<0.02	<0.02
ADIPOCYTE SPECIFIC ANTIBODY COLUMN				
Membrane binding capacity	mg antigen bound per mg IgG on column	0.049	0.014	0.028
	mg antigen bound per mg added membrane protein	0.022	0.022	0.050

by the column and was least for the anti-(sheep APM) antiserum, 1.5% of which could be bound. When binding to APM by the antibody fraction, purified from the APM column, was compared with that of the original antiserum, anti-(APM) activity was found to be increased by 3.4 to 5 fold, when expressed per mg of protein. Adsorption of antibodies with HPM, KPM and EPM resulted in anti-(APM) antibodies of similar specificities for all 3 species. The adsorption removed 20, 23 and 33% of the anti-(APM) antibody fraction for the chicken, sheep and rat respectively. The specific anti-(rat APM) antibody column was able to bind 5% of the total solubilised rat APM protein while the specific anti-(chicken APM) and anti-(sheep APM) antibody columns were able to bind only 2.2% of solubilised chicken and sheep APM.

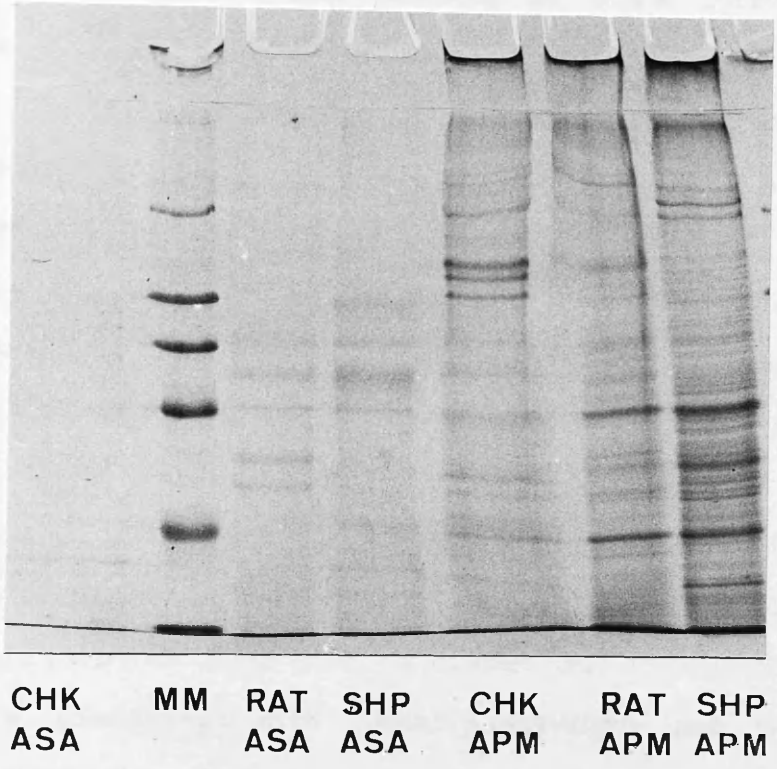
SDS PAGE showed that rat, sheep and chicken APM consisted of at least 19, 18 and 31 major polypeptides respectively (Fig. 6.3), while the ASA of chickens and rats consisted of 5 and 6 major polypeptides respectively, and that of sheep consisted of 7 major polypeptides with the following approximate molecular weights.

SPECIES	MOLECULAR WEIGHT (kD)						
Rat	96	88	72	59	53		
Sheep	112	96	85	72	44	36	30
Chicken	102	88	72	38	36		

The 59 kD protein of the rat ASA was a doublet

Each preparation also included a small number of minor polypeptide bands.

Fig. 6.3. Polyacrylamide gel electrophoresis of rat, sheep and chicken adipocyte plasma membranes and adipocyte specific antigens



Chicken (CHK), rat (RAT) and sheep (SHP) APM and ASA were prepared, as described in Section 2.9 and were separated by SDS PAGE and stained with Coomassie blue, as described in Section 2.10. The molecular weight markers (MM) were carbonic anhydrase, ovalbumin, BSA, phosphorylase 6, β galactosidase and myosin with molecular weights of 29, 45, 66, 97.4, 116 and 205 kD respectively.

6.3.3 Discussion

The APM columns of all 3 species, used for the affinity purification of anti-(APM) antibodies, did not bind as much antibody as would be expected for high titre antisera. Possible reasons for this were discussed in Section 4.3.3.2. Adsorption of the anti-(chicken APM) antiserum using the HPM, KPM and EPM affinity column did not remove as much antibody as did a corresponding adsorption of the anti-(rat APM) antiserum. This contrasts with the effects of adsorption of the whole antiserum with liver homogenate, which removed more anti-(APM) activity of the anti-(chicken APM) than the anti-(rat APM) antiserum. The HPM, KPM and EPM used for the preparation of the affinity column were purified plasma membrane preparations while, as discussed in Section 6.2.3, the liver homogenate may have included non-hepatic components, such as connective tissues.

The rat ASA apparently constituted more than twice as much of the total APM protein as the chicken and sheep ASA. However, this conclusion can only be drawn if it is assumed that the APM of all 3 species were solubilized with equal efficiency and that the anti-(APM) antibodies of the 3 species, used to prepare the affinity columns, were of equal affinity and, once bound to the immuno-absorbant, were equally able to bind antigen. There is no evidence that these conditions were satisfied and, hence, it is not possible to draw conclusions about the relative proportions of APM of the different species which comprised the ASA.

The ASA of the 3 species were all considerably enriched in certain polypeptides, compared with the whole APM. The sheep ASA appeared to contain more major components than the rat and chicken

ASA, although SDS PAGE of chicken ASA was difficult to interpret because, while apparently equivalent amounts of protein were loaded on the gel for the ASA of the 3 species, bands on the chicken ASA were difficult to visualise using Coomassie blue.

6.3.4 Conclusions

1. The affinity purification of APM antigens, using anti-(APM) antibodies from which more than 95% of the cross-reactivity with HPM, KPM and EPM had been removed, resulted in preparations considerably enriched in 5, 6 and 7 polypeptides for the chicken, rat and sheep respectively.

6.4 CROSS-REACTIVITY BETWEEN ADIPOCYTE PLASMA MEMBRANES AND ADIPOCYTE SPECIFIC ANTIGENS OF DIFFERENT SPECIES

6.4.1 Experimental

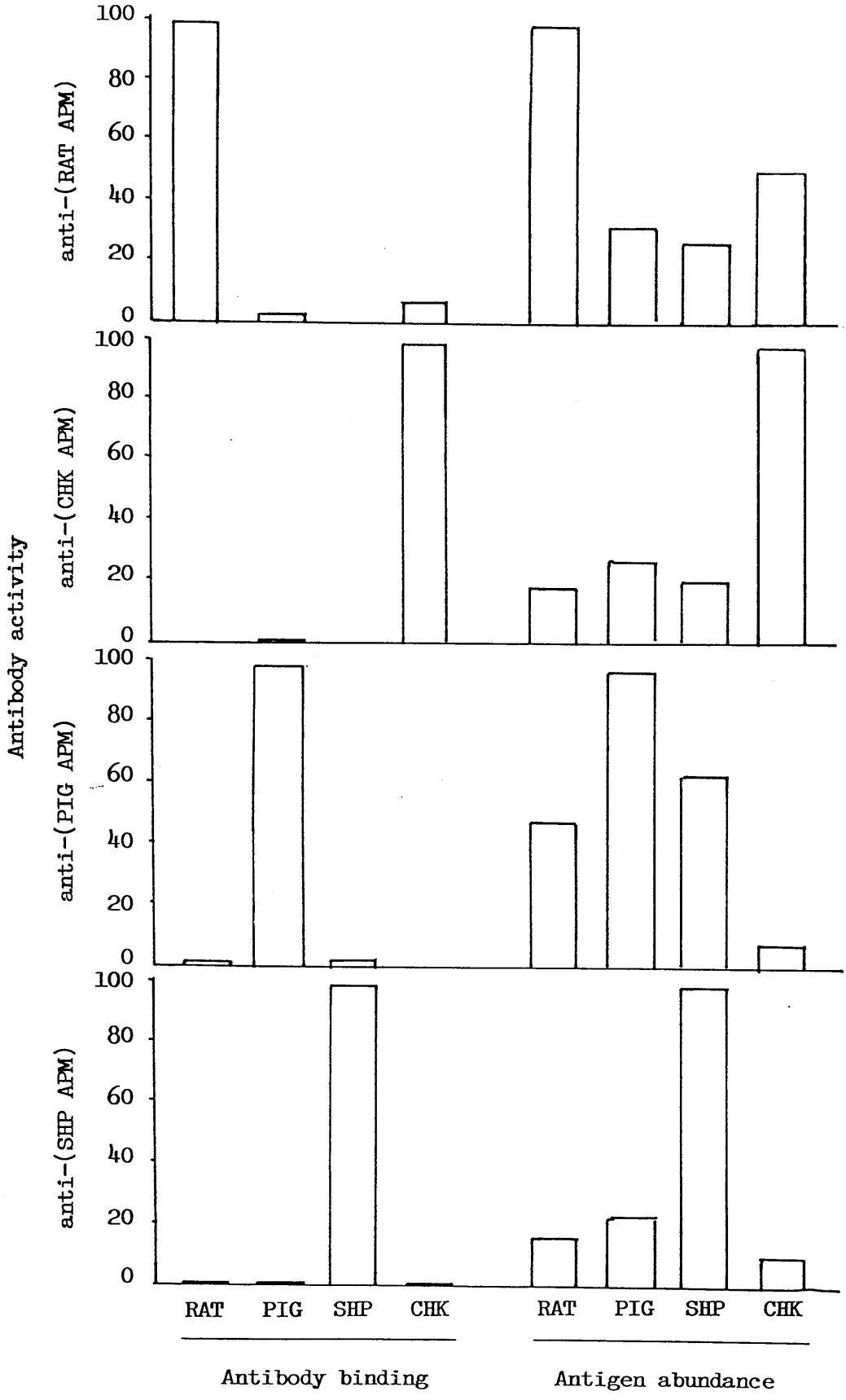
Binding of antisera raised against rat, chicken, sheep and pig APM to APM of the same and of the 3 different species was measured by ELISA. Binding to rat, sheep and chicken ASA of the anti-(rat APM) and the anti-(chicken APM) antiserum was measured by Western blotting. Satisfactory Western blots could not be obtained using the anti-(sheep APM) antiserum.

6.4.2 Results

All anti-(APM) antisera showed less than 5% of the antibody binding to heterologous compared with homologous APM. Relative antigen abundances against heterologous APM, however, ranged from 5 to 60% of those against homologous APM.

Fig. 6.2. Species cross-reactivity of antisera raised against adipocyte plasma membranes of different species, as determined by ELISA

Antibody activity against rat APM (RAT), pig APM (PIG), sheep APM (SHP) and chicken APM (CHK) by antisera raised against rat APM, pig APM, sheep APM and chicken APM was measured by ELISA. The APM used for screening were prepared in the absence of BSA. Relative antibody binding and antigen abundances were determined as described in Section 2.6.3 and are expressed as a percentage of that to homologous APM.


Antibody activity
anti-(RAT APM)
anti-(CHK APM)
anti-(PIG APM)
anti-(SHP APM)
100
80
60
40
20
0
RAT
PIG
SHP
CHK
Antibody binding
Antigen abundance

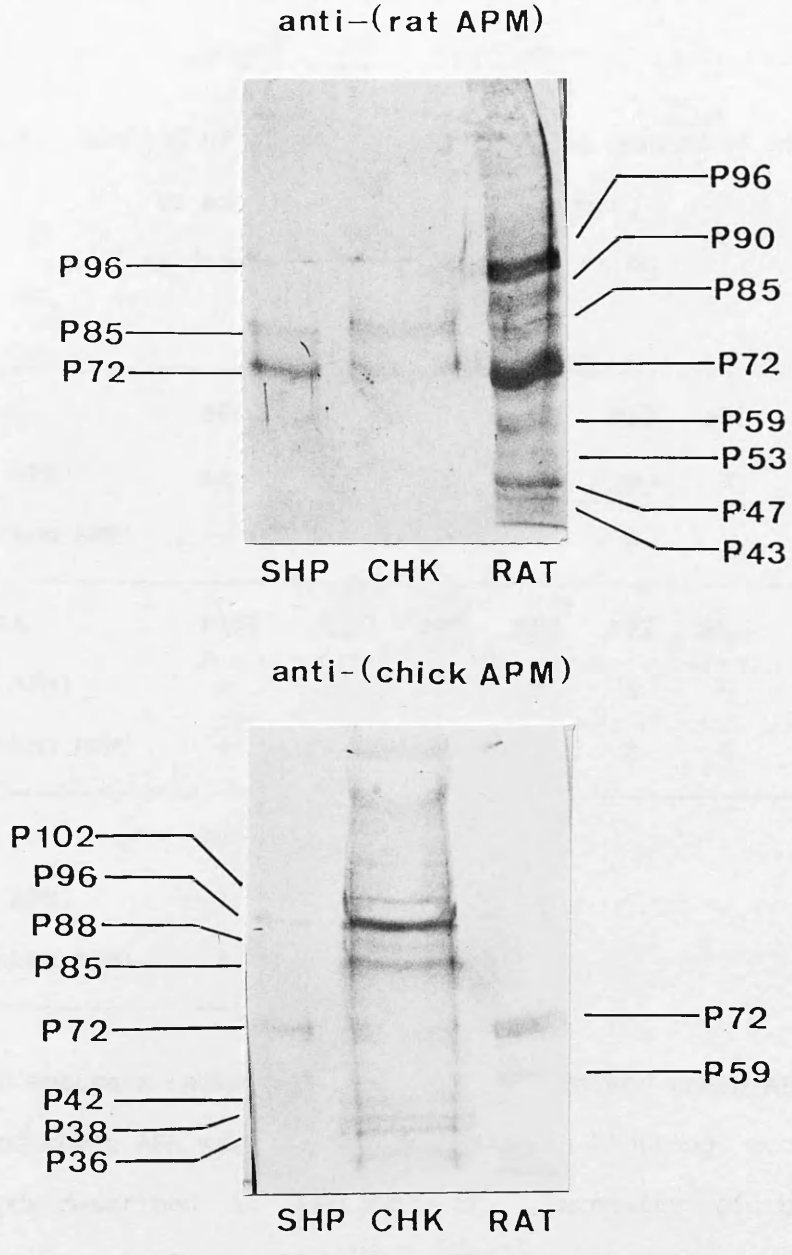
Difficulties experienced in the interpretation of Western blots were discussed in Section 4.3.3. Using the same scale as was used in that Section, the components of the ASA of the 3 species recognised by the anti-(rat APM) and the anti-(chicken APM) are shown in Table 6.3. The antisera bound more strongly and recognised more bands on homologous than heterologous ASA but both antisera recognised some bands on heterologous ASA. Proteins with approximate molecular weights of 96, 85 and 72 kD were recognised by the anti-(rat APM) antiserum in ASA of all 3 species while the 85 kD protein was not recognised by the anti-(chicken APM) antiserum in any of the ASA preparations.

6.4.3 Discussion

Despite the low antibody binding of all the anti-(APM) antisera tested against heterologous APM, the relative antigen abundances showed that there was some cross-reactivity between APM of different species. It is possible that antigens shared between species were present at a low frequency on the APM. Alternatively, minor changes in amino acid sequence of antigens of different species might result in antibodies having a lower affinity for antigens of a different species than for corresponding antigens of the species against which the antibodies were raised.

As was discussed in Section 4.3.3, the use of single antiserum dilutions for Western blotting does not allow conclusive determinations of whether or not a polypeptide is recognised by an antiserum. The Western blots did, however, provide evidence of cross-reactivity between ASA of different species and show additional polypeptides in the ASA preparations, not visible by Coomassie blue

Fig. 6.4. Species cross-reactivity of adipocyte specific antigens of different species, as determined by Western blotting



The binding to sheep (SHP), chicken (CHK) and rat (RAT) ASA by antisera raised against rat APM (A/S 83) and chicken APM (A/S 642) was measured by Western blotting, as described in Section 2.11. Polypeptide bands mentioned in the text have been indicated by P, followed by the approximate molecular weights in kD.

Table 6.3. Binding of anti-(adipocyte plasma membrane) antisera to adipocyte specific antigens, as determined by Western blotting

Rat ASA	P96	P90	P85	P72	P59	P53	P47	P43
Anti-(rat APM)	+++	+	+	+++	+	±	+	+
Anti-(chicken APM)	-	-	-	+	±	-	-	-
Chicken ASA	**P102**	**P96**	**P88**	**P85**	**P72**	**P42**	**P38**	**P36**
Anti-(rat APM)	-	±	-	+	±	-	-	-
Anti-(chicken APM)	+	+++	+	-	±	±	+	+
Sheep ASA	**P96**	**P85**	**P72**					
Anti-(rat APM)	±	±	++					
Anti-(chicken APM)	±	-	+					

Binding of antisera raised against rat, chicken and sheep APM to rat, chicken and sheep ASA was measured by Western blotting, according to the methods described in Section 2.11. Intensity of bands was assessed on a scale from - to +++, as described in Section 4.3.2. Bands are denoted by the letter P, followed by the approximate molecular weight (kD).

staining following SDS PAGE. Western blotting, using A/S 83, revealed 2 low molecular weight proteins in the rat ASA, not clearly visible by coomassie blue staining or on a previous Western blot using A/S 83 (Fig. 4.2). Since the same ASA preparation was used for both blots, differences in the activity of the anti-(sheep IgG) antiserum or the peroxidase-anti-peroxidase preparation, used for detection of antiserum binding, may have contributed towards these differences in sensitivity. Western blotting of the anti-(chicken APM) antiserum against chicken ASA showed clear binding of 5 major polypeptides, one of which had an approximate molecular weight of 96 kD and was not clearly visible by SDS PAGE. Western blotting also revealed at least 2 more minor components of the preparation. Despite the evidence of additional polypeptide components shown by Western blotting, when Western blots of antiserum binding to whole APM and ASA were compared, the ASA preparations still appeared to be considerably enriched in certain polypeptides.

A/S 83 (anti-(rat APM)) recognised a 72 kD protein (P72) strongly on the rat ASA and more weakly in the sheep and chicken ASA. The possibility was raised in Section 4.3.3 that P72 might be albumin. The presence of P72 in the chicken ASA suggests that this is not the case because ovalbumin has a molecular weight of 45 kD. Since the ASA was prepared from APM, isolated in the absence of BSA, the only possible source of a 72 kD albumin in the chicken ASA is ovine albumin which might have contaminated the sheep IgG used to prepare the affinity column, used for the isolation of the ASA. As described in Section 4.3.3, the rat ASA appeared to be contaminated with sheep IgG that had leached off the affinity column during the ASA preparation. While theoretically possible, significant

contamination of the sheep IgG with albumin seems unlikely, as the sheep IgG was affinity purified after ammonium sulphate precipitation. It is possible, therefore, that P72, in addition to P96 and P85, both of which were recognised by A/S 83 on the rat, chicken and sheep ASA preparations, were common to sheep, chicken and rat APM, or at least bore shared epitopes in the 3 species. Since A/S 83 bound less strongly to P72, P85 and P96 of the sheep and chicken ASA than to the rat ASA proteins, these proteins may be present at a lower frequency on the sheep and chicken ASA than on the rat ASA or there may be minor differences in the proteins of the different species, resulting in a lower affinity of A/S 83 for the sheep and chicken proteins, compared with the rat proteins. The anti-(chicken APM) antiserum did not apparently recognise P85 and did not recognise P96 of the rat ASA. This may be because of a difference in titre or affinity of this antiserum, compared with A/S 83, or proteins of the 3 species, while having similar electrophoretic mobilities, may be completely unrelated proteins.

6.4.4 Conclusions

1. While there was some cross-reactivity between APM and ASA of different species, as shown by ELISA and Western blotting, binding of antisera to APM and ASA of heterologous species was very low, compared with that to respective homologous species.
2. While there were proteins of similar molecular weights in the ASA preparations of different species, binding of antisera to such proteins varied in strength between ASA of different species. Proteins of similar molecular weights in the different ASA preparations cannot, therefore, be antigenically identical.

3. It is likely that different antisera and immunogens must be produced for the passive and active immunisation of different species against the adipocyte.

6.5 ACTIVE IMMUNISATION OF LAMBS

6.5.2 Experimental

6.5.1.1 Experiment 1

This experiment was performed in collaboration with Dr. S. Rhind at the Hill Farming Research Organisation (Penicuik, Midothian), using twin Scottish Blackface lambs. One lamb of each pair, when 2-4 weeks old, was immunised, subcutaneously at 3 sites, with 100 μg of Blackface sheep APM conjugated to 100 μg of rabbit serum albumin (RSA) in complete Freunds adjuvant, by the method described in Section 5.2. Lambs were boosted with APM conjugated to RSA in incomplete Freunds adjuvant at 3 and 7 weeks and with unconjugated APM in incomplete Freunds adjuvant at 13 and 21 weeks. The other lamb of each pair received the same treatment, with the exclusion of APM, at 0, 3 and 7 weeks and received no subsequent boosts. Body weights were recorded at 2-weekly intervals. Lambs were bled at 7, 10, 12, 15, 18, 21, 23, 27 and 29 weeks after the first immunisation and the serum was assayed for anti-(APM), anti-(HPM) and anti-(KPM) antibodies by ELISA. Lambs were killed at about 7½ months of age and carcass analysis was performed as described in Section 2.14.4.

6.5.1.2 Experiment 2

Experiment 2 was performed as for Experiment 1, with the following exceptions. Control lambs received no immunisation.

Treated lambs were immunised with sheep APM conjugated to RSA in complete Freunds adjuvant at 2-4 weeks of age and were boosted with the same immunogen in incomplete Freunds adjuvant at week 6. They then received sheep ASA conjugated to RSA in incomplete Freunds adjuvant at week 14 and unconjugated ASA in incomplete Freunds adjuvant at week 25. After weaning, food intake was measured weekly.

The binding of serum from the actively immunised lambs to APM, prepared from adipocytes isolated in the presence of BSA, and to APM, prepared by homogenisation of adipose tissue in the absence of BSA, was measured by ELISA.

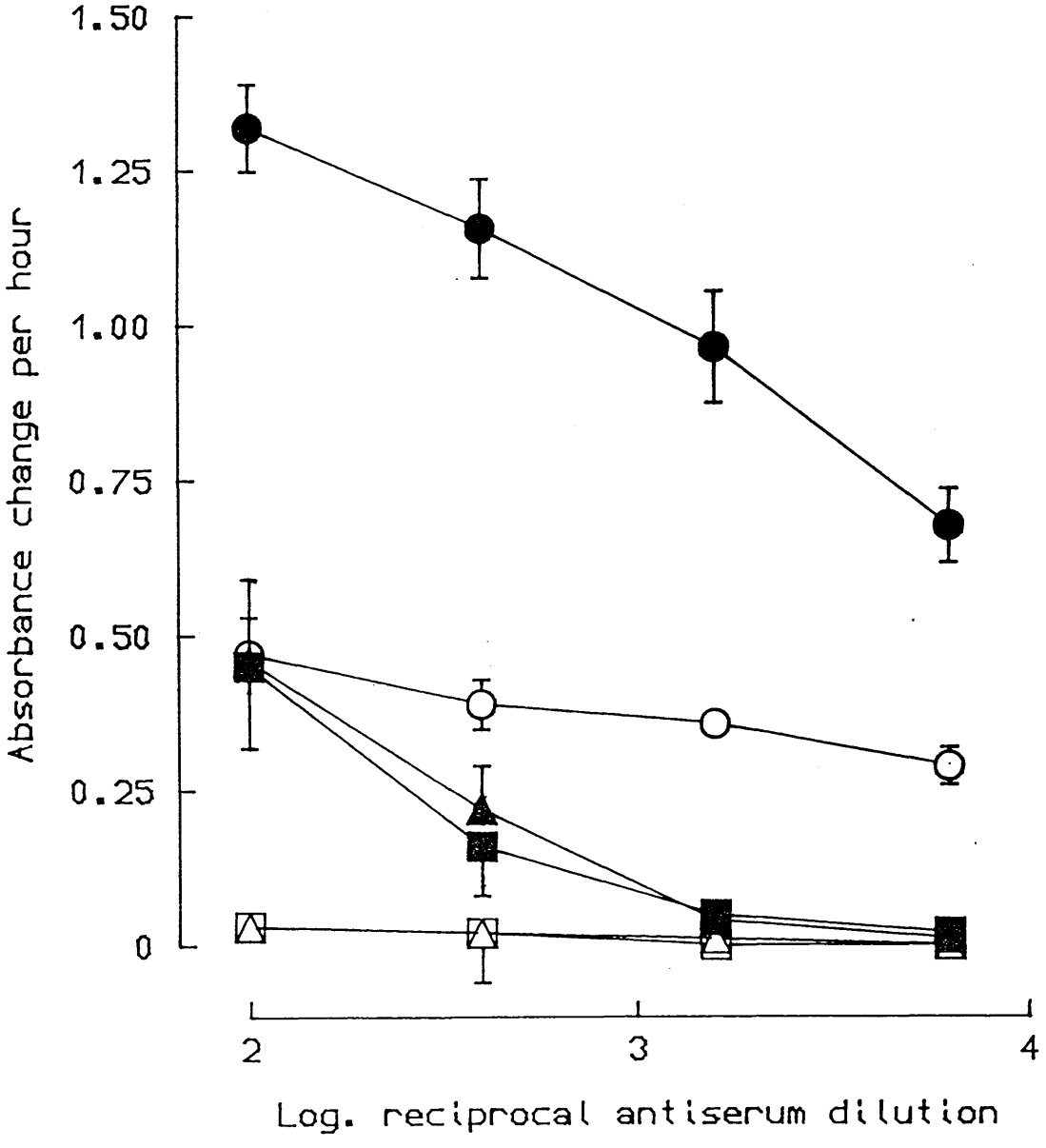
Delayed type hypersensitivity of lambs was tested at week 30, by injecting, intradermally, 100 μl of RSA (1 mg/ml), APM (1 mg/ml) or PBS in a shaved area of the back and measuring the diameter of the swelling 24 and 48 h later.

6.5.2 Results

6.5.2.1 Apparent anti-(adipocyte plasma membrane) responses in actively immunised lambs

In Experiment 1, serum from lambs immunised with APM conjugated to RSA and with RSA alone bound to sheep APM (prepared in the presence of BSA), as determined by ELISA (Fig. 6.5). The use of APM, prepared from adipocytes isolated in the presence of gelatin, for screening of antisera gave similar results (results not shown). The apparent response to APM of lambs immunised with APM was significantly greater, by a factor of at least 100 fold, than that of lambs immunised with RSA alone, 15 weeks after the first immunisation.

Fig. 6.5. The apparent antibody response to adipocyte, hepatocyte and kidney plasma membranes in actively immunised lambs



Lambs were immunised with either RSA (open symbols) or APM conjugated to RSA (closed symbols) in Freunds adjuvant. Antibody binding to APM prepared in the presence of BSA (circles), HPM (squares) and KPM (triangles) of serum from actively immunised lambs 15 weeks after the first immunisation was measured by ELISA. Results are means ± SEM of 8-10 observations.

In Experiment 2, lambs immunised with APM followed by ASA showed similar apparent responses to APM (prepared in the presence of BSA) to those induced by immunisation with APM in Experiment 1 (Fig. 6.6).

Apparent anti-(APM) responses in RSA-treated lambs were relatively constant between 5 and 28 weeks after the first immunisation, while those of APM-immunised lambs of Experiment 1 reached a peak at about 18 weeks after the first immunisation, before gradually declining (Fig. 6.6).

The binding of serum from actively immunised lambs of Experiment 2 to APM, prepared by homogenisation of intact adipose tissue in the absence of BSA, was not significantly above those achieved by sera from untreated lambs (Table 6.4).

6.5.2.2 The tissue specificity of antibody responses in actively immunised lambs

Very little anti-(HPM) or anti-(KPM) antibody responses could be detected, by ELISA, in lambs immunised with RSA alone or with APM followed by ASA in Experiment 2 (Fig. 6.5). A low level of binding to HPM and KPM of sera from lambs immunised with APM in Experiment 1 was detected.

6.5.2.3 Delayed type hypersensitivity of actively immunised lambs

The majority of lambs of Experiment 2 that had been treated with APM conjugated to RSA, followed by ASA, developed 1-1.5 cm swellings in response to RSA at 24 and 48 h after injection and showed virtually no response to APM or PBS.

6.5.2.4 The effects of active immunisation on adipose tissue

There was no difference in mass of any of the adipose depots studied between APM-immunised and RSA-immunised lambs of Experiment 1

Fig. 6.6. The change in apparent antibody response to adipocyte plasma membranes in actively immunised lambs with time

Lambs were immunised with either RSA (open circles), APM conjugated to RSA (closed circles) or APM followed by ASA conjugated to RSA (closed squares) in Freunds adjuvant. Antibody binding to APM, prepared in the presence of BSA, by serum from actively immunised lambs was measured by ELISA. The standard was a goat anti-(sheep APM) antiserum.

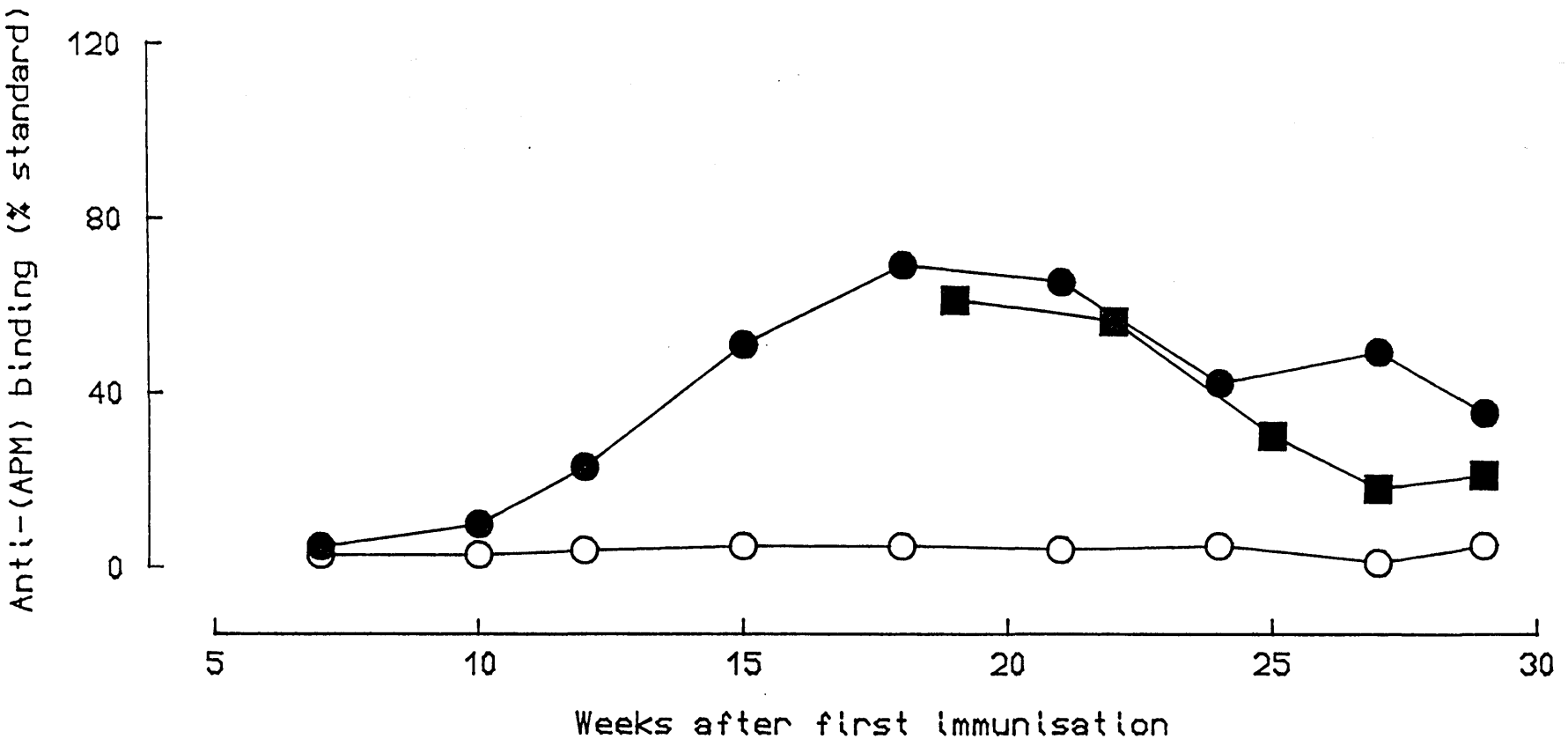

Anti-(APM) binding (% standard)
120
80
40
0
5
10
15
20
25
30
Weeks after first immunisation

Table 6.4. The role of anti-(BSA) antibody responses in apparent anti-(adipocyte plasma membrane) antibody responses of actively immunised lambs

Antiserum	Binding to APM prepared in the absence of BSA as % of binding to APM prepared in the presence of BSA	
	Relative antibody binding	Relative antigen abundance
Goat anti-(sheep APM)	16	100
Actively immunised lambs	0	0

The goat was immunised with sheep APM conjugated to RSA and the lambs were immunised with sheep APM, followed by sheep ASA conjugated to RSA. Binding to APM prepared from adipocytes isolated in the presence of BSA and to APM prepared from whole adipose tissue in the absence of BSA was measured by ELISA. Relative antibody binding and antigen abundances were determined as described in Section 2.6.3.

(Table 6.5). Immunisation with APM followed by ASA in Experiment 2 caused significant reductions in mass of inter-muscular, kidney channel and omental adipose tissue, compared with untreated controls (Table 6.5). When adipose tissue mass was expressed as a percentage of body weight, the differences between the 2 groups were reduced to non-significant levels. While total lean body mass was reduced in immunised lambs compared with controls, when expressed as a percentage of body weight immunised lambs showed a greater percentage of lean tissue than controls, although this difference was not significant.

6.5.2.5 The effects of active immunisation on body weight gain, food intake and food conversion efficiency

There were no significant differences in body weight gain between APM- and RSA-treated lambs of Experiment 1 (Fig. 6.7) In Experiment 2, treated lambs showed significantly reduced body weights, compared with untreated controls, from about 8 weeks after the first immunisation (Fig. 6.7). Food intake was measured between 22 and 28 weeks after treatment. During this period, treated lambs ate significantly less food but also gained less weight, resulting in a similar food conversion efficiency to controls (Table 6.6).

6.5.3 Discussion

6.5.3.1 Apparent anti-(adipocyte plasma membrane) antibody responses of actively immunised lambs

As with the active immunisation of rats, the detection of apparent anti-(APM) antibodies in the sera of lambs immunised with RSA alone raised the question of whether anti-(BSA), rather than anti-(APM) responses were actually being measured. Again the sera

Table 6.5. The effects of immunisation with rabbit serum albumin, adipocyte plasma membranes and adipocyte specific antigens on body composition

Treatment	Live weight (g)	Hot carcass weight (g)	Total lean (kg)		Subcutaneous fat (kg)		Intermuscular fat (kg)		Kidney channel fat (kg)		Omental fat (kg)	
RSA	30.3 ±1.2	13.7 ±0.4	3.37 ±0.14	(24.6) (±0.3)	0.70 ±0.08	(5.1) (±0.5)	0.87 ±0.04	(6.3) (±0.2)	0.24 ±0.03	(1.7) (±0.2)	0.71 ±0.01	(5.1) (±0.5)
APM-RSA	29.6 ±0.9	13.2 ±0.5	3.23 ±0.12	(24.5) (±0.3)	0.72 ±0.10	(5.2) (±0.4)	0.84 ±0.00	(6.3) (±0.3)	0.23 ±0.03	(1.7) (±0.2)	0.71 ±0.01	(5.5) (±0.7)
None	47.1 ±1.3	24.8 ±0.6	5.23 ±0.16	(21.1) (±0.5)	2.40 ±0.20	(9.6) (±0.7)	1.73 ±0.04	(7.0) (±0.2)	0.57 ±0.08	(2.3) (±0.3)	2.32 ±0.13	(9.4) (±0.5)
APM-RSA	40.9**	21.2**	4.73	(22.5)	2.10	(9.7)	1.37***	(6.5)	0.40**	(1.9)	1.88*	(8.8)
ASA-RSA	±2.3	±1.0	±0.18	(±0.5)	±0.24	(±0.6)	±0.06	(±0.1)	±0.06	(±0.2)	±0.19	(±0.6)

Lambs were immunised with either RSA, APM conjugated to RSA or APM followed by ASA conjugated to RSA, all in Fruends adjuvant or were untreated. Carcass analysis was performed abut 7 months after the first treatment. Figures in parentheses are percentages of body weight. Results are means ± SEM of 8-10 observations. *P<0.01, **P<0.005, ***P<0.001, compared with untreated controls (Student's paired t-test, pairing siblings).

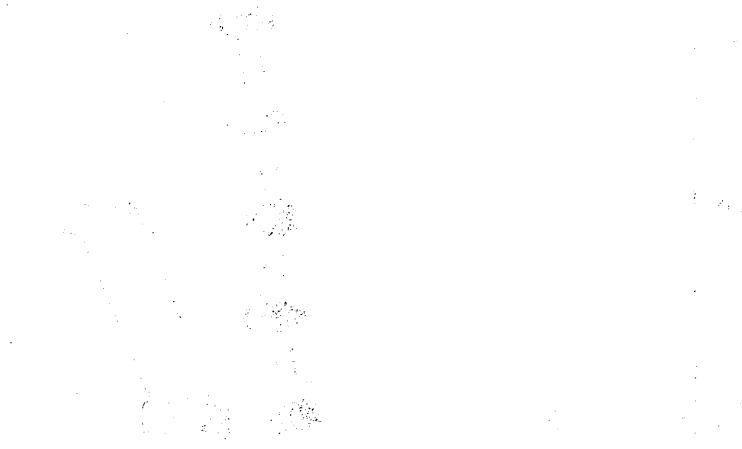


Fig. 6.7. Body weights of actively immunised lambs

Lambs were immunised with either RSA (open circles), APM conjugated to RSA (closed circles) or APM followed by ASA conjugated to RSA (closed squares) or were untreated (open squares).

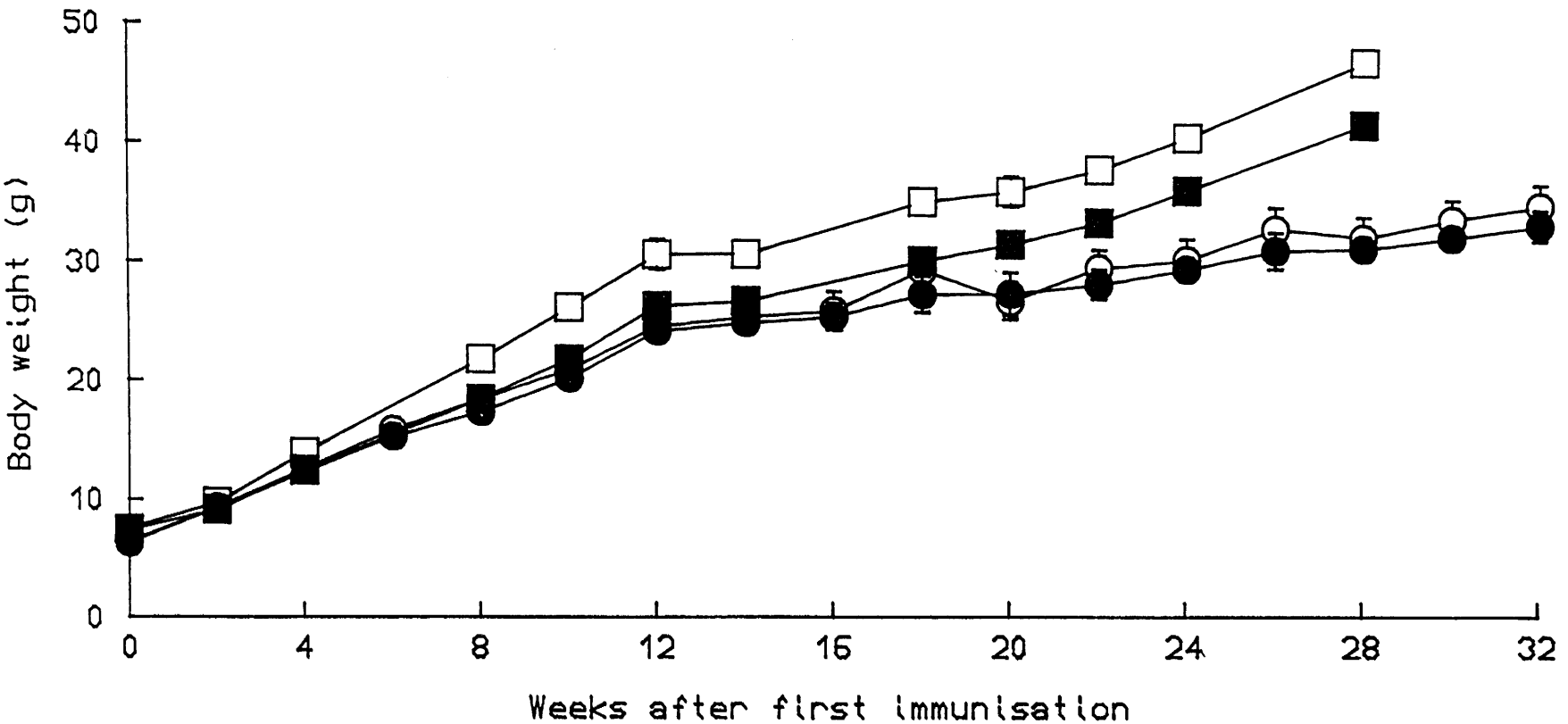

Body weight (g)
50
40
30
20
10
0
0
4
8
12
16
20
24
28
32
Weeks after first immunisation

Table 6.6. The effects of immunisation with adipocyte plasma membranes and adipocyte specific antigens on body weight gain, food intake and food conversion efficiency

Treatment	None	APM-RSA ASA-RSA
Weight gain (kg/day/lamb)	0.21 ±0.012	0.19 ±0.016
Food intake (kg/day/lamb)	1.92 ±0.048	1.72** ±0.077
Food intake - maintenance requirement (kg/day/lamb)	1.41 ±0.044	1.28 ±0.068
$\frac{\text{Intake - maintenance}}{\text{Gain}}$	6.90 ±0.41	6.91 ±0.40

Lambs were immunised with APM followed by ASA conjugated to RSA in Fruends adjuvant or were untreated. Weight gain and food intake were measured between 20 and 36 weeks after the first immunisation. The daily maintenance requirement was assumed to be 12 g/kg body weight. Results are means ± SEM of 8 observations. **$P<0.005$, compared with controls (Student's paired t test, pairing siblings).

bound equally well to APM prepared from adipocytes isolated in the presence of BSA or gelatin (results not shown), so it was assumed that a true anti-(APM) response was being measured. The discovery that serum from actively immunised rats did not bind to APM, isolated from homogenised adipose tissue in the absence of BSA, was not made until after the initiation of the second experiment involving the active immunisation of lambs. Consequently, in the second experiment, the controls were completely untreated. It would, clearly, have been ideal to have included 2 sets of controls, one of which was untreated and one of which received RSA alone. However, the results of the active immunisation experiments with rats suggested that the use of siblings in different treatment groups was important for the demonstration of effects and so twin lambs were used, allowing 2 treatment groups only.

Unlike the corresponding experiments with rats, lambs, immunised with APM and RSA, showed a greater apparent anti-(APM) response than RSA-immunised controls.

The sera of lambs immunised with ASA were subsequently shown to be virtually devoid of antibody activity against APM, prepared from homogenised tissue in the absence of BSA. While SDS PAGE of sheep APM, prepared in the presence of BSA, was not performed, it is likely that, since the method used was identical to that used for rat APM, sheep APM, like rat, were contaminated with BSA. Therefore, the apparent anti-(APM) response in RSA-immunised and RSA with ASA-immunised lambs may have been due to binding of anti-(RSA) antibodies (that cross-reacted with BSA) to BSA contaminating the APM used for screening. The APM-immunised lambs of

Experiment 1 received, in addition to RSA, APM that were probably contaminated with BSA and so a greater anti-(BSA) response than seen in the RSA-immunised lambs may have been induced, resulting in a greater apparent anti-(APM) response. However, it had previously been found that sheep immunised with rat APM, contaminated with BSA, produced a poorer anti-(BSA) response than rats immunised with the same immunogen, suggesting that BSA is poorly immunogenic in sheep. A higher apparent anti-(APM) response in lambs immunised with APM and RSA than in those immunised with RSA alone may, therefore, have been due to a true anti-(APM) response.

Since no convincing anti-(APM) response could be demonstrated in ASA-immunised lambs, sheep ASA may be poorly immunogenic in lambs. However, due to the scarcity of the sheep ASA, the lambs only received 2 immunisations of this material and it was not possible to screen the antisera directly against sheep ASA.

6.5.3.2 The specificity of antibody responses of actively immunised lambs

The serum from lambs immunised with sheep APM, in common with that from rats immunised with rat APM, showed little cross-reaction with other tissues. However, while serum from rats immunised with rat ASA showed considerable cross-reactivity with non-adipose tissues, serum from lambs immunised with sheep ASA showed no demonstrable binding to non-adipose tissues. It was suggested in Section 5.4.2 that the lack of adipose specificity of the rat sera may have been related to the anti-(sheep IgG) response of actively immunised rats. The sheep ASA may have been contaminated with goat IgG but this would be unlikely to be highly immunogenic to sheep. Alternatively, the sheep ASA may have included fewer antigens that

cross-reacted with other tissues than the rat ASA. However, since no convincing anti-(APM) response was measured in ASA-immunised lambs and it was not possible to screen the antisera against ASA, it is not known whether there was any response to the ASA preparation at all.

6.5.3.3 The effects of active immunisation on body composition, body weight gain and food intake

In Experiment 1 there was no difference between body weight gain or body composition between treated and control lambs (Table 6.4). In Experiment 2, the most clear difference between treated and control animals was the reduced body weight of the treated group which was evident from 8 weeks after the first treatment (Fig. 6.7). It is possible that this was a non-specific effect relating to the use of Freunds adjuvant, since both groups of lambs in Experiment 1 received Freunds adjuvant and showed reduced body weights, compared with the untreated group from Experiment 2. However, as the 2 experiments were performed in different years, the difference in the body weights achieved in the 2 experiments may be due to factors unrelated to the experimental treatment. Treatment of rats with Freunds adjuvant had no effect on body weight gain.

The reduction in body weight of the treated lambs of Experiment 2 was reflected in reduced weights of both adipose and lean tissue. However, the percentage mass of lean tissue was increased and the percentage mass of adipose tissue was reduced in the treated group, compared with controls, although these differences were not significant. This suggests that, in addition to a non-specific effect on growth, there was some repartitioning of energy from fat into protein deposition. As in the rat, this could not be correlated with the presence of circulating anti-(APM) antibodies and further

emphasises the importance of devising assays for antibody-producing cells and cell-mediated immune responses. Delayed type hypersensitivity tests did not show a response to APM, but such tests are not always successful using insoluble antigens.

The reduced body weight gain in treated animals could be correlated with a reduction in food intake (Table 6.5). Lipectomy can result in a reduction in food intake in rats when the capacity for fat storage has been reached (Faust, 1977), but, as a reduction in food intake was measured only 8 weeks after the first treatment, the reduction in food intake of actively immunised lambs was unlikely to be due to a reaching of the maximum capacity for fat storage. It is not known whether actively immunised lambs had, like the rat, fewer but larger adipocytes than controls. Mears and Mendel (1974) found that food intake was greater in sheep with larger numbers of adipocytes. These authors suggested that the amount of glucose removed from a central pool might act as a feedback signal, inducing a high long-term food intake. Such a mechanism could contribute towards the reduced food intake in the actively immunised lambs, if those animals had reduced adipocyte numbers. In contrast Graham (1969) found that fatter sheep tended to have a reduced food intake compared with their leaner counterparts. However, these differences were only demonstrable in lambs whose degree of adiposity varied to a much greater extent than that achieved in the active immunisation experiments.

6.5.3.4 Conclusions

1. Immunisation of lambs with sheep APM conjugated to RSA in Freunds adjuvant had no effect on adiposity or body weight, compared with RSA-immunised controls.

2. Immunisation of lambs with sheep APM followed by sheep ASA conjugated to RSA in Freunds adjuvant caused a significant reduction in body weight and fat pad weight compared with untreated controls.

3. Anti-(APM) antibodies could not be convincingly demonstrated in lambs immunised with APM or ASA conjugated to RSA.

CHAPTER 7

FINAL DISCUSSION

a stronger response to BSA and no clearly demonstrable response to rat APM. Gel electrophoresis of the APM used as immunogens showed a high level of contamination with BSA. Rat APM are likely to be highly immunogenic in sheep, whereas BSA probably shows considerable antigenic similarity with ovine albumin and so exhibits poor immunogenicity in this species. In addition, antibodies directed against auto-antigens frequently bind to a very restricted number of epitopes on that antigen, while xenogeneic antisera to the same antigen recognise a much wider range of epitopes. For example, the majority of human anti-(thyroglobulin) antibodies are directed against 2 epitopes of human thyroglobulin, while rabbit anti-(human thyroglobulin) antisera recognise about 40 epitopes on the molecule (Nye, Pontes de carvatho and Ratt, 1980). These authors suggested that some epitopes were less effective than others at inducing tolerance, whether through the direct tolerisation of B cells or through establishment of specific suppressor mechanisms, such as antigen or idiotype-specific T suppressors for the stimulation of anti-idiotype clones.

There were also differences in the immune response of sheep and rats to rat ASA. While the immunisation of sheep with rat ASA induced the production of an antiserum that, according to the results of ELISA and in vivo studies, showed more adipocyte specificity than sheep antisera raised against whole APM, the autoimmunisation of rats with rat ASA induced the production of antisera that, according to the results of ELISA, showed more cross-reactivity with non-adipose tissues than rat antisera raised against whole APM. It was suggested in Section 5.4.2 that the cross-reactivity with non-adipose tissue might, in some way, relate

to the rat immune response to sheep IgG, which was a contaminant of the ASA. Since sheep IgG would not be highly immunogenic in a sheep, this may account for the apparently more tissue-specific response induced by immunisation of sheep with rat ASA, compared with that induced in the rat by the same immunogen.

It should also be borne in mind that the measurement of circulating antibodies in actively immunised rats may not provide an accurate measure of the true antibody responses induced by autoimmunisation since antibodies binding to autoantigens may be sequestered within the target tissues.

7.1.2 Differential effects of passive and active immunisation against the adipocyte on adipocyte numbers and volume

Passive immunisation of rats by intraperitoneal administration of an anti-rat (APM) antiserum caused a 50% reduction in parametrial adipocyte numbers, which persisted for at least 24 weeks after treatment (until 30 weeks of age). Active immunisation against rat APM caused a 40-50% reduction in parametrial adipocyte numbers, which was evident 16 weeks after the first immunisation (at 20 weeks of age). Passive immunisation also caused a reduction in parametrial adipose tissue mass, which comprised of a reduction of 50% at 24 weeks after treatment, while active immunisation did not consistently cause a reduction in parametrial adipose tissue mass. This difference was apparently due to the fact that, in actively immunised rats, fat reduction due to decreased numbers of adipocytes was compensated for by the enlargement of remaining adipocytes to an abnormally large volume, whereas there was no such compensatory increase in the size of remaining parametrial adipocytes of passively immunised rats.

The ability of passive immunisation to inhibit the compensatory increases in adipocyte volume, seen in actively immunised rats, may have been the result of differences in the intensity or nature of the immune effector mechanisms recruited by passive, compared with active, immunisation. The effects of passive immunisation must have been dependent upon the presence of anti-(APM) antibodies and were also shown to be characterised by complement activation and infiltration of adipose tissue with polymorphonuclear leucocytes and lymphocytes. Whilst rat anti-(APM) antibodies arising from activation of the host idiotypic network in response to sheep anti-(APM) could not be detected in passively immunised rats, an increased anti-(sheep IgG) response was measured in these animals. Binding of rat antibodies to sheep anti-(APM) antibodies, already bound to adipocytes, may have resulted in the enhanced activation of immune effector mechanisms and contributed towards the inhibition of compensatory increases in adipocyte size in passively immunised rats. Such a mechanism has been proposed for the production of prolonged kidney disease in rats, passively treated with xenogeneic anti-(kidney) antisera (Unanue and Dixon, 1967). Circulating anti-(APM) antibodies could not be convincingly demonstrated in actively immunised rats, but this does not necessarily indicate the total absence of such antibodies, as already discussed. Alternatively, T-cell mediated immune responses may have been responsible for the reduction in adipocyte number in actively immunised rats. Clearly, the development of cell-mediated cytotoxicity assays and histological and immunohistochemical examination of adipose tissue of both passively and actively immunised rats will be required to determine the location and density

of antibodies, the precise nature of any cellular infiltrates in the tissue and thereby the probable cell-killing mechanisms involved.

There were differences in the responses of different adipose depots to passive immunisation and these differences were found to be due, largely, to the site of injection. Passive immunisation may, therefore, have had a greater effect on adipose tissue mass than active immunisation because a high concentration of antibody could be targeted to the tissue by the mode of administration.

7.1.3 Differential effects of passive and active immunisation against the adipocyte on non-adipose tissues

Passive immunisation with A/S 83 had no effect on body weight with lost fat compensated for by the deposition of protein and water. Body composition analysis was not performed on actively immunised rats. However, in the single active immunisation experiment where a reduction in adipose tissue mass was observed, there was also a reduction in body weight and in subsequent experiments there was neither a reduction in adipose mass nor in body weight. A significant increase in protein deposition in actively-immunised rats seems, therefore, unlikely. It is possible that A/S 83 had direct effects on muscle which were not mimicked by antibodies induced by active immunisation. Alternatively, the increase in protein deposition in passively immunised animals may have resulted from a decrease in competition between adipose tissue and muscle for common biochemical substrates. Since fat deposition was not decreased in actively immunised rats, such a reduction in competition between the 2 tissues would not occur.

No evidence of proteinurea could be found in actively immunised rats, providing further evidence that the transient nephritis observed in passively immunised rats was related to the rat immune response to sheep IgG. However, circulating antibodies of rats actively immunised with whole APM, in contrast to A/S 83, bound very poorly to kidney plasma membranes. Antibodies directed against renal antigens may have been sequestered by the kidney and, thus, would not have been present in the circulation, although it could have represented a truly poor response to kidney antigens since autoimmunisation frequently produces more tissue-specific antisera than cross-species immunisation (Shulman, 1974). Paradoxically, rats immunised with rat ASA did produce circulating antibodies that bound to kidney plasma membranes but no signs of proteinurea were found in these animals. Before it can be concluded that the active immunisation procedures used had no effects on the kidneys, more frequent urine sampling would be required, since nephritis induced by antibody or immune complexes can be of a highly transient nature.

No gross abnormalities of the liver, such as were seen in rats treated with certain anti-(APM) antisera, were ever observed in actively immunised rats. Circulating antibodies binding to hepatocyte plasma membranes were barely detectable in rats immunised with whole APM but were detectable in rats immunised with adipocyte specific antigens. Again, the measurement of circulating antibodies may not give an accurate indication of immune responses in actively immunised rats.

7.2 POTENTIAL USES AND LIMITATIONS OF PASSIVE AND ACTIVE IMMUNISATION AGAINST THE ADIPOCYTE

7.2.1 The reduction of body fat in meat-producing species and humans

7.2.1.1 The requirement for the demonstration of the reduction of adiposity by passive or active immunisation against the adipocyte in a species other than the rat

The majority of the work described in this thesis was performed using the rat, in the hope that this species might prove to be a useful model for the effects of passive or active immunisation against the adipocyte in other species, including man. While much of the reported work aimed at studying the characteristics and control of the development of adipose tissue has been performed using the rat, this species is unusual in that the long bones of the rat continue to grow throughout life and adiposity also increases throughout life. Thus, the rat might prove a useful model for meat-producing species which are generally killed when they are still in a growth phase and adiposity is increasing. It might not, however, prove a good model for the treatment of humans since, in many cases, treatment would be required in the adult human when the growth phase has essentially stopped. Passive immunisation was performed exclusively with young lean animals in the experiments described in this thesis and it would clearly be useful to examine the effects of passive immunisation on mature or obese animals, where large amounts of adipose tissue are available for destruction and/or triglyceride mobilization.

A comparison of the effects of active immunisation in rats and lambs gives some indication of the usefulness of the rat as a model for the effects of active immunisation in sheep. Similar immunisation protocols induced similar apparent antibody responses in the 2 species. There were differences in the effects of active immunisation on body weight gain in the 2 species which may have been because sheep are more susceptible than rats to the non-specific toxic effects of Freunds adjuvant. Unfortunately, it was not possible to measure adipocyte sizes of lambs and so a comparison of the effects of active immunisation of the 2 species on adiposity could not be made. While it was possible to raise antisera against chicken and pig APM with similar binding activities to homologous plasma membranes to A/S 83, time did not permit the evaluation of the in vitro effects on isolated adipocytes or the in vivo effects of these antisera. A comparison of the binding of anti-(APM) antisera to APM of different species showed that there are major antigenic differences between APM of different species, emphasizing the probable requirement for different immunogens and antisera for each species, although conserved and therefore weakly immunogenic antigens may exist which could be used in more than one species.

Although it can never be assumed that the results of passive or active immunisation against the adipocyte will be the same in other species as in the rat, studies with the rat could be extremely useful in the identification of potential problems and promising approaches. For instance, in vitro antiserum screening assays and methods of preparing adipocyte specific antigens could be developed for the rat, before application to other species.

Whatever animal models are chosen for the examination of the effects of passive or active immunisation, efforts should be made to improve the adipocyte specificity of immunogens and antisera and *in vitro* assays able to predict the *in vivo* effects of antisera would greatly improve the screening of potentially useful antibodies. In addition, more information is required about the effects of immunization in the short and long-term, both on adiposity and animal health.

7.2.1.2 The requirement for adipocyte specificity

The failure to reproduce the *in vivo* effects of early bleeds of A/S 83 with later bleeds of A/S 83 emphasizes the potential advantage of monoclonal antibodies against APM determinants, which would provide an unlimited supply of antibodies of a defined titre, specificity and cytotoxicity. Since the whole APM has a complex antigenic composition, attempts were made to identify adipocyte specific antigens which were important in the fat-reducing properties of the polyclonal antisera, so that such antigens could be used for the production of monoclonal antibodies.

It was also hoped that polyclonal and monoclonal antisera, raised against adipocyte specific antigens, might be devoid of the undesirable side effects of antisera raised against the whole APM, such as the immediate effects on food intake and subsequent effects on the kidney and liver. In addition, the use of highly-purified, well-defined adipocyte specific immunogens for active immunisation might eliminate one potential cause of variability in the response induced by immunistion with whole APM.

Adsorption of an antiserum raised against the whole APM (A/S 164) with liver rendered that antiserum more adipocyte specific, as

indicated by ELISA. This antiserum retained its fat-reducing properties *in vivo* but no longer caused liver abnormalities. This was an important discovery because, while the adsorbed antiserum was not totally adipocyte specific, it did indicate that antibodies directed against adipocyte specific antigens could cause a reduction in adiposity and so provided impetus for the preparation of adipocyte specific antigens. The adsorbed antiserum still had anaesthetic-like effects immediately after treatment and caused a reduction in food intake on the first day of treatment. The effects on food intake may be an inevitable consequence of the release of fat from adipose tissue but this is unlikely to be the case since the anaesthetic-like effects were not evident in rats treated with A/S 83, an antiserum with potent fat-reducing properties. It is of great importance that the antigens recognised by antibodies responsible for the anaesthetic-like effects, or at least the tissues on which they are expressed, should be identified so that screening for adipocyte specificity and adsorption procedures can include these tissues. It is also important to determine whether the adsorbed antiserum, which showed very little *in vitro* reactivity against kidney membranes, caused transient nephritis, as seen in A/S 83-treated rats. This would indicate whether the nephritis results from anti-(kidney) antibodies or from the non-specific deposition of immune complexes in the glomerulus.

The methods used to prepare the ASA preparation provided a considerable degree of purification of the APM but the adipocyte specificity of at least the rat ASA must be questioned and this preparation was almost certainly contaminated with sheep IgG. Clearly, further characterisation of all the components of all the

ASA preparations is necessary. The raising of polyclonal antisera against individual polypeptide components of the ASA, excised or eluted from polyacrylamide gels, or the raising of monoclonal antibodies against the ASA, and the determination of their adipocyte specificity and cytotoxicity would indicate the potential use of individual polypeptides as immunogens. It would also be of interest to determine whether any of the components of the ASA are glycoproteins, since rat APM have been reported to include 2 major glycoproteins with molecular weights of 94 and 78 kD which, when incorporated into liposomes, have been shown to exhibit glucose transport activity (Shanahan and Czech, 1977). By incorporation into liposomes, potential transport activity of any of the components of the ASA could be examined. Hormone binding to the ASA could also be studied. High performance liquid chromatography of the ASA would provide pure preparations of the different components for these types of characterisation.

The fact that an antiserum raised against rat ASA was cytotoxic to adipocytes in vitro and had fat-reducing properties in vivo suggests that one or more of the antigens in this preparation may be able to induce the formation of cytotoxic antibodies. However, it is possible that, using the method of antigen purification described in this thesis, other adipocyte specific antigens, capable of inducing a potent cytotoxic response, may have been lost. Since several antisera, raised in both sheep and rabbits, against rat APM are now available, a panel of antisera could be used for antigen purification to minimise the chances of missing potentially useful antigens. The method of antigen preparation may also require modification. The APM affinity column, used for the initial purification of anti-(APM)

antibodies, consistently gave poorer yields than would be expected from high titre antisera, which may have resulted in the loss of antibodies capable of binding to potentially useful antigens. Better results might be obtained using insoluble APM coupled to the affinity support rather than their solubilized counterparts. The questionable adipocyte specificity of some of the antigens prepared suggests that either a different detergent, such as deoxycholate or n-octylglucoside, might be used in order to achieve a more effective solubilization of the APM, or that a more efficient method of adsorption should be devised. Perhaps an initial adsorption of the anti-(APM) antiserum with liver homogenate before affinity purification might yield antibodies showing a higher degree of adipocyte specificity than those used for the ASA preparation. Since an antiserum raised against rat ASA bound to kidney and hepatocyte plasma membranes in vitro and had anaesthetic-like effects in vivo, more rigorous demands in terms of adipocyte specificity should be made of antibodies before their use for antigen purification. If the adipocyte specificity of antigens prepared by affinity chromatography cannot be improved, it may be necessary to adopt the approach, used by Lee et al (1986), of immunoprecipitation of adipocyte specific antigens from solubilized APM using pre-adsorbed adipocyte specific antisera. Such an approach, however, would not yield the quantity of antigen that can potentially be achieved using affinity chromatography because of the ability to re-use affinity columns. It would, however, overcome the inherent problem of affinity chromatography - i.e. that only moderate affinity antibodies are of practical use, since irreversible binding may occur to high affinity antibodies.

Despite doubts about the true adipocyte specificity of all the ASA preparations, an antiserum raised against the rat ASA was considerably more tissue specific than antisera raised against whole APM, as shown by both *in vitro* and *in vivo* experiments and had fat-reducing properties. Some components of the preparations described in this thesis may, therefore, be of use as immunogens for active immunisation or the raising of monoclonal or polyclonal antisera.

7.2.1.3 *The requirement for in vitro assays able to predict the in vivo effects of antisera*

ELISA and *in vitro* cytotoxicity assays, performed in the way described in this thesis, did not reliably predict the *in vivo* effects of an antiserum in terms of the effects on adipose tissue, the liver or the immediate anaesthetic-like effects. The use of polyclonal or monoclonal antisera for passive immunisation of animals or humans requires the development of *in vitro* assays able to predict the results of the *in vivo* administration of an antiserum.

It may be necessary to perform *in vitro* cytotoxicity assays in the presence of complement of the species to be immunised, for more than 24 h, in the presence of lower concentrations of antibody than have previously been used, in order to mimic the *in vivo* situation more closely. No cytotoxicity against adipocytes in the presence of rat complement could be measured using the assays described in Section 2.7.2. Therefore, the main role of complement in mediating the fat-reducing properties of antisera may be the attraction of phagocytic cells, rather than cytolysis through the formation of the membrane attack complex of complement. If this is the case, the critical properties determining the potency of an antiserum may be

its ability to activate complement and the proportion of the antibody able to bind to adipose tissue. Assays of complement activation, rather than complement-mediated cytolysis of target cells, might, therefore, prove to be more appropriate and, at the very least, beneficial. Measurements of antibody binding to whole cells, rather than plasma membranes adsorbed to plastic, might also provide a better indication of the ability of an antiserum to bind to a tissue *in vivo*. Western blotting of antisera against a wide range of tissues, including muscle, the central nervous system, lungs etc., might help to identify critical antigens, the binding to which is responsible for *in vivo* side effects.

7.2.1.4 The requirement for further investigations of the effects of passive and active immunisation on non-adipose tissues

The precise fate of fat released from adipose tissue as a result of passive or active immunisation against the adipocyte must be determined, particularly when considering the treatment of obese humans, since the deposition of released fat into, for instance, the arteries may have long-term undesirable consequences.

Clearly, the effects of passive and active immunisation on non-adipose tissues must be investigated more thoroughly. Adverse effects on the liver and kidney were found in some passively immunised rats suggesting that more detailed studies of liver and kidney function in both passively and actively immunised rats should be made.

The effects of passive and active immunisation on muscle and meat quality have yet to be examined. β-agonists have been successful in increasing the protein content and decreasing the fat content of the carcass (Dalrymple *et al*. 1984; Hanrahan *et al*.,

1986). However, the muscles of treated animals have been shown to have a high pH, increasing the likelihood of early spoilage, a dark colour and reduced intra-muscular fat, resulting in the production of dry meat (Warriss and Kestin, 1988). The effects on these parameters of passive and active immunistion against the adipocyte should also be examined.

7.2.1.5 The relative merits of passive and active immunisation against the adipocyte and alternative methods of modifying body composition

When examining the relative merits of passive and active immunisation against the adipocyte and alternative methods of modifying body composition of meat species, it is necessary to consider the effectiveness of the approach in inducing increased protein deposition and reduced fat deposition, the cost effectiveness and flexibility of the approach, the effects of the approach on animal health and potential harm to the consumer.

While bovine and porcine growth hormones have been shown to improve carcass protein:fat ratios in meat-producing species (Muir et al., 1983; Chung, Etherton and Wiggins, 1985), the use of this approach may be limited by its cost effectiveness. As with other hormones and synthetic β-agonists, the effects of growth hormone on nutrient partitioning and hence body composition are reduced within a few days of cessation of treatment. The administration of an anti-(APM) antiserum on 4 consecutive days caused a reduction in body fat that lasted for at least 6 months in the rat. This approach to modifying body composition does not require frequent administration of antiserum because the effects are achieved, at least in part, by causing a long-term reduction in adipocyte numbers, rather than

causing short-term alterations in metabolism. However, the amounts of antiserum required for even single treatments of large animals are likely to render this approach practical only for the treatment of poultry, unless highly cytotoxic, adipocyte specific monoclonal antibodies, possibly linked to a cytotoxin, can be developed. Active immunisation against the adipocyte may prove a more practical approach than passive immunisation for the treatment of large animals. Even for poultry, active immunisation is an attractive proposition because the immunisation of a single laying hen may result in the treatment of large numbers of progeny through passive transfer of maternal antibodies to the yolk. The active immunisation experiments using rats suggested that, while more effective methods of immunisation need to be devised to increase the intensity of the immune response induced, it is possible to induce an autoimmune response against adipose tissue, as shown by the decreased numbers of adipocytes in immunised animals. Clearly, chemically defined antigens must be used as immunogens for active immunisation of humans or meat-producing species. The use of anti-idiotypic antibodies, raised against anti-(adipocyte) antibodies, as antigen images, may prove useful for the large scale production of immunogens for this purpose. Attempts have been made to utilise such an approach for vaccine production. The administration of anti-idiotypic antibodies, raised against antibodies recognising parasitic (Grzyck, Capron, Lambert, Dissons, Torres and Capron, 1985), bacterial (Stein and Soderström, 1984) and viral (Kennedy, Dressman, Butel and Lanford, 1985) antigens, have been shown to confer protective immunity against the corresponding infectious agents in experimental animals.

Passive immunisation against the adipocyte has the advantage over all existing approaches towards the manipulation of body composition of allowing selection of which adipose depots to reduce, by varying the site of injection or the specificity of the antibodies towards individual depots. This would be useful in the meat industry as a certain amount of subcutaneous fat may be desirable for the maintenance of eating quality of meat (Moran, 1986).

The 3 active immunisation experiments described in Chapter 5 were treated separately in order to illustrate the variation in the effects of the same treatment on body weight gain and adiposity in different groups of rats. Variation in the immune response between individuals of the same breed and between breeds is a potential limitation of the use of active immunisation, unless very effective immunostimulatory protocols can be devised.

One of the major limitations of the use of any method of manipulating carcass composition is the possibility of harmful effects on the treated animals. Information on such effects of existing approaches is limited but sex steroids can have adverse effects on reproductive function and behaviour, while β-agonists may have harmful effects on the cardiovascular system (Heitzman, 1986). Whether reduction of carcass fat content would have potential harmful effects during nutritional stress, for example, is one important consideration. Immunological techniques, such as active immunisation against somatostatin (Spencer, Garssen and Hart, 1983) or against the adipocyte, require the development of non-toxic, metabolizable immunological adjuvants. While progress is being made in this field (Warren et al., 1986), a non-toxic synthetic adjuvant has yet to be found that can mimic all the potent stimulatory effects of Freunds adjuvant.

With regard to the commercial use of passive immunisation against the adipocyte, it would be necessary to identify and eliminate the cause of the adverse effects on the liver and the anaesthetic-like effects of treatment. It may prove impossible to prevent the initial effects on food intake of passive immunisation, since all antisera with in vivo fat-reducing properties caused a dramatic reduction in food intake on the first day of treatment.

The current EEC ban on the use of steroid growth promoters in animal production arose from concern about possible harmful effects of residues on consumers. The only known harmful effects of such agents has been the result of the illegal injection of prohibited anabolic agents into the edible tissues of veal calves which subsequently contaminated batches of baby food (Heitzmann, 1986). β-agonists are clearly orally active hormones and so are likely to be subject to similar attention to that received by steroid growth promoters, which is likely to limit their commercial use. Passive immunisation against the adipocyte would be unlikely to leave harmful residues in the edible tissues, since treatment could be timed to allow clearance of antibody before slaughter and such antibodies would not survive either cooking or digestion in the alimentary tract. However, considerations for the safety of consumers would almost certainly prevent the use of immunotoxins to increase the potency for anything other than their use in human obesity where the condition was life-threatening.

7.2.2 The examination of competition between adipose and mammary tissue during lactation

The discovery that A/S 83 temporarily reduced fat deposition in existing adipocytes, as well as reducing adipocyte numbers, suggests

that the effects on milk production of inhibiting fat deposition in adipose tissue in late lactation could be examined using this approach. It would first, however, be essential to ensure that the antiserum had no effects on mammary tissue and to prevent the inhibitory effects of the antiserum on food intake. Since the reduction in food intake may be an inevitable consequence of the reduction in adiposity, it may be necessary to treat rats before mating, in which case a greater reduction in adiposity than that achieved in the experiments described in this thesis may be required.

7.2.3 The investigation of the regulation of adiposity

In most mammals body fat mass is normally a stable proportion of total body mass, implying that changes in adiposity can be detected and are corrected by alterations in food intake or metabolism. It is not clear whether adipose mass, adipocyte number or adipocyte volume is monitored. The likely response to passive or active immunisation against the adipocyte will depend upon which of these parameters is the 'lipostat'. If adipocyte number or adipose mass is monitored, the response to a reduction in adipocyte number would be the recovery of lost tissue through enlargement of existing adipocytes or proliferation and/or differentiation of pre-adipocytes. If adipocyte volume is monitored, assuming that the reduction in adipocyte number was not accompanied by any change in the mean cell volume of remaining adipocytes, no recovery of lost mass would be expected to occur.

In actively immunised rats 16 weeks after treatment, adipocyte size was increased to compensate for lost adipocyte numbers, so that total dissected adipose mass was similar to that of control rats,

implying that adipose mass rather than adipocyte size is monitored. In contrast, 24 weeks after treatment parametrial adipocytes of passively immunised rats were the same size as those of controls, while the mass of the tissue was 50% of that of controls, suggesting that, at least in this tissue, adipocyte size, rather than adipocyte number or adipose mass is monitored and regulated. Studies of the effects of nutritional manipulations and lipectomy on rats by Faust et al. (1976, 1977, 1978) provide a possible explanation for these apparently contradictory results. Adult rats fed a palatable high fat diet for prolonged periods became obese, initially through an increase in adipocyte size, and then also by increases in adipocyte number (Faust et al., 1978). Upon re-introduction of a normal diet, rats initially lost weight due to the return of adipocyte size to a normal level, but the elevated adipocyte number and, hence, an elevated body weight, was retained. These authors suggested that a given diet predisposes rats to a particular mean adipocyte size, regardless of adipocyte number or adipose mass. However, nutritional manipulations cause a number of metabolic changes, making the results of such studies difficult to interpret. Consequently, the same authors performed a series of lipectomy experiments, whereby adipocyte numbers were reduced without affecting adipocyte size. Removal of the epididymal fat depots of Swiss mice and Sprague-Dawley rats did not result in regeneration of lost tissue or compensatory hypertrophy of remaining fat depots (Faust et al., 1976), again suggesting that adipocyte size is regulated. However, lipectomy of the epididymal fat depot of the Osborne-Mendel rat resulted in compensatory hypertrophy of remaining adipose depots such that total fat mass reached the level of controls, when the rats were fed a

normal diet. When lipectomised rats were fed a high-fat diet, the rats initially showed hypertrophy of remaining depots until a 'critical' adipocyte size was reached, when food intake was reduced and total body fat mass did not reach the level of control rats, fed the same diet (Faust et al., 1977). It would appear that, in this strain of rat, when the animal is lean, adipose mass or adipocyte number is the major regulator. When the animal reaches a particular stage of maturity or 'critical' adipocyte volume, a mechanism whereby adipocyte size is detected and regulated comes into operation. Therefore, while the experiments described above suggest a role for the regulation of adipocyte size in the regulation of adiposity, the time at which this mechanism comes into operation probably depends upon the species and strain of animal. A possible hypothesis to explain the results of passive and active immunization would be that, in Wistar rats, at 19 weeks of age (when the actively immunised rats were examined) adipose mass is the major regulating factor so that loss of adipocytes as a result of active immunisation was compensated for by increases in adipocyte size. At 30 weeks of age, when the passively immunised rats were examined, adipocyte size would have increased so that it may have become the major regulator, resulting in a permanent reduction in adipose mass in passively immunised rats since adipocyte numbers were still decreased. It is worthy of note that in neither form of treatment was there evidence for compensatory increases in cell number. The apparent differences in the response to a decreased adipocyte number in passively and actively immunised rats may have been the result, therefore, of the different stages of maturity at which the animals were killed, which coincided with differences in the way in which adiposity is regulated.

Alternative explanations for the response to passive and active immunisation against the adipocyte, other than that given above, can be proposed. Prolonged exposure of actively immunised rats to low concentrations of anti-(APM) antibodies may have paradoxically stimulated lipid accumulation in adipocytes. A/S 83, at low concentrations, exhibited stimulatory effects on lipid deposition in isolated adipocytes (Flint et al., 1985) so that the elevated adipocyte size in actively immunised rats may have been a direct response to stimulatory antibodies, rather than regulation of body fat mass. However, since the mass of dissectable fat in actively immunised rats was very similar to that of controls, despite a 50% reduction in parametrial adipocyte numbers, this seems unlikely. In passively immunised rats, the surviving parametrial adipocytes took several weeks to recover from the effects of passive immunisation and so 24 weeks after treatment, when the rats were killed, adipocytes may still have been in the process of making compensatory increases in adipocyte size. Had these rats been examined at a later time point, parametrial adipocytes of treated rats may have been found to be larger than those of controls.

Although the response to active immunisation against the adipocyte was uniform in all of the adipose depots studied, passive immunisation by intraperitoneal injection of A/S 83 had a greater effect on parametrial than on other internal and subcutaneous adipose depots. Treatment with A/S 83 caused a significant reduction in subcutaneous and peri-renal adipocyte numbers 1 week after treatment but by 3 weeks after treatment adipocyte numbers in both depots were nearly recovered. The subcutaneous and peri-renal adipose depots may still be in a period of proliferation in female Wistar rats at 6

weeks of age and so lost adipocytes could be replaced. The majority of proliferation in parametrial adipose tissue may be over by 6 weeks of age, reducing the capacity of the tissue for recovery of lost adipocytes. Since most studies of adipocyte development have been performed in male rats, no firm conclusions can be drawn in this regard. Alternatively, since the parametrial adipose depot was initially the depot most affected by treatment with A/S 83, the recovery time for supporting tissues, such as blood vessels necessary for the provision of signals for proliferation or differentiation of preadipocytes, may have been longer for this than for other depots. It cannot be assumed that there was no proliferative response in parametrial adipose tissue in response to the effects of passive immunisation. Proliferation may have been balanced by a gradual loss of damaged adipocytes. Similarly it is not known whether there was any proliferation in adipose tissue of actively immunised rats to replace lost adipocytes. As these rats were examined at only one time point after the initiation of treatment, the time course of loss of adipocytes is unknown, so it is not possible to determine whether adipocyte loss occurred at a time when intense proliferation was still underway in adipose tissue.

In summary, therefore, the results of both active and passive immunisation against the adipocyte are consistent with the regulation of adiposity through the regulation of adipose mass rather than adipocyte size in the female Wistar rat, at least until 19 weeks of age. By 24 weeks of age a mechanism whereby adipocyte size is monitored and regulated may be in operation. This model requires that the animal is able to detect either adipose mass or adipocyte number early in life and adipocyte size later in life. The means

whereby such detection might be achieved are not clear. Liebel (1977) proposed a biological radar system whereby the intensity of a humoral signal, such as insulin or the ratio of insulin to glucose, varied according to the adipose cell surface area. There were no consistent differences in serum insulin or glucose levels in passively immunised rats at any time point measured after treatment. It would be of interest to measure adipsin levels in both passive and actively immunised rats, since this serine protease homologue is synthesized and secreted by adipocytes and levels are decreased in genetically obese rodents where mechanisms regulating adiposity have broken down (Flier et al., 1987).

The model described above also requires that the animal responds early in life to a reduction in adipose mass or adipocyte number by increasing the size of existing adipocytes or increasing proliferation and/or differentiation of preadipocytes. While adipocyte size was clearly increased in actively immunised rats, it is not known whether compensatory increases in adipocyte number also occurred. Later in life, the animal must respond to the attainment of a 'critical' adipocyte size either by recruiting new adipocytes into the population, repartitioning nutrients away from fat deposition or reducing food intake. The rats in the experiments described in this thesis were not allowed to reach a sufficient stage of maturity to answer these questions but Faust (1977) found that lipectomised Osborne-Mendel rats reduced their food intake when a critical adipocyte size had been reached. Vasselli (1986) detected both a reduction in food intake and an increase in adipocyte numbers in Zucker rats upon reaching a 'critical' adipocyte size.

Clearly, a systematic investigation of the effects of passive and active immunisation on adipose tissue at more time points than examined in the experiments described above, including time points beyond 24 weeks after treatment, would clarify the response to these treatments, in terms of adipocyte hypertrophy and hyperplasia, and effects on food intake. However, before conclusions can be drawn from such experiments about the regulation of adiposity in the female Wistar rat, the effects of passive and active immunisation on preadipocytes must be determined in order to discover whether the ability of the rat to respond to lost adipocytes with a proliferative response has been affected. Cryer (1985) found that an antiserum raised against mature rat adipocytes showed little binding to preconfluent adipocyte precursors, but a marked increase in binding was observed after the reaching of confluence, suggesting that the majority of antibodies were directed against adipocyte differentiation antigens, not present or only present at very low frequency on preadipocytes.

The investigation of the very long-term effects of passive or active immunisation against the adipocyte might be aided by the feeding of treated rats with a high fat diet which would promote the reaching of a 'critical' adipocyte size at an early age. Alternatively, a greater reduction in adipocyte numbers might be achieved by repeat treatments with A/S 83, in which case it would first be necessary to tolerise the rats to sheep IgG in order to prevent immuno-neutralisation of antibodies administered subsequent to the primary injection. A combination of passive and active immunisation might also cause a greater reduction in adipocyte numbers than either treatment alone.

It should be noted that any conclusions about the regulation of adiposity drawn from these experiments apply only to the sex and strain of the rat used. However, preliminary experiments showed that A/S 83 bound to APM of rats of a different sex and strain and so this technique might be used for comparisons of the regulation of adiposity of rats of different sex and strains.

7.4 PERSPECTIVE

In the experiments described in this thesis, a long-term reduction in adiposity, accompanied by increased protein deposition, was achieved by passive immunisation against the adipocyte, rendering this a potentially attractive approach towards the manipulation of body composition in meat-producing species. In common with other methods of improving carcass composition, considerations of animal welfare and safety of the consumer may prevent the use of this approach commercially. In addition, lack of knowledge of the long-term effects may prevent its use for the treatment of obese humans. However, both passive and active immunisation against the adipocyte provide a novel and potentially informative means of studying the regulation of adiposity which may provide a greater understanding of the causes of obesity.

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