THE MUCOSAL REGULATION OF THE SYSTEMIC IMMUNE RESPONSE TO CHOLERA TOXIN

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SOME hae meat, and canna eat,

And some wad eat that want it,

But we hae meat and we can eat,

And sae the Lord be thankit.

Robert Burns (1759-1796)

Summary

The mucosal regulation of the systemic immune response to cholera toxin was investigated in inbred adult BALB/c mice.

Specific systemic humoral immunity to parenterally administered toxin and an immunopurified, formalinised, antigenically cross-reactive toxoid was measured using isotype-specific enzyme-linked immunosorbent assays. Systemic delayed-type hypersensitivity (DTH) was shown to occur under similar experimental conditions by direct skin testing and histological examination of the sites of antigenic challenge. The passive transfer of DTH with sensitised, draining, peripheral lymph node cells and the specific inhibition of the **in vitro** migration of these cells in the presence of antigen confirmed that systemic cell-mediated immunity had been induced.

A single feed of either the toxin or toxoid was able to inhibit the induction of systemic DTH but did not tolerise specific systemic antibody levels. Oral tolerance to these antigens was found to be both antigen-specific and dose-dependant. The induction of tolerance was inhibited by the pretreatment of animals with a single dose of cyclophosphamide two days before feeding.

Splenic T lymphocytes, obtained 7 days after feeding, were able to transfer tolerance for systemic DTH to syngeneic recipients. Cells with similar properties were also present in the mesenteric lymph nodes at 7 days but were not detected in either tissue 3 days after a single

feed of antigen. Transferring these cells at various times after the immunisation of recipients revealed that they inhibited the afferent limb of the DTH response. Feeding mice did not suppress mature DTH effector cell function.

Oral tolerance for DTH could also be transferred by serum obtained from animals fed one hour previously. This phenomenon could be abrogated by pretreating recipients with cyclophosphamide. The serum tolerogen did not induce the splenic suppressor T cells found earlier.

These findings are discussed in the light of present knowledge and the methods used in this project are critically reviewed.

Declaration

I declare that this thesis has been composed entirely by myself, and that the work contained within it, except on occasions which are clearly stated, was performed by myself.

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Abreviations

a chain	Immunoglobulin alpha heavy chain
ABS	Alkaline-buffered saline
ACC	Antitoxin-containing cell
ADP	Adenosine diphosphate
APC	Antigen presenting cell
ARC	Absorbed rabbit complement
BALT	Bronchial-associated lymphoid tissue
B cell	Bone marrow-derived lymphocyte
C'	Complement
°C	Degrees centigrade
cAMP	Cyclic adenosine monophosphate
CFA	Complete Freund's adjuvant
CMI	Cell-mediated immunity
CT	Cholera toxin (choleragen)
CTB	Cholera toxin B subunit (choleragenoid)
C3a	Cleavage product of third complement component
C5a	Cleavage product of fifth complement component
dGuo	2'-deoxyguanosine
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
F_1	First generation
g	Unit gravity
GALT	Gut-associated lymphoid tissue
GTP	Guanosine triphosphate

HRP	Horseraddish peroxidase
H-2	Histocompatability locus 2 (mouse)
$H\text{-}2^{d}$,$H\text{-}2^{k}$,etc	Alleles of murine H-2
H-2K	Subregion of murine H-2
$H\text{-}2K^{d}$	Allele of H-2 subregion
Ia	Immune-associated antigen
I-A	Subregion of murine H-2
IFA	Incomplete Freund's adjuvant
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
I-J	Subregion of murine H-2
IL 1	Interleukin 1
IL 2	Interleukin 2
ip	Intraperitoneal
Ir gene	Immune response gene
KLH	Keyhole limpet haemocyanin
LIF	Leukocyte migration inhibition factor
LMIF	Lymphocyte migration inhibition factor
LNPF	Lymph node permeability factor
LP	Lamina propria
Ly/Lyt-1	T lymphocyte associated antigen (mouse - T helper)
Ly/Lyt-2	T lymphocyte associated antigen (mouse - T suppressor/cytotoxic)
M	Molar
MALT	Mucosal-associated lymphoid tissue
McAb	Monoclonal antibody

MCF	Macrophage chemotactic factor
MDP	Muramyl dipeptide
MHC	Major histocompatability complex
MIF	Macrophage migration inhibition factor
MLN	Mesenteric lymph node
MPIF	Macrophage procoagulant inducing factor
μ chain	Immunoglobulin μ heavy chain
NIF-T	Neutrophil migration inhibition factor
OVA	Ovalbumin
p	Probability
PCLF	Antigen-specific T cell-derived factor
PFC	Plaque forming cell
PGE	Prostaglandin E
pH	Reciprocal $\log_{10}$ hydrogen ion concentration
PMNL	Polymorphonuclear leukocyte
PP	Peyer's patch
sIg+	Surface immunoglobulin positive
SRF	Skin reactive factor
SRBC	Sheep erythrocyte
T cell	Thymus-derived lymphocyte
T c/s	T contrasuppressor cell
TD	Formalinised cholera toxoid
TDL	Thoracic duct lymphocyte
Thy-1	T lymphocyte-associated surface antigen
Thy-1.2	Allele of Thy-1 surface antigen
Ts	Suppressor T cell
Ts(aff)	Ts acting on the afferent limb of contact sensitivity
Ts(eff)	Ts acting on the efferent limb of contact sensitivity

V. cholerae	**Vibrio cholerae**

Length

mm	Millimetre
µm	Micrometre
nm	Nanometre

Volume

l	Litre
ml	Millilitre
µl	Microlitre

Weight

kg	Kilogram
g	Gram
mg	Milligram
µg	Microgram
ng	Nanogram

Symbols

<	Less than
>	Greater than
=	Equal to
/	Per
%	Per cent

Introduction to Experiments

Cholera toxin is the soluble exotoxin secreted by the enteric pathogen, **Vibrio cholerae.** As a consequence of this, cholera toxin is normally only encountered via the gastrointestinal tract. However, cholera toxin is by no means a typical antigen. It has certain biochemical properties which separate it from other proteins and induce the profuse diarrhoea which characterises the disease it causes.

The study of this toxin is therefore of great interest to immunologists for several reasons. Firstly, choleragen is an extremely potent antigen capable of evoking a marked immune response with only microgram quantities. Secondly, toxoids exist which are immunologically cross-reacive but lack the other properties of the holotoxin and so allow the examination of the role of its toxicity in the modulation of immune responses. Finally, although a vaccine has been recently developed which confers good protection, at least in the short term, the mechanism by which the onset of diarrhoea is prevented is still uncertain.

Antitoxin antibodies have not been shown to be protective except in the short term and there is evidence that other antigen-specific mechanisms may be responsible for the control of cholereic diarrhoea. Histopathological examination of infected intestine, from both animals and humans, suggests that mucosal cell-mediated immunity (CMI) may be important in the long term protection against this disease but this limb of the potential immune reponse to choleragen has never been

evaluated. By far the most common outcome of feeding soluble protein antigens is the induction of oral tolerance yet choleragen is a notable exception to this rule. If the mechanism(s) by which this disease is prevented is to be elucidated all potentially protective effector mechanisms must be evaluated and the regulation of such mechanisms must also be assessed. Accordingly, the aim of this thesis has been to examine both cell-mediated and humoral immune responses to choleragen, the role of choleragen's toxicity in immunity, the mucosal regulation of such responses and finally their comparison with the results obtained for more usual soluble protein antigens.

What is the systemic immune response to cholera toxin?

It has been established that choleragen can induce good systemic antitoxin antibody responses and, in addition to confirming this, experiments were designed to examine the capacity of this antigen to induce a delayed-type hypersensitivity (DTH) reaction. A simple protocol was employed where adult BALB/c mice were parenterally immunised with toxin in adjuvant and having been challenged intradermally with antigen were examined for DTH by direct skin testing. DTH was assessed in great detail and the ontogeny, histology and specificity of the swelling response was examined.

Serum was obtained for antitoxin antibody analysis throughout the experiment so both effector limbs of the immune response to choleragen could be assessed simultaneously. This has never been done before.

Is cholera toxoid antigenically distinct from the toxin?

As both toxin and a forminalised immunopurified toxoid were used to immunise animals it seemed logical to measure both anti-toxoid and antitoxin antibody responses in these animals. Enzyme-linked immunosorbent assays were developed for this purpose using modifications of existing techniques. Despite the current availability of these assays, no experiments have been done to examine the effects of detoxification on antigenicity in this way and this novel approach has produced interesting findings about the effect of formalinisation on antigenicity.

Does cholera toxin induce cell-mediated immunity?

Besides DTH, other aspects of CMI were examined. Firstly, primed lymphocytes, obtained from the draining lymph nodes of immunised animals, were tested for their ability to transfer DTH to naive syngeneic recipients in the presence of antigen. These cells were also specifically depleted of certain lymphocyte subclasses to ascertain the lineage of the effector cell. Secondly, an **in vitro** assay was developed using primed lymphocytes to measure the production of certain lymphokines.

Does cholera toxin induce oral tolerance?

Varying doses of cholera toxin and toxoid were fed to examine their ability to induce oral tolerance. Using these two forms of the same

antigen, the role of enterocyte binding and enzyme activation could be established in the mucosal regulation of systemic immunity. Furthermore, unrelated protein antigens were used to examine the specificty of observed responses.

Does feeding cholera toxin induce regulatory cells?

The cells regulating the systemic antibody response after feeding choleragen have been characterised elsewhere and experiments were designed to examine the regulation of specific DTH. In order to elucidate the mechanism(s) by which enterically encountered choleragen may alter the subsequent induction of systemic immunity, a series of studies were performed using both the transfer of lymphoid cells and the depletion of certain cell types by pharmacological and immunological means.

Do several mechanisms operate in the induction of oral tolerance to choleragen?

Recent evidence suggests that passage through the gastrointestinal tract may alter the immunogenicity of dietary proteins rendering them tolerogenic. Serum transfer experiments were used to investigate the intestinal 'processing' of choleragen. The relationship between this postprandial serum fragment and the induction of regulatory lymphocytes was also examined.

Chapter 1

IMMUNE RESPONSES TO CHOLERA TOXIN AND ITS DETOXIFIED DERIVATIVES

In this chapter I propose to review the literature on the effect of cholera toxin on the immune system. Some of these effects are due to its antigenic nature but the toxin may also modulate immune responses by its ability to influence cyclic adenosine monophosphate (cAMP) levels within cells. As these actions are related to its structure and biochemical properties, these will also be reviewed.

A number of attempts have been made to produce an immunologically cross-reactive toxoid either by isolating naturally-occurring toxoid (choleragenoid) or by modifying the toxin to render it less toxic. I shall also review these detoxification procedures as well as comparing the immune responses to these toxoids with those to the native toxin.

1.1 The Structure of Cholera Toxin

Cholera toxin is a protein of molecular weight 82,000. The holotoxin is composed of two different subunits, termed A and B. Each toxin molecule has five B subunits each weighing 11,500. The toxic A subunit component is secreted as a single polypeptide but is rapidly cleaved by a proteolytic enzyme into two peptides, A1 and A2 (Mr 22,000 and 5,000 respectively), which are covalently linked by a single disulphide bond. The A subunit is linked to the pentameric B subunit structure through the A2 peptide.

Cross-linking studies, electron micrographs and preliminary x-ray diffraction data suggest that the B subunits form a symmetrical ring

with the A subunit in the midst of them. In this proposed structure the A2 linking peptide lies in the same plane as the B subunits with only the A1 peptide protruding from the rest of the molecule (van Heyningen, 1983).

1.2 The Biochemical Action of Cholera Toxin

Each of the subunits of cholera toxin serve different functions. The B subunit pentamer is responsible for binding the toxin to the surface of cells. Several workers have demonstrated that the B subunits bind preferentially to the GM1 ganglioside component of cell membranes (Cuatrecasas, 1973; Gill & King, 1975; King & van Heyningen, 1973; Moss, Fishman, Manganiello, Vaughan & Brady, 1976). When this receptor is bound, the B subunits undergo a conformational change (Moss, Osborne, Fishman, Brewer, Vaughan & Brady, 1977). Furthermore, van Heyningen (1982) has shown that the conformational change that occurs in B subunits during binding is transmitted to the A subunit.

The A1 peptide is capable of activating adenylate cyclase on its own, but to do so it must penetrate the cell membrane. This process is probably facilitated by the B pentamer. There are several theories regarding the mechanism by which the cell membrane is penetrated. One is that a hydrophillic tunnel is formed between the B subunits and a pre-existing cellular carrier protein which allows entry of the A1 peptide (Gill, 1978). Another is that the binding of the B subunits increases the chance of random entry of the A1 peptide. It has been

suggested that binding may release the peptide from the holotoxin by virtue of the conformational changes induced in the molecule. The most recent theory is that the A subunit may enter cells by endocytosis. Manuelidis and Manuelidis (1976) and Hansson, Holmgren and Svennerholm (1977) have shown that membrane-bound toxin is partly endocytosed. Other workers have shown that drugs inhibiting receptor internalisation and lysosomal processing also inhibit adenylate cyclase activation in intact HeLa cells and hepatocytes (Lin & Taniuchi, 1980; Houslay & Elliott, 1981). Unfortunately, there is no direct evidence to support any of the above ideas.

During cell entry, or soon after it, the disulphide bond connecting the two fragments of the A subunit is split to generate the enzymatically active reduced A1 fragment. This peptide catalyses the mono ADP-ribosylation of the protein Ns, which is a regulatory component of the adenylate cyclase system, present in the plasma membrane of most mammalian cells and displayed on the cytoplasmic surface. This reaction is essentially irreversible under physiological conditions and leads to a series of changes in the protein's properties. Of these, it is the reduced ability to hydrolyse guanine nucleotide triphosphate (GTP) that results in an elevated adenylate cyclase activity and an increase in intracellular cAMP levels. In order for ADP ribosylation to occur, GTP is required to bind to a separate site on the cell membrane, termed 'S', in a cooperative event that involves a soluble protein called cytosolic factor (Gill & Woolkalis, 1985).

The consequence of increased intracellular cAMP levels is determined by the type of cell in which the change occurs. This explains why toxin-treated fat cells release lipid, liver cells convert glycogen into glucose and adrenal cells produce steroid hormone (Greenough, Pierce & Vaughan, 1970; Zieve, Pierce & Greenough, 1970; Donta, 1974). In addition, these biochemical effects have been held responsible for alterations in lymphocyte function but these will be discussed in more detail later on in this chapter.

In the enterocyte, raised cAMP levels, either directly or by the effect on intracellular Ca^{2+} levels, affects electrolyte transport (Frizzell, Field & Schultz, 1979; Ilundain & Naftalin, 1979). **In vitro** data suggest that these mediators, via activation of protein kinases, have two principal effects on electrolyte transport. They inhibit the neutral NaCl absorption mechanism and they create anion conductance channels in the apical membrane of the crypt cell which allows chloride ions to go from the cell to the crypt lumen down an electrochemical gradient (Powell, Berschneider, Lawson & Martens, 1985). It is these processes that are thought to be ultimately responsible for the profuse diarrhoea typical of cholera.

1.3 Immunological Response to Cholera Toxin

In order to give an account of the development of the study of the immunology of cholera toxin, one must first describe how it was appreciated that the disease could be experimentally transferred

without the need for transfer of the causative microbe and that immune responses to the toxin were protective yet separate from the immune response to the vibrio.

In 1883, Koch identified the **Vibrio cholerae** as the causative agent of cholera and a few years later proposed that the disease was toxin-mediated. The confirmation of an exotoxin was supplied independently by two Indian researchers, De and Dutta in 1959 (De, 1959; Dutta, Panse & Kulkarni, 1959).

This disease was shown to cause an immune response in the host, one of the earliest reports being Metchnikoff's observation that there was a rise in the patient's bacteriolytic titre during convalescence (reviewed: Pollitzer & Burrows, 1959). Craig (1965a, 1965b, 1966) reported the presence of a factor in cholera stools and in filtrates of certain broth cultures of **Vibrio cholerae** which produced induration, erythema and increased capillary permeability at the site of intradermal injection in Guinea-pigs or rabbits, reaching a peak at 18-24 hours. He observed that the convalescent sera from confirmed cholera cases were able to neutralise this factor but not human sera with high agglutination titres evoked by commercial cholera vaccine.

Sera from rabbits, obtained after prolonged immunisation with agar-grown living vibrios, were also able to neutralise this toxic factor (Feeley, 1966). Furthermore, it had been noticed that in cholera cases not treated with antibiotics, the time at which antibody appeared in the blood seemed to correlate well with the time that symptoms abated

(Greenough, Gordon, Rosenberg, Davis & Benenson, 1964). Later work questioned whether this toxic factor was separate antigenically from the vibrio which produced it as high toxin-neutralisation titres were noted amongst children under 15 years of age without vibriocidal or agglutinating antibodies (Benenson, Saad, Mosely & Ahmed, 1968). Also, available whole cell vaccines which were toxin-free did not stimulate antitoxin responses in recipients.

The suggestion that antitoxin may play a role in recovery from disease has led to a number of workers examining the humoral responses to cholera toxin in humans and animal models of cholera infection. Central to this investigation has been the development of numerous assays of antitoxic immunity.

1.4 The Measurement of Anti-Cholera Toxin Responses

The plethora of tests of antitoxic immunity described in the literature makes it necessary for a brief summary to be mentioned here.

Assays of the immune response to cholera toxin fall into two main categories:

a. Bioassays of the response to cholera toxin

Cholera toxin's ability to modulate intracellular levels of cAMP produces a number of different effects depending on the biological

system used. The inhibition of these effects by prior incubation of the toxin with antitoxin provides the basis for the measurement of neutralising antibody in an unknown system. This may be quantified either in terms of amount of toxin neutralised or more usually in comparison to a reference serum containing a known amount of antitoxic activity.

This reasoning has been used in the skin permeability factor assay (also called the limit of blueing assay) (Craig, 1965a; Benenson **et al**, 1968; Craig, Eichner & Hornick, 1972), the isolated fat cell assay (Greenough, Pierce & Vaughan, 1970), the mouse adrenal tumour cell assay (Donta, 1974) and the S49-1 lymphocarcinoma cell assay (Ruch, Murphy, Graf & Field, 1978). In all these assays, serum from one animal is assayed using cells or tissues from another, either **in vitro** or **in vivo**.

The intestinal loop assay is different from this. It depends on the measurement of fluid accumulation within a closed loop of bowel after challenge with either cholera toxin or live vibrios (De & Chatterje, 1953; Kasai & Burrows, 1966; Pierce, Banwell, Sack, Mitra & Mondal, 1970). It may be performed using jejunal or ileal loops of bowel and has been used in rabbits, rats and mice (Aziz, Moshin, Hare & Phillips, 1968; Svennerholm, Lange & Holmgren, 1980). The importance of this assay is that it may be performed as an inhibition test, like those above, measuring the antitoxin content of an unknown serum or it may be used as a measure of specific protection to toxin/vibrio challenge within the host animal. This test has several limitations. Firstly,

when used as an inhibition test (and this applies equally to the other inhibition assays) this method does not take into account the isotype or avidity of neutralising antibody and is thus a very indirect measure of humoral response. This assay is an appropriate measure of local protection in the host. However, to infer that the observed protection **in vivo** is a direct consequence of an antibody-mediated response should be exercised with caution.

The footpad oedema model is another bioassay used to measure an antitoxic response in the host animal (Finkelstein & Hollingsworth, 1970).

The most recent assay of local protection at the gut level has been the introduction of an assay for mucosal adenylate cyclase activity (Lönnroth & Lange, 1981). This test measures the response of this enzyme to cholera toxin or other stimulants by the rate of manufacture of cAMP **in vitro.**

b. Immunological assays of the response to cholera toxin

These tests may be used to measure the humoral response directly. They can be broadly subdivided into two groups.

(i) Those tests which measure the limits of antibody directly.

Originally this was done by passive haemagglutination of sensitised sheep red blood cells (Hochstein, Feeley & Richardson, 1970; Hochstein,

Feeley & DeWitt, 1970; Finkelstein & Peterson, 1970) but is being increasingly replaced by radioimmunoassay (Hejtmancik, Peterson, Markel & Kurovsky, 1977) and enzyme-linked immunosorbent assays (Holmgren & Svennerholm, 1973; Young, Levine, Craig & Robbins-Browne, 1980; Svennerholm & Holmgren, 1978).

(ii) Those test which measure antibody producing cells.

This includes assays of isotype specific plaque-forming cells (Lycke, Lindholm & Holmgren, 1983), antitoxin-containing cells (Pierce & Gowans, 1975) or spot-forming cells (Czerkinsky, Nilsson, Nygren, Ouchterlony & Tarkwoski, 1983).

The original antitoxin-containing cell assay employed successive incubations with purified cholera toxoid and then a fluorescein-labelled detecting antibody to detect positive cells. The authors realised that the ability of toxin to bind to most eukaryotic cells would lead to the detection of false positive results if it was used. More recent papers (Lange, Lönnroth & Nygren, 1984a; Lange, Lönnroth & Nygren, 1984b; Lönnroth, Lange & Hansson, 1984) have used purified cholera toxin in this test and thus their estimations of antitoxin-containing cells may be inaccurate. This same criticism applies to the spot-forming cell assay of Czerkinksy et al, 1983.

The final test of humoral immunity is the assay of antigen-specific immunoglobulin production by lymphoid tissues in culture following their removal from an immunised host. This assay has been used in

rabbits (Svennerholm & Holmgren, 1977) and mice (Svennerholm, Lange & Holmgren, 1980) and has the advantage of localising the site of production of any particular antibody isotype. However, all **in vitro** tests must be applied to the **in vivo** situation with caution.

1.5 Immune Responses in Humans after Enteric Exposure to Cholera Toxin

As **Vibrio cholerae** is an intestinal pathogen, it is via the enteric route that cholera toxin is usually encountered. This section will deal with the antibody responses of humans after enteric exposure to cholera toxin as part of a clinical infection. Antitoxic responses in serum and at the mucosal level will both be considered.

Benenson and his co-workers (1968) reported that serum antitoxin neutralisation titres were relatively high amongst Bangladeshi children even in the absence of vibriocidal and agglutinating antibodies. He noted that this titre increased with the presence of cholera infection. This report appears to be at variance with workers in Calcutta, who showed no change in serum IgG or IgA antibodies occurred during disease but a significant rise in IgA titres were seen during convalescence (Waldman, Bencik, Sakazaki, Sinha, Ganguly, Deb & Mukerjee, 1971). However, the antigen specificity of these antibodies was not determined so a rise in neutralisation titres is still possible in these patients. Later studies confirmed that IgG antitoxin titres, although no use in screening a Bangledeshi population for its cumulative exposure to **V. cholerae**, were a sensitive index in the detection of recent infection.

Serum IgA antitoxin titres were of little value in predicting colonisation or disease (Glass, Svennerholm, Khan, Huda, Huq & Holmgren, 1985).

Levine and his co-workers pointed out that interpretation of seroepidemological studies of cholera varies greatly for populations in endemic or non-endemic areas (Levine, Young, Hughes, O'Donnell, Black, Clements, Robbins-Browne & Lim, 1981). Endemic areas have high background titres of antitoxin due both to the prevalence of infection (clinical and subclinical) and due to immunological cross-reactivity with the toxins of other organisms prevalent in those areas (Holmgren, Söderlind & Wadström, 1973). In non-endemic areas few people have background titres of antitoxin. After challenge with **V. cholerae** or natural infection, most become ill and exhibit a rapid rise in antitoxin titres, often to high levels. Antitoxin titres return to baseline in 1 to 2 years.

This is in agreement with a study of American volunteers who had had cholera between 4 and 12 months earlier. These people exhibited higher neutralisation titres than uninfected controls but their haemagglutination antibody levels were not significantly different. This study confirmed earlier reports that antitoxin levels did not help predict the clinical outcome of an oral challenge with live vibrios (Cash, Music, Libonati, Craig, Pierce & Hornick, 1974).

Early analysis of the immunoglobulin content of stools from convalescent patients showed the presence of IgA, IgG and IgM antibodies. Although some of the IgA was of the 11s size most of the IgA was 7s. Smaller fragments of IgA were also detected. In addition, IgG and IgM coproantibody showed signs of enzymatic degradation (Northrup, Bienstock & Tomasi, 1970). Improved enzymatic inhibition by the addition of a soy bean trypsin inhibitor, revealed that the predominant antibody in small intestinal secretions and stool was secretory IgA (Waldman et al, 1971).

Sack and his co-workers analysed intestinal lavage samples collected during the first three weeks after recovery from clinical cholera (Sack, Islam, Holmgren & Svennerholm, 1980). They detected antitoxin in all three classes, but IgA predominated once again, exceeding IgG levels at least 10 fold. IgA antitoxin appeared early and peaked between days 5 to 9 after illness. Levels then declined to a low level by day 17. Further studies revealed that the IgA antitoxin content in lavage samples from convalescent Bangladeshis were still higher than those from healthy age-matched controls 28 days after infection. Furthermore, intestinal challenge with a small dose of B subunit, at this time, caused a significant mucosal IgA antitoxin response in 7/9 of convalescents compared with only 3/8 of controls (Svennerholm, Jetborn, Gothefors, Karim, Sack & Holmgren, 1984).

1.6 Antitoxic Responses to Enteral Toxin in Animal Models

A number of models have been used to examine immune responses to cholera toxin. For the sake of clarity each species of animal model will be considered in turn.

Much work in dogs has been done using Thiry-Vella loops. Intraloop administration of 2 doses of cholera toxin (CT) has been shown to increase systemic antitoxin by a factor of 4 and loop antitoxin titres by a factor of 30. Boosting increases the numbers of antitoxin-containing cells (ACC) in the lamina propria of this loop. The number of ACC in the lamina propria correlates well with the titres of antitoxin found in the loop fluids, and there appears to be an inverse relationship between the prechallenge antitoxin levels in loop fluids and the amounts of fluid secreted in response to toxin challenge (Pierce, Cray & Sircar, 1978). Oral immunisation has been shown to stimulate a serum antitoxin response and protect the animal from the lethal diarrhoea caused by oral administration of live vibrios (Pierce, Cray & Sacci, 1982).

The rabbit model most commonly used also makes use of Thiry-Vella loops and provides very similar results to the canine model. Enteric administration of antigen has been shown to stimulate a systemic IgG and IgA antitoxin response. This procedure also induced a mucosal antibody response mainly of the IgA isotope although some IgG was detected. The capacity of loop fluids to neutralise toxin, in both skin permeability and ileal loop assays, correlated more closely with

IgA than IgG content in these fluids (Yardley, Keren, Hamilton & Brown, 1978; Hamilton, Yardley & Brown, 1979).

Intraduodenal priming of Wistar Lewis rats with CT produced a vigorous jejunal ACC response when the animals were challenged with crude toxoid by the same route. The magnitude of this response varied directly with primary dose used. Response to challenge was maximal by 2 weeks after priming and was maintained when the challenge interval was extended to 16 weeks. By 32 weeks however, the local memory seemed to wane. Enterically administered toxin again produced a systemic antitoxin response but this did not relate to the magnitude of the local ACC response (Pierce, 1978). Similar findings were noted for Lewis strain (Lew/Crl Br) rats which responded best at 7 weeks of age and appeared to have a longer immunological memory (Pierce, 1984). Repeated peroral immunisations of CT in Sprague-Dawley rats stimulated serum and bile antitoxin responses in addition to an increased number of jejunal ACC. In the serum IgG antibodies predominated but IgA and IgM were detectable, if more transient. In the bile IgA was the predominant antitoxic isotope; IgG was present in lesser amounts but continued to increase in parallel with IgA. IgM concentrations remained fairly constant throughout (Lange **et al**, 1984b).

Other workers have described the appearance of specific biliary IgA antitoxin following peroral, intraduodenal, or direct administration of CT into the Peyer's patches. These workers have shown the ability of this biliary antitoxin to neutralise the effect of CT on ileal loop assays after 30 minutes preincubation. But in one report, non-immune

bile was also capable of doing this until bile salts were removed by passage through a AG1 x 8 Biorad resin column (Vaerman, Derijck-Langendris, Rits & Delacriox, 1985; Tamaru & Brown, 1985).

The mouse model provided similar results to those found in other animals. Several workers have reported the presence of specific antitoxin (IgG, IgA and IgM) in the serum of mice fed CT (Svennerholm **et al**, 1980; Lycke **et al**, 1983; Elson & Ealding, 1984a). Furthermore, feeding CT has been shown to produce a local IgA antitoxin response in intestinal secretions (Lycke **et al**, 1983; Elson & Ealding, 1984a), the production of specific IgA and IgG antibody from cultured intestinal tissue (Svennerholm **et al**, 1980), an increase in the number of ACC in the intestinal lamina propria and the appearance of IgA, IgG and IgM antitoxin in bile (Lange **et al**, 1984a).

Protection against CT-induced secretion in a ligated loop assay appeared to correlate with **in vitro** synthesis of IgA antitoxin, IgA levels in gut secretions and the number of intestinal immunisations (Svennerholm **et al**, 1980; Lycke **et al**, 1983). As with the rat model above, the immunological memory for local antibody formation appeared to be dose-dependent (Svennerholm **et al**, 1980). Recent studies have shown that CT-primed mice can produce a brisk, lamina proprial, IgA antitoxin response within 16 hours of antigenic challenge up to 2 years after the initial priming event. Workers have therefore concluded that the administration of choleragen leads to life-long persistence of memory B cells (and perhaps also T cells) in orally

immunised mice (Lycke & Holmgren, 1986a; Lycke & Holmgren, 1987).

1.7 The Cellular Basis of the Antitoxin Response to Enterically Administered Toxin

The ability to demonstrate ACC in various tissues has allowed investigators to follow the migration and isotype production of antigen-specific B cells and has enabled them to dissect tissue-dependent homing of lymphocytes from that which is dependent on the presence of antigen. Most importantly it has enabled workers to study antigenically reactive cells in syngeneic hosts without the need for labelling which may interfere with the very phemonena one is studying. Some of the experiments described below deal with the secondary response to an intraduodenal challenge with toxin of an intraperitoneal immunisation with toxoid. This has been found to be qualitatively, if not quantitatively similar to enteric priming and will be included below for the purpose of clarity.

Rats primed intraperitoneally with toxoid were discovered to have ACC in their thoracic duct lymph (TDL) 5 to 21 days later, this preceeded the appearance of ACC in the jejunal lamina propria which occured 19 to 90 days after priming. If these primed rats intraduodenally challenged, the response was profoundly increased and quickened. Large numbers of ACC appeared in TDL after only 3 days. These cells were shown to migrate to bowel, as cannulation of the thoracic duct prevented their normal appearance (4 to 6 days after challenge) in the

jejunal lamina propria. These ACC were predominantly of the IgA isotype. Immune thoracic duct lymphocytes appeared in the bowel when injected intravenously into naive syngeneic recipients. From this it was concluded that homing to gut of these cells was not antigen-dependent but their distribution within the gut was influenced by the presence of antigen (Pierce & Gowans, 1975).

Elegant experiments using Thiry-Vella loops in the same rats showed the necessity of Peyer's patch (PP) as the source for these thoracic duct ACC, and confirmed that these cells homed to gut but were locally distributed according to the presence of antigen. Furthermore it was shown that the accumulation of ACC at the site of challenge was due both to specific migration of mature ACC (which then died **in situ**) and the local proliferation of ACC precursors (Husband & Gowans, 1978).

Further studies in rats showed that mucosal priming at specific sites primed for a secondary response both at the site of priming and in other areas throughout the mucosa-associated lymphoid tissue (MALT). Challenge at the site of primary antigen exposure always produced the largest response which suggested that a resident population of antigen-responsive cells was induced in addition to the migratory population. However, experiments further subdivided the migratory population into two subgroups. One was shown to recirculate until attracted by the presence of antigen and the other was thought to undergo limited recirculation and then settle in distant MALT. The authors speculated that the first population were probably T cells but the cells which seeded the lymphoid tissue were thought to be B cells (Pierce & Cray,

1978). Experiments in mice showed that indraduodenal CT primed for a B cell response that was disseminated from the PP to the mesenteric lymph nodes (MLN), spleen and bronchial-associated lymphoid tissues (BALT). Using a modification of the Klinman clonal precursor assay, Fuhrman and Cebra (1981) also showed that immunisation with CT produced a preponderance of IgA +ve ACC. Although the frequency of IgM +ve cells declined initially, to be replaced by IgA-producing cells, IgM was once more expressed by 75% of clones after 12 weeks. Confirmation of this proposed migration was demonstrated by Lycke **et al**, 1983, who showed the sequential appearance of IgA plaque-forming cells in the PP, MLN and spleens of orally immunised mice, 3, 5 and 7 days after challenge, respectively.

Elson and Ealding (1984a) demonstrated B cells capable of producing IgA and IgG anti-CT in the lamina propria (LP), PP, MLN and spleen of orally immunised mice. They also demonstrated that feeding induced helper T lymphocytes for IgG and IgA production in the PP, MLN and spleens of these mice. It might be suspected that cells that migrate via the thoracic duct may be found in the peripheral blood of animals and antigen-specific B cells have indeed been discovered in the blood after oral cholera immunisaton in men and mice. But these cells required **in vitro** cultivation to fully develop into antibody-secreting cells (Lycke, Lindholm & Holmgren, 1985). These peripheral blood lymphocytes have been found to secrete IgA antitoxin when cultured from orally immunised hosts but generally produce IgG from parenterally primed subjects. This would appear to confirm the local origin of these cells.

Finally, humans who had been repeatedly orally immunised more than 12 weeks earlier had a population of antigen-responsive cells which secreted IgM when cultured with mitogen (Lycke **et al,** 1985). These IgM +ve memory cells appear to agree with earlier studies (Fuhrman & Cebra, 1981) that demonstrated the appearance of IgM +ve clones 12 weeks after oral immunisation with choleragen in mice.

1.8 Immune Response to Parenterally Administered Cholera Toxin

The immune response to parenteral choleragen initially stimulated interest because immunisations were originally given systemically. These regimes produced high titres of serum antibody but had an unknown effect on mucosal immunity. Furthermore, the role of humoral antibody in protection at mucosal surfaces had not been fully established (Pierce & Reynolds, 1975). These workers demonstrated that passive immunisation with IgG antitoxin protected against cholera toxin-induced fluid accumulation in Thiry-Vella loops in mongrel dogs, even in the presence of repeated flushing of the loops. Using passive immunisation, the workers were able to protect intact mongrel dogs against orogastric challenge with live vibrios.

The potential of this route for inducing protective immunity led to further studies on the immune response to parenterally administered cholera toxin.

Subcutaneous immunisation produced good serum antitoxin titres (Peterson, 1979) but also stimulated low levels of IgG and IgA antitoxin in the intestinal washings of rabbits (Holmgren, Svennerholm, Ouchterlony, Andersson, Wallenström & Westerberg-Berndtsson, 1975; Svennerholm & Holmgren, 1975). Culture of tissue **in vitro,** revealed that immunisation increased synthesis of specific IgM and IgG by the spleen, IgG and especially IgA by the Peyer's patches, and IgA antitoxin by the intestine (Svennerholm & Holmgren, 1977).

Repeated intravenous administration of choleragen produced a good serum antitoxin response on the rat. IgG antibody levels continued to rise with each successive boost but IgA and IgM peaked after 2 or 3 injections and thereafter began to fall. No IgG or IgM was found in the bile of these animals, but a small transient IgA response was seen after the fourth immunisation (Lange **et al,** 1984b).

Intravenous immunisation produced similar results in mice. The animals responded with increased systemic IgG, IgM and IgA antitoxin levels. IgG predominated. Administration of antigen by this route also led to some IgA, but particularly IgG antibody production by cultured intestinal tissue (Svennerholm **et al,** 1980). In addition, parenteral toxin stimulated plaque-forming cells (PFCs) in the Peyer's patches, MLNs and spleens of these animals. PFC responses were in all these immunoglobulin classes but paradoxically declined with repeated immunisations despite a continued rise in antitoxin titres. This unexplained phenomenon recurred after repeated feeds of cholergen in mice (Lycke **et al,** 1983).

The serum IgG antitoxin response appears to be under the control of the I-A region of the H-2 complex. It has been shown that mice of the H-2^b and H-2^q haplotypes are high responders but H-2^k, H-2^s and H-2^d haplotypes respond poorly to intraperitoneal injections of low doses of antigen. Systemic IgA levels after intraperitoneal injection of cholergen did not appear to be affected by the H-2 haplotype of the host in this study (Elson & Ealding, 1985).

1.9 Modulation of Immunity by the Biochemical Actions of Cholera Toxin

Cyclic AMP plays an important role in the functions of many cells and so it is not surprising that cholera toxin may influence the activity of immunological cells by virtue of its effect on cAMP. In particular there are many reports of the toxic effects of choleragen affecting immunological responses both **in vitro** and **in vivo.** These effects would appear to be independent of the possible immune response to this protein as an antigen.

Amongst its many actions cholera toxin has been shown to inhibit natural killer (NK) activity in mouse and human lymphocytes (Fuse, Sato & Kuwata, 1981; Goldfarb & Herberman, 1981; Fuyama, Sendo, Watabe, Seiji & Arai, 1981; Fuyama, Naiki & Sendo, 1982) as well as inhibit the lectin-induced DNA synthesis in both T and B cells (Sultzer & Craig, 1973; Holmgren, Lindholm & Lönnoth, 1974). It can also inhibit the cytotoxic action of immunised mouse spleen cells against an allogenic

mastocytoma cell line and the antigen-induced IgE-mediated release of histamine from human leukocytes (Lichtenstein, Henney, Bourne & Greenough, 1973).

It has been shown to have both inhibitory and stimulatory effects on the PFC response of mice to sheep red blood cells (SRBC) (Northrup & Fauci, 1972; Lindholm, Holmgren, Lange & Lönnroth, 1976). The adjuvant action of CT when administered prior to immunisation was thought to be due to inhibition of suppressor cells (Lindholm **et al**, 1976) and the immunosuppression seen when CT is administered concurrently with SRBC has been attributed to its action on macrophages and helper T cells (Lyons & Friedman, 1978; Kately, Holderbach & Friedman, 1978).

All these effects have been attributed to an increase of the intracellular cAMP levels of lymphocytes (Lichtenstein **et al**, 1973; Fuyama **et al**, 1982; Fuse **et al**, 1981; Holmgren **et al**, 1974; Kateley, Kasarov & Friedman, 1975, Lindholm **et al**, 1976). It is fair to say that although these biochemical changes have been observed a causal relationship between the biochemistry and immune function of these cells has not yet been established.

Tsuru and his co-workers demonstrated that cholera toxin could also augment the delayed footpad reaction to SRBC in mice when given parenterally 1, 7 or 10 days before immunisaton (Tsuru, Zinnaka, Nomoto & Takeya, 1981). They reasoned that this reaction was not due to toxin's effect on adenylate cyclase as the effect could be minimised by

a formalin-inactivated cholera toxoid (Tsuru, Namoto, Oka, Kitani, Zinnaka & Takeya, 1983). This may well not be the case for two reasons, formalinised toxoid may well revert to toxicity **in vivo** and, secondly, the authors used a hundred fold increase in the dose of toxoid to achieve this effect. The residual toxicity of such a dose may well be enough to stimulate the necessary cAMP response. Both these properties of toxoids will be discussed in greater detail in this chapter.

In addition to the modulatory effects of systemic toxin, cholera toxin may affect immune responses when administered orally. When fed with keyhole limpet haemocyanin (KLH), CT was able to prevent the induction of oral tolerance for systemic IgG and simultaneously induce a secretory IgA response to KLH. It has been suggested that either the binding of CT to the intestinal mucosa, or activation of adenylate cyclase or a combination of the two may be responsible for the alteration in the regulatory environment that cholera toxin seems to be able to induce (Elson & Ealding, 1984b; Lycke & Holmgren, 1986b). It was interesting, in the light of this discovery, that the B subunit of cholera toxin is capable of inducing increased secretory IgA and serum IgG responses to orally administered horseradish peroxidase (HRP), but that the HRP must be covalently bound to the B subunit for this to occur (McKenzie & Halsey, 1984). Once again, these results have been attributed to the lectin-like actions of B subunit but these properties have not been causally related.

1.10 Modulation of Immunity by the Immunological Actions of Cholera Toxin

Choleragen is a highly immunogenic protein and as with other antigens may alter the immune response to itself and other antigens by virtue of its immunological properties. Yardley and his co-workers (1978) showed that combined subcutaneous and intraintestinal immunisaton with cholera toxin in rabbits suppressed the local IgA antitoxin response seen after intraintestinal immunisation alone. This effect has been confirmed by Pierce and Koster (1980) who showed that parenterally administered toxoid could also suppress both primary and secondary mucosal immune responses to toxin. As subcutaneous immunisations alone produced little IgG or IgA antitoxin in loop fluid (Yardley **et al,** 1978) it is unlikely that this phenomenon was due to the neutralisation of antigen by antibody before it could bind to bowel. Studies of the effect of antibody on the mucosal immune response would seem to agree with this conclusion. Whereas, hyperimmune serum was able to suppress primary mucosal response, the development of specific memory and, to some extent, the secondary mucosal response, this was not so for 'primary' immune serum. This 'primary' immune serum was obtained from intraperitoneally immunised rats whose mucosal immune response to intraduodenal toxin had been suppressed (Pierce, 1980).

It was suggested that the suppression of secretory IgA antitoxin in rabbit was not due to the 'toxigenic' effects of choleragen as it could be simulated by moderate doses of a gluteraldehyde-inactivated toxoid given by the same route. The authors concluded that the suppressive

effect was therefore probably by an 'immunogenic' mechanism (Hamilton **et al**, 1979). This has been confirmed by Koster and Pierce (1983) who demonstrated the appearance of nylon wool-nonadherent splenic suppressor cells after parenteral immunisation of rats with toxoid. The suppressive effect of these cells became greater with time and they could be found in the thoracic duct lymph and thymus 2 to 4 months after immunisation.

1.11 The Preparation and Antigenicity of Cholera Toxoids

Cholera toxin is a potent immunogen but its toxic properties make it totally unsuitable as a candidate for a potential vaccine. As a result, means have been sought whereby the antigenicity of the protein may be preserved but its toxicity eliminated. Three approaches to this problem will be considered and the immune responses to each type of toxoid produced will be discussed. Ideally, a toxoid should be immunologically cross-reactive with the parent molecule but without any residual toxicity. It will become apparent that only B subunit fulfils the latter of these criteria. Nevertheless, the result of any detoxificatition procedure will be referred to as a toxoid for the purposes of this discussion.

a) Chemical detoxifications

It was found that treatment of cholera toxin with formaldehyde **in vitro** erradicated its toxicity. This resulted in a toxoid which exhibited little biological activity but that stimulated antitoxic antibodies after parenteral immunisation in monkeys. Unfortunately, this toxoid was also shown to revert to partial toxicity causing local reactions at the site of inoculaton (Northrup & Chisari, 1972).

Inactivation by ammonium sulphate precipitation of a formalin-treated culture medium produced a crude toxoid with some toxin and somatic antigen contamination. Alternatively, the culture filtrate could be passed down an equine antitoxin-bound sepharose column and the eluate neutralised, filter sterilised, incubated with formalin for 21 days, dialysed and sterilised once more to produce a purified formalinised toxoid. Both these toxoids have been prepared by Dr R O Thompson of the Wellcome Research Laboratories, Kent and have been used extensively in animals.

The purified toxoid was capable of inducing a good systemic antitoxin response after subcutaneous administration in rabbits (Peterson, 1979) and intraperitoneal immunisation in rats (Pierce, 1980). Intraperitoneal immunisation produced mucosal ACC priming in rats (Pierce & Gowans, 1975) and stimulated the development of mainly IgG but also IgA ACC in the Peyer's patches, mesenteric lymph nodes and spleens of mice (Fuhrman & Cebra, 1981). Crude toxoid administered subcutaneously, stimulated a systemic antitoxin response in dogs (Sinha

& Bhaskaran, 1973; Pierce & Reynolds, 1975). In these animals, the presence of serum antibody correlated with the appearance of antitoxin in their Thiry-Vella loop washings. It has been suggested that under these conditions most of the gut antibody is derived from serum (Pierce & Reynolds, 1974; Pierce & Reynolds, 1975). Crude toxoid was also able to induce a local secretion of dimeric IgA antitoxin when instilled in canine Thiry-Vella loops (Pierce & Reynolds, 1975). Furthermore this toxoid could also stimulate an ACC response in the jejunum of intraduodenally primed rats.

In contrast, the purified toxoid did not prime for a local humoral response in these rats but was as effective as crude toxoid or CT itself in intraduodenally boosting a primed animal (Pierce & Gowans, 1975; Pierce, 1978).

Chemical detoxification has also been accomplished by treating the purified toxin with gluteraldehyde (Rappaport, Bonde, McCann, Rubin & Tint, 1974). The resultant toxoid was further treated by ultrafiltration to remove any contaminating somatic antigen (Rappaport, Pierzchala, Bonde, McCann & Rubin, 1976). This final step eliminated the development of systemic vibriocidal antibodies in intramuscularly immunised mice but appeared to maintain the antitoxin response in these animals.

Subcutaneously administered toxoid appeared able to produce a good systemic IgG response in rabbits concurrent with the appearance of small amounts of IgG and IgA antitoxin in the Thiry-Vella loops of

these animals (Hamilton et al, 1979). This toxoid appears to stimulate very similar systemic antitoxin levels after parenteral immunisation in rabbits to those produced by the formalinised purified toxoid (Peterson, 1979). The gluteraldehyde toxoid evoked high titres of circulating antitoxin in human volunteers when administered parenterally (Peterson, Verway, Craig, Guckian, Williams & Pierce, 1974) but was also able to produce a systemic humoral response, elicit higher rates of seroconversion and stimulate higher geometric mean antitoxin titres than saline immunised controls when multiple doses were given orally (Levine, Hughes, Young, O'Donnell, Craig, Holley & Bergquist, 1978).

Studies in dogs revealed that although parenterally adminstered toxoid primed for both a systemic and mucosal antitoxin response, enteric administration of this toxoid failed to induce an ACC response or the secretion of antitoxin into the Thiry-Vella loops of these animals (Pierce et al, 1978).

Both the gluteraldehyde- and formalin-treated purified toxoids appeared to be poor at priming an immune response, either mucosal or systemic, when administered enterically compared to the parent toxin. This has been attributed both to their inability to bind to gangliosides and their failure to activate adenylate cyclase (Pierce, 1978; Pierce et al, 1978).

b) Production of isolated choleragenoid (B subunit)

Choleragenoid was originally found to be produced by the **Vibrio cholerae** in addition to CT (Finkelstein & LoSpalluto, 1969). It was discovered that it could be produced in greater quantities from the parent toxin, and that isolated choleragenoid was antigenically identical to the toxin but completely devoid of its toxic properties (Finkelstein & LoSpalluto, 1970).

Choleragenoid retains its ability to bind to GM1 gangliosides and provokes an ACC response in the jejunal lamina propria after oral administration to dogs or intraduodenal injection in rats. In both these cases the ACC counts were less than those observed in animals immunised with holotoxin (Pierce, Cray & Sacci, 1982; Pierce, Cray, Sacci, Craig, Germanier & Fürer, 1983). Oral immunisation with B subunit produces systemic PFC and antitoxin responses in mice of all three classes. It also produces less secretory IgA and less protection against toxin-induced fluid secretion than oral immunisation with choleragen (Lycke **et al**, 1983). Feeding choleragenoid fails to tolerise the induction of systemic IgG antitoxin by its subsequent intraperitoneal administration (Elson & Ealding, 1984b).

A single dose of B subunit either orally (500 μg) or intramuscularly (150 μg) in Bangladeshi volunteers produced a local intestinal secretory IgA response. The magnitude of the response was similar by both routes but the antitoxin response persisted longer after oral immunisation. Levels of antitoxin seen in these volunteers were

comparable to those observed after clinical infection (Svennerholm **et al,** 1984).

c) Heat inactivation

Finkelstein, Fujita and LoSpalluto (1971) described the production of a high molecular weight polymer, designated "Procholeragenoid", when cholera toxin was heated. It was an intermediate formed optimally by heating CT for 5 to 10 minutes at 56° C. If the toxin was heated further purified B subunit was isolated. This intermediate had 5% of choleragen's initial toxicity, by the limit of blueing assay, and was found not to revert to toxicity **in vivo.**

Workers concerned about the residual toxicity further inactivated this polymer by treatment with formalin. Intramuscular immunisation with this formalinised procholeragenoid produced good antitoxin titres but also a weaker vibriocidal antibody response in rabbits. It also produced good titres of neutralising antibodies in Guinea-pigs when injected by the same route (Germanier, Fürer, Varalley & Inderbitzin, 1976). This toxoid induced good antitoxin titres after intramuscular injection into human volunteers (Germanier, Fürer, Varalley & Inderbitzin, 1977).

Unaltered procholeragenoid has also been enterically administered to rats and dogs. In rats it produced a marked response after intraduodenal immunisation. The magnitude of this response was almost

equal to purified toxin but was appreciably reduced by prior incubation with GM1 ganglioside or by treatment with formalin. In dogs, procholeragenoid administration markedly protected them from the lethal diarrhoea following live vibrio challenge and increased the jejunal ACC count in these animals (Pierce, Cray, Sacci, Craig, Germanier & Fürer, 1983).

The residual toxicity of all these toxoids have been compared by the limit of bluing assay. Crude formalinised toxoid has the most residual toxicity. Purified formalinised toxoid, gluteraldehyde-inactivated toxoid and procholeragenoid all had similar toxicities of approximately 5% of the holotoxin. The residual activity of procholeragenoid could be further reduced by formalinisation. Only B subunit is totally non-toxic as it does not contain the A subunit necessary for adenylate cyclase activation (Pierce, 1978; Pierce **et al**, 1983)

1.12 Other Factors which may Prevent Choleragen-induced Intestinal Hypersecretion.

As can be seen from the foregoing account, many authors have correlated the protection observed against CT-induced intestinal secretion (or the lethal diarrhoea after live vibrio challenge) with the presence of antitoxin. The ability of antitoxin to neutralise the biological activities of choleragen (in particular the induction of fluid secretion) after periods of incubation has fostered the assumption that protection against cholera is mediated by antibody.

It is difficult to reconcile this with the observation that convalescent volunteers who had antibody titres similar to vaccinated, or in some cases unimmunised controls, had significantly greater protection against live vibrio challenge (Cash **et al,** 1974). Furthermore, it was observed that there was significant protection against live vibrio challenge in immunised dogs over 6 months after jejunal ACC were last detectable and serum antitoxin titres were less than twice pre-immunisation levels. The authors concluded "it remains possible that protection ..... was due to an immune response other than, or in addition to, the production and secretion of antitoxin" (Pierce **et al,** 1978).

More recently it has been shown that protection is greater in toxin-immunised dogs (Pierce **et al,** 1983) or mice (Lange **et al,** 1984a) than those receiving enteric B subunit even though both animals have equivalent mucosal or systemic antitoxin responses. Furthermore, it has been demonstrated that mucous release, toxin binding or toxin internalisation is not affected by multiple oral immunisations with toxin and the desensitisation of mucosal adenylate cyclase (thought to be responsible for this down-regulation of intestinal secretion) is unrelated to antibody levels in the intestine, bile or serum of both rats and mice. The fluid response to adenylate cyclase stimulation is only depressed for 4 to 7 days when it is elicited by prostoglandin E1 but appears to remain inhibited for more than a month when CT is used (Lange **et al,** 1984b; Lönnroth **et al,** 1984).

A protein factor, of molecular weight 30,000, has been isolated from the brain, pituitary and intestine of animals orally immunised with cholera toxin (Lange & Lönnroth, 1984). This factor, which has lectin-like properties, is able to prevent intestinal hypersecretion only if given shortly before challenge with choleragen. It has been found in the bile and breast milk of immunised animals and has been thought to have hormone-like actions (Lange & Lönnroth, 1984; Lönnroth & Lange, 1984; Lange & Lönnroth, 1986). Unfortunately, the authors have not been able to detect its presence in serum or to demonstrate its ability to desensitise adenylate cyclase. Most significantly, animals exhibiting mucosal adenylate cyclase desensitisation do so in an antigen-specific manner (Lönnroth **et al**, 1984), and these authors have not attempted to explore the specificity of this factor or to explain the mechanism by which a putative hormone may exhibit this property.

Other protective mechanisms may also be responsible for the prevention of cholereic diarrhoea. Sprinz (1962) observed that patients suffering from cholera exhibited crypt hypertrophy and villous atrophy in intestinal biopsies. Increased eosinophil counts have also been observed in the intestines of choleragen-fed rats (Lange **et al**, 1984b) and both these phenomena are thought to be indicative of a T cell-mediated immunological response within the intestinal mucosa (Basten & Beeson, 1970; Colley, 1973; reviewed: Ferguson, 1987).

In the light of this, it is interesting that Russian workers have reported the presence of "specific delayed-type hypersensitivity" in the intestine and lymph nodes of previously germ-free rats chronically infected with cholera vibrios (Gorskaya, Chakkava, Rubstov & Gailonskaya, 1980). Also, other workers have claimed that protection from the vibrio is associated with decreased adhesion and colonisation and that this is accompanied by an increase in the number of intraepithelial lymphocytes (especially "granular" lymphocytes) and the number of lymphoid and plasma cells in the lamina propria (Efremov, Polotsky, Samiostrelsky, Vasser & Seliverstova, 1982). Finally, nude mice do not produce specific IgA when orally immunised with choleragen but both specific IgA and protection against toxin-induced hypersecretion could be restored by subcutaneous thymus-grafting (Lycke, Ericksen & Holmgren, 1987).

Chapter 2

ORAL TOLERANCE TO ENTERIC ANTIGENS

2.1 The Immunological Effects of Feeding Antigen

A number of different immune responses have been reported after the enteric administration of antigen. These may affect both local and systemic immunity and include anergy, tolerance, immunity and hypersensitivity. These reactions are not mutually exclusive and may occur in the same animal depending on the nature and anatomical location of the immune response in question.

Feeding antigen may induce a systemic humoral immune response and circulating antibodies have been found after the ingestion of a number of different antigens including bacteria, viruses, contact sensitising agents, heterologous red blood cells and food antigens (Waldman & Ganguly, 1974; Bradley, Kim & Watson, 1963; Asherson, Zembala, Perera, Mayhew & Thomas, 1977; David, 1979; Rothberg, 1969). In addition to humoral immunity, feeding may also induce a systemic cell-mediated immune reaction (Perrotto, Hang, Isselbacher & Warren, 1974; Asherson et al, 1977). As well as systemic immunity, lumenal exposure to a variety of antigens may also result in a local antibody response especially of the IgA isotype (reviewed, Bienenstock & Befus, 1980).

Although one result of the enteric encounter with antigen may be the induction of specific immunity, an alternative outcome is the induction of tolerance. Oral tolerance has been defined as the induction of a state of specific systemic immunological hyporesponsiveness following the oral administration of antigen and appears to occur more commonly than active immunity after feeding. The remainder of this chapter will

be addressed to a brief overview of the mucosal downregulation of systemic immunity and will include descriptions of the conditions under which oral tolerance may be induced, techniques by which it can be abrogated and the mechanisms thought to be responsible for its occurrence.

2.2 Oral Tolerance

One of the earliest descriptions of oral tolerance was by Dakin in 1829 who described a practice amongst North American Indians of feeding Rhus leaves to their young in order to prevent the development of poison ivy contact dermatitis later in life. In 1911, Wells studied this phenomenon scientifically (Wells, 1911; Wells & Osborne, 1911) and since then downregulation of systemic immunity has been shown to occur after feeding a wide variety of antigens including contact sensitising agents (Chase, 1946; Asherson et al, 1977), protein antigens (Thomas & Parrott, 1974; Hanson, Vaz, Maia, Hornbrook, Lynch & Roy, 1977; Ngan & Kind, 1978) and heterologous red blood cells (Kagnoff, 1978a; Kagnoff, 1978b; Mattingly & Waksman, 1978).

Although oral tolerance may be induced by a wide variety of antigens other factors play an important role in its development including the doses of antigen used, the frequency of administration and the timing of feeding relative to subsequent challenge (reviewed, Bienenstock & Befus, 1980). In addition to these, the age at which antigen is first encountered also appears to determine whether tolerance ensues.

Feeding ovalbumin (OVA) to mice during the neonatal period or at the time of weaning fails to induce the systemic tolerance normally encountered after such a procedure (Hanson, 1981; Strobel & Ferguson, 1984).

The role of the major histocompatibility complex (MHC) in the control of immunity is well-established and recent evidence suggests that the H-2 locus in mice is important in the induction of tolerance to parenterally administered antigen (Lowy, Drebin, Monroe, Granstein & Greene, 1984; Robbins, Thomas, Jensen & Kapp, 1984). Stokes and his colleagues have already demonstrated that differing strains of mice differ in their ability to be tolerised (Stokes, Swarbrick & Soothill, 1983). Tomasi and his colleagues have confirmed these findings but failed to link this difference to the H-2 haplotype of these animals (Tomasi, Barr, Challacombe & Curran, 1983). Unfortunately this work was not done on congenic strains of mice so that other genetic differences may be interfering with the interpretation of results.

2.3 Inhibition of Oral Tolerance

The inhibition of tolerance by pharmacological and immunological procedures has allowed workers to examine this immune process in more detail.

The induction of oral tolerance by feeding OVA has been shown to be abrogated by pre-treatment with cyclophosphamide (Hanson & Miller,

1982; Mowat, Strobel, Drummond & Ferguson, 1982). It was postulated that this phenomonen was related to the effect of cyclophosphamide on suppressor T cells (Röllinghoff, Starzinski-Powitz, Pfizenmaier & Wagner, 1977; Schwartz, Askenase & Gershon, 1978). Experiments, performed at that time, appeared to rule out the possible effect of this drug on the intestinal mucosa as a reason for this alteration in the immune response (Sobhon, Wanichanon & Sretarugsa, 1977; Mowat **et al**, 1982; Mowat, 1981).

Experiments using the drug 2'-deoxyguanosine (dGuo) have recently confirmed these findings (Mowat, 1986). This drug is converted to toxic metabolites by the action of many different purine degradation enzymes (Carson, Kaye & Seegmiller, 1977; Cohen, Lee, Dosch & Gelfand, 1980, Spaapen, Rijkers, Staal, Rijksen, Wadman, Stoop & Zegers, 1984) and selectively prevents the generation of suppressor T cells **in vivo** (Dosch, Mansour, Cohen, Shore & Gelfand, 1980; Varey, Lelchuk, Hutchings & Cooke, 1983; Bril, Van den Akker, Molendijk-Lok, Bianchi & Benner, 1984; Bril, Van den Akker, Hussarts-Odijk & Benner, 1985). Both intestinal epithelial cells and DTH effector cells do not contain the necessary kinase enzymes and are resistant to the actions of dGuo (Carson **et al**, 1977; Bril **et al**, 1984; Bril **et al**, 1985). The use of dGuo after feeding OVA also abrogates the induction of tolerance and is further compelling evidence for the involvement of suppressor T cells in this phenomenon (Mowat, 1986).

Pre-treatment of mice with either oestradiol or muramyl-dipeptide (MDP) was also able to reverse the usual suppression of serum antibody and DTH responses normally observed after feeding OVA (Mowat & Parrott, 1983; Strobel & Ferguson, 1986).

Introduction of a graft-versus-host reaction in F_1 mice once again prevented the enteric downregulation of systemic DTH and anti-OVA IgG antibody responses. The abrogation of tolerance was associated with an augmented ability of spleen cells to present OVA to primed T cells while the phagocytic activity of the reticuloendothelial system of these animals appeared to remain unaltered (Strobel, Mowat & Ferguson, 1985). Preliminary experiments performed by Dr Mowat suggest that activation of antigen-presenting cells (APC) may also occur following pre-treatment of mice with oestrodiol and MDP (personal communication).

It would appear from this data, that gut-associated APC may well play a role in the mucosal regulation of systemic immunity.

2.4 Mechanisms Responsible for Oral Tolerance

A number of processes have been implicated in the immunological suppression of systemic immunity following feeding. These include both serum and cellular fractions which may act independently or in combination.

These mechanisms have been elicited after feeding a variety of antigens and it has become apparent that while some mechanisms may be shared others appear peculiar to one particular substance. Some of these differences have been attributed to the physical nature of the antigen i.e. whether the antigen was particulate or soluble, but most particulate antigens would be rendered soluble when administered by gavage. The two antigens used most frequently in the study of the mechanisms of oral tolerance are sheep red blood cells (SRBC) and OVA and this section will primarily include descriptions of the results obtained from the use of these. One limitation of this work is that these studies use antigens which are relatively large and may be expected to contain more than one antigenic epitope. All the immune reactions have been measured to the antigen as a whole and so the fine regulation of particular idiotypic responses still remains to be elucidated.

Some of the variety of mechanisms involved in oral tolerance are illustrated by feeding SRBC to mice. The tolerance observed in serum antibody and splenic plaque-forming cell (PFC) responses has been attributed to circulating antigen-antibody complexes (André, Heremans, Vaerman & Cambiaso, 1975). Later work, although not confirming the tolerance observed with IgG and IgA anti-SRBC PFC responses, attributed the downregulation of specific splenic IgM responses to anti-idiotypic antibody (Kagnoff, 1978b). Other workers have isolated an IgG fraction from the serum of fed animals which suppresses anti-SRBC PFC responses **in vitro** (Chalon, Milne & Vaerman, 1979).

In addition to serum-borne suppressive factors, feeding SRBC to mice also induces a feedback suppressor pathway where Ly-1+ 23-/I-J+ T cells can suppress specific splenic PFC responses by causing normal T cells to become suppressor effectors (MacDonald, 1982a; MacDonald, 1982b).

It is interesting to note that although feeding SRBC may suppress specific PFC responses, removal of Peyer's patch suppressor T cells can reveal the priming of B cells in the presence of oral tolerance which has been induced by feeding ovalbumin (Titus & Chiller, 1981a). Other experiments using human gammaglobulin (HGG) have revealed that B cell anergy may also occur after feeding (Vives, Parks & Weigle, 1980). This serves to emphasise the problems of extrapolating the results obtained with one antigen to that obtained from another.

Feeding SRBC not only tolerises humoral immunity but the induction of systemic CMI may also be suppressed. This phenomenon has been ascribed to splenic suppressor cells which appear to inhibit the development of DTH effector cells (Kagnoff, 1978a).

Many of the above findings elicited after feeding SRBC have been confirmed and extended using the soluble protein, ovalbumin. Suppressor T cells that inhibit anti-OVA IgG antibody and IgE antibody responses have been demonstrated after feeding. These cells appear first in the Peyer's patches (PP) and migrate from the bowel, appearing next in the mesenteric lymph nodes (MLN) and later in the spleen (Ngan & Kind, 1978; Richman, Graeff, Yarchoan & Strober, 1981). A similar migration pattern for T suppressor cells (Ts) has also been described

after feeding SRBC. T cells capable of suppressing anti-SRBC IgM and IgG PFC responses and antigen-specific DTH were detected in the PP and MLN of rats within 2 days of feeding. After 4 days of feeding these cells were no longer present in the PP or MLN but could be found instead in the thymuses and spleens of these animals (Mattingly & Waksman, 1978).

Oral administraton of OVA also induces splenic T cells capable of preventing the development of specific DTH effector cells (Miller & Hanson, 1979). While it would appear from the foregoing account that mucosal regulation of systemic immunity is most commonly accomplished by Ts, B suppressor cells have been additionally identified as responsible for the oral tolerance observed after feeding contact sensitivity agents (Asherson **et al,** 1977). Feeding OVA appears to induce Ts for both humoral and cell-mediated immune responses but there is evidence to suggest that these regulating mechanisms may operate independently. Studies have shown that whereas feeding 2 mg OVA to BALB/c mice only tolerises sytemic CMI and IgM anti-OVA antibody responses, a larger feed of 25 mg inhibits the development of specific systemic IgG and IgM antibody as well as CMI. Furthermore, pre-treatment with cyclophosphamide completely abrogates the tolerance induced by feeding 2 mg OVA but only partially inhibits the induction of tolerance seen after oral administration of the larger dose of antigen (Mowat **et al,** 1982).

Further evidence of the separate nature of the regulatory mechanisms controlling different limbs of systemic immunity has come from the study of 'gut processed' antigen. 'Processing' is the term used to describe the ability of the gut to generate a tolerogenic form of antigen which is detectable in the serum of mice one hour after feeding. These studies have been carried out after feeding OVA, and have revealed that the tolerogenic serum fragment is antigen-specific but is only capable of inducing tolerance in systemic CMI. Experiments using cyclophosphamide suggest that this may be achieved by inducing the development of suppressor T cells (Strobel, Mowat, Drummond, Pickering & Ferguson, 1983; Bruce & Ferguson, 1986a). Further characterisation of the serum tolerogen revealed that it has B cell determinants in common with the fed antigen and has a similar molecular weight (Bruce & Ferguson, 1986b). This is the first description of the transfer of tolerance by serum antigen from protein-fed mice and serves to underline the close association between the absorptive and immune functions of the intestine.

Experiments studying the effect of feeding native or denatured OVA have served to highlight the dissimilarities that exist between orally- and parenterally-induced tolerance (Mowat, 1985). Suppressor T cells are believed to recognise intact antigens (Feldmann & Kontiainen, 1976) whereas helper T cells respond to determinants within the primary structure of protein antigens that have been processed and presented by APC. It is therefore surprising that both antigenic forms induced oral tolerance which was cross-reactive. Although splenic suppressor T cells were only found after feeding the native antigen, it has been

shown that pulsed macrophages can induce Ts that cross-react with both forms of OVA **in vitro** (Takatsu & Ishizaka, 1977).

Earlier studies, however, have inferred that the antigen-presenting cell may inhibit the induction of oral tolerance (Mowat & Parrott, 1983; Strobel **et al**, 1983; Strobel & Ferguson, 1986). Although the APC examined in these studies were isolated from spleens, other workers have examined the ability of Peyer's patch accessory cells to present antigen. Peyer's patch cells were found to be good stimulators of primary and secondary mixed lymphocyte reactions but were poor at presenting soluble antigens to sensitised lymph node cells (Tomasi **et al**, 1983). Furthermore, feeding these soluble antigens did not induce a T-cell proliferative response in the PP lymphocytes and this could not be reversed by drugs shown to be effective against suppressor cells.

Peyer's patches have been shown to contain helper T (Kiyono, Babb, Michalek & McGhee, 1980; Richman **et al**, 1981; Elson & Ealding, 1984a), suppressor T (Ngan & Kind, 1978; Richman **et al**, 1981; MacDonald, 1983) and even contrasuppressor T cells (Green, Gold, St Martin, Gershon & Gershon, 1982) after feeding and it has been assumed that the APC present in the PP were responsible for their induction. The evidence presented above would suggest that this is not the case for the cells involved in oral tolerance. This has been recently confirmed by Enders and his colleagues who were able to demonstrate the induction of oral tolerance in rats whose PP had been surgically removed (Enders, Gottwald & Brendel, 1986).

A series of experiments performed by Bland and Warren have suggested an alternative site for the presentation of antigen in the gastrointestinal tract. Using isolated villous, columnar, epithelial cells in short-term cultures they have shown that these cells are capable of binding antigen and presenting it to T cells (Bland & Warren, 1986a). Rat lymph node cells exposed to antigen and epithelial cells were able to mediate antigen-specific suppression of proliferative T cell responses. The generation of suppressor activity was accompanied by an increase of the expression of suppressor phenotype as shown by monoclonal antibodies (Bland & Warren, 1986b). Caution must be exercised in the extrapolation of **in vitro** phenomena to the **in vivo** situation but this work presents an intriguing alternative to the PP APC as a source of suppressor T cell induction in oral tolerance.

2.5 Contrasuppression and Oral Tolerance

Challacombe and Tomasi (1980) have demonstrated that feeding either ovalbumin or **Streptococcus mutans** to mice induced specific salivary IgA antibodies as well as tolerance in systemic humoral immunity. Increased secretory immunity in the presence of systemic tolerance has also been shown after intranasal inoculation with poliovirus (Ogra & Karzon, 1969). Mice fed OVA were shown to have decreased antigen absorption as well as oral tolerance and this was assumed to be additional evidence for the presence of mucosal immunity (Swarbrick, Stokes & Soothill, 1979).

This phenomenon appeared to be explained by a study which demonstrated the simultaneous induction of antigen-specific IgA T helper cells and IgG T suppressor cells in the PP of mice fed ovalbumin 6 days previously (Richman **et al,** 1981). Similar results were obtained after feeding sheep red blood cells (Mattingly, 1983).

The description of an **in vitro** contrasuppressor circuit in which a Peyer's patch T cell appeared capable of blocking the effects of a locally-induced suppressor T cell seemed to provide further evidence of a mechanism maintaining local immunity in the presence of concurrent systemic tolerance (Green **et al,** 1982). In this proposed circuit an I-J+/Ly-2+ inducer cell found in the PP was thought to interact with an I-J+/Ly-1,2+ transducer cell in the spleen to generate an I-J+/Ly-1+ contrasuppressor (c/s) effector cell. This c/s effector cell was thought to be a resident component of the PP which masked the effects of an I-J-/Ly-2+ suppressor effector T cell induced at the same time. Thus, suppressor effectors migrating to the spleen would tolerise systemic responses but the action of the resident PP c/s cell would maintain local immunity (Green & St Martin, 1983).

Recent experiments have challenged these results. Measurement of intestinal antibodies in mice fed KLH showed that there was no local immune response in the presence of tolerance. Furthermore, simultaneous feeding of CT with KLH both abrogated the induction of oral tolerance and stimulated a local response (Elson & Ealding, 1984b; Lycke & Holmgren, 1986). This situation seems to be in agreement with studies performed in this laboratory which could not detect a mucosal

DTH response in animals who were orally tolerised. Only procedures which abrogated oral tolerance appeared to induce a local DTH response (Mowat & Ferguson, 1981; Mowat & Ferguson, 1982b; Mowat **et al**, 1982; Mowat & Parrott, 1983; Strobel & Ferguson, 1984).

Chapter 3

SYSTEMIC DELAYED-TYPE HYPERSENSITIVITY

3.1 Introduction

Delayed-type hypersensitivity (DTH) represents one of the first immunological phenomena to be described (Jenner, 1798; Zinsser, 1925). It has been induced in mice using a number of different antigens including microbes or their products, heterologous proteins, polysaccharides and simple chemical contactants (Crowle, 1962; Asherson & Ptak, 1968; Kettman, 1972; Crowle, 1975; Vadas, Miller, Gamble & Whitelaw, 1975; Clark & Azar, 1977; Ruddle, 1978; Titus & Chiller, 1981b). The reaction, which becomes visible 16 to 24 hours after antigenic challenge, is specific and is mediated by T lymphocytes - antibody and complement not being necessary (Zembala & Asherson, 1973; Vadas, Miller, McKenzie, Chism, Shen, Boyse, Gamble & Whitelaw, 1976; Moorhead, 1978).

Hypersensitivity to antigens in mice has been assessed following elicitation with specific antigen by measuring swelling in either footpad (Kettman, 1972; Clark & Azar, 1977) or ear (Asherson & Ptak, 1968; Ruddle, 1978) or by the incorporation of radiolabel (Vadas, Miller, Gamble & Whitelaw, 1975). The value of these DTH measurements is that they represent a means of quantifying this reaction **in vivo.**

The DTH lesion has a characteristic histology and time-course and although T lymphocytes are fundamental to the phenomenon they are not the only cell involved in the ontogeny of this lesion. All these points will be considered in greater detail below.

3.2 The Development of the Swelling and Histology of the DTH Lesion

After challenge with specific antigen the earliest change that occurs is an immediate swelling response. This localised oedema is most marked 2 hours after challenge and has largely disappeared by 4 hours after the application of the contact sensitising agent (Van Loveren, Meade & Askenase, 1983).

After approximately 4 to 8 hours small and medium sized lymphocytes begin to infiltrate the perivenular and perivenous areas of the superficial vascular plexus at the junction of the papillary and reticular dermis. In addition some macrophages and basophils can be detected at this time but they are small in number. Over the next hours and days the mononuclear infiltrate increases progressively reaching a maximum at 24 to 48 hours in the mouse and 3 days in man (Dvorak, Mihm, Dvorak, Johnson, Manseau, Morgan & Colvin, 1974; Scovern & Kantor, 1982). As the lesion matures, the infiltrating lymphocytes appear to be larger but fully developed immunoblasts (of the type seen in draining lymph nodes) are rare.

As the mononuclear infiltrate increases and spreads to involve the intervascular areas of both the dermis and epidermis the number of polymorphmononuclear cells (PMNLs) also increase following the distribution of the lymphocytes. By 24 hours there is a dense infiltrate of PMNLs both in the dermis and epidermis. These cells consist chiefly of basophils but the other leucocytic component depends on the means of antigenic challenge. When contact sensitising agents

are used eosinophils are the second most frequent PMNL. Neutrophils take their place when antigen is intradermally injected (Dvorak et al, 1974).

Electron microscopy has revealed that although there are signs of surface activation of tissue mast cells and basophils as early as 6 hours after challenge, degranulation of these cells does not appear to occur until 18 hours after challenge. It should be noted, however, that no ultrastructural studies have been performed on DTH lesions earlier than 6 hours after challenge. It is also approximately 18 hours after challenge that ultrastructural changes are occuring in endothelial cells of the postcapillary venules of the superficial vascular plexus. These include separation of the intercellular junctions and thinning of the endothelial cell cytoplasm which allow the migration of leucocytes through these cell gaps by diapedesis (Dvorak et al, 1974; Dvorak, Mihm & Dvorak, 1976; Askenase, Bursztajn, Gershon & Gershon, 1980).

This increase in vascular permeability assists in the processes responsible for the cellular infiltration, increasing dermal and epidermal oedema, vascular compaction and fibrin deposition which is characteristic of a fully developed DTH lesion and which causes the measurable increase in footpad or ear thickness which occurs between 24 and 72 hours after antigenic challenge depending on the species studied (Dvorak et al, 1974).

3.3 Cellular Interactions in DTH

In DTH, local challenge with antigen elicits an antigen-specific delayed inflammatory action that depends on the ability of sensitised T lymphocytes to recruit non-immune, blood-borne cells into the extravascular tissues (Kosunen, Waksman, Flax & Tihen, 1963; Najarian & Feldman, 1963; Cohen, McCluskey & Benacerraf, 1967; Asherson & Ptak, 1968; Lubaroff & Waksman, 1968). It had been held that DTH depended solely on the ability of these lymphocytes to release lymphokines and sequester circulating leucocytes into the site of antigen challenge but there is now some controversy over the mechanism by which circulating primed lymphocytes are themselves brought into contact with antigen. In particular, the role of the tissue mast cell has recently been the subject of conflicting reports.

T lymphocytes have a central role in DTH reactions. Transfer of DTH to heterologous erythrocytes and soluble protein in the mouse is mediated by the Lyt-1+ 2-/Ia- subset of circulating T cells (Huber, Devinsky, Gershon & Cantor, 1976; Vadas **et al**, 1976). DTH against allogeneic cells and viral antigens can be mediated by an Lyt-1- 23+ subpopulation (Smith & Miller, 1979a; Smith & Miller, 1979b; Leung & Ada, 1980). Recent reports of mouse cell lines indicate that DTH may be mediated by both Lyt-2- and Lyt-2+ cells and that these cells may display considerable flexibility in their function (Bianchi, Hooijkaas, Benner, Tees, Nordin & Schreier, 1981; Dennert, Weiss & Warner, 1981; Weiss & Dennert, 1981; Kaufmann & Hann, 1982; Thomas, Mottram, & Miller, 1982; Milon, Marchal, Seman, Truffa-Bachi & Zilberfarb, 1983;

Tamura, Chiba, Kojima & Uchida, 1983).

The first event required for the manifestation of DTH responses is the induction of antigen-specific T cells which requires two signals: antigens presented in association with the appropriate Class II major histocompatability antigens on the macrophage or dendritic cell surface, and a nonantigen-specific mediator, with the properties of interleukin I, produced by macrophages (Shevach & Rosenthal, 1973; Katz, Graves, Dorf, Dimuzio & Benaceraff, 1975; Schwartz, David, Sachs & Paul, 1976; Thomas, Yamashita & Shevach, 1977; DeFreitas, Chesnut, Grey & Chiller, 1983; Jakway & Shevach, 1983). These events may result in the expression of interleukin II (IL2) receptor sites on T cells, the production of IL2 by T cells and the production of lymphokines (Abramson, Brown, Puck & Rich, 1983; DeFreitas **et al**, 1983). The final manifestation of a DTH response is controlled by the Ir-genes of the responding animal and by the activity of regulatory suppressor cells or mediators (Strassmann, Eshhar & Mozes, 1980a; Strassmann, Eshhar & Mozes, 1980b; reviewed: Turk, 1980; reviewed: Geczy; 1984).

T-T cell interactions may mediate the effector phase of DTH (Strassman **et al**, 1980b; Van Loveren, Kato, Meade, Green, Horowitz, Ptak & Askenase, 1984). These interactions may involve the amplification of the action of one cell by another or the more efficient distribution of a second effector cell due to the action of the first. Furthermore, it has been suggested that the second of these two interactions is itself mediated via a non-lymphoid cell, the tissue mast cell (Miller & Butler, 1983; Van Loveren **et al**, 1983; Van Loveren **et al**, 1984).

Van Loveren and his colleagues have described an attractive model for the initial stage of the DTH reaction which attempts to explain the process by which circulating antigen-specific T cells may be brought into contact with antigen deposited in the tissues after challenge (Askenase & Van Loveren, 1983).

They have demonstrated an effector T cell which produces a lymphokine (PCLF) which is able to transfer, in an antigen-specific non-H2-restricted manner, the early swelling phase of DTH. They have also shown that this swelling may be inhibited by serotonin antagonists and the subsequent late phase of DTH is not present in mast cell-deficient mice. They have therefore suggested that serotonin release from PCLF-sensitised tissue mast cells (in the presence of antigen) allows the ingress of the second DTH effector T cell which then mediates the late-phase reaction of DTH (Askenase, Rosenstein & Ptak, 1983; Van Loveren **et al**, 1984; Gershon, Askenase & Gershon, 1975; Schwartz, Askenase & Gershon, 1977; Askenase **et al**, 1980; Askenase, Van Loveren, Kraeuter-Kops, Ron, Meade, Theoharides, Nordlund, Scovern, Gershon & Ptak, 1983).

Recently, however, several authors have reported that although serotonin antagonists may inhibit the delayed-phase of the DTH reaction, both mast cell-deficient and serotonin-deficient animals are capable of expressing normal, or even increased, classical DTH reactions (Thomas & Schrader, 1983; Galli & Hammel, 1984).

Although this casts doubt on the role of the mast cell in the early ingress of DTH effector cells to the site of antigenic challenge, it does not exclude the possibility of this phenomenon occurring altogether - perhaps mediated by another resident population of cells. This is further corroborated by the fact that although PCLF can transfer the early phase of DTH it cannot directly sensitise mast cells either **in vivo** or **in vitro** (Askenase **et al,** 1983).

This model still remains the most attractive explanation for the efficient approximation of effector T cell and challenge antigen. Although some T cell clones may lack the cell surface determinants which react with endothelial cells in lymphoid organs and thus have abnormal migratory patterns, this model goes some way to explain why other clones can transfer DTH when injected locally but are unable to do so when given systemically (Bianchi **et al,** 1981; Dennert **et al,** 1981; Lin & Askonas, 1981; Weiss & Dennert, 1981; Marchal, Seman, Milon, Truffa-Bachi & Zilberfarb, 1982; Minami, Okuda, Sunday & Dorf, 1982; Thomas **et al,** 1982; Carroll, Palladino, Oettgen & De Sousa, 1983; Gallatin, Weissman & Butcher, 1983; Tamura **et al,** 1983).

3.4 The Role of Lymphokines in the inflammatory phase of the DTH Reaction

Since the description of the first lymphokine, MIF in 1966 it is clear that these soluble products had a role to play in the expression of

DTH. This next section will be a brief review of the effect of these substances on the development of the inflammatory phase of the delayed skin reaction.

Before presenting a more detailed discussion of lymphokine function, it is as well to highlight some limitations of research in this area. Lymphokine activities have generally been derived from supernatants of heterogeneous cellular populations. Recent evidence suggests that Lyt-2- and Lyt-2+ lymphocytes, B lymphocytes, lymphoid cell lines and even non-lymphoid cells can produce lymphokines in addition to the more commonly described Lyt-1+ 2- T cells and their often obligatory macrophage (Geczy, Farram, Moon, Meyer & McKenzie, 1983; Guerne, Piguet & Vassalli, 1983; Bigazzi, Yoshida, Ward & Cohen, 1975; Kuhner, Cantor & David, 1980; Landolfo, Herberman & Holden, 1978; Miura, Shimokawa, Honda & Hayashi, 1983; Nelson & Leu, 1975; Newman, Gordon, Hämmerling, Senik & Bloom, 1978; Sorg & Geczy, 1978; Rosenstreich & Wahl, 1979). Studies using T lymphomas and T cell hybridomas have shown that they may produce supernatants with multiple lymphokine activities and it is still not clear whether a single lymphokine molecule may have multiple activities (Farrar, Fuller-Farrar, Simon, Hilfinker, Stadler & Farrar, 1980; Higushi, Asada, Kobayashi & Osawa, 1983; Gemsa, Debatin, Kramer, Kubleka, Deimann, Kees & Krammer, 1983; Papageorgiou, Henley & Glade, 1972; Prystowsky, Ely, Beller, Eisenberg, Goldman, Goldman, Goldwasser, Ihle, Quintans, Remold, Vogel & Fitch, 1982; Prystowsky, Ely, Vogel, Goldwasser & Fitch, 1983; Remold, Mednis, Kawaguchi, Bersch & Golde, 1983; Schrader & Clark-Lewis, 1981; Schreiber, Altman & Katz, 1982; Schreiber, Pace, Russell, Altman &

Katz, 1983; Smith, Gilbride & Favata, 1980). Furthermore, several molecules may have the same biologic activity and these functions may be altered by enzymatic modification (Huff, Uede, Iwata & Ishizaka, 1983; Männel & Falk, 1983; Nathan, Murray, Wiebe & Rubin, 1983; Schreiber **et al**, 1983). Finally, the study of the molecular mechanisms of lymphokine-induced responses is often complicated by the heterogeneity of responding cell populations, the complexity and semi-quantitive nature of the bioassays and the requirements for second signals (Geczy, 1984).

The processes by which lymphokines may promote the development of the inflammatory phase of the DTH reaction may be divided into three areas. They may affect the permeability of cutaneous vessels, exert chemotactic effects on lymphoid and non-lymphoid cells and inhibit the migration of cells away from the site of the DTH lesion. These actions may be accomplished both directly and indirectly and have been summarised in table 3.1.

Three lymphokines have been directly implicated in increasing vascular permeability. Lymph node permeability factor (LNPF) was the first to be described but is present in the extracts from both non-immune lymphoid and non-lymphoid cells leaving its role in DTH open to question (Walters & Willoughby, 1965; reviewed: Willoughby, 1973). Skin reactive factor (SRF) induces skin lesions typical of DTH 3 to 5 hours after intradermal injection in Guinea-pigs (Bennett & Bloom, 1968). This factor is as yet not fully characterised and its effects **in vivo** may be due to a number of mediators in unfractionated

supernatants (Morley & Williams, 1973; Pick, Krejci, Cech & Turk, 1969; Pick, Krejci & Turk, 1970; Schwartz, Cantanzaro & Leon, 1971; Warrington, Buehler & Roberts, 1976; Yoshida & Cohen, 1974). Most recently, PCLF, an antigen-specific factor produced by Lyt-1+ 23- murine T cells, has been shown to transfer the early swelling phase of DTH. This factor which has T cell but no B cell antigenic determinants, weighs less than 70,000 daltons and has some biological actions similar to IgE (Askenase et al, 1983; Van Loveren et al, 1984). Its role in DTH has been discussed earlier in this chapter (section 3.3).

Lymphokines may also produce changes in vascular permeability indirectly. Several factors produced as a consequence of coagulation cascade activation may also participate in inflammatory reactions. Thrombin-activated platelet-derived products may contribute to vascular permeability changes in DTH (reviewed: Osler & Siraganian, 1972; Ryan & Majno, 1977). Thromboplastic factors, fibrin and fibrinopeptides also behave as vasoactive peptides (reviewed: Belew, Gerdin, Porath Saldeen, 1978; Jansco, 1961; Ratnoff, 1969). In addition lymphokines induce plasminogen-activator from macrophages and so permeability changes as a result of plasmin degradation of fibrin are possible (Ratnoff, 1969). Plasmin may also induce the formation of the vasoactive kinins, C5a and C3a (reviewed: Ryan & Manjo, 1977; Larsen & Henson, 1983). Finally, lymphokines may mediate a change in vascular permeability through the production of oxygen-free radicals or by the induction of the release of prostaglandin E from macrophages (Johnston, Godzik & Cohn, 1978; Nathan, Nogueira, Juangbhanich, Ellis & Cohn,

1979; Murray & Cohn, 1980; Hopper & Cahill, 1983; Clement & Leymeyer, 1983).

Circulating leukocytes are recruited from the intravascular compartment to the site of antigenic challenge. This is the result of local generation of lymphocyte-derived chemotactic factors which direct cell migration along an increasing concentration gradient of the factor to the site (reviewed: Rocklin, Bendtzen & Greineder, 1980; Snyderman & Goetzl, 1981).

The first chemotactic lymphokine was described by Ward and his colleagues as chemotactic for macrophages (MCF) (Ward, Remold & David, 1969). Chemotactic factors for neutrophils, basophils, eosinophils, lymphocytes and fibroblasts have been reported (Cohen & Ward, 1971; Ward, Unanue, Goralnick & Schreiner, 1979; Postlethwaite, Snyderman & Kang, 1976). Recent studies indicate that murine Lyt-1+ 2- T cells stimulated with antigen or mitogen produce chemotactic factors for lymphocytes and macrophages (Miura, Shimokawa, Honda & Hayashi, 1983; Shimokawa, Miura, Hifumi & Hayashi, 1983). In addition, chemotactic factors have been extracted from the sites of DTH reactions indicating that these factors may be involved in cell recruitment **in vivo** (Cohen, Ward, Yoshida & Burek, 1973; Honda, Miura, Kuratsu & Hayashi, 1982).

Murine Lyt-1+ 2- T cells and Ia+ accessory cells when stimulated with Concanavalin A, periodate or antigen produce macrophage procoagulant-inducing factor (MPIF) (Geczy, 1984). MPIF may activate coagulation and products of the coagulation sequence may also participate in

chemotaxis. Thrombin may be directly chemotactic for monocytes and macrophage cell lines, may induce the production of platelet factor 4 (a powerful chemoattractant for moncytes and neutrophils) or cleave fibrinopeptide B from fibrinogen (Deuel, Senior, Chang, Griffin, Heinrickson & Kaiser, 1981; Bar-Shavit, Kahn, Fenton & Wilner, 1983). Fibrinopeptide B is chemotactic for neutrophils and eosinophils (Kay, Pepper & McKenzie, 1974). Other lymphokine-activated enzymes including plasminogen activator and kallikrein are chemotactic for monocytes, neutrophils and basophils (reviewed: Kay & Kaplan, 1975). Recent experiments have shown that a T cell-derived lymphokine which induces macrophage aggregation is fibronectin (Godfrey & Purhoit, 1982). Fibronectin is chemotactic for fibroblasts and its fragments attract monocytes (Prydz & Lyberg, 1980; Norris, Clark, Swigart, Huft, Weston & Howell, 1982).

Following the attraction of mononuclear cells to the site of antigenic challenge, the migration inhibition lymphokines presumably retain the emigrated cells in the area. The best characterised of these factors are migration inhibition factors for macrophages (MIF) and for leukocytes (LIF). These lymphokines have been the subject of several reviews detailing their production, measurement, chemical characteristics and mode of action (David, 1971; Pick, 1979; Rocklin, 1981; Schrader & Clark-Lewis, 1981). Other lymphokines capable of inhibiting the migration of neutrophils (NIF-T), lymphocytes (LMIF) and macrophages (MIF and MaggF/fibronectin) have been described (Weissbart, Lusis, Kacena, Spolter, Eggena & Golde, 1982; Mowat & Ferguson, 1982a; Mowat & Ferguson, 1982b; Godfrey & Purhoit, 1982). Although the

mechanism of migration inhibition is still unresolved, recent experiments suggest that one mechanism whereby inhibition occurs is by activation of the coagulation cascade resulting in the crosslinking of surface-bound fibrinogen, thereby immobilising the cells (Hopper, Geczy & Davies, 1981). This reaction is mediated by MPIF (Geczy, 1984).

This section has dealt only with the role of lymphokine in the development of the inflammatory phase of the DTH reaction. Lymphokines may also be involved with the induction of T cells (as alluded to earlier), the deposition of fibrin (which results in the induration typical of DTH) and the regulation of the process itself.

Factors affecting cutaneous vessels

a. Lymphokines acting directly

Lymph node permeability factor (LNPF)
Skin-reactive factor (SRF)
Antigen-specific factor transferring early swelling response (PCLF)

b. Lymphokines acting indirectly

Macrophage procoagulant inducing factor (MPIF) - activation of coagulation:
    Thrombin-induced platelet activation-vasoactive products
    Vasoactive fibrinopeptides

Plasmin fibrinolysis: vasoactive fibrinopeptides
                      kinins
                      C5a, C3a

Lymphokine-induced (MAF) oxygen-free radical production by macraphages
Lymphokine-induced (MAF) prostoglandin production by macrophages

Factors affecting chemotaxis

a. Lymphokines acting directly

Chemotactic factor for neutrophils (NCF)
                       macrophages (MCF)
                       lymphocytes (LLF)
                       lymphoblasts
                       eosinophils (ECF)
                       basophils (BCF)
                       fibroblasts

b. Lymphokines acting indirectly

MPIF-induced activation of coagulation:
    Thrombin: chemotactic for macrophages
    Fibrinopeptide B/fibrin: chemotactic for eosinophils and neutrophils
    Thrombin-induced platelet activation: platelet factor 4, chemotactic for monocytes and neutrophils

Plasminogen activator: chemotactic for monocytes, neutrophils, and basophils
Plasmin-fibrinolysis: fibrinopeptides chemotactic for monocytes, neutrophils, and eosinophils

Fibronectin (MaggF): chemotactic for fibroblasts
Fibronectin fragments: chemotactic for monocytes

Factors affecting cell migration

Leukocyte migration inhibition factor (LIF)
Lymphocyte migration inhibition factor (LMIF)
Neutrophil migration inhibition factor (NIF-T)
Macrophage migration inhibition factor (MIF)
Macrophage procoagulant inducing factor (MPIF)
Macrophage aggregation factor (MaggF) (Fibronectin)

Table 3.1. Direct and indirect effects of lymphokines in DTH reactions

Chapter 4

MATERIALS AND METHODS

4.1 Animals

Adult BALB/c inbred mice were used normally between the ages of 6 to 12 weeks. Both sexes of mice were employed but within each separate experiment animals of one gender only were used.

Mixed strain Guinea-pigs and rabbits (Scottish Antibody Production Unit) were used as a source of complement.

All animals, with the exception of the rabbits, were bred and maintained in the Animal Unit, Western General Hospital, Edinburgh.

4.2 Diet

Animals were maintained on a standard ovalbumin-free rodent diet (Labsure CRM (X)) and had access to tap water **ad libilum.**

4.3 Anaesthesia

Procedures such as footpad injection were carried out under light ether anaesthesia. Bleeding of mice from axillary vessels was performed under heavier ether anaesthesia as this was a pre-terminal event.

4.4 Sacrifice of Animals

Mice were killed by cervical dislocation.

4.5 Bleeding of Mice

Small quantities of blood were obtained routinely from the retro-orbital plexus under ether anaesthesia using heparinised haematocrit tubes (Propper Ltd). In this way, approximately 300 µl could be obtained from each mouse at regular intervals. When large quantities of blood were required, mice were bled out from the axillary vessels.

4.6 Removal of Mouse Feet

Mouse feet were removed for histology immediately after sacrifice. The specimens were excised above the ankle joint.

4.7 Histology

After removal, mouse feet were immediately placed in 10% phosphate buffered formalin. The tissues were left in this fixative for 7 to 10 days. At the end of this time, two thin longitudinal strips of tissue were excised on the medial and lateral aspects of the foot, which included the innermost and outermost toes.

The specimens were then placed in a neutral EDTA solution to decalcify the osseus material. This process took between 4 to 6 weeks and the progress was monitored by the radio-opacity of the bones on x-rays. An x-ray unit (Watson R600) was used for this purpose.

When the bones were of the same density as the surrounding soft tissues, the specimens were embedded in paraffin wax and longitudinal sections of 4 µm thickness were cut.

Tissues processed in this way were stained with Haematoxylin and Eosin.

4.8 Cyclophosphamide

Mice were given 100 mg/kg cyclophosphamide (Endoxana W B Ltd) in saline, intraperitoneally.

4.9 Antigens

Purified cholera toxin (CT) and toxoid (TD) were the antigens most frequently used throughout this work.

Purified cholera toxin was obtained from both the Sigma Chemical Company, Dorset, England and from List Biological Laboratories Inc., California, USA.

The immunopurified formalinised toxoid (lot No: VT2216/7A) was a kind gift from Dr R O Thompson of the Wellcome Research Laboratories, Beckenham, Kent. To prepare this toxoid, formalinised vibrio culture supernatants were concentrated by ammonium sulphate precipitation. This concentrate was dialysed and then applied to an equine antitoxin-bound affinity column. This adsorbed toxoid was eluted with acid, neutralised and further incubated with formalin to prevent toxic reversion. This resultant toxoid was then concentrated and the residual formalin removed by ultrafiltration.

Purified cholera toxin B subunit or CTB (List Biological Laboratories Inc.) and ovalbumin or OVA (chicken egg albumin, Grade V; Sigma Chemical Co) were also used.

4.10 Parenteral Immunisation of Mice

Mice were immunised for studies of systemic immunity with antigen in complete Freund's adjuvant (CFA: H37Ra - Difco Ltd) intradermally into one rear footpad. Occasionally, mice were also immunised with antigen in incomplete Freund's adjuvant (IFA: 0639-59 - Difco Ltd).

When lymphocytes were required for assays of CMI, mice were immunised with either 1 µg CT, 100 µg OVA or saline in CFA into both rear footpads and the draining lymph nodes were subsequently removed.

4.11 Oral Immunisation of Mice

Unanaesthetised mice were fed antigens in a solution of PBS pH 7.6 containing 6% $NaHCO_3$ (alkaline-buffered saline: ABS) intragastrically using a rigid, steel feeding tube with a rounded end.

4.12 Direct Skin Testing for Systemic DTH

Mice were tested by measurement of footpad thickness before and 24 hours after an intradermal injection of antigen in 50 µl of saline, using a pair of skin calipers (Pocotest A - Carobronze Ltd). In each case, immunised mice were also tested with 50 µl of saline as controls. There was normally no net increase in footpad thickness 24 hours after this control injection.

4.13 Footpad Challenge with Antigen

Cholera toxin has been shown to cause oedema after intradermal injection into rat (Finkelstein & Hollingsworth, 1970) and mouse footpads (Lexomboon, Goth & Finkelstein, 1971). As any extranous swelling would interfere with the measurement of systemic DTH, intradermal injections of various doses of cholera toxin and toxoid were given into the rear footpads of unimmunised mice. The incremental change in footpad thickness at 24 hours was compared to that observed in saline-inoculated animals.

As can be seen from figure 4.1, an injection of saline did not cause any footpad swelling. However, all doses of toxin and the highest dose of toxoid (50 µg) did ($p<0.001$ in all cases), although the toxoid caused considerably less footpad oedema than the choleragen inoculates ($p<0.001$).

The two lower doses of toxoid and the unrelated protein antigen, ovalbumin, did not significantly alter footpad thickness 24 hours after injection. For this reason, 5 µg of toxoid was used to elicit DTH responses in toxin- and toxoid-immunised animals.

4.14 Enzyme-linked Immunosorbent Assays for the Detection of Circulating Antibody

a) IgG anti-cholera toxin ELISA

EIA microtitration plates (M129A; Dynatech Ltd) were coated at 4° C for 16 hours with 5 µg/ml solution of purified cholera toxin in a 0.05 M carbonate buffer pH 9.6. These plates were then sequentially incubated with serum samples or with a hyperimmune mouse serum diluted 1/200 with serum diluent for 2½ hours at room temperature, 1/5000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (heavy and light chain) affinity-purified IgG antibody (Jackson Immunoresearch) in serum diluent for 3½ hours at room temperature, and finally, p-nitrophenylphosphate substrate (Sigma) in a 10% Diethanolamine buffer pH 8.6 for approximately 30 minutes at room temperature.

The wells were extensively washed between incubations with an isotonic saline solution containing 0.5% Tween 20 (Sigma) and 2% horse serum.

The plates were read in a MR 580 micro ELISA autoreader (Dynatech Instruments). Mouse IgG antitoxin levels were recorded as light absorbance readings at 405 nm, all results were adjusted by a correction factor so that the positive hyperimmune serum raised in BALB/c mice gave a value of 1.00.

b) IgM anti-cholera toxin ELISA

The same procedure as above was employed with minor modifications. The plates were coated as before, but the serum to be assayed was diluted 1/100. All incubation conditions and times were as above except that the detecting antibody used in this ELISA was a 1/2000 dilution of alkaline phosphate-conjugated goat anti-mouse μ chain IgG antibody. The remaining steps are as described above.

c) IgA anti-cholera toxin ELISA

Microtitre plates were coated with a 5 μg/ml solution of purified toxin in a carbonate buffer for 2 hours at 37° C and were then incubated with a 1/20 dilution of the serum to be tested for 16 hours at 4° C. Any bound antibody was detected by incubating the plate with a rabbit anti-mouse alpha chain antibody (Lutton Bionetics Inc) at a 1/1000 dilution followed by an alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (Northeast Biomedical) also diluted 1/1000.

Both these incubations were for 6 hours at room temperature. Substrate and washes were as for the other ELISAs.

d) IgG anti-cholera toxoid ELISA

EIA plates were coated with a 5 µg/ml solution of cholera toxoid in carbonate buffer for 16 hours at 4° C. Sequential incubations were performed with a 1/200 dilution of mouse serum and a 1/500 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (heavy and light chain) affinity purified IgG antibody for 2½ and 3½ hours, respectively, at room temperature.

e) IgG anti-ovalbumin ELISA

Ovalbumin was used as the solid phase antigen by coating EIA plates with a 10 µg/ml solution in carbonate buffer for 16 hours at 4° C. Mouse serum was diluted 1/400 and incubated as above. The remaining steps are also as described above.

Details of these assays are summarised in table 4.1.

4.15 Preparation of Cell Suspensions

Spleens and mesenteric and peripheral lymph nodes were removed immediately after sacrifice. After washing in RPMI 1640 (Flow Labs) they were dissected free of surrounding material and cut into small

pieces with scissors. The pieces were then gently pushed through a fine gauge wire mesh using the plunger of a 5 ml syringe (BD Ltd). The resulting cell suspension was washed x 3 at 400 **g** in unaltered RPMI 1640 for cell transfer experiments and in supplimented medium for lymphocyte migration inhibition assays. Cell viability was assessed by the Trypan blue exclusion method and was normally greater than 90%.

4.16 Detection of sensitisation in peripheral lymph nodes by lymphocyte migration inhibiton

Popliteal and inguinal lymph nodes were taken from animals immunised bilaterally into the rear footpads with antigen in CFA. After processing, cell suspensions were adjusted to 1.5 x 10^8 /ml in complete RPMI 1640 and 10 µl capillary tubes (Drummond Microcaps) filled with the cells. After sealing one end with parafin wax (Cristoseal - Hawksley Ltd), these were pelleted at 400 **g** for 4 minutes and the tubes cut just above the cell pellet. The parts containing the cells were then placed in wells of migration inhibition plates (Sterilin Ltd), being held in place with silicone vacuum grease (Edwards Ltd). The wells were then filled with 0.5 ml complete RPMI 1640 alone or medium containing various concentrations of antigen, sealed with a coverslip and incubated for 21 hours at 37° C.

After culture, the areas of migration were drawn using a camera lucida (Zeiss Ltd) attached to a Zeiss Spectromicroscope and measured by planimetry. The results are expressed as a migration index obtained as

follows:

$$MI = \frac{\text{Area of migration in wells containing antigen}}{\text{Area of migration in medium alone}}$$

A minimum of 6 wells were used for each antigen concentration.

4.17 Reduction of Toxicity in Guinea-Pig and Rabbit Serum

2×10^7 lymphoid cells/ml of complete RPMI 1640 were suspended in 1/10 dilution of fresh Guniea-pig or rabbit serum and incubated at 37° C for 40 minutes. Cells were washed 3 times and viable cells were counted using Trypan blue exclusion.

Both Guinea-pig and rabbit serum are non-specifically toxic to both splenic lymphocytes and thymocytes (table 4.2).

In order to reduce this toxicity, a crude suspension of BALB/c thymocytes and spleen cells were suspended in the fresh serum in a proportion of 1 part cells to 9 parts serum, by volume. This mixture was agitated gently for 1 hour at 4° C. At the end of this time, the suspension was centrifuged at 800 **g** for 10 minutes, the 'absorbed' supernatant was decanted and incubated with splenocytes at 37° C to assess residual toxicity. As can be seen from table 4.3, this proceedure dramatically reduced the cytotoxicity of the Guinea-pig and rabbit serum.

4.18 Elimination of T Cells from Cell Suspensions

2×10^7 lymphoid cells/ml of complete RPMI 1640 were incubated for 30 minutes at room temperature in 1/1000 dilution of monoclonal IgM anti-Thy-1.2 antiserum (F7D5 - Serotech Ltd). The cells were then washed 3 times in medium, resuspended in 1/10 dilution of 'absorbed' Guinea-pig or rabbit serum as a source of complement and incubated at 37° C for 40 minutes. Cells were washed 3 times before use. In cytotoxicity assays, killing was assessed by Trypan blue exclusion.

As can be seen in table 4.4, both sources of complement became lethal to lymphoid cells when used in conjunction with monoclonal antibody. The combination appears to be particularly effective at removing T cells as shown by the depletion of thymocytes.

4.19 Identification of Lymphocyte Classes by an Immunoperoxidase Technique

To identify surface immunoglobulin-positive (sIg+) cells, 15 drops of a 5×10^5 lymphoid cell suspension in medium were cytocentrifuged at 1000 RPM for 10 mins.

The resultant preparation was fixed for 10 minutes in a solution of acetone with 0.5% H_2O_2. The slide was washed in phosphate buffered saline pH 7.6 and incubated with a 1/10 dilution of horseraddish peroxidase-labelled rabbit anti-mouse immunoglobulins (Dako Ltd) for 30

minutes at room temperature in a damp box. The slide was washed twice in PBS and the peroxidase stain was developed with a filtered 3,4-diaminobenzidine solution (DAB: BDH Ltd) for approximately 10 minutes. The cells were counterstained with Harris' haematoxylin and the slide was coverslipped using a 90% glycerine-in-water mounting medium. Cells were examined under an Ortholux microscope (Leitz Ltd) at 400 x magnification.

Thy-1.2-bearing cells were identified indirectly. A cytocentrifuged lymphoid cell suspension was prepared as above. The cells were incubated with a 1/20 dilution of monoclonal anti-Thy-1.2 for 60 minutes at room temperature in a damp box. The slides were washed twice in PBS and then incubated with a 1/10 dilution of HRP-labelled rabbit anti-mouse immunoglobulins. Further steps were as above. The proportion of Thy-1.2-positive cells were determined as follows:

% Thy-1.2-positive = % staining after (anti-Thy-1.2 + anti-mouse Ig) - % staining with anti-mouse Ig alone.

4.20 Determination of Efficiency of T Cell Elimination from Spleen Cell Suspensions

The above immunoperoxidase technique was used to assess the efficiency of absorbed rabbit serum, anti-Thy-1.2 monoclonal antibody or the combination of the two at removing the T cell component of splenic

lymphocytes.

Table 4.5 shows that the serum or antibody alone have no effect but the combination destroys 98.5% of splenic Thy-1.2-positive cells.

4.21 Statistics

Results are expressed ± 1 standard deviation, unless otherwise stated. Student's t-test was used to compare differences between groups in most cases. In experiments including ELISA assays, non-parametric distributions were observed and results were compared by Wilcoxon's Rank Sum test in addition to the Student t-test. In practice, similar significance levels were usually obtained.

4.22 Solutions and Buffers

10% phosphate buffered formalin: (1 litre)	Formaldehyde 100 ml Distilled water 900 ml $NaH_2PO_4.2H_2O$ 4 g Na_2HPO_4 6.5 g
Neutral EDTA solution: (1750 mls)	Na_2 EDTA (BDH Ltd) 250 g Distilled water 1750 ml pH 7.0

Phosphate buffered saline: (5 litres)	NaCl 36.0 g Anhydrous Na_2HPO_4 7.49 g Anhydrous KH_2PO_4 2.15 g Distilled water 500 ml pH 7.6
10% diethanolamine buffer: (900 mls)	DEA (diethanolamine: BDH Ltd) 100 ml Distilled water 800 ml $MgCl_2.6H_2O$ (BDH Ltd) 0.1015g Sodium azide 0.2 g pH 8.6
Tris-HCl buffer: (100 mls)	0.2 M tris [hydroxymethyl] aminomethane (BDH Ltd) 25 mls 0.1 N HCl 75 mls
3,4-diaminobenzidine solution:	DAB (3,4-diaminobenzidine: BDH Ltd) 10 mg 30% u/v H O 10 µl Tris-HCl buffer pH 25 ml
ELISA reagents:	
Carbonate buffer:	Carbonate buffer 0.05 M pH 9.6 (North East Biochemicals Ltd)

Washing solution:	0.05% Tween 20 (BDH Ltd) in physiological saline
Serum diluent:	0.05% Tween 20 in physiological saline 0.2% sodium azide (BDH Ltd)
Complete RPMI 1640 medium:	RPMI 1640 (Flow Labs) 100 ml Penicillin (5000 u/ml)/Streptomycin (5000 µg/ml) (Flow Labs) 2 ml Hepes buffer 1 M (Flow Labs) 1.5 ml Glutamine 0.2 M (Flow Labs) 2 ml

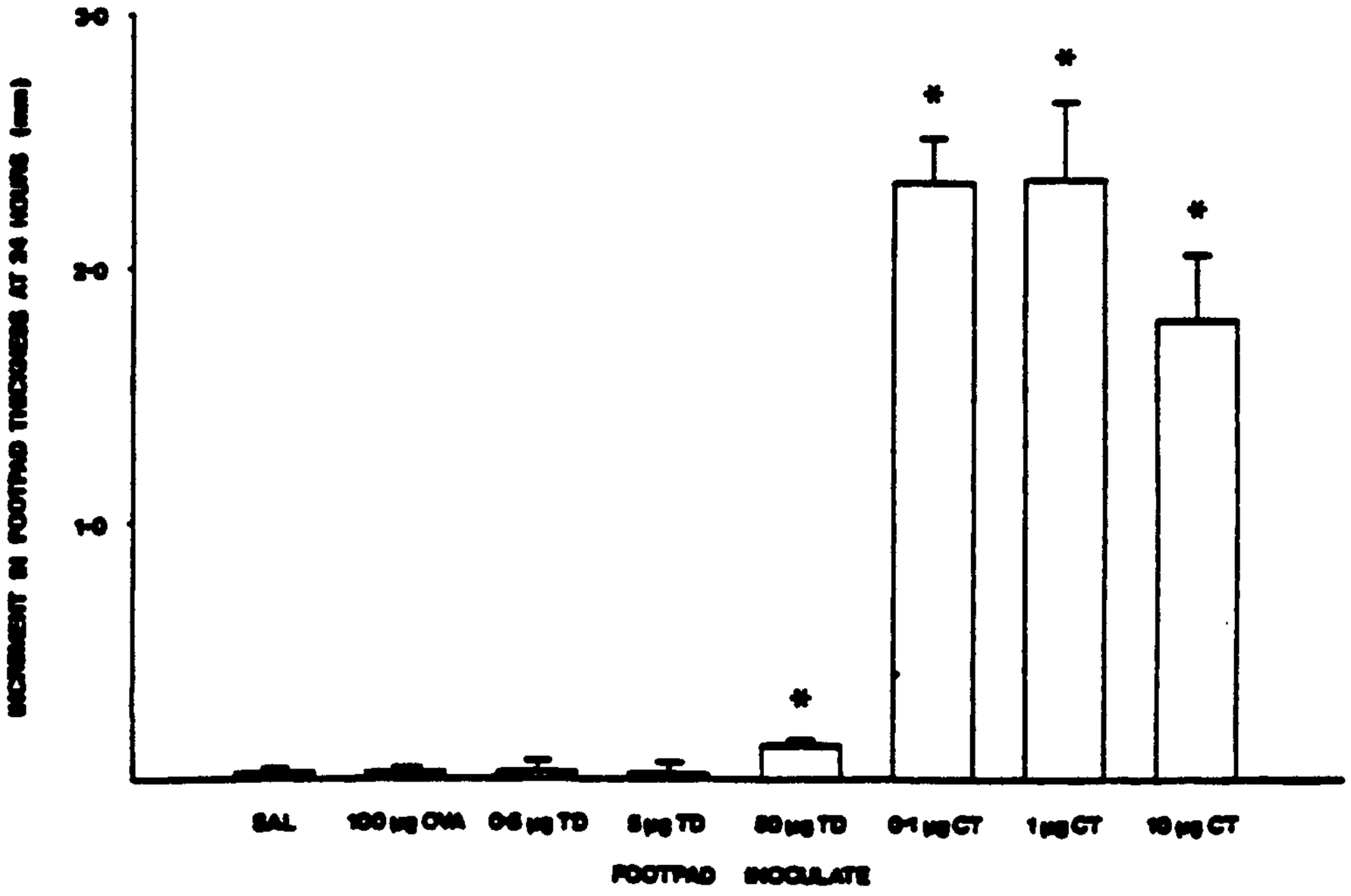

Figure 4.1 The non-specific footpad swelling caused by intradermal injection of antigen

This graph shows the increment in footpad thickness 24 hours after the injection of saline or various antigens dissolved in saline. The volume of inoculate was 50 µl. The bars represent the mean increment in footpad thickness ± standard deviation for each group.

All results were compared to those obtained from mice injected with saline only.

* $p<0.001$

Table 4.1 Protocols for ELISA assays

ELISA	Coating Antigen	△Serum Dilution	△2nd Antibody	△Alkaline Phosphatase conjugated antibody
IgG antitoxin	CT 5 µg/ml○	1/200□	-	GaM IgG (H+L chain)◇ (1/5000)
IgM antitoxin	CT 5 µg/ml○	1/100□	-	GaM µ chain◇ (1/2000)
IgA antitoxin	CT 5 µg/ml●	1/20■	RaM a chain◆ (1/1000)	GaR IgG◆ (1/1000)
IgG anti-toxoid	TD 5 µg/ml○	1/200□	-	GaM IgG (H+L chain)◇ (1/500)
IgG anti-OVA	OVA 10µg/ml○	1/400□	-	GaM IgG (H+L chain)◇ (1/500)

GaM: goat anti-mouse
RaM: rabbit anti-mouse
GaR: goat anti-rabbit
a chain: alpha chain

△ diluted in serum diluent
○ incubated in carbonate buffer pH 9.6 for 16 hours at 4° C
● incubated in carbonate buffer pH 9.6 for 2 hours at 37° C
□ incubated for 2½ hours at 25° C
■ incubated for 16 hours at 4° C
◇ incubated for 3½ hours at 25° C
◆ incubated for 6 hours at 25° C

% Cytotoxicity of lymphoid cells

	Spleen	Thymus
Guinea-pig serum	67.7	56.0
Rabbit serum	30.3	71.0

Table 4.2 Cytotoxicity of fresh Guinea-pig and rabbit sera on various lymphoid cells

	% Cytotoxicity of splenic lymphocytes
Absorbed Guinea-pig serum	5.0 (0.2 - 9.8)
Absorbed rabbit serum	2.0 (0.0 - 6.0)

Table 4.3 Cytotoxicity of 'absorbed' Guinea-pig and rabbit serum to splenocytes (mean and range of 3 experiments)

Treatment	% Cytotoxicity to lymphoid cells		
	Peripheral LN cells	Splenic lymphocytes	Thymocytes
anti-Thy-1.2 abs + Guinea-pig C'	46.0	43.6	95.9
anti-Thy-1.2 abs + rabbit C'	NT	45.4	97.0

Table 4.4 The effect of anti-Thy-1.2 monoclonal antibody in combination with two sources of complement on the mortality of various lymphoid cell populations.

NT not tested

Treatment	% Thy-1.2-positive cells in splenic lymphocytes
None	41.2
anti-Thy-1.2 alone	40.2
abs rabbit C' alone	41.8
anti-Thy-1.2 + abs rabbit C'	0.6

Table 4.5 The effect of various incubations of the T cell content of a splenic lymphocyte population.

Chapter 5

SYSTEMIC HUMORAL IMMUNITY TO PARENTERALLY AND ORALLY ADMINISTERED CHOLERA TOXIN AND TOXOID

5.1 Introduction

Systemic humoral immunity has been extensively studied in a number of animal models following the parenteral administration of cholera toxin and toxoid. Both the holotoxin and its immunopurified formalinised derivative have been shown to be powerful antigens but generally the toxoid has been employed at much higher doses to give equivalent results (Peterson, 1979; Fuhrman & Cebra, 1981; Pierce & Gowans, 1975). The B subunit is the major antigenic determinant against which antitoxin is directed (Pierce, 1978) and so the following experiments have employed doses of toxin and toxoid with a similar B subunit content so that the relative antigenic potency of these two preparations may be more accurately compared.

Experiments have been designed which examine the systemic antibody response to parenterally administered toxin and toxoid, but as it is the purpose of this project to also examine the state of cell-mediated immunity in these animals, the conditions used to look at humoral immunity are also those in which DTH is likely to occur, and will be repeated in the following chapter which deals solely with systemic CMI.

As mentioned above, cholera toxoid is generally used in greater quantities than choleragen in order to obtain a similar immune response. This requirement is usually explained in terms of poorer binding to gangliosides or reduced adenylate cyclase activation (Pierce, 1978) but the possibility that formalinisation limits the antigenic cross-reactivity between these two proteins has never been

examined. To this end, an anti-toxoid ELISA has been developed so that both anti-toxoid and antitoxin responses can be measured following the administration of these antigens by both of the above routes.

Animals have also been prefed both toxin and toxoid in order to examine the effect of initial enteric exposure on the subsequent development of specific systemic antibody responses.

5.2 Systemic Humoral Immunity to Cholera Toxin

BALB/c mice were immunised with either saline or 1 μg CT in CFA into the hind footpad. Two weeks later the animals were given a second intradermal injection of 5 μg TD in saline into the contralateral footpad. IgG, IgM and IgA antitoxin antibody levels were measured 7, 13 and 22 days after the initial injection and comparison was made between the two groups of animals.

IgG antitoxin levels (figure 5.1) were undetectable in saline-primed animals at all the times tested. Similarly low IgA antitoxin levels were obtained from these animals (figure 5.2). Although positive readings were detected from this group when the specific IgM isotype was measured, this probably reflects non-specific binding by this class of antibody (figure 5.3). When one considers the results of the CT-primed animals, IgG antitoxin levels were significantly greater than those detected in the saline-immunised controls at all times tested ($p<0.05$ on day 7, $p<0.01$ on days 13 and 22). The largest difference

between the two groups was seen on day 22 of the experiment, eight days after the intradermal injection of toxoid. The IgA antitoxin responses of the toxin-primed mice were only significantly greater than control values ($p<0.01$) on day 22 of the experiment, that is eight days after the toxoid booster injection. Specific IgM antibody was not detected as the ELISA absorbance readings did not significantly differ between choleragen- and saline-primed animals at any time during the experiment.

5.3 The Influence of Antigen Dose, both Immunisation and Boosting, on the Systemic Antitoxin Antibody Response.

As can be appreciated from the above results, the greatest levels of antitoxin antibody detected in CT-primed animals were found 8 days after these mice were given an intradermal booster of toxoid (day 22 of the experiment). This time was therefore chosen as the most appropriate at which to examine the effects of varying the immunising and boosting doses of these two respective substances. Accordingly, mice were intradermally primed with either saline or three differing doses (0.1 µg, 1.0 µg and 10.0 µg) of CT in CFA. Two weeks later these mice were intradermally boosted into the contralateral footpad with one of three differing doses of toxoid (0.5 µg, 5.0 µg and 50 µg). Eight days after this, on day 22 of the experiment, the animals were bled and their serum IgG and IgA antitoxin antibody levels were measured.

Unfortunately there were not enough animals to perform a statistical comparison between the groups boosted with 0.5 µg TD but the trends observed in the other results also appear to hold true for these animals. IgG antitoxin levels were greatest in animals immunised with 1 or 10 µg CT regardless of the footpad challenge dose of toxoid used. The levels observed, in these two groups, did not significantly differ from each other but were significantly greater ($p<0.05$) than those measured in animals immunised with 0.1 µg CT which were, in turn, significantly greater than the antitoxin responses ($p<0.05$) of saline-immunised controls when challenged with either 5.0 or 50.0 µg TD (figure 5.4).

Results for IgA antitoxin levels were similar in that all toxin-primed animals had significantly greater ($p<0.05$) responses than saline-immunised controls. IgA antitoxin antibody levels did not significantly differ between groups of toxin-primed animals (figure 5.5).

5.4 Comparison of Systemic Antitoxin Humoral Immunity after Toxin or Toxoid Immunisation

As has been mentioned earlier, both cholera toxin and toxoid have been shown to be powerful antigens capable of inducing similar antibody responses in a variety of animals. Unfortunately, no such comparison has been made using equivalent doses of antigen. The major antigenic determinants on the holotoxin have been shown to reside on the B

subunit, and so the following experiment employed doses of toxin and toxoid with a similar B subunit content. As the predominant humoral response to parenterally administered toxin is of the IgG isotype, the following experiment was confined to assaying this specific class of antibody as a measure of the animals' humoral response to priming with these two antigens.

Mice were intradermally primed with either saline, 1 µg CT or 5 µg TD in CFA on day 0 of the experiment. As before these animals were intradermally boosted with 5 µg TD two weeks later. The mice were bled on days 7, 13 and 22 of the experiment and the IgG antitoxin content of their sera was measured. The results of this experiment are shown in figure 5.6.

Once again, saline-primed animals failed to produce significant IgG antitoxin at any time during the experiment. Just as before, the toxin-primed animals had significantly higher levels of IgG antitoxin antibody ($p<0.01$) than the saline controls at all times.

The results of the animals primed with toxoid fell between these two extremes. IgG antitoxin levels in toxoid-primed mice did not significantly differ from the saline controls until after receiving the booster injection ($p<0.01$ on day 22), but were significantly less than specific IgG levels in toxin-primed animals at all times ($p<0.05$ on day 7, $p<0.01$ on days 13 and 22).

5.5 Comparison of Antitoxin and Anti-toxoid Humoral Immunity after Parenteral Immunisation with Toxin or Toxoid

Toxoid-primed animals may produce poorer antitoxin responses for two reasons. Toxoid may be a poorer antigen or anti-toxoid antibody may only partially cross-react with the holotoxin. If the toxoid is a poorer antigen, then one would expect a lesser humoral response regardless of whether antitoxin or anti-toxoid antibody levels were measured. However, if the above result represents limited cross-reactivity between the two forms of antigen, animals primed and boosted and then tested against the same antigenic form should give the highest responses, i.e. toxin primed and boosted mice should have the highest serum antitoxin antibody levels.

To examine this, groups of animals were immunised with either 1 µg CT or 5 µg TD in CFA as before. Two weeks later half of each group of mice received 5 µg TD into the contralateral footpad and half were intradermally boosted with 1 µg CT in saline. Mice were bled the day before and 8 days after recieving booster injection (days 13 and 22 of the experiment).

Figures 5.7 and 5.8 show the antitoxin and anti-toxoid IgG responses, respectively, observed in these animals. Toxin-immunised animals had significantly greater ($p<0.01$) IgG antitoxin responses than mice primed with toxoid both before and after boosting. Toxoid was equally effective at boosting antitoxin responses as the native holotoxin.

After challenge, IgG anti-toxoid levels were not significantly different between any of the groups, although the mean absorbance (405 nm) level was higher in toxoid-primed animals. Similarly, there was no statistical difference between the anti-toxoid levels measured in the groups before they received their booster injections. However, if these day 13 results are pooled (a statistically valid procedure as the animals had not been subdivided at this stage), the toxoid-primed animals have a significantly increased anti-toxoid response compared with the combined results of the two groups of mice primed with toxin ($p<0.01$).

5.6 Investigation of the Effect of Feeding Choleragen or Toxoid on the Subsequent Induction of Systemic Humoral Antitoxin Immunity

Previous work has shown that one can obtain good antibody responses to cholera toxin by priming with antigen in CFA on day 0, boosting with toxoid on day 14 and measuring the antitoxin responses on day 22. Adding a single antigenic feed to a similar protocol, one week before priming (day -7), has been shown to be effective at tolerising both systemic CMI and antibody responses to ovalbumin when performed in this strain of mice (Mowat **et al,** 1982). A single feed of either choleragen or toxoid was therefore added to the above regime to see if either would tolerise systemic antitoxin responses.

Animals were fed either alkaline-buffered saline (ABS) alone or ABS containing either 1 µg CT or 5 µg TD. Seven days later the animals were immunised with 1 µg CT in CFA and then boosted with a footpad injection of 5 µg toxoid fourteen days after this. The animals were bled for serum IgG and IgA antitoxin estimations 8 days after receiving the toxoid booster (day 22 of experiment).

The serum obtained from the animals on day 22 revealed that neither feeding toxin nor toxoid significantly inhibited the induction of an IgG or IgA antitoxin response compared with sham-fed controls (figure 5.9), i.e. oral tolerance was not induced for this antigen.

5.7 Investigation of the Ontogeny of the Systemic IgG Antitoxin Antibody Response after Prefeeding Varying Doses of Choleragen

In order to ascertain the effect of feeding differing doses of choleragen on the induction of systemic immunity, mice were fed either 0.1, 1 or 10 µg CT in ABS as well as ABS alone 7 days before priming with an intradermal injection of 1 µg CT in CFA. Animals were boosted with 5 µg TD two weeks after this and antitoxin estimations were made on serum obtained on days -1 (pre-immunisation), 7, 13 and 22 of the experiment. The results of this experiment are shown in figure 5.10.

Unfortunately, animals fed 0.1 or 10 µg CT were not bled before immunisation, but IgG antitoxin antibody was not detected in animals fed either ABS or 1 µg CT. Seven days after immunisation, there was no significant difference in the levels of specific antibody found in animals fed either ABS or 0.1 or 1 µg CT. However, the IgG antitoxin antibody responses of animals fed 10 µg CT were significantly greater than those observed in all other groups at this time ($p<0.02$). By day 13, there was no significant difference in the systemic IgG antitoxin levels of any these animals and the same was true on day 22 of the experiment.

These results suggest that varying the dose of choleragen fed does not induce oral tolerance for antibody but that the oral administration of 10 µg CT initially appears to induce significantly more priming of systemic humoral immunity.

5.8 Investigation of the Induction of Oral Tolerance for Systemic Humoral Anti-toxoid Reaponses by Feeding Choleragen or Toxoid

The foregoing experiments have shown that feeding cholera toxin or toxoid does not tolerise the induction of systemic antitoxin antibody responses. A further experiment was performed to assess the effect of prefeeding on the induction of anti-toxoid humoral immunity.

Animals were fed either ABS, 1 µg CT or 5 µg TD one week before immunisation. Fed mice were immunised with 5 µg TD in CFA as was a group of unfed animals. Other unfed animals were immunised with saline in CFA at the same time. After 14 days all animals were intradermally boosted with 5 µg TD and serum anti-toxoid levels were measured 8 days after this.

The results of this experiment are shown in figure 5.10. They show that saline-primed animals gave low IgG anti-toxoid responses even 8 days after receiving an intradermal booster injection of toxoid. Toxoid-primed animals had significantly greater anti-toxoid responses ($p<0.01$). Feeding either ABS, 1 µg CT or 5 µg TD failed to tolerise systemic anti-toxoid antibody levels.

5.9 Summary

Intradermal immunisation with CT induces significant IgG and IgA antitoxin responses especially after boosting with toxoid. Optimal IgG and IgA antitoxin responses were obtained after immunisation with either 1.0 or 10.0 µg toxin regardless of the booster dose of toxoid employed.

Parenterally administered toxoid also induces significant IgG antitoxin levels but these are significantly less than those obtained with the holotoxin. Toxoid is as effective as choleragen at boosting antitoxin antibody levels but is superior to the native toxin in inducing pre-

challenge IgG anti-toxoid responses. It would appear that each antigen is superior at priming for antibodies against its own antigenic determinants but that each are equally capable of boosting primed responses regardless of the agent used to stimulate the B cells initially.

Finally, feeding cholera toxin or toxoid failed to tolerise the induction of either antitoxin or anti-toxoid humoral immunity. Work performed independently around this time, using a slightly different experimental protocol, confirmed the failure of fed choleragen to induce oral tolerance for systemic antitoxin antibody responses (Ealding & Elson, 1984b). Not only does a single feed of 10 µg CT fail to induce oral tolerance but experiments described above suggest that it may prime for systemic antitoxin responses.

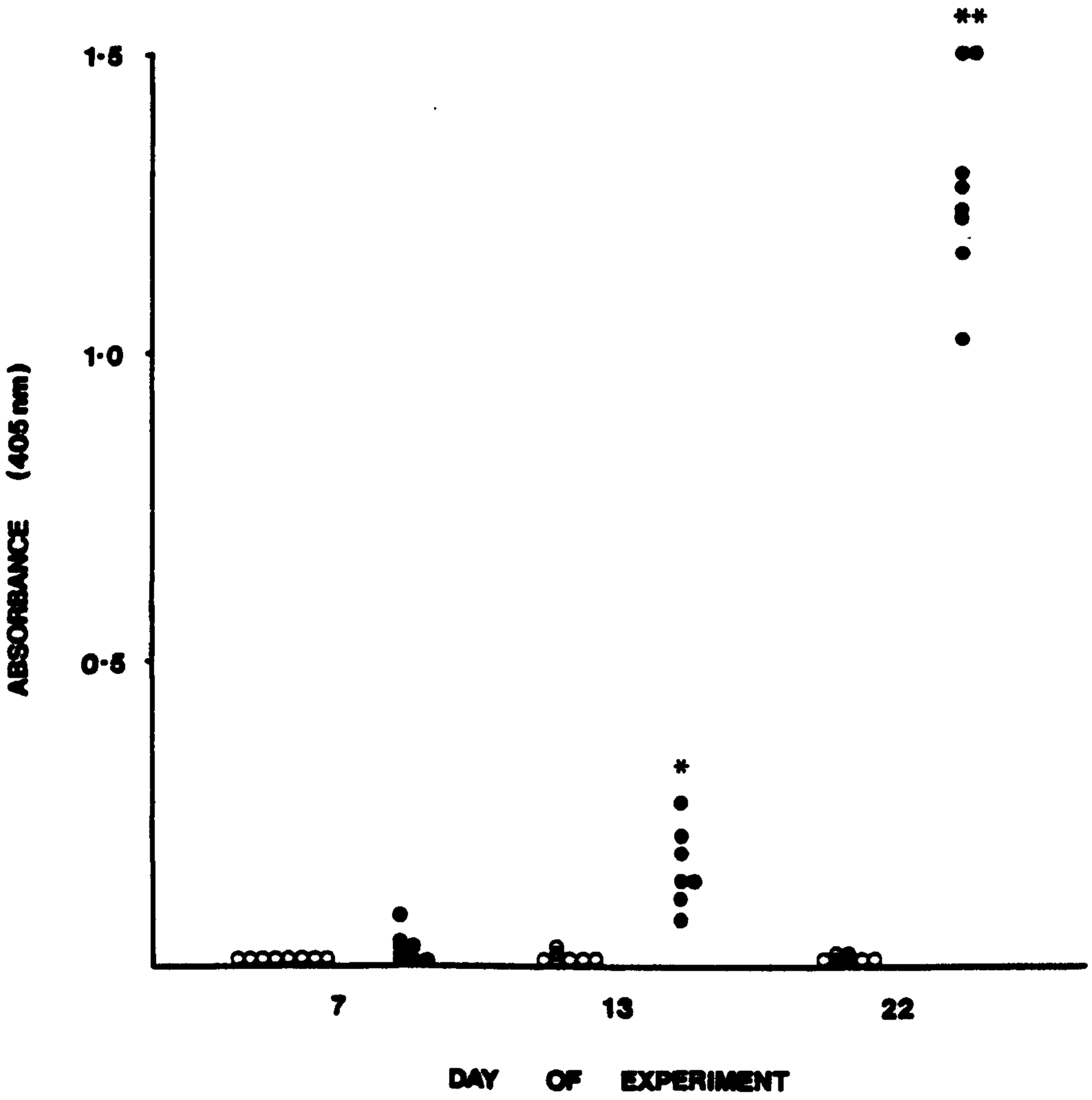

Figure 5.1 Systemic IgG antitoxin antibody levels after immunisation with cholera toxin

The above graphs show the serum IgG antitoxin antibody levels obtained from mice immunised with either saline (○) or CT (●) in CFA. Animals were intradermally immunised on day 0 and boosted with 5 µg TD in saline on day 14. Individual animals were bled on day 7, 13 and 22 of the experiment and their serum was measured in an IgG antitoxin ELISA. The above points represent the absorbance readings of these sera in this assay.

Statistical comparisons were made using the Wilcoxon rank sum test between saline- and choleragen-primed animals.

* $p<0.05$

** $p<0.01$

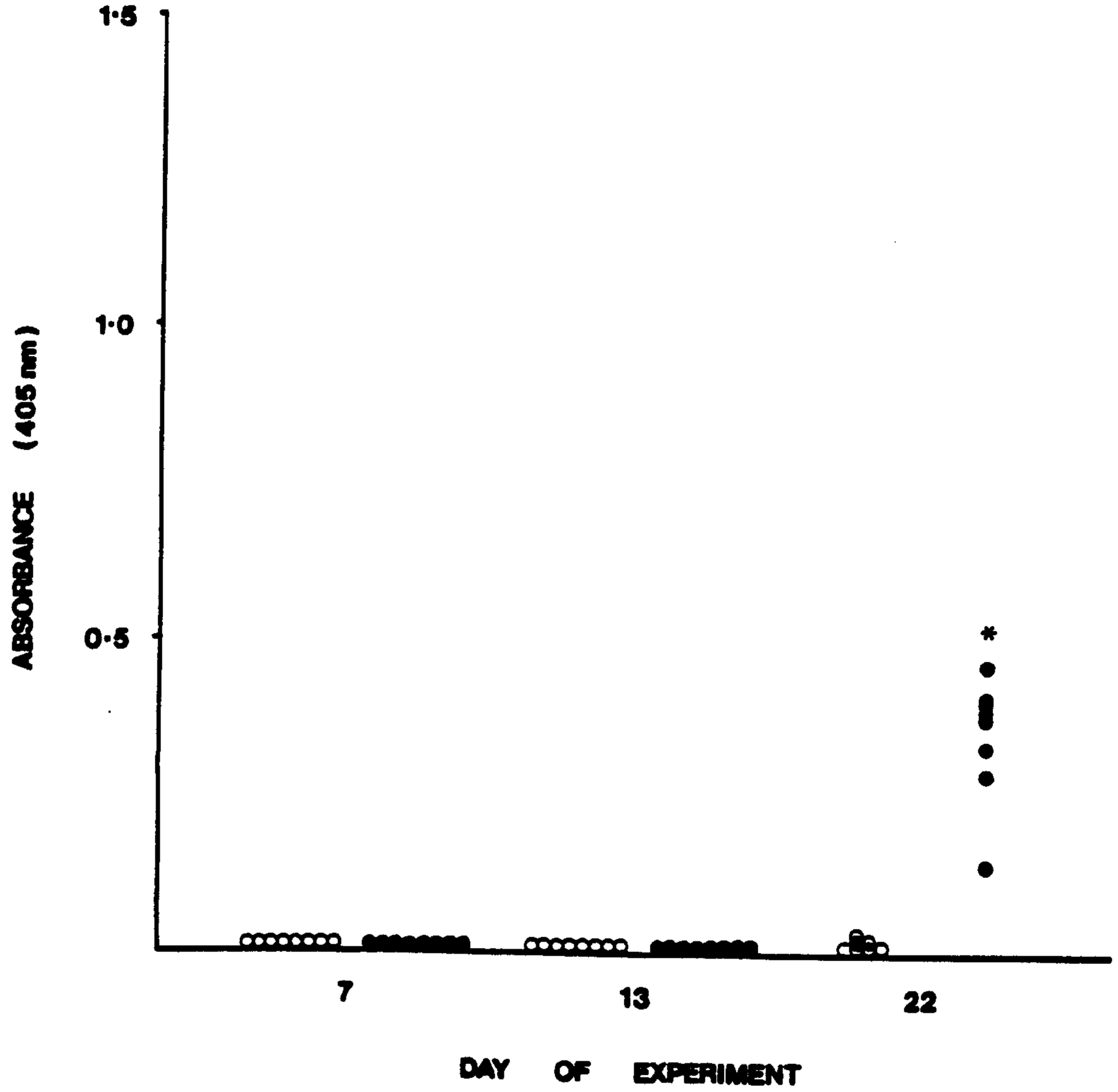

Figure 5.2 Systemic IgA antitoxin antibody levels after immunisation with cholera toxin

The above graphs show the serum IgA antitoxin antibody levels obtained from mice immunised with either saline (○) or CT (●) in CFA. Animals were intradermally immunised on day 0 and boosted with 5 µg TD in saline on day 14. Individual animals were bled on day 7, 13 and 22 of the experiment and their serum was measured in an IgA antitoxin ELISA. The above points represent the absorbance readings of these sera in this assay.

Statistical comparisons were made using the Wilcoxon rank sum test between saline- and choleragen-primed animals.

* $p<0.01$

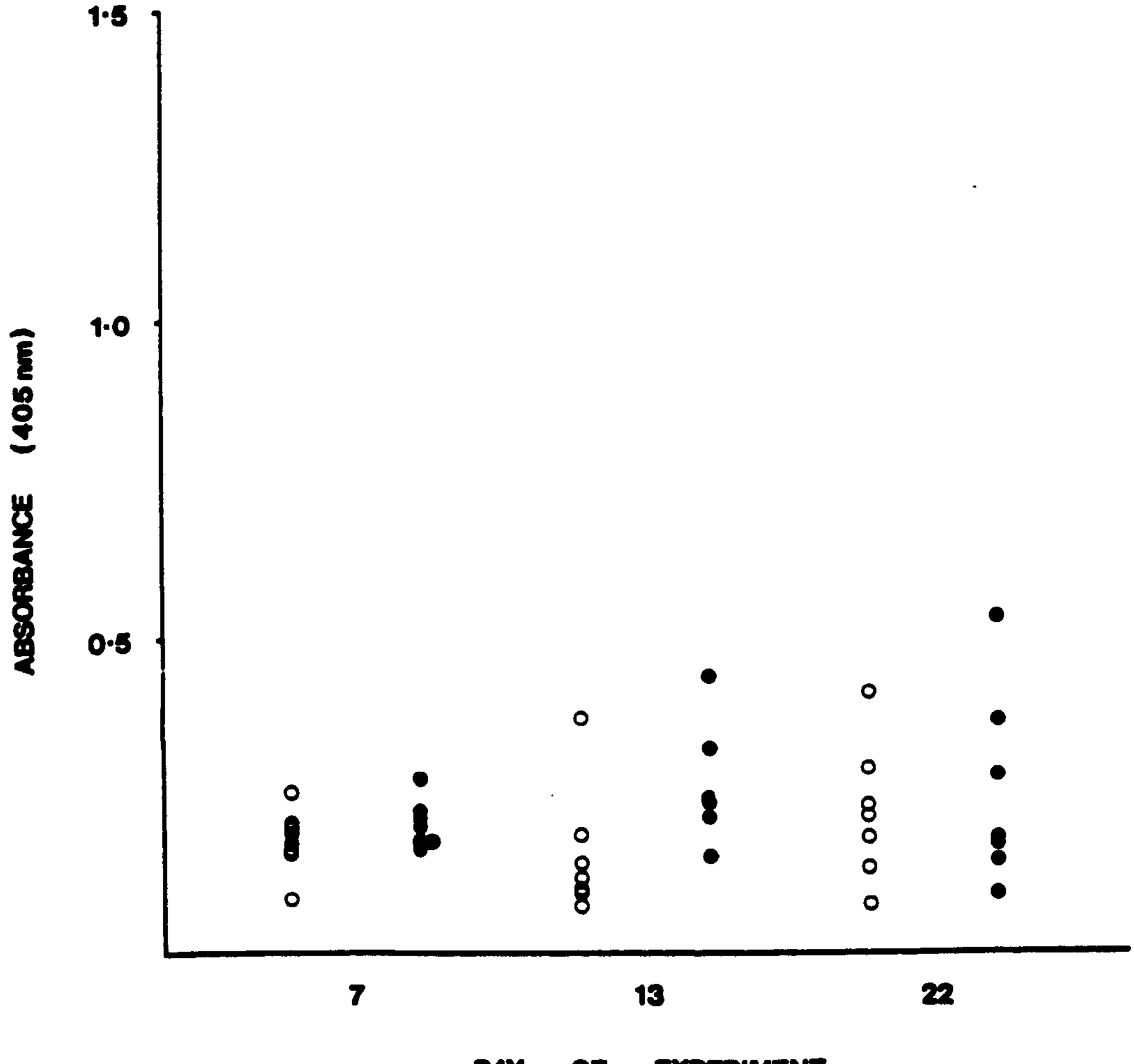

Figure 5.3 Systemic IgM antitoxin antibody levels after immunisation with cholera toxin

The above graph shows the serum IgM antitoxin antibody levels obtained from mice immunised with either saline (○) or CT (●) in CFA. Animals were intradermally immunised on day 0 and boosted with 5 μg TD in saline on day 14. Individual animals were bled on day 7, 13 and 22 of the experiment and their serum was measured in an IgM antitoxin ELISA. The above points represent the absorbance readings of these sera in this assay.

Statistical comparisons were made using the Wilcoxon rank sum test between saline- and choleragen-primed animals.

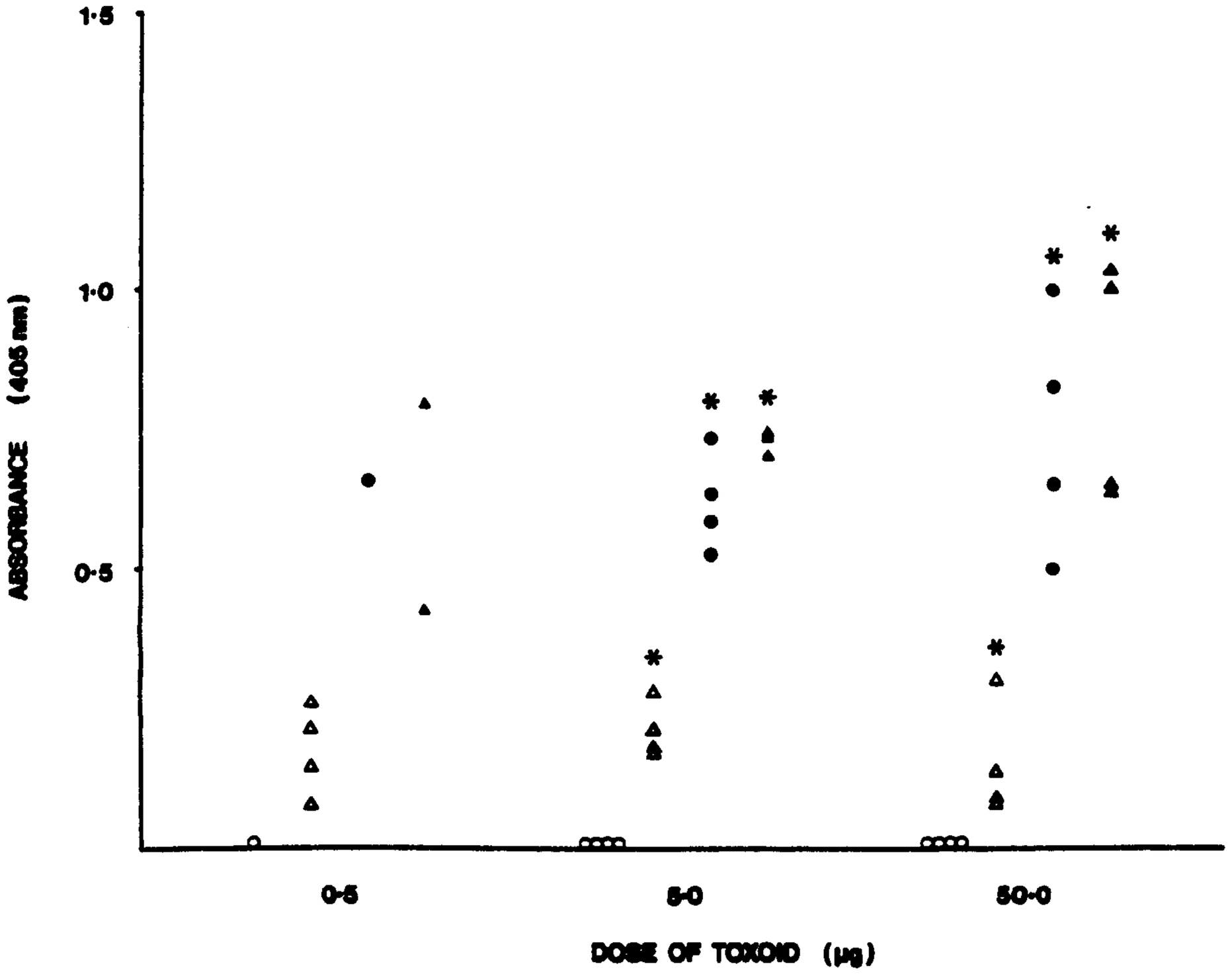

Figure 5.4 The effect of varying the immunisation and boosting doses of antigen on the induction of systemic IgG antitoxin antibodies

The above graph shows the serum IgG antitoxin antibody levels in mice immunised with either saline (○), 0.1 µg CT (△), 1.0 µg CT (●) or 10.0 µg CT (▲) in CFA. Mice were intradermally immunised on day 0 and boosted into the footpad with either 0.5 µg, 5.0 µg or 50.0 µg TD on day 14 of the experiment. The points above represent individual antitoxin results obtained on day 22 of the experiment.

Statistical comparison was made between saline- and toxin-immunised animals for each dose of toxoid used.

* $p<0.05$

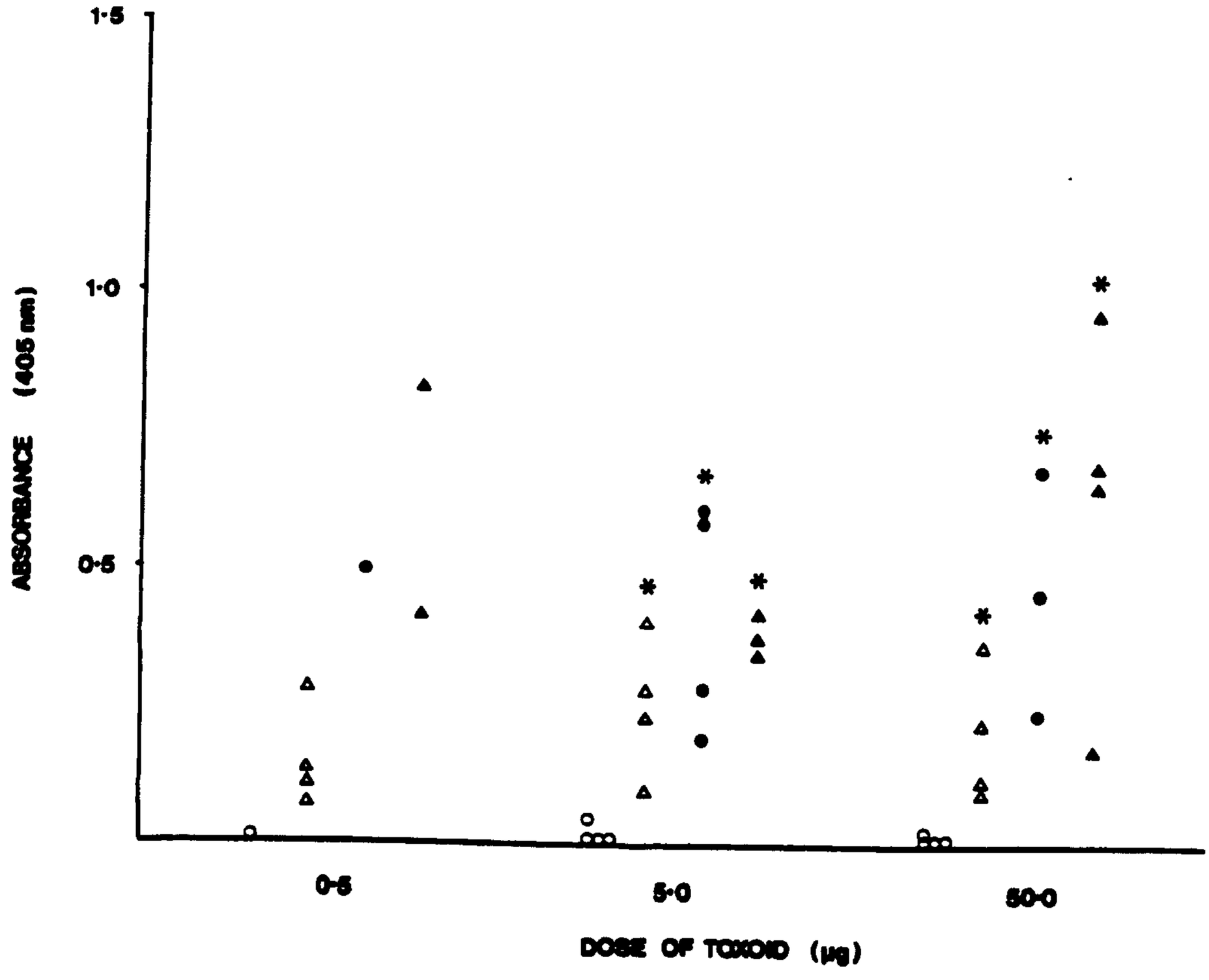

Figure 5.5 The effect of varying the immunisation and boosting doses of antigen on the induction of systemic IgA antitoxin antibodies

The above graph shows the serum IgA antitoxin antibody levels in mice immunised with either saline (○), 0.1 µg CT (△), 1.0 µg CT (●) or 10.0 µg CT (▲) in CFA. Mice were intradermally immunised on day 0 and boosted into the footpad with either 0.5 µg, 5.0 µg or 50.0 µg TD on day 14 of the experiment. The points above represent individual antitoxin results obtained on day 22 of the experiment.

Statistical comparison was made between saline- and toxin-immunised animals for each dose of toxoid used.

* $p<0.05$

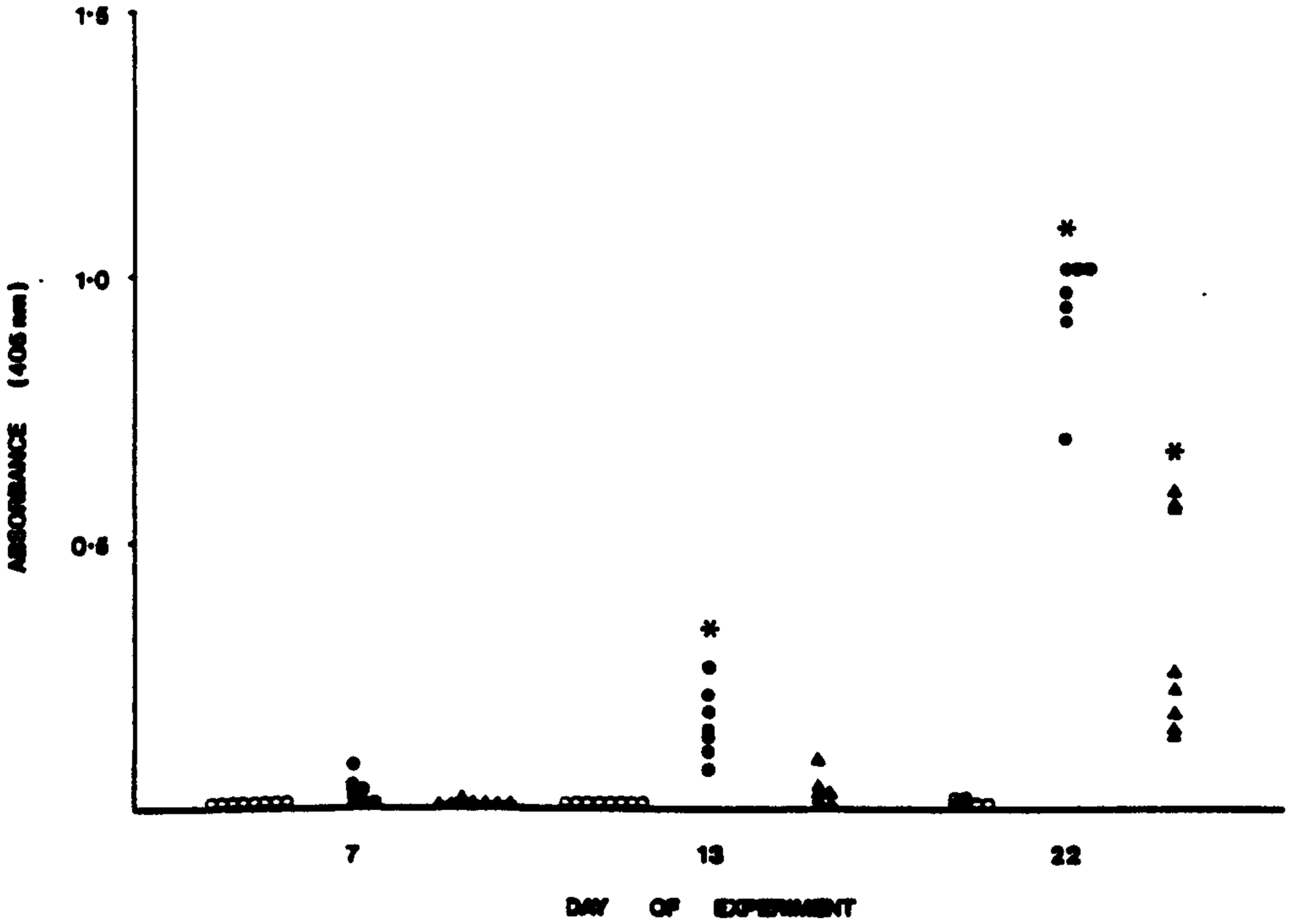

Figure 5.6 The effect of immunisation with either cholera toxin or toxoid on the induction of systemic IgG antitoxin antibody levels

The above graph shows the serum IgG antitoxin levels obtained from mice immunised with either saline (○), 1 µg CT (●) or 5 µg TD (▲) in CFA. Animals were intradermally immunised on day 0 and footpad boosted with 5 µg TD on day 14 of the experiment. The points above represent individual antitoxin results obtained on days 7, 13 and 22 of the experiment.

Statistical comparison were made between saline- and antigen-immunised animals at each time point.

* $p<0.01$

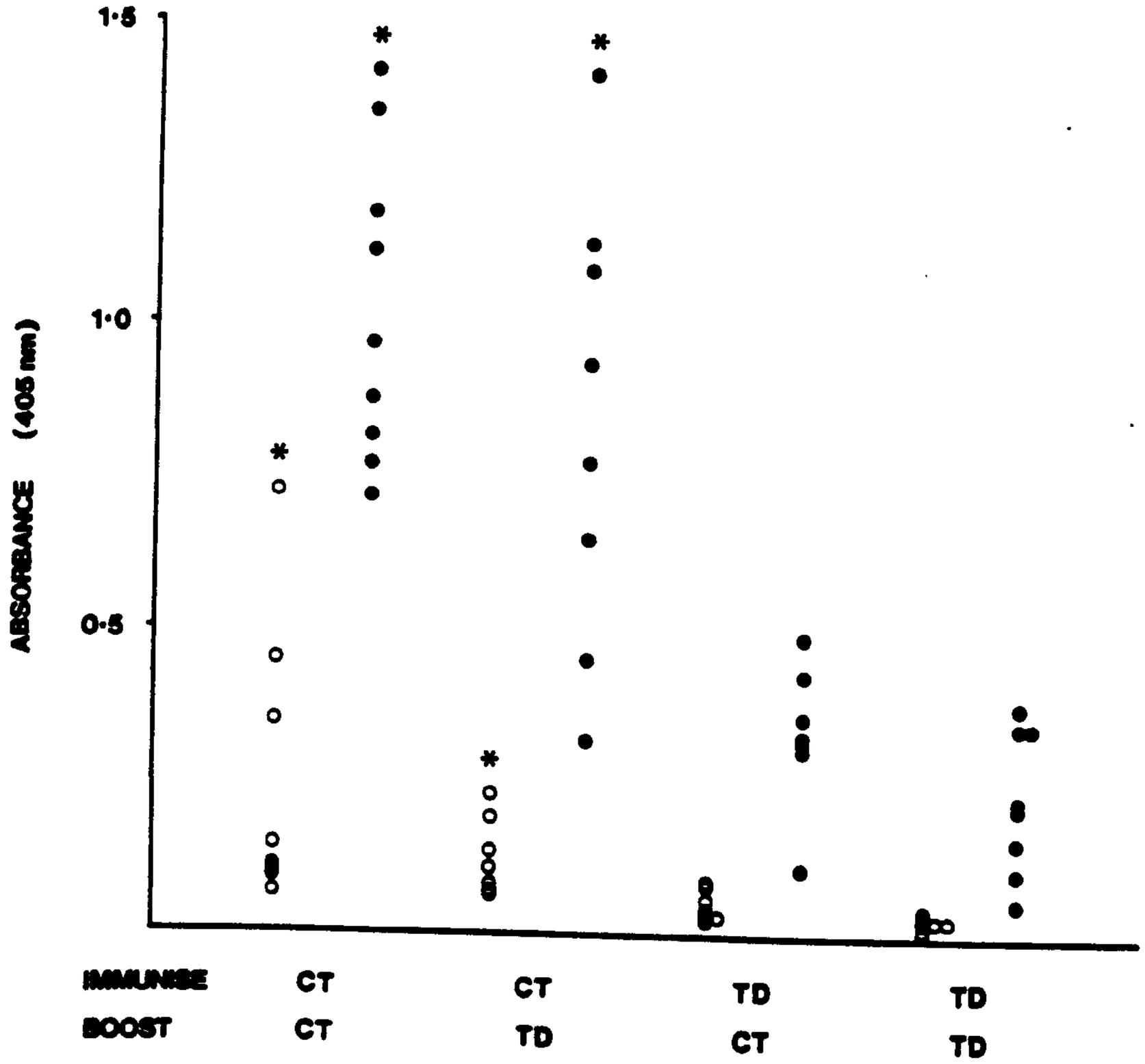

Figure 5.7 The influence of immunising or boosting with either choleragen or toxoid on systemic IgG antitoxin antibody responses

This graph shows the systemic IgG antitoxin levels obtained from mice immunised with either 1 μg CT or 5 μg TD in CFA on day 0 and boosted with either 1 μg CT or 5 μg TD on day 14 of the experiment. Individual mice were bled on day 13 (○) and day 22 (●) and their sera were measured in an IgG antitoxin ELISA. The above points represent the absorbance readings of these sera in this assay.

Statistical comparisons were made between identically boosted groups at the same time point after immunisation with either toxin or toxoid.

* $p<0.01$

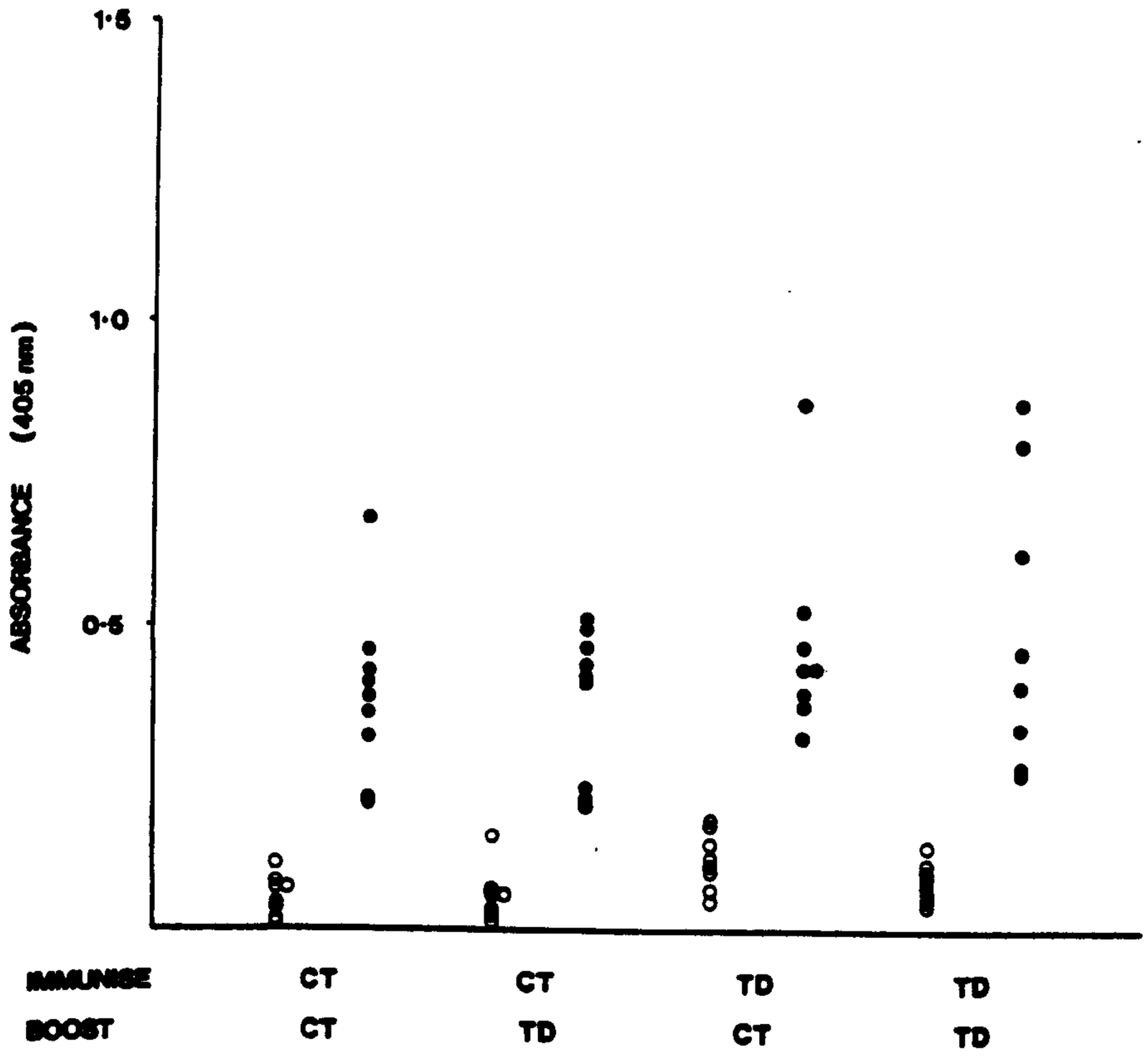

Figure 5.8 The influence of immunising or boosting with either choleragen or toxoid on systemic IgG anti-toxoid antibody responses

This graph shows the systemic IgG anti-toxoid levels obtained from mice immunised with either 1 µg CT or 5 µg TD in CFA on day 0 and boosted with either 1 µg CT or 5 µg TD on day 14 of the experiment. Individual mice were bled on day 13 (○) and day 22 (●) and their sera were measured in an IgG anti-toxoid ELISA. The above points represent the absorbance readings of these sera in this assay.

Statistical comparisons were made between identically boosted groups at the same time point after immunisation with either toxin or toxoid.

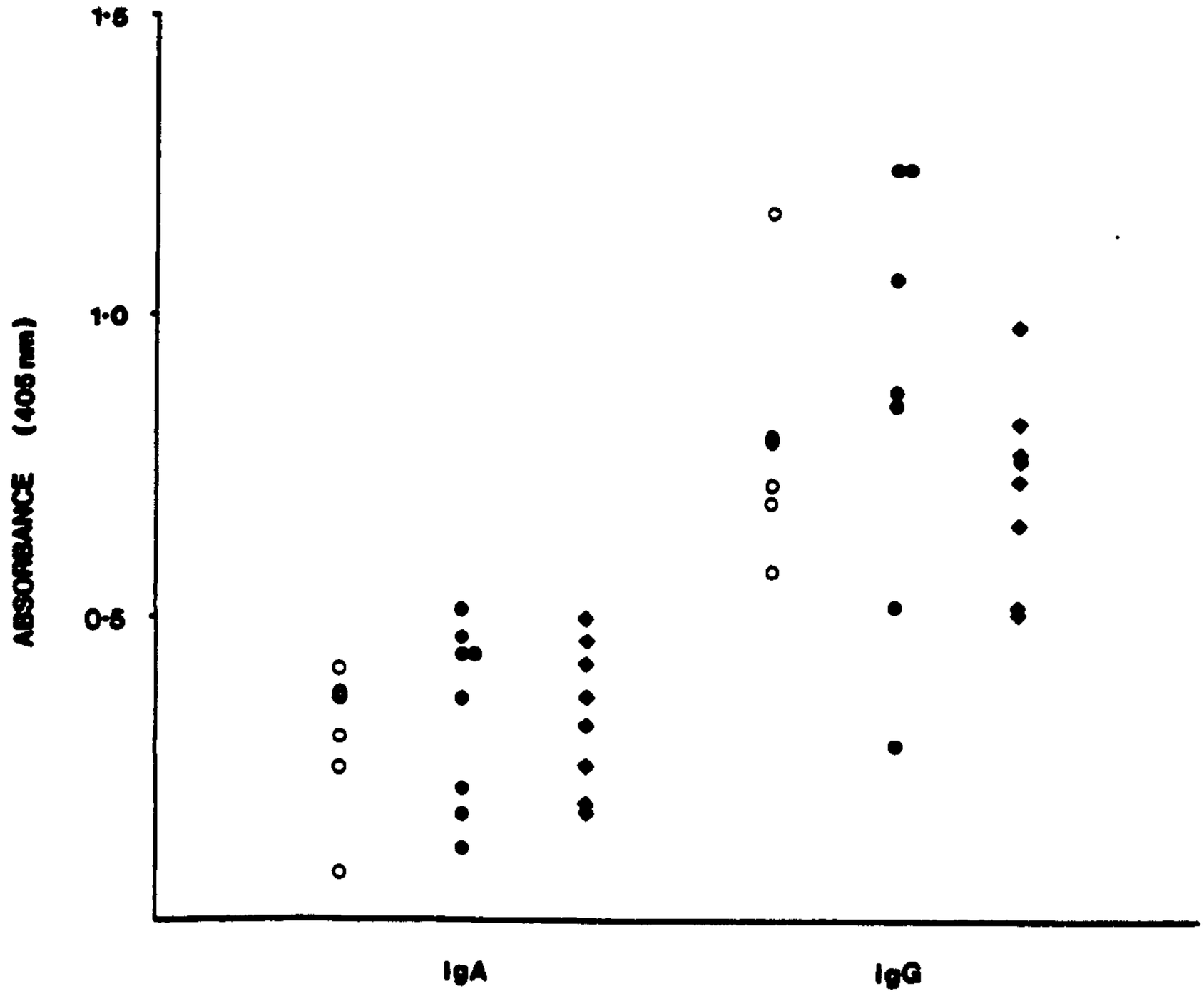

Figure 5.9 The effect of prefeeding choleragen or toxoid on the induction of systemic IgG and IgA antitoxin antibodies

This graph shows the effect of feeding ABS (○), 1 µg CT (●) or 5 µg TD (◆) on the induction of systemic IgG and IgA antitoxin after immunisation with 1 µg CT in CFA. Animals were fed seven days before immunisation. Mice were boosted into the footpad with 5 µg TD two weeks after immunisation and were bled for serum antibody measurement 8 days after this injection. The above points repesent the absorbance readings of these sera in these isotype-specific assays.

Statistical comparison was made between sham- and antigen-fed groups of animals.

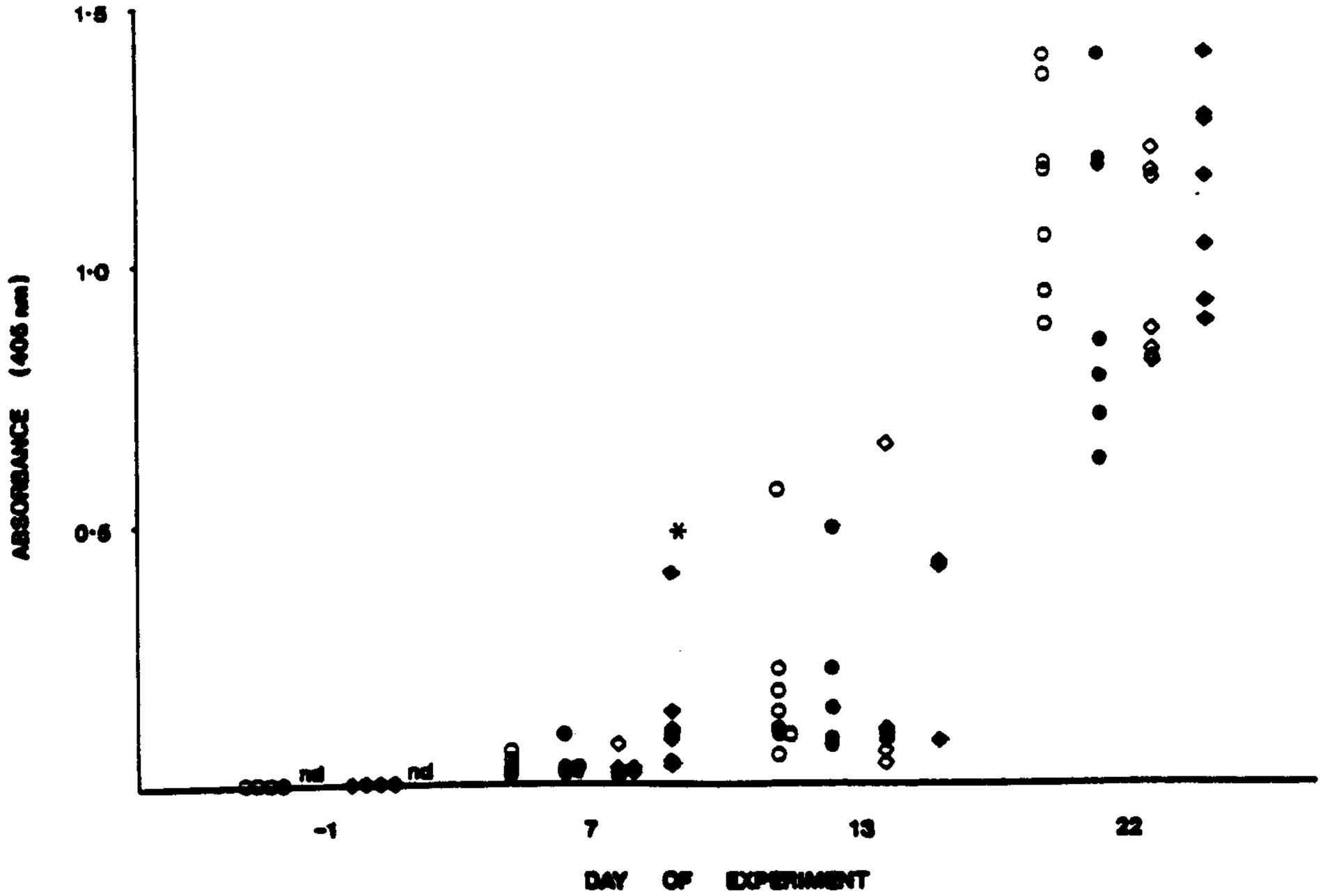

Figure 5.10 The effect of feeding varying doses of choleragen on the development of the IgG antitoxin response to a choleragen immunisation

This graph shows the IgG antitoxin antibody responses of mice fed either ABS (○), 0.1 µg CT (●), 1 µg CT (◇) or 10 µg CT (◆) seven days before intradermal immunisation with 1 µg CT in CFA. The animals were fed on day -7, immunised on day 0 and footpad boosted with 5 µg TD on day 14. The animals were bled on days -1, 7, 13 and 22 of the experiment and their sera tested in an IgG antitoxin ELISA. The above points represent the absorbance of these sera in this assay.

Statisical comparison was made between saline- and choleragen-fed animals for each time point.

* $p<0.02$

nd not tested

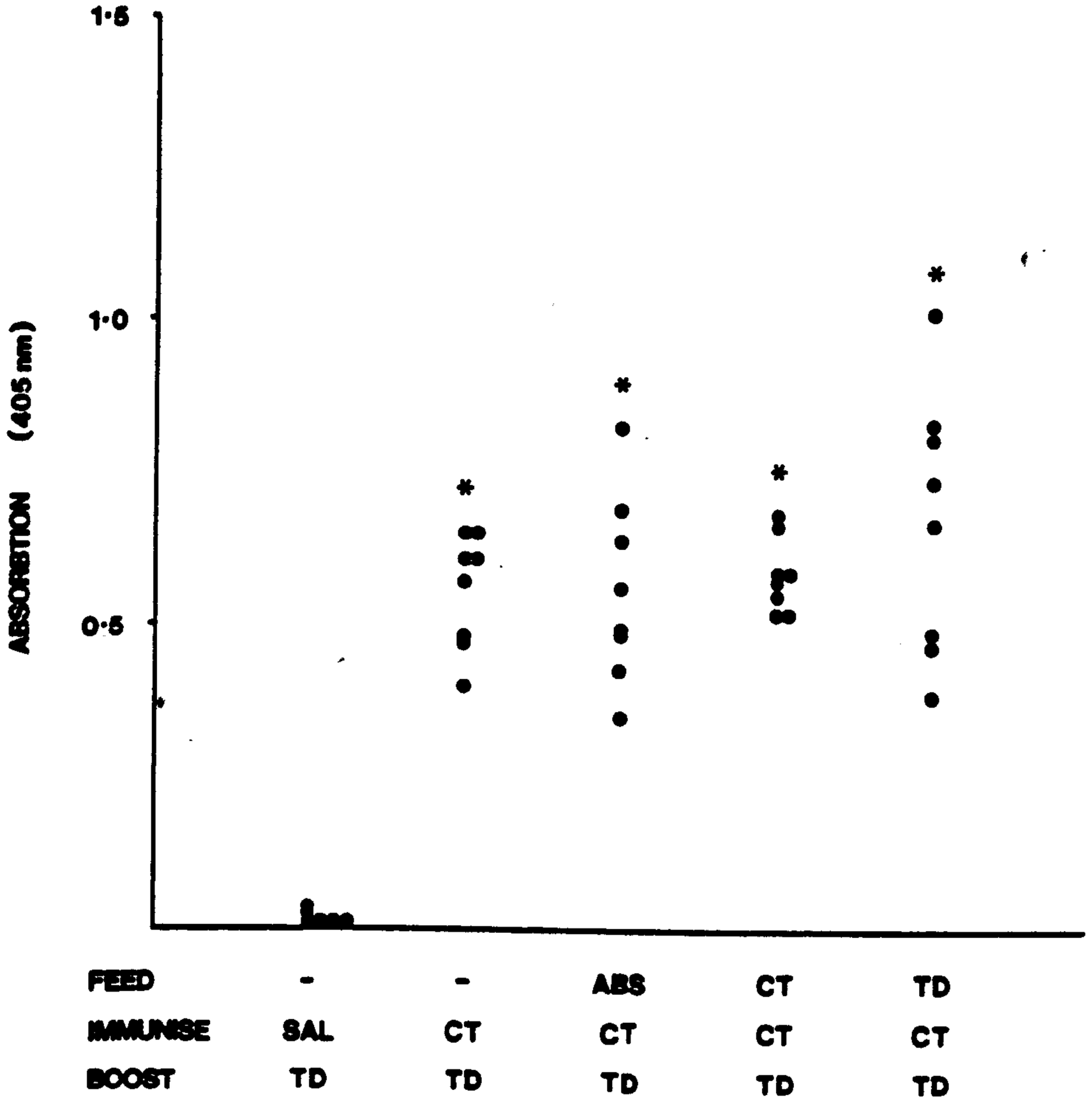

Figure 5.11 The effect of prefeeding cholera toxin or toxoid on the subsequent induction of systemic IgG anti-toxoid responses by toxoid immunisation

Animals were either fed ABS, 1 µg CT or 5 µg TD seven days before immunisation. Fed mice were immunised with 5 µg TD in CFA as was a group of unfed animals. Other unfed animals were immunised with saline in CFA at the same time. Two weeks later all animals were boosted into the footpad with 5 µg TD and the serum anti-toxoid levels were measured 8 days after this.

The points shown above represent individual animal's IgG anti-toxoid antibody response and experimental groups were statistically compared to those unfed animals immunised with saline in CFA.

* $p<0.01$

Chapter 6

SYSTEMIC CMI TO CHOLERA TOXIN AND TOXOID

6.1 Introduction

Antibody responses to both choleragen and its detoxified derivatives have been extensively studied but the possibility of a cell-mediated reaction to these antigens has been virtually ignored by comparison. Experiments have therefore been designed to investigate the state of systemic cell-mediated immunity (CMI) under conditions shown previously to induce a specific serum antibody response to both cholera toxin and toxoid.

There have been reports in the literature that suggest that DTH reactions have an obligatory early component in which an 'immediate-type' swelling occurs (Schwartz, Askenase & Gershon, 1977; Askenase, van Loveren, Kraeuter-Kops, Ron **et al**, 1983). In 1970, Finkelstein and Hollingsworth described a bioassay for antitoxic immunity. They showed that the footpad oedema normally associated with an intradermal injection of cholera toxin could be lessened if the animal had first been intravenously immunised with the substance. They also reported that an inoculation of formalinised toxoid failed to produce footpad oedema. However if the animal had been immunised previously, the toxoid then caused an increase in footpad thickness within 30 minutes of intradermal administration. This may have been the earliest description of DTH to cholera toxin had a later time-point been used to measure the increment in footpad thickness.

Experiments performed in chapter 4 confirmed some of the findings of Finkelstein and Hollingsworth and so experiments were designed to investigate this phenomenon more fully. In particular, swelling responses were measured 24 hours after antigenic challenge in order to establish whether DTH had occurred.

As in 1970, toxoid was used as the antigen to recall a possible DTH reaction because it does not cause footpad oedema which would interfere with the interpretation of skin testing. Using the simple protocol described in Chapter 5, the responses of mice immunised with antigens in CFA were examined. Various parameters of DTH were investigated, including the specificity, dose-response and time-course of the response. The histology of the footpad swelling was also investigated.

Since much of the work in this thesis will relate to **in vivo** skin testing of animals, it was important to establish the presence of systemic immunity by tests other than the careful examination of the footpad response. An **in vitro** assay of lymphocyte migration inhibition factors and an adpotive transfer proceedure, both of which appear to be T cell-mediated phenomena (Marchal, Milon Hurtrel & Lagrange, 1978; Titus & Chiller, 1981b; Scovern & Kantor, 1982; Mowat & Ferguson, 1982a), have also been used to investigate the presence of systemic CMI in toxin-primed mice.

Section A: DTH Responses in Host Animals

6.2 Systemic DTH Responses to Differing Immunisation Doses of Cholera Toxin

The previous chapter described the systemic antibody responses in BALB/c mice immunised with toxin in CFA and boosted intradermally with toxoid a few weeks later. This protocol has been adopted in the following experiments but the second exposure to antigen has been used as a challenge for the presence of DTH. In this way, the activity of both effector limbs of the immune response may be assessed in the same animal - something completely novel in the study of the immune response to choleragen.

Groups of mice were intradermally immunised with either saline or 0.1, 1.0 or 10.0 µg CT in CFA. Three weeks later, these groups were subdivided and challenged with either 0.5, 5.0 or 50.0 µg TD into the contralateral hind footpad. Twenty-four hours later, the increment in footpad thickness was measured and the results are displayed graphically in figure 6.1.

A footpad injection of 0.5 or 5.0 µg TD in saline-primed animals failed to produce an increase in footpad thickness. However an injection of 50.0 µg TD caused a significantly greater amount of non-specific footpad swelling in similarly immunised mice ($p<0.001$ in both cases).

Regardless of the immunising dose used, toxin-primed mice did not have significant antigen-specific footpad swelling when challenged with 0.5 µg TD suggesting that this is too small a dose to elicit **in vivo** skin reactivity.

On the other hand, when footpad challenged with 5.0 µg TD, all the CT-immunised animals had significantly greater footpad swelling than saline-primed controls ($p<0.01$ for groups immunised with 0.1 and 10.0 µg CT; $p<0.001$ for 1 µg CT-immunised animals). Furthermore, the group immunised with 1 µg CT in CFA had a significantly greater response to that observed in the other two positive groups ($p<0.05$ and $p<0.001$ compared to groups immunised with 10.0 µg and 0.1 µg CT, respectively).

Despite the considerable non-specific swelling reaction with the 50.0 µg dose, a further, statistically significant increment in footpad thickness could be elicited from animals immunised with 1.0 µg and 10.0 µg CT ($p<0.001$ and $p<0.002$, respectively). Animals primed with 0.1 µg CT did not have significantly increased footpad responses when compared with mice primed with saline.

As animals had the largest footpad responses when immunised with 1 µg CT and challenged with 5 µg TD, this regime was employed in all the following experiments.

6.3 The Specificity of Systemic DTH to Choleragen

The previous section showed that mice immunised and challenged with two apparently cross-reactive antigens were capable of giving a positive footpad response. Experiments were designed to show that this response was specific for toxin-derived antigens.

Accordingly, groups of mice were immunised with saline, 100 µg OVA or 1 µg CT in CFA. In addition, a group of mice were also immunised with 1 µg CT in IFA.

The mice immunised with saline or OVA in CFA or CT in IFA were footpad challenged with 5 µg TD while the animals immunised with choleragen in CFA were challenged with either saline or 100 µg OVA. As a positive control, animals primed with CT in CFA were challenged with 5 µg TD. Animals were challenged either 2 or 3 weeks after immunisation. The results of these experiments are shown in figure 6.2.

In the groups tested 21 days after immunisation, the animals immunised with saline and challenged with toxoid did not have a significantly positive response and just as before the toxin-primed, toxoid-challenged mice mounted significant DTH swelling ($p<0.001$). Not unsurprisingly, toxin-primed animals challenged with saline failed to mount a significant response and did not differ statistically from the saline-immunised, toxoid-challenged group of mice.

Both the animals immunised with OVA and challenged with TD and the CT-primed mice challenged with OVA failed to give a significantly positive response. These groups did not significantly differ from the saline-primed mice.

Finally, the group of animals immunised with CT in IFA were able to mount a significant DTH response when challenged with toxoid. Although the response of these animals was significantly less than the one observed in positive controls ($p<0.001$), it was significantly greater at the 5% level than the swelling seen in saline-primed, toxoid-challenged controls.

The trends observed above also hold true for animals challenged two weeks after immunisation. Once again, mice immunised with saline and challenged with toxoid and toxin-primed animals challenged with saline both failed to give significantly positive responses. As before, toxin-primed, toxoid-challenged mice gave a good DTH response which was significantly greater than the results observed in the above two groups ($p<0.001$).

The footpad responses observed in the OVA-primed, TD-challenged and the CT-primed, OVA-challenged groups did not significantly differ from those observed in the saline-primed animals but were significantly less than the responses of the positive controls ($p<0.001$).

These results indicate that the footpad swelling response observed in CT-primed animals is specific for toxin-derived antigens. Furthermore, there appears to be no significant quantitative or qualitative difference in the results observed either two or three weeks after priming. As the shorter of the two protocols was the more convenient, this time interval between immunisation and challenge was adopted for almost all the following experiments.

6.4 Time Course of the DTH Response

Groups of mice were intradermally immunised with either saline or 1 µg CT in CFA. Two weeks later, half the toxin-primed mice were challenged with 5 µg TD and half with saline only. The saline-primed mice were footpad challenged with 5 µg TD at the same time. Over the next 48 hours, these animals' footpads were measured at various intervals in order to determine the time-course of the DTH swelling response.

The results of this experiment (figure 6.3) show that the footpad swelling response to antigen challenge in immunised mice is biphasic in nature.

The control animals, either mice immunised with CT and challenged with saline or immunised with saline and challenged with TD, exhibited similar footpad responses at all times. Both these groups had swollen feet within 30 minutes of the injection of 50 µl of inoculate but

this had largely disappeared by 2 hours and was virtually gone at 24 hours.

In the responsive animals (immunised and challenged with antigen) the DTH curve is different. In the first place, these animals mounted significantly greater footpad swelling than controls at all times ($p<0.001$ in all cases except one; $p<0.01$ between this and the saline-primed group, 9 hours after footpad injection).

The shape of this response curve is also different. The initial peak, present at 30 minutes, declined rapidly at first and then gradually reached its lowest point 9 hours after injection. By 18 hours, the mice footpads were again swollen and reached a second peak at 24 hours. The swelling present at both 18 and 24 hours after injection was significantly greater than that present at 9 hours ($p<0.01$ in both cases). At 48 hours, the incremental change in footpad thickness had once again declined.

Figure 6.4 shows the composite curve of net footpad swelling when the result means of the saline-primed animals was subtracted from the means obtained from the positive group. It is now appreciable that it is the second phase of the response which is responsible for the largest amount of swelling when the administraton of fluid into the footpad is taken into account.

6.5 The Histology of the Footpad Response to Choleragen

DTH reactions have a characteristic histology (Dvorak **et al**, 1974) and experiments were designed to see whether the microscopic appearance of the footpad reaction was consistent with earlier descriptions. In order to observe the development of the lesion, mice immunised with either toxin or toxoid were challenged with 5 µg TD fourteen days later. The mice were then sacrificed either at 30 minutes or 2, 4, 8, 18 or 24 hours after antigen challenge and the histology of their footpads examined. Control animals primed with antigen and challenged with saline were also sacrifed as above. The results may be seen in figure 6.5.

The earliest change observed was sub-papillary oedema. This could be seen in both antigen- and saline-challenged footpads. This oedema settled in control footpads and was not detectable in the sections taken at 4 hours.

In the footpads undergoing DTH, mononuclear cells could be seen within the venules of the superficial dermal plexus after 4 hours. Perivenular lymphocytes were not obvious at this time. By 8 hours, however, the superficial dermal plexus was crammed with mononuclear cells, mainly small- to medium-sized lymphocytes. The epidermis and deeper dermal layers are relatively free of infiltrate but perivenular 'cuffs' of lymphocytes were prominent.

Later changes, observed 18 to 24 hours after antigenic challenge, were typical of a classical DTH reaction, with a prominent, widespread, mononuclear cell infiltrate occurring mainly in the deep dermis. This infiltrate consisted not only of lymphocytes but other leucocytes as well, the most common being basophils and neutrophils. Macrophages were not commonly seen at any time.

6.6 The Systemic DTH Response to Cholera Toxoid

The previous chapter compared the antibody responses of BALB/c mice to doses of parenterally administered toxin and toxoid with equivalent B subunit content. Using an appropriate dose of toxoid (with a similar B subunit content to the optimal DTH priming dose of toxin), the DTH responses to this modified antigen were examined.

Groups of mice were immunised with 5 μg TD in CFA. These animals were footpad challenged with either saline, 100 μg OVA or 5 μg TD either 14 or 21 days after immunisation. The results of this experiment are displayed in figure 6.6.

Regardless of the time elapsed after priming, challenging toxoid-primed animals with either saline or OVA failed to produce a significant footpad response. In contrast, challenge with the appropriate antigen (5 μg TD), produced a footpad swelling response which was significantly greater than that observed in the other two groups ($p<0.001$).

6.7 The Effect of Challenging Toxin- and Toxoid-primed Animals with Cholera Toxin B Subunit

Experiments in chapter 5 suggest that cholera toxin and toxoid may not be totally immunologically cross-reactive. One reason for the similarity in the footpad swelling responses of CT- and TD-primed animals may be that only shared determinants are recalled with a toxoid challenge.

In order to investigate this, animals were intradermally immunised with either saline, 1 µg CT or 5 µg TD in CFA. Two weeks later, these mice were footpad challenged with either 5 µg TD or 1 µg cholera toxin B subunit (CTB) and the increment in footpad thickness measured 24 hours after this. Using these doses, the TD and CTB challenges had an equivalent B subunit content. The result of this experiment is shown in figure 6.7.

A small swelling response was observed in saline-primed animals but this was comparable in mice challenged with either TD or CTB. CT- and TD- primed animals mounted good DTH responses, of similar magnitude, to a toxoid challenge. This response was significantly greater than that observed in saline-primed and toxoid-challenged controls ($p<0.001$, in both cases). When footpad challenged with CTB, both CT- and TD-primed animals exhibited significantly increased footpad swelling compared with saline-primed controls ($p<0.02$, in both cases). The DTH reaction did not significantly differ regardless of the antigen used for immunisation, but was significantly less than that observed in

similarly primed but toxoid-challenged counterparts ($p<0.001$, in both cases).

This result suggests that both toxin and toxoid are equally good at priming for a DTH reaction and that TD is superior to CTB as a recall antigen. This finding is in agreement with the observation that TD is in a partially aggregated form (Thompson, 1984) and that aggregated proteins elicit greater DTH reactions (Titus & Chiller, 1981b).

SECTION B: Other Assays of Systemic CMI

6.8 The Passive Transfer of DTH

This section will deal with the first corroborative test for the presence of systemic CMI. Other workers have shown that the transfer of DTH to naive syngeneic recipients is mediated by T cells (see chapter 3), the experiments that will be described examine the ability of draining lymph node cells from toxin-primed animals to reproduce this phenomenon. These cells were obtained under conditions where DTH has been shown to exist.

This section will be subdivided into three parts which will, in turn, contain descriptions of experiments to determine the number of cells necessary to transfer DTH, the specificity of this phenomenon and the phenotype of the cell responsible.

a: The DTH Responses Transferred by Varying Numbers of Primed Lymphocytes

Draining popliteal and inguinal lymph node cells were obtained from animals immunised two weeks earlier with either saline or 1 µg CT in CFA and were transferred in varying numbers with 5 µg TD to the footpads of naive syngeneic recipient mice.

As can be seen from figure 6.8, saline-primed lymph node cells all transferred a small swelling response except when 10^8 cells were injected. A transfer of 10^5 toxin-primed cells/animal did not produce a significant DTH response, but all other toxin-primed inoculates did ($p<0.001$). 10^8 saline-primed cells caused a large amount of non-specific footpad swelling. This was significantly greater than the swelling responses seen in all other groups except that transferred by the equivalent number of antigen-primed cells ($p<0.001$).

10^7 cells was chosen as the optimal dose to employ in further experiments as that number provided a good positive response, a low background of non-specific swelling and required only a small number of donor animals.

b: The Specificity of the Passive Transfer of DTH

Groups of mice were intradermally immunised with either saline, 100 µg OVA or 1 µg CT in CFA. Two weeks later, these animals were sacrificed and their draining lymph nodes removed and made into single cell suspensions.

The saline- and OVA- primed cells were mixed with 5 µg TD and the toxin-primed cells were mixed with saline or 100 µg OVA prior to transfer. Either 5 µg TD or 10^7 toxin-primed cells alone were injected into recipients as negative controls and a combination of the two was transferred as a positive control. The increment in the recipients' footpad thickness was measured 24 hours after transfer and

the results are displayed in figure 6.9.

Positive control animals receiving toxin-primed cells with toxoid had the largest footpad response. This swelling response was significantly greater than the responses measured in the other groups ($p<0.001$).

The smallest footpad response was seen in those animals given toxoid alone. The swelling measured in the other negative control groups, recieving either 10^7 saline-primed cells with antigen or 10^7 toxin-primed cells alone, was significantly greater ($p<0.002$) but still much less than the positive control. The swelling response measured in the remaining two groups did not differ from those seen in the negative controls. This confirms the specificity of the DTH response described in section 6.3.

c: The Effect of the Depletion of THY-1.2 +ve Cells on the Ability of Toxin-primed Lymphocytes to Transfer DTH

Toxin-primed lymphocytes (10^7/animal) were treated with either anti-THY-1.2 monoclonal antibody, absorbed Guinea-pig serum or a combination of the two. Cells which survived this procedure were transferred to recipients mixed with 5 µg TD. Toxin-primed cells were transferred with antigen as a positive control and either cells or antigen alone were transferred as negative comparisons. The results may be seen in figure 6.10.

Untreated toxin-primed lymphocytes again transferred a good DTH response in the presence of toxoid. Treating these lymphocytes with either anti-THY-1.2 monoclonal antibody or absorbed Guinea-pig serum alone did not alter the ability of these cells to transfer DTH.

However, when toxin-primed cells were treated with monoclonal antibody followed by the absorbed serum, they became incapable of transferring a positive reaction ($p<0.001$). The footpad response that these cells caused was not significantly different from the non-specific swelling caused by an injection of lymph node cells or antigen alone.

6.9 The Investigation of Migration Inhibition by Choleragen-primed Lymphocytes In Vitro

The experiment described in this final section is a further attempt to substantiate the presence of CMI in toxin-primed animals. It concerns the measurement of a lymphocyte migration inhibition factor. This factor, produced **in vitro**, has been shown to correlate well with the presence of systemic CMI and is most easily obtained from T cell-enriched populations (Mowat & Ferguson, 1982a).

Draining lymphoid cells from BALB/c mice primed two weeks previously with either saline, 100 µg OVA or 1 µg CT in CFA were tested in a lymphocyte migration inhibition assay (see chapter 4.16). The migration out of capillary tubes of these three differently sensitised groups of cells was measured after culture for 21 hours in either

medium alone or in the presence of various antigens. Antigens used were OVA at concentrations of 1.0 and 0.1 mg/ml and TD at concentrations of 1.0, 0.1, 0.01 and 0.001 µg/ml. The areas of migration of these cells were measured by planimetry and the results are expressed as a migratory index, where uninhibited migration of cells in medium alone is given a value of 1.0. The results of this experiment may be seen in figure 6.11.

Lymph node cells from saline-primed animals were able to migrate fully in the presence of both toxoid and ovalbumin at the concentrations used. OVA-primed cells were inhibited from migrating by medium containing OVA at 0.1 mg/ml ($p<0.01$) but were unaffected by the higher concentration of OVA or by toxoid at any concentration.

Conversely, CT-primed lymphocytes exhibited significant migration inhibition at a concentration of 0.01 µg TD/ml ($p<0.05$). Ovalbumin, at both the above concentrations, and the remaining concentrations of TD did not significantly affect the migration of these cells.

6.10 Summary

Experiments described in this section have shown that mice primed with cholera toxin produce an antigen-specific footpad swelling response 24 hours after intradermal challenge with toxoid. A similar response was obtained after immunisation with toxoid.

Histological examination of the footpad 24 hours after challenge revealed a mononuclear cell infiltrate which consisted mainly of small to medium-sized lymphocytes and, to a lesser degree, basophils and neutrophils. These findings are indicative of a DTH reaction. Serial measurements of footpad thickness and footpad histology were also consistent with the ontogeny of a DTH lesion as reported by other workers (Dvorak **et al**, 1974; Van Loveren **et al**, 1983) and confirms that a DTH reaction does include an early oedematous swelling component.

Both TD and CTB were able to successfully recall DTH responses, suggesting that T-cell determinants are shared between choleragen and its formalinised toxoid. Furthermore, the greater response observed after footpad challenge with TD is additional evidence of its partially aggragated nature.

Toxin-primed lymph node cells were able to inhibit their own migration **in vitro** when cultured with toxoid and the T cell component of these lymphocytes were able to passively transfer DTH to naive syngeneic recipients. Both these results confirm that systemic CMI is primed under the same conditions that induce DTH. This is the first description of systemic CMI to cholera toxin and shows that it occurs as part of an immune response which includes the production of circulating antitoxic antibodies.

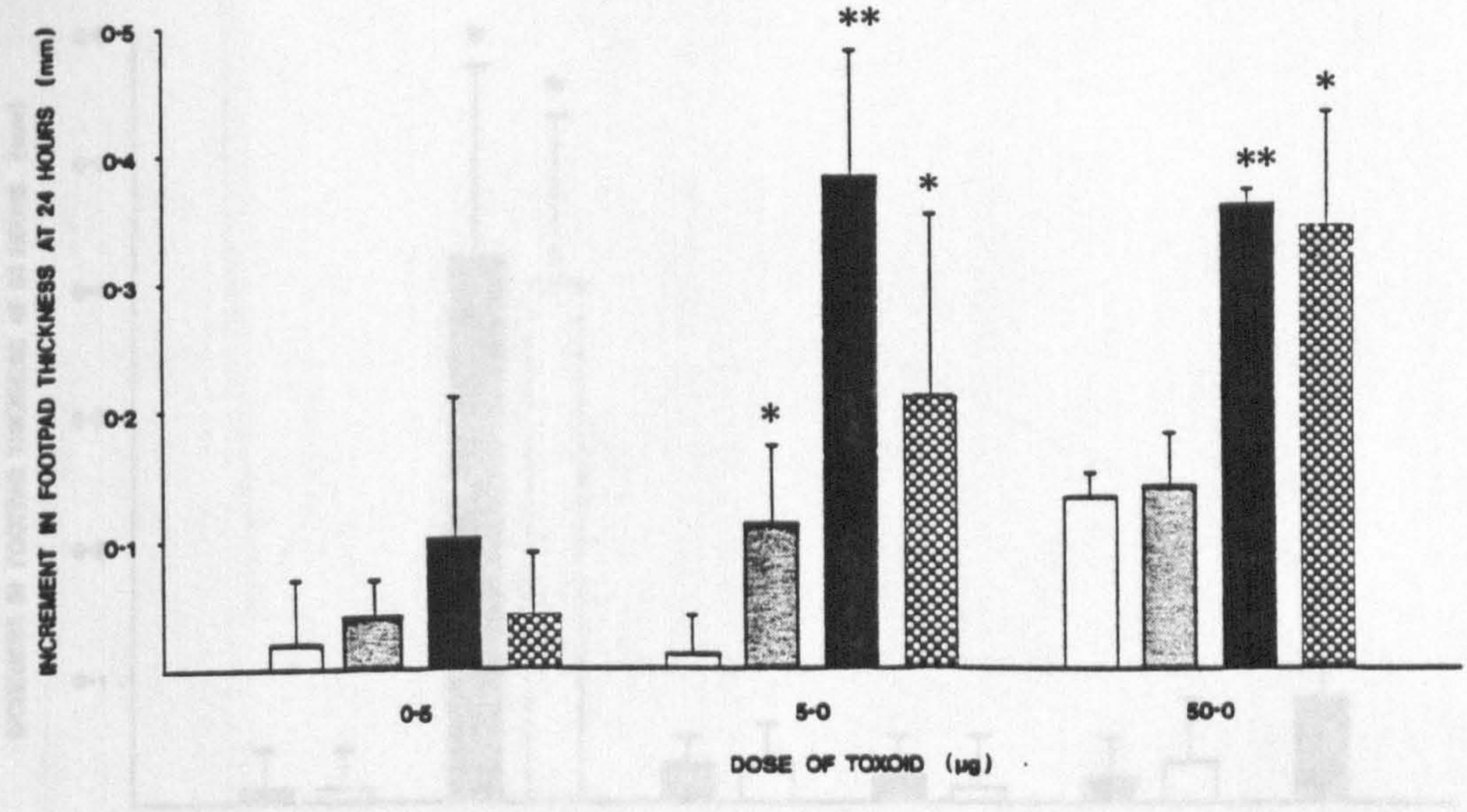

Figure 6.1 The effect of varying the doses of priming and challenge antigens on the induction of DTH to choleragen

This graph shows the DTH responses of mice intradermally immunised with either saline (□) or 0.1 µg (▩), 1.0 µg (■) or 10.0 µg (▩) CT in CFA. Three weeks later, these mice were footpad challenged with either 0.5, 5.0 or 50.0 µg TD. The bars represent the mean increment in footpad thickness ± 1 standard deviation 24 hours after footpad challenge.

Statistical comparison was made between the results obtained for saline-primed mice and the other groups of animals for each different challenge dose of toxoid.

*$p<0.01$

**$p<0.001$

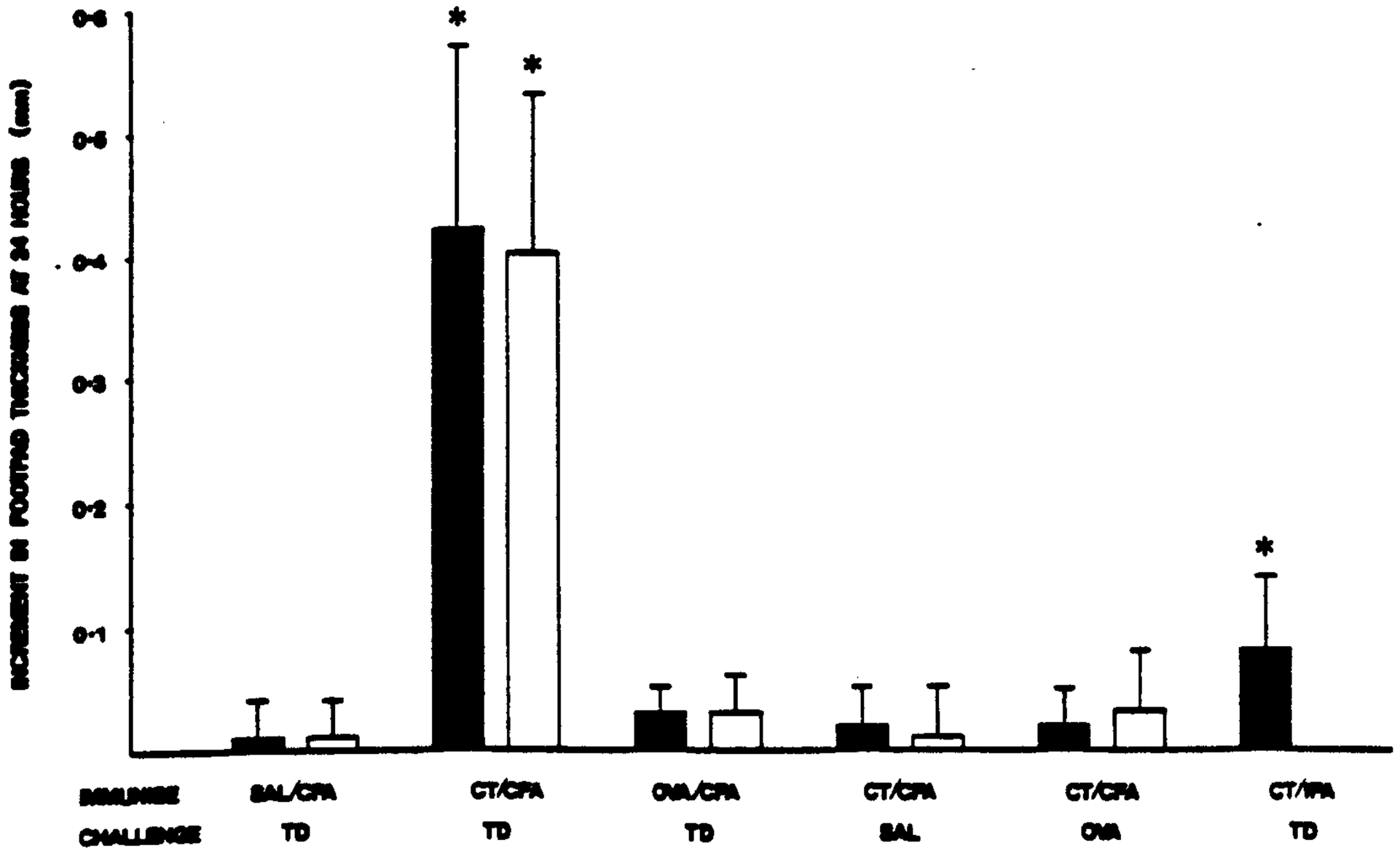

Figure 6.2 The antigen-specificty of the DTH response to choleragen

Groups of mice were intradermally immunised with either saline, 100 µg OVA or 1 µg CT in CFA. One group of mice was also immunised with 1 µg CT in IFA. Two (□) or three (■) weeks later, these animals were footpad challenged with either 5 µg TD, 100 µg OVA or saline as shown above. The bars represent the mean increment in footpad thicknesses ± 1 standard deviation measured 24 hours after footpad challenge.

The results of the other groups were statistically compared to those obtained from the saline-primed, TD-challenged animals, either 2 or 3 weeks after immunisation as appropriate.

*$p<0.001$

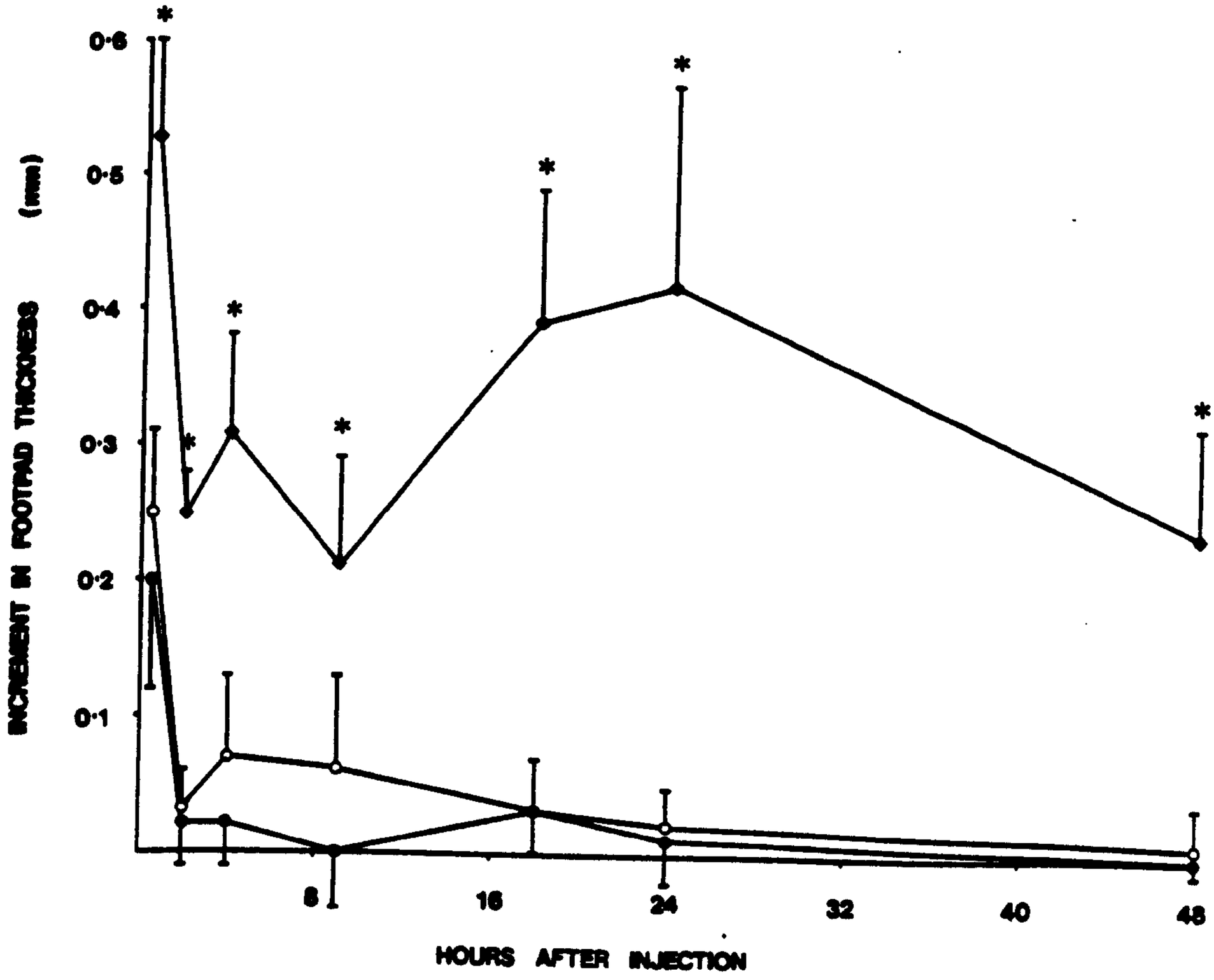

Figure 6.3 The footpad swelling response of choleragen-primed animals at various time after footpad challenge with toxoid

This graph shows the DTH responses of mice intradermally immunised with either saline (●) or 1 µg CT in CFA. Fourteen days after this, the saline-primed animals were intradermally challenged with 5 µg TD and CT-primed animals were challenged with either 5 µg TD (◆) or saline (○). The incremental changes in footpad thickness were measured at various intervals over a 48 hours period. Each point represents the mean increment in footpad thickness ± 1 standard deviation.

Statistical comparisons were made between the results obtained for toxin-primed animals challenged only with saline and those obtained for the other two groups.

*$p<0.001$

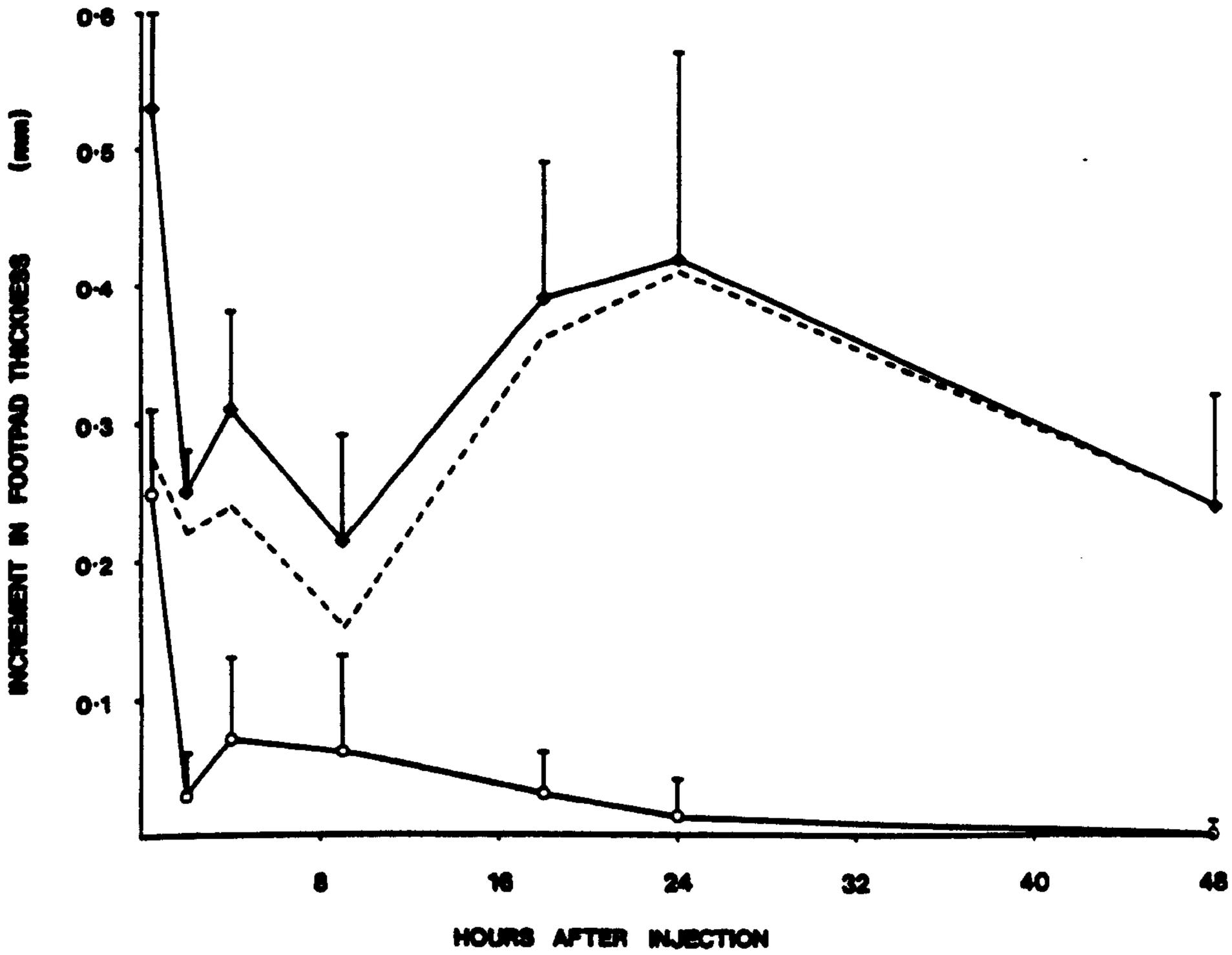

Figure 6.4 The ontogeny of the DTH swelling response in choleragen-primed animals

This graph shows the net footpad swelling over a 48 hour period caused by a DTH reaction in cholera toxin-primed animals. The line showing net swelling (- - -) is obtained by subtracting the mean incremental changes in footpad thickness caused by an injection of 5 µg TD (◆) from those caused by a saline injection (○) in toxin-primed animals. Each point represents the mean increment in footpad thickness + 1 standard deviation.

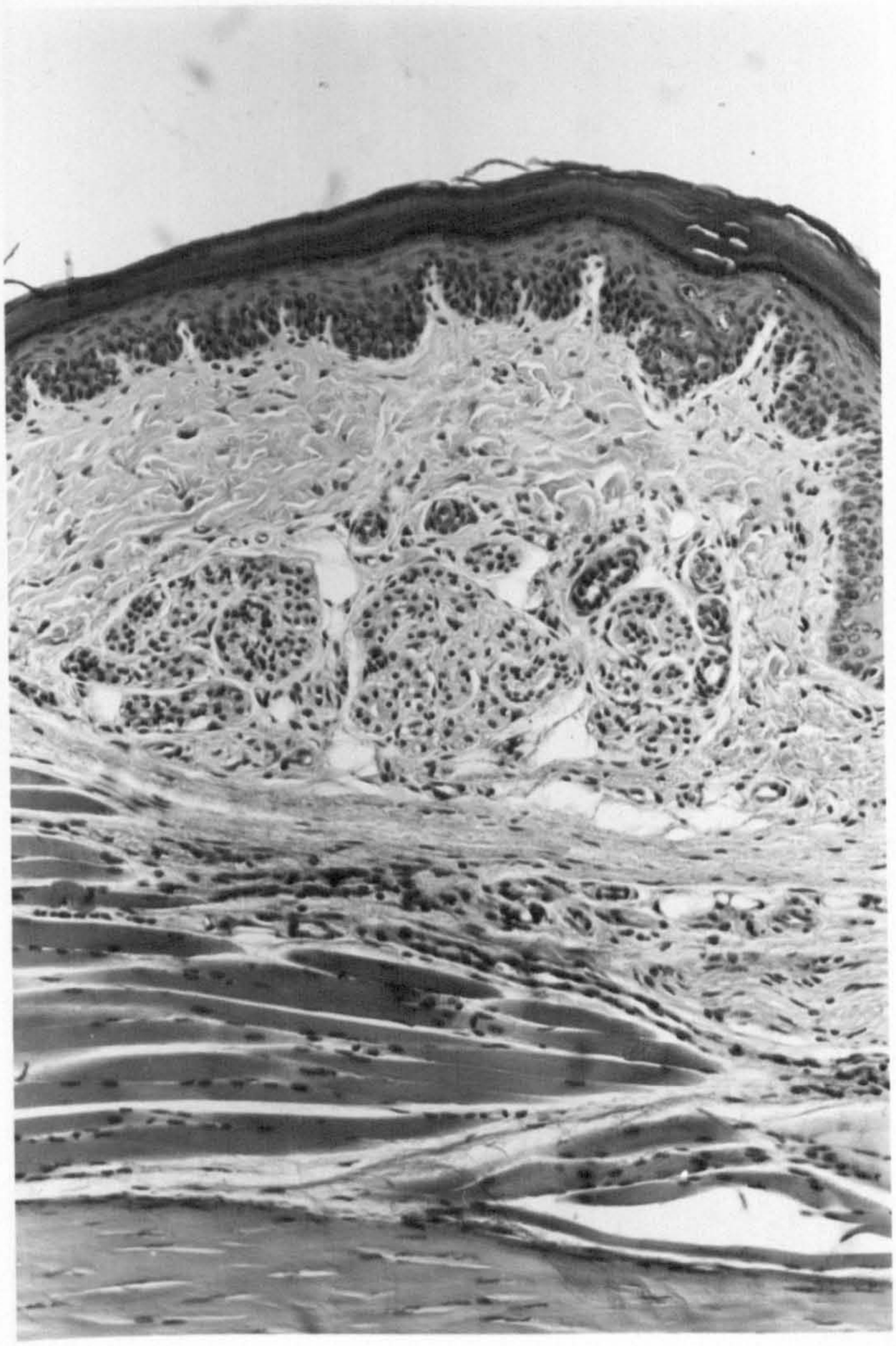

Figure 6.5 The histology of the footpad swelling response in toxin-primed animals

a: Subpapillary oedema seen two hours after footpad challenge with toxoid.

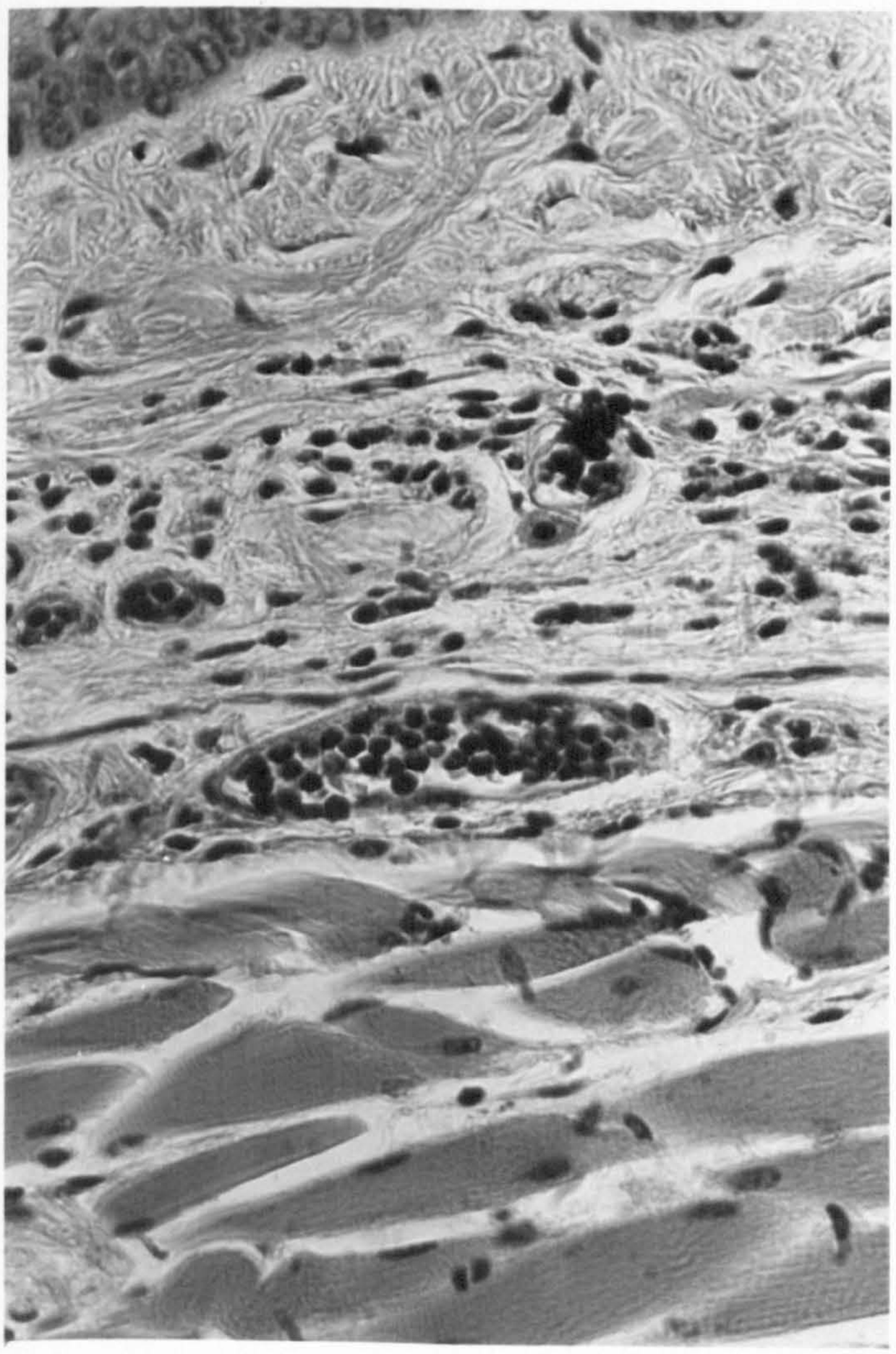

Figure 6.5 The histology of the footpad swelling response in toxin-primed animals

b: Mononuclear cells filling venules of the superficial dermal plexus at 4 hours after footpad challenge.

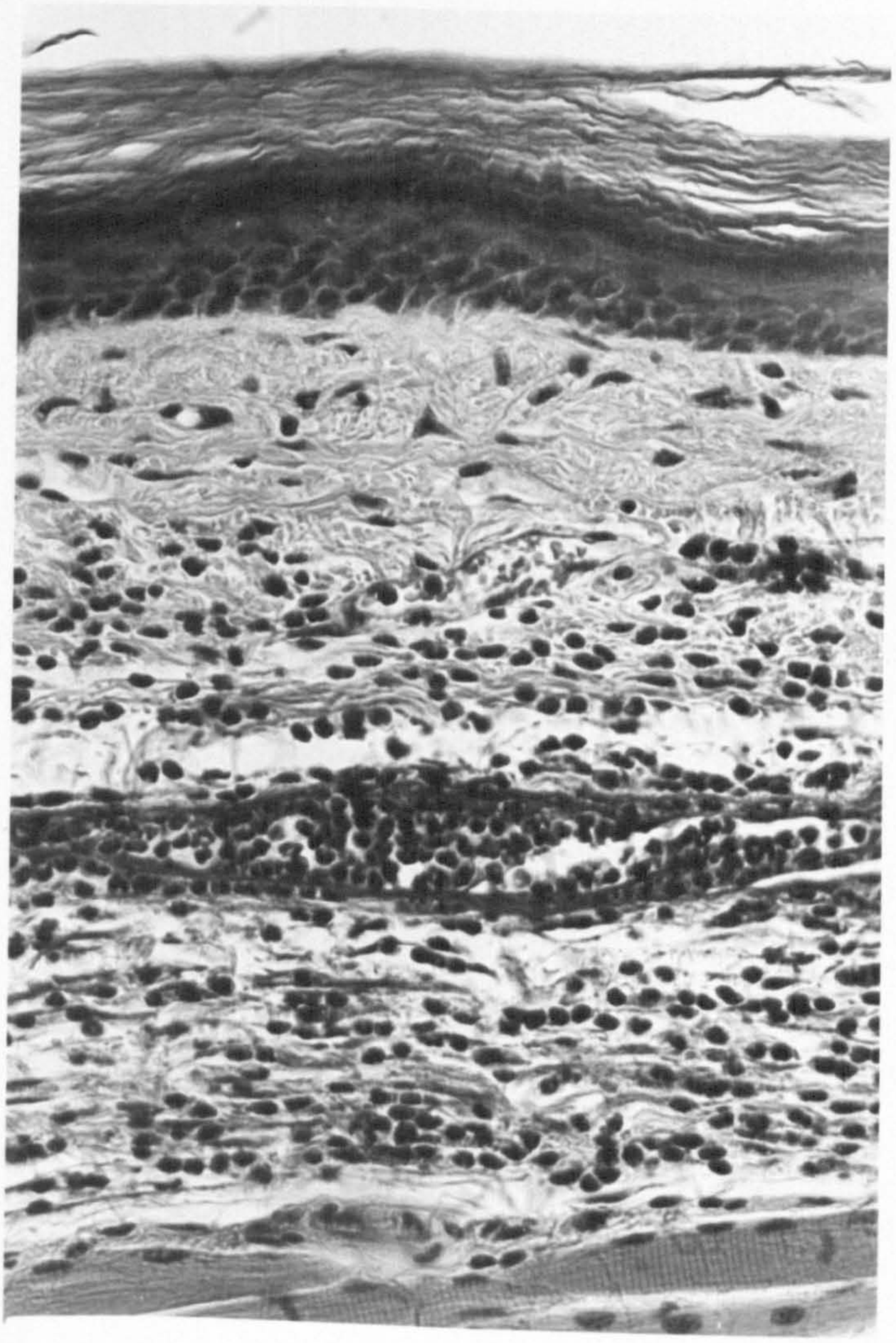

Figure 6.5 The histology of the footpad swelling response in toxin-primed animals

c: Perivenular 'cuffing' with lymphocytes at the dermo-epidermal junction at 8 hours after intradermal challenge.

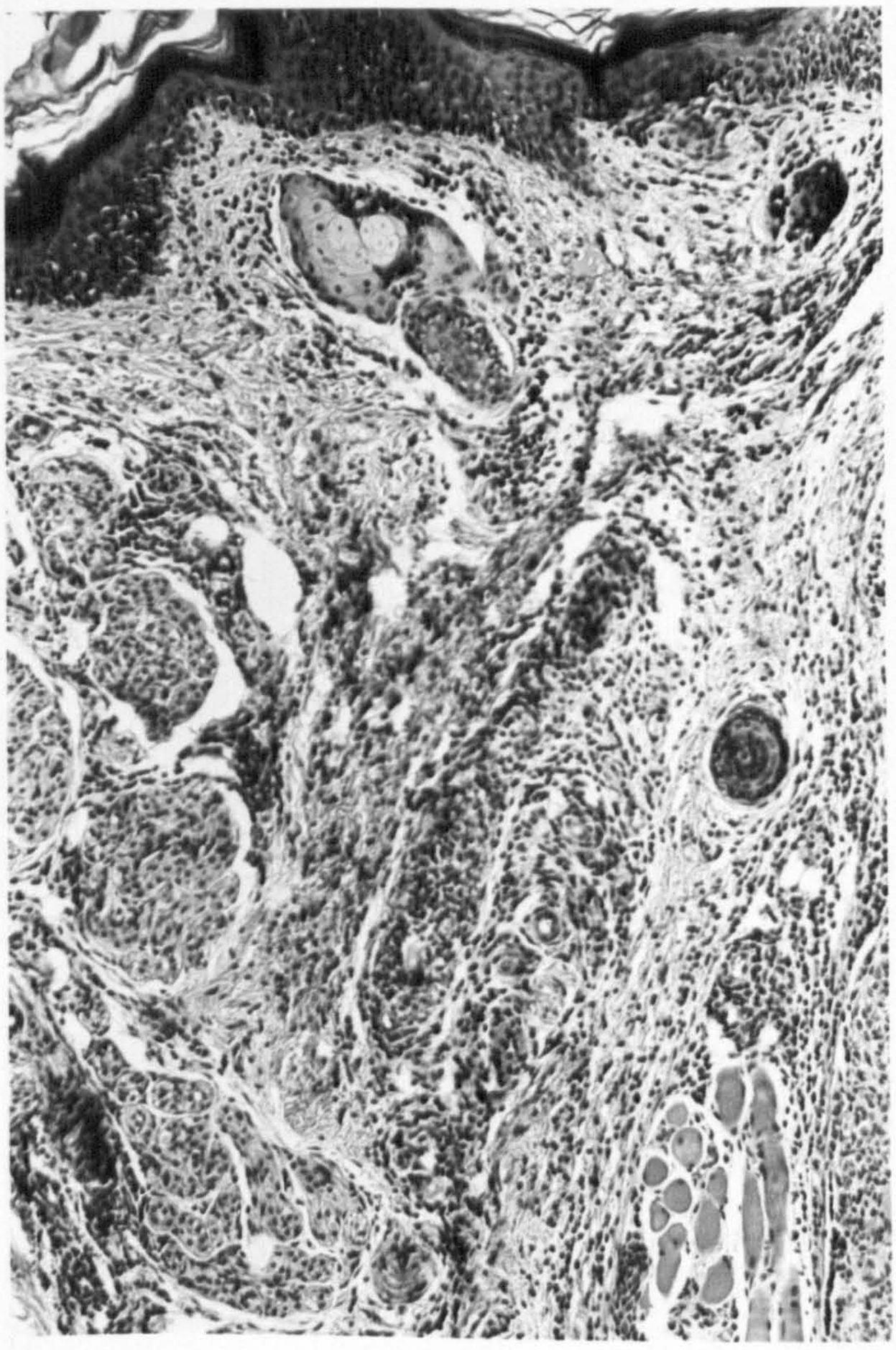

Figure 6.5 The histology of the footpad swelling response in toxin-primed animals

d: Profound mononuclear cell infiltrate in the epidermis and deep dermis 24 hours after footpad challenge.

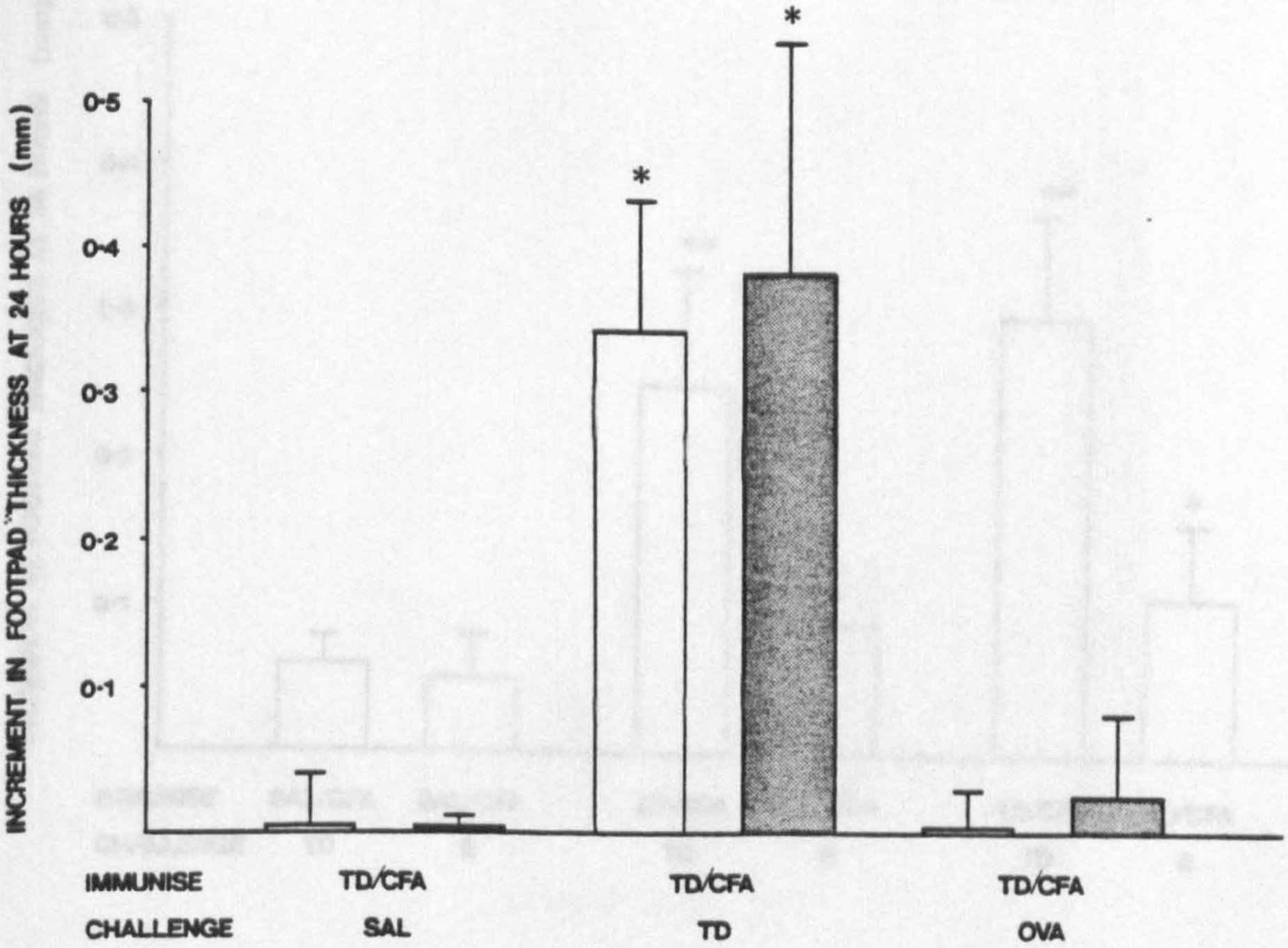

Figure 6.6 The footpad swelling response in animals primed with cholera toxoid

This graph shows the systemic DTH responses observed in mice two (□) or three (▩) weeks after being intradermally immunised with 5 µg TD in CFA. These animals were footpad challenged with either saline, 100 µg OVA or 5 µg TD. The bars represent the mean increment in footpad thickness ± 1 standard deviation 24 hours after intradermal challenge.

Statistical comparison was made between the group challenged with saline and the other two experimental groups.

*$p<0.001$

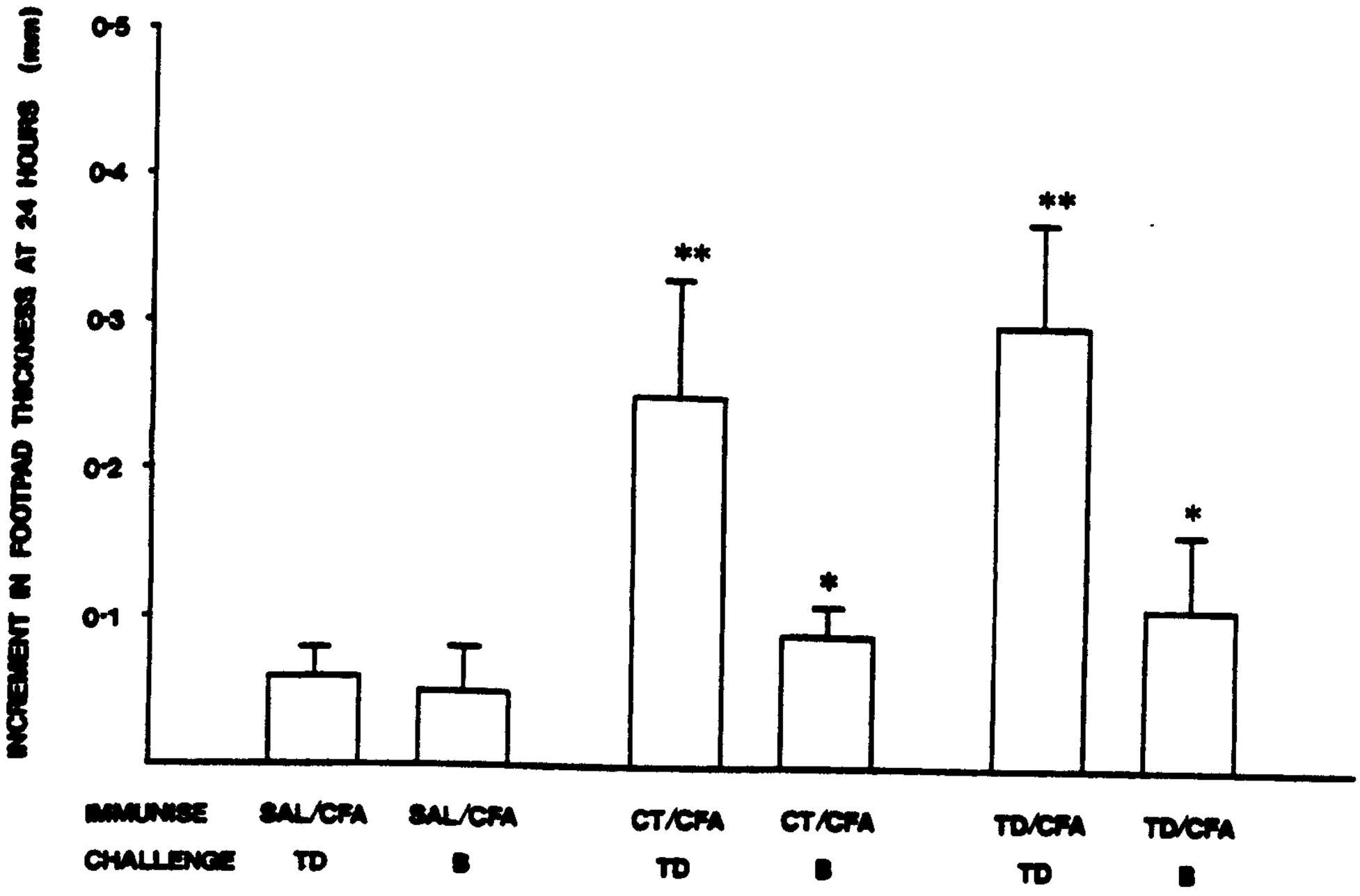

Figure 6.7 The effect of a challenge with cholera toxin B subunit on the DTH responses of toxin- and toxoid-pried mice

This graph shows the DTH responses of mice intradermally immunised with either saline, 1 µg CT or 5 µg TD two weeks before footpad challenge with either 5 µg TD or 1 µg CTB. The bars represent the mean increment in footpad thickness 24 hours after footpad challenge.

Statistical comparisons were made between saline-primed animals and the other experimental groups for each challenge antigen.

*p<0.02

**p<0.001

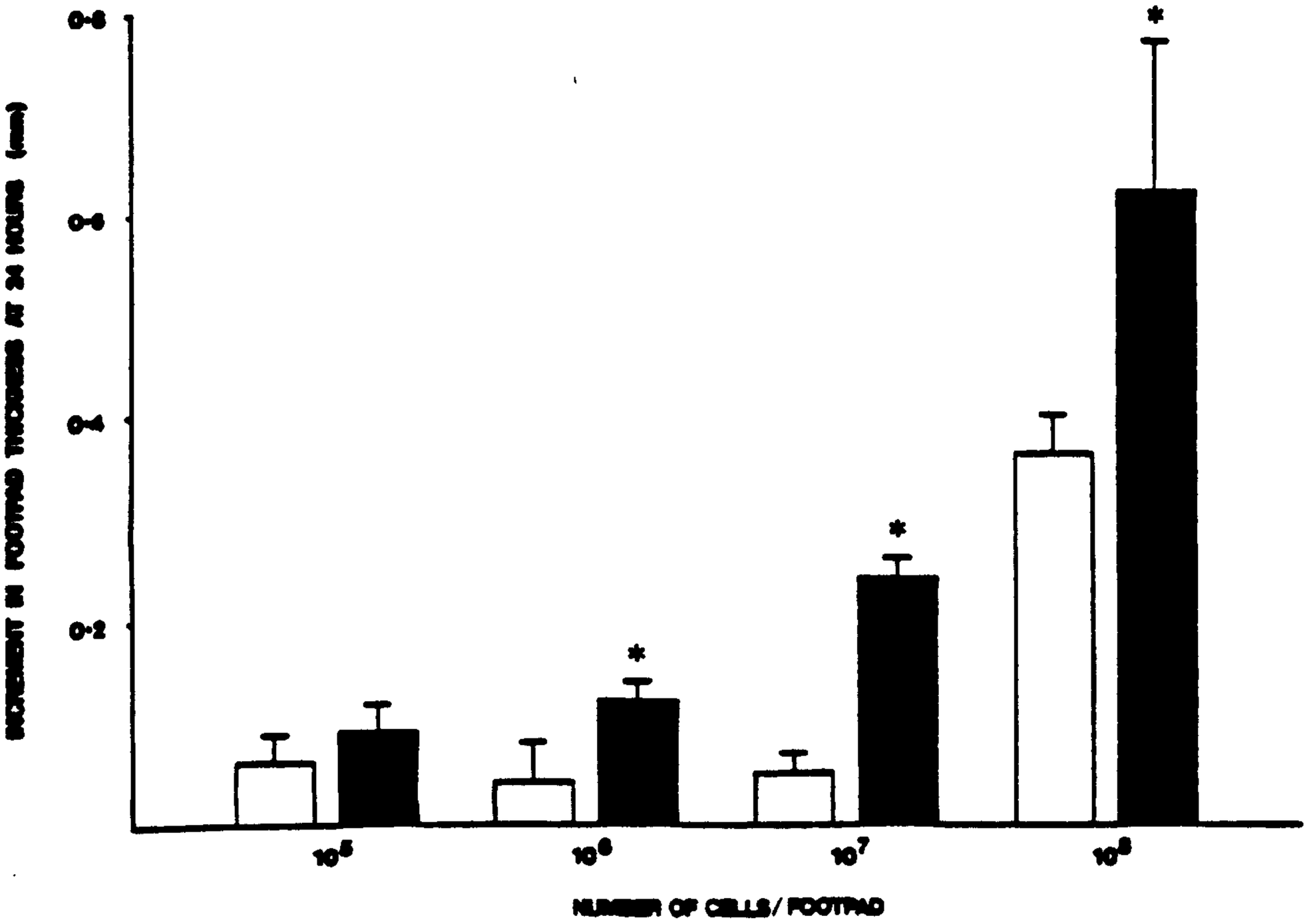

Figure 6.8 The ability of varying numbers of toxin-primed lymphocytes to passively transfer DTH

This graph shows the footpad swelling responses of mice receiving various numbers of lymphoid cells plus 5 µg TD into their hind footpads. Donor animals were bilaterally intradermally immunised with either saline (□) or 1 µg CT (■) in CFA. After 2 weeks, their draining lymph nodes were removed and made into single cell suspensions. Varying numbers of lymphoid cells from each donor group were mixed with antigen and the final volume of 50 µl of inoculate was intradermally injected into the footpads of naive syngeneic recipients. The bars represent the mean increment in footpad thickness ± 1 standard deviation measured 24 hours after cell transfer.

Statistical comparisons were made between the saline-primed lymphoid cell recipients' responses and the results obtained from those mice receiving toxin-primed lymphocytes, for each concentration of cells transferred.

*$p<0.001$

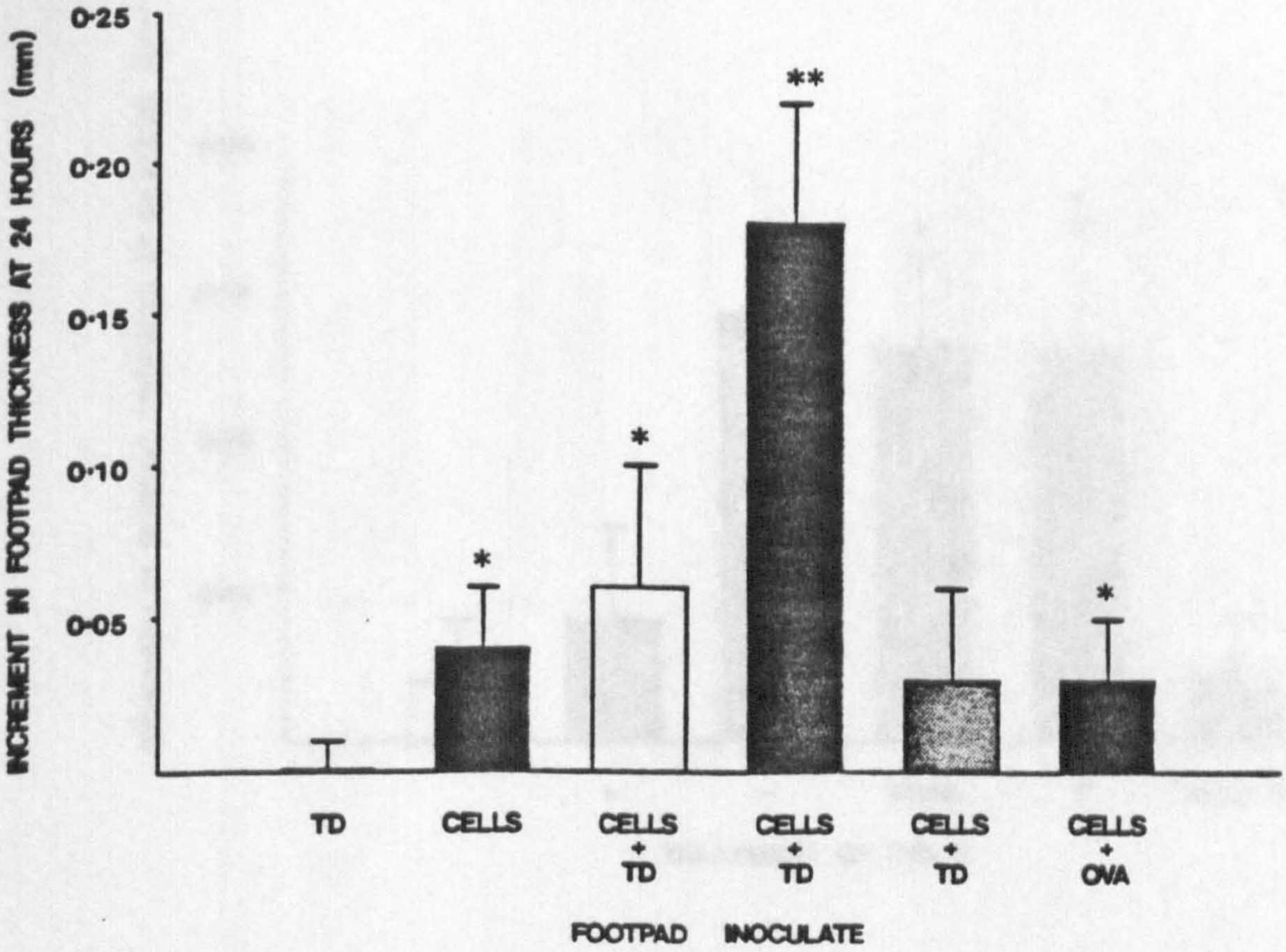

Figure 6.9 The specificity of the passive transfer of DTH

This graph shows the footpad swelling responses of mice receiving 10^7 primed lymphocytes from animals immunised two weeks earlier with either saline (□), 1 µg CT (■) or 100 µg OVA (▩) in CFA. These cells were either transferred alone or with 5 µg TD or 100 µg OVA as shown above. One group of animals received a footpad inoculate of 5 µg TD alone as a negative control.

Statistical comparison was made between the animals receiving toxoid alone and the other experimental groups.

*$p<0.02$

**$p<0.001$

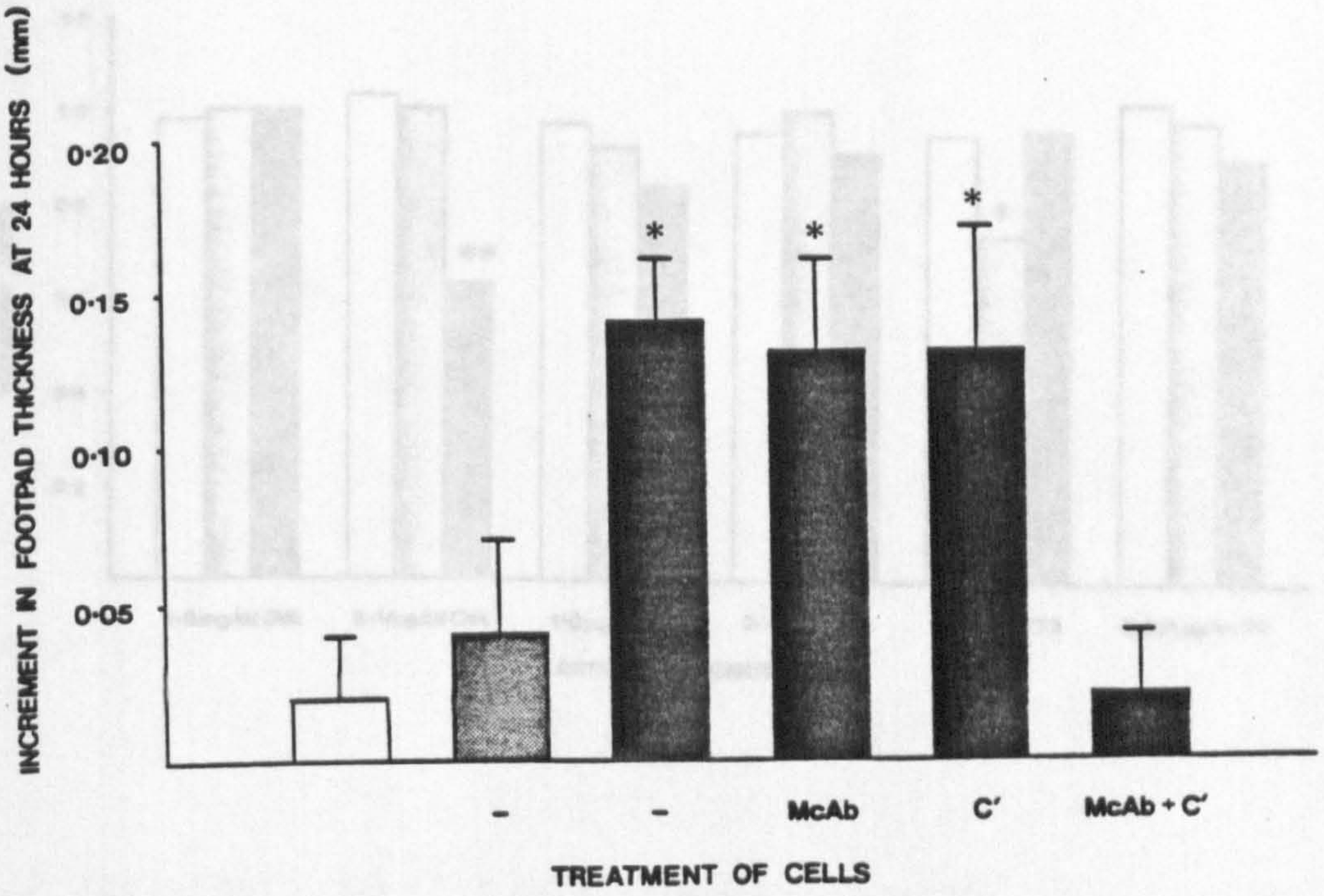

Figure 6.10 The effect of depletion of THY-1.2-positive cells on the passive transfer of DTH

This graph shows the footpad swelling responses of mice receiving either 5 µg TD alone (□) or 10^7 CT-primed lymphocytes in the presence (■) or absence (▩) of 5 µg TD. The toxin-primed cells were either left untreated or treated with anti-THY-1.2 monoclonal antibody (McAb), absorbed Guinea-pig complement (C') or McAb followed by C'. The bars represent the mean increment in footpad thickness ± 1 standard deviation 24 hours after footpad injection.

Statisitcal comparisons were made between the experimental groups and those animals receiving an injection of 5 µg TD alone.

*$p<0.001$

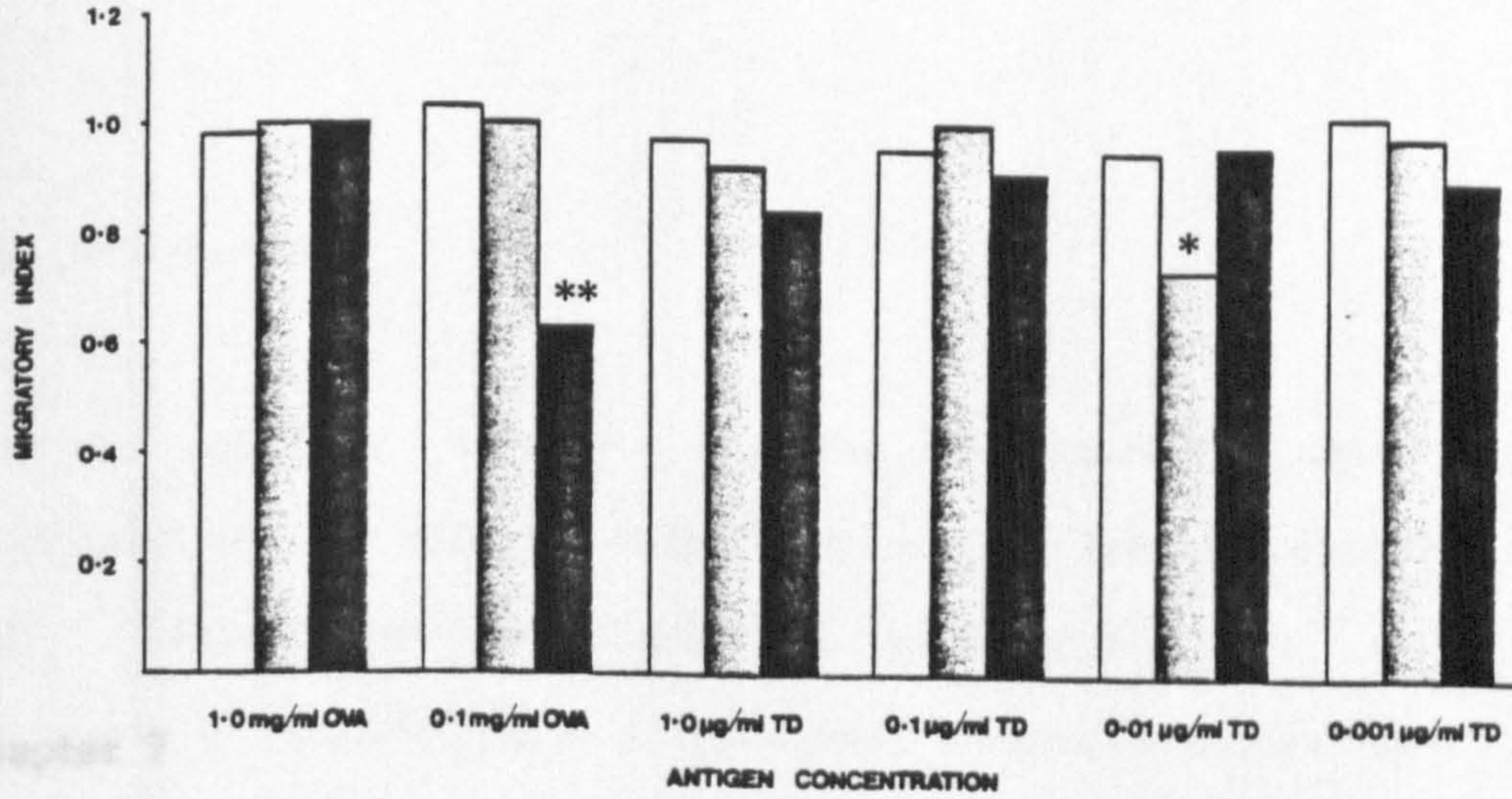

Figure 6.11 The effect of various antigens on the ability of choleragen-primed lymph node cells to inhibit their own migration in vitro

This figure shows the migratory indices of draining lymph node cells from saline- (□), OVA- (■) or CT- (▩)primed donors in the presence of either OVA or TD at varying concentrations. A migratory index of 1.0 is taken as that area occupied by lymphoid cells in antigen-free medium after 21 hours in culture at 37 °C. The migratory is calculated according to the formula shown in chapter 4.16.

Statistical comparison is made between the migration observed in antigen-free medium and that in the presence of antigen for each population of lymphoid cells from the donors described above.

*$p<0.05$

**$p<0.01$

Chapter 7

ORAL TOLERANCE TO CHOLERA TOXIN OR TOXOID

7.1 Introduction

In the previous chapter it was demonstrated that parenteral administration of cholera toxin and toxoid induces specific systemic CMI. Experiments in Chapter 5 demonstrated that under similar conditons a specific systemic humoral immune response may be also detected. It was also shown that prefeeding choleragen or toxoid failed to tolerise the induction of this antitoxin response which is in agreement with the findings of Elson and Ealding (1984b).

This chapter reports the results of studies, performed in parallel with those in Chapter 5, that examine the effect of feeding on the induction of specific CMI.

Using the protocol employed in Chapter 5, sytemic CMI was assessed after feeding various doses of toxin and toxoid. As mentioned in chapter 1, the oral administration of toxin and toxoid produces different effects on the induction of systemic antibody and this has been attributed to the difference in the biochemical properties of these two substances (Pierce, 1978). Prefeeding both choleragen and its toxoid allowed the role of these biochemical properties in the induction of oral tolerance to be assessed.

The specificity of the effect of prefeeding these antigens was once again studied by using the unrelated protein, ovalbumin.

7.2 The Effect of Feeding Cholera Toxin on the Subsequent Induction of Systemic DTH

In order to investigate the effect of prefeeding choleragen, the experimental protocol, illustrated in figure 7.1, was devised.

Essentially, two groups of animals fed alkaline-buffered saline (ABS) were immunised one week later with either 1 µg CT or saline in CFA to act as positive and negative controls, respectively. Two other groups of mice were fed 1 µg CT and subsequently immunised with either 1 µg CT or saline in CFA to see whether feeding affected the induction of DTH or was able to induce DTH directly. All animals were footpad challenged with 5 µg TD two weeks after immunisation. The results of this experiment are shown in figure 7.2.

As might be expected, animals fed ABS and immunised with saline did not exhibit significant footpad swelling. The positive control group (fed ABS and immunised with CT) gave a good response which was significantly greater than that observed in the negative controls ($p<0.001$).

Feeding toxin tolerised the induction of DTH, the footpad responses being significantly less than that measured in the positive control group ($p<0.02$). However, tolerance was not complete as the DTH swelling in this group was still greater than that of negative controls ($p<0.02$).

Feeding choleragen did not induce DTH directly as the responses observed in this group did not differ statistically from those of the negative controls.

7.3 The Effect of Feeding Cholera Toxoid on the Subsequent Induction of DTH

In order to see whether the biochemical properties of choleragen were important in the induction of oral tolerance, the same protocol was used here as has been described in section 7.2 with the exception that 5 μg TD was fed in place of the holotoxin.

The results of this experiment are shown in figure 7.3. Once again, negative controls (fed ABS and immunised with saline) did not exhibit DTH but positive controls (fed ABS and immunised with CT) gave a good response ($p<0.001$).

Feeding toxoid partially tolerised the induction of DTH ($p<0.01$ compared with positive controls, $p<0.02$ compared with negative controls) but was unable to induce DTH directly.

7.4 The Effect of Feeding Different Doses of Toxin and Toxoid on the Subsequent Induction of DTH

Previous experiments showed that prefeeding either toxin or toxoid was able to induce oral tolerance. Experiments with ovalbumin have shown this phenomenon to be dose-dependent (Mowat **et al**, 1986). In order to see whether the same applied to these proteins, animals were fed either ABS, CT (0.1, 1.0 and 10.0 µg) or TD (0.5, 5.0 and 50.0 µg) one week before intradermal immunisation with 1 µg CT in CFA. Systemic DTH responses were assessed 14 days after immunisation.

The results of this experiment are shown in figure 7.4. Feeding 1.0 or 10.0 µg CT tolerised the induction of systemic DTH compared with ABS-fed controls ($p<0.001$ and <0.05, respectively). 50 µg and 5.0 µg TD administered orally also significantly suppressed the induction of subsequent DTH responses ($p<0.05$ and <0.01, respectively).

The animals fed either 0.1 µg CT or 0.5 µg TD were not tolerant, having DTH responses which were not statistically significantly different from ABS-fed controls.

7.5 The Specificity of Oral Tolerance Induced by Feeding Toxin and Toxoid

This was assessed in two ways. First of all, mice immunised with 100 µg OVA in CFA were fed ABS, 1 µg CT or 5 µg TD one week

earlier. Two weeks after immunisation these animals were footpad challenged with 100 µg OVA, and their DTH responses were measured 24 hours later.

As can be seen in figure 7.5, a pre-feed of either toxin or toxoid did not suppress the induction of DTH to OVA compared to that observed in sham-fed controls.

Secondly, animals were fed either 25 mg OVA, 1 µg CT or 5 µg TD one week before immunisation with 1 µg CT in CFA. Systemic DTH responses were tested two weeks later.

OVA-fed animals had antitoxin DTH responses which were significantly greater than those observed in both the toxin and toxoid-fed mice ($p<0.001$ and <0.05, respectively). These results are displayed graphically in figure 7.6.

7.6 The Effect of Feeding Toxin or Toxoid on the Subsequent Induction of Toxoid-primed DTH Responses

Both choleragen and its toxoid are able to tolerise the induction of specific systemic CMI. However, as has been shown in chapter 5, these two proteins are not totally immunologically cross-reactive. It was decided, therefore, to examine the effects of feeding these antigens on the induction of systemic CMI to cholera toxoid.

Accordingly, mice were fed either ABS, 1 µg CT or 5 µg TD. One week later these mice were immunised with 5 µg TD in CFA along with unfed controls. Other unfed mice were immunised with saline in CFA at the same time as negative controls. All these animals were footpad challenged with 5 µg TD two weeks later. The DTH responses measured 24 hours after footpad challenge are shown in figure 7.7.

Once again, negative controls did not exhibit DTH. Feeding ABS did not depress DTH responses in toxoid-primed mice compared to unfed positive controls. Both these groups had footpad responses which were significantly greater than the saline-primed mice ($p<0.001$, in both cases).

Feeding CT or TD significantly suppressed the induction of DTH ($p<0.01$ in both cases) but once again the tolerance was incomplete as these responses were significantly greater than those seen in the negative controls ($p< 0.001$ and $p<0.01$, respectively).

7.7 Summary

Feeding either choleragen or its toxoid inhibits the induction of systemic DTH to either of these antigens. This tolerance is incomplete as there is still a residual DTH reaction. This is in accordance with the findings for other antigens.

The oral tolerance induced is both antigen-specific and dose-dependent. Feeding OVA failed to suppress systemic antitoxin CMI and the oral administration of either CT or TD did not tolerise the induction of systemic DTH to OVA. In addition, the lowest doses of toxin (0.1 μg) and toxoid (0.5 μg) fed failed to suppress the induction of specific DTH.

What appears to be novel in this system is the totally divergent effect of enterically applied antigen on the two limbs of systemic immunity. A dose of toxin (10 μg) which has been used to induce systemic antitoxin is also capable of tolerising systemic DTH. This is the first time such an obvious separation in the mucosal regulation of different effector mechanisms of systemic immunity has been reported.

Finally, as both toxin and toxoid are capable of suppressing the induction of DTH, it suggests that the ability to induce oral tolerance is not related to the toxic properties of choleragen.

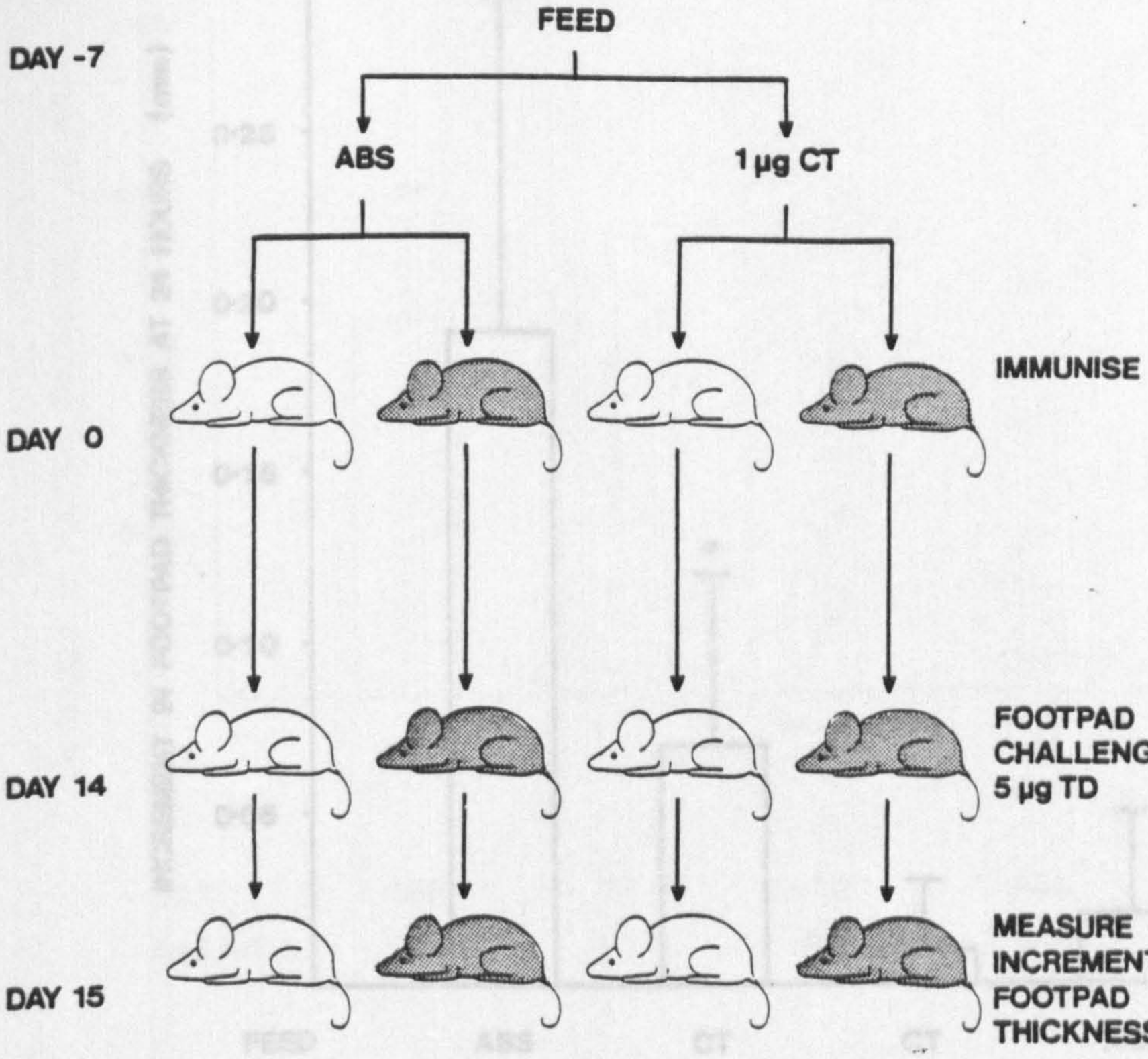

Figure 7.1 Experimental protocol to examine the effect of prefeeding cholera toxin on the induction of systemic DTH

The shaded mice have been immunised with 1 µg CT in CFA. The others have been immunised with saline in CFA.

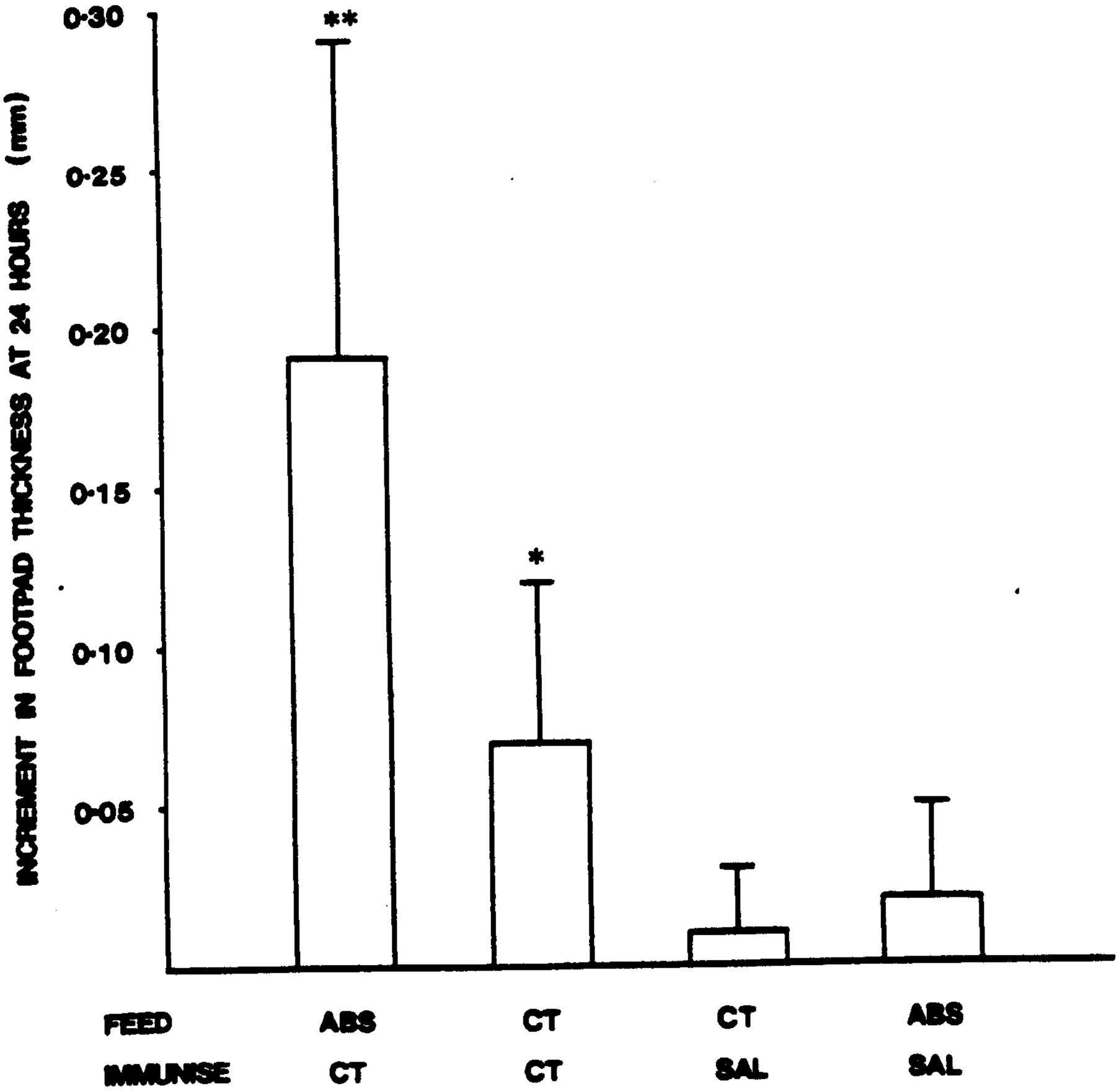

Figure 7.2 The effect of feeding choleragen on the induction of systemic DTH

This graph shows the DTH responses of mice fed either ABS or 1 μg CT one week before intradermal immunisation with either 1 μg CT or saline in CFA. All animals were footpad challenged with 5 μg TD two weeks after immunisation. The bars represent the mean increment in footpad thickness ± 1 standard deviation 24 hours after footpad challenge. Experimental groups were compared statistically to those mice fed ABS and immunised with saline.

* $p<0.02$

** $p<0.001$

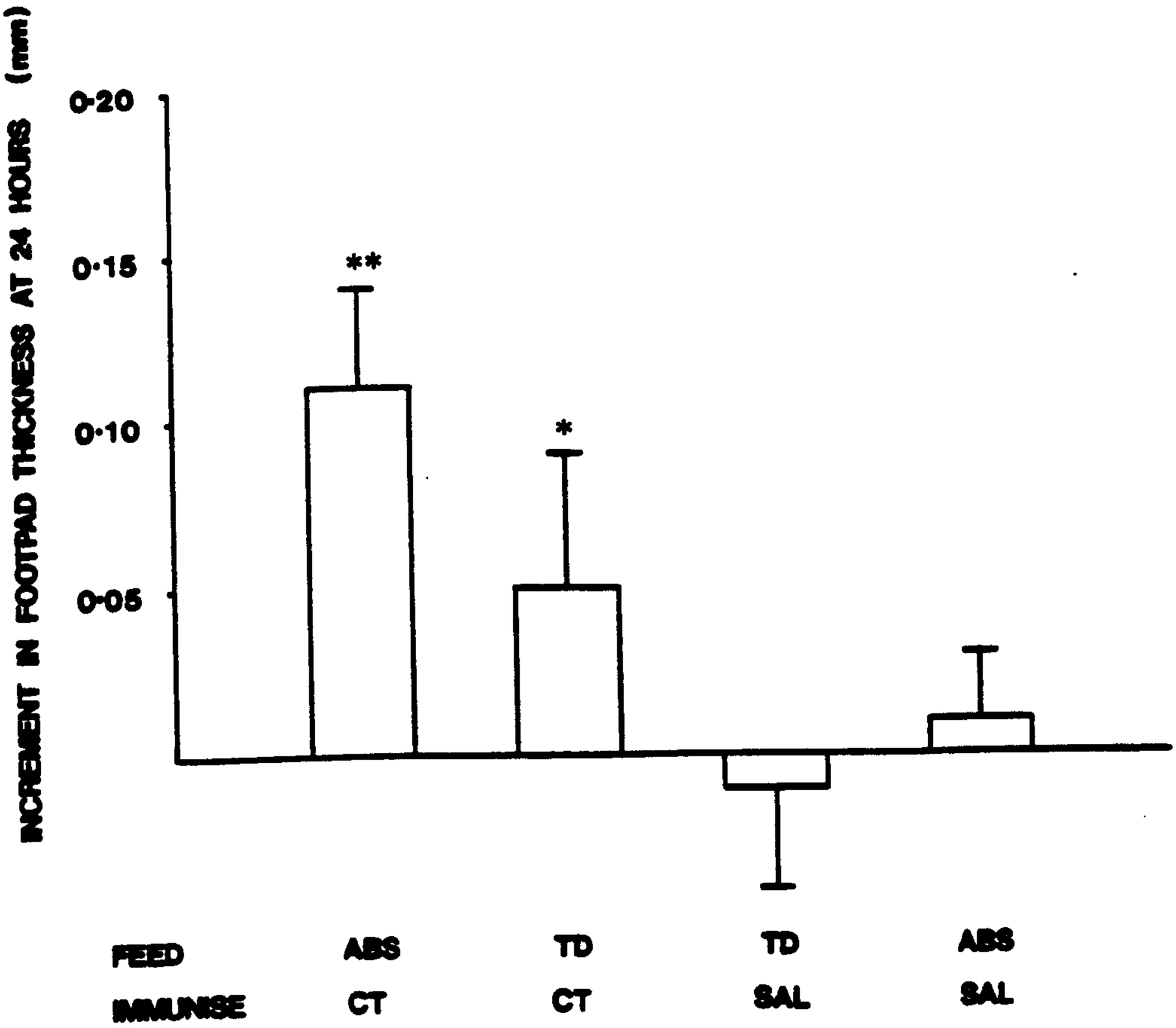

Figure 7.3 The effect of feeding cholera toxoid on the induction of systemic DTH

This graph shows the DTH responses of mice fed either ABS or 5 μg TD one week before intradermal immunisation with either 1 μg CT or saline in CFA. All animals were footpad challenged with 5 μg TD two weeks after immunisation. The bars represent the mean increment in footpad thickness ± 1 standard deviation 24 hours after footpad challenge. Experimental groups were compared statistically to those mice fed ABS and immunised with saline.

* $p<0.02$

** $p<0.001$

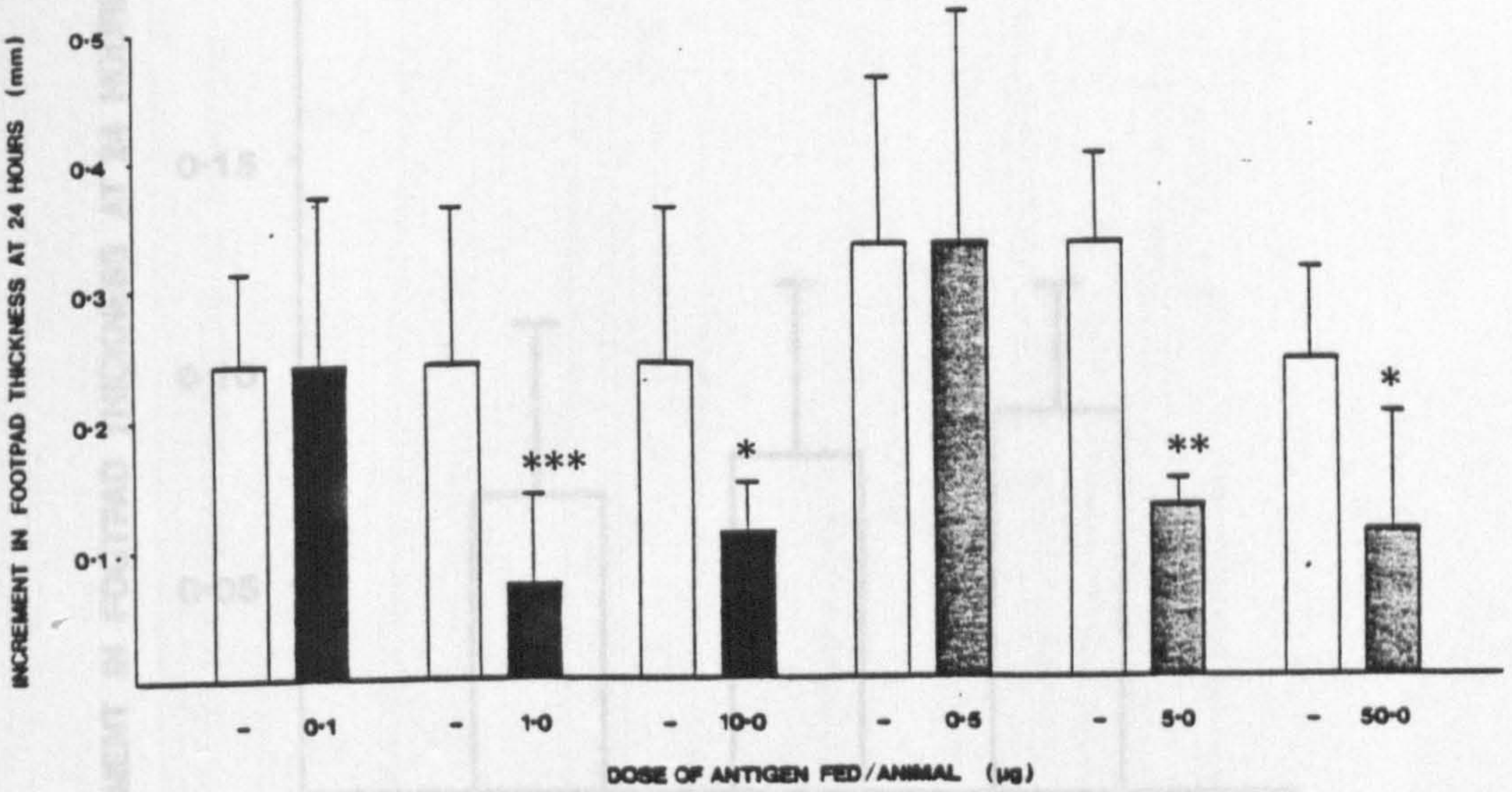

Figure 7.4 The effect of feeding various doses of cholera toxin or toxoid on the subsequent induction of specific systemic DTH

This graph shows the DTH responses of mice fed ABS (□) or various doses of CT (■) or TD (▩) one week before intradermal immunisation with 1 µg CT in CFA. These mice were footpad challenged with 5 µg TD two weeks after immunisation. The bars represent the mean increment in footpad thickness ± 1 standard deviation 24 hours after footpad challenge. The experimental groups were compared statistically to their neighbouring ABS-fed controls.

* $p<0.05$

** $p<0.01$

*** $p<0.001$

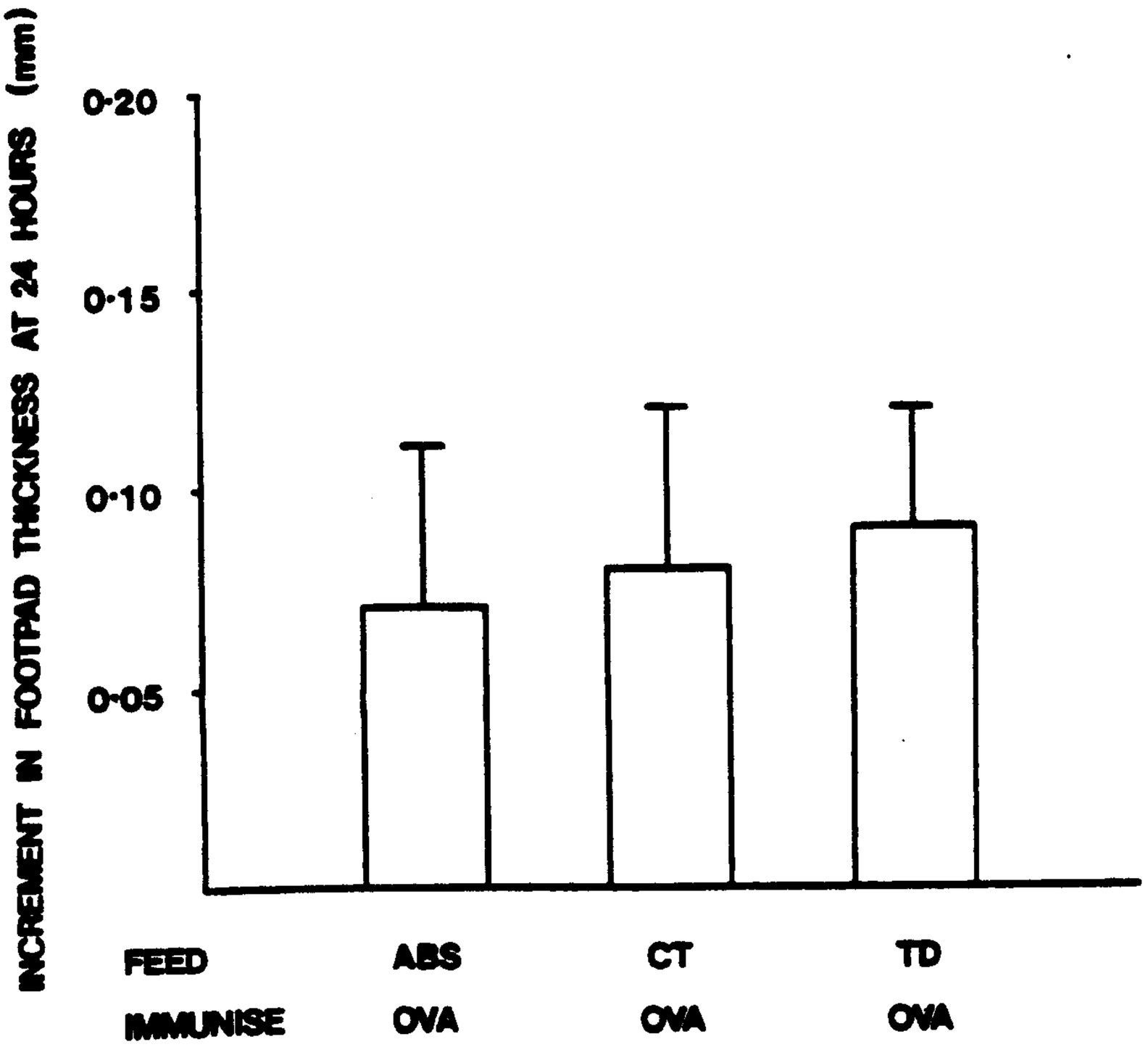

Figure 7.5 The effect of feeding cholera toxin or toxoid on the subsequent induction of systemic DTH to an unrelated protein antigen, ovalbumin

This graph shows the DTH responses of mice fed ABS, 1 µg CT or 5 µg TD one week before intradermal immunisation with 100 µg OVA in CFA. Two weeks after immunisation, the animals were footpad challenged with 100 µg OVA. The bars represent the increment in footpad thickness ± 1 standard deviation 24 hours after footpad challenge. Experimental groups were compared statistically to those mice fed ABS.

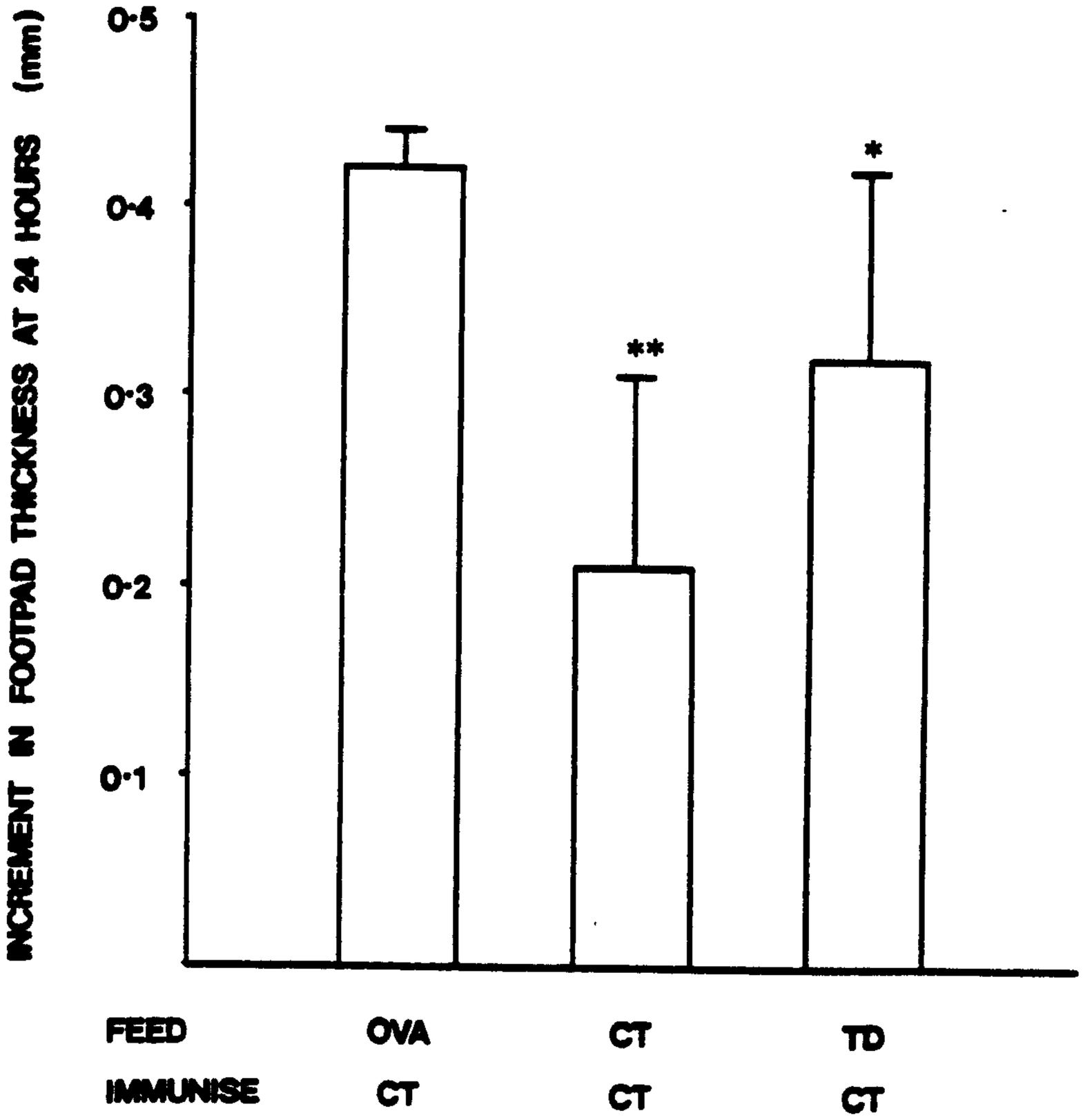

Figure 7.6 The effect of feeding an unrelated protein antigen, ovalbumin, on the subsequent induction of DTH to choleragen

This graph shows the DTH responses of mice fed 25 mg OVA, 1 µg CT or 5 µg TD one week before intradermal immunisation with 1 µg CT in CFA. Two weeks after immunisation, the animals were footpad challenged with 5 µg TD. The bars represent the mean increment in footpad thickness ± 1 standard deviation 24 hours after footpad challenge. The other experimental groups were compared statistically to those mice fed OVA.

* $p<0.05$

** $p<0.001$

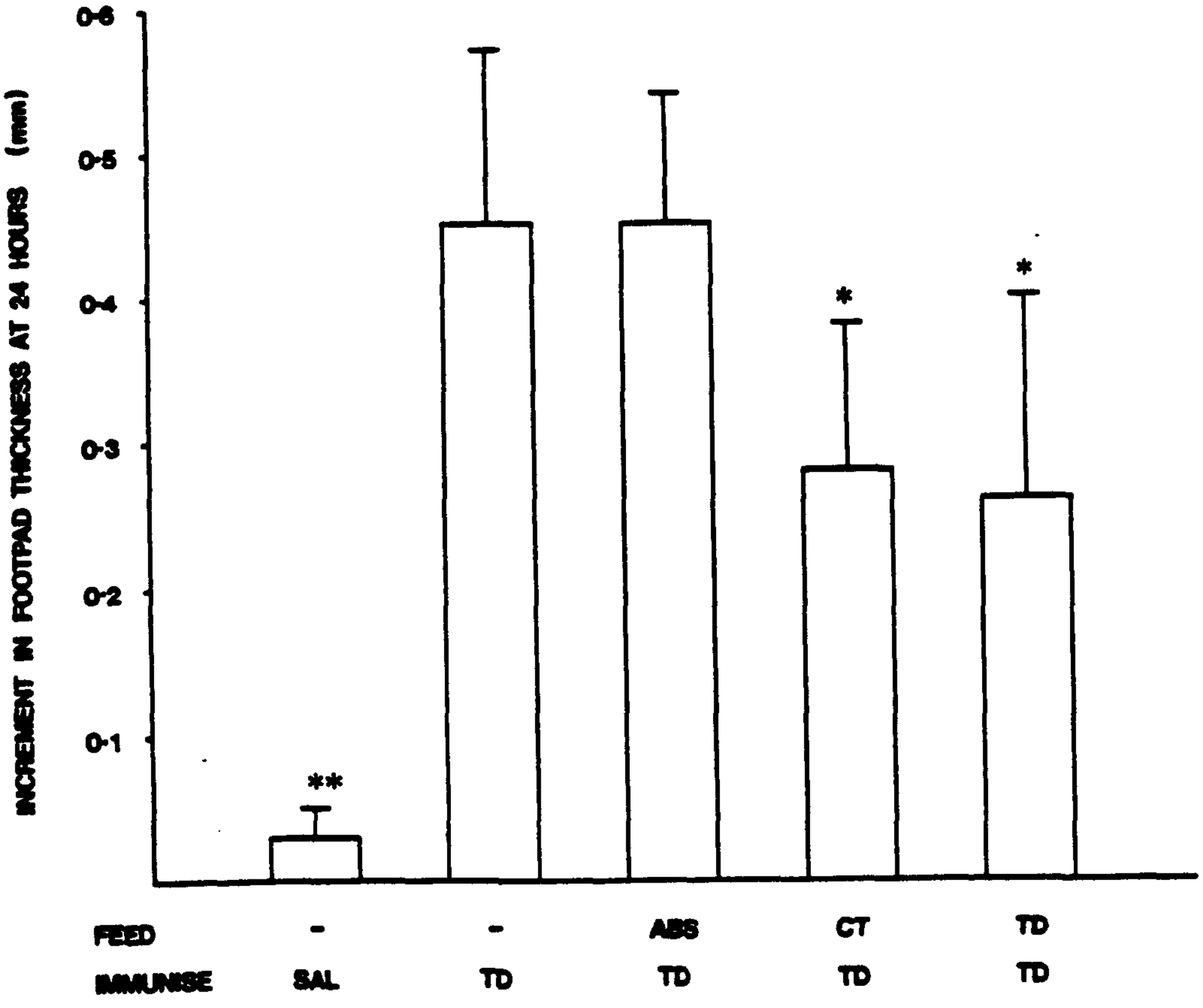

Figure 7.7 The efect of feeding cholera toxin or toxoid on the subsequent induction of systemic DTH to cholera toxoid

This graph shows the DTH responses of mice fed ABS, 1 µg CT or 5 µg TD one week before intradermal immunisation with 5 µg TD in CFA. Other unfed groups of mice were immunised with either 5 µg TD or saline in CFA, at the same time, to act as positive and negative controls, respectively. All animals were footpad challenged with 5 µg TD two weeks after immunisation. The bars represent the mean increment in footpad thickness + 1 standard deviation 24 hours after footpad challenge. All results were compared statistically to those observed in the positive control group.

* $p<0.01$

** $p<0.001$

Chapter 8

THE EFFECT OF CYCLOPHOSPHAMIDE AND ORALLY-PRIMED SPLEEN CELLS ON THE INDUCTION OF ORAL TOLERANCE

8.1 Introduction

Cyclophosphamide (CY) is a potent alkylating agent which acts principally on cells actively synthesising DNA. CY may have differing effects on the immune responses of the host depending on the dose used and the timing of administration relative to immunisation (reviewed: Mowat, 1981). It would appear however, that T suppresor and B cells are more sensitive to its action than helper or cytotoxic T cells (Kaufmann, Hahn & Diamantstein, 1980; Shand & Liew, 1980).

Suppressor T cells are thought to be responsible for the down-regulation of CMI responses after feeding OVA (Miller & Hanson, 1979; Challacombe & Tomasi, 1980; Titus & Chiller, 1981). The sensitivity of murine suppressor T cells to certain doses of CY (Askenase, Hayden & Gershon, 1975; Ferguson & Simmons, 1978; Gill & Liew, 1978) has been used to elucidate mechanisms responsible for oral tolerance after feeding OVA. Pretreatment of BALB/c mice with CY (100 mg/kg) before feeding abrogated the induction of tolerance normally observed with orally administered OVA (Mowat **et al**, 1982). CY pretreatment also prevented the induction of splenic suppressor cells normally associated with feeding OVA (Hanson & Miller, 1982).

Feeding cholera toxin appears only to tolerise the induction of the cell-mediated limb of systemic immunity but it may well be that choleragen induces similar suppressor mechanisms to that found with more usual protein antigens.

In order to examine this, the ability of CY to abrogate the induction of oral tolerance associated with feeding cholera toxin and toxoid was tested. The effect of CY pretreatment on the induction of systemic humoral immunity was also investigated. Furthermore, transfers of spleen cells from donors fed either toxin or toxoid were performed to elucidate their effects on the systemic immune response of parenterally immunised recipients.

8.2 The Effect of Cyclophosphamide on the Induction of Oral Tolerance by Cholera Toxin

Mice were given an intraperitoneal injection on day -9 of either cyclophosphamide (100 mg/kg) or the equivalent volume of saline. Half the cyclophosphamide-treated mice were fed 1 µg CT and half were fed ABS on day -7. Saline-injected animals were treated in the same way. After 7 more days, mice were immunised with CT in CFA and assessed for systemic DTH 2 weeks later. On day 22 of the experiment the animals were bled and their serum antibody levels measured. The experimental protocol may be seen in figure 8.1.

The effects of cyclophosphamide may be seen in figure 8.2. There was no difference in the footpad responses of ABS-fed animals with or without cyclophosphamide pretreatments. Feeding toxin significantly suppressed subsequent DTH responses ($p<0.001$). Cyclophosphamide pretreatment abrogated the tolerance induced by feeding choleragen. The footpad swelling seen in these animals did not significantly differ

from that observed in ABS-fed controls.

The systemic IgG and IgA antitoxin levels of these animals are shown in figure 8.3. As noted earlier in chapter 4, feeding toxin fails to tolerise serum antibody levels and, furthermore, pretreatment with cyclophosphamide does not significantly alter these levels.

8.3 The Effect of Cyclophosphamide on Oral Tolerance Induced by Cholera Toxoid

The same experimental protocol was employed as described in the previous section, except that animals were fed 5 µg toxoid in place of toxin.

Once again, toxoid-fed animals had significantly reduced footpad swelling responses ($p<0.01$). Pretreatment with cyclophosphamide also abrogated the induction of tolerance in these animals. The results of this experiment are shown in figure 8.4.

8.4 The Effect of Spleen Cells Transferred from Orally Immunised Animals on the Induction of Systemic Immunity in Recipients

Groups of mice were fed 1 µg CT, 5 µg TD or ABS alone. One week later these mice were sacrificed and a suspension of their spleens intraperitoneally injected into syngeneic recipients. 10^8 spleen cells

were transferred on the same day as recipients were intradermally immunised with CT in CFA. Two weeks later systemic DTH responses were assessed as before and 8 days after this (22 days after footpad immunisation) the recipient mice were bled and their serum antitoxin levels (IgG and IgA) were measured. As a control, animals were sham-injected with medium only and immunised, footpad challenged and bled as for the spleen cell recipients.

ABS-fed animals had identical footpad swelling to unfed animals who received spleen cells from ABS-fed donors (figure 8.5). Recipients of spleen cells from either toxin- or toxoid-fed donors had significantly suppressed DTH responses ($p<0.01$ and $p<0.05$, respectively).

There was no significant difference between the IgG and IgA serum antitoxin levels measured in any of these groups (figure 8.6).

8.5 Summary

Cyclophosphamide abrogated the induction of tolerance for systemic DTH responses normally observed following feeding cholera toxin or toxoid. At a dose of 100 mg/kg cyclphosphamide did not affect serum antitoxin levels observed in these animals.

Tolerance for DTH induction could be transferred by 10^8 spleen cells obtained from mice one week after feeding either CT or TD.

Transferred spleen cells did not affect the induction of systemic humoral antitoxic immunity.

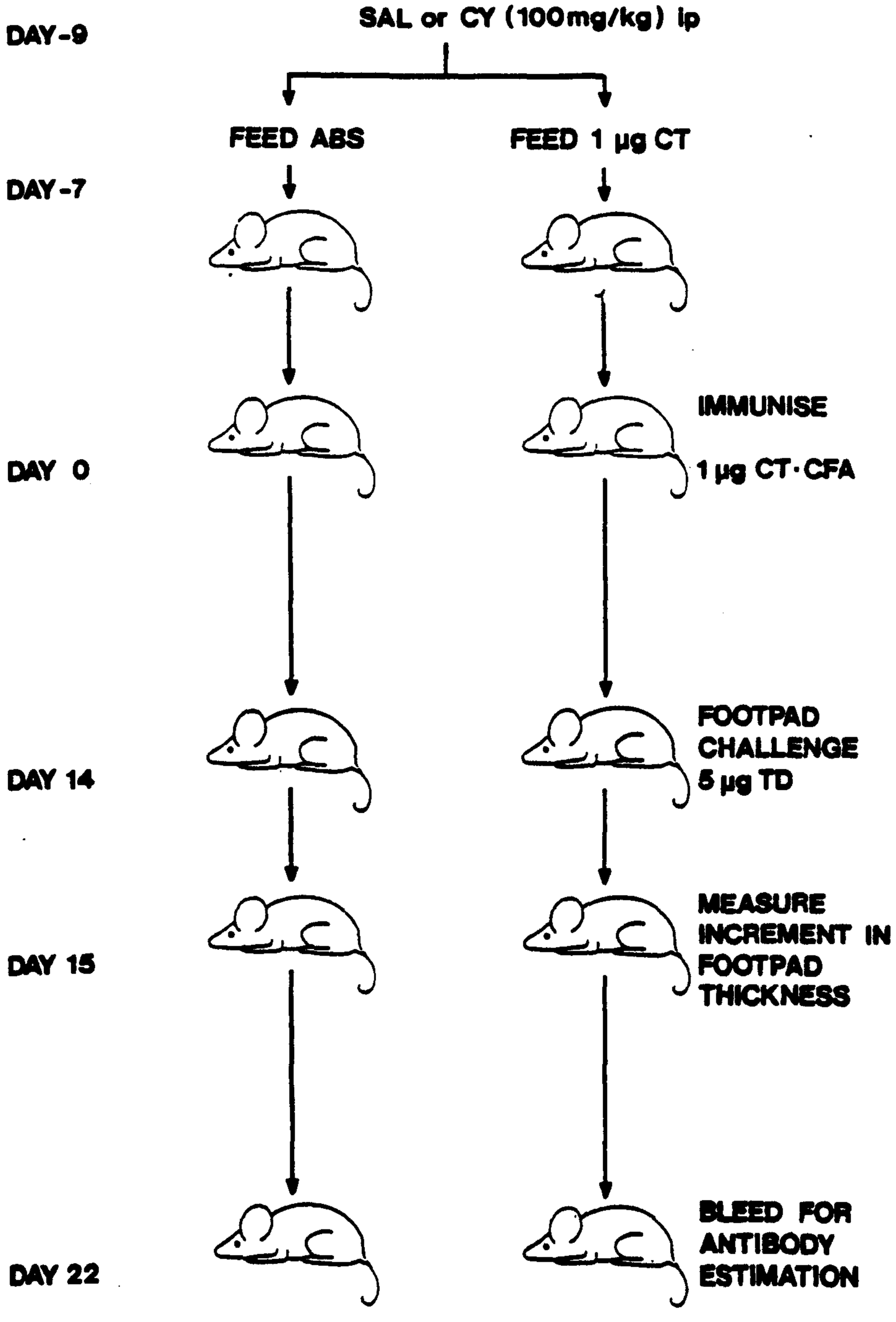

Figure 8.1 Experimental protocol to test the effect of pretreating mice with cyclophosphamide (100 mg/kg) on the induction of oral tolerance

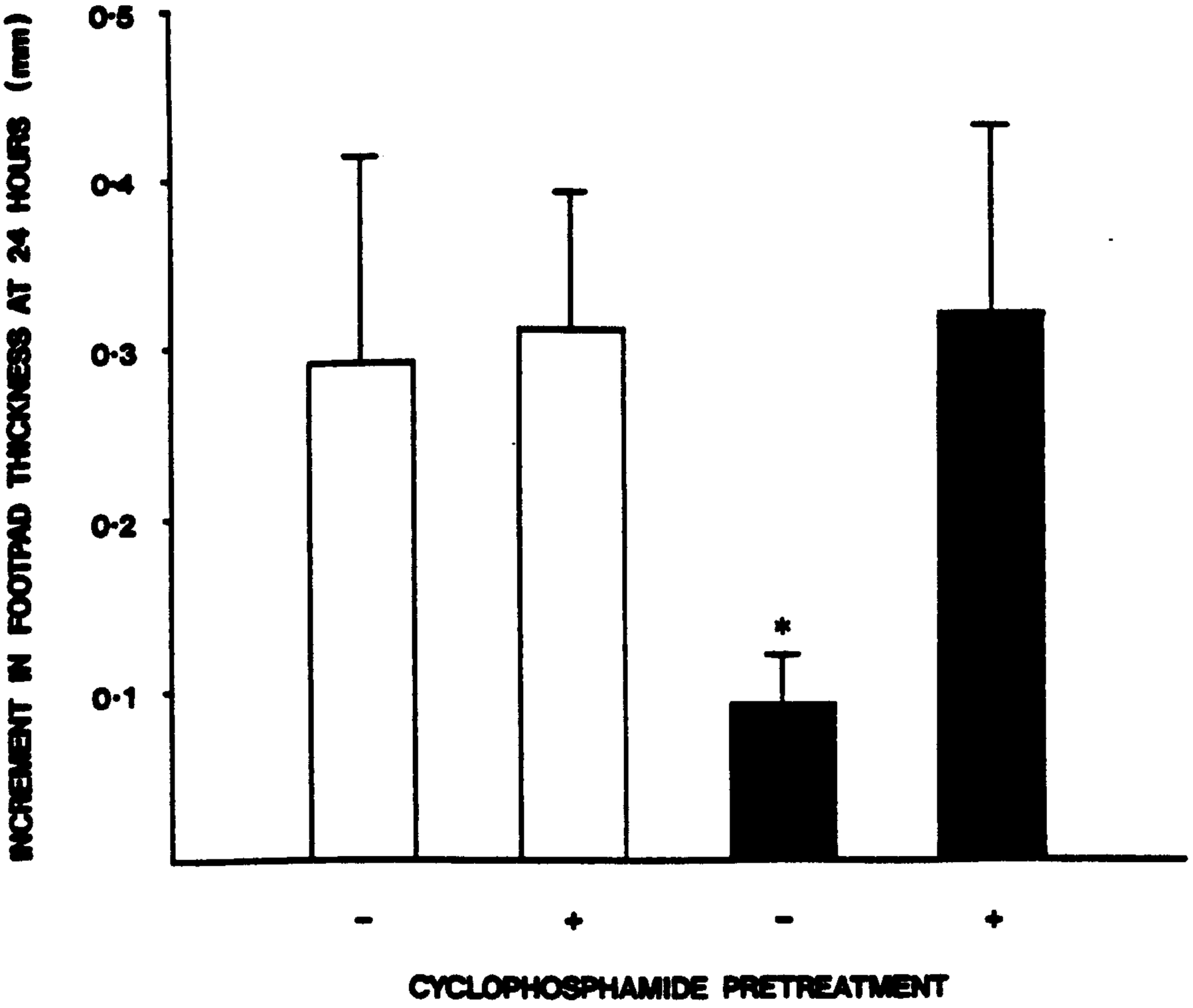

Figure 8.2 The effect of cyclophosphamide pretreatment on the induction of tolerance associated with feeding cholera toxin

This graph shows the effect of intraperitoneally administered CY (100 mg/kg) on the induction of oral tolerance to cholera toxin. Animals received CY two days before being fed 1 µg CT (■) or ABS (□) and were intradermally immunised with 1 µg CT in CFA one week after feeding. Animals were footpad challenged two weeks after immunisation. The bars represent the mean increment in footpad thickness ± 1 standard deviation 24 hours after footpad challenge with 5 µg TD. Experimental groups were statistically compared to those mice fed with ABS and not injected with CY.

* $p<0.001$

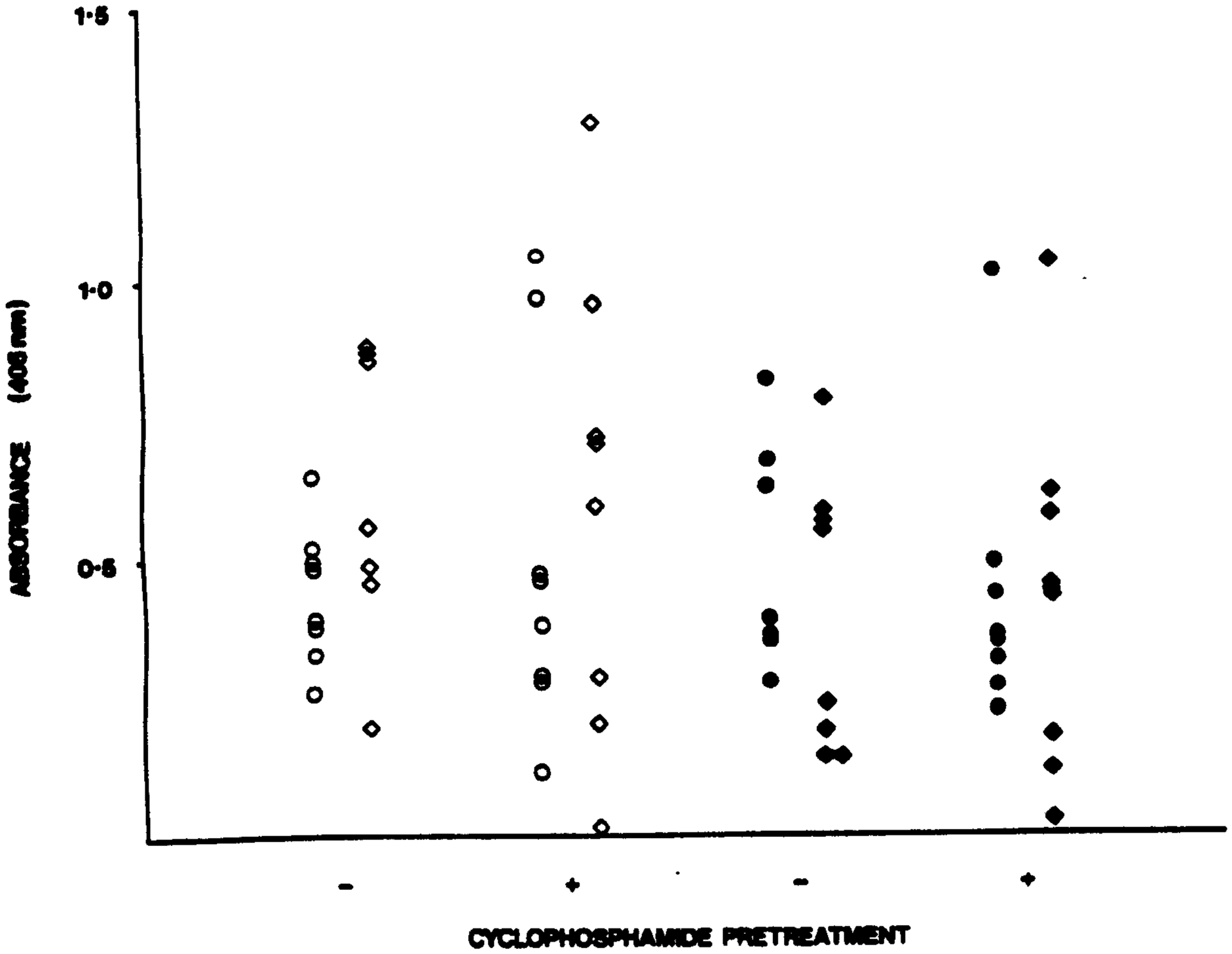

Figure 8.3 The effect of cyclophosphamide pretreatment on the induction of systemic humoral antitoxin responses

This graph shows the effect of intraperitoneally administered CY on subsequent systemic antitoxin antibody responses. Animals were pretreated with CY two days before being fed either 1 µg CT (■) or ABS (□) and were intradermally immunised with 1 µg CT in CFA one week after feeding. Two weeks after immunisation, mice were footpad challenged with 5 µg TD and were bled for serum antibody determination 8 days after this.

The points represent the IgG (circles) or IgA (diamonds) antitoxin levels obtained from individual mice expressed as the absorbance at 405 nm in an isotype-specific ELISA.

Experimental groups were compared statistically to the results from those mice fed ABS but not treated with CY.

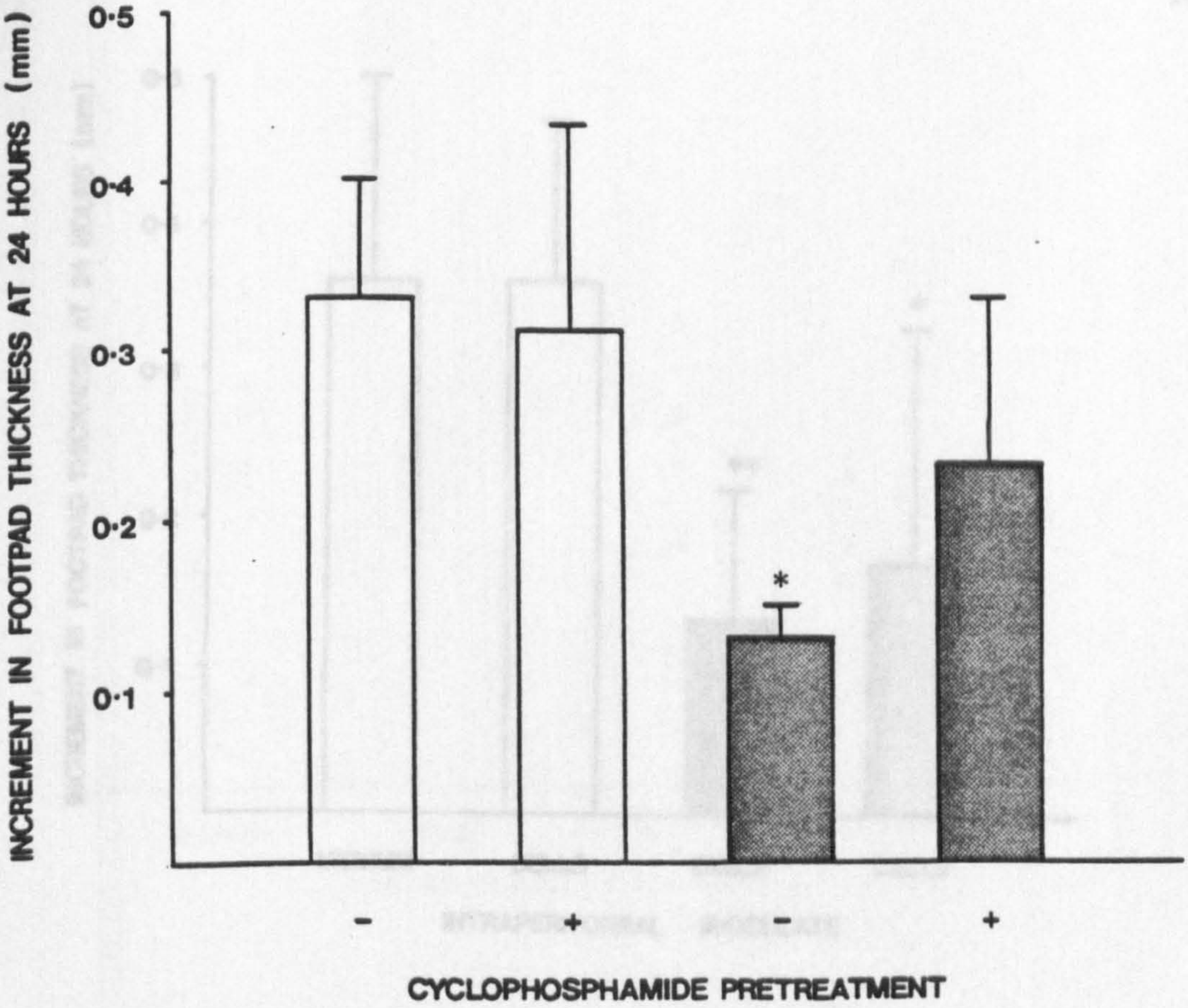

Figure 8.4 The effect of cyclophosphamide on the induction of oral tolerance to cholera toxoid

This graph shows the effect of intraperitoneally administered CY (100 mg/kg) on the induction of oral tolerance to cholera toxoid. CY was administered two days before either 5 µg TD (▩) or ABS (□) was fed. Seven days after feeding, animals were immunised with 1 µg CT in CFA and footpad challenged two weeks later with 5 µg TD. The bars represent the mean increment in footpad thickness ± 1 standard deviation 24 hours after footpad challenge. Statistical comparison was made between the group that was fed ABS but did not recieve CY and the other groups of animals.

* $p<0.01$

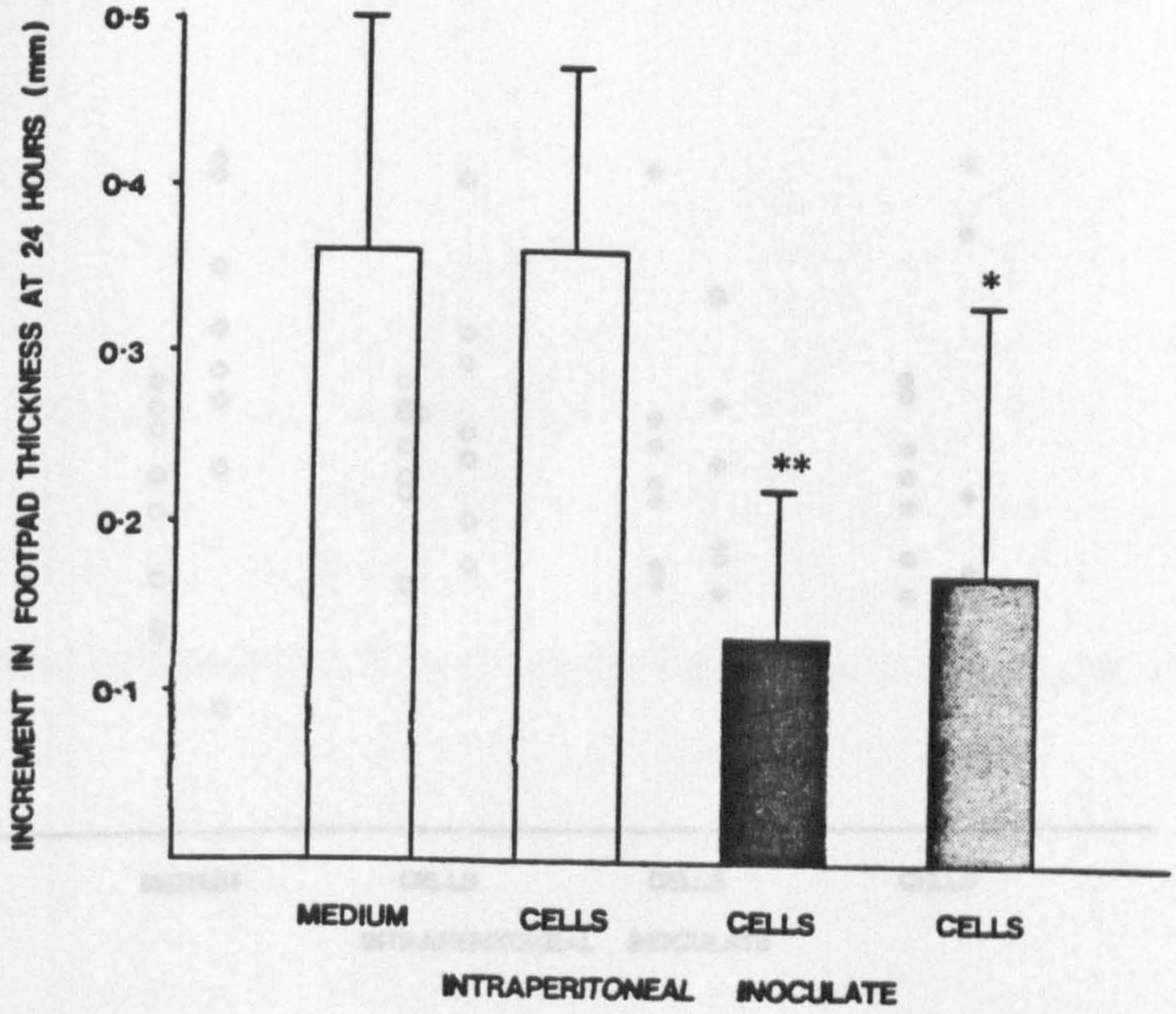

Figure 8.5 The effect of orally immunised spleen cells on the induction of systemic DTH in parenterally immunised syngeneic recipients

This graph shows the effect on the induction of DTH of the transfer of 10^8 spleen cells from ABS-, CT- or TD-fed syngenic donors. Donor mice were fed either ABS (□), 1 µg CT (■) or 5 µg TD (▩). One week later these mice were sacrificed, and 10^8 of their spleen cells were injected intraperitoneally (ip) into syngeneic recipients. Spleen cell recipients were intradermally immunised with 1 µg CT in CFA on the same day as cell transfer. Two weeks later they were footpad challenged with 5 µg TD. The group of mice that did not receive spleen cells, were given a sham ip injection of an equivalent volume of medium only on the day of immunisation and were otherwise treated like the other animals. The bars represent the mean increment in footpad thickness 24 hours after footpad challenge ± 1 standard deviation.

Results of experimental groups were statistically compared to those obtained from the sham-injected animals.

* $p<0.05$

** $p<0.01$

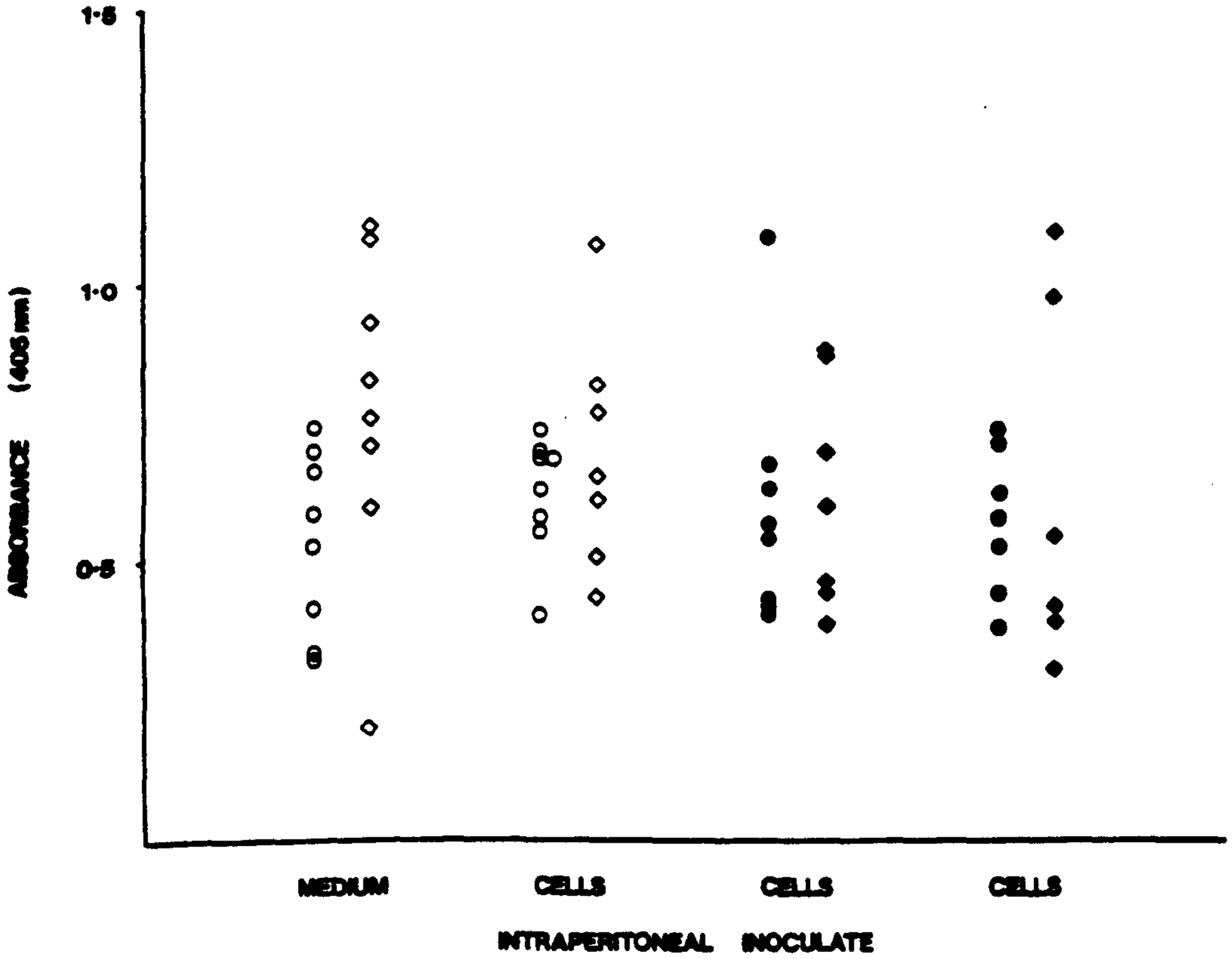

Figure 8.6 The effect of orally immunised spleen cells on the systemic humoral response induced by the parenteral administration of choleragen

This graph shows the serum IgG (circles) and IgA (diamonds) antitoxin antibody responses of CT-primed mice who received either a sham ip injection of medium or 10^8 spleen cells from syngeneic donors fed ABS (□), 1 µg CT (■) or 5 µg TD (■) one week earlier. Recipient animals were intradermally immunised on the same day as the ip injection and were footpad challenged with 5 µg two weeks later. Eight days after footpad challenge the animals were bled for serum antibody estimations. The points represent the antitoxin levels of individual mice expressed as the absorbance at 405 nm in an isotype-specific ELISA.

Experimental groups were compared to sham injected controls.

Chapter 9

CHARACTERISATION OF THE ORALLY-INDUCED SUPPRESSOR CELL

9.1 Introduction

The previous chapter has suggested that the mucosal downregulation of the induction of systemic CMI is achieved by the induction of cyclophosphamide-sensitive suppressor cells. These cells were found in the spleen seven days after feeding. Previous work with OVA has produced similar findings (Miller & Hanson, 1979; Mowat et al, 1982), and so experiments were designed to examine whether other similarities exist between the suppressor cells induced by the oral administration of these two dissimilar protein antigens. Accordingly, several facets of the choleragen-induced splenic suppressor cell were examined.

Feeding OVA results in a THY-1 +ve suppessor cell which inhibits the induction of systemic CMI. Depletion of this population of cells from the spleens of toxin-fed animals was performed to see if this abrogated the ability of these cells to transfer oral tolerance to recipient mice.

Earlier studies have shown that after feeding both soluble and particulate antigens, suppressor cells may be found in the Peyer's patch (PP), mesenteric lymph node (MLN), spleen and thymus. Transfer of lymphoid cells from these tissues various times after feeding antigens have suggested that orally-induced suppressor cells appear first in the PP and then subsequently migrate to MLN, spleen and thymus (Ngan & Kind, 1978; Mattingly & Waksman, 1978; Richman et al, 1981). Cell transfer protocols with cells obtained from MLN and spleen

were used to examine whether similar migration patterns applied to the suppressor cells induced by feeding toxin.

Finally, the suppressor cell induced by feeding OVA or SRBC has been shown to inhibit the afferent limb of cell-mediated immunity (Kagnoff 1978a; Miller & Hanson, 1979). Transfer protocols of splenic cells from CT-fed donor to recipients on the same day as or various days after immunisation was undertaken to see whether these lymphocytes functioned in the same manner.

All these experiments were performed to see if the mucosal regulation of systemic CMI is achieved by a common mechanism despite the differences in the control of humoral immunity that are apparent after feeding dissimilar protein antigens.

A: A PHENOTYPIC STUDY OF THE SPLENIC SUPPRESSOR CELL

9.2 The Effect of Depletion of Thy-1.2 +ve Cells on the Suppressive Function of Toxin-primed Spleen Cell Suspensions

Donor mice were fed 1 µg CT or ABS alone. One week later, the animals were sacrificed and their spleens were made into a cell suspension. The toxin-primed spleen cells were either left untreated or treated with absorbed rabbit complement (ARC), anti-Thy-1.2 monoclonal antibody (McAb), or McAb followed by ARC. The procedure of cell depletion is as described in Chapter 4.18.

10^6 control splenocytes (donors fed ABS) along with the treated and untreated CT-primed spleen cells were transferred intraperitoneally to syngeneic recipients. Recipient animals were immunised with 1 µg CT in CFA, on the same day as cell transfer, and then footpad challenged 2 weeks later with 5 µg TD in saline. The increment in footpad thickness was measured 24 hours after footpad challenge.

Untreated toxin-primed spleen cells were able to transfer significant suppression of the induction of systemic DTH compared to spleen cells from ABS-fed donors ($p<0.01$). Treating toxin-primed cells with either McAb or ARC alone did not alter their ability to transfer suppression as both of these cell populations were able to convey significant tolerance ($p<0.05$ and $p<0.01$, respectively).

Treatment of toxin-primed spleen cells with the McAb followed by ARC abrogated their ability to suppress the induction of systemic DTH. The DTH responses of recipients of Thy-1.2-depleted toxin-primed spleen cells were not significantly different from those seen in animals receiving control cells. The response observed in this group was, however, significantly greater than those observed in the recipients of untreated toxin-primed cells ($p<0.01$), McAb-treated cells ($p<0.05$) and ARC-treated cells ($p<0.01$). The results of this experiment may be seen in figure 9.1.

B: STUDY OF THE MIGRATION PATTERN OF THE ORALLY-INDUCED SUPPRESSOR CELL

9.3 The Effect of Transferring MLN and Spleen Cells Various Days after Feeding ABS or CT on the Induction of Specific DTH in Syngeneic Recipients

Groups of BALB/c mice were fed either ABS or 1 µg CT. Either three or seven days after feeding, these animals were sacrificed and their MLNs and spleens removed. 10 cells from each separate lymphoid source were transferred intraperitoneally into syngeneic recipients on the same day as they were immunised with 1 µg CT in CFA. Two weeks later, the recipient animals were footpad challenged with 5 µg TD in saline and the increment in footpad thickness was measured one day after this. This experimental protocol is shown diagrammatically in figure 9.2. The results of this experiment are shown in figures 9.3 and 9.4.

Neither the MLN nor the spleen cells transferred from CT-fed donors significantly suppressed the induction of DTH when removed 3 days after feeding. It is worth noting, that of the 8 animals that received CT-primed MLN cells, 4 exhibited statistically significantly reduced DTH responses compared to control animals by some 74% ($p<0.001$). However, the remaining 4 animals in this group failed to respond in this way to their MLN inoculate with DTH results that were similar to, or even greater than, the mean control response. It is this divergence which is responsible for the large standard deviation seen in these results.

Similarly curious results were obtained when this experiment was repeated twice more.

MLN and spleen cells removed from donor animals 7 days after feeding toxin both transferred significant inhibition compared to ABS-fed control cells ($p<0.05$ in both cases)(see figure 9.4).

C: THE MODE OF ACTION OF THE ORALLY-INDUCED SPLENIC SUPPRESSOR CELL

9.4 The Effect of Transferring CT- and TD-primed Spleen Cells on or after the Day of Immunisation on their Ability to Convey Suppression to Syngeneic Recipients

Donor animals were fed either ABS, 1 µg CT or 5 µg TD and sacrificed one week later and their spleens removed. 10^8 spleen cells were then transferred intraperitoneally to syngeneic recipients. The recipients were immunised with 1 µg CT in CFA and were given the spleen cells either on the same day as immunisation or 3 or 6 days afterwards. All recipient animals were footpad challenged with 5 µg TD two weeks after immunisation and the increment in footpad thickness was measured 24 hours after this. This experimental protocol is illustrated in figure 9.5.

Toxin-primed cells were able to produce significant suppression when transferred on the same day or 3 days after immunisation ($p<0.001$ and $p<0.05$, respectively). However, the DTH responses of recipients were not significantly different from controls receiving cells from ABS-fed donors when the cell transfer was 6 days after immunisation. These results can be seen in figure 9.6.

Figure 9.7 shows the results of the experiments using toxoid-primed cells. As with CT-primed cells, TD-primed spleen cells transferred tolerance to syngeneic recipients when given intraperitoneally on the same day as or 3 days after immunisation ($p<0.05$ and $p<0.01$,

respectively). However, when these cells were transferred 6 days after immunisation they failed to suppress the induction of systemic DTH.

9.5 The Effect of Feeding Toxin or Toxoid on the Function of DTH Effector Cells

The previous experiment suggested that orally-induced suppressor cells were only effective in the prevention of the induction of DTH. Their inability to suppress DTH when transferred 6 days after immunisation implied that they inhibit the afferent limb of the CMI response. In order to confirm this an experiment was designed whereby primed DTH effector cells were transferred into orally-primed animals.

Animals were fed either ABS, 1 µg CT or 5 µg TD and one week later were given a footpad injection of 10^7 CT-primed cells plus 5 µg TD. As a control, ABS-fed animals were inoculted with only 5 µg TD or 10^7 primed lymph node cells into their footpads. The increment in footpad thickness was measured 24 hours after footpad injection. The primed DTH effector cells were obtained from the draining lymph node cells of animals intradermally primed with 1 µg CT in CFA two weeks earlier.

The results shown in figure 9.8 show that neither 1 µg CT or 5 µg TD suppressed the DTH transferred by primed effector cells in the presence of antigen. The control animals receiving either 10^7 lymph node cells or 5 µg TD alone did have significantly less footpad

swelling than the similarly fed group of animals receiving a combination of the two ($p<0.001$ in both cases).

9.6 Summary

The results of these experiments suggest that the cell transferring oral tolerance is Thy-1.2 +ve and is therefore presumably a T cell. This cell appears to be present in the MLN as well as the spleen 7 days after feeding, however, the data concerning its presence or absence in the MLN 3 days after feeding is inconclusive. It would seem that the cell has not yet reached the spleen after 3 days.

Spleen cell transfer experiments indicated that the suppressor cell was only capable of inhibiting the induction of DTH effector cells. Feeding mice with either CT or TD one week earlier (which allows sufficient time for the induction of both MLN and splenic suppressor cells) failed to inhibit the adoptive transfer of DTH by mature effector cells which would seem to confirm that the suppressor cell inhibits the afferent limb of the DTH response.

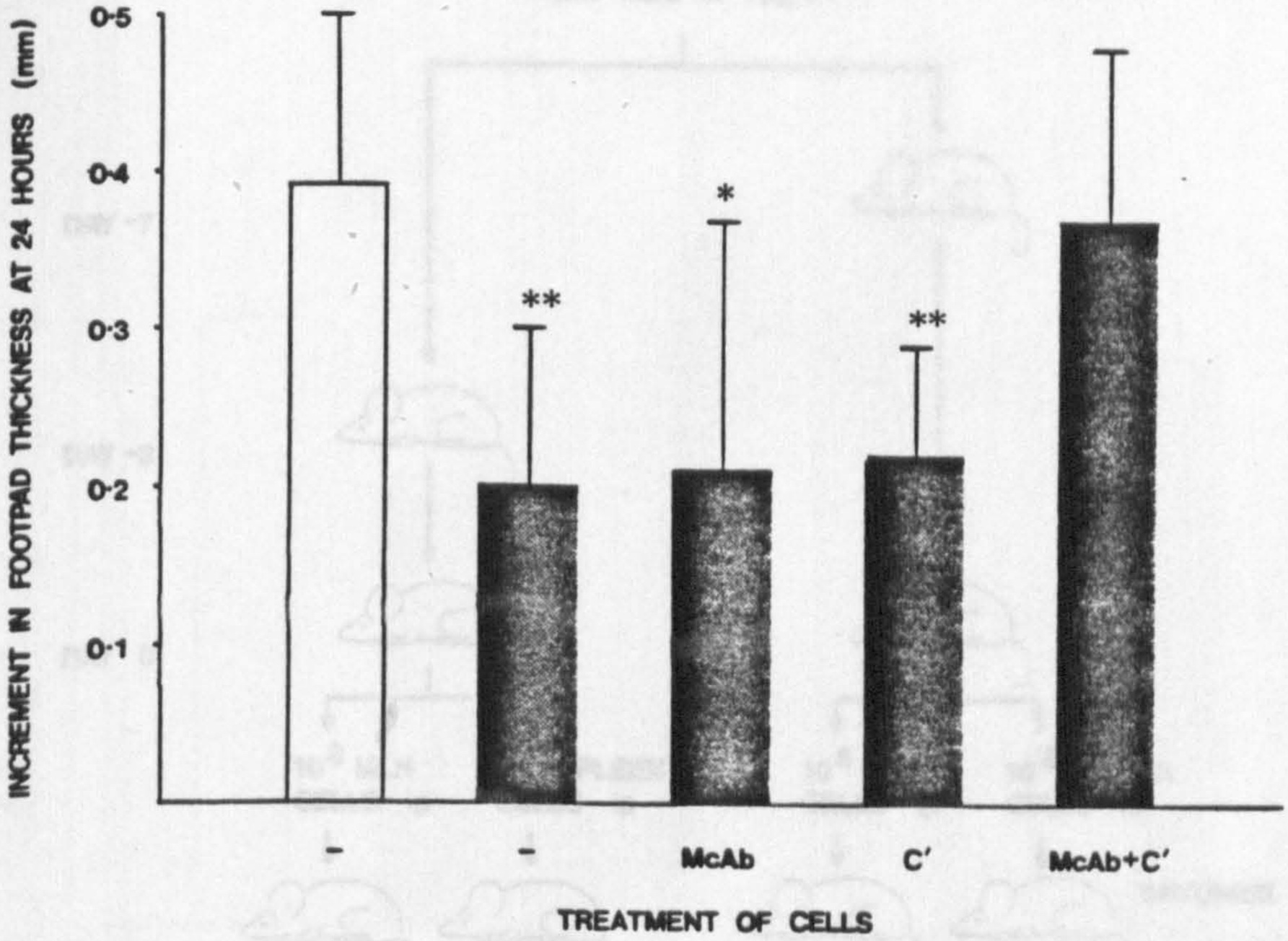

Figure 9.1 The effect of depletion of Thy-1.2 +ve splenic lymphocytes on the ability of those cells to transfer oral tolerance

This graph shows the DTH responses of mice receiving spleen cells from CT- (■) or ABS-fed (□) donors on the same day as they were intradermally immunised with 1 µg CT in CFA. These animals were intradermally footpad challenged with 5 µg TD in saline two weeks later and the increment in footpad thickness 24 hours after this.

Spleen cells were obtained from donors fed 1 week previously and were transferred to recipients intraperitoneally. Two groups of mice received either 10^8 untreated ABS- or CT-fed spleen cells respectively. As further controls two groups received 10^8 spleen cells from CT-fed donors treated with either absorbed rabbit complement (ARC) or anti-Thy-1.2 monoclonal antibody (McAb) alone and the experimental group received the cells that remained after 10^8 CT-fed splenocytes had been treated with first McAb and then ARC.

The bars represent the mean increment in footpad thickness ± 1 standard deviation 24 hours after footpad challenge. Statistical comparison was made between the group receiving ABS-fed splenocytes and the others.

* $p<0.05$

** $p<0.01$

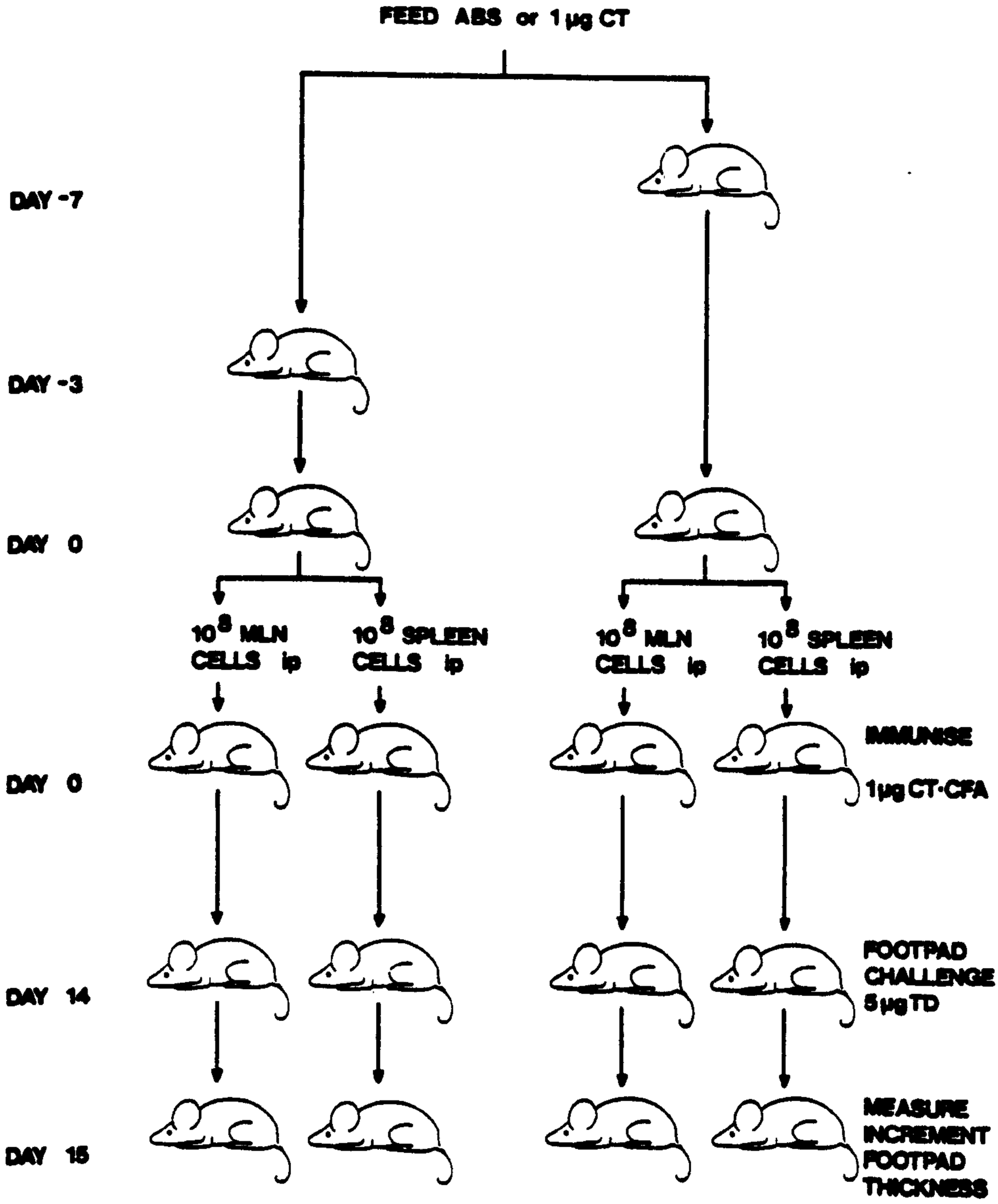

Figure 9.2 Experimental protocol to elucidate the migration pattern of suppressor cells

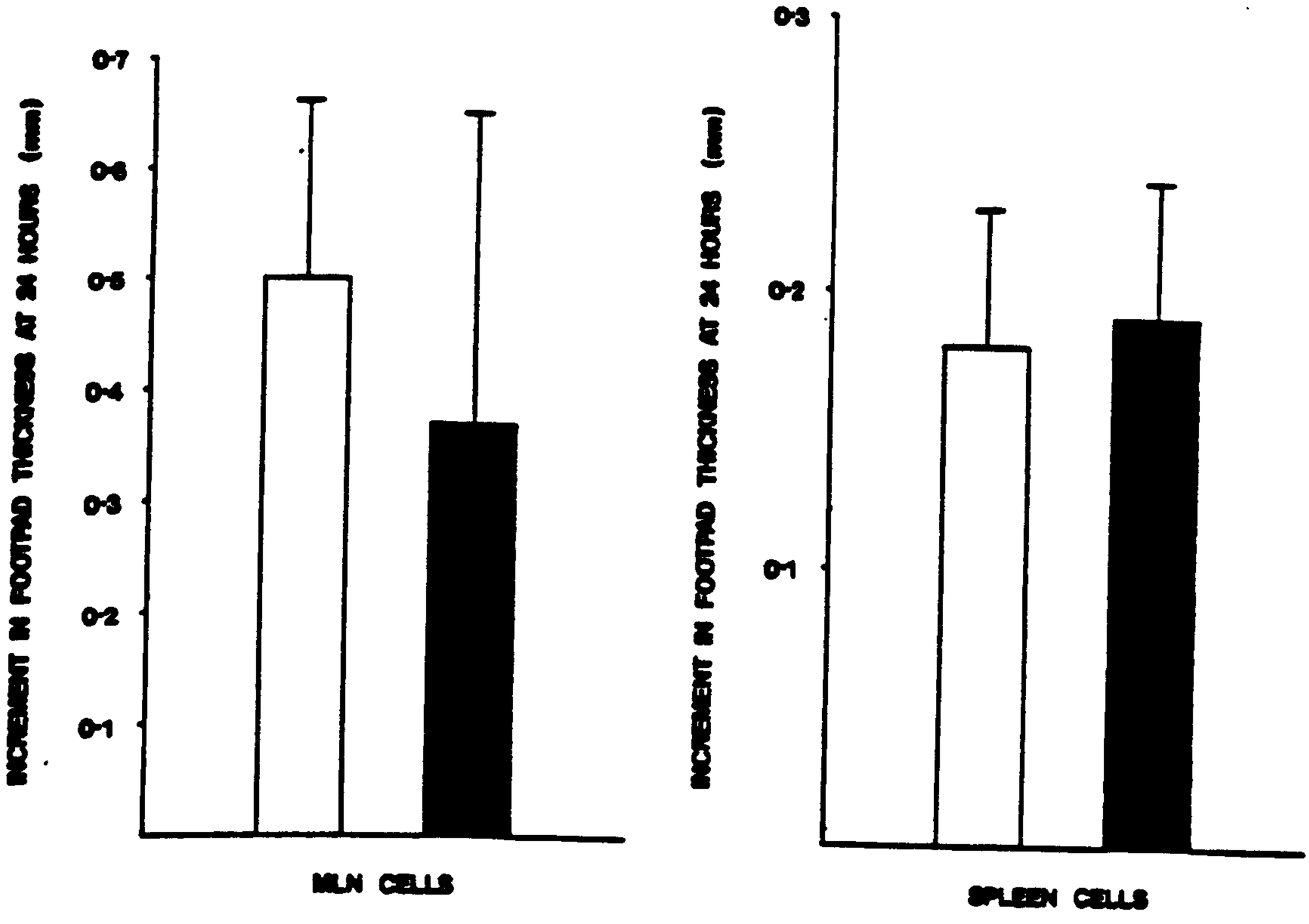

Figure 9.3 The effect of the transfer of MLN or spleen cells obtained 3 days after feeding CT or ABS on the induction of specific DTH in syngeneic recipients

Groups of mice were fed either ABS (□) or 1 µg CT (■). Three days later these animals were sacrificed and a suspension of 10 MLN or spleen cells were transferred intraperitoneally to syngeneic recipients. The recipient mice were immunised intradermally with 1 µg CT in CFA on the day of transfer and footpad challenged with 5 µg TD in saline two weeks after this. This protocol is illustrated diagrammatically in figure 9.2. The bars shown represent the mean increment in footpad thickness ± 1 standard deviation measured 24 hours after footpad challenge.

Statistical comparison was made between recipients of antigen-fed cells and recipents of control suspensions for each source of lymphocyte.

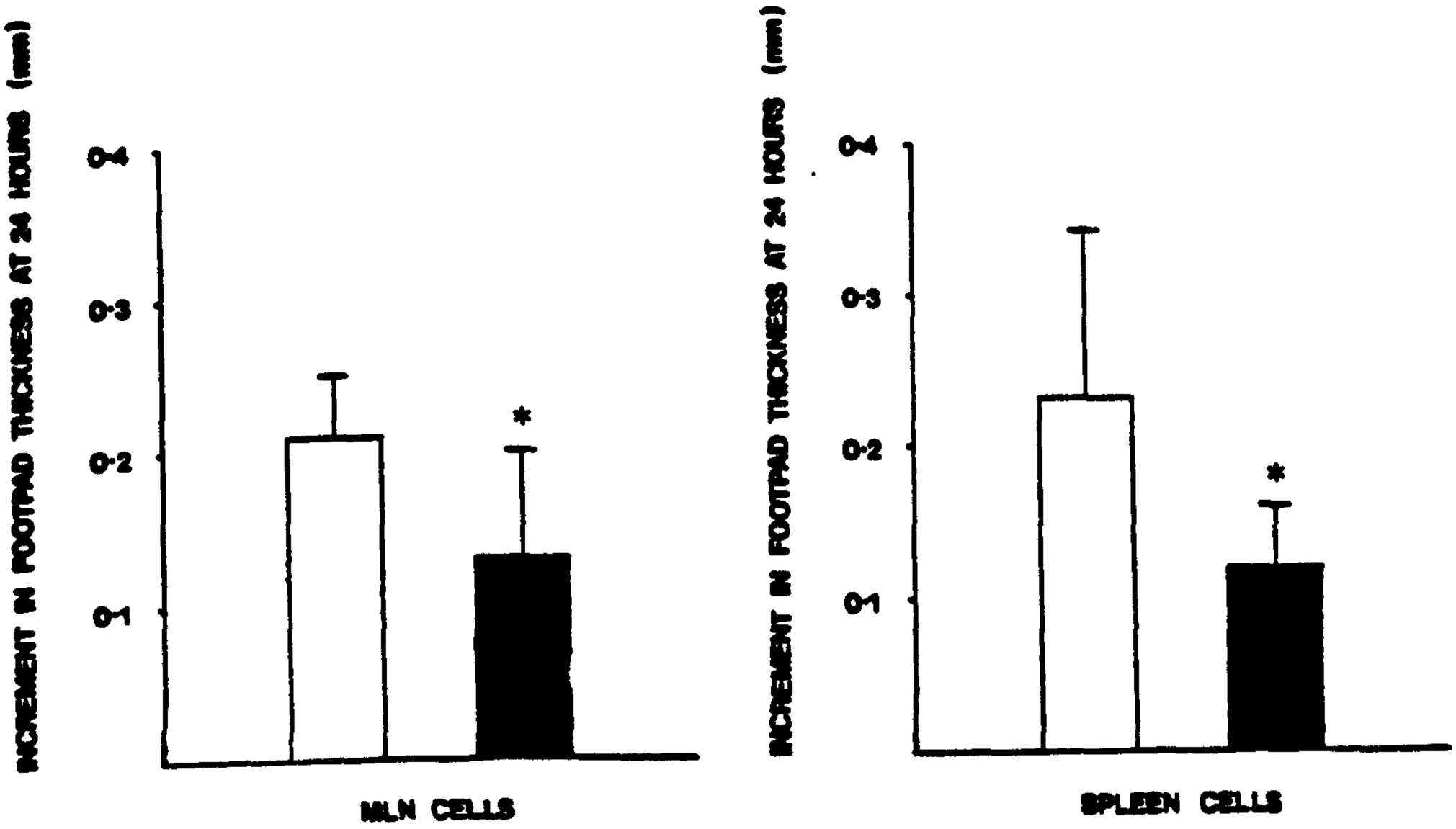

Figure 9.4 The effect of the transfer of MLN or spleen cells obtained 7 days after feeding CT or ABS on the induction of specific DTH in syngeneic recipients

Groups of mice were fed either ABS (□) or 1 µg CT (■). One week later these animals were sacrificed and a suspension of 10 MLN or spleen cells were transferred intraperitoneally to syngeneic recipients. The recipient mice were immunised intradermally with 1 µg CT in CFA on the day of transfer and footpad challenged with 5 µg TD in saline two weeks after this. This protocol is illustrated diagrammatically in figure 9.2. The bars shown represent the mean increment in footpad thickness ± 1 standard deviation measured 24 hours after footpad challenge.

Statistical comparison was made between recipients of antigen-fed cells and recipients of control suspensions for each source of lymphocytes.

* $p<0.05$

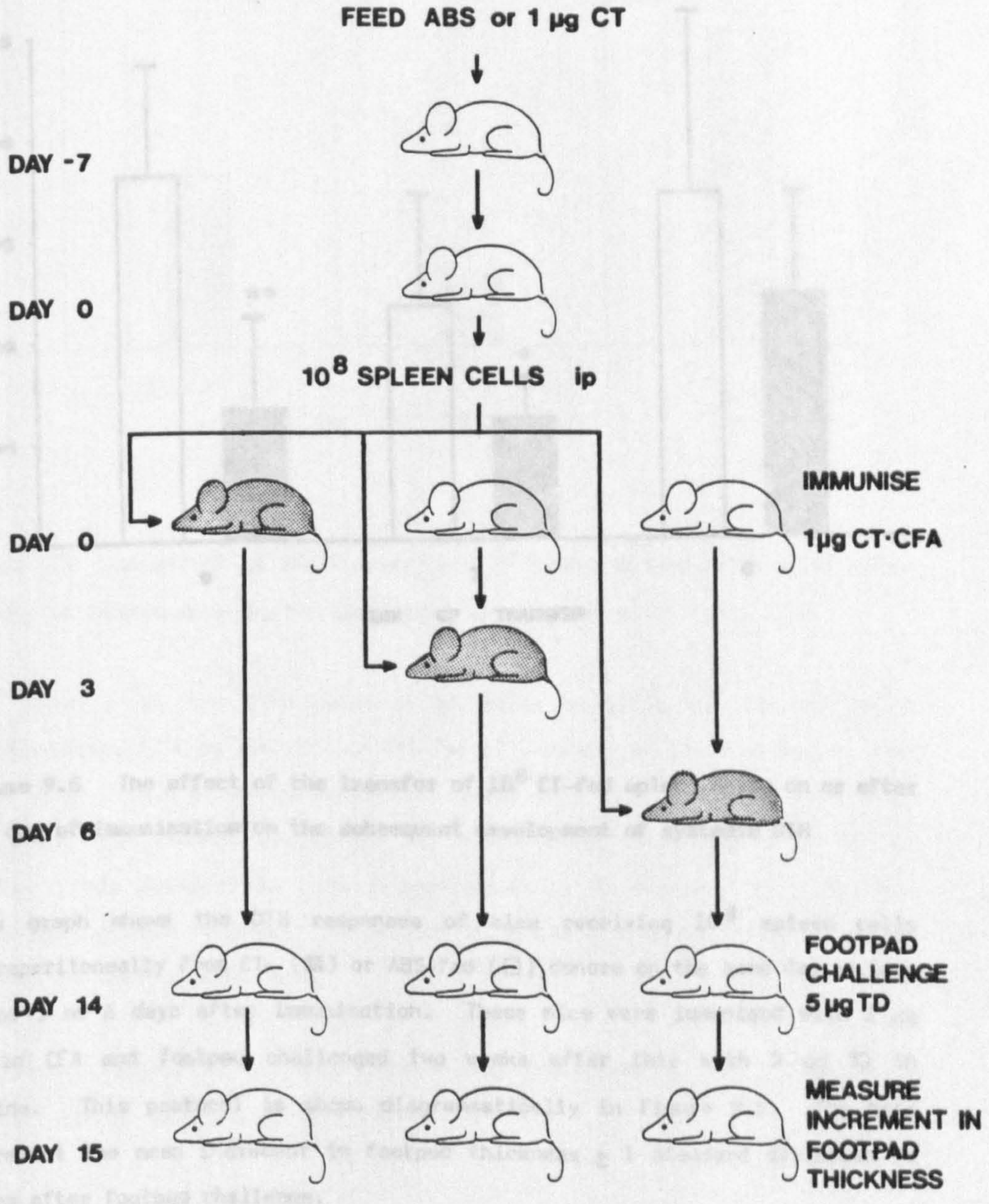

Figure 9.5 Experimental protocol to assess the mode of action of the splenic suppressor cell

The shaded mice denote the day of experiment that the spleen cells were transferred to the recipient animal

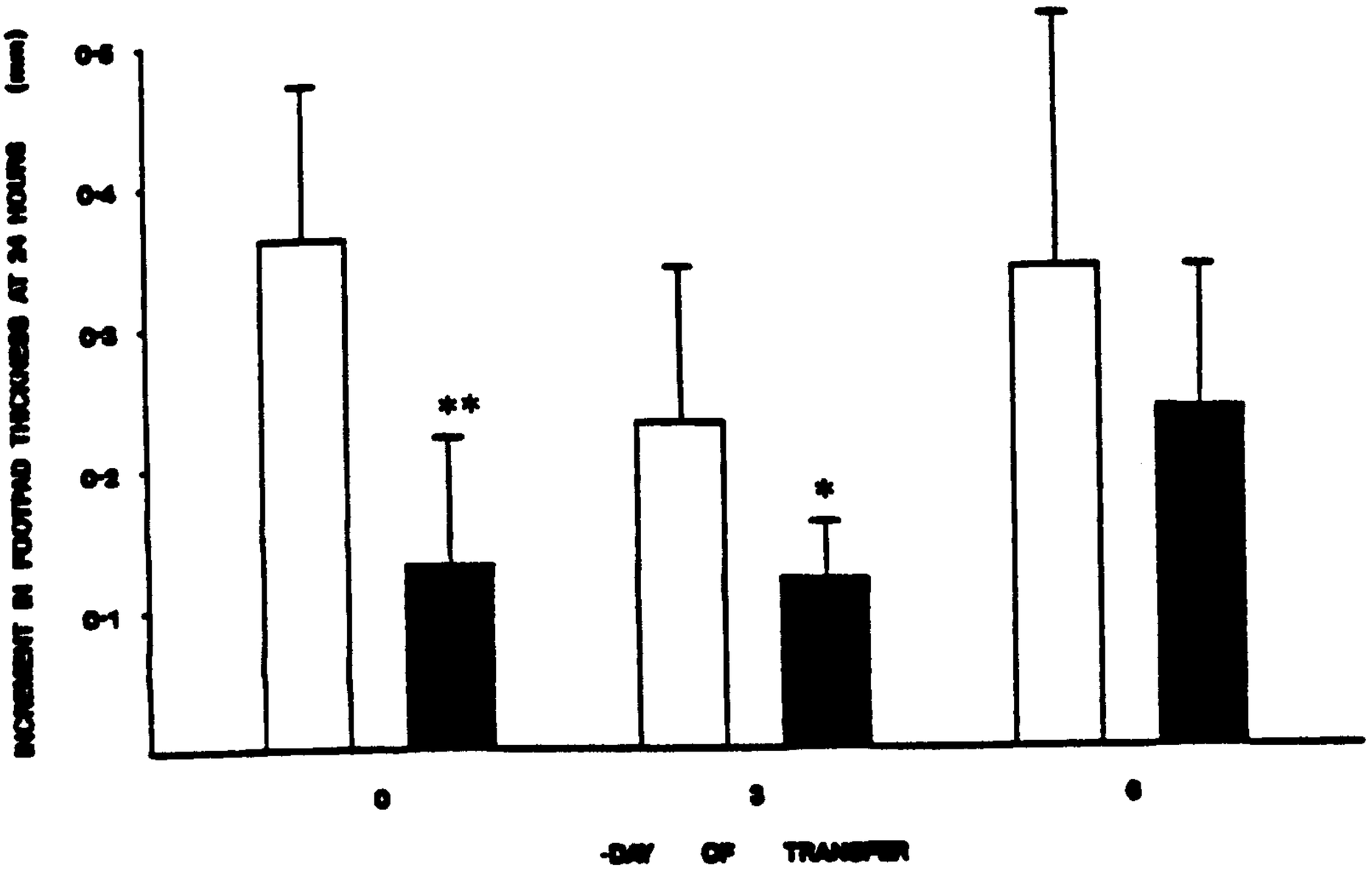

Figure 9.6 The effect of the transfer of 10^8 CT-fed spleen cells on or after the day of immunisation on the subsequent development of systemic DTH

This graph shows the DTH responses of mice receiving 10^8 spleen cells intraperitoneally from CT- (■) or ABS-fed (□) donors on the same day as (day 0) or 3 or 6 days after immunisation. These mice were immunised with 1 μg CT in CFA and footpad challenged two weeks after this with 5 μg TD in saline. This protocol is shown diagrammatically in figure 9.5. The bars represent the mean increment in footpad thickness ± 1 standard deviation 24 hours after footpad challenge.

Statisitical comparison was made between the recipients of the two types of spleen cell for each day of transfer.

* $p<0.05$

** $p<0.001$

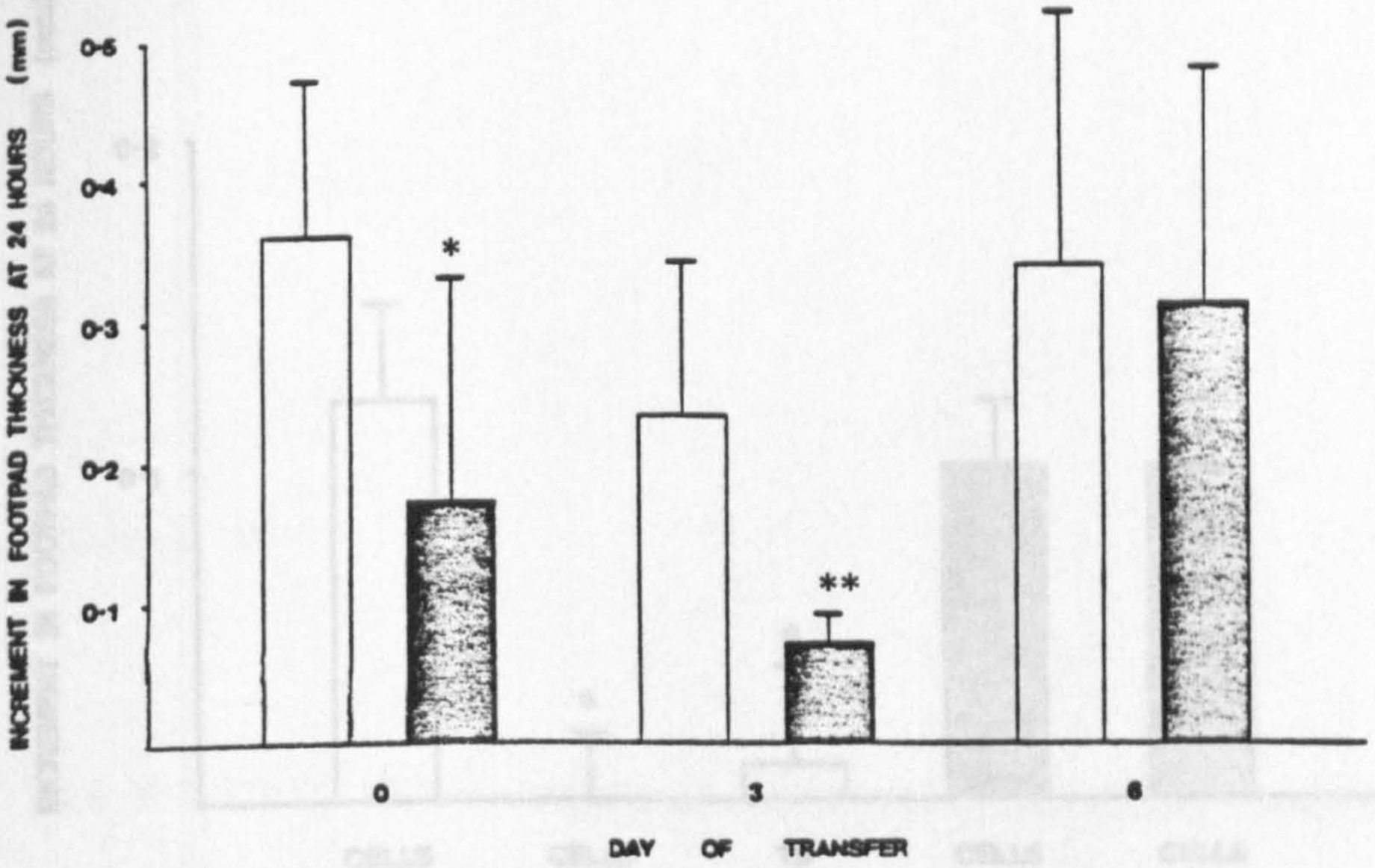

Figure 9.7 The effect of the transfer of 10^8 TD-fed spleen cells on or after the day of immunisation on the subsequent development of systemic DTH

This graph shows the DTH responses of mice receiving 10^8 spleen cells intraperitoneally from TD- (▩) or ABS-fed (□) donors on the same day as (day 0) or 3 or 6 days after immunisation. These mice were immunised with 1 µg CT in CFA and footpad challenged two weeks after this with 5 µg TD in saline. This protocol is shown diagrammatically in figure 9.5. The bars represent the mean increment in footpad thickness ± 1 standard deviation 24 hours after footpad challenge.

Statistical comparison was made between the recipients of the two types of spleen cell for each day of transfer.

* $p<0.05$

** $p<0.01$

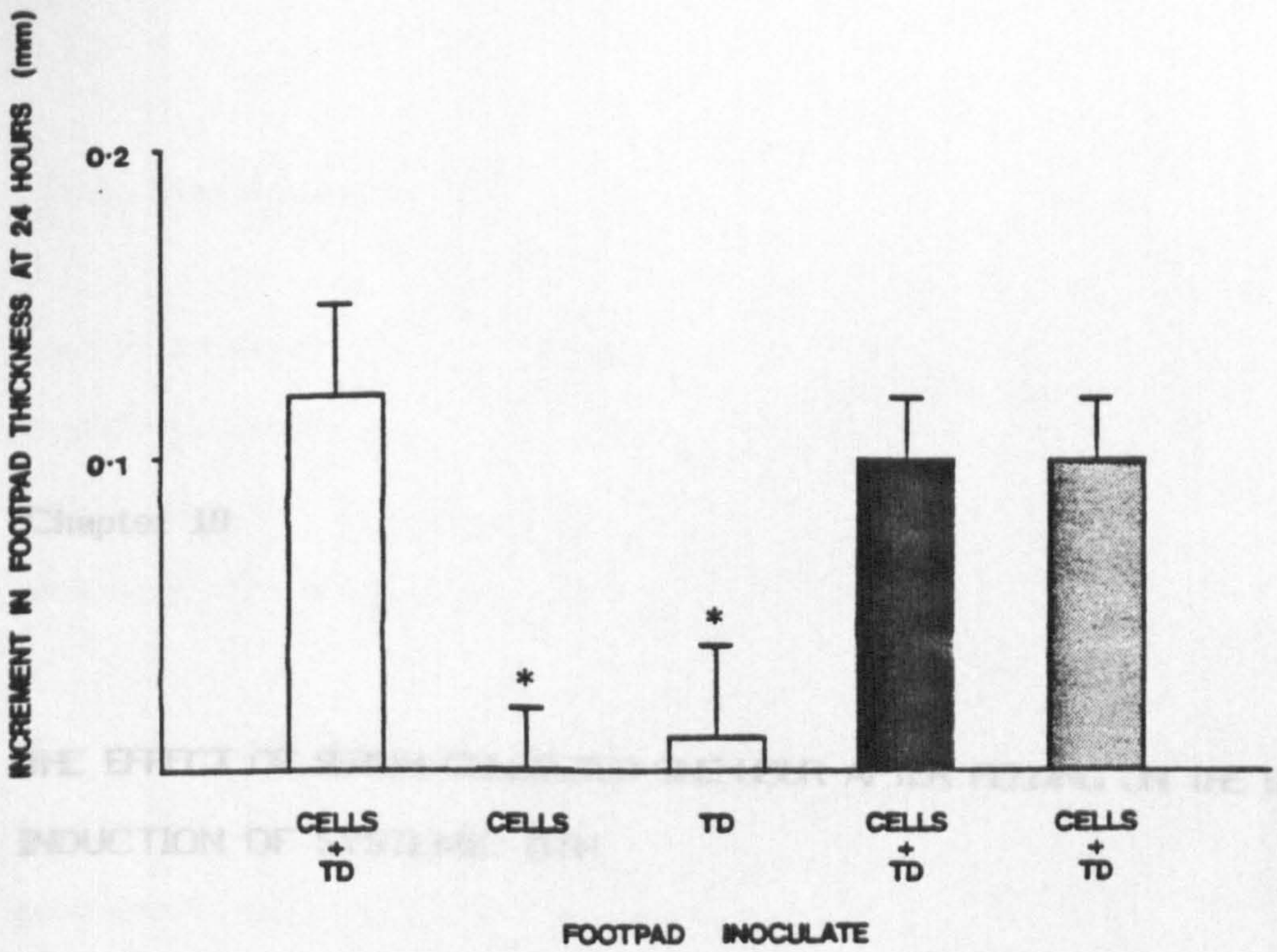

Figure 9.8 The effect of prior oral administration of CT or TD on the passive transfer of DTH by primed effector cells

This graph shows the DTH responses observed in groups of BALB/c mice fed either ABS (□), 1 μg CT (■) or 5 μg TD (▩). One week later these animals received 10^7 draining lymph node cells from toxin-primed donors in the presence of 5 μg TD. Draining lymphocytes were obtained from syngeneic donors immunised intradermally with 1 μg CT in CFA two weeks earlier.

ABS-fed mice also received either 5 μg TD or 10^7 lymph node cells alone, as controls. The bars shown represent the mean increment in footpad thickness ± 1 standard deviation measured 24 hours after the intradermal injection of cells or toxoid or both. Statistical comparison was made between the results of the ABS-fed mice receiving primed cells and antigen and the results of the other groups.

* $p<0.001$

Chapter 10

THE EFFECT OF SERUM COLLECTED ONE HOUR AFTER FEEDING ON THE SUBSEQUENT INDUCTION OF SYSTEMIC DTH

10.1 Introduction

Serum collected from mice fed OVA one hour previously has been shown to transfer suppression for the induction of antigen-specific DTH responses in syngeneic recipients. Tolerance for systemic antibody cannot be transferred in this way.

As the suppression transferred by serum may be abrogated by the pretreatment of the recipient but not the donor with CY, it has been postulated that serum transfer induces tolerance by the induction of a T suppressor cell (Strobel **et al**, 1983).

Analysis of this serum, reveals that a protein fragment bearing determinants for anti-OVA antibody and having the same or approximately the same molecular weight as the native antigen is responsible for this phenomenon (Bruce & Ferguson, 1986a; Bruce & Ferguson, 1986b). A CY-sensitive splenic T suppressor cell capable of inhibiting the induction of DTH following OVA feeding has been described previously (Miller & Hanson, 1979; Hanson & Miller, 1982). It is possible that the tolerogenic serum fraction described above induces this cell.

The OVA-specific suppressor T cell described above has characteristics in common with the splenic suppressor cell induced by feeding cholera toxin described in the previous chapters. Serum obtained one hour after feeding choleragen was therefore examined for its capacity to transfer tolerance. Furthermore, experiments employing either CY or cell transfer protocols were used to investigate the capacity of this

serum tolerogen to induce the splenic suppressor cell described earlier.

10.2 The Effect of Serum Transfer on Systemic DTH Responses

A protocol was devised to see if serum obtained one hour after feeding toxin could suppress systemic DTH responses as has been observed with ovalbumin. Donor mice were fed either 1 µg CT or ABS alone. One hour later, the mice were bled out and their serum was separated and pooled. Groups of syngeneic recipients were injected intraperitoneally with 0.8 mls of toxin-fed serum, ABS-fed serum or an equal volume of saline as a sham control. One week later, all groups were intradermally immunised with 1 µg CT in CFA and were challenged with 5 µg TD into the contralateral rear footpad 14 days after this. The increment in footpad thickness was measured 24 hours later.

The results of this experiment are shown in figure 10.1. Mice receiving serum from ABS-fed donors had similar DTH responses to those receiving a sham intraperitoneal injection of saline. Serum obtained from animals fed CT one hour previously was able to significantly suppress the induction of DTH after intraperitoneal transfer ($p<0.01$).

10.3 The Effect of Serum Tolerogen on the Splenic Suppressor Cell

Work with ovalbumin has suggested that gut processed antigen induces a specific suppressor cell for DTH responses. Such a cell has been found in the spleens of toxin-fed animals, so experiments were designed to see if serum obtained one hour after feeding toxin could induce this splenic lymphocyte.

The experimental protocol is shown diagrammatically in figure 10.2. Mice were fed either 1 µg CT of ABS alone. One hour later these mice were bled out and their serum was pooled. 0.8 mls of ABS- or toxin-fed serum were each injected into two groups of animals. The first group of animals received the appropriate serum intraperitoneally and 7 days later were immunised as before.

The second group of serum recipients were intermediate hosts. These mice were intraperitoneally injected with 0.8 mls of the appropriate serum, 7 days later they were sacrificed and their spleens removed. 10^8 spleen cells were then intraperitoneally transferred to a third set of animals who were immunised on the same day as cell transfer. All mice were footpad challenged two weeks after immunisation and the increment in footpad thickness noted after 24 hours.

The results of this experiment are displayed in figure 10.3. Splenic lymphocytes from ABS-fed serum recipients do not alter the systemic DTH responses significantly when compared with the responses of animals receiving ABS-fed serum directly. As in the previous experiment,

toxin-fed serum was able to significantly suppress the induction of systemic DTH ($p<0.001$). However, toxin-fed serum did not induce suppressor cells in the spleens of recipient mice after 7 days. The DTH responses in these spleen cell recipients were not significantly different from those observed in ABS-fed serum recipients.

10.4 The Effect of Cyclophosphamide on Serum-induced Tolerance

As the serum tolerogen did not induce the splenic suppressor cell normally found after feeding cholera toxin, an experiment was performed to see if this serum transfer phenomenon was sensitive to cyclophosphamide as has been reported for ovalbumin.

In these experiments groups of mice received an intraperitoneal injection of either cyclophosphamide (100 mg/kg) or saline. Two days later these mice received another intraperitoneal injection - 0.8 mls of either ABS- or CT-fed serum. Seven days after serum transfer the mice were immunised and subsequently footpad challenged as has been described earlier.

Earlier experiments have shown that cyclophosphmide alone does not alter the DTH responses in saline fed mice. It is not suprising, therefore, that the results, displayed in figure 10.4, show that toxin-fed serum significantly suppresses the induction of DTH compared with responses observed in cyclophosphamide-pretreated ABS-fed serum recipients ($p<0.002$). The tolerance observed in toxin-fed serum

recipients is abrogated by pretreatment with cyclophosphamide.

10.5 Summary

Serum collected one hour after feeding BALB/c mice with cholera toxin was able to suppress the induction of DTH when transferred intraperitoneally to syngeneic recipients. This phenomenon was abrogated by pretreatment of the recipients with cyclophosphamide (100 mg/kg) two days before serum transfer.

This serum fragment did not appear to induce the splenic suppressor normally found one week after feeding cholera toxin to BALB/c mice. However, further experiments are required to establish this with certainty and to define the alternate mechanism(s) by which the serum tolerogen transfers cyclophosphamide-sensitive suppression.

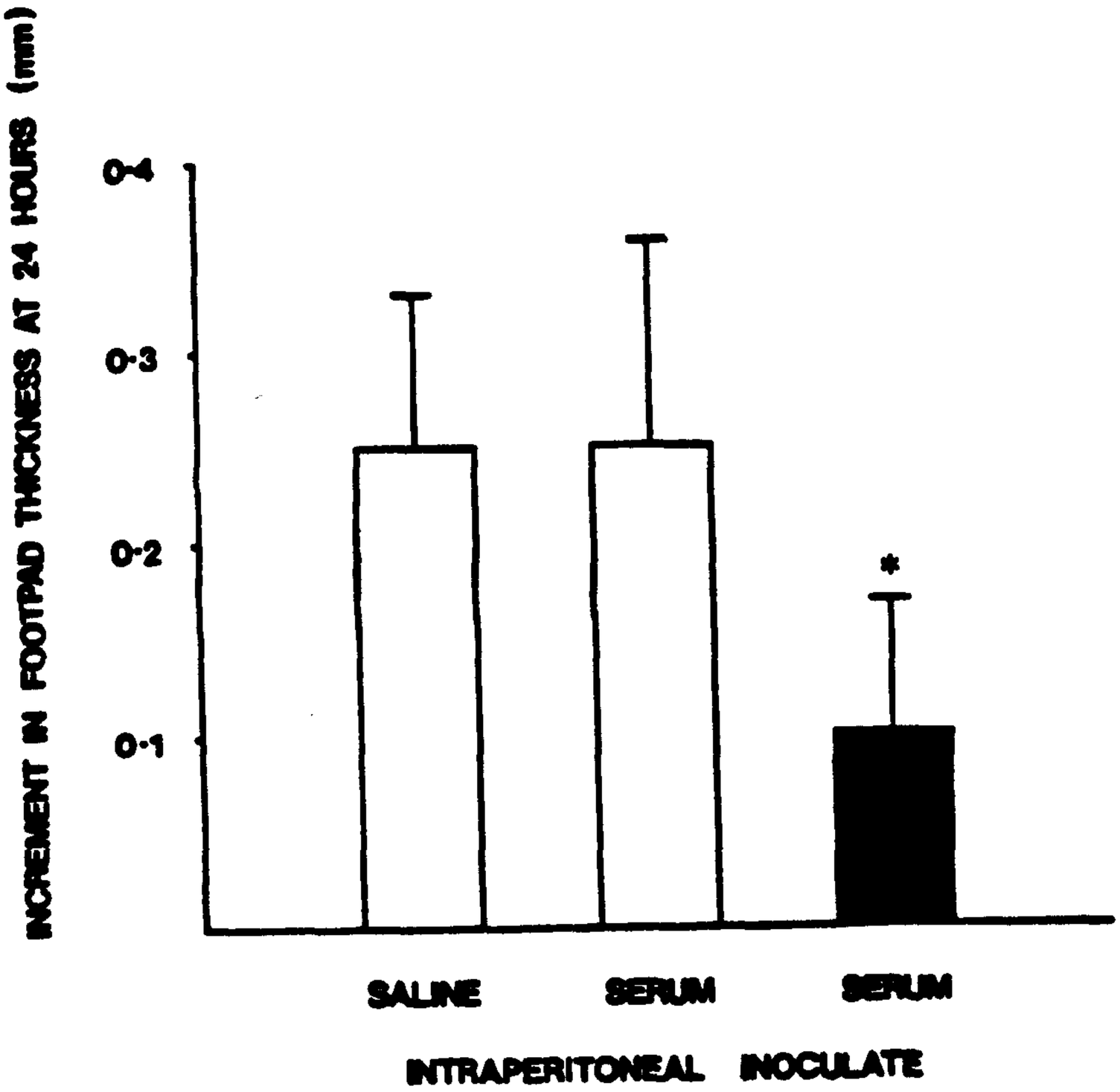

Figure 10.1 The effect of transfer of choleragen-fed serum on the subsequent induction of systemic DTH

This graph shows the DTH responses of three groups of mice intradermally immunised with 1 μg CT in CFA one week after an intraperitoneal injection of saline or serum from syngeneic mice fed either ABS (□) or 1 μg CT (■) one hour previously. Immunised mice were footpad challenged with 5 μg TD two weeks later. The above bars represent the mean increment in footpad thickness ± 1 standard deviation observed 24 hours after footpad challenge. Results were compared to those obtained from the mice receiving the sham intraperitoneal injection of saline.

* $p<0.01$

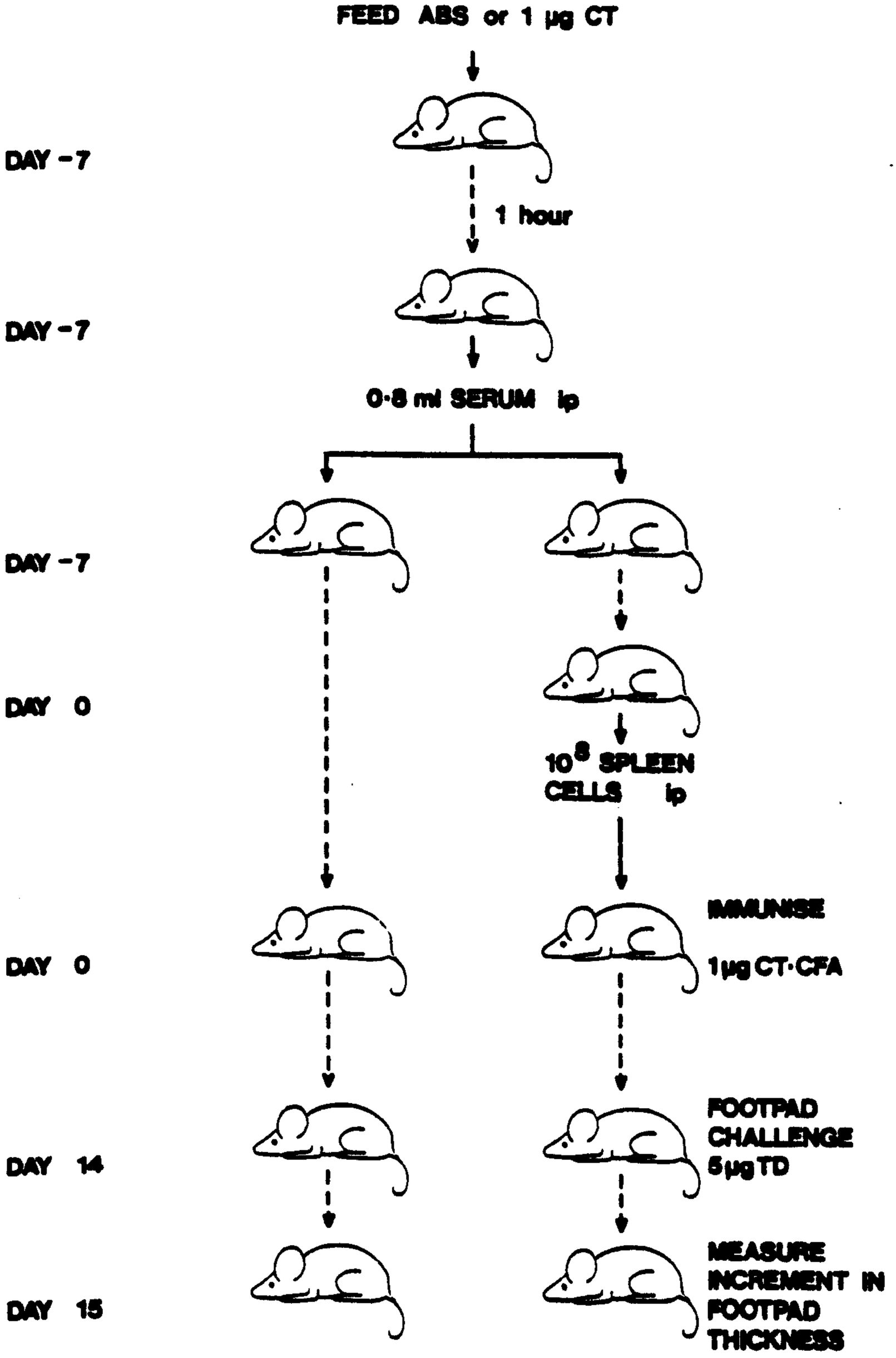

Figure 10.2 Experimental regime to examine whether serum toleragen induces a splenic suppressor cell 7 days after transfer

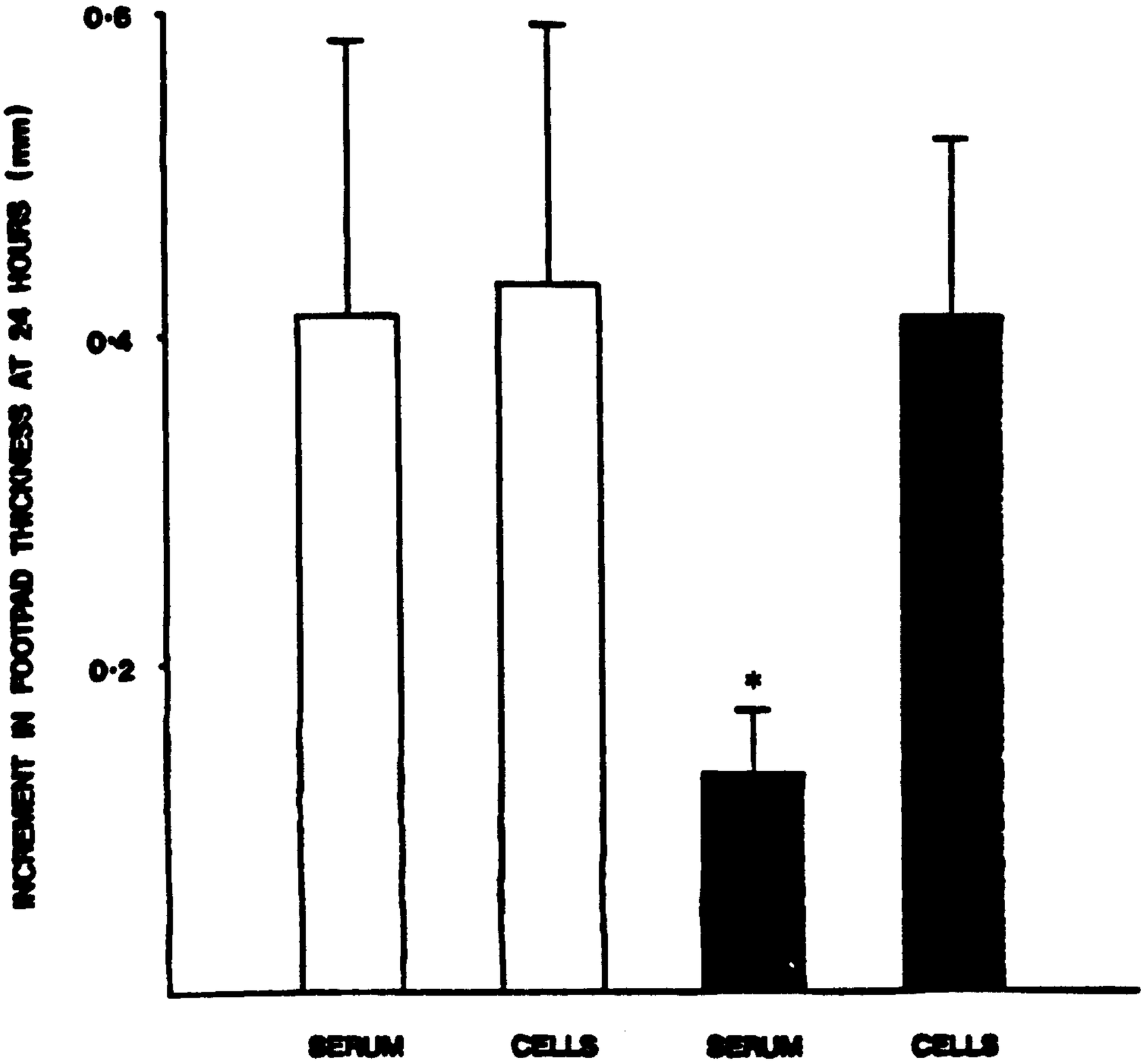

Figure 10.3 Experiment to assess the capacity of choleragen-fed serum to induce a splenic suppressor cell

The above graph shows the DTH responses of groups of mice receiving either spleen cells from serum recipients of serum itself from ABS- (□) or CT-fed (■) donors. Serum was transferred one hour after feeding. Animals receiving serum were immunised one week later, spleen cell donors received serum at the same time but their splenocytes were transferred one week later to recipient mice. These mice were intradermally immunised on the same day as cell transfer. This regime ensured that all recipient groups were immunised on the same day and footpad challenged two weeks later. A diagrammatic representation of this protocol may be seen in figure 10.2. The bars represent the mean increment in footpad thickness ± 1 standard deviation, 24 hours after footpad challenge.

Experimental results are compared to those obtained in mice receiving serum from ABS-fed donors.

* $p<0.001$

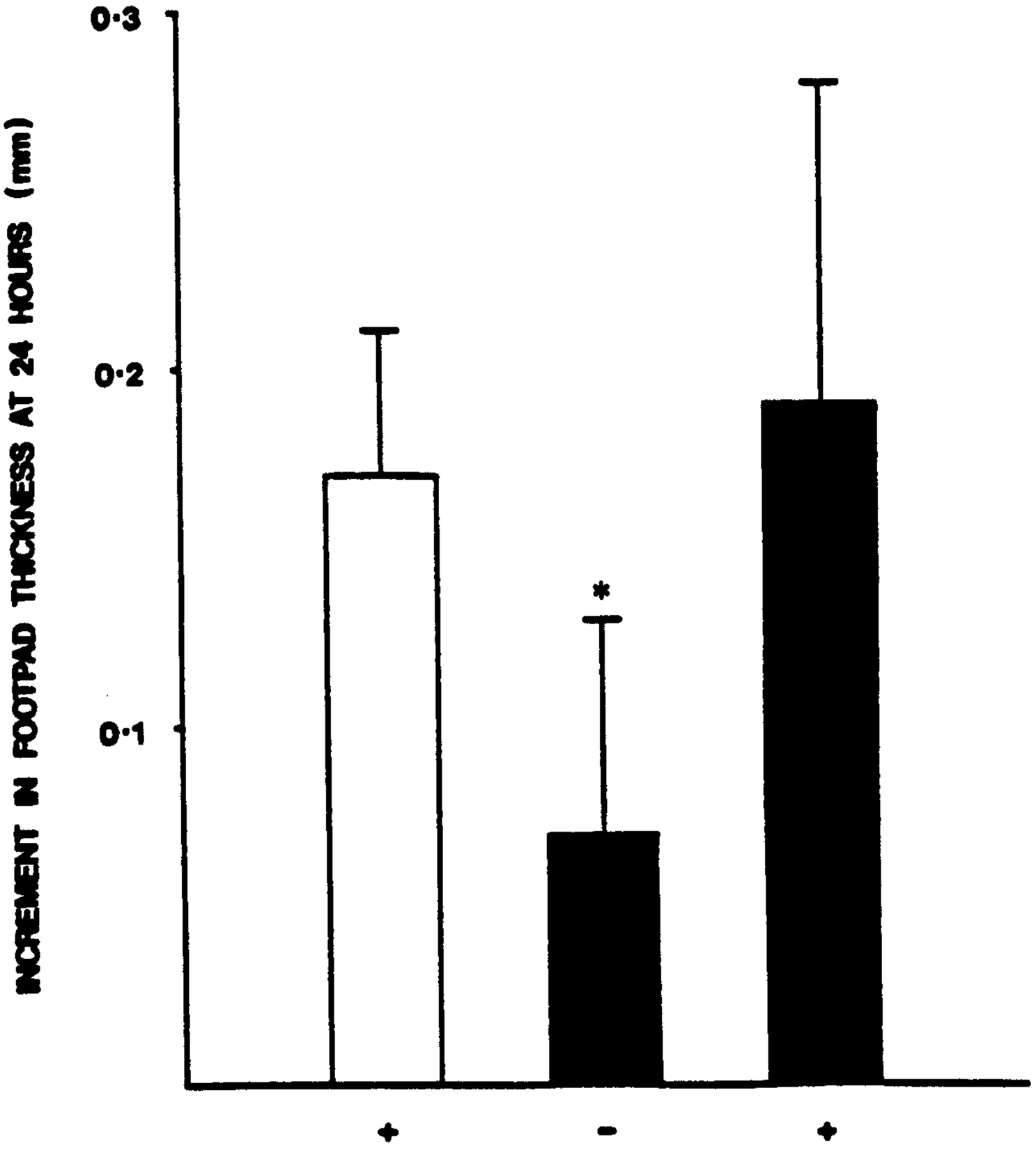

Figure 10.4 The effect of cyclophosphamide on the tolerance transferred by choleragen-fed serum

This graph shows the DTH responses of mice pretreated with cyclophosphamide (100 mg/kg) or given a sham injection of saline two days before receiving an intraperitoneal injection of serum from syngeneic donors fed ABS (□) or CT (■) one hour previously. These mice were immunised with CT in CFA one week after receiving serum and were footpad challenged two weeks after immunisation. The bars represent the mean increment in footpad thickness ± 1 standard deviation, 24 hours after footpad challenge.

Experimental results are compared to those obtained from cyclophosphamide-pretreated mice receiving serum from ABS-fed donors.

* $p<0.002$

Chapter 11

GENERAL DISCUSSION

11.1 Introduction

The objective of this thesis has been to study the systemic immune response of BALB/c mice to cholera toxin and to examine the consequences for this response when the antigen was prefed. This objective has been achieved. I have been able to show that a cell-mediated immune response is induced by a parenteral immunisation of toxin in addition to the production of specific antitoxin antibodies. Studies in which choleragen was prefed have confirmed that this antigen does not induce oral tolerance for systemic antibody. However, these experiments did reveal that choleragen suppresses the induction of systemic cell-mediated immunity. Further investigation revealed that cyclophosphamide pretreatment reversed the induction of tolerance and cell tansfer studies showed that feeding induced a splenic suppressor cell.

There is evidence that choleragen can modulate immune responses by its biochemical properties as well as by virtue of its antigenicity (chapter 1). In order to examine whether the above observations were due to its inherent 'toxicity', a toxoid was substituted for the toxin in several experiments. These studies revealed that the toxoid was inferior at priming for antitoxin antibody responses but that some of this effect was due to limited cross-reactivity of these two antigens. However, both these proteins were equally effective at boosting antitoxin antibody responses, priming for systemic DTH and inducing oral tolerance.

Further investigation of the nature of the toxin-induced splenic suppressor cell revealed that it had many of the characteristics of suppressor cells induced by feeding other more classical enteric protein antigens. The role of the gut in immunologically 'processing' antigen was also examined by serum transfer and showed that a great similarity exists between the handling of different protein antigens. Furthermore, this work suggests that several mechanisms participate in the induction of oral tolerance for systemic CMI.

Each of these findings will be discussed in turn, beginning with the systemic immune responses to parenterally administered choleragen.

11.2 Systemic Antibody Levels in Parenterally Primed Mice

Systemic antibodies to cholera toxin have been discovered after specific priming of mice (Svennerholm **et al**, 1980; Lycke **et al**, 1983; Elson & Ealding, 1984a; Elson & Ealding, 1985), rats (Lange **et al**, 1984b), rabbits (Peterson, 1979; Yardley **et al**, 1978; Hamilton **et al**, 1979) and dogs (Pierce **et al**, 1978; Pierce **et al**, 1982). IgG and IgA antitoxin antibody levels have been most frequently measured but specific IgM has also been detected. This study has confirmed some of these findings in that parenteral immunisation with choleragen has been shown to induce systemic IgG and IgA antibodies. However, circulating IgM antitoxin antibodies were not consistently present. There are several possible reasons for this which lie both in the particular assay used and the phenomenon of IgM

production **in vivo.**

ELISA assays were used to measure specific antibody levels in this study. ELISA is a technique commonly employed to assay levels of specific antibody and has been frequently used to measure anti-choleragen antibody levels in both humans and animals (Holmgren & Svennerholm, 1973; Svennerholm & Holmgren, 1978; Young **et al,** 1980). This particular assay was set up using a hyperimmune serum raised in adult BALB/c mice. This serum contained significant amounts of all the three principal isotypes of antitoxin and was used as the positive standard against which experimental sera were evaluated. While the other two assays worked consistently, the IgM antitoxin ELISA did not regularly detect significant levels in specifically immunised animals. Background absorbance levels were higher in this assay than in the other two. It appeared that the isotype-specific antiserum was able to non-specifically bind to the toxin-coated plates despite blocking with protein. The conjugate only bound non-specifically to toxin-coated wells. This phenomenon was also observed when an anti-mouse immunoglobulin antiserum (Miles Ltd) was used in this assay. IgM is not the predominant isotype in the antitoxin response and thus has to compete in this assay for binding spaces with more numerous and perhaps more avid antibodies of other classes. It is possible that under these circumstances not enough specific IgM antitoxin is bound to fall within the detectable range of the ELISA system.

There are other reasons why low levels of specific IgM may be observed in these animals. There are no studies of the isotypic response to different doses of parenteral choleragen but virtually all the studies examining the humoral immune response to choleragen in mice have used 10 μg toxin either orally or parenterally (Svennerholm et al, 1980; Elson & Ealding, 1984a; Elson & Ealding, 1984b; Lycke et al, 1985; Lycke & Holmgren, 1986a). Furthermore, workers raising anti-choleragen monoclonal antibodies of the IgM isotype found that large doses of toxin (50 μg) were needed and that clones were detected 4 days after immunisation (Remmers, Colwell & Goldsby, 1982). Therefore, another explanation may be that the dose of antigen used was too small and the timepoint examined (7 days) too late to either stimulate or detect significant IgM antitoxin responses.

A number of workers have compared the antigenicity of formalinised toxoid to that of the holotoxin (Pierce & Gowans, 1975; Pierce, 1978; Peterson, 1979; Pierce, 1980; Fuhrman & Cebra, 1981). Varying conclusions have been drawn from these studies, but none of these papers have used these two substances in comparable amounts. Comparative experiments using these two proteins at the mucosal surface have always attributed differences in immune response to the particular biochemical properties of the toxin (binding to cell surface gangliosides and adenylate cyclase activation) (Pierce, 1978; Pierce at al, 1978; Lycke & Holmgren, 1986a) but more subtle changes in antigenicity due to the detoxification procedure have never been examined. The majority of antibodies raised by immunisation with the holotoxin are directed against B subunit determinants (Holmgren &

Svennerholm, 1979; Svennerholm, Holmgren, Black, Levine & Merson, 1980; Lindholm, Holmgren, Wilkström, Karlsson, Andersson & Lycke, 1983). It is for that reason that B subunit is generally thought of as being the immunodominant portion of choleragen (Holmgren & Svennerholm, 1983). Experiments described in chapter 5 have therefore employed doses of toxin and toxoid with a similar B subunit content.

The results obtained show that toxin is superior to toxoid at priming for an antitoxin response, but that both substances are equally efficient at recalling an anamnestic response especially of the IgG isotype. Boosting of a specific IgG response has been shown to be a T cell phenomenon (Schrader, 1975; Okumura, Metzler, Tsu, Herzenberg & Herzenberg, 1976) and it is therefore possible that this result reflects cross-reactivity at the T cell level whereas B cell determinants are not shared between these two substances. An anti-toxoid ELISA was therefore developed in order to examine whether differences exist in the response to both forms of this antigen. The ELISA used solid M129A microtitre plates in place of the flexible M 29 plates used in the method of Cruce and his colleagues (Cruce, Wachsmuth & Feeley, 1983). In contrast to their findings we found that solid plates were able to bind sufficient antigen and gave more consistent results than the flexible plates.

Results from this assay were intriguing, the toxoid-primed animals had significantly higher levels of circulating IgG anti-toxoid than animals immunised with choleragen. However this difference was not as great as in the reverse situation and significance was only found when all the

prechallenge animals were compared (i.e. those destined for challenge with either toxin or toxoid). This is a fair statistical comparison to make as the original two groups had not been further subdivided at this stage of the experiment. This difference was not maintained after the second antigenic challenge where all groups gave statistically similar results. It could be that although there are specific differences in the antigenic determinants on these two substances only shared determinants are 'helped' by specific T cells. The antitoxin results suggest that some antigenic determinants have been lost from the holotoxin during the process of formalinisation.

Formalinisation has been used for the conversion of toxins to toxoids for some time. Formaldehyde has been shown to effect a reductive alkylation of lysine (Pancake & Nathenson, 1973) and tyrosine residues (Habeeb, 1969). The effect of such changes on antigenicity is variable with some authors reporting decreases in antigenicity (Bradley & Barnes, 1972; Pancake & Nathenson, 1973; Gatti, Östborn & Fagraeus, 1974; Pierce, 1978; Peterson, 1979) and others reporting no change (Jacobs & Sommers, 1939; Grasset & Zoutendyk, 1933; Zinsser & Catteneda, 1931; Pappenheimer Jr, 1938; Hewitt, 1930; Bradley & Barnes, 1972; Habeeb, 1969) or even increased reactivity (Warren, Spero & Metzger, 1973). Crude formaldehyde is able to polymerise antigens and the observed difference in responses has been attributed both to polymerisation (Warren et al, 1973) and the alteration of lysine-containing B cell determinants (Pancake & Nathenson, 1973). While all the foregoing studies have measured the humoral immune response to formalinised antigenic determinants, the T cell response to such

antigens has only recently been critically evaluated. Some work employing cellular antigens is relevant to the consideration of this point.

Elegant studies using formalin-fixed target cells have shown that chemical treatment removes the determinants recognised by the majority of anti-H-2 monoclonal antibodies. However, these cells are still capable of stimulating the appropriate response from H-2-specific cytotoxic T lymphocytes. Work with mutant cell lines has shown that target cells must possess a lysine residue within the B cell determinant for formalinisation to prevent the binding of monoclonal antisera (Hua, Langlet, Bufferne & Schmitt-Verhulst, 1985). These studies clearly demonstrate that formalinisation can selectively alter the ability of B cells and antibodies to recognise specific determinants and suggests that it may be this phenomenon which is responsible for the observed differences in immunogenicity between toxin and toxoid (especially when assayed against the holotoxin) rather than the biochemical properties of choleragen.

11.3 Systemic Cell-Mediated Immunity in Parenterally Primed Mice

It is common for antigens to induce both humoral and cell-mediated immune responses. Experiments described in chapter 6 have established that choleragen is capable of inducing systemic CMI under conditions where specific IgG and IgA have also been shown to exist (chapter 5). This is the first description of CMI to choleragen.

DTH was assessed by direct skin testing whereby the incremental change in footpad thickness 24 hours after intradermal challenge with antigen is measured using engineers' calipers. This is a reliable and well-established method for quantifying DTH responses in experimental animals (Kettman, 1972; Clark & Azar, 1977; Titus & Chiller, 1981b). However, the assay as used in this project had two drawbacks. Firstly, preliminary results (chapter 4) showed that an intradermal injection of choleragen causes a large amount of non-specific footpad oedema. They also showed that toxoid, in amounts up to 5 µg, did not, both of which are in agreement with earlier studies (Finkelstein & Hollingsworth, 1970; Lexomboon **et al**, 1971). As the non-specific oedema would interfere with the measurement of DTH, toxoid was chosen as the challenge antigen regardless of the immunising protein. This meant that differential responses to a challenge with the two forms of cross-reacting antigen could not be assessed. This was not felt to be of major importance as other studies have confirmed that formalinisation does not appear to alter T cell reactivity (Hua at al, 1985) and that limited studies in which animals were challenged with purified B subunit gave similar responses in both choleragen- and toxoid-primed mice.

The second problem in this assay was the wide scatter of results obtained from mice that had been identically treated i.e. the intra-group variation was large and this resulted in large standard deviations about the mean. This was not due to poor technique because repeating this assay on OVA-primed and challenged animals gave results which agreed well with those obtained by other workers in our

laboratory. The possible reasons for this variation are two-fold. Toxoid (like toxin) has a limited shelf-life. The DTH responses obtained one or two months after being kept at 4 to 8 °C were not as great as was obtained when the preparation was 'fresh'. As the toxoid was a gift, in limited supply and could not be frozen to maintain antigenicity, extra internal controls were always included. Secondly, this formalinised immunopurified toxoid has been shown to have a small amount of residual toxicity in the limit of bluing assay (Pierce, 1978). Furthermore, other formalinised cholera toxoids have been shown to revert to toxicity **in vivo** (Northrup & Chisari, 1972) and it might be this that is responsible for the observed mouse-to-mouse variation.

The DTH skin test confirmed that toxin-primed mice gave positive responses both 24 and 48 hours after specific antigen challenge. Histological examination of the lesions induced showed a gradual accumulation of mononuclear cells. Initially, these were lymphocytes but basophils and neutophils were additonally involved at later timepoints. These findings are also consistent with earlier descriptions of DTH (Dvorak **et al**, 1974; Scovern & Kantor, 1982; Pellitier, Forget, Bourassa & Skamene, 1984). In addition to the later swelling response, an 'immediate-type' oedematous response was also observed. This is in agreement with the findings of other workers (Van Loveren **et al**, 1983) who have suggested that the early swelling is an obligatory component of the DTH response brought about by the release of serotonin from tissue mast cells specifically armed with a T cell factor (Askenase at al, 1983a; Askenase at al, 1983b; Askenase & Van Loveren, 1983; Van Loveren **et al**, 1984). There are studies

which have shown DTH to occur in mast cell-deficient mice but these have not examined whether this early swelling component still occurs (Thomas & Schrader, 1983; Galli & Hammel, 1984). The necessity or exact nature of this phenomenon has yet to be established but these experiments do confirm its existence.

Dose-response studies have shown that optimal DTH is induced by priming with 1 μg of choleragen, whereas other work suggests that priming of specific antibodies is better with 10 μg of toxin. Work with cloned T cells has shown that they can both passively transfer DTH and provide help for specific antibody production (Bianchi **et al**, 1981; Dennert **et al**, 1981; Milon **et al**, 1983). Earlier studies in SRBC-primed animals revealed that low doses of antigen preferentially induced DTH whereas higher doses primed most favourably for specific humoral immunity. It was suggested that the T cell mediating both these functions may be specifically trapped in the spleen by B cells induced by the larger doses of antigen (Milon **et al**, 1983).

The foregoing experiments were specifically designed to look at the activation of T cells by choleragen. DTH is an established measure of T cell reactivity but other facets of T cell activation were also assessed.

Antigen administered intradermally into the hind footpad has been shown to drain to the popliteal and inguinal lymph nodes of the recipient (Hall, 1985). Draining lymph node cells from choleragen-primed mice were shown to have reduced migration when cultured in the presence of

cholera toxoid at a concentration of 0.01 µg/ml. Antigen concentrations both ten-fold higher and lower failed to induce migration inhibition in these cells and cells from saline- or OVA-primed donors were not inhibited by any of the concentrations of toxoid in culture. This method is a modification of the technique described by Mowat and Ferguson (1982a) and has been used to assess the cell-mediated immune response of mesenteric lymph node cells after the enteric administration of antigen (Mowat & Ferguson, 1982b; Mowat, 1986). The assay was originally described using the peripheral draining lymph node cells from mice primed intradermally with ovalbumin and experiments performed in this thesis have confirmed that OVA-primed cells exhibit migration inhibition only in the presence of specific antigen. Although best seen in T cell-enriched populations the phenomenon could also be observed with T cell-depleted lymphocyte preparations but T cell contamination could not be ruled out as a possible source of error (Mowat & Ferguson, 1982a).

Lymphocytes have been shown to release many soluble products capable of inhibiting the migration of a number of differing cells including macrophages, lymphocytes and neutrophils (David, 1972; Pick, 1979; Rocklin **et al**, 1980; Schrader & Clark-Lewis, 1981; Weissbart **et al**, 1982; Godfrey & Purhoit, 1982). Lymphoid cells from a number of tissues have been used in direct migration inhibition assays including spleen (Rich & Lewis, 1932; Carpenter, Barsales & Ganghan, 1968; Likhite, Haasz, Algom & Richter, 1972; Hughes, Preece & Light, 1980), thymus (Bakker, Englehart, Mulder & Hoedemaeker, 1975; Hughes **et al**, 1980), tonsil (Mazuran, Rabatic, Sabioncello & Dekaris, 1979) and lymph

nodes (Thor & Dray, 1968; Likhite **et al**, 1972). Migration inhibition has also been described in peripheral blood lymphocytes (Kowalczyk & Zembala, 1978; Bradsher, Sutcliffe & Alford, 1979). Positive results in these assays have been correlated with the presence of DTH **in vivo** (Thor & Dray, 1968; Likhite **et al**, 1972; Mazuran **et al**, 1979; Hughes **et al**, 1980) and other measures of CMI (Kowalczyk & Zembala, 1978; Bradsher **et al**, 1979). Although other workers have shown that immune complexes may inhibit cell migration **in vitro** (Bloom & Bennett, 1966; Carpenter **et al**, 1968), Mowat and Ferguson (1982a) have shown that their migration inhibition assay closely paralleled DTH **in vivo** and was unaffected by the level of humoral immunity.

Finally, the corner stone of CMI has been the description of immune responses that could be transferred with cells but not serum. Many authors have been able to transfer DTH to naive syngeneic recipients using primed lymphoid cells (Marchal **et al**, 1978; Titus & Chiller, 1981b; Scovern & Kantor, 1982; Milon **et al**, 1983; Van Loveren **et al**, 1984). These experiments have established that T cells transfer this reaction. Work with T cell clones has further confirmed the lineage of the DTH effector cell. It has also revealed that some of these clones are only capable of transferring a DTH reaction if injected locally in the presence of antigen (Bianchi **et al**, 1981; Dennert **et al**, 1981; Lin & Askonas, 1981; Weiss & Dennert, 1981; Marchal **et al**, 1982; Minami **et al**, 1982; Thomas **et al**, 1982; Carroll **et al**, 1983; Gallatin **et al**, 1983; Tamura **et al**, 1983).

The local passive transfer of DTH using primed lymphoid cells admixed with antigen has been used successfully to demonstrate the presence of CMI to bacteria (Lin & Askonas, 1981; Kaufmann & Hahn, 1982), contact sensitising agents (Thomas **et al**, 1982; Van Loveren **et al**, 1984), heterologous red blood cells (Marchal **et al**, 1978; Bianchi **et al**, 1981; Marchal **et al**, 1982; Milon **et al**, 1983), and protein antigens (Scovern & Kantor, 1982). Cells transferring DTH have been obtained from mucosal as well as peripheral lymphoid tissues (Shields & Parrott, 1985). In all cases the effector cells have been shown to be THY-1 positive. This technique has the obvious advantage that it does not require the immune cells to migrate to the site of antigenic challenge and that it appears to require fewer primed lymphocytes than passively sensitising the whole animal intravenously. For these two reasons the passive transfer of DTH was used as a measure of CMI in toxin-primed animals. Draining lymph node cells, obtained from mice primed with toxin, were able to transfer DTH to naive syngeneic recipients in the presence of specific antigen. Depletion of the Thy-1.2 positive lymphocytes from this population abrogated their ability to transfer DTH and confirmed the T cell lineage of these effector cells.

11.4 Systemic Immunity after Prefeeding with Antigen

Previous studies have demonstrated that the induction of systemic immunity may be inhibited by prefeeding with a number of protein

antigens (Vaz, Maia, Hanson & Lynch, 1977; Ngan & Kind, 1978; Richman, Chiller, Brown, Hanson & Vaz, 1978; Swarbrick **et al**, 1979; Challacombe & Tomasi, 1980; Titus & Chiller, 1981a; Mowat **et al**, 1982). Few of these studies have examined the consequences of feeding on both cell-mediated and humoral limbs of the effector response. A protocol which does is the one routinely employed in this laboratory to assess the effect of feeding OVA (Mowat **et al**, 1982; Mowat & Parrott, 1983; Mowat, 1985; Strobel **et al**, 1985; Mowat, 1986). This was adopted as it also allowed a more direct comparison of the responses to choleragen and the more frequently studied enteric antigen, OVA. BALB/c mice have been shown, by a number of workers, to be a strain capable of exhibiting oral tolerance (Mowat **et al**, 1982; Mowat & Parrott, 1983; Strobel & Ferguson, 1984; Mowat, 1985) and have been used in the examination of the murine response to orally and parenterally administered choleragen (Remmers **et al**, 1982; Robb, Nichols, Whoriskey & Murphy, 1982; Lindholm **et al**, 1983; Lycke **et al**, 1987) and so were a natural choice for these experiments.

A single feed of either 1 or 10 µg of cholera toxin was able to tolerise the induction of systemic CMI but had no effect on the subsequent induction of systemic antitoxin levels by parenteral immunisation. A smaller feed of choleragen (0.1 µg) failed to tolerise the induction of either limb of the immune response. Several workers have shown that the induction of oral tolerance is dose-dependent and it is usually the higher doses of antigen which successfully induce tolerance (Asherson, Zembala, Thomas & Perera, 1980; Mowat, Thomas, MacKenzie & Parrott, 1986). The inability of

choleragen to induce oral tolerance for specific antitoxin responses has been reported independently by other workers (Svennerholm **et al**, 1980; Elson & Ealding, 1984b; Lycke & Holmgren, 1986a). Indeed, earlier studies have shown that feeding cholera toxin can prime for both local and systemic antitoxin responses (Lange & Holmgren, 1978; Pierce, 1978; Svennerholm **et al**, 1980; Lange **et al**, 1984a; Elson & Ealding, 1984a). Unfortunately, pre-parenteral immunisation bleeds were not performed routinely on the above animals, but the IgG antitoxin levels obtained 7 days after parenteral immunisation were consistently and statistically significantly higher in those animals which had been fed 10 µg choleragen (chapter 5). By day 22 of the experiment there was no difference in the antitoxin levels in any of the groups. This is consistent with the findings of Lange & Holmgren (1978) who observed that antitoxin levels were similar in animals who had received between 2 and 5 boosters with toxin and that further significant elevation in specific antibody levels required additional exposure to antigen.

Experiments using other proteins have shown that different immune effector mechanisms have differing susceptibilities to the induction of oral tolerance. Mowat and his co-workers have shown that feeding 5 mg OVA tolerised the induction of both specific DTH and IgM antibody levels whereas higher doses of fed OVA (25 mg) were required to additionally tolerise specific IgG antibody levels (Mowat **et al**, 1982). Furthermore, studies in which mice were pretreated with cyclophosphamide revealed control mechanisms with different sensitivities to this drug (Mowat **et al**, 1982). It has been

inferred from these results that each effector limb of systemic immunity is under the control of separate mechanisms. Several workers have suggested that such regulation is accomplished by T cells and have been able to demonstrate B cell priming in animals who had been tolerised by the oral administration of OVA (Titus & Chiller, 1981a; Mowat **et al,** 1986). Experiments in which human gammaglobulin has been fed has revealed that B cell anergy may also occur (Vives **et al,** 1980).

A possible explanation for this clearly divergent response to the oral administration of choleragen may lie in its 'toxic' biochemical properties. Choleragen has been shown to alter the activity of lymphocytes **in vitro** (Lichtenstein **et al,** 1973; Holmgren **et al,** 1974; Kateley **et al,** 1975; Lindholm **et al,** 1976; Fuse **et al,** 1981; Fuyama **et al,** 1982) and both suppress (Northrup & Fauci, 1972; Yardley **et al,** 1978; Hamilton **et al,** 1979; Pierce & Koster, 1980; Koster & Pierce, 1983; Fuse **et al,** 1981; Goldfarb & Herberman, 1981; Fuyama **et al,** 1981; Fuyama **et al,** 1982) and enhance (Lindholm **et al,** 1976; Tsuru **et al,** 1981; Tsuru **et al,** 1983; Lycke & Holmgren, 1986) immune responses to related and unrelated antigens **in vivo.** It may be that the suppressor cells which are induced by feeding protein antigens and have been shown to control both antibody levels and plaque-forming cell responses (Hanson **et al,** 1977; Ngan & Kind, 1978; Richman **et al,** 1978; Challacombe & Tomasi, 1980; Titus & Chiller, 1981; Cowdery & Johlin, 1984) are more sensitive to the actions of choleragen than those which regulate the induction of specific DTH.

In order to examine the relevance of the biochemical properties of choleragen, experiments were performed whereby cholera toxoid was fed which lacked both the ability to bind to gangliosides and activate adenylate cyclase. Feeding a dose of toxoid with an equivalent B subunit content to that of the fed choleragen had similar effects on oral tolerance. The two highest doses of toxoid (5 and 50 µg) suppressed the induction of DTH but not specific antibody responses and the lowest dose fed (0.5 µg) suppresed neither. It would seem therefore that the inability to induce tolerance for antibody is an inherent antigenic property of these preparations. However the same may not be true for the ability of these preparations to stimulate antibody when administered enterically.

These two preparations differ in their ability to stimulate both local and systemic specific humoral responses when fed. The holotoxin stimulates several orders of magnitude more antitoxin-containing cells and it has been suggested that this difference is a reflection of the 'toxic' actions of choleragen (Pierce, 1978). Some evidence for this has been accrued by feeding other proteins simultaneously with choleragen. When cholera toxin is fed at the same time as keyhole limpet haemocyanin (KLH), it both abrogates the systemic tolerance for antibody usually observed and induces a local humoral immune response to KLH (Elson & Ealding, 1984b; Lycke & Holmgren, 1986a). There is no difference between the effect of feeding KLH alone or in the presence of purified cholera B subunit on anti-KLH responses (Lycke & Holmgren, 1986a). Feeding horseraddish peroxidase (HRP) does not induce a local or systemic antibody response, but both may be induced if HRP is

covalently linked to choleragenoid (McKensie & Halsey, 1984). Both binding and enzymatic actions of choleragen may therefore play a role in the enteric priming of mucosally-associated B cells. This is further supported by the observation that isolated B subunit is a much more powerful oral immunogen than the formalinised toxoid (Pierce, 1978) but is still inferior to the native holotoxin at inducing local antitoxin-containing cells (Pierce **et al**, 1983). Procholeragenoid, a high molecular weight, heat-aggregated polymer of choleragen which has less than 5% of the residual toxicity of the holotoxin, is equally as good as choleragen and better than isolated B subunit at stimulating local ACC responses (Pierce **et al** 1983). This is in agreement with experiments which demonstrated that small amounts of orally administered choleragen (100 ng) had adjuvant properties. When fed simultaneously with either KLH or isolated B subunit it stimulated enhanced local antibody production to them even though this dose was insufficient to provoke an antibody response to itself (Lycke & Holmgren, 1986a). It is of interest that although choleragen and cholera toxoid differ in their ability to prime for antibody, they are both equally capable of boosting specific humoral immune responses when applied enterically (Pierce, 1978) suggesting that this phenomenon may be mediated by T cells.

11.5 Cellular Suppressor Mechanisms Induced by Feeding Choleragen

A single feed of choleragen has been shown to tolerise systemic DTH. Feeding other protein antigens has also resulted in the suppression of systemic CMI (Miller & Hanson, 1979; Challacombe & Tomasi, 1980; Titus & Chiller, 1981a; Mowat **et al**, 1982) and this has been most frequently associated with the induction of suppressor cells (Miller & Hanson, 1979; Challacombe & Tomasi, 1980; Mowat **et al**, 1982; Mowat, 1986). Some of the evidence for suppressor cell involvement in oral tolerance has indirectly come from the use of pharmacological agents (Mowat **et al**, 1982; Mowat, 1986).

Preliminary experiments (chapter 8) investigated the sensitivity of oral tolerance to pretreatment with cyclophosphamide. Cyclophosphamide (CY) is an alkylating agent used in cancer chemotherapy. It has been shown to reverse the tolerance resultant from suppressor cell activation (Sy, Miller & Claman, 1977; Turk, Parker, Poulter, 1972) and when used within the dose range of 20 to 200 mg/kg has been found to inhibit short-lived suppressor cells affecting DTH responses (Attallah, Ahmed & Sell, 1979; Diamantstein, Klos, Hahn & Kaufmann, 1981). Pretreatment with CY (100 mg/kg) two days before feeding has been found to abrogate the induction of tolerance by OVA (Mowat **et al**, 1982). CY has the potential to damage the intestinal mucosa (Sobhon **et al**, 1977) but studies have shown that this dose of CY has minimal effects on the integrity of the gut mucosa (Ecknauer & Löhrs, 1976; Hartwich, Weisshaar & Domschke, 1978; Mowat, 1981) and experiments which have examined the tolerogenic properties of absorbed antigen have shown it

to be unchanged by this procedure (Mowat, 1981; Strobel **et al**, 1983). Using the protocol described above, I found that cyclophosphamide abrogated the induction of tolerance by choleragen. This suggested that suppressor cells may also be implicated in oral tolerance to choleragen and so a cell transfer protocol was employed to examine this further.

Spleen cells were obtained from donors fed cholera toxin (1 µg) one week previously and were transferred intraperitoneally to syngeneic recipients on the same day as they were parenterally immunised with choleragen in adjuvant. One hundred million viable spleen cells were transferred as this was the average content of an adult BALB/c mouse spleen. The intraperitoneal route of transfer was preferred over the intravenous one as it gave the same results in preliminary experiments but abolished the loss of animals through pulmonary embolism.

Spleen cells from toxin-fed donors were able to suppress the induction of DTH as did cells from animals fed cholera toxoid (5 µg). Splenic suppressor cells capable of inhibiting CMI have also been demonstrated after feeding OVA (Miller & Hanson, 1979), SRBC (Kagnoff, 1978a) and contact sensitising agents (Asherson **et al**, 1980). The similarity between the mechanism controlling DTH after feeding choleragen and other protein antigens was remarkable considering the unique action of the holotoxin on the humoral immune response when administered enterically. Experiments were designed (chapter 9) to investigate the phenotype, migration and mode of action of these cells to see if any further similarities were apparent.

Suppressor cells induced by feeding antigen are usually of T cell lineage (Mattingly & Waksman, 1978; Ngan & Kind, 1978; Richman **et al**, 1978; Miller & Hanson, 1979; Asherson **et al**, 1980; MacDonald, 1982a; MacDonald, 1982b; Silverman, Peri, Fitch & Rothberg, 1983; Mowat **et al**, 1986). However, suppressor B cells have also been found after the enteric administration of contact sensitising agents (Asherson **et al**, 1977; Asherson, Perera, Thomas & Zembala, 1979). Treatment of spleen cells from choleragen-fed donors with anti-THY-1.2 monoclonal antibody and complement abrogated their ability to inhibit the induction of DTH confirming that these cells were also T lymphocytes.

Suppressor cells have been found to migrate from the PP to MLN and then to the spleen after feeding OVA (Ngan & Kind, 1978; Richman **et al**, 1981). A similar pattern of migration was found after feeding SRBC but the thymus was colonised in addition to the spleen (Mattingly & Waksman, 1978). Experiments were designed to study this phenomenon in choleragen-fed animals and migration was followed using functional characteristics; in other words, the capacity of different lymphoid tissues to transfer suppression was examined at various times after feeding.

Suppressor activity was present in both the spleen and MLN one week after feeding. Spleen cells did not transfer suppression when obtained 3 days after feeding but the results for MLN cells at this time were curious. Although the experiment was repeated 3 times, the same result was obtained in that half the toxin-fed MLN recipients had tolerant DTH

responses (<50% of control respnses) and half remained unsuppressed by the cell transfer. These results occurred despite pooling and mixing donor cells. Suppressed and unsuppressed responses did not occur sequentially (suggesting that mixing was adequate) and transferred cells were always greater than 95% viable. These cells were transferred intraperitoneally for the reasons given before and this unusual but repeatable result may reflect a defect in their capacity to migrate at this stage of their ontogeny. The experiment would need to be repeated using the intravenous route of transfer but there are several examples of effective T cells which fail to function until experimentally located at the site of antigen challenge (Weiss & Dennert, 1981; Lin & Askonas, 1981; Kaufmann & Hahn, 1982; Minami **et al,** 1982; Van Loveren **et al,** 1984). It may also be that 3 days is not the most appropriate time after feeding to examine the MLN for the presence of suppressor cells. No conclusive evidence for a migration of cells regulating DTH has been accrued from these experiments and further timepoints and other lymphoid cells (PP and thoracic duct lymph) need to be examined. However, antitoxin-containing and regulatory cells have been shown to disseminate throughout the MALT in toxin-fed rats (Pierce & Gowans, 1975; Pierce & Cray Jr, 1981) and helper T cells for IgA and IgG have been found in the PP, MLN and spleen of mice fed choleragen (Elson & Ealding, 1984a).

Suppressor cells have been demonstrated which work either on the afferent or efferent limbs of the effector T cell response (Zembala & Asherson, 1973b; Asherson & Zembala, 1974; Asherson **et al,** 1977; Kagnoff, 1978a; Miller, Sy & Claman, 1978a; Miller, Sy & Claman, 1978b;

Miller & Hanson, 1979; Perera & Asherson, 1981; Battisto, Beckman & Yen-Lieberman, 1985). In additon to their functional characteristics, these suppressor cells have other distinct properties which allow their separation. Suppressor cells (Ts aff) working on the afferent limb of the T cell response appear to be sensitive to CY and resistant to adult thymectomy (Asherson **et al**, 1980; Thomas, Watkins & Asherson, 1981) which is the opposite to that found for suppressors (Ts eff) of T effector cells (Asherson **et al**, 1980). Although a Ts eff has been found which is sensitve to CY (Gill & Liew, 1978), this may reflect the sensitivity of a second auxilliary T cell which is necessary for the normal operation of the first (Sy, Miller, Moorhead & Claman, 1979; Asherson **et al**, 1980). It might be supposed, from the results of cyclophosphamide pretreatment, that the choleragen-induced suppressor cells may work by inhibiting the afferent limb of DTH and experiments were designed to examine this.

Spleen cells, obtained seven days after feeding, were transferred to recipients either on the same day as or 3 or 6 days after immunisation. If the suppressor cells inhibited the mature DTH effector T cell it would be expected that similar suppression would be observed at all of these timepoints. However, if the orally-primed lymphocytes were only effective at preventing the induction of DTH effector cells, it would be expected that the observed suppression would decline the later the transfer was performed after immunisation. The results of this experiment showed that the suppression declined the longer after immunisation the cells were transferred. Significant suppression occurred when toxin- or toxoid-primed cells were transferred on the

same day as or 3 days after immunisation. There was no significant suppression when either cell was injected after 6 days.

In order to confirm these findings, mice which had been fed either antigen or ABS seven days previously were given a footpad inoculate of mature toxin-primed DTH effector cells and toxoid. There were no differences in the DTH responses observed in animals which were sham fed or which received enteric choleragen or toxoid. This would appear to confirm the action of these orally-induced suppressor cells on the afferent limb of DTH. Such suppressor cells have been found in the spleens of animals fed either contact sensitising agents, SRBC or OVA (Asherson **et al**, 1977; Kagnoff, 1978a; Miller & Hanson, 1979). It is interesting to note that recent experiments have revealed that animals which are fed OVA after being parenterally immunised also have suppressed DTH responses. In this model spleen cells obtained from these animals were also ineffective at transferring tolerance (Lamont, Bruce, Watret & Ferguson, 1987). It would therefore appear that several mechanisms are active in the control of systemic DTH.

11.6 Serum Tolerogen after Feeding Cholera Toxin

The work in this thesis has highlighted the divergence in the mucosal regulation of the two effector limbs of the systemic immune response to choleragen. Not only does feeding not induce oral tolerance for antibody but B cell priming (Lange & Holmgren, 1978; Pierce, 1978; Svennerholm **et al**, 1980; Lycke & Holmgren, 1986a; Lycke & Holmgren,

1986b) and antigen-specific IgG and IgA helper T cell activity have been demonstrated (Elson & Ealding, 1984a). On the other hand, experiments described earlier have shown that oral tolerance for systemic DTH may be induced and is associated with the appearance of CY-sensitive T suppressor cells. While some of these differences may reflect the particular sensitivities of immune regulatory cells to the 'toxic' actions of this protein antigen (Pierce, 1978; Elson & Ealding, 1984b; Lycke & Holmgren, 1986a), some may also be explained by a differential response of these cells to 'intestinally processed' antigen.

Intestinal processing is the term used to describe the alterations that occur in antigenicity of foodstuffs consequent on their passage through the gastrointestinal tract. One approach to the study of gut-processed antigen has been its collection in the serum of fed animals within a short time of feeding and its transfer to recipients whose functional responses are then studied (Strobel **et al,** 1983).

Other facets of oral tolerance have also been studied using serum transfer protocols. However, serum transfer studies have been uniformly unsuccessful in tolerising systemic antibody when collected between 1 hour or 14 days after feeding (Kagnoff, 1978b; Hanson **et al,** 1979; Strobel at al, 1983; Bruce & Ferguson, 1986a). Tolerance for DTH, on the other hand, has been successfully transferred by serum from mice given a single feed of OVA (Strobel **et al,** 1983; Bruce & Ferguson, 1986a).

Using the protocol devised in this laboratory by these workers, the effect of gut processing on choleragen was studied. Serum was collected and pooled one hour after feeding 1 µg choleragen to BALB/c mice and was transferred to syngeneic recipients who were parenterally immunised one week later. The development of specific antitoxin antibody and DTH responses were measured as before. Eight hundred microlitres of serum were transferred as this was felt to be representative of the circulating serum volume of an adult mouse. Serum obtained as above was capable of inducing specific suppression of DTH but had no effect on systemic antibody. This is in agreement with results obtained after feeding OVA (Strobel **et al**, 1983; Bruce & Ferguson, 1986a).

Experiments in which tolerance has been transferred by serum collected from mice after several feeds of particulate antigen has shown that the serum contains either antibodies (Kagnoff, 1978b) which were thought to be anti-idiotypic in specificity (Kagnoff, 1980) or immune complexes (André **et al**, 1975). Serum in the above experiment was not examined for specific antibody or immune complexes for several reasons. No specific antibodies were detected in serum obtained one hour after feeding OVA (Bruce & Ferguson, 1986a) or one week after its transfer to recipients (Strobel **et al**, 1983). Furthermore it was felt extremely unlikely that a naive animal could mount a specific immune response of detectable magnitude to choleragen in one hour. Earlier experiments (chapter 5) in which serum had been examined for the presence of specific antibody one week after feeding 1 µg cholera toxin had failed to detect levels which were significantly different

from sham-fed controls.

The serum fragment obtained after feeding OVA induced tolerance which was sensitive to CY. Treatment of donors did not alter intestinal epithelial cell structure (Ecknauer & Löhrs, 1976; Hartwich **et al**, 1978; Mowat, 1981) or affect their ability either to take up antigen or generate serum tolerogen (Mowat, 1981; Strobel **et al**, 1983) but treatment of recipients abrogated the induction of oral tolerance by serum transfer (Strobel **et al**, 1983; Bruce & Ferguson, 1986a). Pretreatment of recipients with cyclophosphamide (100 mg/kg) also abrogated the induction of tolerance with serum from choleragen-fed donors (chapter 10).

The inference from work with cyclophosphamide and OVA was that the serum tolerogen induced a distinct class of suppressor cell which did not act on antibody responses (Bruce, 1985) and this appeared to be substantiated by the report of the induction of DTH suppressor cells after feeding OVA (Miller & Hanson, 1979) and the description of a mouse suppressor cell for DTH which did not affect plaque-forming cell responses (Whisler & Strobo, 1978). It might be expected from the foregoing account that the suppressor T cell induced by feeding choleragen (chapters 8 & 9) results from activation by the serum tolerogen above. In order to test this, serum obtained from toxin-fed mice was pooled and transferred to recipient animals. The recipients were left for 7 days and then half were immunised and challenged as before and half were sacrificed. Spleen cells were obtained from the sacrificed serum recipients and transferred intraperitoneally to

another set of syngeneic mice who were parenterally immunised at the same time. Once immunised, these cell recipients were treated identically to the surviving serum tolerogen recipients. In this way the quality of the serum tolerogen could be assured while testing its ability to induce suppressor cells. These experimental mice were accompanied by the appropriate sham-fed controls (chapter 10). Results showed that animals who received spleen cells from serum recipients of toxin-fed donors were not tolerised, yet the toxin-fed serum was able to tolerise recipients. This suggests that the splenic suppressor cell described earlier is not induced by the serum tolerogen obtained after feeding cholera toxin although further work is required to confirm this.

The apparent dissociation between the presence of serum tolerogen and the induction of splenic suppressor T cells does not rule out the possibility that the post-prandial serum fragment does induce a suppressor cell. Certainly the ability of CY to prevent the transfer of tolerance to recipients would be an argument in its favour. Other sites of migration for orally-induced suppressor cells such as PP, MLN or thymus (Mattingly & Waksman, 1978; Ngan & Kind, 1978; Richman et al, 1981; MacDonald, 1982a, MacDonald 1982b) have not yet been examined and the possibilty of a circulating suppressor cell which migrates to the site of antigenic challenge has not been excluded. Indeed, both resident and circulating lymphocytes which act together in a synergistic fashion to produce antitoxin have been described after the oral administration of choleragen. It was postulated that the B cells disseminate from the site of initial exposure to more distant

regions of the MALT and then become resident. The circulating lymphocytes, thought most likely to be T cells, migrate continuously until recruited to the site of antigen challenge (Pierce & Gowans, 1975; Pierce & Cray Jr, 1981).

Further characterisation of the serum tolerogen in OVA-fed mice revealed that the fragment was in the molecular weight range of native OVA and had B cell determinants in common with the native protein (Bruce & Ferguson, 1986b). This serum fragment did not appear to be either deaggregated, denatured or native as injections of all three of these preparations in the quantity found after feeding did not reproduce the effects of the gut processed antigen (Bruce & Ferguson, 1986a). It has been postulated that the 'processed' OVA may be able to stimulate Ts directly as earlier work has shown that both B lymphocytes and T suppressor cells respond preferentially to tertiary antigenic structures (Feldmann & Kontiainen, 1976; Endres & Grey, 1980a; Endres & Grey, 1980b; Chesnut, Endres & Grey, 1980; Chesnut & Grey, 1985). Helper T cells, however, have been shown to respond to short lengths of protein primary structure presented in the context of class II MHC antigens (Ishizaka, Okudaira & King, 1975; Takatsu & Ishizaka, 1975; Chesnut et al, 1980; Unanue, Beller, Lu & Allen, 1984). Experiments have shown that procedures which increase macrophage function are associated with the abrogation of oral tolerance and this has been taken as additional support for the above hypothesis (Mowat & Parrott, 1983; Strobel et al, 1985; Strobel & Ferguson, 1986).

Superficially, the experimental findings of this thesis would appear to be in conflict with the above hypothesis. The two forms of the toxin antigen (holotoxin and formalinised toxoid) do not appear to be equally efficient at priming for antibody responses. Anti-toxoid and antitoxin ELISA results suggest that this may be due to partial cross-reactivity between these two antigenic forms. However, these two proteins are equally good at boosting specific IgG antibody responses, shown to be mediated by antigen-specific T helper cells (Okumura **et al,** 1975; Schrader **et al,** 1976), and are equally good at stimulating DTH responses. It would not be unreasonable to suggest from the foregoing account that these two antigenic forms have an identical primary structure and are therefore totally cross-reactive at the T helper/DTH effector T cell level. Indeed work using cells generated from single clones has suggested that for the murine immune response they may be one and the same thing (Bianchi **et al,** 1981; Milon **et al,** 1983). It would appear from the results on specific antibody responses that choleragen and the toxoid have different tertiary structures. It might also be expected, that if orally-induced suppressor T cells recognise tertiary structures of protein antigens, these two antigenic forms would not be equally efficient at inducing oral tolerance and that the tolerance so induced would not be immunologically cross-reactive. In fact, the reverse has been the case in that both choleragen and toxoid are equally capable of tolerising systemic DTH induced by either protein. While it is clear that formalinisation effects a reductive alkylation of tyrosine and lysine residues and alters the antigenicity of proteins for B cells (Habeeb, 1969; Pancake & Nathenson, 1973; Peterson, 1979) it does not necessarily alter tertiary structures

recognised by T cells. Indeed, Hua and his colleagues demonstrated that formaldehyde treatment of cytotoxic target cells inhibited the binding of H-2-specific monoclonal antibodies but did not affect the ability of H-2-specific cytotoxic T cells to lyse them (Hua et al, 1985). Work with mutant targets has shown that the cytotoxic clones used above respond to conformation-dependent determinants involving contributions from both the alpha 1 and alpha 2 domains of $H\text{-}2K^{b}$ (Sherman, 1982) and this suggests that formaldehyde treatment may only selectively destroy B cell determinants leaving T cell conformational determinants, such as seen by suppressor T cells, relatively intact.

Other studies using native and denatured OVA have shown that these two forms of the antigen produced different effects when administered intravenously but produced totally cross-reactive tolerance when fed (Mowat, 1985). In addition to highlighting the difference between 'classical' and oral tolerance this also would appear to suggest that tertiary antigenic structure may not be of importance in the induction of mucosally-associated suppressor mechanisms. Recent evidence suggests that accessory cells may be important in the induction of suppressor cells (Usui, Aoki, Sunshine & Dorf, 1984) and workers have demonstrated OVA-pulsed macrophages that induce suppressor cells which are reactive to both native and denatured forms of the antigen (Takatsu & Ishizaka, 1977). Interestingly, feeding native OVA was associated with the induction of splenic suppressor cells which induced tolerance to either antigenic form. The denatured protein also induced tolerance to both forms of antigen but was not associated with the induction of

splenic suppressor cells (Mowat, 1985). This would appear to be yet further evidence to suggest that several systems control the induction of systemic DTH.

The study of the nature of gut-processed antigen is thus of fundamental importence in the understanding of oral tolerance. There are several approaches to this problem, some authors have examined the biochemical properties of fed antigen and tried to modify native proteins to reproduce the effect phenomenologically (Mowat, 1985; Bruce & Ferguson, 1986a; Bruce & Ferguson, 1986b). Other workers have characterised suppressor and helper epitopes on proteins with a known sequence and have immunised mice with these epitopes in order to uncover the hierarchy in which they are recognised in responder and non-responder strains (Oki & Sercarz, 1985; Krzych, Fowler & Sercarz, 1985). This approach has not yet been used in the study of oral tolerance but it appears to be a promising technique.

I am currently pursuing a different course by raising a series of monoclonal antibodies to choleragen and examining the presence or absence of antigenic epitopes on fed or native toxin. Furthermore, by raising anti-iditoypic antibodies to promising epitopes one may able to examine the precise nature of this process more fully.

11.7 Areas for Further Investigation

This thesis has highlighted two further areas of cholera immunity that warrant further investigation.

This thesis has been the first to demonstrate choleragen's ability to stimulate cell-mediated immunity. While the presence of systemic CMI is of great interest in examining the processes of oral tolerance, it would be more appropriate, in view of the nature of this disease, to examine the state of cellular immunity within the intestinal mucosa. Now that several techniques exist to measure systemic CMI, it should be possible to employ these and other established morphometric and histological analyses of mucosal parameters (Ferguson & Parrott, 1973; MacDonald & Ferguson, 1977; Marsh, 1980; Marsh, 1981; Mowat & Ferguson, 1982c) to study the presence of mucosal CMI in choleragen-fed animals. Furthermore, studies have shown that other mechanisms not related to specific antibody production may be responsible for protection against toxin-induced diarrhoea (Pierce **et al**, 1978; Pierce **et al**, 1983; Lange **et al**, 1984a; Lange **et al**, 1984b; Lönnroth **et al**, 1984). Workers have attributed this protection to the presence of a hormone-like factor which has been isolated from brain, pituitary and intestinal tissue (Lönnroth & Lange, 1984; Lange & Lönnroth, 1984; Lange & Lönnroth, 1986) unfortunately they have not been able to demonstrate its presence in the circulation or explain the mechanism by which it exhibits antigen specificity (Lange **et al**, 1984b; Lönnroth **et al**, 1984). There is evidence to suggest that local CMI may be induced in animals who are chronic carriers of the

cholera vibrio (Gorskaya **et al,** 1980; Efremov **et al,** 1982). In addition, the protection afforded by multiple feeds of choleragen is antigen-specific and is associated with the presence of various T-cell dependent phenomena (Lange **et al,** 1984b; Lönnroth **et al,** 1984). CMI reactions occurring within the mucosa have been shown to alter a number of physiological processes within the gastrointestinal tract (Ferguson & Parrott, 1973; MacDonald & Ferguson, 1977; Castro, 1982; Mowat & Ferguson, 1982c; Ferguson, 1987). Therefore a project is underway to study the ability of orally administered choleragen to induce local CMI and whether specific mucosal CMI is involved in the downregulation of the enterocyte's sensitivity to choleragen-induced adenylate cyclase activation.

There are many circumstances in which it would be of benefit to investigate the primary mucosal immune response to neoantigens in humans. At present it is difficult to accrue direct evidence of abnormalities of immune function within the gastrointestinal tract. Indirect evidence is normally obtained from morphometric, histological or immunohistochemical analyses of biopsy specimens, analysis of circulating antibody levels and specificites or skin test responses.

It would be preferrable to study gut immune function directly and one approach would be the use of test antigens. These materials could then be fed and the resulting immune responses could be measured in terms of specifically reactive cells and/or by the presence of products of a specific immune response. In order to be a successful candidate as a test antigen a substance would have to be immunogenic, non-toxic and

whose specifically-induced reactive cells or immune products could be easily measured. The B subunit of choleragen is such an antigen and this has been the rationale for characterising the mucosal regulation of the systemic immune response to the holotoxin in the first place. If a model for the effect of feeding choleragen can be established and cells and other products, which have been stimulated enterically, can be detected peripherally then the basis exists for similar critical analysis of gastrointestinal immune function in man. Many of these criteria have been satisfied for choleragenoid in that both local and systemic antibodies directed against the B subunit have been detected after feeding (Svennerholm, Sack, Holmgren & Bardhan, 1982; Svennerholm, Gothefors, Sack, Bardhan & Holmgren, 1984) and circulating lymphocytes capable of producing IgA antitoxin after short term culture have been isolated from the peripheral bloodstream after enteric administration of antigen (Lycke **et al**, 1985). These results have been obtained from both humans and mice illustrating how closely, to date, the murine model has resembled the immune responses observed in the human. Experiments performed in this thesis suggest that cells controlling systemic CMI disseminate from the intestine after feeding choleragen, so the possibility exists that either regulatory or effector cells of CMI or both may also be found in peripheral blood of subjects fed choleragen. Further work on the migration of regulatory cells from GALT is therefore needed. There is the possibilty that, unlike for humoral immunity, different cellular immune responses may occur locally from those observed systemically after feeding choleragen. It would therefore be necessary to examine whether this divergence exists and to see to what extent the reactions of peripheral

blood lymphocytes reflect the immune response at different anatomical locations throughout the immune system.

11.8 Conclusion

This work represents a comprehensive investigation of the systemic immune effector response to parenterally administered cholera toxin and one of its detoxified derivatives. It has demonstrated that choleragen is able to modulate the induction of specific systemic immunity when prefed. While feeding this antigen induces systemic antibodies, it also tolerises the induction of DTH. Although atypical in many respects, choleragen would appear to behave like other, more classical, enteric protein antigens with respect to the induction of oral tolerance for systemic DTH.

These results have important implications. It is now clear that mucosal immune effector mechanisms, other than antibodies, must be considered as candidates for the protective process which prevents cholereic diarrhoea. The totally divergent effect of this antigen on the regulation of systemic immunity now allows us to carefully dissect both the physiology and anatomical location of antigen presentation within the gastrointestinal tract. It is apparent from other studies that rodents and humans respond with remarkable similarity to choleragen and its associated antigens, therefore finally, and perhaps most importantly, this project has laid the foundation for the specific analysis of oral tolerance and mucosal immunity in humans without the

need for invasive procedures.

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