THE REGULATION OF NECROSIS IN MYCOBACTERIAL LESIONS

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ABSTRACT

Tuberculosis is characterised by necrotic immunopathology in the lesions and tuberculin skin-test sites (Koch phenomenon), and persistence of the organisms. Previous work from this laboratory suggested that immunotherapy with killed *M. vaccae* can reduce this tissue damage, and that TNF plays a role in its pathogenesis. The purpose of this thesis was to study the mechanism of action of *M. vaccae* in man and in a rodent model.

Sera from patients in Kuwait who received *M.vaccae* or saline were screened by immunoblotting and laser scanning to seek changes in the pattern of mycobacterial antigens recognised, and by an immunoassay for changes in levels of agalactosyl IgG (a correlate of T cell-dependent immunopathology). A fall in agalactosyl IgG and a selective increase in antibody to a 30kDa antigen was noted. This antigen was identified as the fibronectin-binding secreted protein, and was purified to homogeneity from culture filtrate by column chromatography. The increased antibody binding was confirmed by ELISA.

A murine model of TNF-induced tissue damage in sites of T cell-mediated inflammation was then devised, to mimic aspects of the Koch phenomenon using the 30 KDa and the 65 KDa heat shock protein (hsp). This model allows simultaneous assessment of both swelling and haemorrhage caused by injection of TNF into delayed hypersensitivity (DTH) sites. It was found that some DTH responses evoked by mycobacterial antigens are as sensitive to subsequently injected TNF as are sites prepared by injection of LPS (local Shwartzman reaction) though for mycobacterial antigens CD8+ T cells

are required. This phenomenon also involved CR3+ cells, phagocytic cells, complement, and perhaps IL-6, and could be diminished by PAF inhibitors and a prostaglandin analogue. However, by testing different immunisation schedules using *M. vaccae*, it was discovered that it is also possible to evoke DTH responses in which subsequent injection of TNF has no effect at all. The heat shock proteins may play a role in this effect. The implications of the existence of two qualitatively different types of DTH response and the consequences of neutralizing TNF, using an anti-murine TNF antibody, in a murine tuberculosis model are discussed in relation to the immunopathology of tuberculosis and autoimmune diseases such as rheumatoid arthritis where mycobacterial antigens, cytokines and T cell dependent inflammation are important.

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LIST OF ABBREVIATIONS

ABTS	2,2`-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
BCG	Bacillus-Calmette Guerin
BCG85	Mycobacterial 31-29 kDa complex antigen
BSA	Bovine Serum Albumin
DTH	Delayed Type Hypersensitivity
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Fetal Calf Serum
FIA	Freund's Incomplete Adjuvant
FRIE	Fused Rocket Immunoelectrophoresis
Hb	Haemoglobin
H & E	Hematoxylin and Eosin
HLA	Human Leukocyte Antigen
HN	Haemorrhagic Necrosis
HRP	Horse Radish Peroxidase
IFN-gamma	Interferon-gamma
IL-1	Interleukin-1
IL-6	Interleukin-6
i.p.	Intraperitoneally
i.v.	Intravenously
LAM	Lipoarabinomannan
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
LSR	Local Shwartzman Reaction
mAb	Monoclonal Antibody

M	Murabutide
mRNA	messanger Ribonucleic Acid
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide
PAF	Platelet Activating Factor
PBS	Phosphate Buffered Saline
PG	Prostaglandins
PPD	Purified Protein Derivative
rpm	Revolutions per minute
s.c.	Subcutaneously
SDS-PAGE	Sodium-dodecyl Sulfate Polyacrylamide Gel Electrophoresis
spp	Species
T	New Tuberculin
Tb	Tuberculosis
TNF	Tumor Necrosis Factor

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1. GENERAL INTRODUCTION

1.1. THE THREAT OF TUBERCULOSIS INFECTION

Although tuberculosis is considered to be a severe health problem in the developing world, the high incidence of AIDS (Acquired Immunodefeciency Syndrome) has made the mycobacterial infections of an increasing threat in the developed world too. A special study carried out in 1989/1990, by the World Health Organization (Tuberculosis Unit) estimated that 1,700 million people (one third of the world's population) are infected with *Mycobacterium tuberculosis* with 8 million new cases and 2.9 million deaths in 1990 (Kochi, 1991). Moreover, almost 5% of the tuberculosis patients throughout the world are dually infected with AIDS. Thus, AIDS is one of the reasons for the increasing problem of mycobacterial infections and there is an urgent need for specific and highly sensitive diagnostic tests and a reliable treatment for both tuberculosis and AIDS.

1.1.1. BCG vaccination and the various possible explanations for its low efficacy in some parts of the world :

Another major reason for the increasing threat of tuberculosis is the failure of BCG vaccination in some parts of the world (Stanford *et al*, 1981). BCG trials showed that 78% protection was afforded to the population of the United Kingdom. However, only 14% and 0% of the population of Alabama and Georgia and south India were

protected, respectively. There are several possible explanations for the failure of BCG to protect against tuberculosis in South India and Georgia and Alabama (Grange, 1985). These include the variation of the immunogenicity of the vaccine strains, differences in the host's genetic background, differences in the virulence of *M.tuberculosis* strains in different parts of the world or the previous immunisation with environmental mycobacteria. In this context, we will only discuss the last theory.

1.1.2. The existence of two patterns of cell-mediated immune responses in mycobacterial infections :

Based on both human and animal studies, it was postulated that there are two patterns of cell mediated responses to mycobacteria (Stanford *et al*, 1981). One of these responses is a necrotic, tissue-damaging nonprotective response known as the Koch phenomenon. It was given this name because Koch was the first to describe such a reaction. He observed, in the 1890's, that an intradermal injection of live or killed mycobacteria or even the culture supernatant of these bacteria into tuberculous guinea pigs, 4-6 weeks after infection results in swelling, necrosis and sloughing of the challenged area. Therefore, the Koch type of response to the New Tuberculins is a necrotic tender reaction that peaks at 72-96 hours. The other type of response is a protective one and was originally postulated to be similar to the immune response of mice to *Listeria monocytogenes* described by Mackaness. Hence, it was called the listeria-type of response. This type of response to the new tuberculins peaks at 48 hours. It is usually

softer than the Koch type of response and it disappears rapidly without any necrosis. Furthermore, it was suggested that some of the mycobacterial species such as *M.vaccae*, *M.nonchromogenicum* and *M.leprae* induce the listeria (non-necrotising) type of response. However, other species such as *M.tuberculosis* and *M.kansasii* induce either type of response depending on their frequency in the environment. Therefore, the weak continuous exposure of an individual to environmental mycobacteria induces either type of response. BCG vaccination induces the non-necrotising pattern of response. However, in an adult with a pre-existing response to environmental mycobacteria, BCG administration only boosts the already exisiting response. Furthermore, skin test studies carried out in the United Kingdom and East Africa showed that BCG vaccination enhances the non-necrotising of response to both the Old and New Tuberculins. In contrast, in Burma where the Koch type of response to environmental mycobacteria is induced early in life, BCG vaccination affords 60% protection to children presenting a small reaction to PPD (purified protein derivative) whereas those with large positive response to PPD were provided with a minimal protection. Injecting various mycobacterial species into mice provided more evidence for the presence of two qualitatively different responses induced by different species (Rook *et al*, 1981). Interestingly, these responses can also modulate each other in the mouse. It has been shown that the preimmunisation of mice with an organism that induces the non-necrotising type of response blocks the production of the Koch type of response induced by a

subsequent challenge with *M.kansasii*, a Koch type response inducer (Rook *et al*, 1981).

1.1.3. The choice between the immunotherapy and chemotherapy for controlling tuberculosis infection :

The first attempt at immunotherapy was made by Robert Koch (1890) when he challenged, intracutaneously, guinea pigs infected with live *M.tuberculosis* 4-6 weeks earlier, with the same live bacilli (Stanford, 1989; Grange, 1990). This resulted in a local response characterized by swelling, necrosis and ulceration of the challenged skin (the Koch phenomenon) and organisms did not grow at the new challenge site. Therefore, he thought of trying the induction of increased necrosis in human tuberculosis by injecting Tb patients, subcutaneously, with Old Tuberculin. This treatment was useful for skin lesions which underwent necrosis and sloughed off but it was abandoned because it led to the liquifaction and cavity formation in the deeper lesions, in the lungs or vertebrae. The best evidence for the curing effect of Old Tuberculin was provided by a clinical trial in which this preparation of denatured proteins was used for the immunotherapy of chronic skin tuberculosis (*Lupus vulgaris*)(Rook, 1988a). This treatment was also abandoned because it produced the same disastrous side effects that Koch saw in his patients. Immunotherapy trials stopped when streptomycin was discovered in 1947. Multiple drug chemotherapy used, nowadays, for treating tuberculosis infection reduces the number of the acid fast bacilli to a significant level which enables the patient's immune system to eleminate the remaining bacilli. However, the long term

chemotherapeutic regimen used is associated with several problems such as the high cost of the drugs, the non-compliance of patients and the emergence of drug resistence. In addition, there is always the problem of relapses and reactivation of the disease caused by the persisters, dormant or low metabolizing bacilli. This has resulted in renewed interest in the immunotherapy which aims at improving the ability of the patient's immune system to eradicate the persisters. Moreover, the recent attempts of immunotherapy differ from those of Koch because they aim at eliminating the necrotic component of the immune response in Tb patients.

1.1.4. The use of *M.vaccae* for immunotherapy :

Mycobacterial antigens are complex and hence they were classified or described, by different research groups, according to the system used for studying them. The use of immunodiffusion studies facilitated the classification of these antigens into 4 major groups: group i is the common antigens shared by all mycobacterial species (spp.); group ii antigens are shared by all of the slowly growing spp.; group iii antigens are shared by the fast growing spp. while group iv antigens are species specific (Stanford, 1989). The fact that BCG vaccination protects against both tuberculosis and leprosy provided evidence for the association of mycobacterial common antigens with protection since BCG was shown to share only these antigens with *M.leprae* .

Further evidence for the importance of common antigens is provided by the finding that BCG vaccinated healthy subjects respond positively when skin tested with the antigenic preparations

of various mycobacterial spp. (the New Tuberculins) including species they are very unlikely to have encountered reflecting their recognition of shared antigens. Conversely, tuberculosis patients lack the ability to respond to these antigens or even to the slow-growers associated antigens.

A new approach to immunotherapy has been introduced recently by Stanford (1989). Killed *M.vaccae* combined with chemotherapy was used for treating tuberculosis patients. *M.vaccae* is a rapidly growing non-pathogenic environmental mycobacterium. The strain used for immunotherapy is R877R (NCTC 11659) and it was originally isolated from the mud on grass roots collected from Uganda. There are several reasons for choosing this organism as an immunotherapeutic agent:

1) It is rich in the common antigens. These never evoke necrotic skin-test (Koch) responses, and patients with Tb or leprosy tend to have negative skin test responses to them implying that they may be important for non-necrotic protective responses. It is now clear that the common antigens include the heat shock proteins and these antigens, discussed in detail later, may play a role in some of the regulatory effect of *M.vaccae* noted in the early work.

2) It lacks the species specific ones which evoke the necrotic tissue damaging reactions (the Koch type).

3) It maintains its immunogenicity after autoclaving it.

4) Rook *et al* (1981) showed that the immunisation of mice with an inducer of non-necrotising responses such as *M.vaccae* blocked the subsequent induction of the Koch type of response by *M.kansasii*. Therefore, it was theoretically possible that the non-protective necrotizing response (Koch response) seen in tuberculosis patients could be blocked and converted into a protective non-necrotizing response by immunotherapy with a mycobacterium that is rich in the common (protective) antigens.

5) The addition of soluble antigens of fast growers to those of the slow growing spp. for use in skin testing demonstrated the ability of fast growers to suppress the response to the slow growers injected with them locally or even those injected at a distant site, in the other arm (Nye *et al*, 1983 & 1986). One of the fast growers tested was *M.vaccae* and these results may implicate that it contains immunoregulatory antigens.

6) Another experimental evidence was provided when mice were fed with live *M.vaccae* in their drinking water and then given a human dose of BCG Glaxo or given irradiated *M.vaccae* simultaneously with the BCG Glaxo challenge (Stanford *et al*, 1978). Compared with mice treated only with BCG, the test animals presented an enhanced rapidly appearing response to tuberculin injected in their foot-pads. This could parallel environmental contact in human populations where protection is thought to be derived from this source.

7) The inclusion of killed *M.vaccae* with the BCG Glaxo vaccine given to children in leprosy endemic areas in Iran improved their recognition of leprosin A (Stanford *et al*, 1987a).

1.1.5. Previous preliminary studies conducted using *M.vaccae* :

Irradiated *M.vaccae* bacilli (10^9) were injected intradermally into healthy volunteers (Torres *et al*, 1987). Compared to the saline treated group, this resulted in a local scar, a slight increase in their antibody response to various new tuberculin preparations and a significant increase in their tuberculin test reaction.

An immunotherapy study was then carried out in Spain where bacteriologically negative, fully treated lepromatous leprosy patients were given, i.d., either saline or various doses of irradiated *M.vaccae* (Stanford *et al*, 1987b). Forty percent of the patients who received the immunotherapy at a dosage of 10^9 bacilli showed an enhanced response to leprosin A. Other than the slight fever and malaise seen in few patients for few days, the only side effect was the formation of a scar at the site of the injection.

A small study was also carried out, at the Middlesex hospital (U.K.) on 10 tuberculosis patients treated with multiple drug chemotherapy for 3 months (Stanford, 1989). These patients were given a dose of 10^9 of *M.vaccae* . Skin testing of these patients showed qualitative changes in their reactions to New Tuberculins. The immunotherapy induced the conversion of their necrotic large (Koch type) respone into a smaller non-necrotic one.

1.2. THE HOMOLOGY BETWEEN THE KOCH PHENOMENON AND THE SHWARTZMAN REACTION

1.2.1. The Koch phenomenon in man :

A necrotic delayed type hypersensitivity reaction, similar to the previously described Koch phenomenon, is induced in past or present tuberculosis patients when tested with PPD or a sonicated preparation of *Mycobacterium tuberculosis* (New Tuberculin). Necrosis is also seen in tuberculous lesions. Such tissue damage can not be attributed to the toxicity of the antigens used because the same preparations produce a non-necrotic induration when BCG-vaccinated healthy subjects are tested. Moreover, a preparation of sonicated *M.leprae* does induce a necrotic response in tuberculosis patients but not in the tuberculoid leprosy ones in spite of their possession of an intact cell mediated immunity. Therefore, it is of considerable interest to investigate the possibility of eliminating the necrotic component of the reaction in tuberculosis patients. Recently, it has been suggested that both the Koch phenomenon and the Shwartzman reaction are similar with respect to the pathological picture, particularly tissue damage and necrosis observed (Rook, 1990).

1.2.2. The Shwartzman reaction :

The Shwartzman reaction was first described by Shwartzman himself in 1928, as a haemorraghic necrosis of the skin of rabbits, at sites injected intracutaneously with *Salmonella typhi* culture filtrate followed 24 hours later by a second intravenous injection of the same preparation (Mori, 1981). A few years before Shwartzman's

finding, Sanarelli had described a state of systemic reaction characterised by dissiminated intravascular coagulation in several vital organs followed by death of the challenged rabbits. This reaction was produced by injecting, intravenously, a sublethal dose of *Vibrio cholerae* followed 24 hours later by another intravenous injection of *Escherichia coli* culture filtrate. This phenomenon is now known as the generalised Shwartzman reaction. Lipopolysaccharide (LPS) is known now to be the active component in the injected gram-negative bacilli culture filtrate. Rothstein & Schreiber (1988) have recently shown that a recombinant preparation of TNF injected locally in murine skin that was treated 24 hours previously with LPS or *Corynebacterium parvum* results in haemorraghic necrosis in the challenged skin. Therefore, this confirms that the second intravenous injection of bacterial organisms or LPS is mainly associated with TNF production in the Shwartzman reaction .

1.2.3. Experimental evidence for the homology between the Koch phenomenon and the Shwartzman reaction :

Previous reports have indicated, using different experimental models, that mycobacteria can prepare animals for a Shwartzman reaction. Systemic infection of mice with *M.bovis* BCG, that is known to induce T-cell dependent granulomas in the liver, renders them hyperreactive to the lethal effects of endotoxin (Shands & Senterfitt, 1972). When these mice were challenged with a small dose of LPS, that is non toxic to normal animals, two weeks after the infection, acute hepatic damage was demonstrated by morphological and histochemical studies. Furthermore, similar findings were seen in Guinea pigs injected in their foot-pads with heat killed tubercle

bacilli emulsified in Freund's adjuvant (Nagao & Tanaka, 1985). Muramyldipeptide (MDP, the smallest unit of bacterial cell wall peptidoglycan that possesses the adjuvant activity of the cell wall) given intracutaneously at the flank to these animals 3-5 weeks after the sensitisation induced severe necrosis in the foot-pad where the tubercle bacilli were injected. Some of the challenged animals even died of generalised shock. In addition, the immunotherapy treatment (Old Tuberculin) that Koch used also resulted in necrosis in the skin lesions of tuberculous patients.

1.3. THE PRODUCTION OF SHWARTZMAN REACTION

The first injection in the Shwartzman reaction is described as being preparative for the site while the second injection is known to be provocative for the reaction. Despite all the work done since the phenomenon was first described, we do not yet know, quantitatively or qualitatively, the nature of the changes induced by these two injections. The substances required for preparing or provoking the reaction do not need to be the same. The induction of various functional changes in the endothelial cells by cytokines (e.g. TNF) were suggested to play a major role in the preparation of a site for a LSR (it will be discussed in detail in a separate section). It is also important to mention that Rothstein and Schreiber's work (1988) demonstrated that TNF can replace the provocative but not the preparatory injection of LPS. Moreover, in a normal rabbit, a preparatory injection of endotoxin given intradermally results in edema and erythema at the challenged site with infiltration of inflammatory cells while provocation is

followed by local haemorrhage and necrosis of tissues with fibrin thrombi seen in the small blood vessels. An interesting observation that has been made by several workers is that both tumors and pregnancy prepare for a Shwartzman reaction and therefore a single injection is enough to elicit the reaction in the prepared site (Mckay, 1962; Mori, 1981). Recently, various investigators have demonstrated the complexity of these reactions reflected, in the involvement of several cellular and humoral mediators leading to an amplified cascade of events. The role of some of these mediators will be discussed in the following sections.

1.4. THE ROLE OF TUMOR NECROSIS FACTOR (TNF)

Haemorrhagic tumor necrosis was first reported by Coley, in the 19th century in cancer patients treated with culture broths of *streptococcus* and *Serratia* (Beutler & Cerami, 1988). However, this therapy was abandoned because of its toxicity. Later, O'malley & Shear (1962) purified the lipopolysaccharide (LPS) from bacterial broths and described it as being responsible for the tumor necrosis. They also showed that a biological factor producing the necrosis was in the serum of LPS-treated animals and therefore could be transferred to tumor bearing mice. Carswell *et al* (1975) documented that serum of BCG-infected mice treated with LPS contained a high concentration of the tumor necrotising biological factor. Since they proved that it was selectively toxic to tumor cells both *in vivo and in vitro*, they named it Tumor Necrosis Factor (TNF). Furthermore, they put forward the suggestion that the endotoxin-induced tumor necrosis is mediated by TNF.

Cerami *et al* , in the 1970's, were investigating cachexia (wasting) in chronic diseases (Beutler & Cerami, 1988). They demonstrated that in the terminal stages of trypanosomal infection of rabbits, these animals lose more than half of their initial body weight despite their low level of parasitemia. Wasting was also accompanied by high levels of plasma triglycerides caused by the systemic suppression of the lipoprotein lipase (LPL) enzyme. Furthermore, endotoxin- treated animals showed low levels of LPL and the suppression of the enzyme could be transferred by a serum factor, given the name Cachectin. Cachectin was also shown, *in vitro*, to inhibit both LPL expression in adipocytes and the production of the enzymes required for de novo triglyceride synthesis. The homology in the amino-terminal sequence and the biological activities proved the identity of TNF and Cachectin (Beutler & Cerami, 1986).

TNF/Cachectin is a 17 KDa (157 amino acids) polypeptide hormone encoded by a gene lying within the major histocompatibility complex in chromosome 17 in the mouse and chromosome 6 in man (Beutler, 1985; Aggarwal, 1985; Spies, 1986). TNF aggregates *in vitro* to form dimers, trimers or pentamers depending on the species and method of isolation, but it is the trimer that is found to be the active form of the protein (Smith *et al*, 1987). Both mouse and human TNF are derived from a propeptide which contains 76 extra amino acids in the human and 79 in the mouse protein, attached to the amino-acid terminus (Pennica *et al*, 1985). Moreover, there is 86% homology

between the murine and the human propeptide and 79% homology in the mature proteins, reflecting a high degree of conservation.

1.4.1. TNF biosynthesis; cellular sources and inducing signals :

Various cell types produce TNF, macrophages being the major known source (Matthews, 1981; Decker *et al*, 1987). In addition to bacterial endotoxin, the previously described potent TNF inducer, MDP, derived from bacterial cell wall peptidoglycan (Noso *et al*, 1988), Lipoteichoic acid of gram positive cocci (Usami *et al*, 1987), mycobacterial lipoarabinomannan (LAM, Moreno *et al*, 1989), the staphylococcal toxin (Jupin *et al*, 1988) and many other bacterial products are also capable of inducing massive levels of TNF. The long list of inducers includes also viruses like Sendai virus (Aderka *et al*, 1986), Influenza virus (Beutler *et al*, 1986) and parasites like Trypanosomes (Beutler & Cerami, 1986) and Malaria (Bate *et al*, 1988). T lymphocytes were also shown to produce TNF in response to the calcium ionophore A23187 in conjunction with Phorbol Myristate Acetate (Cuturi *et al*, 1987), mitogens like concavalin A and phytohaemagglutinin and Interlukin-2 (Nedwin *et al*, 1985). Interferon-gamma, which on its own had no effect, augmented the production by stimulated macrophages and T lymphocytes. Recent reports have demonstrated the ability of natural killer cells (Beutler & Cerami, 1988), mast cells (Gordon & Galli, 1990) to secrete TNF.

TNF binding sites are widely distributed, virtually in all somatic tissues reflecting its multiple biological activities in the body. Previous reports have demonstrated the presence, in both

normal and malignant cells, of a single class of high affinity receptors for TNF with a dissociation constant ranging from $13x10^{-9}$ M to $71x10^{-11}$ M (Kull *et al*, 1985; Tsujimoto *et al*, 1985; Munker *et al*, 1987; Stauber *et al*, 1989). However, other investigators have shown the expression of both high (K_d=$2.6x10^{-13}$M) and low binding affinity (K_d= $1.5x10^{-10}$ M) receptors on human monocytes (Imamura *et al*, 1987). Furthermore, more recent work has shown, using radiolabelled TNF and cross-linking experiments, that TNF receptor expressed on myeloid cells (HL-60) had a K_d of $7.14x10^{-11}$ M whereas cells of epitheloid origin (HEp 2) had binding sites of K_d= $3.26x10^{-10}$ M (Hohmann *et al*, 1989). These two types of receptors were shown to be different in molecular masses , N- and O-linked glycosylation and their peptide maps. Binding of TNF to its cell surface receptor is followed by internalisation and intracellular degradation (Tsujimoto *et al*, 1985; Imamura *et al*, 1987). Since cell lines resistant to TNF cytotoxicity do iternalise and degrade TNF, so these events may be necessary for TNF action but not sufficient to result in a cytotxic effect. Recently, two distinct TNF receptors with a molecular weight of 55 and 75 kDa were cloned and shown to be expressed on various cells (Loetscher *et al*, 1990; Scall *et al*, 1991).

1.4.2. The beneficial versus the pathological effects of TNF :

TNF seems to manifest both beneficial and toxic effects *in vivo* depending on its concentration and the presence of other mediators in the cellular environment (Tracey *et al*, 1989). One of the beneficial biological effects of TNF is tissue remodelling following

wound healing . Therefore, TNF acts as a growth factor by stimulating the proliferation of fibroblasts and mesenchymal cells and inducing the production of various growth factors such as the colony stimulating factors. In addition, the combined administration of TNF and IL-1 was found to protect mice against the lethal effects of ionizing radiation (Neta *et al*, 1991). TNF was also shown to confer protection against various viral, bacterial and parasitic infections (reviewed in Rook *et al*, 1991c). As an example, the neutralisation of TNF in mice renders them more susceptible to the lethal effects of an infection with *Listeria monocytogenes* or *Mycobacterium bovis* BCG.

On the other hand, high levels of circulating TNF have been associated with various pathological and infectious diseases. Grau *et al* (1987) have investigated the role of this cytokine in the development of cerebral malaria as a complication of *Plasmodium berghei* infection in a murine model. Interestingly, this acute neurological manifestation was not associated with high parasitemia but with a high serum TNF level resulting in a focal accumulation of macrophages in the cerebral blood vessels. Similarly, TNF has been recently shown to be responsible for the lesions seen in the intestinal tract and the epidermis in the acute phase of graft-vs-host disease (Piguet *et al*, 1987). In this model, TNF was not actually detected in the serum but protection was seen in animals challenged with an allogeneic graft and treated simultaneously with anti-TNF antibody. In addition, it has been proved that TNF is directly involved in the pathogenesis of endotoxic shock (Tracey

et al, 1986). Human recombinant TNF infused into rats caused hypotension, metabolic acidosis, diffuse pulmonary inflammation, haemorraghic necrosis in the gastrointestinal tract and kidneys and finally death, similar to what is seen after the administration of endotoxin. Moreover, complete protection against septecaemic shock and death was conferred to baboons by passive immunisation with anti-TNF monoclonal antibodies (Tracey *et al*, 1987).

1.4.3. TNF production in tuberculosis :

Macrophages within the well developed granulomas in tuberculosis could be activated by a product of T lymphocytes, Interferon-gamma (IFN-gamma, Rook *et al*, 1986a). These macrophages, as a result, will express a high level of 1-hydroxylase enzyme which converts the circulating inactive form of vitamin D_3 (25-(OH)) into the active form $(1,25\text{-}(OH)_2)$ or calcitriol. A recent report has shown that IFN-gamma and calcitriol had an additive effect on the expression of anti-tuberculosis activity in human macrophages. Both were also found to prime macrophages for the enhanced release of TNF in response to LPS (Nedwin *et al*, 1985; Rook *et al*, 1987). Interestingly, the same report demonstrated the ability of both viable and heat killed *M.tuberculosis* bacilli to induce the secretion of TNF from those primed macrophages. Recently, such an *in vivo* activated state of macrophages has been confirmed by showing that alveolar lavage cells from both tuberculoid and sarcoid patients release more TNF *in vitro* than lavage cells from normal donors (Rook *et al*, 1990). In addition, LAM, a mycobacterial cell wall component, has been shown to induce TNF release, both *in vitro* by human macrophages and *in vivo* by macrophages from

Propionibacterium acnes -primed mice (Moreno *et al*, 1989). Despite all these observotions, TNF activity has never been detected in tuberculosis patients sera. The short plasma half life (6-7 minutes) and the rapid clearance of TNF, as shown in rabbits injected intravenously with radioiodinated TNF, might be possible explanations (Beutler, Milsark & Cerami, 1985). False negative results could also be obtained because the level of the integral transmembrane form of TNF (26 kDa) has not been taken into consideration (Kriegler *et al*, 1988). The detection of an inhibitor of TNF activity in these patients sera, however, could serve as an indirect evidence for the *in vivo* secretion of TNF (Foley *et al*, 1990).

Therefore, it has been hypothesised that in a tuberculin skin test, the tuberculin prepares the skin site and recruits specific T cells which will, in turn, activate macrophages by the IFN-gamma/calcitriol pathway. Then, LAM (and possibly other mycobacterial components with TNF inducing properties) will induce the secretion of TNF, and other cytokines, leading to the production of a necrotic delayed type hypersensitivity (DTH) (Rook, 1990).

1.5. INTERFERONS (IFN)

Interferons have also been implicated in the modulation of various inflammatory reactions (Billiau, 1988). Heremans *et al* (1987) have developed a murine model for a local Shwartzman-like reaction using NMRI mice. In their model, the injection of a single dose of LPS in

the foot-pads of mice resulted in the production of two peaks of swelling occuring on days 2 to 3 and 6 to 8, respectively. The first peak was characterised by edema and local mononuclear cell infiltration whereas the second peak was associated with the production of intravascular thrombosis in the foot-pads. The systemic administration of recombinant preparations of IFN-gamma or IFN-alpha inhibited the development of the murine local reaction. However, when mice were pretreated with neutralising antibodies to IFN-gamma, a modulated monophasic response was produced indicating that the endogenous IFN-gamma is playing a major role in the production of this reaction. Therefore, it seems that although locally produced IFN-gamma is involved in the production of this reaction, systemic IFN-gamma release imposes a negative regulation. The regulatory effect of systemic IFN-gamma in inflammatory reactions was also demonstrated in another recent report which gave evidence for the abrogation of streptococcal cell wall-induced arthritis in rats by the systemic administration of the cytokine (Wahl *et al*, 1991). Moreover, they showed that IFN-gamma suppressed this pathological reaction by inhibiting the recruitment of leukocytes to the synovium and that this effect on the inflammatory cells was associated with the reduction in their expression of C5a receptors.

On the other hand, the systemic production of IFN-gamma seems to play a pathological role in the murine generalised Shwartzman-like reaction (Billiau *et al*, 1987; Heremans *et al*, 1990). The generalised reaction characterised by dissiminated haemorrhagic lesions seen in the mouth, conjuctiva, anus and nose tip was

produced when NMRI mice were challenged with a local injection of LPS in the foot-pads followed 24 hours later with another injection of LPS given intravenously. Most of the challenged mice died within 24-48 hours. High serum IFN-gamma levels were detected in the challenged animals 2 hours after provocation. Pretreatment of the mice with a systemic injection of a neutralising monoclonal antibody to IFN-gamma significantly reduced both the morbidity and the mortality. In addition, the systemic administration of recombinant IFN-gamma on its own or in combination with a small preparatory dose of LPS did enhance the mortality rate.

1.6. THE ROLE OF VASCULAR ENDOTHELIUM

In the last few years, it has been observed that endothelial cells are not just a passive lining for the blood vessels. They undergo, in response to cytokines amongst other stimuli, several alterations in their metabolism, function and structure (Cotran, 1987). These sepcific alterations were descibed as endothelial activation and were found to influence the outcome of the host response to inflammation. The following sections will discuss the overlapping various functions of activated endothelial cells.

1.6.1. Haemostasis :

One of the main functions of vascular endothelium is the maintenance of vascular fluidity and to act as a barrier controlling the passage of cells and protein molecules from the blood vessel to the surrounding tissues. Therefore, several observations have prompted various groups to investigate the effect of inflammatory cytokines

on endothelial cells. First, the Shwartzman reaction is known to be associated with the formation of fibrin deposits, either locally (in the local reaction) or systemically (in the generalised reaction) resulting in necrosis and organ damage. Second, TNF-induced haemorrhagic necrosis of tumors produced *in vivo* was shown to be associated with endothelial damage and platelet aggregation leading to the diminution of blood flow to the tumor cells (MacPherson & North, 1986). That may explain the state of preparation of tumors for a Shwartzman reaction. The damaging effects of TNF on endothelial cells, in the two previously mentioned events, is partially due to the interference with the role of these cells in maintaining haemostasis. Endothelial cells have both procoagulant and anticoagulant properties. However, when these cells are treated with IL-1 or TNF, they demonstrate enhanced procoagulant activity reflected in the increased production of cell surface tissue factor and plasminogen activator inhibitor (PAI)(Nawroth & Stern, 1986; Schleef *et al*, 1988). Simultaneously, these cells express reduced production of thrombomodulin, activated protein C and the tissue plasminogen activator which comprise the anticoagulant activity of endothelial cells (Nawroth & Stern, 1986; Schleef *et al*, 1988; Moore *et al*, 1989). Tissue factor acts by activating the extrinsic pathway of coagulation which results in the production of thrombin and fibrin deposits while the PAI reduces the fibrinolytic activity of endothelial cells. However, when thrombomodulin is highly expressed on the surface of endothelial cells, it acts by binding to thrombin and thus reducing its ability to catalyze the clot formation (Esmon, 1987). Moreover, the thrombomodulin-thrombin complex activates, in

the presence of protein S, protein C which inactivates factors Va and VIIIa of the coagulation pathway. Activated protein C also activates fibrinolysis by neutralising the PAI. Fibrinolysis is carried out by tissue plasminogen activator which binds fibrin deposits and thus induces the activation of plasmin. Patients with dissiminated intravascular coagulation demonstrate low to undetectable levels of protein C activity (Marlar *et al*, 1985). In addition, the infusion of activated protein C prevented the coagulopathic and the lethal effects of gram-negative septicaemia in baboons while the administration of an antibody to protein C to animals infected with sublethal doses of *Escherichia coli* resulted in a lethal effect (Taylor *et al*, 1987). Moreover, the glomerular fibrin deposition and the consequent renal cortical necrosis seen in the generalised Shwartzman reaction, resulting from the sequential challenge with endotoxin, was prevented in rabbits treated with tissue plasminogen activator a few hours after the provocative injection of LPS (Bergstein & Riley, 1987).

1.6.2. Acute inflammation and cellular migration :

An important role of endothelial cells in the inflammatory responses is their increased adhesion to leukocytes. Various investigators found that cytokines, namely TNF, IL-1 and IFN-gamma induce the surface expression of adhesion receptors which mediate the attachment of monocytes, neutrophils and T cells and thus enhance their migration through the vascular wall. Human neutrophils preincubated with TNF demonstrated increased surface expression of CR3 and hence they showed an enhanced adhesion to human umbilical

vein endothelial cell monolayer within a few minutes (Gamble *et al*, 1985). Human monocytes incubated with LPS or TNF showed similar rapid adhesion (Doherty *et al*, 1989). However, the increased expression of both ELAM-1 and ICAM-1 adhesion receptors, induced by TNF and IL-1, on the surface of endothelial cells required *de novo* RNA and protein synthesis and thus, their expression peaked at 4 and 8 hours, respectively (Gamble *et al*, 1985; Pober *et al*, 1986). TNF-stimulated human endothelial cells were also found to optimally bind T lymphocytes within 4 hours (Cavender *et al*, 1987). Adhesion of various cell types to vascular endothelium was found to be a complex process that requires the cooperation of various adhesion molecules (Springer, 1990). Therefore, blocking one of these molecules results in only a partial inhibition of the adhesion process.

IL-1 and TNF-activated endothelium secretes IL-1, Interleukin-6, colony stimulating factor, platelet activating factor and monocyte chemoattractant factors which can influence the interaction of leukocytes with the endothelium and therefore, amplify the inflammatory response (Cotran & Pober, 1989; Rollins *et al*, 1990).

1.6.3. The immune response :

Endothelial cells show an increased expression of the major histocompatibility complex (MHC)- class I (A and B) antigens within several days in response to TNF and IFN-gamma (Pober *et al*, 1986; Collins *et al*, 1986). This may indicate a role for endothelial cells in antigen presentation to cytotoxic T cells. In response to IFN-gamma, endothelial cells also express class II antigens and thus,

these cells may play a role in the production of delayed type hypersensitivity reaction.

1.7. OTHER MEDIATORS

The production of the Shwartzman reaction involves a number of other participants such as the platelet activating factor (PAF), neutrophils and macrophages, IL-1, the complement pathway and platelets. It is possible that TNF, probably in the presence of IL-1, initiates the reaction which is then amplified by the production of PAF, the activation of the complement components and the recruitment of inflammatory cells. The role of some of these factors will be discussed in detail in the following chapters.

1.8. AIMS OF THE STUDY :

The increasing threat of tuberculosis infection associated with increased incidence of AIDS, the failure of BCG vaccination in parts of the world where there is a high risk of mycobacterial infections, and the incomplete effectiveness of chemotherapy, has renewed the interest in developing an immunotherapy. The immunotherapy studies aim at first, reducing the time taken to treat the disease and therefore reducing the problem of cost and compliance. Second, they aim at reducing the cases of relapses and reactivation of the disease by enhancing the ability of the patient's immune system to recognise and eliminate the persisters. We hypothesised that first, rapid recognition of persisters might require the recognition of certain subset of antigens, perhaps the secreted ones. Second, this recognition must lead to the non-necrotising pattern of response.

Third, the necrotising pattern of response is related to the Koch phenomenon and Shwartzman reaction, and can be switched off by an effective immunotherapeutic agent. Therefore, the present study aimed to:

1. Screen the sera of tuberculosis patients who received various preparations of immunotherapy for both qualitative and quantitative changes in their antibody responses.

2. Look for changes in the antibody responses to BCG85, an antigen secreted by various mycobacterial species. This is in view of our increasing knowledge about the importance of proteins secreted from live bacteria,

3. Measure, in those patients sera, the changes in the proportion of agalactosyl IgG, a marker of disease activity in tuberculosis infection and several other immuno-pathological events.

4. Test the hypothesis that the Koch phenomenon and the Shwartzman reaction are similar. That was carried out by :

   (a) Improving the murine model of LSR introduced by Rothstein & Schreiber (1988) by developing reliable quantitative measures for the pathological changes detected in the local Shwartzman reaction (LSR).

   (B) Testing for the ability of mycobacterial antigens of various species to prepare for a local Shwartzman reaction.

(c) Detecting the involvment of various cellular and humoral mediators (the complement, PAF, prostagandins, phagocytic cells and adhesion molecules) in LSR.

5. Investigate the possiblity of eliminating the necrotic component of the LSR since only tuberculosis patients and not healthy subjects produce a necrotic skin test response.

6. The association of the pathology of tuberculosis infection with the abnormal secretion of cytokines prompted us to investigate the role of TNF in such immunopathology using a murine model of tuberculosis.

CHAPTER TWO

Changes in the humoral immune response of tuberculosis patients induced by the immunotherapeutic agent, *Mycobacterium vaccae*

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2.1. INTRODUCTION

In spite of the effectiveness of multiple drug chemotherapy, used for treating tuberculosis, in eliminating most of the bacilli, there are several problems associated with this treatment regimen. These include the high cost of the drugs, the emergence of drug resistent bacteria and the patient's non-compliance. Furthermore, the chemotherapeutic drugs are usually used for 6-9 months to prevent relapses or reactivation of the disease caused by the persisters, low metabolising organisms. Therefore, immunotherapy aims at improving the ability of the patient's immune system to recognise and destroy these persisters by changing the nature of the immune response and perhaps its fine specificity. This could allow us to shorten the period of chemotherapy.

Mycobacterium vaccae is a non-pathogenic bacterium that has been isolated from the soil of Uganda. Previous animal and human studies provided evidence for its immunomodulatory effects (disussed in detail in the previous chapter) which led to its experimental use for the immunotherapy of tuberculosis.

In a preliminary study carried out in Kuwait, patients included in the study were sputum-positive pulmonary tuberculosis and were treated with isoniazid, rifampicin and streptomycin for 9 months (Stanford *et al*, 1990). In a double blinded manner, these patients received an intradermal injection, over the deltoid muscle, of either saline (placebo) or irradiated *M.vaccae* (10^9) one month after starting the chemotherapy. Over a 4-month period, the immunotherapy had no effect on the clinical, bacteriological, biochemical, haematological and radiological parameters of the tested patients. This is mainly because chemotherapy given at the

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antigens might be expressed on or released by live actively metabolising pathogens. Therefore, we looked at changes in the patient's responses after immunotherapy, to secreted antigens, particularly the fibronectin binding BCG85 complex antigen.

The BCG85 complex is a major component of the culture supernatant of both BCG and *M.tuberculosis* and it consists of three closely realted antigens, the A, B and C components (Abou-Zeid *et al*, 1988; Wiker *et al*, 1990a). These differ in their molecular weight, electrophoretic mobility and immunological properties (Wiker *et al*, 1986). Furthermore, this antigen is released by various mycobacterial species such as *M.bovis* BCG, *M.avium*, *M.leprae*, *M.tuberculosis* and *M.kansasii* (Wiker *et al*, 1990b). Interestingly, this antigen was found to have high affinity for fibronectin (FN) and culture fluid of mycobacteria inhibits the attachment of BCG to FN-coated surfaces suggesting a possible role for this antigen in mycobacterial diseases (Abou-Zeid *et al*, 1988; Ratliff *et al*, 1988). Cloning of the BCG85-A demonstated the existence of a signal peptide sequence proving it to be a secreted protein (Matsuo *et al*, 1988; Borremans *et al*, 1989).

Furthermore, it was interesting to measure the changes in the proportion of serum agalactosyl IgG (G0) after therapy. Patients with tuberculosis, rheumatoid arthritis and Crohn's disease were shown previously to have high serum levels of IgG with heavy chains lacking terminal galactose from the biantennary oligosaccharide on the conserved glycosylation site on the C_H2 domain (Rook, 1988c). The proportion of G0 in normal sera decreases from 30% in children to 20% at the age of 25 years and then increases to 40% at the age of 70 years. The abnormal increased levels of G0 were found to be associated with the presence of chronic T cell-dependent

inflammation, acute phase reaction, fever and weight loss. Interestingly, the levels of G0 returns to normal during remission in patients with rheumatoid arthritis and Crohn·s disease. In diseases, such as osteoarthritis, acute rheumatic fever, most cases of sarcoidosis, and a number of viral infections, in which there is either an acute phase reaction or chronic T cell dependent pathology, but not both, no changes in G0 values can be detected. The present findings indicate the changes in the levels of G0 before and after therapy of tuberculosis.

2.2.1. Preparation of sonicated and secreted mycobacterial antigens :

To prepare the sonicated antigens of *M.tuberculosis* (H37Rv), the bacilli were grown on Sauton's medium solidified with 1.5% agar. Harvested organisms were suspended in saline and sonicated in a 100 Watt ultrasonic disintegrator for 15 minutes with the wave peak distance set at 8-9 um, spun at 13,000 g for one hour and then the supernatant was sterilised by filtration through Millipore filters (Millex-GV, 0.22 um). Protein concentration was measured by the method of Lowry *et al* (1951). This preparation of soluble antigens is known as New Tuberculin.

Secreted antigens of mycobacteria were obtained as described by Abou-Zeid *et al* (1986). In brief, *M.tuberculosis* (H37Rv) was grown as a pellicle on liquid Sauton's medium at 37°C for 3 weeks. The bacteria were then removed by centrifugation at 10,000 rpm (J2-21M/E Beckman centrifuge) for 30 minutes. Culture supernatants were then passed through Millipore filters (Millex GV, 0.22 um) and stored at -20°C.

2.2.2. Tuberculosis patients :

These patients, as described before in Bahr *et al* (1990), were admitted to the Chest Diseases Hospital in Kuwait and were confirmed to have a sputum positive pulmonary tuberculosis. Different groups of patients were given, intradermally over the deltoid muscle, either the immunotherapeutic preparation (detailed in the section 2.2.3.) or saline (the control group). In principle, the physicians were expected to allocate the patients randomly, though our data

prove that they did not. Details of the various bacteriological, haematological, clinical, biochemical and skin test results of these patients used were described in Bahr *et al* (1990).

2.2.3. The immunotherapeutic preparations :

Suspensions of various concentrations of *M.vaccae* /ml were dispensed into vials and were then either irradiated (by exposure to 2.5 Mrads from a ^{60}Co source) or autoclaved (for 15 minutes at 121°C (Bahr *et al*, 1990). Details of the immunotherapeutic preparations used and the number of patients tested are shown in Table 2.1. Two blood samples were drawn from the patients tested, the first one taken after 1 month of treatment just before injecting the immunotherapy. The second blood sample was collected 4 weeks later (protocol is shown in Fig.2.1). Serum samples were stored at -20°C.

2.2.4 Sodium dodecyl sulphate-polyacrylamide gel electophoresis (SDS-PAGE) :

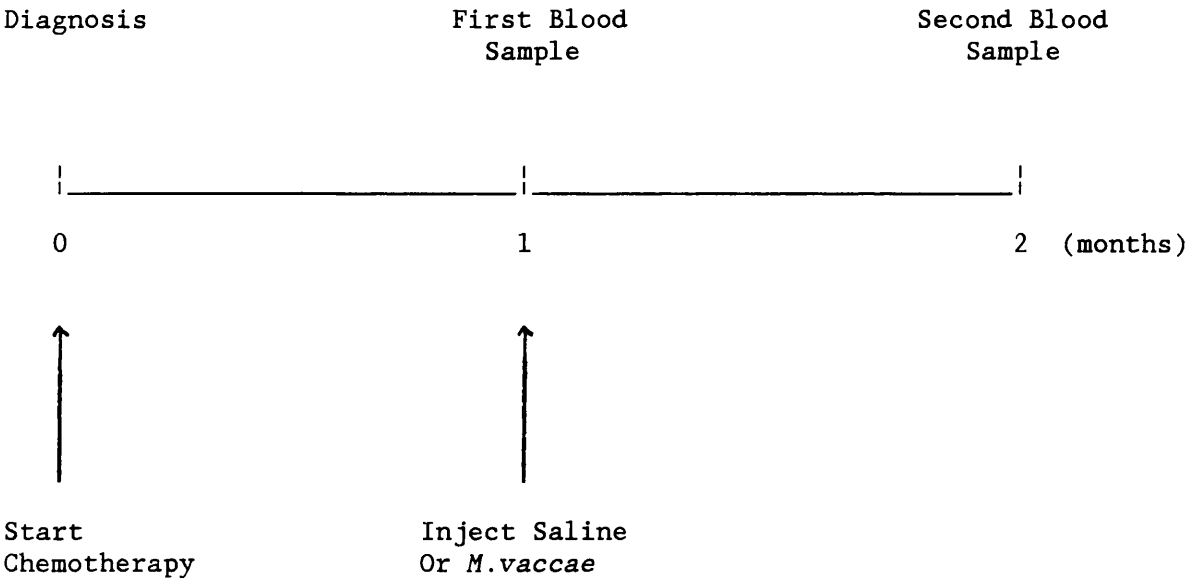
Both the sonicate and culture filtrate preparations of *M.tuberculosis* were separated on 12-well slab gels of 12.5 % resolving gel using the discontiuous buffer system of Laemmeli (Abou-Zeid *et al*, 1986). A mixture of standard protein markers (MW-SDS-200, Sigma SDS-6H) was used for the determination of molecular mass. Proteins were transferred onto nitrocellulose paper (0.2um, BA 83 Schleicher and Shuell, Dassel, FRG) using a semi-dry electroblotter (Ancos, Olsbykke, Denmark). Visualization of proteins

Table 2.1. The number of tuberculosis patients tested in the various immunotherapeutic groups.

Group	Number of patients [2] tested
1. 10^8 irradiated *M.vaccae* .	8
2. 10^9 irradiated *M.vaccae* plus New Tuberculin (T)[3].	13
3. 10^9 irradiated *M.vaccae* plus murabutide (M)[3].	13
4. 10^9 irradiated *M.vaccae* plus T plus M.	26
5. $2X10^9$ irradiated *M.vaccae* .	11
6. 10^9 autoclaved *M.vaccae* .	24
7. Saline	24
8. Healthy subjects	16

1. The preparations were injected intradermally at 0.1 ml.
2. The age of the patients tested ranges between 15-56 years.
3. T and M were used at a concentration of 0.2 and 500 ug/ml, respectively.

Fig.2.1. The time schedule for the administration of therapy and the collection of blood samples from Tb patients.



was acheived by staining with Aurodye, a colloidal gold solution (Jansen Life Sciences Products, Belgium). For immunoblotting, Western blots were cut and transferred into a 24-well plastic tray and then soaked, at room temperature, in phosphate-buffered saline (PBS, 10 mM, pH 7.4) made up with 0.1 % Tween 20 (v/v) and 2 % bovine serum albumin (BSA, w/v), to block non specific binding. Nitrocellulose strips were then incubated overnight with sera from patients or normal donors at 1/16 at 4°C and probed with peroxidase-conjugated rabbit anti-human immunoglobulins (Dako P212) at 1:300 dilution for two hours at room temperature. Peroxidase activity was visualized using 4-chloro-1-naphthol (Sigma C-8890). The antibody response of tuberculosis patients to sonicate or culture filtrate before and after immunotherapy was compared. To quantitate the antibody respone, the protein band pattern was examined with an CAMAG TLC/HPTLC scanner (Switzerland).

2.2.5. Purification of BCG 85 antigen :

The purification of BCG85 protein was partially carried out at the Institute of Immunology and Rheumatology, University of Oslo, Norway, with the kind help of both Dr. M. Harboe and Dr. H. Wiker. The purification procedure was performed as described previously by Wiker *et al* (1986). Fourteen days old culture of *M.bovis* strain BCG grown on Sauton's medium was used. The BCG culture fluid was centrifuged and then sterile filtered to remove the bacterial cells. It was then concentrated by ammonium sulphate precipitation (50%) overnight at 4°C. After being dialysed, the precipitated proteins were then passed through hydroxyapatite column (Bio-Gel HTP code No.

130-0420, Bio-Rad Lab., Richmond, USA) equilibrated with 10mM sodium phosphate buffer, pH 5.8. BCG 85 with very few contaminants usually passes through the column. This preparation was then run through a hydrophobic interaction chromatography, phenyl sepharose CL-4B (code No. 17-0810-02, Pharmacia Fine Chemicals, Uppsale, Sweden) equilibrated with 20 mM sodium phosphate buffer, pH6.8. BCG 85-B was eluted first from the column with 10 mM Tris-glycine buffer, pH 8.9 followed by BCG 85-A eluted with a 1:1 mixture of the previously used Tris-glycine buffer and ethylene glycol. To remove contaminants of the B component from the BCG 85-A preparation and vice versa, the fractions were separately passed through DEAE-sephacel (Pharmacia, code No. 17-0500-01) ion exchange chromatography. The A component was eluted with 20 mM sodium phosphate buffer, pH 6.8 while the B component was eluted with the same buffer mixed with 50 mM sodium chloride.

2.2.6. Fused Rocket Immunoelectrophoresis (FRIE) :

FRIE was used to monitor the different steps of the purification of BCG85 (Wiker *et al*, 1986). Agarose (Litex HSA, Glostrup, Denmark) was used as a 1% solution prepared in barbiturate-Tris buffer, pH8.6. Clean glass plates (9X11 cm) were used. The lower gel layer was made by pouring warm 1% agarose solution on 3.8X11 cm of the plate. Then the top layer was prepared by mixing 400ul of either polyclonal rabbit anti-BCG serum (Dako B124, Copenhagen, Denmark) or K1020 (a rabbit anti-BCG85 polyclonal serum provided generously by H. Wiker) with 8 mls of the agarose solution. This mixture was then applied to the top part of the plate. Two rows of circular wells were then punched in the lower layer. Starting with the lower row of

wells, 10 ul of each fraction was applied per well. Electrophoresis was then carried out overnight. The plates were washed, dried and stained with Coomasie brilliant blue R-250 (Sigma B0149).

2.2.7. Enzyme-Linked Immunosorbent Assay (ELISA) :

ELISA assay was used to measure the concentration of purified BCG 85 according to the method described by Wiker *et al* (1990b). ELISA plates (Nunc Immuno-plates) were coated with the mouse monoclonal antibody, HYT 27 (kindly provided by Dr. J. Bennedsen, Denmark) at 1:8000 in PBS. The plates were then incubated for 48 hours at 4°C. Serial dilutions of the samples to be tested for its content of the antigen were prepared in PBS-Tween 20 (0.05 %) and then added to the plate. Serial dilutions of a lyophilized MPT 59 (BCG 85-B) antigen preparation (kindly donated by Dr. S. Nagai, Japan) were used as a standard and was included in each assay. The plates were then incubated at 37°C for 45 minutes. The antigen was then detected by adding rabbit anti-BCG 85 polyclonal serum (K1020, provided generously by Dr. H. Wiker, Norway) at 1:1000 and the plates were incubated at 37°C for 45 minutes. Finally, swine anti-rabbit immunoglobulin conjugated to horse raddish peroxidase (Dako P217) was added. The reaction was then developed using 2,2`-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma A-1888) dissolved in citrate phosphate buffer, pH 4.1 and hydrogen peroxide added just before use. The reaction was then stopped using sodium fluoride and the optical density was read at 630 nm on a MicroElisa AutoReader (Dynatech).

An ELISA assay was also used to screen the tuberculosis patients for their response to BCG 85. Therefore, 96-well plates were coated with either the A or the B component of the antigen at 5 ug/ml or with desalted *M.tuberculosis* (H37Rv) culture filtrate at 1 ug/ml. After coating the plates overnight at 4°C, the plates were washed with PBS containing 0.05 % Tween 20 and then blocked using the same buffer for one hour at room temperature. Sera of tuberculosis patients or normal donors were added in a dilution of 1:200 to the BCG 85 coated wells and 1:500 to the culture filtrate coated ones. The plates were then incubated for two hours at room temperature. A horse radish peroxidase conjugated anti-human IgG (Dako P214) was added to the washed plates and incubated overnight at 4°C. The reaction was then developed as described previously.

2.2.8. Immunoassay for agalactosyl IgG :

ELISA plates (Nunc-Immunoplate Maxisorp F-96) were coated overnight at 4°C, with 2.5 ug/ml of protein A (Sigma P-6031) made up in PBS (This assay has been developed in this lab, Rook *et al*, 1991d). Next day, the plates were washed and then blocked with PBS containing 1% BSA and 0.05% Tween 20. Serum samples were diluted 1/100 with a buffer consisting of 0.1 M glycine and 0.16 M sodium chloride adjusted to pH 7.0 with sodium hydoxide. Patient·s sera (50 ul) were then added to the coated wells and incubated at 37°C for 2 hours. A set of standard human sera of which the G0 value was determined by biochemical procedures were included in each assay. The plates were washed with PBS and then carefully placed on the surface of water in a waterbath (85°C) for 7-8 minutes. This is to denature the IgG and

hence expose the sugars. A solution of 2 ug/ml of biotinylated anti-GlcNAc GN7 (a monoclonal antibody that was produced by immunising mice with group A streptococcal cell wall peptidoglycan/polysaccharide mixture and it was selected for it·s specificity to terminal N-acetylglucosamine, GlcNAc) was made up in PBS/BSA/Tween and added to the wells. After an overnight incubation, peroxidase-conjugated streptavidin (1 ug/ml) made up in PBS/BSA/Tween was added to the plate and incubated at 37°C for 1 hour. The reaction was then developed using ABTS and hydrogen peroxide. The optical density was measured at 630 nm using a MicroElisa AutoReader (Dynatech). The absorbance values produced by the standard sera were plotted against the percentage of G0 determined biochemically and that plot was then used to calculate the G0 percentage for the unknown sera. The biochemically determined standards were provided by Dr. T Rademacher, Glycobiology Institute, Department of Biochemistry, Oxford.

2.3.1. The analysis of the patient's antibody response on Western blots of *M.tuberculosis* antigens :

Examination of both the aurodye stained blot of the fractionated antigens of the sonicate and its scan showed that it has a very complex band pattern (Fig.2.2A). The sera of tuberculosis patients treated with various preparations of immunotherapy were incubated with the western blots of the sonicated antigen of *M.tuberculosis* H37Rv. Thus, we compared the antibody responses to this antigenic preparation, before and after immunotherapy. The analysis of the antibody responses showed that tuberculosis patients, of the different groups, respond strongly to most of the protein bands of the sonicate (Fig.2.3). Therefore, neither the western blots of these patients nor their scans could demonstrate any clear quantitative or qualitative change after therapy. On the other hand, the examination of the aurodye-stained blot of the culture filtrate preparation and it's scan showed that it's protein band pattern is less complex than that of the sonicated antigen (Fig2.2B). This is mainly because it contains proteins excreted to the culture medium, those secreted to be attached to the outer cell wall and a few of the cytoplasmic proteins released from dead bacteria at the end of the logarithmic growth phase (Andersen *et al*, 1991). In Fig.2.4, the pattern of response of the same Tb patients of various groups, to the sonicate can be compared with their response to the culture filtrate. The serum samples of the group which received the irradiated bacilli plus Tuberculin (T) plus Murabutide (M),

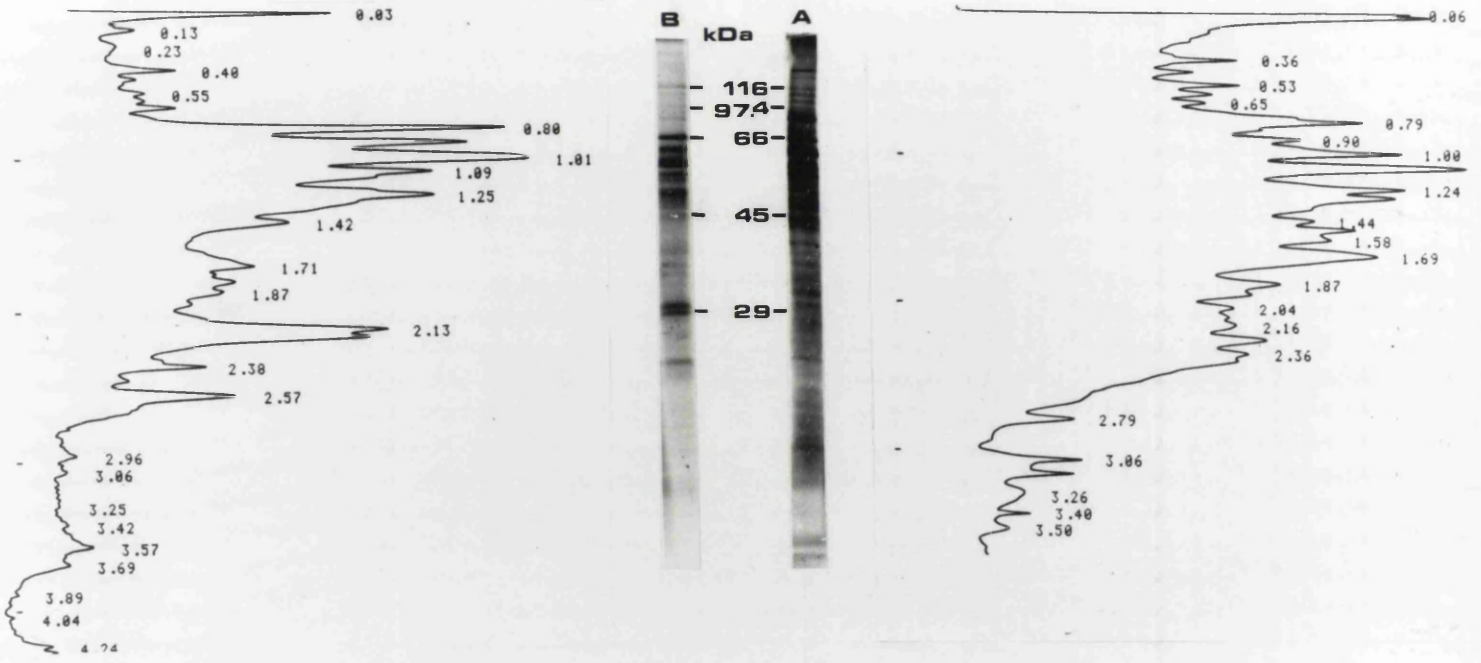


Fig.2.2. An aurodye-stained western blot of the sonicated antigens of the M.tuberculosis H37Rv and its scan (A). The major 6 proteins discussed in the text have a retention time of (70,0.79; 65,1.00; 50, 1.51; 38,1.87; 31-29,2.11-2.16; 23,2.36).

The western blot of the culture supernatant of the same mycobacterium and its scan is shown in (B). The 6 proteins have a retention time of (70,0.8; 65, 0.9; 50,1.25; 38,1.71; 31-29,2.13-2.18;23,2.57).

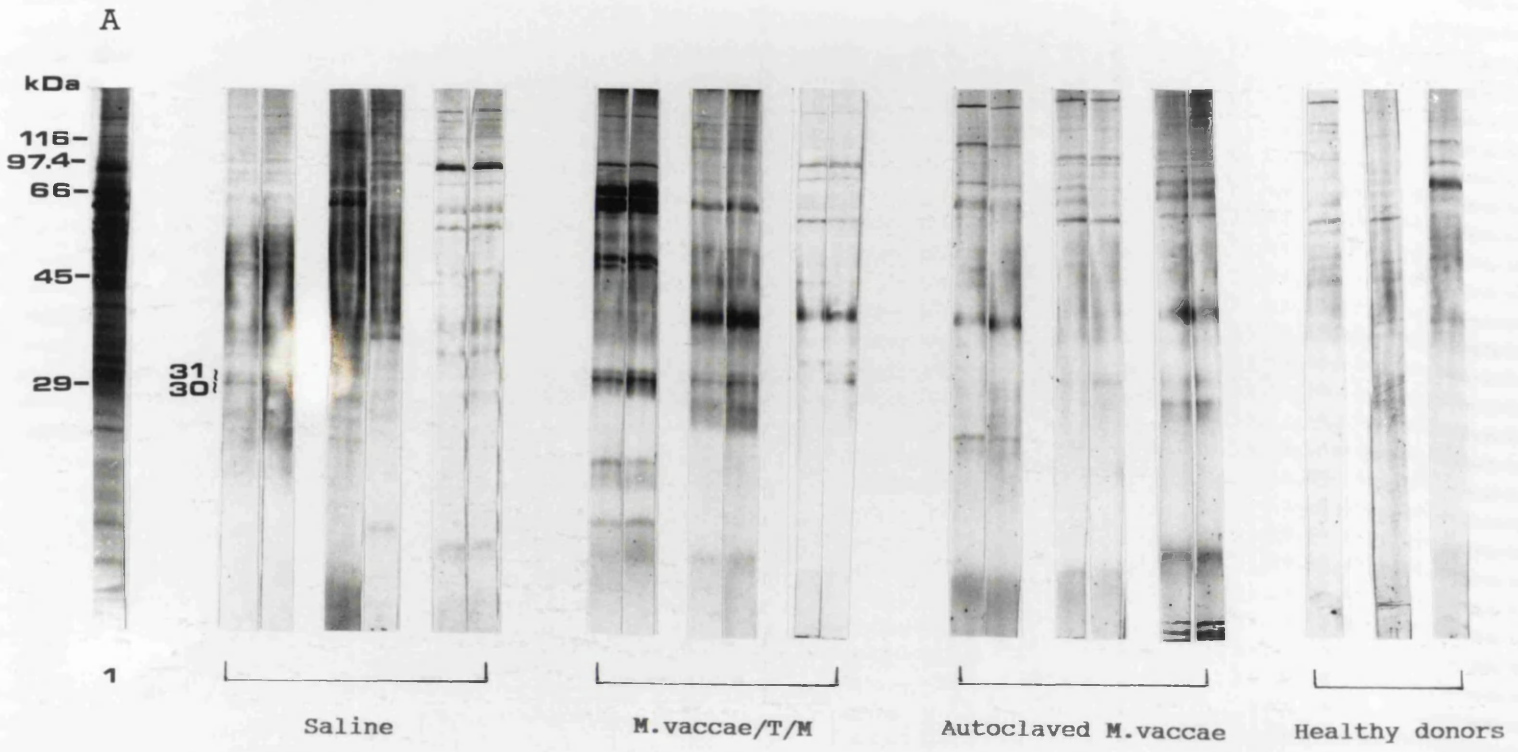


Fig.2.3. The antibody responses of Tb patients of various groups (saline, M.vaccae+T+M and autoclaved M.vaccae) and healthy donors to western blots of the sonicated antigens of M.tb (1).

Each pair of lanes represents the response of one patient, before and after therapy.

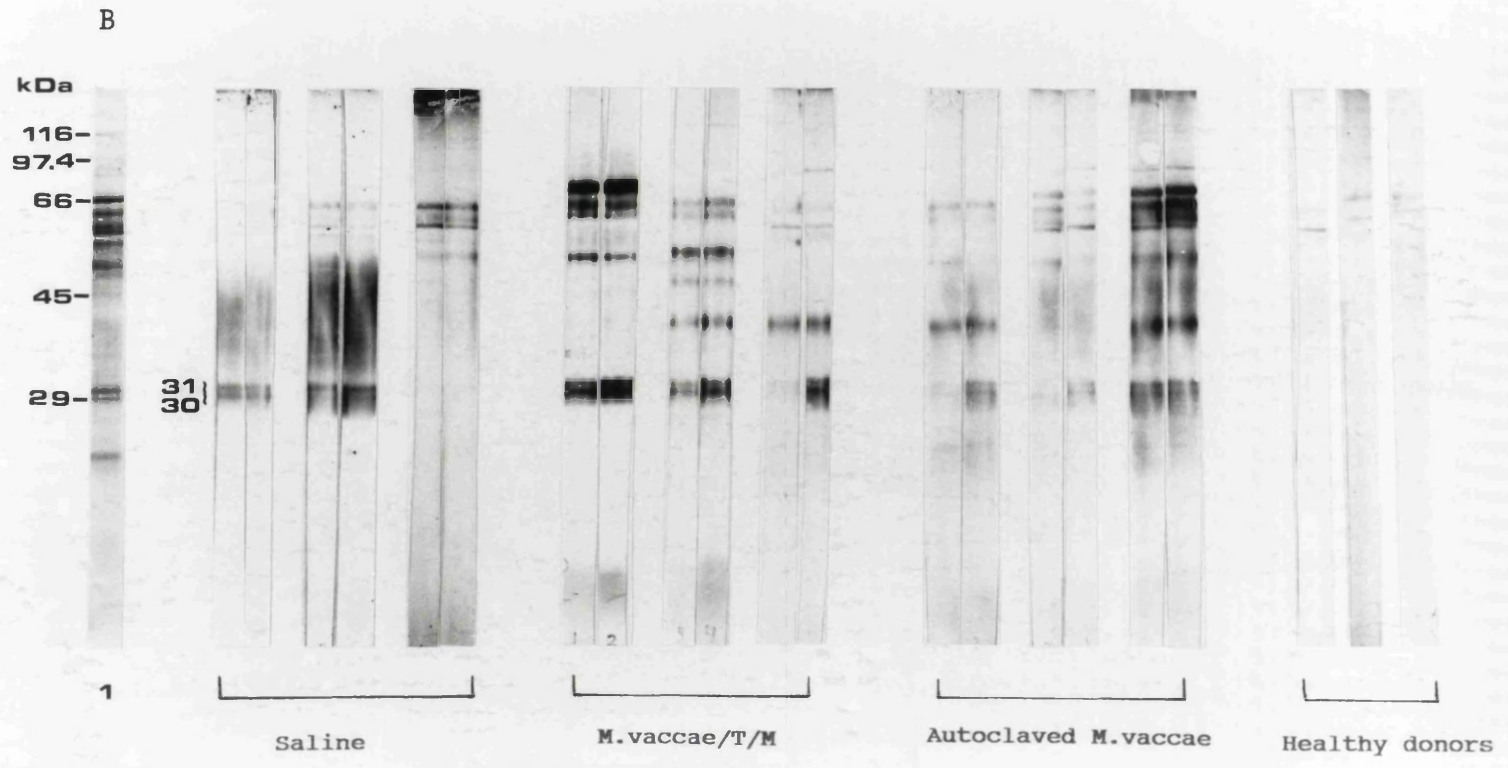


Fig.2.4. The antibody responses of the same Tb patients (presented in Fig2.3) and healthy donors to western blots of the culture supernatants of M.tuberculosis (1).

incubated with blots of culture filtrate demonstrated, in comparison to the saline treated group, a striking increase in their antibody response to the BCG85 complex (30-31 kDa) (Fig.2.4).

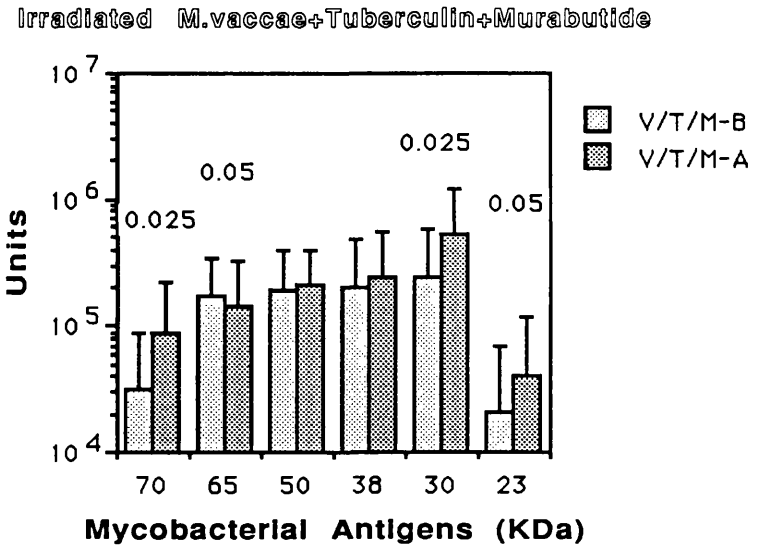
The antibody responses of the tuberculosis patients of various immunotherapy groups to the culture filtrate were measured on the CAMAG TLC/HPTLC scanner. Figure 2.5. demonstrates the mean of responses, calculated by measuring the area under the peaks to 6 major bands of the culture filtrate, the 70, 65, 50, 38, 30-31 doublet and the 23 kDa antigens. The saline-treated group showed a slight increase in their response to the 38, 30 and 23 kDa whereas the patients who received the irradiated mycobateria mixed with the New tuberculin (T) and Murabutide (M) demonstrated a significant increase in their antibody response to the 70, 30 and the 23 kDa proteins and a decrease in their response to the 65 kDa antigen. Note that in view of the 3 weeks half life of IgG, 4 weeks is quite early to look for falls in antibody levels. Furthermore, patients injected with the autoclaved mycobacteria showed a significant decrease in their response to the 65 and 50 kDa antigens while those injected with the low dose of irradiated mycobacteria (10^8) or the higher dosage of it ($2X10^9$) showed no major differences in their antibody responses. However, based on the clinical data of the patients before therapy, it was noticed that the groups were not evenly divided since the saline-treated group contained more advanced cases.

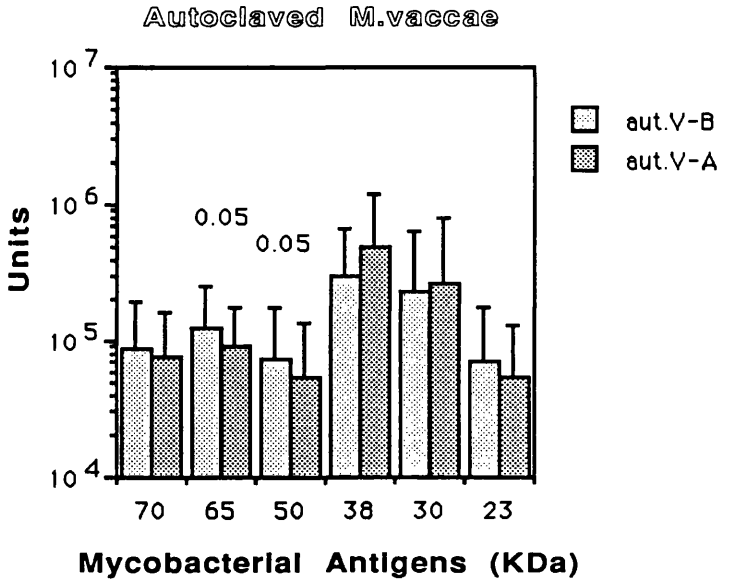
Serum samples of healthy subjects reacted with very few bands on either the sonicate or culture filtrate blots. Scans of their antibody responses to the culture filtrate indicated that they mainly recognise the 65 kDa heat shock protein and the 50 kDa. They

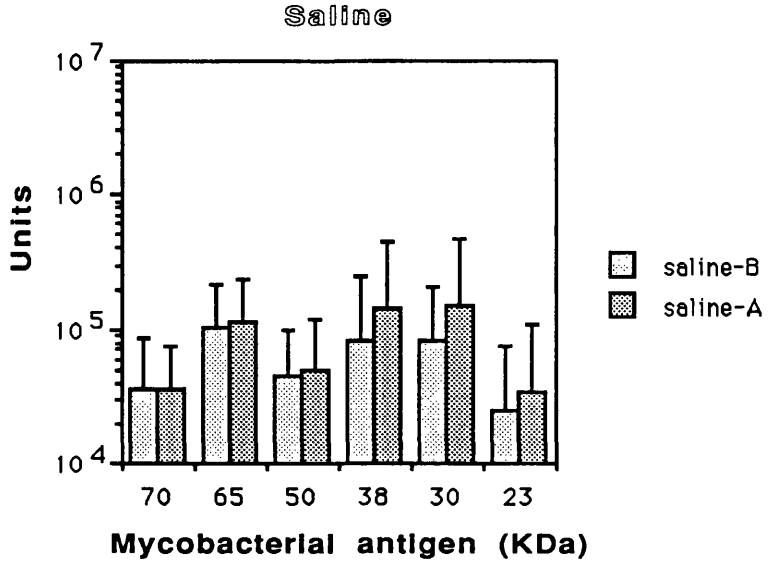
Fig.2.5.The scans of the antibody responses (western blots) of healthy subjects or tuberculosis patients of various groups to 6 major proteins in the culture fluid of M.tuberculosis.

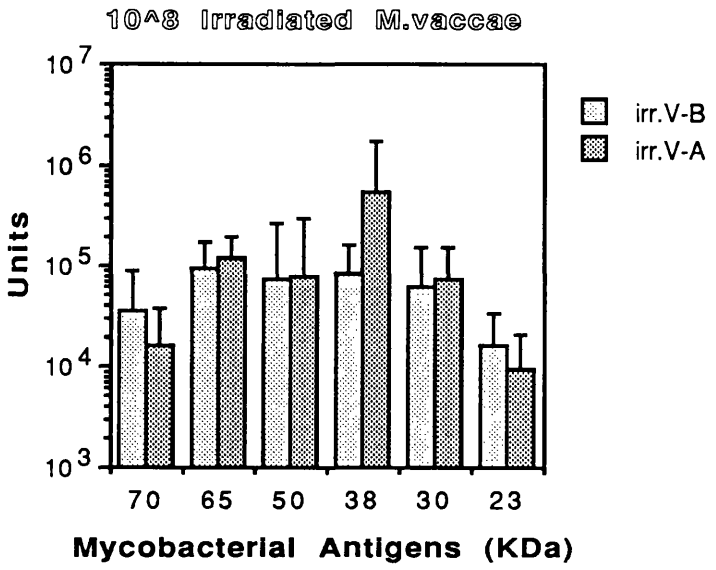
1-each pair of columns represent the antibody response before (B) and after (A) therapy.

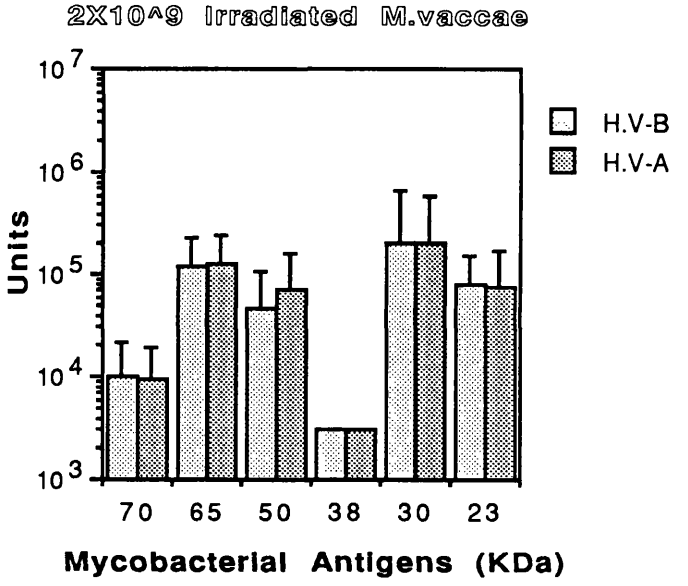
2- p values compare the antibody response before and after therapy to a single antigen (paired-t test).

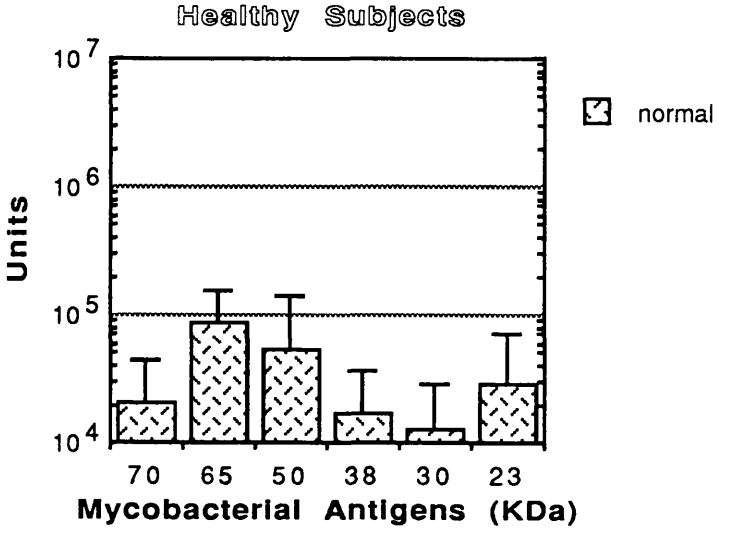

Irradiated M.vaccae+Tuberculin+Murabutide
Units
10 7
10 6
10 5
10 4
0.025
0.05
0.025
0.05
V/T/M-B
V/T/M-A
70
65
50
38
30
23
Mycobacterial Antigens (KDa)


Autoclaved M.vaccae
Units
10 7
10 6
10 5
10 4
0.05
0.05
aut.V-B
aut.V-A
70
65
50
38
30
23
Mycobacterial Antigens (KDa)


Saline
Units
10 7
10 6
10 5
10 4
saline-B
saline-A
70
65
50
38
30
23
Mycobacterial antigen (KDa)


10^8 Irradiated M.vaccae
Units
10^7
10^6
10^5
10^4
10^3
70 65 50 38 30 23
Mycobacterial Antigens (KDa)
irr.V-B
irr.V-A


2X10^9 Irradiated M.vaccae
Units
10^7
10^6
10^5
10^4
10^3
70 65 50 38 30 23
Mycobacterial Antigens (KDa)
H.V-B
H.V-A


Healthy Subjects
Units
10^7
10^6
10^5
10^4
70 65 50 38 30 23
Mycobacterial Antigens (KDa)
normal

also show a weaker response to the 70 kDa heat shock protein and the 23 kDa protein. Interestingly, very few of the normal donors respond to the 38 and 30 kDa secreted antigens.

2.3.2. Changes in the antibody responses to the purified BCG85 complex, induced by therapy :

Live metabolising mycobacteria release proteins into their surrounding environment (Abou-Zeid *et al*, 1988; Andersen *et al*, 1991). These secreted antigens have been desribed as good targets for the immune system and therefore they would induce the rapid recognition of live metabolising mycobacteria by the host. This is one possible explanation for the fact that immunisation with live and not killed mycobacteria generates a protective immune response (Orme, 1988). Because the immunotherapy aims at improving the ability of the immune system of the tuberculosis patient to recognise the remaining slowly metabolising live bacilli (the persisters) and based on the fact that the scanner results suggested an increase in the anti-BCG85 antibody in the immunotherapy group, we attempted to purify the fibronectin-binding BCG85 secreted antigen and examine the changes in the antibody response of Tb patients to this antigen, before and after therapy.

The BCG85 was described by Wiker *et al* (1986), as a complex of 3 antigens known as the A, B and C components. As described in the materials and methods section, we purified the BCG85-A and BCG85-B from the culture supernatant of *M.bovis* BCG strain by ammonium sulphate precipitation followed by column chromatography. The chromatographic separation started by passing the precipitated proteins through a hydoxyapatite column resulting in the separation of the BCG85 and few other contaminants (peak A) which do not bind

to the column from those which bind (the second peak) to the hydoxyapatite (Fig.2.6a). Figure 2.6b demonstrates the analysis of the differnent fractions on Fused Rocket Immunoelectrophoresis (FRIE) in the presence of anti-BCG culture filtrate serum in the top layer gel and indicates the separation of the BCG85 from the other antigens in the culture filtrate. This is further confirmed when anti-BCG85 serum (K1020) was included in the top gel layer (Fig.2.6c). The BCG85 antigen-containing fraction was then passed through a phenyl-sepharose (hydrophobic) column which allowed for the partial separation of the B (peak A) from the A component (peak B) (Fig.2.7a). The the analysis of the fractions containing the two peaks (components) is shown in Fig.2.7b. This was finally followed by the DEAE-sephacel ion exchange chromatography used to obtain a better separation of the two components from each other and to get rid of all the remaining contaminants (Fig.2.8a & b). The purified BCG85-A and B containing fractions were finally tested on aurodye stained-SDS-PAGE blots and detected with the polyclonal anti-BCG85 (K1020) serum (Fig2.8c). On aurodye stained blots, the BCG85-A component shows a band at 31 kDa while the BCG85-B component shows a major band at 29 Kda and a weaker band at 27 kDa. The concentration of the purified components was then estimated by ELISA, as described in the materials and methods, using a lyophilised preparation of MPT59 (BCG85-B) as a standard.

Although all the immunotherapy-treated patients and the control group showed significant rises in their antibody responses to the culture filtrate of *M.tuberculosis* after therapy (Fig.2.9), those treated with the irradiated bacilli combined with tuberculin and murabutide presented the most significant increase in their antibody response to both components of the BCG85 (Fig.2.10A & B). The

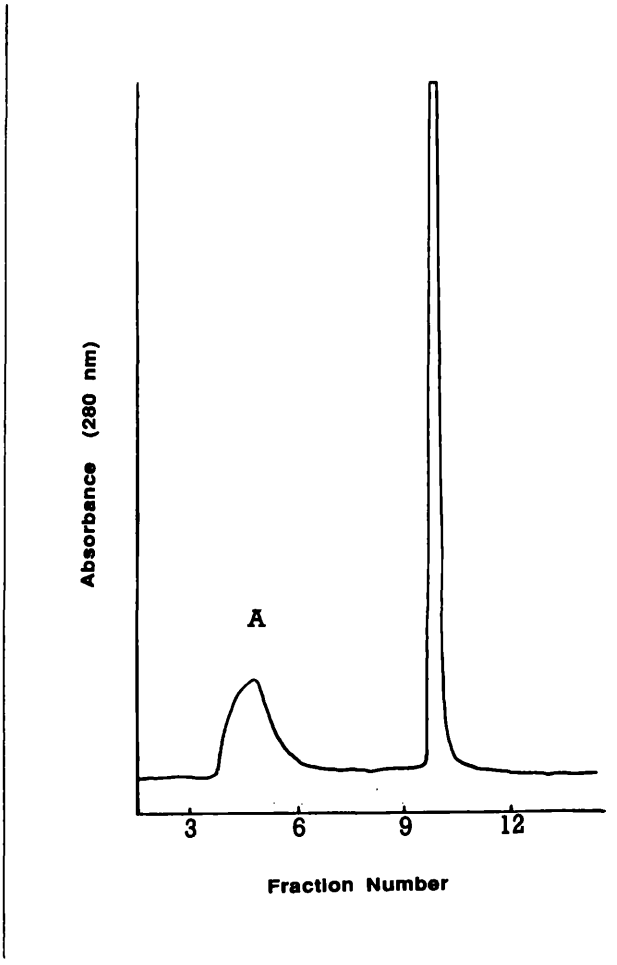
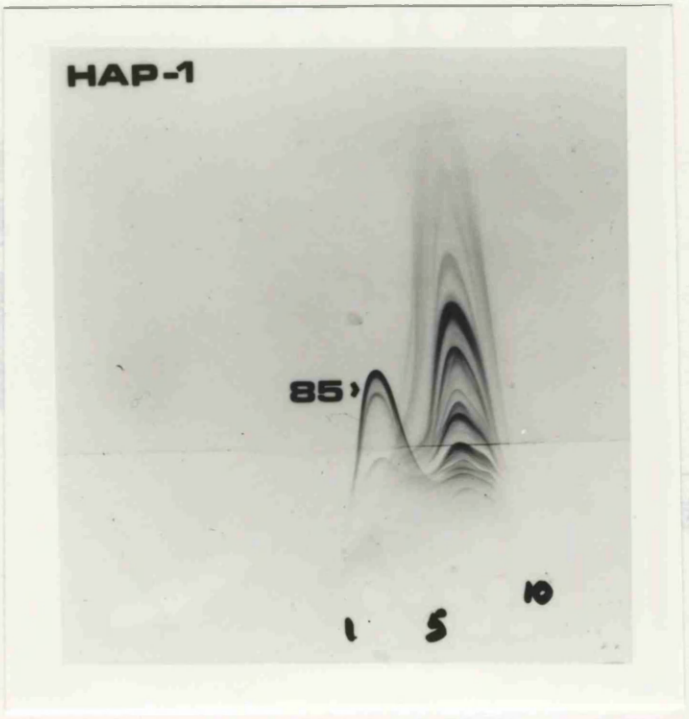


Fig.2.6a. BCG culture filtrate fractionated by hydoxyapatite column.

A



B

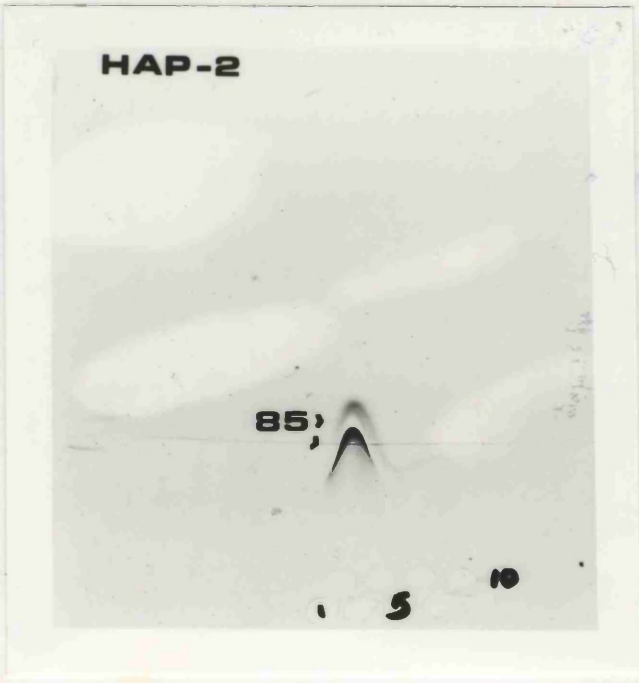
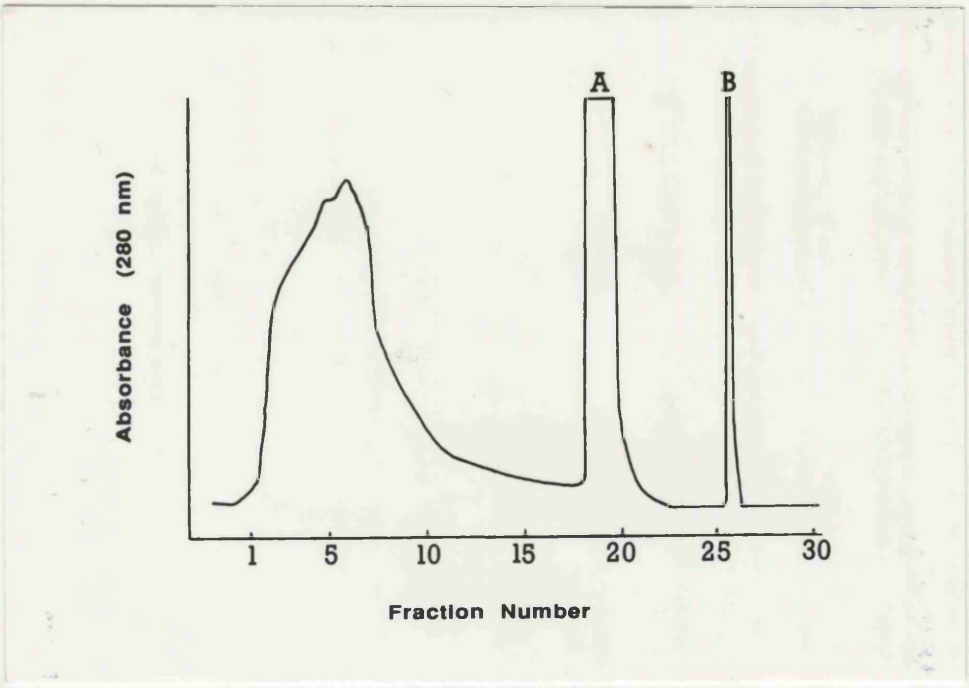


Fig.2.6 (b) FRIE analysing the separation of BCG culture filtrate by hydoxyapatite chromatography using the polyclonal anti-BCG serum or the anti-BCG85 serum (c) in the top gel layer.



B

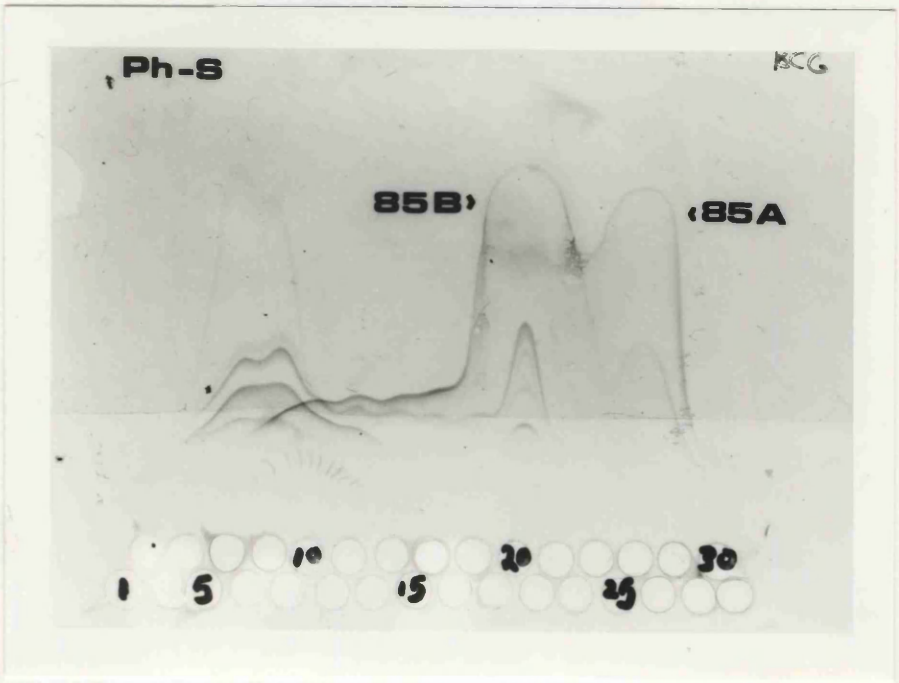
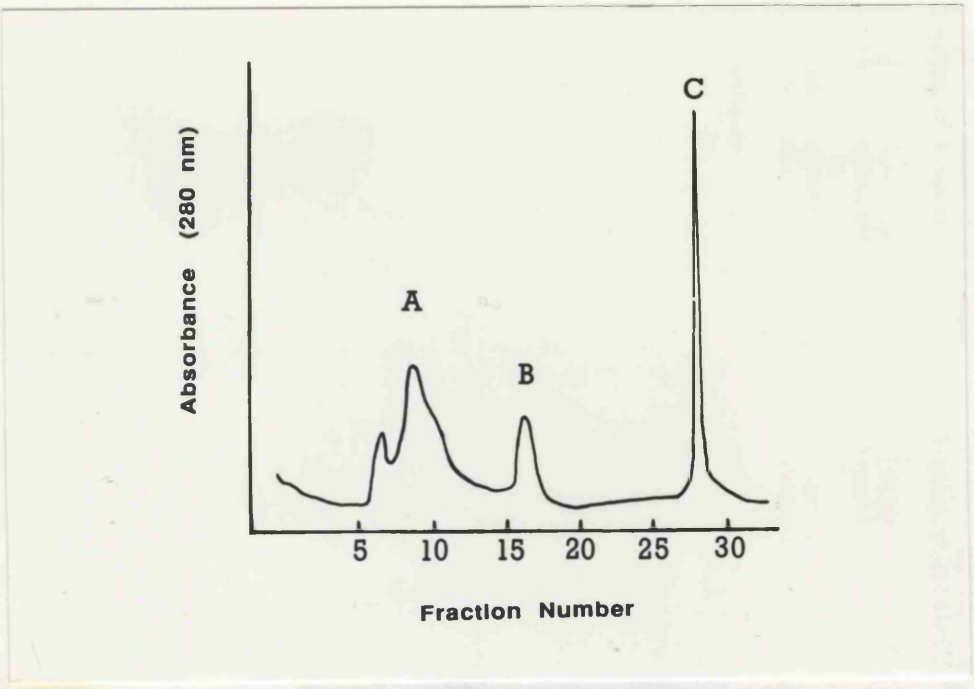


Fig.2.7. The separation of the BCG85 -containing fraction collected from the hydroxyapatite column on a hydrophobic column chromatography (a) and the FRIE analysis of this column using the anti-BCG polclonal serum (b).



B

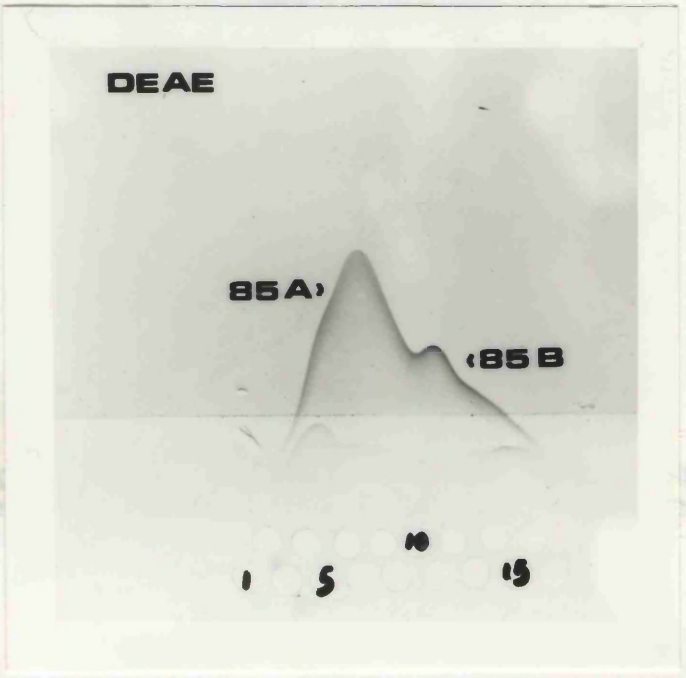


Fig.2.8. (a) The fractionation of the BCG components (collected from the phenyl sepharose column) on the DEAE-sephacel column. (b) shows the FRIE analysis of the collected fractions using the anti-BCG polyclonal serum.

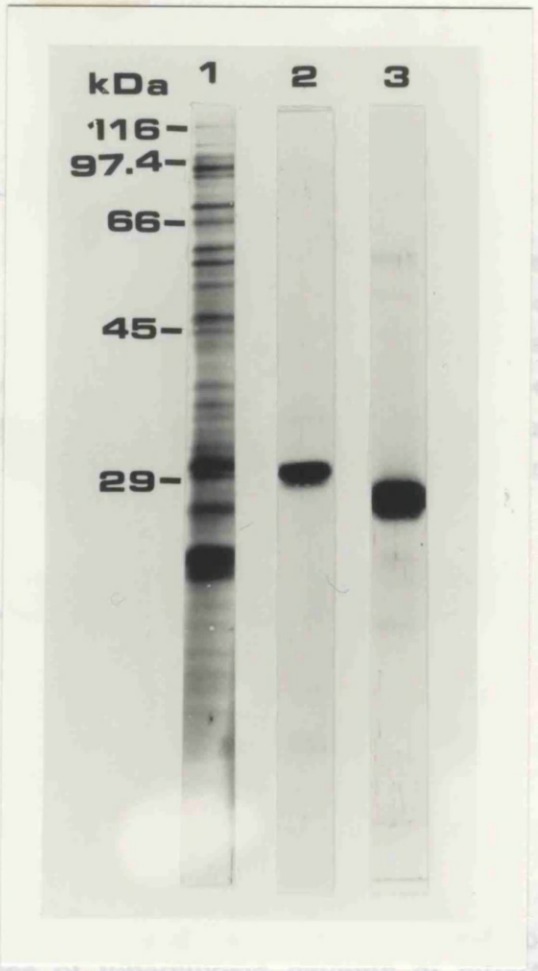


Fig.2.8. (c) An aurodye-stained blot of the culture supernatant of BCG (1) and the purified components of the BCG85 complex, A (2) and B (3).

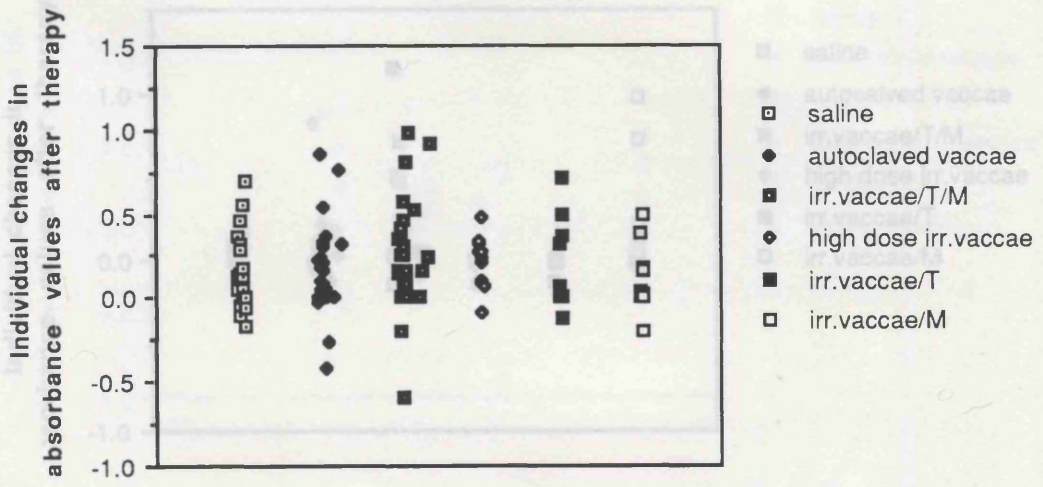


Fig.2.9.The individual changes in the antibody responses of tuberculosis patients of different groups to the culture filtrate of M.tuberculosis.

-All groups showed significant rises in their responses (paired-t test).

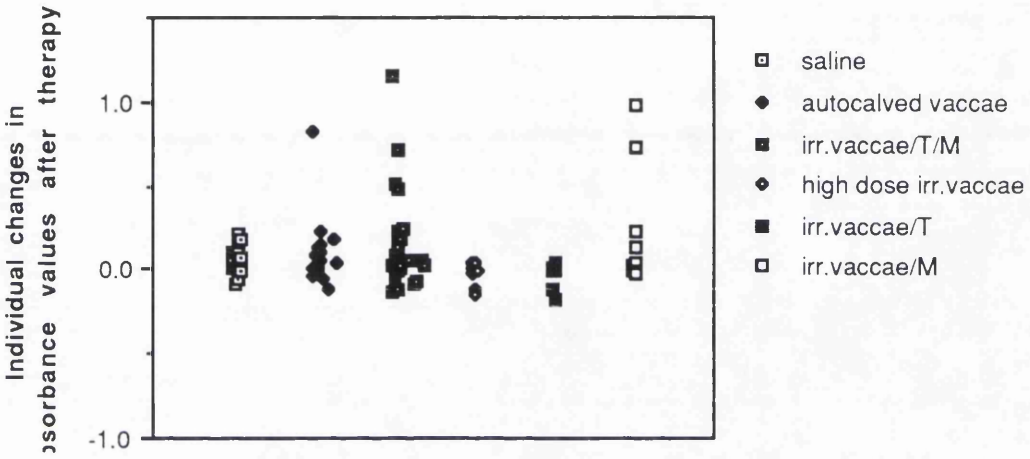


Fig.2.10A. The individual changes in the antibody response of tuberculosis patients of different groups to the purified BCG85-A.

p<0.025	for the saline group	(paired-t test)
p<0.005	for the irr.vaccae/T/M	(paired-t test)
p<0.05	for the aut.vaccae	(paired-t test)

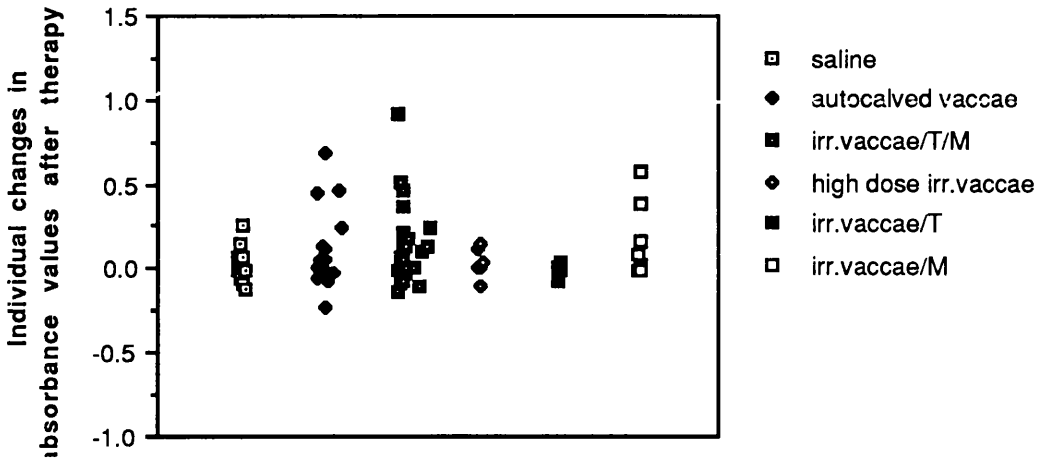


Fig.2.10B. The individual changes in the antibody response of tuberculosis patients of different groups to the purified BCG85-B.

p<0.05	**for the aut.vaccae group**	**(paired-t test)**
p<0.01	**for the irr.vaccae/T/M**	**(paired-t test)**
p<0.0025	**for the irr.vaccae/T**	**(paired-t test)**
p<0.05	**for the irr.vaccae/M**	**(paired-t test)**

patients injected with the autoclaved bacilli also demonstrated a significant increase in their responses to the two components. In addition, the saline-treated group had a significant increase in their reponse to the BCG85-A while those who received the irradiated mycobacteria with either the Tuberculin or Murabutide showed an enhanced response to the BCG85-B antigen.

2.3.3. Changes in agalactosyl IgG after therapy :

Tb patients with active disease are known to have high G0 level (Rook, 1988c). However, the screening of our patient's sera before receiving therapy showed their uneven distribution since most of the patients in the saline group had normal levels of G0 while most of those who received immunotherapy had abnormally high levels (Fig.2.11). Screening the patient's sera, before and after therapy, for changes in the G0 values showed, in comparison to the control group, a decrease in the G0 levels in some of the patients who received the irradiated bacilli/T/M, the autoclaved bacteria, or the high dose of irradiated bacteria (Fig2.12A). However, these changes are difficult to interpret since the groups were not evenly divided. Thus, in order to make the groups more comparable, the data were replotted using only the patients who had G0 levels more than 7% higher than normal-age corrected values before therapy. This confirmed the fall induced by the most successful immunotherapy regimens when compared to the saline group, and the recepients of the uneffective preparations (Fig2.12B).

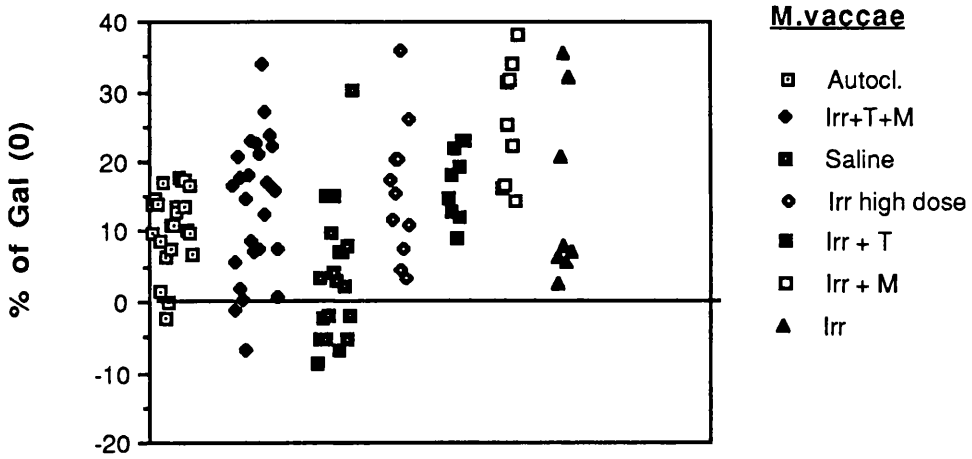


Fig.2.11. The percentage of agalactosyl IgG in the serum of Tb patients before therapy.

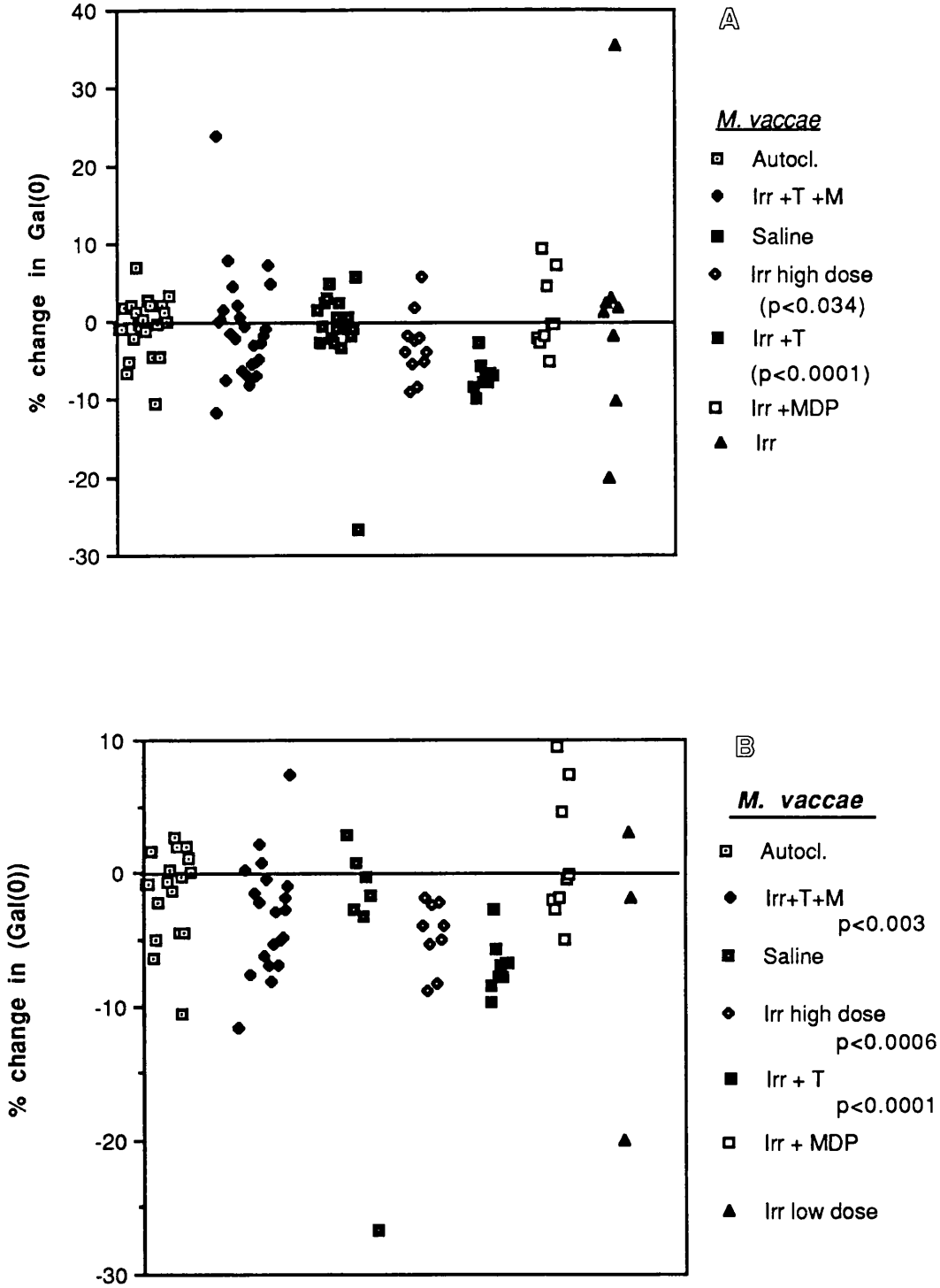


Fig.2.12. The percentage change in agalactosyl IgG (Gal 0) one month after immunotherapy of all Tb patients of different groups (A) and those who had a Gal (0) value that was higher than the control value by 7% at time of immunotherapy (B).

2.4. DISCUSSION

Examination of the Western blots of the culture filtrate of *M.tuberculosis* H37Rv incubated with the sera of Tb patients who received various formulations of immunotherapy demonstrated that the administration of irradiated *M.vaccae* plus New Tuberculin (T) plus Murabutide (M), in conjunction with chemotherapy enhanced their antibody response to the BCG85 and 23 kDa secreted antigens and significantly reduced their anti-hsp65 antibody response within one month of treatment (see Table 2.2). In addition, patients treated with the autoclaved bacteria showed significant diminution in their anti-50 kDa and the anti-hsp65 antibodies while the saline-injected patients showed insignificant changes in their antibody responses. In comparison, healthy donors were shown to have antibody responses to the hsp70, hsp65 and the 23 kDa, similar to those of Tb patients, but little antibody to the 38 and 31-29 kDa proteins.

Further evidence for the role played by the secreted antigens was provided by a recent study which compared the proliferative responses of spleen lymphocytes of mice immunised with killed or live mycobacteria (Andersen *et al*, 1991). Their study indicated that although the two groups responded similarly to a number of mycobacterial antigens (e.g. 17-19 and 65 kDa), only those injected with live bacilli could recognise the secreted antigens, particularly the 31-29 kDa. The secreted antigens of *M.tuberculosis* were divided into 3 goups (Andesen *et al*, 1991). First, those released (or excreted) in massive amounts early in the culture

Table 2.2. The statistical significance of the changes in the antibody responses detected treated Tb in patients, to various mycobacterial antigens and agalactosyl IgG.

Group	Antigens Tested					
	65[1]kDa	23[1]kDa	31[2]kDa	29[2]kDa	Tb culture filtrate[2]	Gal 0
Saline	NS[3]	NS	0.025[4] ↑	NS	0.005	NS
Irradiated *M.vaccae* /T/M	0.05 ↓	0.05	0.005 ↑	0.01	0.0005	0.003 ↓
Autoclaved *M.vaccae*	0.05 ↓	NS	0.05 ↑	0.05	0.0025	NS[6]
High dose irradiated *M.vaccae*	NS	NS	NS	NS	0.0005	0.0006 ↓
Irradiated *M.vaccae* /T	ND[5]	ND	NS	0.0025	0.025	0.0001 ↓
Irradiated *M.vaccae* /M	ND	ND	NS	0.05	0.05	NS

1. results from Laser scans of Western blots
2. results from ELISA using purified antigens
3. Not Significant
4. p value using paired *t*- test
5. Not Done
6. Although not significant overall, 5/17 patients showed e significan fall in Gal (0).

medium, represented by the 23 kDa. Second, slowly released proteins which are also present in the SDS extraction of mycobacterial bacilli and these include the hsp70, 17-19 and the 31-29 kDa antigens. These are probably secreted, except for the hsp70, by viable bacteria to be attached to the outer cell wall. The third group includes cytoplasmic antigens which are released from dead bacteria during the late logarithmic growth phase and it is represented by the hsp65.

Therefore, these previous reports prompted us to investigate whether there are qualitative or quantitative changes in the antibody responses of Tb patients to the constituents of short-term mycobacterial culture fluid, particularly the ones identified as being secreted. Examination of the Western blots and the scans of the Tb patients agrees with the previous observation (Grange, 1989) that they have highly variable antibody responses to mycobacterial antigens. However, most of these patients showed significant changes in their antibody responses to two secreted antigens, the BCG85 and 23 kDa.

Various antigens that exist in the culture filtrate of mycobacteria were shown to be sticky and therefore it is difficult to obtain the the purified antigens in sufficient amounts for further studies. However, the combination of several purification methods that use different principles was previously shown to allow for a better separation of these proteins (Wiker *et al*, 1986). Therefore, the application of a series of different chromatographic procedures to purify the BCG85 antigen made this protein available for further studies. The significant rises in the anti-BCG85 (31-29 kDa) antibodies in some of the immunotherapy groups,

particularly the recipients of irradiated vaccae/T/M and the autoclaved bacteria, were confirmed by ELISA using the purified antigen (Table 2.2). However, the saline group also showed significant increase in their anti-BCG85A response although the changes in their responses were much smaller than those of patients who received immunotherapy. Furthermore, the present findings demonstrate that chemotherapy alone or in combination with effective immunotherapy significantly enhances the antibody response of Tb patients to the culture filtrate of *M.tuberculosis* H37Rv, as shown by ELISA (Table 2.2).

Tb patients who had agalactosyl IgG (Gal 0) levels 7% higher than the normal values before therapy and received an injection of *M.vaccae* with T, M or both showed a significant reduction in the proportion of the agalactosyl antibody after treatment (Table 2.2). Moreover, 5/17 recipients of autoclaved *M.vaccae* also showed a significant fall whereas the *M.vaccae* +M or saline recipients did not. Thus, there is some agreement between the different ways of assessing immunotherapy.

Since the Tb patients under study were bled 4 weeks after receiving immunotherapy, in view of the half life of IgG (3 weeks) it seems to be early to look for changes in the humoral response. However, the significant changes in the antibody responses (an increase in the antibody responses to the BCG85 and the 23 kDa proteins and a decrease in the anti-hsp65 and % Gal (0) response) of patients given the irradiated bacteria/T/M or the autoclaved bacteria correlates with the improvement seen in their immunological parameters (lymphocyte proliferation and antibody responses to mycobacterial sonicated antigens) (Bahr *et al* , 1990). This is

rendered more striking by the observation that % Gal (0) does not normally fall in Tb patients for 6 months (F. Onyebujoh, personal communication) and further attempts to check the validity of an accelerated fall in % G (0) as a measure of recovery, and the success of immunotherapy, are in progress.

Tuberculosis patients (77%) were shown to have positive Interferon-gamma (IFN-gamma) production, lymphocyte proliferative and antibody responses to the BCG85-A (Huygen *et al*, 1988a). However, tuberculin-positive normal donors showed specific lymphocyte proliferation and IFN-gamma production but no antibody response to this antigen. Furthermore, spleen cells from BCG-sensitised mice secrete IFN-gamma in response to the BCG85-A antigen (Huygen *et al*, 1988b). Although the BCG85 antigen was shown to mediate the adherence of mycobacteria to fibronectin with high affinity, the significance of this finding or its relation to the biological role of this antigen is still unkown. However, this adherence could lead to the invasion of the host cells. Fibronectin binding proteins were shown to play a role in the virulence of pathogenic bacteria such as *Streptococcus pyogenes* and *Staphylococcus aureus* (reviewed by Young *et al*, 1990). Thus, it is possible that the early recognition of this antigen is important for the effective elimination of the infecting bacteria.

The 23 kDa of *M.tuberculosis* has been recently identified as a superoxide dismutase (SOD) and therefore it·s extracellular location may contribute for the resistance of these intracellular pathogens to the lethal effect of oxygen radicals produced by the host phagocytes (Andersen *et al*, 1991; Zhang *et al*, 1991).

There is good evidence that protection in mycobacterial

infections is correlated with the cellular immune response whereas antibodies are assumed to play no major protective role in this infection. However, the development of an antibody response to antigens secreted in massive amounts and which have a biological importance for the survival of the pathogenic bacteria can probably aid in the early recognition and elimination of these bacteria. Alternatively, the rise in the antibody response could be a reflection of a change in the nature of the T cell response into one which is providing an activation signal for B cells. The rapid rise in the antibody response could also be an indication of the enhanced recognition and destruction of live bacteria leading to the sustemic release of the bacterial antigens in large amounts.

The mycobacterial hsp65 is a highly conserved antigen that shares sequence homology with self antigens (reviewed by Young *et al*, 1990). Furthermore, this antigen was described as an immunodominant molecule inducing both T and B cell responses. The antibody response to the hsp65 may be correlated with the dysregulation in cytokine production as seen in Rheumatoid arthritis. Thus, the fall in the anti-hsp65 response in the immunotherapy-treated patients could be attributed to the regulatory effect imposed by *M.vaccae* used in the immunotherapy and it is tempting to relate this effect to the fall in G (0), as suggested in rheumatoid arthritis though no link has benn identified at the molecular level (Rook *et al*, 1991).

Finally, the increase in the Gal 0 values in Tb patients correlates with necrotising T cell-dependent pathology. Therefore, the significant diminution in its levels may indicate the achievment of one of the aims of immunotherapy, that is the conversion of the response of these patients into a protective non-necrotising one.

In the following chapters, we describe our attempts to further examine the role of mycobacteria in the preparation of experimental animals for the TNF-mediated necrotic tissue damage and testing the various possiblities of switching off such resoponse. In addition, the present results prompted us to further investigate the role of the BCG85 and hsp65 in the production or the modulation of this tissue damage, in a murine model.

CHAPTER THREE

The role of soluble mycobacterial antigens in the preparation of tissues for tumor necrosis factor-mediated local Shwartzman reactions.

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3.1. INTRODUCTION

Shwartzman, in 1928, used the rabbit as an animal model for studying the phenomenon of local tissue damage, described later as the local Shwartzman reaction (LSR). The local reaction resulted from an intradermal injection of a culture filtrate of gram-negative bacilli followed 24 hours later by intravenous administration of the same preparation of culture filtrate. Other animal spieces such as the mouse, the guinea pig and the horse were found by various investigators to be refractory to this reaction. On the other hand, Arndt *et al* (1960) demonstrated, using various mouse strains, the irregular incidence of LSR in very few inbred strains such as C-57 and ICR-swiss, suggesting the importance of the genetic make-up as a determinant for their reactivity.

In 1988, Rothstein & Schreiber described the induction of a local Shwartzman-like reaction in pathogen-free mice (C3H/HeN & BALB/cAn) which had been prepared subcutaneously with bacterial endotoxin and then challenged 24 hours later with endotoxin-free TNF (Rothstein & Schreiber, 1988). The reaction could also be prepared with a gram-positive bacterium, *Corynebacterium parvum*, or myco-plasma (cell wall deficient organism)-infected cell lysate. The ability of a purified preparation of IL-1 produced by murine macrophages to both prepare and provoke a local Shwartzman reaction demonstrated previously in rabbits (Beck *et al*, 1986) is question-able because of the possibility of its contamination with TNF. Later on, neither the injection of an endotoxin-free highly purified recombinant preparation of IL-1, nor recombinant TNF alone were able to prepare a site for subsequent TNF-mediated necrosis.

Administration of both was required to prepare for such a reaction in rabbits (Movat *et al*, 1987). Thus, a mixture of both cytokines can replace the preparatory injection of LPS. Moreover, Recombinant TNF has been proved, in Rothstein & Schreiber's report, to substitute, on its own, for the gram-negative bacteria culture filtrate used for provocation in the classical local Shwartzman reaction. On the other hand, TNF used for provocation could not be replaced by other recombinant cytokine preparations like Interlukin-1 (IL-1) or IFN-gamma. In this chapter, we have addressed the question whether mice develop LSR, and if they do so, whether it is restricted to certain strains. In addition, some improvements to the animal model introduced by Rothstein & Schreiber for studying LSR were made. First, much smaller doses of both the preparatory (LPS) and provocative (TNF) injections were used. Second, we used two different quantitative measures for a precise evaluation of the reaction using the foot-pad skin as a target instead of the skin of the back used in their model. In order to test the hypothesis suggesting the analogy between the Koch phenomenon and the local Shwartzman reaction, we have attempted to investigate the ability of mycobacteria, both pathogenic (*M.tuberculosis* and *M.leprae*) and non-pathogenic (*M.vaccae*) to prepare for LSR in mice. The effect of immunisation with a mycobacterium, *M.vaccae*, on the intensity of LSR and the role of various purified mycobacterial antigens, in this reaction, is also examined.

3.2. MATERIALS AND METHODS

3.2.1. Mice :

8-10 weeks old female mice of the C57/Bl/T, Balb/c and CBA strains were inbred colonies maintained at the animal unit in the School of Pathology, Middlesex Hospital Medical School.

3.2.2. Cytokines and bacterial antigens :

Recombinant murine TNF (lot no. 4296-17) a gift from Genentech (South San Francisco,CA), had a specific activity of $1.2x10^7$ units (U)/mg and endotoxin level of 0.51 endotoxin units (EU)/mg, as measured by *Limulus* amebocyte lysate assay. Recombinant human TNF (specific activity of $3x10^7$ U/ml) was also used. Lipopolysaccaride, LPS of *E.coli* 055:B5 (Cat.No. L-2880, Sigma Chemical Co., st. Louis ,MO) was diluted in pyrogen-free saline. Lipoarabinomannan (LAM), a mycobacterial cell wall component purified from *M.tuberculosis* (H37Rv) was kindly provided by Dr. C. Moreno (MRC Tb unit, Hammersmith). *Mycobacterium vaccae* strain R877R (NCTC 11659), originally isolated from Ugandan mud by Dr J.L. Stanford (Microbiology Department, Middlesex Hospital Medical School), was grown on Sauton·s medium solidified with 1.5% agar. Harvested organisms were suspended in saline and sonicated for 15 minutes in a 100 Watt ultrasonic disintegrator for 15 minutes with the wave peak distance set at 8-9 um, spun at 13,000 g for one hour and then the supernatant was sterilised by filtration through Millipore filters (Millex-GV, 0.22 um). Protein concentration was measured by the method of Lowry *et al* (1951).

Soluble antigens of *M.tuberculosis* (H37Rv) (New Tuberculins) were prepared in the same way. The sonicate of armadillo-grown

M.leprae (batch CD 99) was kindly provided by Dr. R.J.W. Rees, Clinical Research Centre, U.K. Autoclaved *M.vaccae* NCTC 11659 (for 10 minutes at 116°C), which was generously provided by Dr. J.L. Stanford, was used for the immunisation of C57/Bl/T mice at a concentration of 10^9 bacilli/mouse, given by various routes.

3.2.3. The assay for local Shwartzman reaction :

The lower backs of mice were shaven using a chemical depilator (Immac, Whitehall Laboratories, New York) a few hours before starting the experiment. Mice were prepared by injecting LPS at 10 ug or *M.vaccae* sonicated antigen (Vaccin) at 17 ug given subcutaneously (s.c.) in 40 ul of pyrogen free saline either on their lower back (at the base of the tail) or in their foot-pads (four mice were used per group). They were then challenged, 24 hours later, with 1.2×10^4 U/1 ug of r-TNF, given s.c. at the the same site. Haemorrahgic necrosis (HN) was examined 24 hours later macroscopically, seen as dark red lesion when the lower back was tested and was given arbitary score. When the foot-pad skin was challenged, the increase in its thickness was measured using a Mitutoyo thickness guage (code no. 7308, Japan). The challenged foot was then cut and treated with 2 ml of a 1:4 mixture of 10% Cetrimide (Mixed AlkyltriMethylammon-ium Bromide, Sigma, M-7635) and 1N Sodium hydroxide. These feet were then left for 48 hours in a water bath set at at 60°C for the extraction of the haemoglobin (Hb) derivative, the concentration of which was then measured on the spectrophotometer at 570 nm. The final concentration of the Hb (or the haeme) derivative was then read off a standard curve constructed using a purified human haemoglobin preparation (Sigma, H-7379 dissolved in the cetrimide/sodium hydroxide mixture).

3.2.4. Statistical analysis :

Student's *t* -test and Mann-Whitney U test were used throughout the whole study (except when otherwise mentioned) to compare the test groups with the control ones. Data are expressed as mean±standard deviation.

3.3.1. Local Shwartzman reaction (LSR) in different mouse strains :

The ability to induce LSR in CBA, C57/Bl/T and Balb/c mouse strains was tested. Mice (4 per group) were injected s.c., at the base of the tail, with 10 ug of LPS in 40 ul of pyrogen free saline, followed 24 hours later, by another injection of rhu-TNF at a dosage ranging from $6x10^5$ U (9.9 ug) to $2.2x10^4$ U (0.36 ug). Massive haemorrhagic necrosis (HN) was seen in C57/BL/T mice with a 100% incidence of necrosis at a wide range of TNF doses used (Fig. 3.1 & 3.2). In contrast, CBA and Balb/C showed much lower incidence of necrosis (50% and 60% respectively) and smaller lesions even at the highest TNF dose used. Neither the preparatory (LPS) nor the provocative (TNF) injection alone could produce such a positive reaction. Therefore, it was decided to use C57/BL/T mice for the following experiments and a dose response curve was constructed to choose the optimum dose of TNF. Doses of rhu-TNF ranging from $2x10^5$ U (3.3 ug) to $3.6x10^3$ U (0.033 ug) were used for provocation. As shown in Fig. 3.3, these mice showed a 100% incidence of necrosis at a dose as low as $7.3x10^3$ U (0.11 ug).

3.3.2. The mouse foot-pad as a target for LSR :

The mouse foot-pad skin was chosen as an alternative for the skin of the back for two main reasons. First, swelling of the foot was measured by comparing the thickness of the foot-pad before and 24 hours after preparation and/or provocation whereas no corresponding measurement was possible using the skin of the back. Second, the skin of the challenged foot-pad remains intact (in contrast to the skin of the back which becomes completely necrotic) and hence

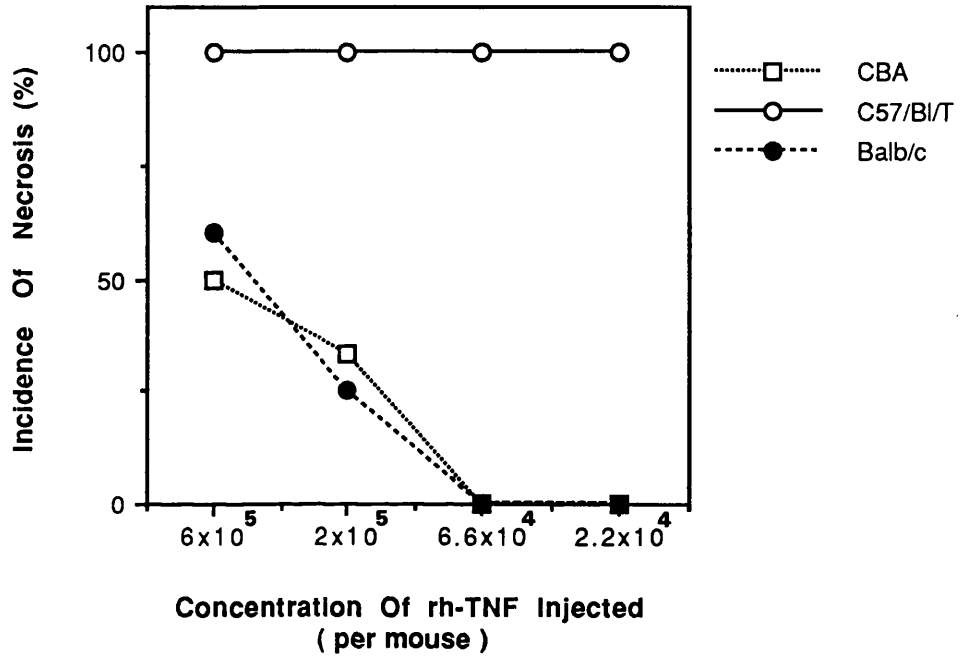


Fig.3.1. The response of various mouse strains to a local Shwartzman-like reaction.

Fig.3.2. Haemorrhagic lesions (LSR) in the skin at the base of the tail of mice detected after sequential subcutaneous injections of M.vaccae and TNF.

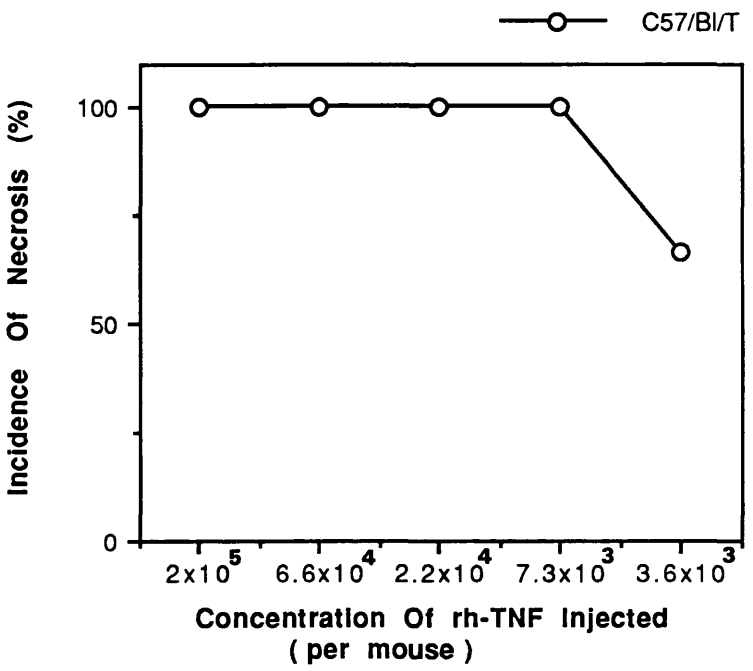


Fig.3.3. Skin lesions induced in C57/Bl/T mice induced by sequential injections of LPS and various doses of rHu-T NF.

allows for the measurement of the haemorrhagic component of the reaction (Fig.3.4). Therefore, after the thickness of the challenged foot is measured, it is cut off and treated with 2 mls of a mixture of cetrimide and Sodium hydroxide to dissolve the tissues. A clear suspension that is yellow to brown in colour was obtained after an incubation period of 48 hrs at 60°C. In an attempt to identify the nature of this extract, its absorption spectrum was measured on a scanning spectrophotometer at wave lengths of 400-700 nm. As shown in Fig. 3.5A, two major peaks were seen, one at 400-450 nm and the other at 560-620 nm. The absorption spectrum of the extract was found to be similar to that produced by several haemoglobin pigments (Dacie & Lewis, 1991; Gowenlock, McMurray and McLauchlan, 1988) and by a lyophilised human haemoglobin preparation dissolved in the cetrimide/NaOH mixture at 4 mg/ml (Fig. 3.5A). Therefore, a standard curve was constructed using a purified preparation of human haemoglobin dissolved at various concentrations in the same mixture used for dissolving the foot tissues. Then the optical density of such preparations was measured on the spectrophotometer at 570 nm, within the second peak (Fig. 3.5B) since although most of the Hb pigments are known to show maximum absorbance at 400-450 nm, the second peak is more specific for these pigments. The concentration of the Hb derivative in the tissue extract is then read off the standard curve.

3.3.3. Kinetics of the local Shwartzman reaction in the foot-pad :

A time course for changes detected in both the thickness and the Hb derivative concentration in the challenged feet was followed. In

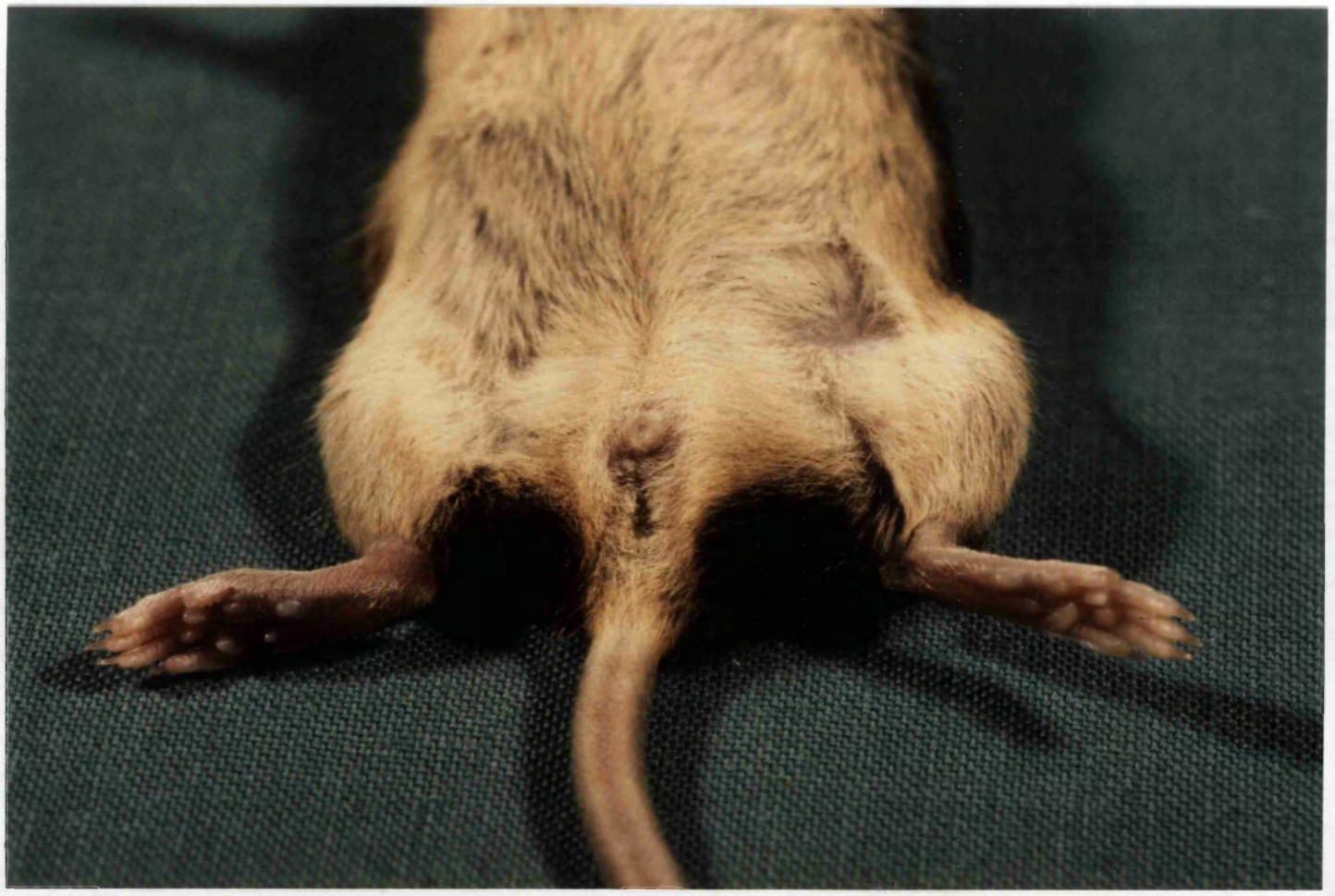


Fig3.4. Haemorrhage and swelling seen in the right foot-pad of an animal challenged for a LSR.

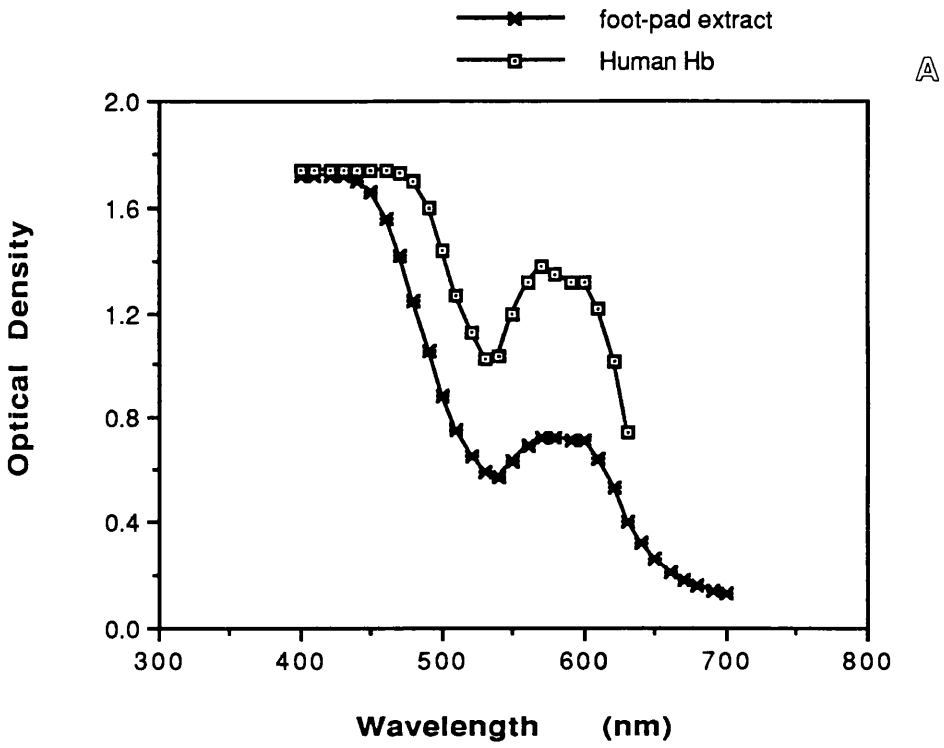


Fig.3.5A. The absorbance spectrum of the challenged foot-pad extract, compared to that of the purified human haemoglobin.

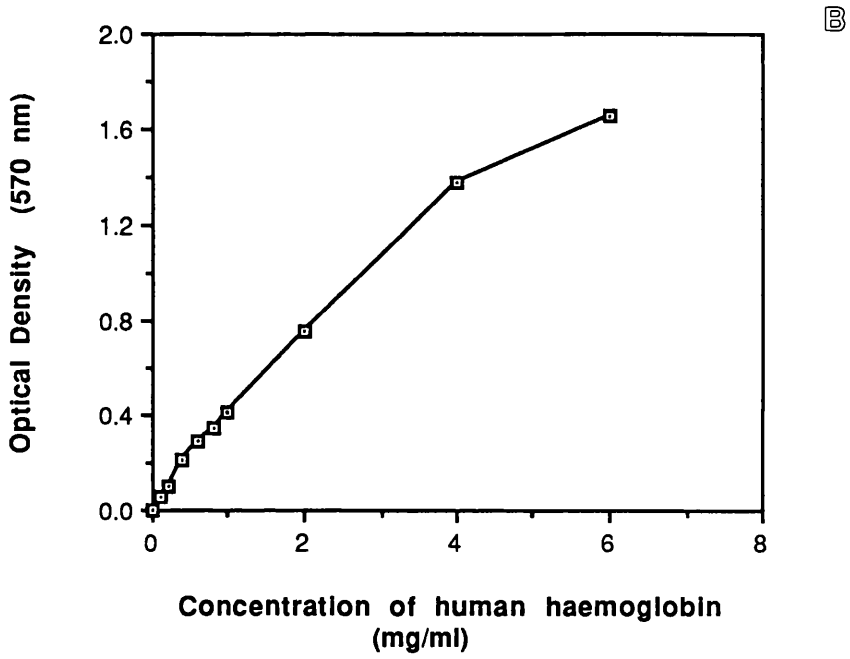


Fig3.5B. The standard curve used for the estimation of the concentration of haemoglobin derivative extracted from the challenged feet.

mice given only a subcutaneous injection of LPS, a small increase in the thickness of their feet was seen by 24 hours (0.0015+0 inches, mean+standard deviation) (Fig. 3.6A). A larger response was seen after the injection of TNF with a peak at 16 hours (0.0045+0.001 inches). An enormous swelling of the foot was seen after sequential s.c. injections of LPS and TNF, reaching its maximum at 16 hours after TNF administration (0.0145+0.001 inches). Likewise, they also synergised to produce more haemorrhage, with a peak at 20 hours after provocation (0.72+0.025 mg) compared to that produced by LPS (0.4+0 mg) or TNF (0.24+0.005 mg) alone (Fig.3.6B). Based on these results, it was decided, for the subsequent experiments, to compare the thickness of the foot-pad before the preparation and 16 hours after provocation while the haemoglobin derivative is extracted 20 hrs after TNF administration.

3.3.4. Classical local Shwartzman reaction :

Since most of the previous investigators have reported that mice are resistant to the local Shwartzman reaction, we have attempted to test this notion in our model. LPS was injected at 10 ug under the skin of foot-pads, followed 24 hrs later with various doses (10, 40 and 80 ug) of the same preparation of LPS given intaperitoneally (i.p.). Not only was a negligible response (both swelling and haemorrhage) obtained in these mice but also the swelling induced by the preparatory injection was inhibited when the provoking injection was given i.p. (Fig. 3.7) confirming the previously described results. The same observation was made when both injections were given subcutaneously (data not shown).

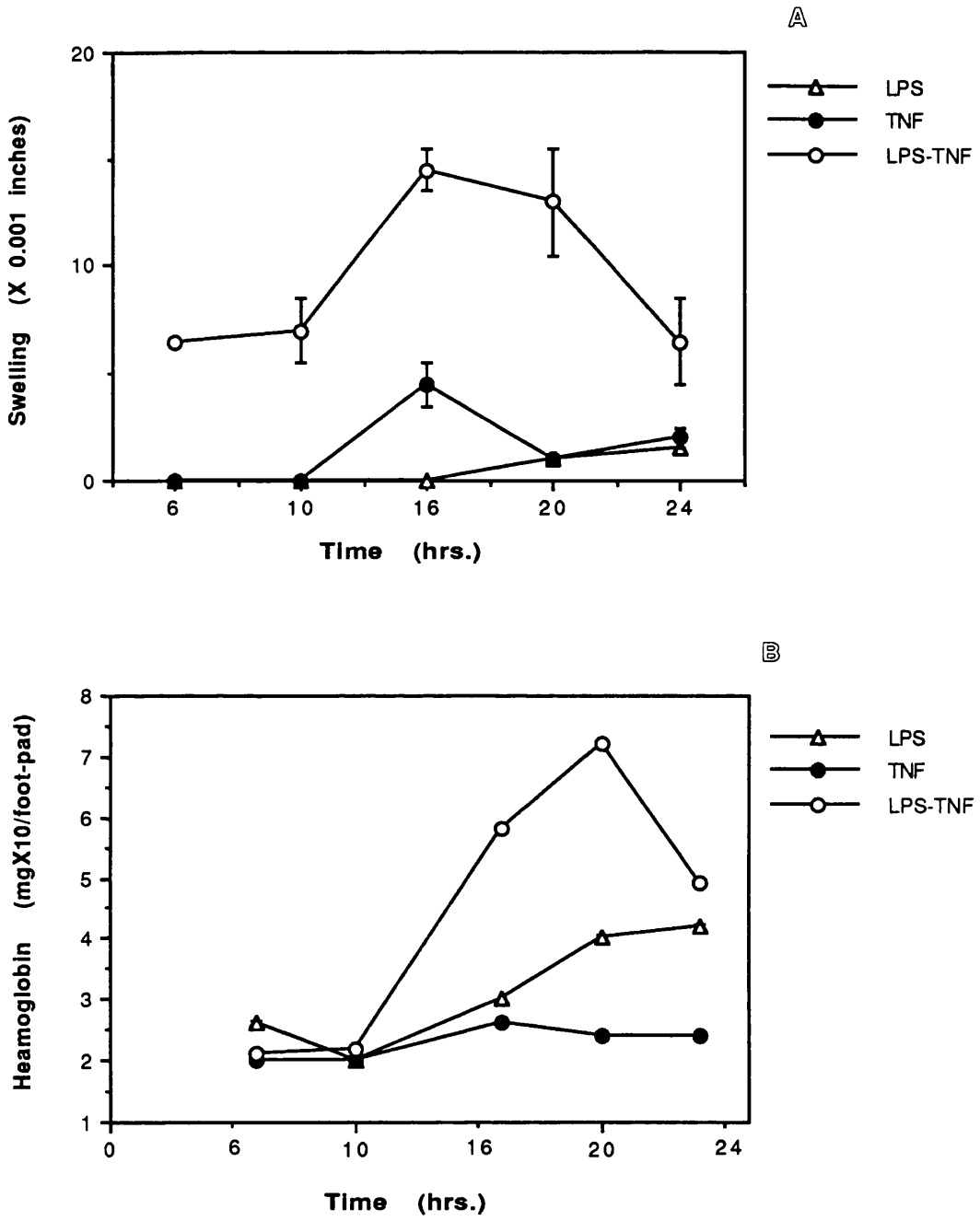


Fig.3.6.The time course of the swelling (A) and the haemorrhage (B) detected in the foot-pads of challenged mice.

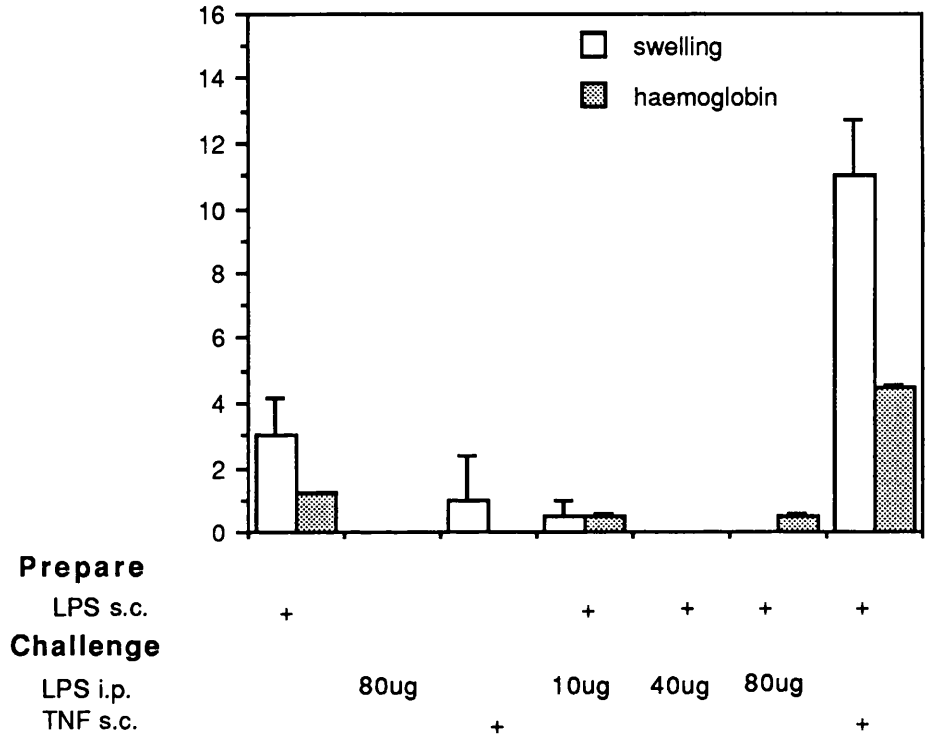


Fig.3.7. The failure of mice to mount a classical local Shwartzman reaction.

3.3.5. The ability of sonicated antigens of various mycobacterial species to prepare for LSR :

LPS was replaced by a preparation of sonicated antigens of *M.vaccae* (used at a range of 1-43 ug/mouse) given under the skin of normal C57/BL/T mouse strain, on their back. TNF (6.6x104 U/ 1ug) was also given s.c., at the same previously prepared site, after 24 hrs. An antigen dose correlated response was observed with a maximum response seen at 12.25 ug of *M.vaccae* antigenic preparation (Fig. 3.8).

Soluble antigens of pathogenic mycobacteria, *M.tuberculosis* (H37Rv) and *M.leprae*, were also tested. Like *M.vaccae*, these two mycobacterial spp. were also capable of preparing for a Shwartzman reaction (shown in Fig. 3.9), in a comparable way to LPS. However, animals that were prepared but not challenged with TNF produced a negative reaction.

3.3.6. Seasonal variation in the intensity of LSR in C57/Bl/T mice:

Another remarkable observation was that mice showed variable intensity in their response to preparation with both LPS and *M.vaccae* in different seasons (Fig.3.10, each point represents the results of 4 challenged animals). The strongest LSR was detected in December in response to *M.vaccae* and in December-January in response to LPS. Therefore, we addressed the question whether immunisation with *M.vaccae* bacilli would enhance their LSR in the seasons in which they show low reactivity.

3.3.7. The effect of immunisation with autoclaved *M.vaccae* on LSR :

C57/BL/T mice were immunised with an injection of 10^9 autoclaved *M.vaccae* bacilli given subcutaneously (s.c.) at the base of the tail, intravenously (i.v.), intraperitoneally (i.p.) or orally

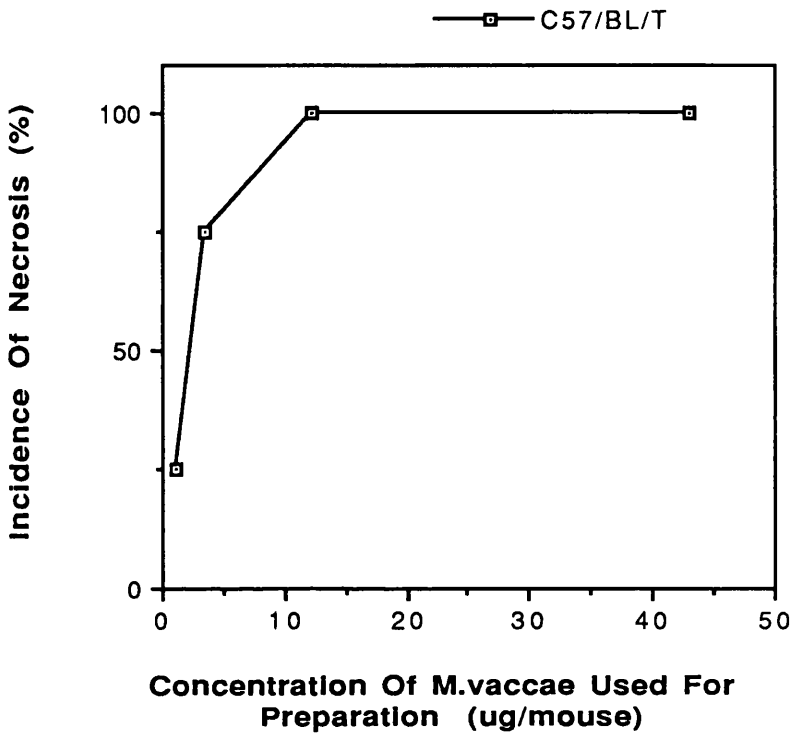


Fig.3.8. TNF-induced local skin damage in sites prepared 24 hours earlier by a wide range of M.vaccae sonicate doses.

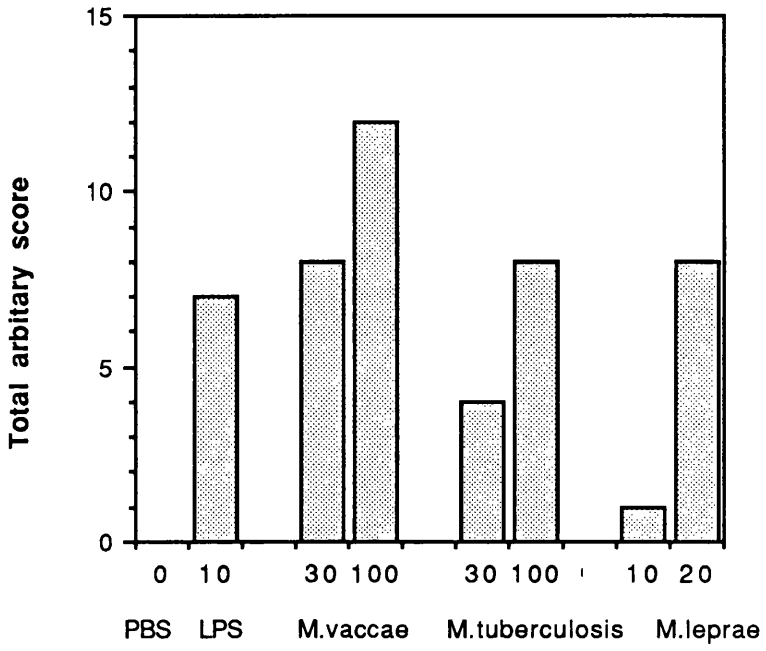
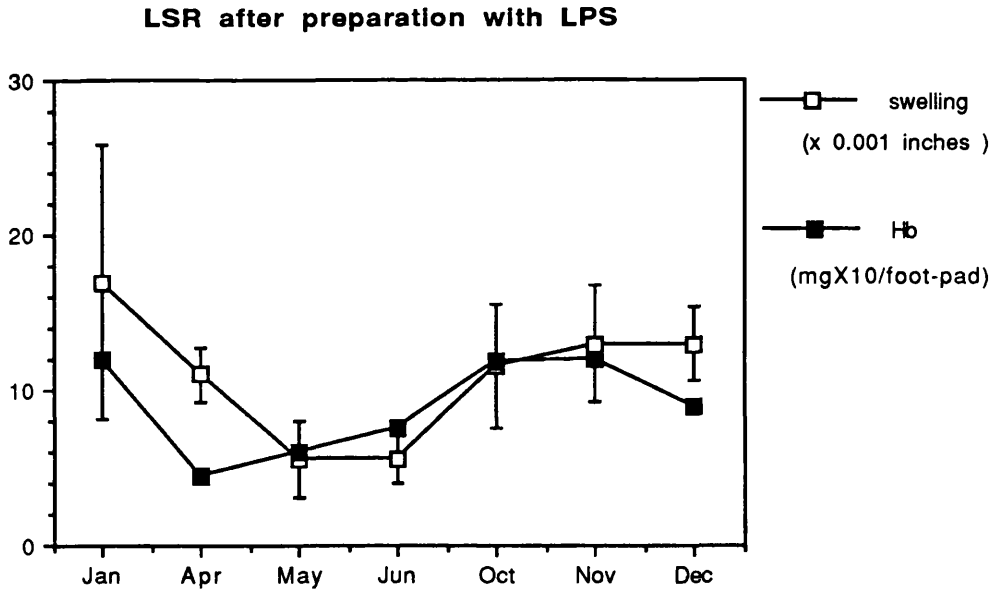


Fig.3.9. Local skin necrosis at sites prepared with the sonicates of various mycobacterial species.

scores: -~0 ; +~1 ; ++~2 ; +++~3



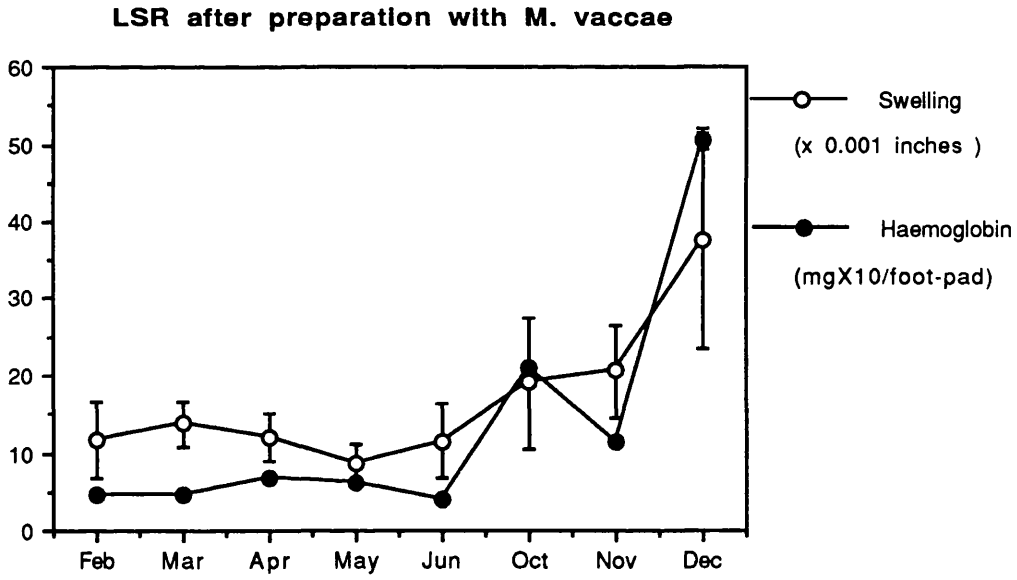


Fig.3.10. Seasonal variation in the TNF-mediated skin necrosis in sites prepared by LPS or M.vaccae sonicate.

(p.o.). Ten day later, the immunised mice were prepared with an injection of *M.vaccae* sonicated antigens (17 ug) and subsequently challenged with 1 ug of TNF given s.c. Compared to the control non-immunised animals, the subcutaneously immunised ones had enormous increase in both the swelling (0.0205+0.006 vs 0.0637+0.017 inches, respectively) and haemorrhage (1.14+0.4 vs 10.95+2.7 mg, respectively) (Fig. 3.11). Conversely, other routes of immunisation had almost no effect on either of the reaction components. In order to prove the specificity of the immune response mounted to *M.vaccae*, mice pretreated subcutaneously with killed *M.vaccae* bacilli were prepared with either LPS (10 ug) or *M.vaccae* sonicate (17 ug) and then challenged with TNF (1 ug) 24 hrs later. Although an enhanced LSR was seen in animals prepared with *M.vaccae* sonicate, no such effect was seen when a different unrelated antigen such as LPS is used for preparation (Fig. 3.12).

3.3.8. The role of LAM in inducing LSR :

Since LAM, a mycobacterial cell wall component, has been shown previously to induce TNF production both *in vitro* and *in vivo* models, it seemed of considerable interest to investigate its ability to induce LSR. C57/Bl/T mice were either prepared with LPS (10 ug, s.c.) and then provoked with LAM (10ug, s.c.), or prepared with LAM (10 ug, s.c.) and then challenged with TNF (1 ug, s.c.) and compared to those treated sequentially with LPS and then TNF. Feet treated with LPS and then LAM had significantly more haemorrhage than those challenged with only LPS or LAM but they had insignificantly lower swelling (0.0102+0.0086 inches) and haemorrhage (0.85+0.3 mg Hb) than those treated with LPS and TNF (0.017+0.0088 inches, 1.2+0.5 mg) (Fig.3.13). Moreover, feet challenged sequentially with LAM and then TNF had more haemorrhage

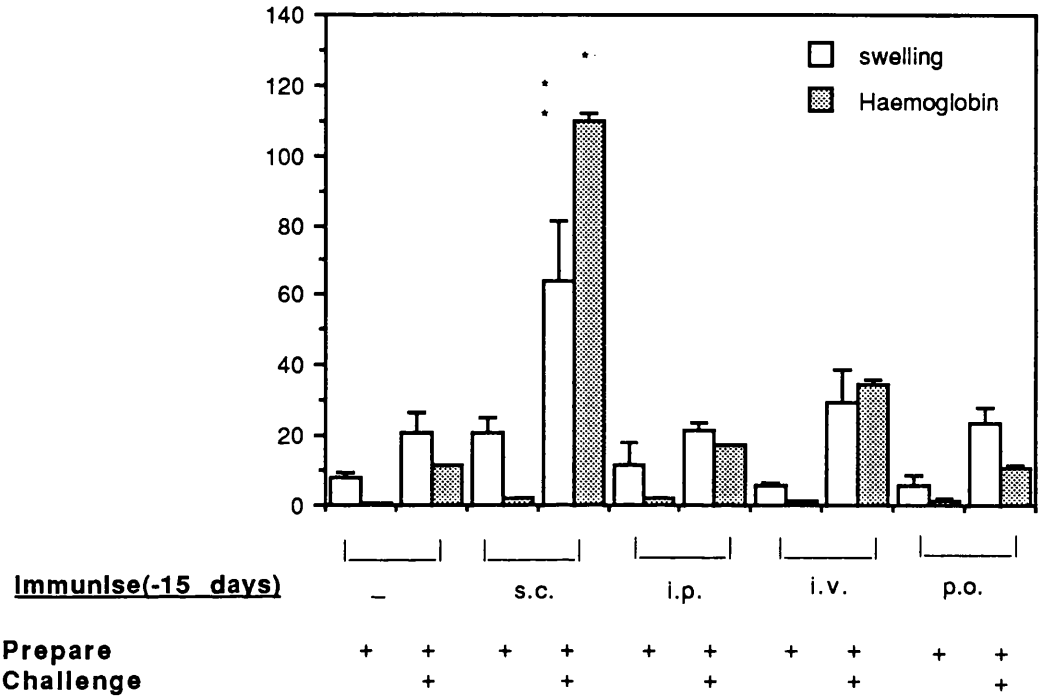


Fig.3.11. The effect of immunisation with autoclaved M.vaccae baciIli on the swelling and the haemorrhage at sites prepared with M.vaccae sonicate.

* $p<0.025$ (Students t-test)
** $p<0.01$ "

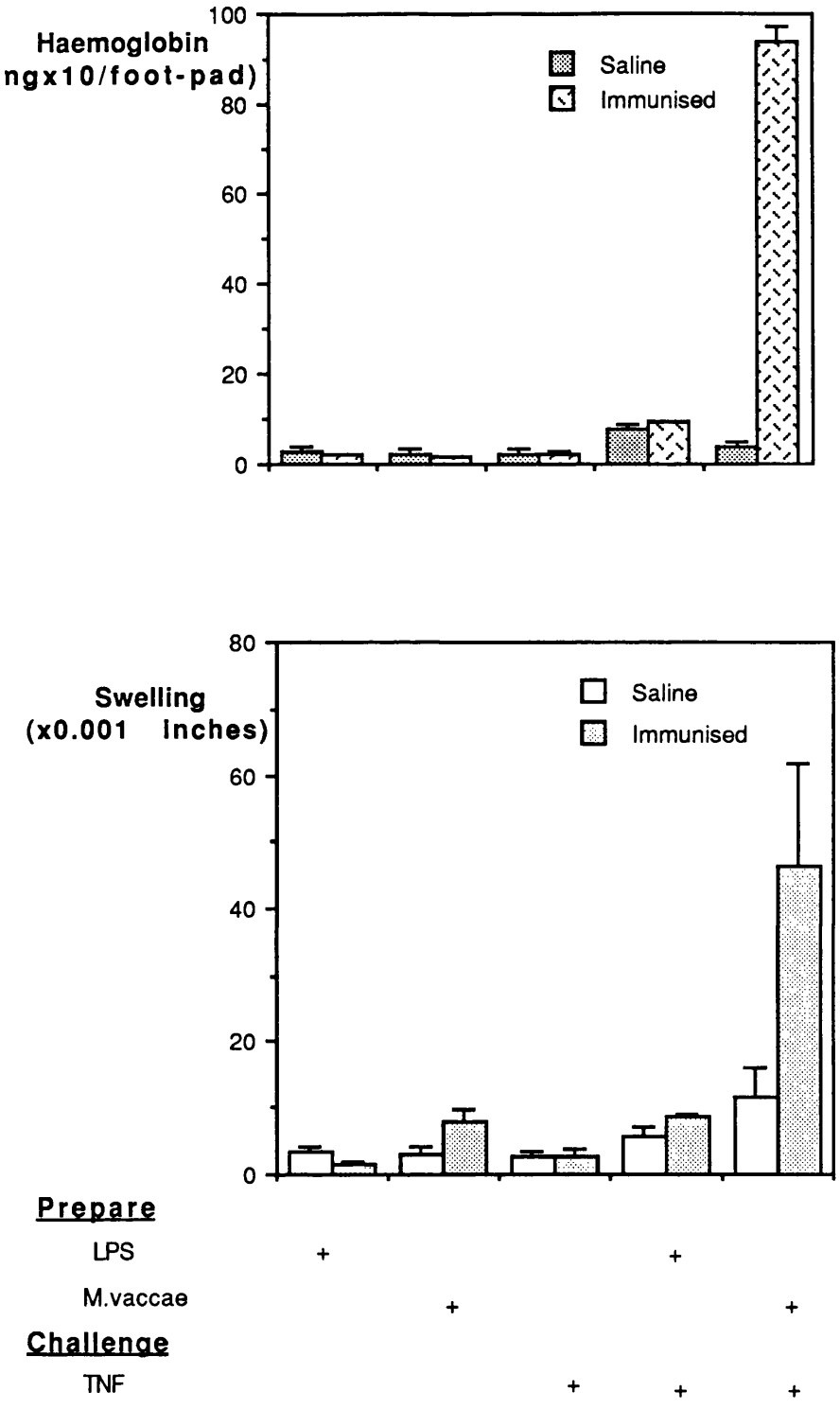


Fig.3.12. The enhancement of LSR at sites prepared by M.vaccae sonicate but not by LPS following immunization with autoclaved M.vaccae bacilli.

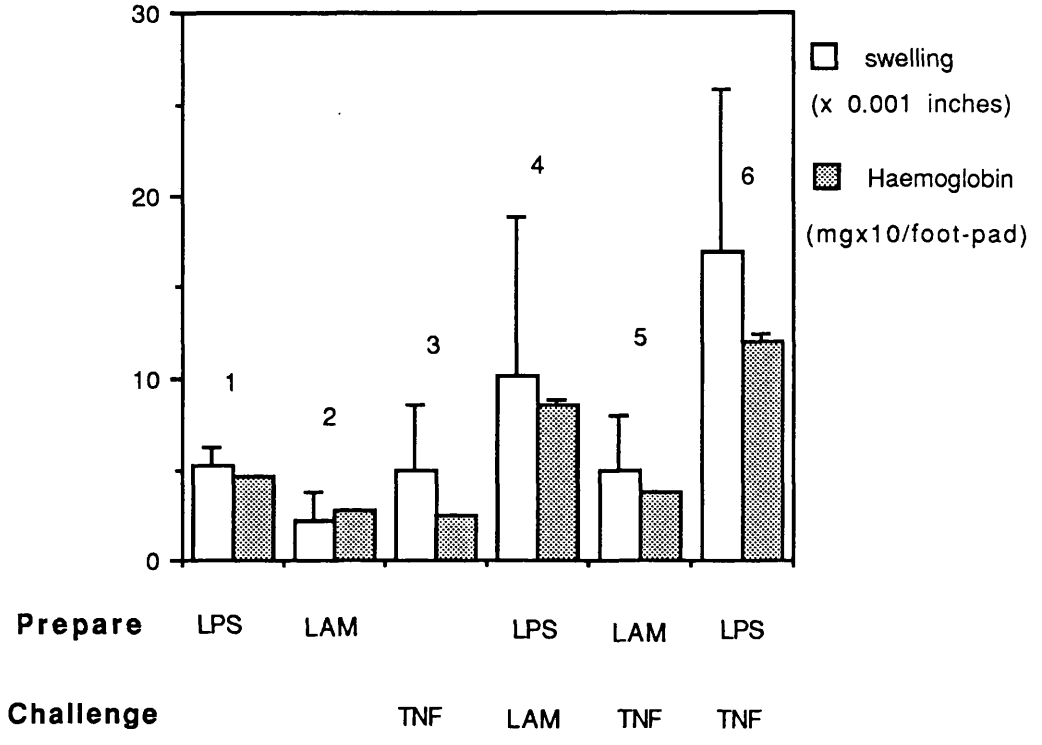


Fig.3.13. The role of the mycobacterial LAM in inducing a local Shwartzman reaction.

For swelling: 4 vs 2 $p<0.05$ (Mann-Whitney test)

For Hb: 5 vs 2,3 $p<0.05$ "
4 vs 1,2 $p<0.05$ "

than those injected with only LAM or TNF but they showed significantly less swelling and blood ($p<0.05$) than those treated with LPS and then TNF. These findings demonstrate that LAM does participate in preparing the skin site for a local tissue damage and more importantly they emphasize the role of LAM in inducing TNF production locally.

3.4. DISCUSSION

The present results confirm the work of Rothstein and Schreiber (1988) concerning the induction of a local necrotic skin tissue damage in mice, previously considered to be refractory to the local Shwartzman reaction (LSR). On the other hand, using much smaller doses (by 10 fold) for preparation and provocation than those used by Rothstein and Schreiber, we have demonstrated, that different mouse strains show variable intensity in their response to TNF provoked damage in LPS-prepared site, with C57/BL/T mice being the most reactive strain compared to CBA and Balb/c strains. Moreover, the use of the skin of the foot-pad, instead of the lower back used by Rothstein & Schreiber, as a target for LSR has provided us with a sensitive quantitative measurement of two separate components of the reaction, swelling and haemorrhage, in the challenged feet. The reaction in the foot-pads was also more reproducible than that of the lower back. Rabbits that were prepared with an intradermal injection of LPS and then challenged with another injection of LPS given intravenously, revealed an accumulation of leukocytes, diffuse haemorrhage and intra-vascular aggregation of platelets and leukocytes in the dermis (Movat *et al*, 1987). In our foot-pad model, this is reflected in a swelling followed by haemorrhage detected 16 and 20 hrs, respectively, after provocation. Further, we have confirmed the inability of mice to develop a classical local Shwartzman reaction in which LPS is used for both preparation and provocation.

Results described in this chapter demonstrate, also, the ability of both pathogenic (*M.tuberculosis* and *M.leprae*) and non-pathogenic (*M.vaccae*) mycobacterial sonicated antigens to

synergise with TNF to induce necrotic tissue damage. In addition, an immune response mounted to *M.vaccae* in mice that had been injected with autoclaved *M.vaccae* bacilli strongly enhanced the damage seen in the foot-pads. Immunisation with *M.vaccae* had, however, no effect on the LSR at LPS-prepared sites. The possible mechanism of the effect of immunisation on LSR, whether it is cell or antibody mediated, will be discussed in the next chapter. This effect allowed us to overcome the problem of getting weak responses in certain seasons. Mice showed a maximum response in December when they were prepared with *M.vaccae* and in December-January after preparation with LPS. The seasonal variation in the response could, in part be attributed to the exposure of these animals to environmental mycobacteria present in their food and drinking water though this would not explain the similar findings in LPS-prepared animals.

Necrotic tissue damage can be seen clinically in patients with past or present tuberculosis infection, in their internal lesions or when skin tested with PPD or New Tuberculins. However, when tuberculoid leprosy patients and BCG vaccinated healthy donors are skin tested, a non-necrotic induration is seen. Since necrotic tissue damage can be produced in experimental animals in sites prepared by both leprosy and tuberculosis bacilli, leprosy patients may have immunoregulatory mechanisms which are missing or suppressed in tuberculosis. Therefore, the existence of such regulatory mechanisms implies that there is a possibility of eliminating the necrotic component of the reaction in challenged animals and perhaps in human tuberculosis.

Macrophages within the well developed granulomas in tuberculosis are activated by IFN-gamma and will therefore convert the circulating 25-(OH) Vitamin D_3 to its active form, 1,25-$(OH)_2D_3$ or Calcitriol (Rook *et al*, 1986a). IFN-gamma and Calcitriol showed an additive anti-tuberculosis activity when added to cultures of human monocytes infected with *M.tuberculosis* bacilli. Both mediators have been also shown to prime macrophages for TNF release in response to LPS and live virulent *M.tuberculosis* bacilli (Rook *et al*, 1987). These previous findings and our results that demonstrate the production of local tissue damage by a direct injection of TNF into a site that has been prepared with a non-toxic preparation of mycobacterial antigens reinforce the hypothesis put foreward by Rook (1990). He suggested that necrotic lesions seen in tuberculosis and in the Koch phenomenon are Shwartzman like reactions in which mycobacterial antigens prepare a site and interact with T cells to induce IFN-gamma production amongst other lymphokines. This results then in macrophage activation and production of calcitriol which will prime these macrophages for an enhanced production of TNF in response to LAM and probably other mycobacterial components. The role of LAM in provoking a Shwartzman reaction at an LPS-prepared site has been proved in this chapter. The results discussed here and those presented in the previously mentioned reports could also explain the findings of two early clinical studies of immunotherapy of *Lupus Vulgaris* (chronic granulomatous skin tuberculosis). In one of these studies, large doses of *M.tuberculosis* culture supernatant given to the patients subcutaneously resulted in necrosis and healing of skin lesions but aggravation of the deeper ones (Rook, 1988a). In the second study, vitamin D_3 was used as an

immunotherapeutic agent. This resulted in fever, raised erythrocyte sedimentation rate, inflammation and necrosis of the chronic granulomatous lesions followed by their healing (Rook, 1988b).

Therefore, necrotic tissue damage seen in those patients suggests a role for TNF in the immunopathology rather than the immunoprotection although recent reports have demonstrated the importance of TNF production as a first line defence in the host. It has been recently shown that in spite of the lack of functional mature T and B cells in mice with SCID (severe combined immunodeficiency) mutation, these mice could resist an infection with live *Listeria monocytogenes* (Bancroft *et al*, 1989). Natural killer cells from these mice released high levels of IFN-gamma in response to heat-killed *L.monocytogenes* and TNF, secreted by macrophages. This provided a rapid pathway for T cell-independent macrophage activation and resistance to infection. Moreover, this resistence could be attributed to the finding that murine macrophages make nitric oxide in the presence of IFN-gamma and TNF and this controls the growth of mycobacteria in these cells (Dr. B. Bloom, personal communication to Dr. G. Rook). However, human macrophages have not been shown to make nitric oxide because they are unable to synthesise tetrahydrobiopterin (Dr. C. Nathan, personal communication to Dr. G. Rook). In addition, the neutralisation of endogenous TNF exacerbated BCG infection in mice (Kindler *et al*, 1989).

However, TNF may have two conflicting roles, a protective one via the production of nitric oxide and tissue damaging because of the necrosis produced at prepared sites as described in this chapter. Furthermore, there is another mechanism which enhances the

cytotoxic effect of TNF. Mouse L929 fibroblasts infected with various pathogenic bacteria such as *Shigella flexeneri*, *Salmonella typhmurium* and *Listeria monocytogenes* were more susceptible to the cytotoxic effect of TNF than untreated cells (Klimpel *et al*, 1990). This effect was dependent on the bacterial invasion of their host cells since fibroblasts treated with non-invasive strains of these bacteria or their culture supernatants were not different from the control ones indicating a role for TNF in eliminating infected cells. Interestingly, the infection of fibroblasts with live *M.tuberculosis* H37Rv (or even its soluble sonicated antigens) increased their sensitivity to the toxic effects of TNF (Filley & Rook, 1991). However, an infection with *M.avium* or *M.tuberculosis* H37Ra had only an intermediate effect while the infection with *M.vovis* bacillus Calmette-Guerin had almost no effect. The authors suggested that the increased sensitivity of infected cells to TNF cytotoxicity explains the absence of *M.tuberculosis* from non-phagocytic cells *in vivo* in spite of the ability of this mycobacteruim to infect various types of cells *in vitro* . This could also, partially, explain the immunopathology seen in tuberculosis infection.

The following chapters will discuss the involvement of various types of cells and biological mediators in the production of LSR. In addition, the results concerning the immunomodulation of such a reaction will also be demonstrated.

CHAPTER FOUR

The role of leukocytes and their adhesion receptors in the production of local Shwartzman reaction.

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4.1. INTRODUCTION

Both LPS and TNF have been shown to alter the number of circulating leukocytes. Previous reports have shown that exogenously administered LPS or TNF induce peripheral lymphopenia and initial neutropenia followed by neutrophilia, similar to the haematologic changes seen during bacterial infection (Remick *et al*, 1986; Ulich *et al*, 1989). It has been also shown that the neutropenia was a result of margination of neutrophils, possibly, to facilitate their migration to sites of inflammation. Neutrophilia, however, resulted from the demargination and recirculation of these cells and the production of immature neutrophils by the bone marrow. In addition, TNF modulates the function of neutrophils and macrophages and hence influences their role in inflammatory reactions. Human peripheral blood monocytes and polymorphonuclear leukocytes can be induced, by TNF, to migrate across polycarbonate and nitrocellulose filters (Ming *et al*, 1987). Human neutrophils were also shown to express enhanced phagocytic activity, respiratory burst and degranulation when treated with a recombinant preparation of TNF (Shalaby *et al*, 1985; Ferrante *et al*, 1988). These findings together with the observation that neutrophils migrate first to an inflammatory lesion produced by an intradermal injection of *Escherichia coli* into the skin of rabbits followed by monocytes (Issekutz *et al*, 1981) suggest a possible involvement of these two cell types in the production of local tissue damage provoked by TNF. Movat *et al* (1987) have demonstrated the accumulation of leukocytes, predominantly neutrophils in the dermis of animals prepared intradermally by injecting a combination of IL-1 and TNF and then challenged intravenously with endotoxin. Furthermore, polymophonuclear

leukopenia induced by nitrogen mustard was reported to protect animals against both local and systemic Shwartzman reactions (Good & Thomas, 1952).

Adhesion receptors expressed on the cell surface and mediating cell to cell or cell to extracellular matrix close contact have been shown by various researchers to play a vital role in the organisation of cells in differentiated organs and in the conduction of various functions of the immune system (reviewed by Springer, 1990). There are three main families of adhesion receptors. First, the immunoglobulin superfamily which includes the antigen-specific receptors expressed on the surface of T and B lymphocytes. Second and third are the integrin and the selectin families involved in mediating the adhesion and trans-endothelial migration of leukocytes amongst other functions. The integrins are formed of various combinations of non-covalently associated alpha and beta subunits. The integrins were, however, divided into three subfamilies according to their beta subunit, the beta-1 (CD29), the beta-2 (CD18) and the beta-3 (CD61) integrins. The beta-1 subfamily receptors are expressed on the surface of both leukocytes and non-haematopoietic cells and they mediate their interaction with both cells and extracellular matrix. These molecules were given the name, very late activation (VLA) antigens because some of them are expressed 2-4 weeks after stimulation of T lymphocytes with an antigen *in vitro*. Few members of this family such as VLA-4 were described to be important for the mediating the migration and localization of leukocytes in inflammatory sites. A major surface adhesion receptor family that has been identified on both human and murine leukocytes is the leukocyte integrin (CD18-CD11) family and it includes LFA-1, Mac-1 and P150,95. These antigens share a common beta chain (CD18)

linked noncovalently to one of three different alpha chain types (CD11 a,b and c, respectively). LFA-1 binds ICAM as a co-receptor and it is involved in the mediation of various T and B lymphocytes responses, antibody-dependent cellular cytotoxicity (ADCC) and the adherence of leukocytes to endothelial cells. Mac-1 and p150,95 mediate the adherence of myeloid cells to other cells and products of the clotting system. LECAM-1 (Mel-14/LAM-1), a member of the selectin family and a calcium dependent lectin, also mediates the adherence of leukocytes to the endothelial cells at inflammatory sites (Brandley *et al*, 1990). Patients with congenital leukocyte adhesion deficiency (LAD) due to a genetic mutation in their beta-2 subunit and presenting a history of recurrent life-threatening bacterial infections have highlighted the importance of myelomonocytic surface adhesion molecules in facilitating the migration of these cells to sites of inflammation (Ross, 1986; Rosen & Gordon, 1990). It has also been recently shown that the blockage of leukocyte integrins via the infusion of specific monoclonal antibodies can reduce tissue damage and mortality in bacterial meningitis in rabbits (Tuomanen *et al* , 1989) and prevents the splenic T cell-mediated-transfer of insulin-dependent diabetes mellitus from diabetic donors to sub-lethally irradiated recipient mice (Hutchings *et al*, 1990).

In this chapter, the contribution of macrophages and T cells in the production of a local Shwartzman reaction (LSR) is assessed. In addition, the effect of a monoclonal antibody to the myelomonocytic type 3 complement receptor (CR3 or Mac-1) on the development of LSR is discussed. Subcutaneous immunization with an autoclaved preparation of *M.vaccae* was shown, in the previous chapter to enhance the LSR at sites prepared with *M.vaccae* sonicate. Thus, we

have investigated whether this effect is mediated by cellular or humoral components. Histological examination of the murine skin inflammatory sites is also described.

4.2.1. *In vivo* macrophage depletion :

The drug dichloromethylene diphosphonate (Cl_2MDP), one of the most toxic diphosphonates to macrophages, encapsulated into multilamellar liposomes has been used for the temporary complete elimination of macrophages from the circulation, liver and spleen. PBS-encapsulated liposomes were used as a control (prepared liposomes were kindly provided by Dr N. Van Rooijen, Free University, The Netherlands). Groups of four C57/Bl/T mice were injected with 0.2 ml of Cl_2MDP-encapsulated liposomes (containing 2 mg of the drug), PBS containing liposomes or PBS, intravenously, 24 hours before they were prepared with LPS (10 ug/mouse) followed 24 hrs later with TNF (1 ug/mouse). Macrophage depletion was shown to be complete, by this method, within 24 hrs followed by slow repopulation of the spleen within 1 week (Van Rooijen & Van Nieuwmegen, 1984).

4.2.2. The assay for LSR :

The assay has been described, in details, previously in chapter 3.

4.2.3. The effect of 5C6 mAb on the development of LSR :

5C6, a rat IgG2b directed against the murine CR3 (a generous gift from Dr H. Rosen, University of Oxford) was injected (at 1 mg/mouse) intravenously, in the tail vein, into C57/Bl/T mice one hour before preparation or provocation. The control animals were given either pyrogen free saline or IC5.5H10.A11, an isotype matched control mAb. These mice were then prepared with *M.vaccae* sonicate (17 ug/mouse) and provoked with TNF (1 ug/mouse) for the induction of LSR.

4.2.4. *In vivo* T cell depletion :

The rat hybridoma cell line secreting the YTS 191.1 IgG2b mAb (anti-murine L3T4) and the YTS 169.4 IgG2b mAb (anti-murine Lyt2) were a kind gift from S.P. Cobbold, Dept. Pathology, University of Cambridge. The cell lines were grown in RPMI 1640 (Gibco, Scotland) supplemented with 20 % heat inactivated FCS, 100 ug/ml penicillin, 100 ug/ml streptomycin, o.1 mM/ml sodium pyruvate, 5 x 10^{-5} M 2-mercaptoethanol. Cells (5 x 10^7) were injected into pristane primed (LOU x DA)F_1 male rats. The mAb was then purified from the ascitic fluid by precipitation at 40 % of ammonium sulfate. Mice to be depleted were first injected, i.v., with 0.2 ml (400 ug) of either mAb and then given, i.p., the same dosage again 3 days later. Both the T cell-depleted and the control group receiving only saline were challenged for the induction of LSR 2 days after the second dosage of mAb.

4.2.5. Adoptive transfer experiments :

The possibility of transferring the *M.vaccae* -enhanced LSR by humoral or cellular components was tested. Spleens were obtained from C57/Bl/T mice immunized ten days previously with 10^9 autoclaved *M.vaccae* given s.c. at the base of the tail. The spleens were homogenised by passing them through a fine stainless steel mesh which removed the splenic capsule and dispersed the cells into a suspension. The cells were then washed once in RPMI 1640 and suspended in ammonium chloride to lyse the red blood cells. Spleen cells were also obtained from control animals. Recipients were injected i.v. with 0.3 ml serum or 10 x 10^6 spleen cells of either immunized or control animals 30 min before they received a s.c. injection of *M.vaccae* sonicate (17 ug) in their feet as a preparation for LSR.

4.2.6. Histological studies :

Skin of challenged foot-pads was frozen in cold isopentane and stored in liquid nitrogen. Sections of 6 um thickness were prepared from these tissue samples using an AS600 cryotome, fixed in methanol for 10 min, air dried for 24 hrs and then stained with Haematoxylin and Eosin. The sections were examined by light microscopy.

4.2.7. Enzyme histochemistry :

Cryostat sections of 6 um thickness were prepared as described in the previous section. Non-specific esterase activity of the macrophages was demonstrated with alpha-naphthyl acetate (Sigma, N 8505) with pararosanilin as a diazonium salt. The sections were then washed with PBS, stained with haematoxylin and examined for the distribution of macrophage cells.

4.3.1. The effect of macrophage depletion on the development of the local Shwartzman reaction :

Macrophages do not ingest small molecules but they do phagocytose liposomes. Therefore, liposomes containing the toxic drug, dichloromethylene diphosphonate (Cl_2MDP), were injected intravenously into test animals 24 hrs prior to their challenge for a local Shwartzman reaction. Once the liposomes are phagocytosed, the drug is released inside the macrophages, and the cells will be eliminated from the circulation and spleen. The results were compared to those of control animals given PBS containing liposomes. This technique was employed to elucidate the role of macrophages in the production of LSR. As shown in Fig.4.1., although insignificant, the toxic drug-containing liposomes considerably reduced both the swelling and haemorrhage when compared to the PBS-liposomes effect. The LSR was similar in magnitude (both swelling and haemorrhage) in both the group that has been given the combined treatment (LPS and TNF) and the one that has been only prepared (LPS). This indicates that macrophage, and possibly neutrophil, depletion due to the administration of the toxic drug has led to the complete elimination of the synergistic effect exerted by the combined treatment, LPS/TNF.

4.3.2. The ability of 5C6 to abolish LSR :

5C6 mAb, specific for an epitope on the CR3 receptor of murine myelomonocytic cells, was shown to inhibit the emigration of neutrophils and monocytes from blood vessels to inflammatory foci (Rosen, Gordon & North,1989). Thus, we explored the ability of this antibody preparation to interfere with the development of the

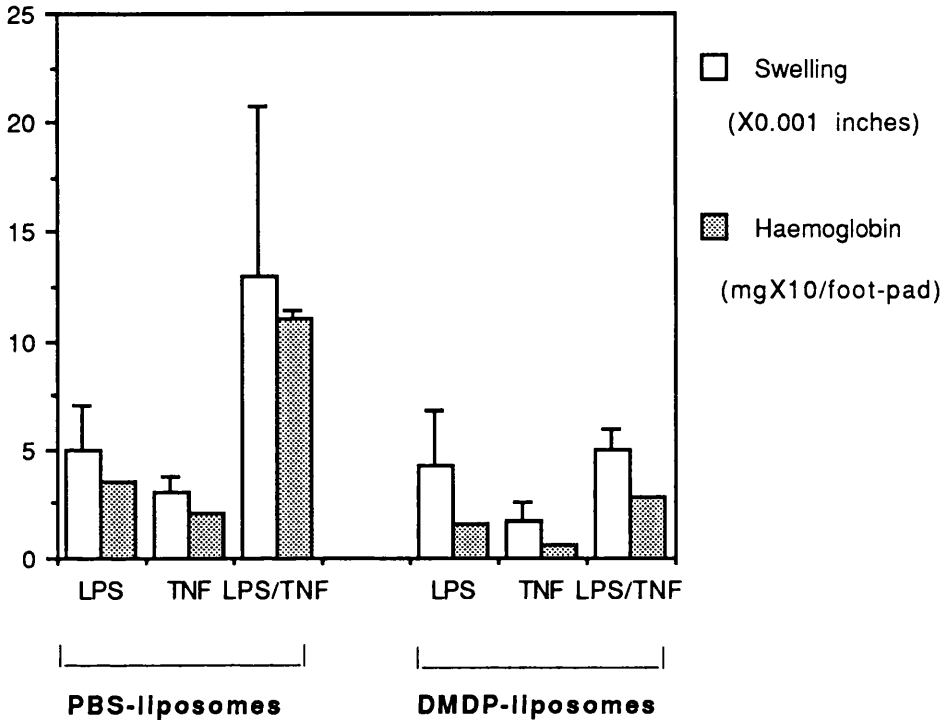


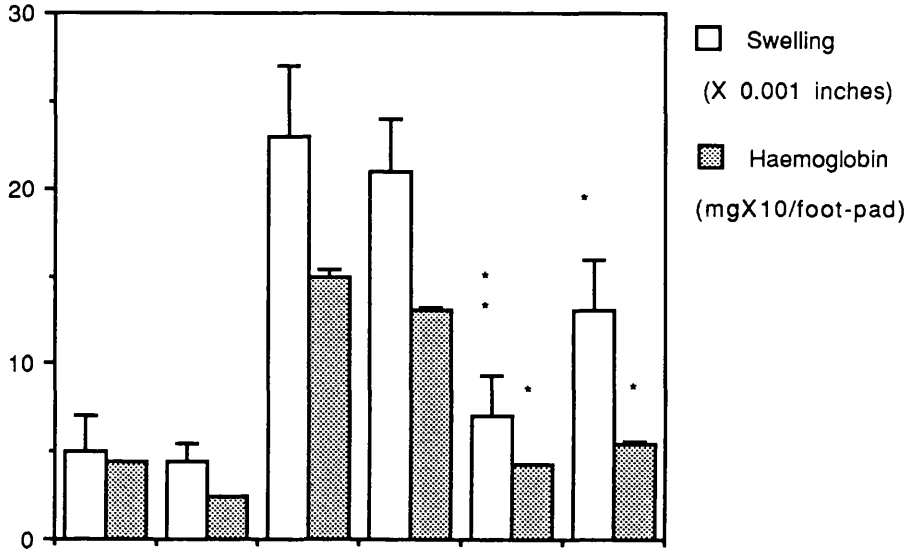
Fig.4.1. The effect of macrophage depletion on the induction of LSR.

local Shwartzman reaction. Injecting 5C6 mAb i.v. one hour before preparation with *M.vaccae* sonicate almost completely inhibited the LSR (0.007 ± 0.0024 inches of swelling, 0.43 ± 0.03 mg of Hb derivative) compared to the control group (0.023 ± 0.004 inches, (1.5 ± 0.3 mg) and the control mAb, IC5.5H10.A11-treated group (0.021 ± 0.003 inches, 1.3 ± 0.2 mg), shown in Fig. 4.2. In addition, when mice were given the mAb one hour before provocation with TNF, a weaker but significant effect was seen on both the swelling (0.013 ± 0.003 inches) and the Hb pigment concentration (0.55 ± 0.05 mg). This demonstrates the importance of CR3 expressed on the activated inflammatory cells surface in facilitating their extravasation during the development of LSR.

4.3.3. The effect of T cell depletion on LSR :

We have examined the effect of T lymphocyte depletion, using specific monoclonal antibodies. The depletion of $Lyt2^+$ cells had no effect on the local tissue damage at sites prepared with LPS (Fig.4.3). However, the depletion of $L3T4^+$ cells enhanced the haemorrhagic component of the reaction without affecting the swelling.

The role of T cells in the development of LSR, at sites prepared with *M.vaccae* sonicate, was also investigated. The administration of anti-murine $Lyt2^+$ antibody significantly reduced the haemorrhage without affecting the swelling (Fig.4.4A). On the other hand, anti-murine $L3T4^+$ had an enhancing effect on the tissue damage produced. Mice were also immunised, subcutaneously, with autoclaved *M.vaccae* bacilli (10^9) and then depleted, ten days later, of their $L3T4^+$ or $Lyt2^+$ cells. They were then prepared for a LSR with *M.vaccae* sonicate. In contrast to our findings in the non-



Control	mAb	(-1hr)				+		
5C6	mAb	(-1hr)					+	
M.vaccae		(0hr)	+		+	+	+	+
5C6	mAb	(23hrs)						+
TNF		(24hrs)		+	+	+	+	+

Fig.4.2. CR3-mediated adhesion of leukocytes is required for the extravasation of these cells to inflammatory foci.

** p<0.025 (Student's t-test)
* p<0.05 "

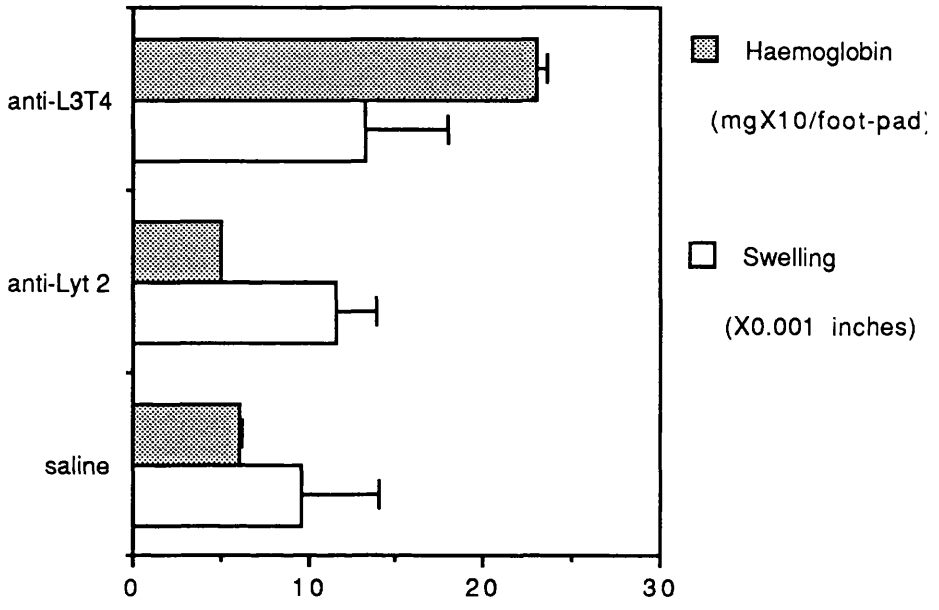


Fig.4.3. The effect of T cell depletion on the LPS-mediated local tissue damage.

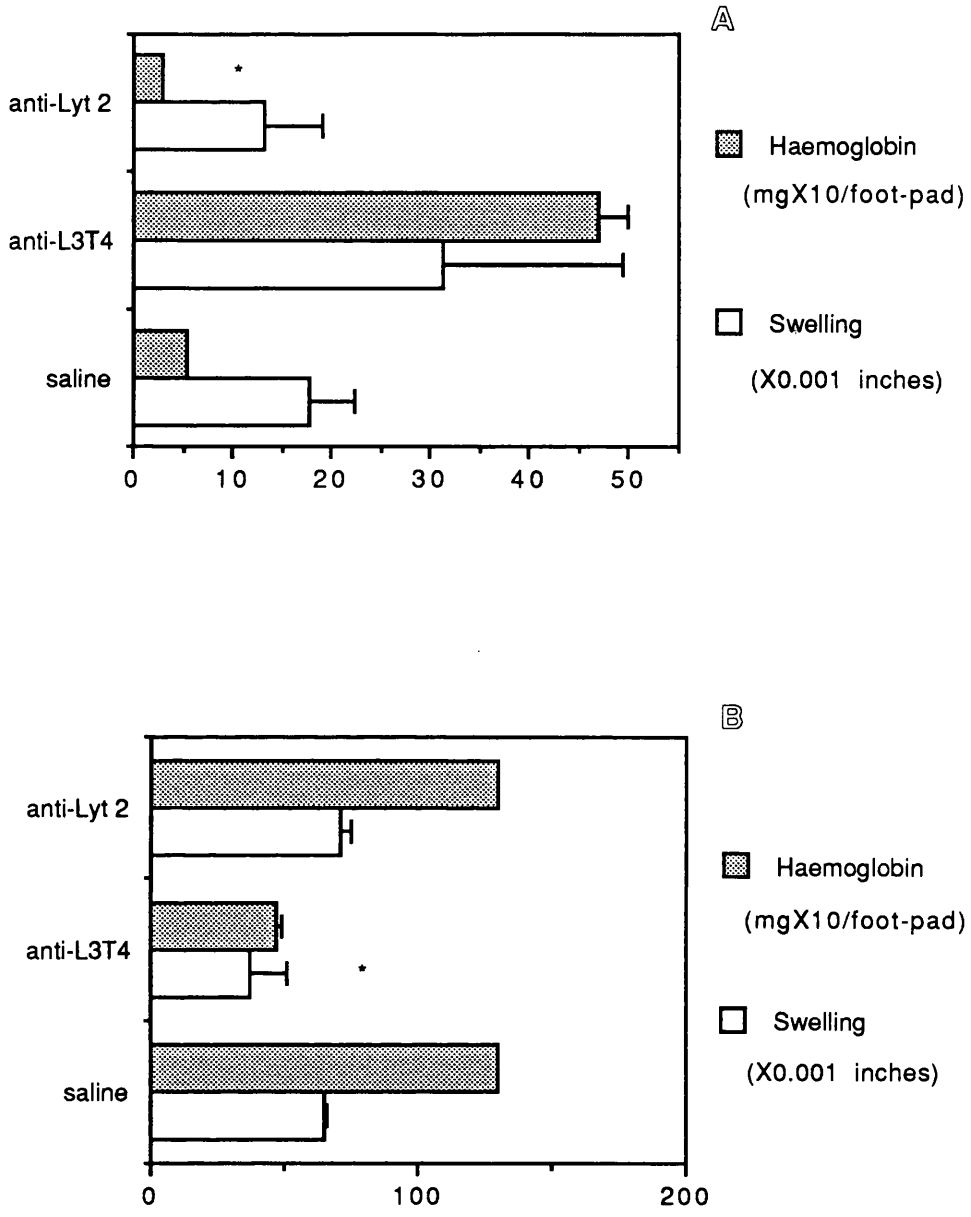


Fig.4.4. The effect of T cell depletion on the LSR at sites prepared with M.vaccae sonicate in saline-treated (A) or M.vaccae-immunised (B) mice.

*** $p<0.05$ (Mann-Whitney test)**

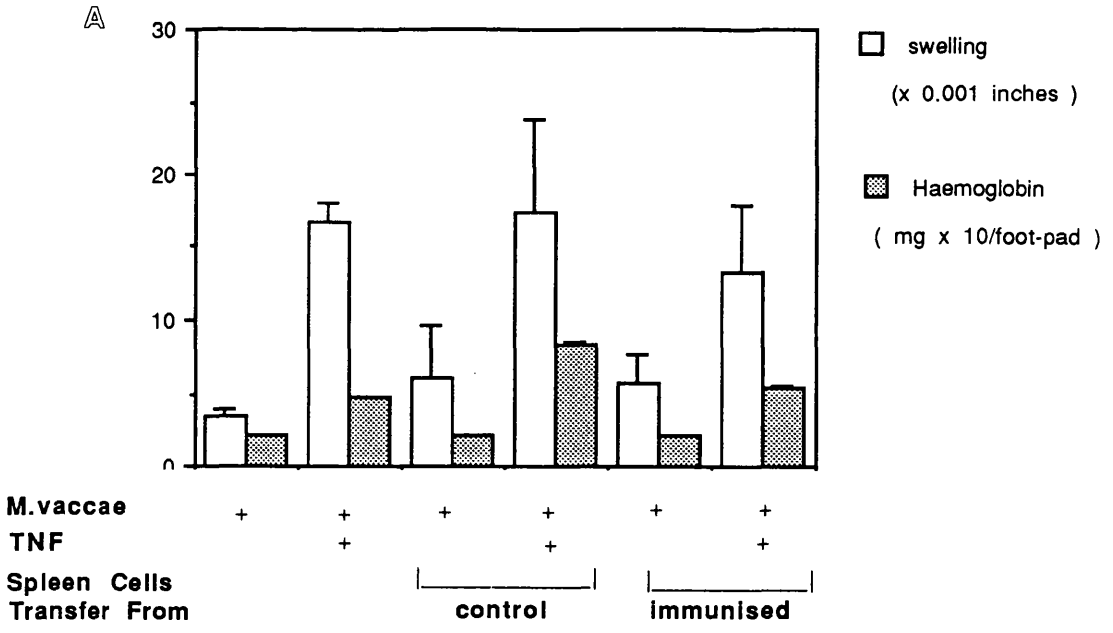
immunised animals, the anti-L3T4$^+$ antibodies significantly inhibited both the swelling and the haemorrhage whereas the anti-Ly2$^+$ antibodies had no effect on the reaction (Fig.4.4B).

4.3.4. Does immunization enhance LSR by cellular or humoral-mediated mechanism :

Spleen cells (10 x 10^6) or serum (0.3 ml) were obtained from autoclaved *M.vaccae* immunized or saline treated animals and injected intravenously into age matched group of mice. Recipients given either cells or serum from either immunised or control animals were prepared 30 min later with 17 ug of *M.vaccae* sonicate and then challenged with 1 ug of TNF. Neither the spleen cells (Fig.4.5A) nor the serum transfer (Fig.4.5B) could show any enhancement of the reaction seen in the immunized donor animals (Fig.4.5C).

4.3.5. Characterisation of the various infiltrating cells in the challenged feet :

Fixed skin tissue sections from mice immunised with autoclaved *M.vaccae* were stained with hematoxylin (H) and eosin (E) and, later, were examined with light microscopy. *M.vaccae* prepared footpads, compared to the normal skin sections (Fig.4.6) were edematous and showed massive infiltration of inflammatory cells, including both neutrophils (identified by their doug-nut shaped nuclei in the H & E sections) and macrophages (identified by their brownish cytoplasm in the sections stained for the non-specific esterase enzyme) and hence causing an increase in the thickness of the dermis layer (Fig.4.7 & Fig.4.8). On the other hand, there was a very slight increase in the thickness of the dermis layer in TNF-injected feet reflecting the infiltration of fewer cells (Fig.4.9). No tissue damage or haemorrhage was detected in the *M.vaccae* or TNF-injected



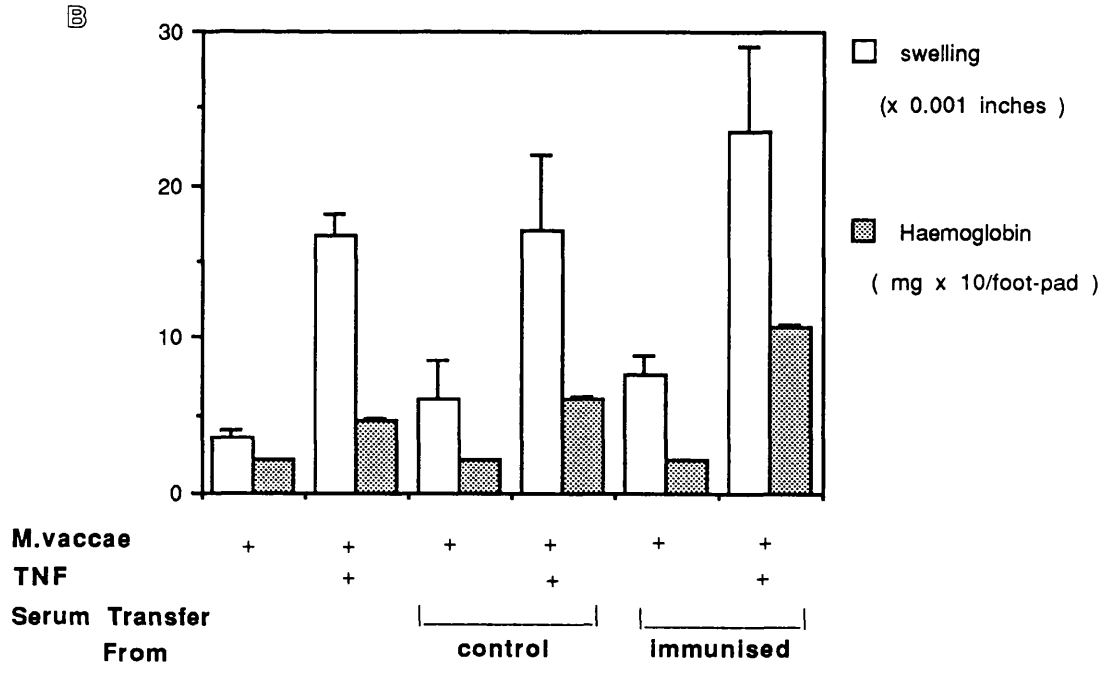


Fig.4.5. Enhancement of the LSR is transferred by neither the spleen cells (A) nor the serum (B) of immunised mice (C).

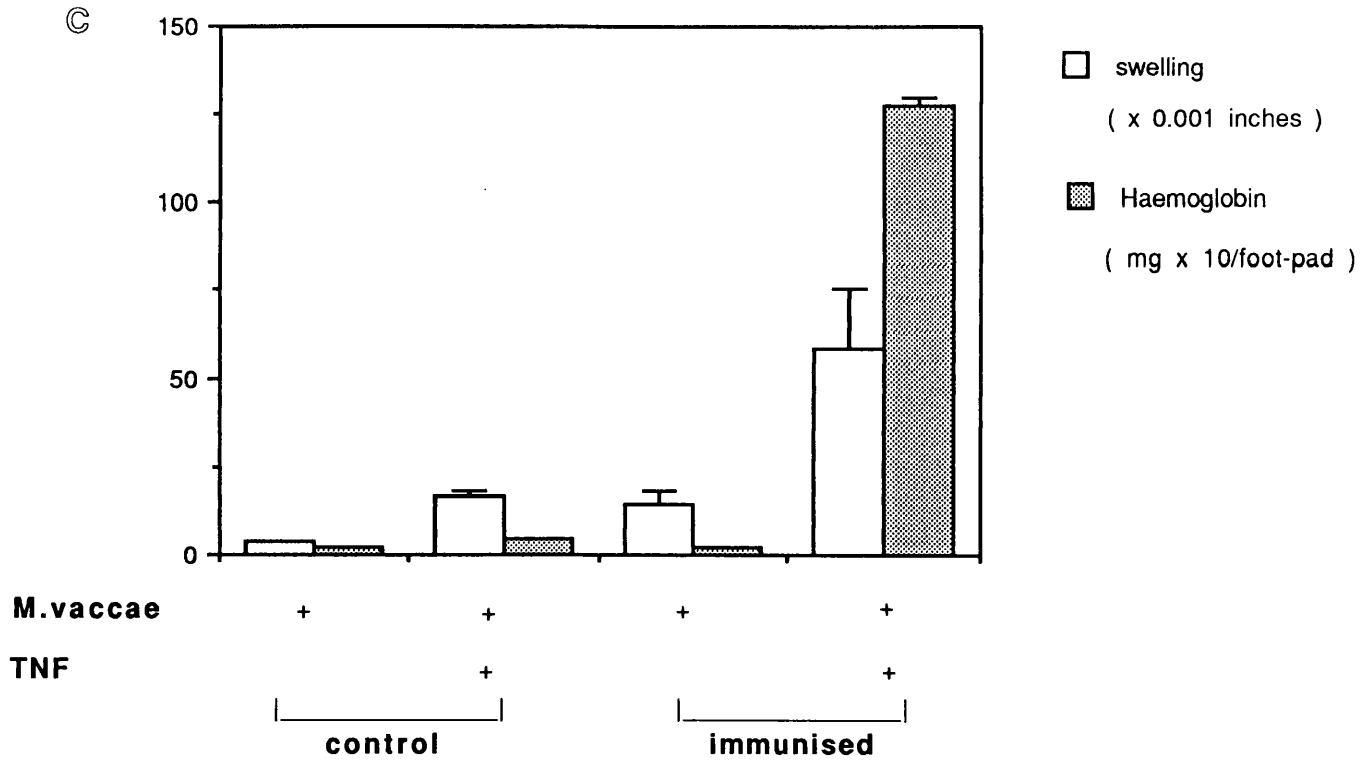


Fig.4.5C. The swelling and the haemorrhage in the foot-pads of mice immunised with autoclaved M.vaccae, as compared to the saline-treated control group.

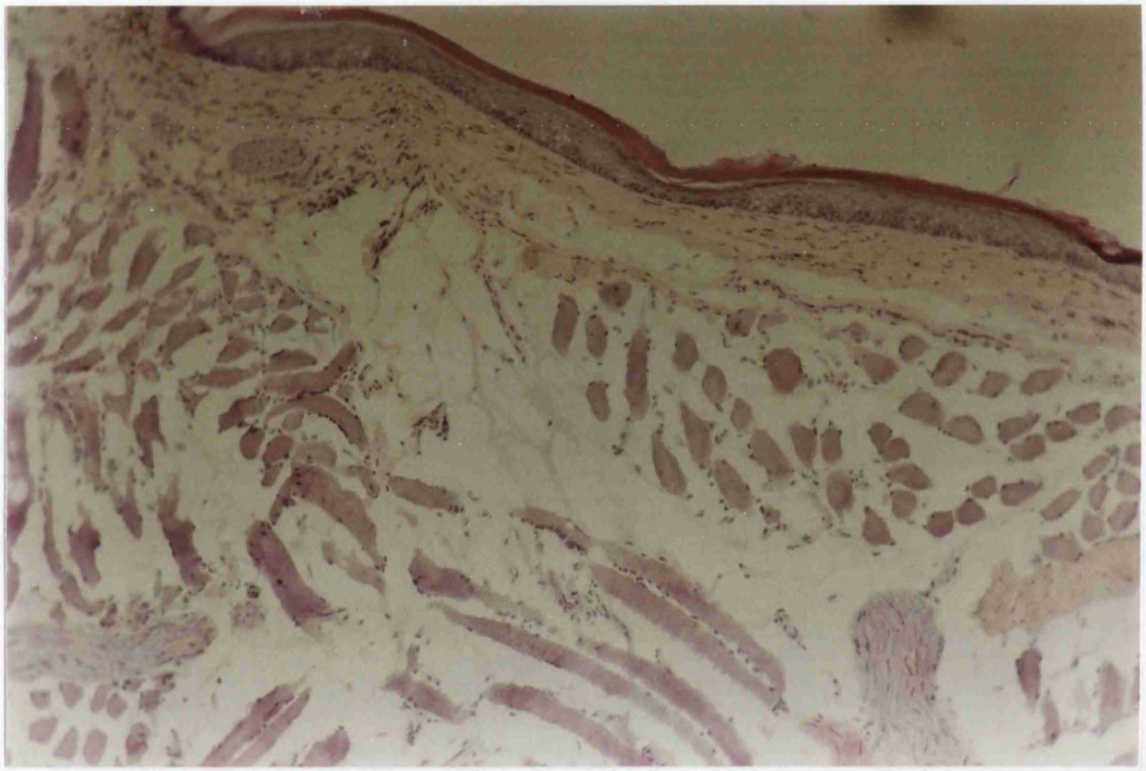


Fig.4.6. Hematoxylin and Eosin stained section of normal skin (magnification, X 10).

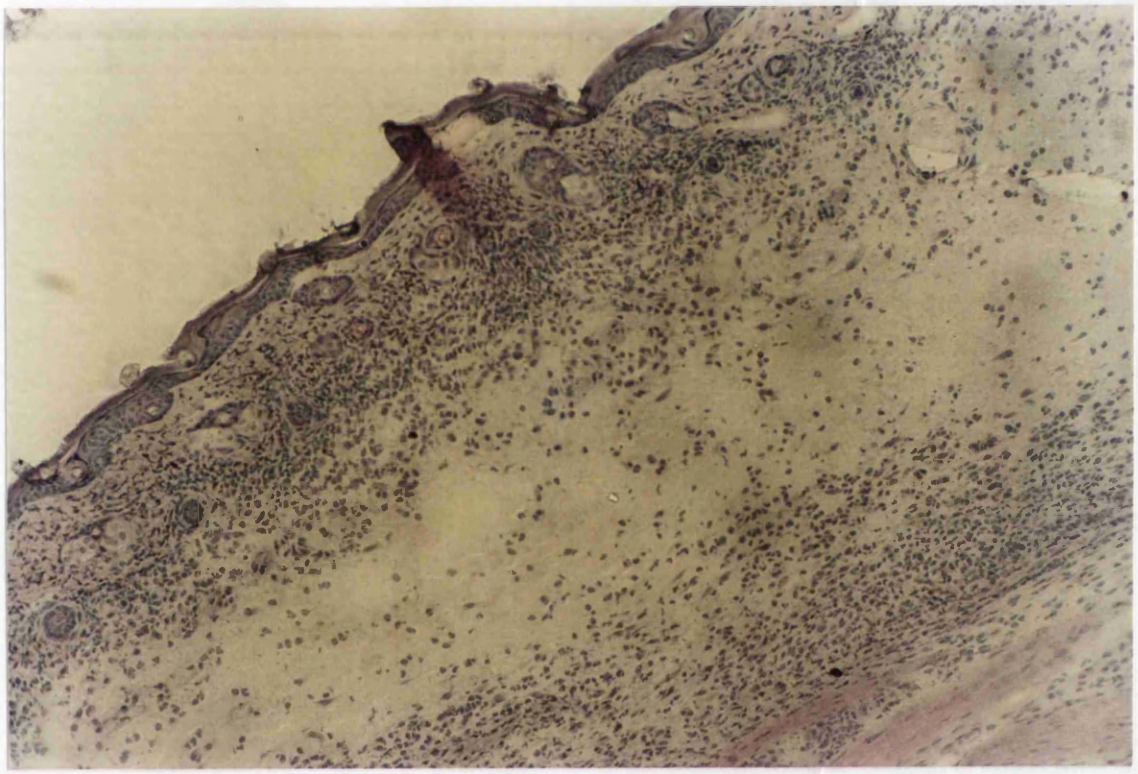


Fig.4.7. The histology of a skin site prepared with M.vaccae sonicate 24 hours earlier, note the difference in the thickness of the dermis layer, compared to the normal skin in Fig.4.6., (H & E staining; magnification, X 10).

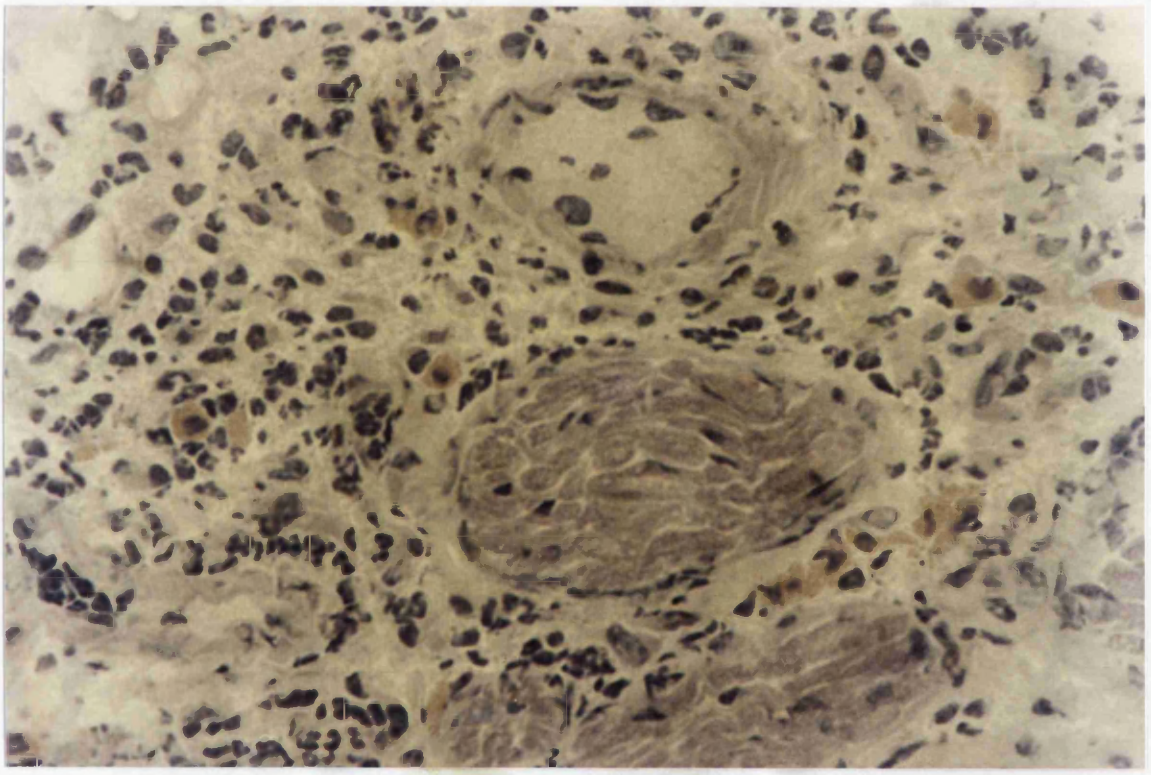


Fig.4.8. M.vaccae-injected skin stained for the non-specific estrase enzyme to visualise the infiltrating macrophages (brownish cytoplasm), seen around a blood vessel. The section was counterstained with hematoxylin (magnification, X 40).

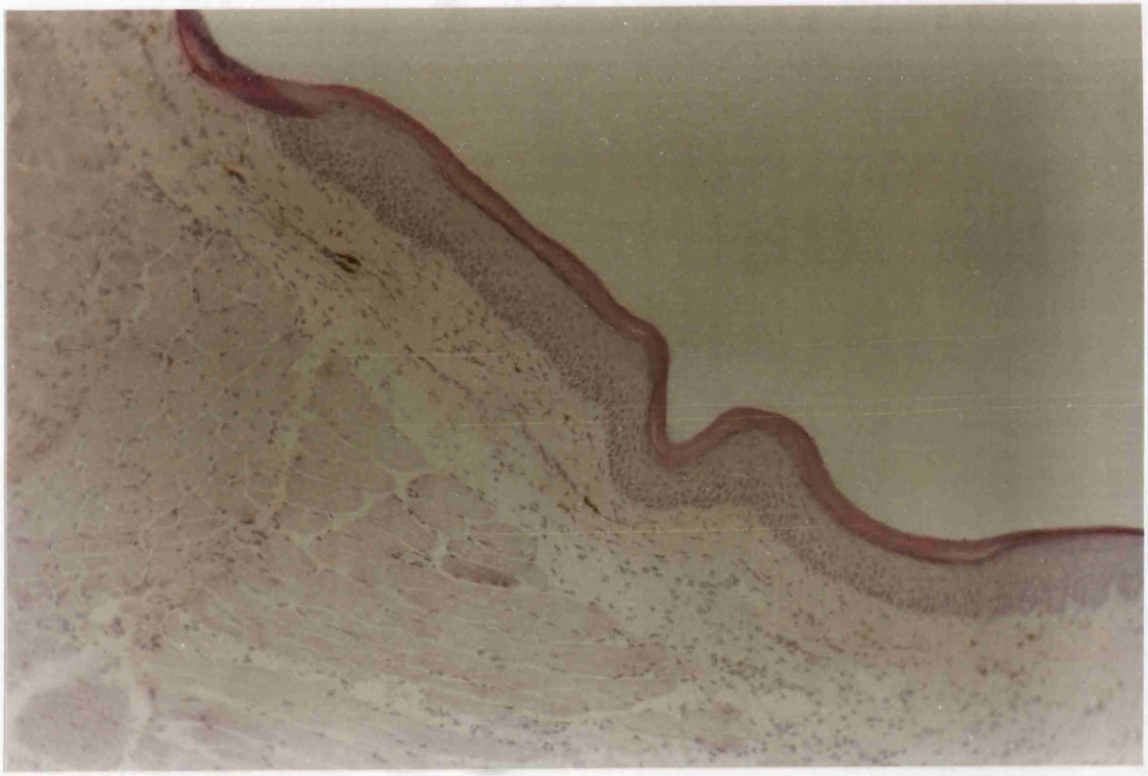


Fig.4.9. The histology of TNF-injected skin of a normal mouse (H & E staining; magnification, X 10).

feet. The skin of foot-pads challenged with both *M.vaccae* and TNF was highly infiltrated with inflammatory cells (neutrophils and macrophages), massively distributed in the dermis and seen inside and around the blood vessels (perivascular cuffing), shown in Fig.4.10A & B, accompanied with edema, haemorrhage and increased thickness of the dermis and the hypodermis layers (Fig.4.11). Comparing the slides stained for macrophages (using the non-specific estrase enzyme as a marker) following various treatments did not show any major differences in the number of infiltrating macrophages. Moreover, in the foot-pads injected with both *M.vaccae* and TNF, the epidermis, the keratin and the muscular tissue seen in normal skin were completely destroyed. Therefore, although the challenged feet do not, macroscopically, show signs of tissue damage, the H & E stained sections demonstrate the haemorrhagic necrosis seen in the foot-pads with positive LSR.

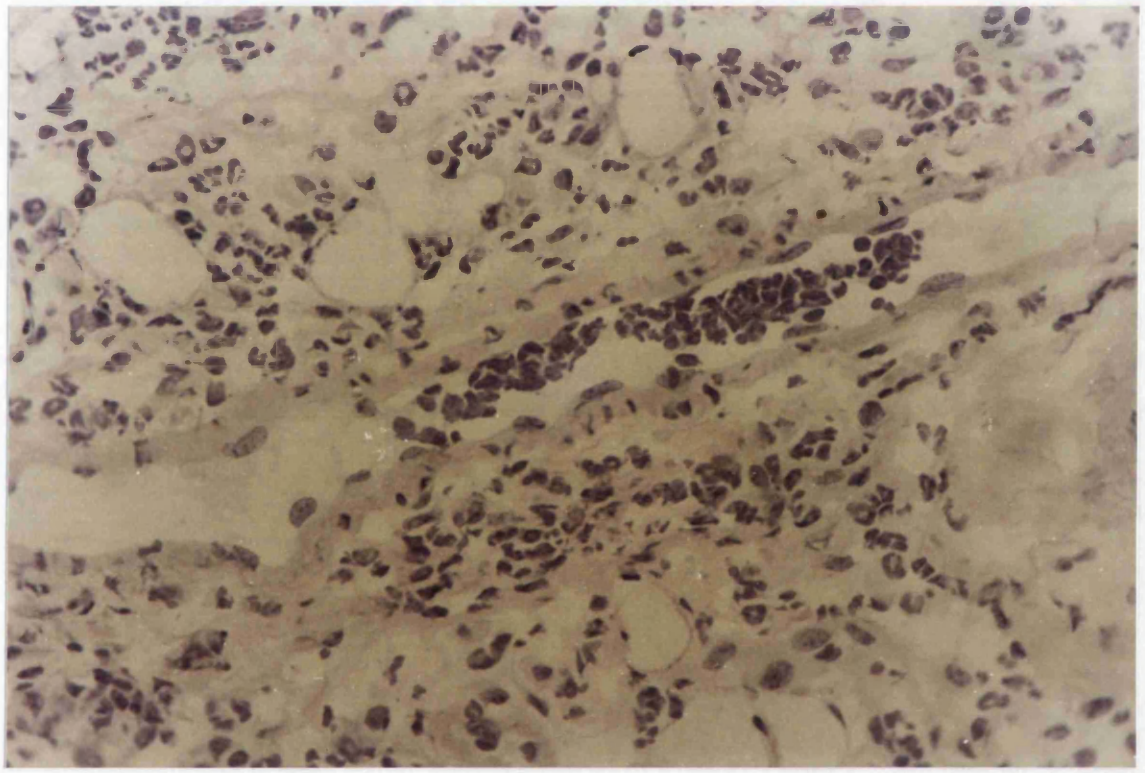


Fig4.10A. A blood vessel in a H & E stained section of skin injected with M.vaccae+TNF, showing the a massive number of cells infiltrating into the site of inflammation. (magnification, X 40).

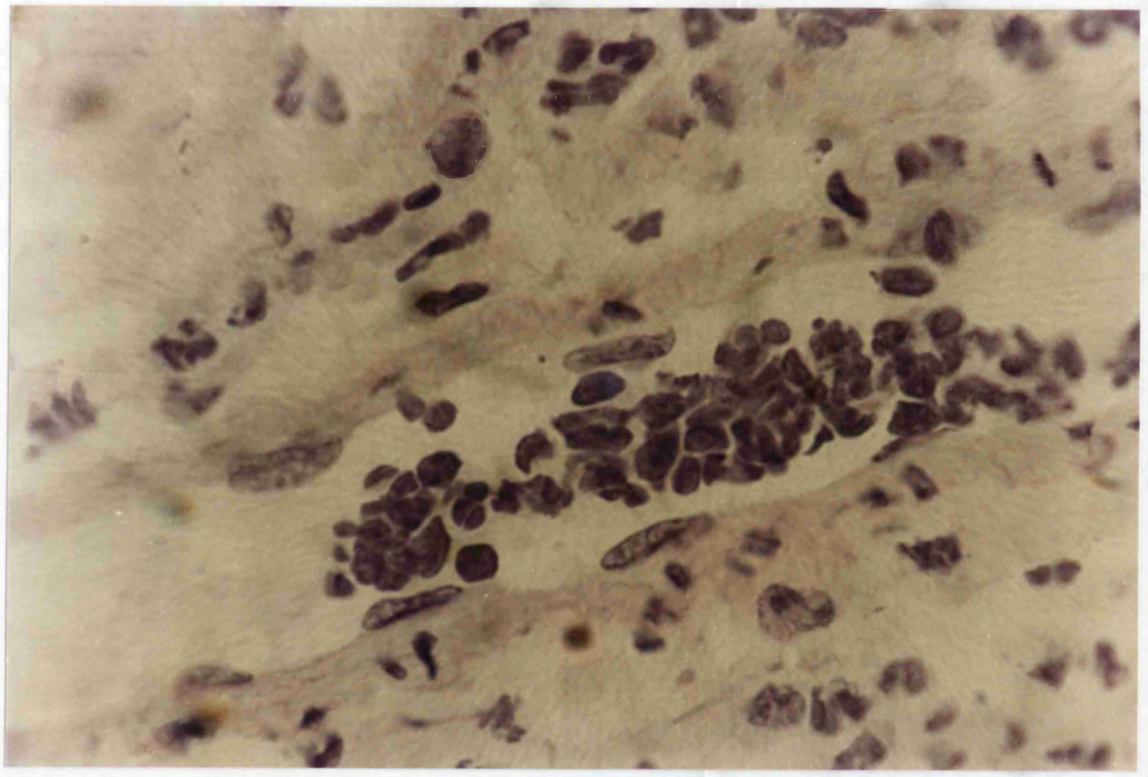


Fig4.10B. A higher magnification(X 100) of the same blood vessel (Fig.4.10A) shown to visualise the types of infiltrating cells. Cells making contact with the endothelium or migrating through the vessel wall can be seen.

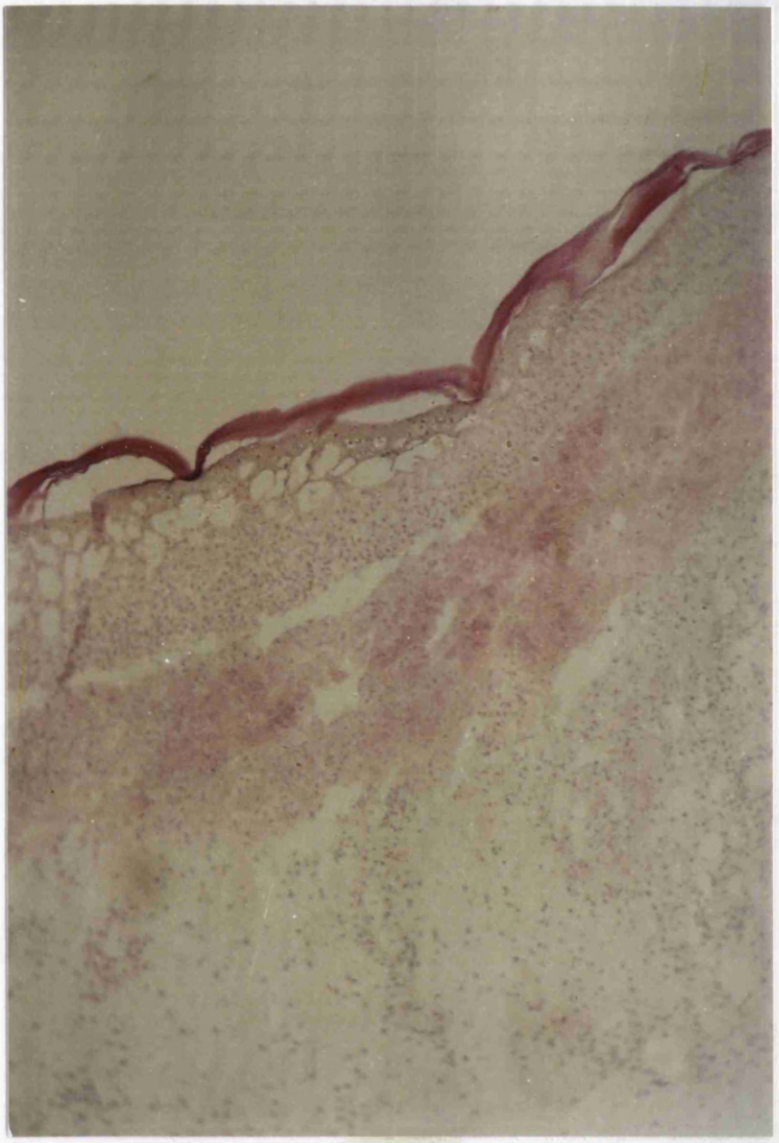


Fig.4.11. H & E stained section of skin injected with M.vaccae+TNF showing the haemorrhagic necrosis, a characteristic of LSR (magnification, X 10).

4.4. DISCUSSION

Leukocytes, mainly neutrophils, were shown to migrate early to the site of inflammation followed by monocytes which continue to accumulate for at least 24 hrs, becoming the predominant cell type in *Escherichia coli* injected rabbit skin (Issekutz *et al*, 1981). We attempted to investigate the role played by phagocytic cells in LSR since they form one of the primary lines of defence against foreign organisms and substances. The use of multilamellar liposomes made up from a mixture of phospholipids, phosphatidyl-choline and cholestrol, and containing toxic drugs is a new approach used for studying the functional aspects of macrophages *in vivo* (Van Rooijen, 1989). Once the liposomes are ingested by phagocytes, the liposomal membranes are digested by lysosomal enzymes and the toxic drug is released inside the cell (Van Rooijen and Van Nieuwmegen, 1984). The intravenous administration of dichloromethylene diphosphonate-containing liposomes to the test animals is a method proved previously to completely deplete the circulation, spleen and liver of their macrophages within 24 hrs and they remain depleted for almost a whole week. Such an effect was confirmed by the absence of macrophage lysosmal acid phosphatase activity and surface markers and by the absence, in the circulation, of cells capable of ingesting carbon particles. Since neutrophils are also major phagocytic cells, they might also be destroyed by this drug although this has not been investigated. In our model, the i.v. injection of the toxic drug encapsulated in liposomes partially inhibited the swelling and haemorrhage produced by the sequential injection of LPS and TNF into the foot-pads, while the mice which received the PBS-containing liposomes presented the same intensity

of reaction seen in untreated animals. The tissue damage seen in an inflammatory reaction could be partially attributed to various lysosomal enzymes released when the macrophages or neutrophils undergo phagocytosis. Human polymorphonuclear leukocytes (PMN) lysosomal releasates injected intradermally, in rabbits, resulted in microvascular injury reflected by increased vascular permeability, haemorrhage, platelet aggregation and PMN cell accumulation (Movat and Wasi, 1985). Free reactive oxygen intermediates (e.g. superoxide), Cytokines (TNF) and nitric oxide can also be responsible for the pathological consequences of an immune response (Henson & Johnston, 1987; Liew & Cox, 1991). Thus, macrophage depletion, in our test animals, resulted in a reduced number of cells available to migrate to the site of inflammation, reduced swelling and haemorrhage and therefore, less tissue damage produced.

The Mac-1 cell surface receptor, also known as the type three complement receptor (CR3), is a member of the leukocyte integrin family of adhesion receptors (Rosen & Gordon, 1990). It consists of non-covalently linked heterodimer; a common beta chain (CD18) of 95 KDa and a specific alpha chain (CD11b) of 165 KDa. The CR3 receptor is expressed only on myeloid cells and it binds the complement cleavage product C3bi and fibrinogen (Springer, 1990). Recombinant human TNF has been recently shown to increase the expression of the CD11b antigen to 182 % of the control polymorphonuclear cells. Moreover, CR3 has been recently shown to contribute to the adherance of myelomonocytic cells both *in vitro* and *in vivo* (Rosen & Gordon, 1987). The intravenous injection of 5C6 mAb, specific to an epitope on CR3 distinct from the C3bi binding site, inhibited the recruitment of both PMN cells and macrophages into the peritoneal cavity of a thioglycolate challenged animals. In our murine model,

the injection of 5C6 abolished the local tissue damage in the challenged foot-pads even if injected in prepared animals, as late as one hour before provocation. It is important to note that the 5C6 completely abolished the reaction in spite of the expression, on the surface of leukocytes, of two other adhesion receptors, the LFA-1 and LECAM-1, that can mediate their binding to the endothelial cells (Springer, 1990). The LFA-1 receptor binds to the ICAM-1 receptor of which the expression is induced by LPS and various inflammatory cytokines such as IL-1 and TNF. LECAM-1 is a lectin receptor that binds a carbohydrate-like ligand and it is constitutively expressed on the surface of leukocytes (Brandley *et al*, 1990). This indicates that the binding and the trans-endothelial migration of leukocytes is a cooperative process and that the inhibition of CR3 binding to its ligand is enough to abolish the whole reaction. The role of CR3 receptor in mediating the adherence to vascular endothelium and then the migration of myelomonocytic cells has been previously described, using the 5C6 mAb, in various pathological experimental models such as murine Listeriosis (Rosen *et al*, 1989) Insulin-dependent diabetes mellitus (Hutchings *et al*, 1990) and pulmonary inflammatory responses (Rosen & Gordon, 1990).

Rothstein & Schreiber (1988) reported that athymic nude mice develop a positive LSR, after sequential injections of LPS and TNF, equal to that seen in normal animals indicating, that functional T lymphocytes are not required. Moreover, they evaluated the skin lesions macroscopically and considered those of 0.5-2.5 cm in diameter as positive and then calculated the incidence of necrosis. However, measuring both the swelling and the haemorrhage as two separate markers in our model was more accurate for evaluating the LSR lesions. Thus, the depletion of the test animals of their $CD4^+$

cells resulted in the enhancement of the haemorrhage in both the LPS- and *M.vaccae* -prepared feet. The role of T cells in the mediation of LSR at LPS-prepared sites could be attributed to a cellular immune response to a protein contaminant in our LPS preparation.

However, the *in vivo* depletion of $CD8^+$ lymphocytes significantly inhibited the haemorrhagic component of the LSR in the *M.vaccae* -prepared mice. The role of $CD8^+$ lymphocytes in the production of haemorrhagic necrosis was previously reported by Leveton *et al* (1989). They showed that tuberculosis infected mice depleted of $CD8^+$cells had much less haemorrhagic necrosis in their lesions than the control undepleted animals. This could also explain the enhanced haemorrhage in mice depleted of their $CD4^+$ cells which may play a regulatory role in this reaction. Since these mice were not deliberately immunised, the role of T cells in the production of LSR could be attributed to their sensitisation by environmental mycobacteria. This correlates with our previous finding showing the seasonal variation in the intensity of the reaction in animals prepared with *M.vaccae*.

Furthermore, mice subcutaneously immunised with killed *M.vaccae* bacilli showed a different picture, an inhibition of the LSR reaction following a depletion of the $CD4^+$ lymphocytes. This could be explained by the recent finding that $CD4^+$ T cell clones derived from healthy purified protein derivative positive donors were found to be cytotoxic to monocytes pulsed with mycobacterial antigens or infected with live *M.tuberculosis* bacilli (Boom *et al*, 1991). Thus, in our model, mice probably acquire, after immunisation, $CD4^+$ cells that can kill macrophages pulsed with mycobacterial antigens during preparation for the LSR.

A local Shwartzman-like reaction elicited in rabbits by preparing the skin with an intradermal injection of both rHu-TNF and rHu-IL-1 or endotoxin followed by an intravenous injection of endotoxin 18 hrs later was histologically examined using radiolabelled erythrocytes, platelets and neutrophils (Movat *et al*, 1987). Extravasated erythrocytes were seen around the blood vessels and within the skin tissue and aggregates of platelets, leukocytes and fibrin were seen extensively in the blood vessels. In our model, the H & E stained sections of TNF-injected feet show a small increase in the thickness of the dermis with few infiltrating cells while those injected with *M.vaccae* had thicker dermis and much more inflammatory cells and they were more edematous. Our results confirm the previous findings and demonstrate both neutrophils and macrophages in haematoxylin and eosin stained skin tissue sections of feet treated with both *M.vaccae* and TNF. They also show the increased thickness and the extensive haemorrhagic necrosis produced in the dermis layer of mice foot-pads challenged for a LSR.

This section confirms a role for phagocytic cells, CR3 and T cells in the LSR primed by mycobacterial antigens. The role of T cells is a novel finding, which leads to the possibility that preparation of a site for LSR is a stage in the development of necrosis in mycobacterial diseases which may be susceptible to immuno-modulation. Further evidence for this is outlined in chapter 6.

CHAPTER FIVE

The therapeutic intervention in LSR using a platelet activating factor antagonist, a synthetic prostaglandin E_1 analogue and cobra venom factor.

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5.1. INTRODUCTION

The aim of this study was not only to investigate the mechanism of the local tissue damage produced in the Shwartzman phenomenon but also to establish potential therapeutic approaches to reverse such damage. Thus, in this chapter we examined the effect of several pharmacological agents expected to modulate the inflammatory process such as WEB 2170, a specific platelet activating factor antagonist and misoprostol, a synthetic prostaglandin E_1 analogue.

Platelet-activating factor (PAF) is a phospholipid mediator produced by various cells including the neutrophils, macrophages, platelets, endothelial cells and mast cells (Braquet & Rola-Pleszczynski, 1987). PAF is derived from membrane phosphatidyl choline in two-step synthetic pathway (Sha·afi and Molski, 1988). The first step involves the removal of arachidonate from the phospholipid by phospholipase A_2 producing lyso-PAF (biologically inactive) (Fig 5.1). In the second step, lyso-PAF is converted by an acetyl transferase to the active form PAF-acether (or PAF). PAF has been described as a proinflammatory mediator that is capable of modulating the activities of macrophages, neutrophils, platelets and T cells. The development of specific PAF-antagonists has facilitated the investigation of the pathophysiological significance of this mediator in the production of endotoxic shock and various inflammatory and allergic reactions. PAF induces the aggregation, superoxide production, and degranulation of neutrophils and the aggregation and superoxide anion release from monocytes (Braquet & Rola-Pleszczynski, 1987; Yasaka *et al*, 1982). Doebber *et al* (1985) has shown that endotoxin-induced hypotension is mediated, in the

rat, by the endogenous production of PAF and that it can be reversed by the infusion of a specific PAF-receptor antagonist, Kadsurenone. The ability of cytokines such as TNF and IL-1 to induce PAF production (Valone & Epstein, 1988; Stewart *et al*, 1991) and vice versa (Poubelle *et al*, 1991) can amplify such effects in the endotoxic shock. Recently, small doses of PAF were found to synergize with non-toxic minute doses of LPS to produce an ischemic bowel necrosis in the rat (Gonzalez-Crussi & Hsueh, 1983). In man, intradermally injected PAF results in an early weal and flare response followed by a late-onset erythema at the site of challenge (Archer *et al*, 1984). WEB 2086, a specific PAF antagonist was shown to inhibit the PAF-induced bronchoconstriction, systemic hypotension and the lethal effects in guinea pigs (Casals-Stenzel *et al*, 1987). WEB 2086 reduced also, in a dose-dependent manner, the increase in cutaneous vascular permeability due to intradermally injected PAF. WEB 2086 has been also demonstrated to interfere with the production of anaphylactic shock in guinea pigs (Pretolani *et al*, 1987). WEB 2170 is another specific PAF-antagonist that is structurally similar to WEB 2086 but it is biologically more potent and it has a longer *in vivo* duration of action. Thus, WEB 2170 was used to investigate the involvement of PAF in the development of LSR.

Prostaglandins (PG) are naturally occurring substances produced by various effector cells of the immune system such as macrophages, mast cells and basophils. They are derived from arachidonic acid released from membrane phospholipids by the action of phospholipase A (or C) enzymes (Fig.5.1). Arachidonic acid is then converted by cyclooxygenase into prostaglandins (e.g. PGE_2 and PGI_2) or thromboxanes (TxA_2 and TxB_2)(Bach, 1988). Alternatively, the lipooxygenase enzyme will convert the arachidonate into leukotrienes. Prostacyclin

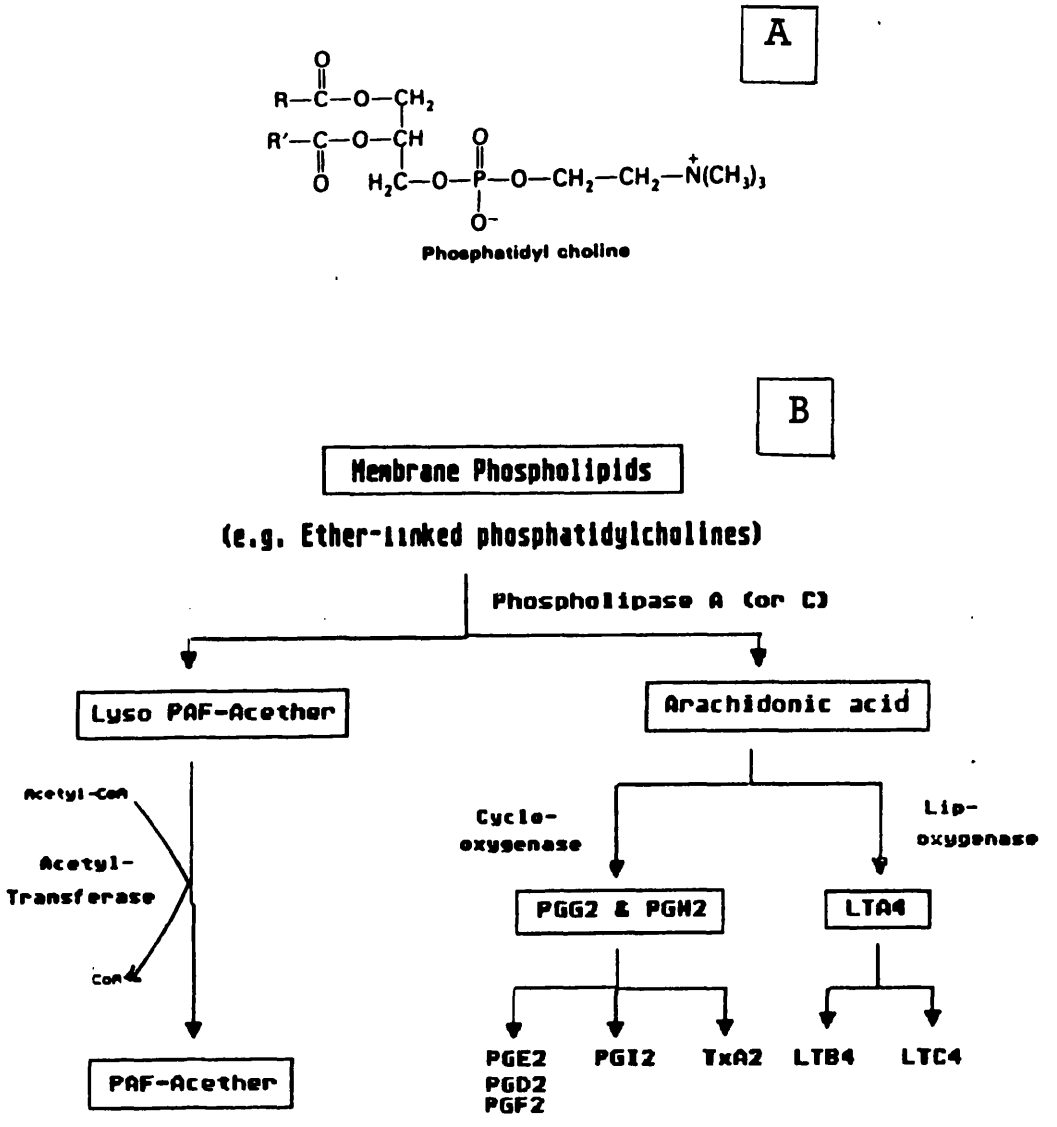

Fig.5.1. The structure of phosphatidylcholine (a membrane phospholipid) (A) and the metabolic pathway of membrane phospholipids leading to the production ob both PAF and arachidonic acid (B) (Stryer, 1981); Shaafi et al, 1988; Vane et al, 1990).

(PGI_2) and PGE_2 were described to be co-mediators of inflammation. PGI_2 and PGE_1 have also been implicated in the prevention of multiple organ failure associated with sepsis and the adult respiratory distress syndrome (ARDS) (Bihari and Tinker, 1988). The reduction of tissue damage produced by these two mediators is possibly due to their ability to induce vasodilation of the microcirculation as well as increasing the blood flow. They also inhibit platelet aggregation and reduce neutrophil and monocyte activation. Therefore, we have attempted to assess the effect of a synthetic PGE_1 analogue, misoprostol, on the induction of local skin tissue damage. Both animal and human studies have shown that misoprostol prevents gastric damage by inducing mucus and bicarbonate production (Bauer, 1985; Steiner, 1985; Leung *et al*, 1986; Wilson *et al*, 1986). In addition, it was also proved to inhibit the acidic secretions in the stomach without affecting the gastric blood flow.

Although the plasma complement has evolved as an antimicrobial mechanism, its activation in inflammation, trauma and sepsis can result in tissue damage. The clinical observation that Cardiopulmonary dysfunction and sudden death can be produced by hemodialysis has prompted a great interest in the possible mechanism (Jacob *et al*, 1980). Activation of the alternative pathway of complement resulted when the patient's plasma came in contact with the cellophane membrane of the dialyzer. This resulted in leukopenia, increased sequestration and aggregation of leukocytes in the blood vessels, particularly the pulmonary ones, causing microvascular damage. These effects could be attributed to the generation of the chemotactic peptides, C3a and C5a and then the predominant chemotactic peptide in humans, $C5a_{des\ Arg}$ (Fernandez *et*

al, 1978). The pathological role of C5a was demonstrated when a substantial damage to cultured endothelial cells was produced by superoxide anion and hydrogen peroxide released by C5a-activated granulocytes (Jacob *et al*, 1980). Prolonged circulation of C5a was detected in the early stages of the adult respiratory distress syndrome. Complement activation is not only responsible for the activation and chemoattraction of leukocytes but also the rapid *in vitro* adherence of these cells to umbilical vein endothelial cells which possibly play a role in facilitating their migration to sites of inflammation (Marks *et al*, 1989). This report also gave the evidence for the mere requirement of purified C3 component and factors B and D for the induction of this adhesion. Further tissue damage can be produced by the complement mediated activation of the coagulation system resulting in the deposition of fibrin clots in the blood vessels. Cobra venom factor (CVF) is a glycoprotein that has been purified from the venom of *Naja naja* and was found to be capable of activating the alternative pathway of complement (Muller-Eberhard & Fjellstrom, 1971). Previous studies have proved the participation of complement in the production of LSR (Fong and Good, 1971; Rothstein *et al*, 1988). Both reports indicated that the use of anti-complementary agents *in vivo* prevents the induction of Shwartzman lesions. Using radiolabelled red blood cells, Rothstein *et al* have also described the significant inhibition of haemorrhage in the challenged skin sites. Thus we tested the effect of CVF on LSR in our mouse model.

A recent report has demonstrated the ability of IL-6 to significantly inhibit TNF production by the human U937 histiocytic lymphoma cell line and by cultured human peripheral blood monocytes (Aderka *et al*, 1989). The *in vivo* production of TNF in response to

LPS in BCG-primed animals was also reduced suggesting a role of IL-6 in the negative regulation of TNF production. Interlukin-6 (IL-6) is a 23-26 KDa glycoprotein that is known to possess pleiotropic biological activities (Kishimoto, 1989; Le & Vilcek, 1989). It is also known as B cell stimulatory factor 2, IFN-beta 2, hybridoma/plasmacytoma growth factor and hepatocyte stimulating factor. IL-6 shares some of its biological activities such as the induction of thymocyte proliferation, pyrogenecity and the production of some acute phase proteins, with IL-1 and TNF. This observation together with the fact that both IL-1 and TNF can induce the release of IL-6 suggested the involvement of IL-6 in the mediation of many of the activities of IL-1 and TNF. However, IL-1 and TNF were found to stimulate the generation of limited types of the acute phase reactants whereas IL-6 induced a wide spectrum of these hepatic proteins. In addition, IL-6 was found to stimulate the production of the glucocorticoid hormones that can further enhance the synthesis of the acute phase proteins (Fuller & Grenett, 1989). Interestingly, high levels of these hormones down-regulate the synthesis of the inflammatory cytokines IL-1, TNF and IL-6 and thus providing a regulatory negative feedback. Von Asmuth *et al* (1990) have detected, using a murine zymosan-induced shock model, a maximum serum TNF level, one hour after the injection of zymosan followed one hour later by a peak of IL-6. Therefore, the *in vivo* induction of IL-6 by both IL-1 and TNF, the ability of IL-6 to inhibit TNF release, together with the fact that IL-6 is a major acute phase proteins and cortisol inducer, probably reflect a possible regulatory pathway involving the monocytes, hepatocytes and the adrenal glands operating during inflammation. Recently, murine peritoneal macrophages stimulated with zymosan activated serum

(containing activated complement components) were shown to release massive amounts of IL-6 (Von Asmuth *et al*, 1990) Therefore, we investigated whether the injection of CVF into experimental animals leads to excessive early production of IL-6 which might provide a second pathway, in addition to the depletion of complement itself, for the elimination of the haemorrhagic necrosis seen in LSR.

5.2.1. The *in vivo* effect of WEB 2170, a PAF antagonist on LSR :

To investigate the involvement of platelet activating factor (PAF) in the production of the local Shwartzman reaction, we used WEB 2170, a selective PAF antagonist (a generous gift from Dr H. Heuer, Dept. Pharmacology, Boehringer Ingelheim KG, FRG). The *in vivo* duration of action of WEB 2170 in mice has been estimated to be 5-6 hours after oral administration and it is less after intravenous injection (Dr. Heuer, personal communication). Because there were no reports describing the production of PAF in LSR, a pretreatment regime consisting of two injections was used to ensure that PAF production is inhibited for the longest possible period of time. The first group was given, intravenously, a dosage of 0.024 mg/kg 40 minutes before preparation (*M.vaccae*) followed by another dosage of 10 mg/kg given orally one hour after preparation. The second group was given the same treatment regime, but 40 minutes before and one hour after provocation (TNF). These two groups were compared to saline treated control group. The scheme used is shown in Fig. 5.2.

5.2.2. The effect of misoprostol , a synthetic prostaglandin E_1 analogue on LSR :

Misoprostol, a synthetic PGE_1 analogue (a kind gift from G.D. Searle & Co. Ltd) is stabilised by a 1:100 dispersion on hydroxypropyl methylcellulose (HPMC) which does not alter the pharmacology of misoprostol. Misoprostol was administered, orally, by mixing it with drinking water at a concentration of 0.5 mg/ml, three hours before preparation (*M.vaccae*) and they were maintained on that treatment during the whole experiment. Their water-drug mixture was changed regularly with a fresh preparation. Another

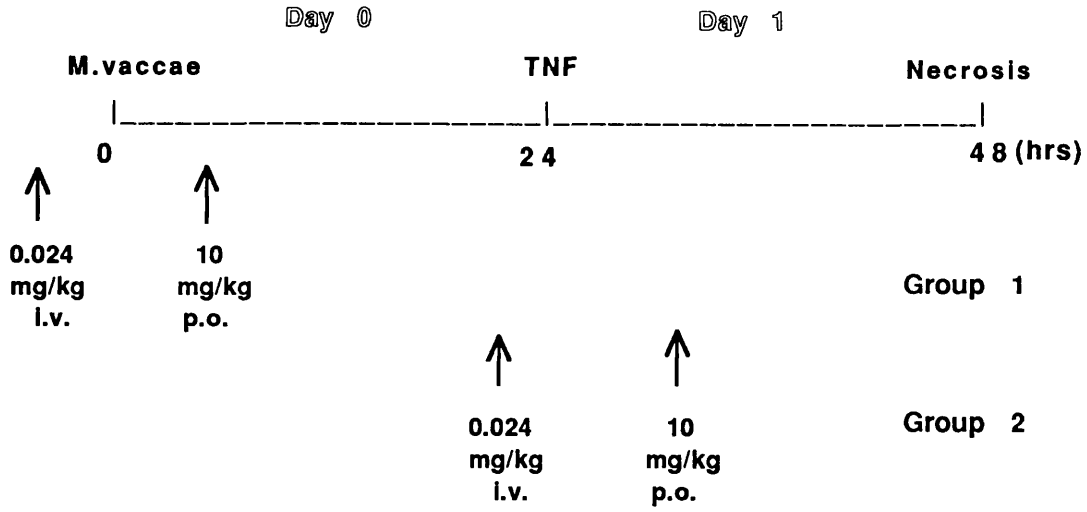

Fig5.2. The treatment regime used for WEB 2170.

group of animals were pretreated with the drug three hours before provocation (TNF). The control groups were given either saline or HPMC alone (0.5 mg/ml) mixed with their drinking water three hours before starting the experiment.

5.2.3. The effect of complement depletion on the development of LSR :

C57/BL/T mice were injected intravenously with 10 ug of cobra venom factor (CVF) purified from *Naja naja kaouthia* (Sigma, C-8406), one hour or eight hours before preparation with *M.vaccae* sonicate (17 ug). They were then challenged, 24 hrs later, with TNF (1 ug). The effect of complement depletion, exerted by CVF, on LSR in these mice was compared to LSR in saline-treated control group.

5.2.4. Measurement of serum complement :

CVF-treated animals were bled from the tail vein one hour before the injection of CVF, one hour later, and then once a day for 2-3 days. Blood was allowed to clot for 30-60 min at 37°C and the serum was then separated and stored at -20°C until assayed for complement level. This was done by radial immunodiffusion. Agarose Type I (Sigma, A-6013) was used as a 1 % solution prepared in barbiturate-Tris buffer (pH 8.6). A mixture of 100 ul of sheep anti-mouse complement C3 (The binding site, PE280) in 3 ml of pre-warmed agarose was poured on clean glass plate (7.5 X 7.5 cm). Rows of circular wells were punched and were filled with 5 ul of the test sera. Serial dilutions of normal mouse serum were included in the assay for reference. The plates were incubated for 48 hrs at room temperature, in a humid chamber. They were then washed with saline and pressed several times to get rid of excessive unbound serum.

Finally, they were completely dried and stained with Coomassie brilliant blue, destained and dried. The diameter of the precipitate was then measured.

5.2.5. IL-6 Bioassay :

A murine hybridoma B9 cell line (a kind gift from Dr. L. Aarden, University of Amsterdam) is IL-6 dependent and therefore was used for measuring IL-6 levels in mice sera (Helle *et al*, 1988). The cells were maintained in RPMI 1640 (Imperial laboratories) supplemented with L-glutamine, 5 % fetal bovine serum and 25 pg/ml of IL-6 (supernatant of MG63 osteosarcoma line, a kind gift from Dr. A. Meager, NIBSC, South Mimms, Potters Bar, U.K.). For the assay, B9 cells were washed in IL-6 free medium and then seeded in 96-well microtiter plates (Nunc) at 5 x 10^3 cells per well in the presence of serial dilutions of the sera to be tested. After an incubation period of 4 days at 37°C, 5 % CO_2 atmosphere, proliferation was measured by adding 10 ul/well of 5 mg/ml of (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide; Thiazolyl blue)(MTT, Sigma, M-2128) prepared in PBS. The plates were then incubated for 45 min at 37°C after which they were treated with 100 ul per well of 10 % Triton X 10 (Sigma, T-6878) in 0.01 M HCl. Optical density was read at 530 nm using the MICROELISA Auto-reader (Dynatech). A standard curve (shown in Fig.5.3) using serial dilutions of IL-6 (supernatant of MG63 cell line) was included in each assay and used to calculate its concentration in the test sera.

5.2.6. Histological studies :

It was carried out as described in chapter 4.

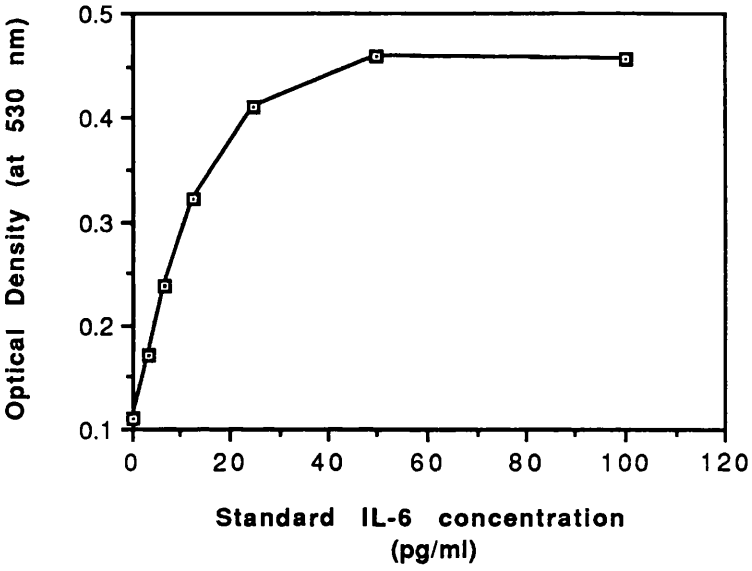

Fig.5.3. The standard curve used to estimate the concentration of IL-6 (pg/ml) in the murine test sera.

5.3.1. The effect of WEB 2170 on the production of LSR :

WEB 2170 is a specific PAF antagonist that has been proved to inhibit the various biological *in vitro* and *in vivo* effects of PAF. Therefore, we used this drug to investigate the possible role of PAF in the production of tissue damage in LSR. C57/BL/T mice were given the first dose of the drug (0.024 mg/kg) intravenously either before preparation (group 1) or before provocation (group 2). The second dose (10 mg/kg) was given, orally, one hour after preparation (group 1) or provocation (group 2). A partial inhibition of the haemorrhage but not the swelling of the foot-pads was detected in both groups (Fig. 5.4) providing an evidence for the release of PAF in LSR and its participation in the induction of tissue damage.

5.3.2. The effect of prostaglandin E_1 analogue on LSR :

Misoprostol is a synthetic prostaglandin E_1 analogue that has been described to be protective in septic shock. One group of animals was given the drug (0.5 mg/ml) in a mixture with their drinking water three hours before preparation (*M.vaccae*) and they were maintained on it until the end of the experiment. The second group was given the same treatment but, 21 hours after preparation and three hours before provocation (TNF). Both of these groups have demonstrated only a partial inhibition of the haemorrhagic component of the reaction when compared to the saline and the HPMC-treated groups (Fig. 5.5).

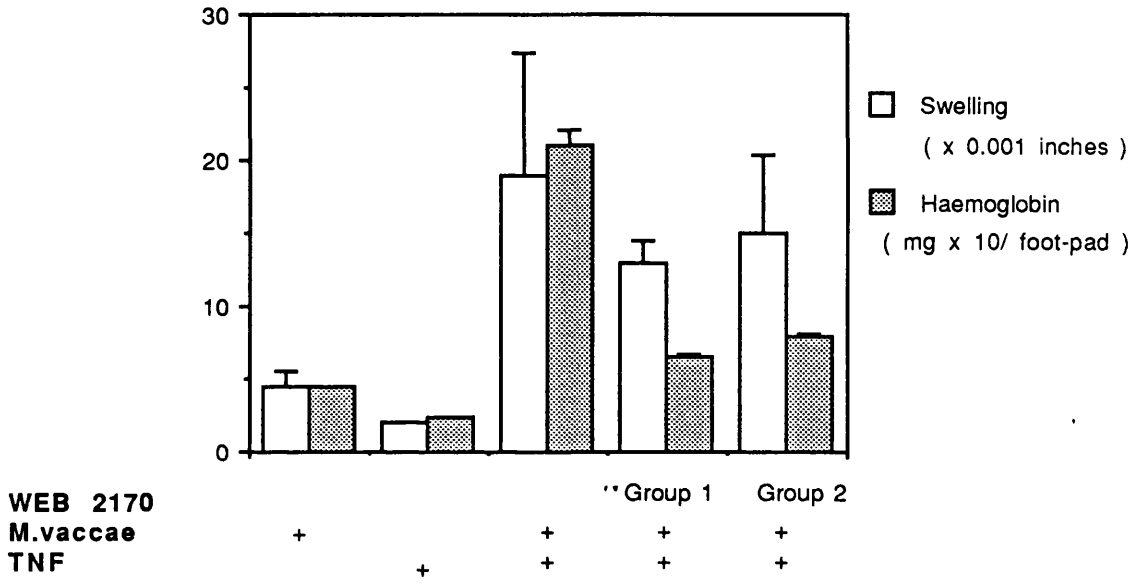

Fig.5.4. The effect of WEB 2170, a specific PAF antagonist on the production of LSR.

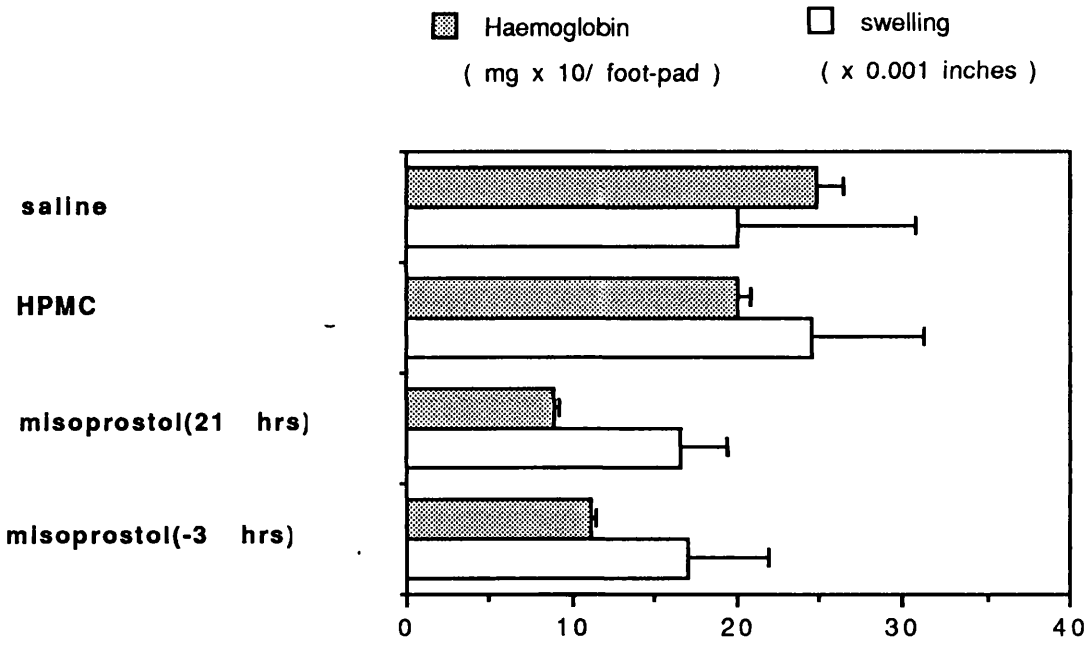

Fig5.5. The role of Misoprostol on the production of local Shwartzman reaction.

5.3.3. The role of complement in the production of local tissue damage :

Because of its ability to decomplement animals, purified cobra venom factor (CVF) was previously used in various experimental models to assess the involvement of the complement pathway in TNF-induced LSR. Test animals were injected intravenously with 10 ug of CVF either one or eight hours prior to challenge. This treatment insignificantly suppressed the swelling of the foot-pads of both groups and completely eliminated the haemorrhagic component of the reaction (Fig. 5.6). Complement regeneration, detected by radial immunodiffusion, in the group of mice that has been treated with CVF 8 hrs prior to their challenge provide an explanation for the small amount of blood seen in their feet. Sera from CVF-treated animals were collected immediately before preparation, 24 hours later (before injecting TNF) and 20 hours after provocation (before cutting off the feet). Serum complement level was measured, using an anti-murine C3 serum, by radial immunodiffusion. The relative concentration of C3 in the test sera is expressed as a percentage of the diameter of the immunoprecipitate reaction produced by these sera compared to that produced by normal mouse serum. In mice pretreated with CVF eight hours prior to their challenge, complement was reduced to 33.3 % of the normal level at the time of preparation (Fig. 5.7). Their complement level, however, increased to 47 % of normal serum complement level on day 2, before the injection of TNF into their feet. On the other hand, mice injected with CVF just one hour before preparation demonstrated a higher complement level (58.8 %) at the time of challenge. On day 2, their complement was lower (35 %) and started increasing on day 3 demonstrating 44.7 % of the normal level. These findings indicate that the injection of a minute

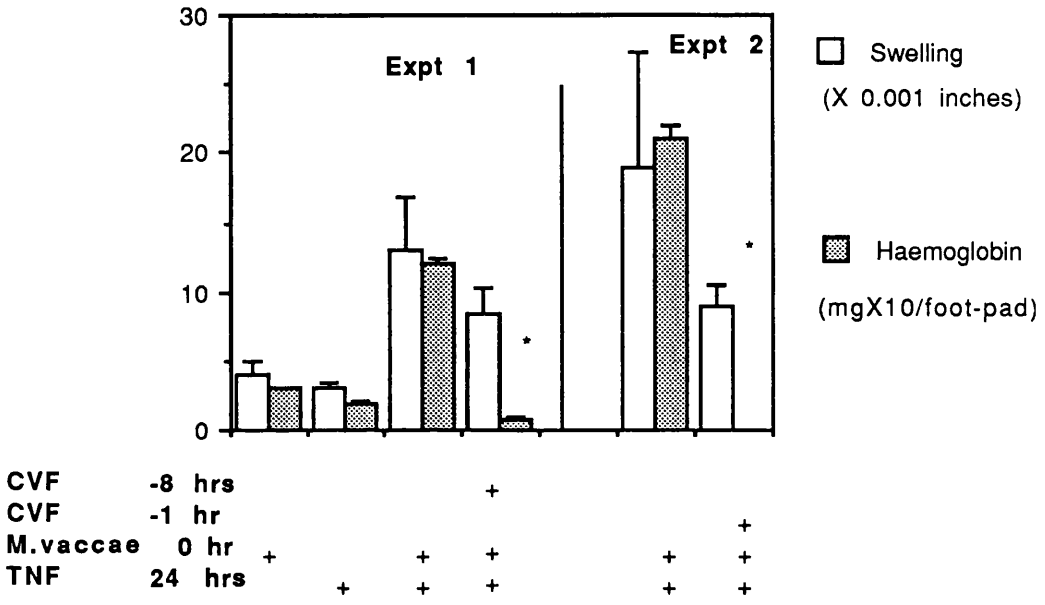

Fig.5.6. Does complement play a role in the production of TNF-mediated local tissue damage.

- **The figure presents the results of 2 separate experiments.**

* **$p<0.05$ (Mann-Whitney U test)**

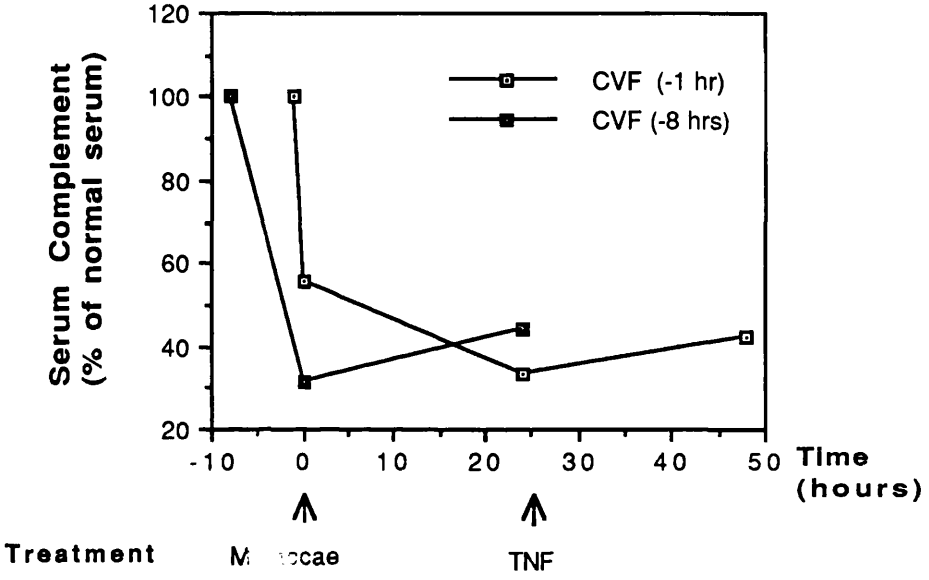

Fig.5.7. The level of the C3 complement component in the CVF-treated mice when challenged for the production of LSR.

dose (10 ug) of CVF eight hours before preparation results in the regeneration of complement before the end of the experiment. In contrast, animals pretreated with the same dose of CVF one hour prior to preparation remain depleted until the end of the experiment.

When compared to the control group foot-pads (shown in the previous chapter, Fig.4.11), histological examination of the feet of decomplemented-challenged mice showed almost a similar increased thickness of the dermis caused by edema and massive infiltration of the skin tissue by inflammatory cells (both neutrophils and macrophages) but without any tissue damage or red blood cell extravasation (Fig. 5.8).

CVF-treated mice were bled one hour before and after the treatment and two hours following preparation (*M.vaccae*) and provocation (TNF). Sera were collected and the IL-6 level was measured using a specific bioassay. As shown in Figure 5.9., the intravenous injection of CVF, on its own, induced a significant level of the cytokine (1064.6 $\pm$ 206 pg/ml), compared to the saline injected group (359.5 $\pm$ 212 pg/ml) ($p < 0.005$). The subcutaneous injection of either *M.vaccae* or TNF, in both the saline and CVF-treated group could also induce massive similar levels of IL-6 cytokine. However, decomplemented and control animals produced the same vast levels of IL-6 when the combined treatment, *M.vaccae* +TNF was given.

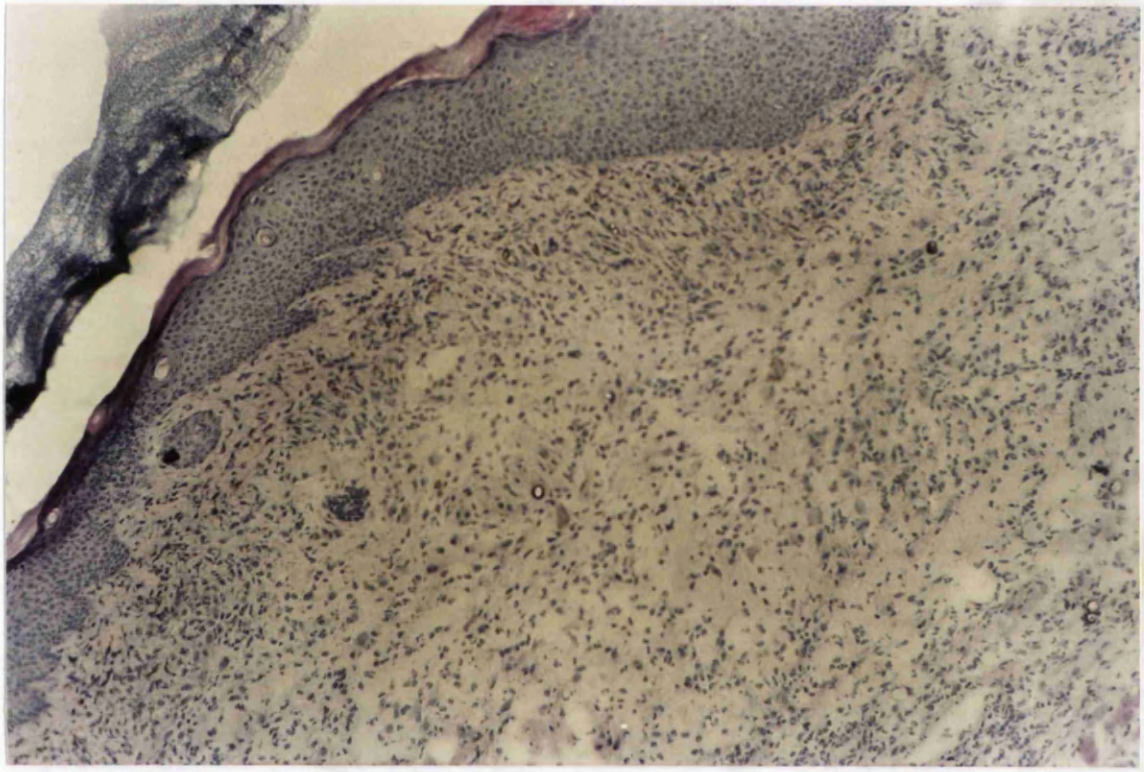

Fig.5.8. H & E stained skin section of a CVF-treated mouse that was challenged for a LSR. Compared to Fig.4.11, there is increased thickness of the skin, massive cellular infiltration but with no haemorrhage or necrosis.

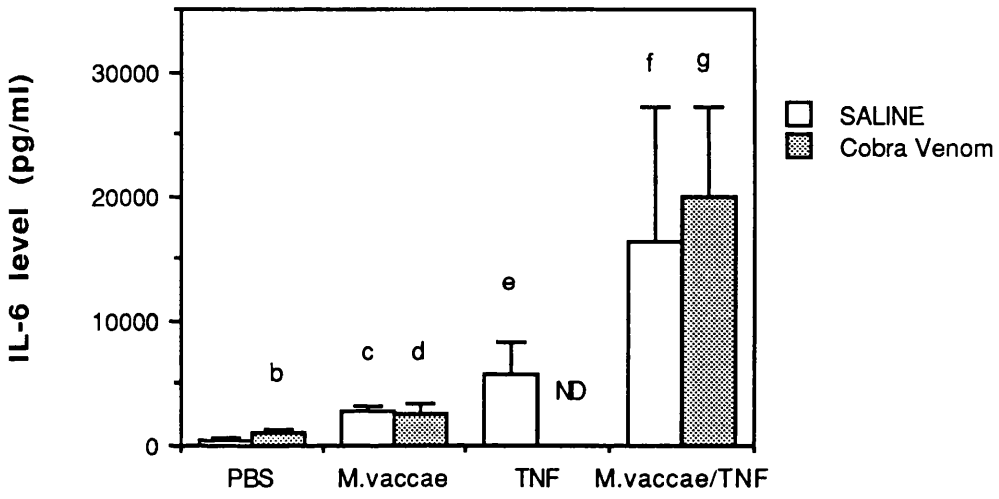

Fig.5.9. IL-6 level in the sera of cobra venom factor-treated mice.

ND: not done

b: p vs controls <0.005 (Student's t-test)
c: " " "
d: " b " "
e: " " <0.01 "
d: " g <0.025 "

5.4. DISCUSSION

Platelet activating factor (PAF) is a phospholipid mediator that has been proved to be a potent stimulator of acute inflammatory reactions (Braquet & Rola-Pleszczynski, 1987). PAF is derived from a membrane phosphatidyl choline by the action of phospholipase A2 enzyme and it has a short half life due to its inactivation by serum deacetylase enzyme (Bach, 1988). The availability of specific PAF antagonists has made it possible to test the involvement of this mediator in the pathogenesis of inflammation. Among the most important effects of PAF is its role in the induction of microvascular injury (Braquet *et al*, 1989). The *in vivo* infusion of PAF into guinea pigs induces cytoskeletal changes and retraction of endothelial cells. This results in the accumulation and adherence of platelets to the retracted endothelium, followed by neutrophils and monocytes leading to thrombus formation and fibrin deposition. PAF production has been demonstrated in experimental endotoxemia and in the plasma of patients with sepsis (Braquet *et al*, 1989). We have demonstrated, in this chapter, that the administration of WEB 2170 twice, before and after the preparatory or provocative injections can reduce the haemorrhage without affecting the swelling produced in LSR. The partial inhibition of haemorrhage could be explained by the fact that other inflammatory mediators such as IL-1, TNF and IL-8, are capable of activating both leukocytes and endothelial cells and promoting their adherence (Ferrante *et al*, 1988; Colditz *et al*, 1989; Smith *et al*, 1991). These results, however, provide more evidence for the therapeutic usefulness of WEB 2170 and other specific PAF antagonists in various pathological reactions where PAF involvement is detected. Such

antagonists did not only inhibit but also reversed the endotoxin-induced hypotension and the lethal effects seen in challenged rats (Terashita *et al*, 1985; Casals-Stenzel, 1987).

Free arachidonic acid released from membrane phospholipids is oxidised by either the cyclooxygenase enzyme, to produce the prostaglandins (PG) and thromboxanes (Tx), or the lipooxygenase enzyme to produce leukotrienes (LT) (Bach, 1988). Prostaglandins causing vasodilation and inhibiting aggregation while thromboxane B_2 and leukotrienes C_4 and D_4 causing vasoconstriction, platelet aggregation and vascular permeability. Since these products have a wide range of opposing biological activities and short half lives, the effect seen *in vivo* depends on the profile and the concentration of the different mediators produced in a specific reaction. Vasoconstriction, micro-embolism and edema associated with sepsis results in cell damage and multiple organ dysfunction (Bihari & Tinker, 1988). Recently, both animal and human studies have provided the evidence for a possible therapeutic usefulness of prostacyclin (PGI_2) and PGE_1 reflected in a reduction of the tissue damage seen in this pathological situation. Similar to what is seen in sepsis, necrotic tissue damage, edema and haemorrhage are observed in a LSR induced in the mice foot-pads. This prompted us to examine the effect of misoprostol on LSR in our murine model. Misoprostol (or Cytotec) is a synthetic PGE_1 analogue that has been shown to prevent the production of gastric damage by reducing mucus and bicarbonate production while inhibitting the gastric acid secretions. Misoprostol was also found to undergo rapid de-esterification to misoprostol acid, its main and pharmacologically active metabolite (Karim, 1987). Misoprostol acid is then further metabolised in a similar way to the naturally occurring

prostaglandins. The present results demonstrate the ability of this drug to inhibit the haemorrhagic component of the reaction. Its partial effect is probably due to the complexity of the reaction and the involvement of various biological mediators. An intravenous injection of human recombinant TNF into rats resulted in hypothermia, hypoglycemia, diarrhea, metabolic acidosis and then death within 2-4 hours (Kettelhut *et al*, 1987). The administration of cyclooxygenase inhibitors, known to block PGE_2, reduced the lethality by 70%. Thus, the role of the arachidonic acid metabolites in septic shock depends on which mediators are produced and the timing of their release.

Activation of the complement system causes tissue damage directly by the cytolytic membrane attack complex (C5b-C9) or indirectly by the activated fragments, C3a and C5a that stimulate a pro-inflammatory response. These two activated components and the C5a $_{des\ Arg}$, an end product of C5a, were shown to be chemotactic to leukocytes (Fernandez *et al*, 1978). In addition, both C5a and C5a $_{des\ Arg}$ stimulate the secretion of both IL-1 and TNF from human mononuclear cells *in vitro* (Okusawa *et al*, 1988). C5a is also responsible for the induction of a transient neutropenia followed rapidly by neutrophilia caused mainly by the mobilization of neutrophils from the bone marrow pools (Kajita & Hugli, 1990). The *in vivo* administration of zymosan-activated plasma or purified C5a into animals provokes granulocyte aggregation and endothelial damage reflected by the extravasation of plasma proteins (Hammerschmidt *et al*, 1981). Moreover, C3bi complement component induces the CR3-mediated neutrophil-endothelial adhesion (Marks *et al*, 1989). Such adhesion was shown to reach its peak within 20 minutes. On the other hand, various adhesion receptors can be involved in mediating

the adhesion and migration of inflammatory cells. Gamble *et al* (1985) demonstrated that TNF enhances the adherence of human peripheral blood neutrophils to human umbilical vein endothelial cell monolayers by acting on both cell types. Its effect on neutrophils did not require protein synthesis and was maximal within 5 minutes. This effect could be explained by the notion that TNF rapidly induced the surface expression of CR3. However, its effect on the endothelial cells took 4 hours due to the requirement of protein synthesis. CR3 receptor expression itself can be induced by the predominant chemotactic peptide, $C5a_{des\ Arg}$ (Fearon & Collins, 1983). Cobra venom factor (CVF) is a glycoprotein purified from the cobra venom. It has been demonstrated that CVF is functionally analogous to the activated complement component, C3b and therefore, it can bind to factor B to form the C3-C5 convertase enzyme (Vogel *et al*, 1984). The intravenous injection of CVF into experimental animals, transiently, depletes them of their complement via the activation of the alternative pathway. In aggreement with the previous findings, we show in this chapter that such treatment blocks the tissue damage produced in LSR. Rothstein *et al* (1988) have provided the evidence for the requirement of the complement C5 component for the induction of TNF-mediated damage. They demonstrated that mice deficient in C5 component due to a genetic defect can develop a local Shwartzman reaction only when they were reconstituted with a normal mouse serum or a partially purified C5a. C5-deficient mice were also protected from the synergistic (TNF-LPS)-induced shock and bowel injury Hsueh *et al*, 1990). Using our model, however, it was interesting to further prove the possibility of separating the two components of the reaction shown by the complete elimination of the haemorrhagic necrosis but not the swelling. This finding give us the chance to explore various ways of

manipulating this pathological reaction. These results were also confirmed by the histological examination of the challanged foot-pads which showed an edematous skin heavily infiltrated with inflammatory cells. Unlike the control group foot-pads, no blood or tissue damage could be detected. An insignificant amount of haemorrhage detected in mice that had been pretreated with a minute dosage of CVF, 8 hours prior to their challenge was presumably due to the regeneration of complement components. On the other hand, the massive cellular accumulation contradicts the suggestion that complement is required merely for the chemoattraction, activation and migration of cells. The vast infiltration of cells in spite of complement depletion could be parially explained by the fact that other biological mediators such as TNF, IL-1 and IL-8 (Ferrante *et al*, 1988; Colditz *et al*, 1989; Smith *et al*, 1991) do have similar effects on leukocytes. Therefore, although activated complement peptides might act in concert with other inflammatory mediators, the precise role of complement in inflammation remains to be elucidated. Complement has been implicated in the pathogenesis of various events such as experimental arthritis in rats (Schwab *et al*, 1982) and myocardial infarction following coronary artery occlusion (Maroko *et al*, 1978).

Interlukin-6 (IL-6) is a 26 KDa glycoprotein produced by various cell types including monocytes/macrophages, fibroblasts, T and B lymphocytes, endothelial cells and tumor cells (Kishimoto, 1989; Le & Vilcek, 1989). It is a pleiotropic cytokine that exerts different biological effects such as the induction of immunoglobulin production in B cells and acute phase proteins in hepatocytes, the growth of plasmacytoma cells and the activation of hematopoietic stem cells. The ability of both IL-1 and TNF to induce IL-6

production together with the observed similarity in some of the effects of the former cytokines to those of the latter mediator have prompted various investigators to suggest the involvement of IL-6 in the mediation of these effects. Abnormal IL-6 production has been recently reported in several pathological events such as cardiac myxoma which is a benign intraatrial heart tumor. Patients with this tumor have high IL-6 levels accompanied with autoantibody production which disappeared after surgical removal of the tumor. Patients with active rheumatoid arthritis also have vast amounts of the cytokine in their synovial fluid. On the other hand, a recent report has revealed the ability of this mediator to inhibit TNF production in U937 cell line and human peripheral blood monocytes in response to LPS (Aderka *et al*, 1989). In addition, pretreatment of monocytes with recombinant human IL-6 abrogated their *in vitro* cytotoxicity to tumor cells. IL-6 also induces the release of wide spectrum of acute phase reactants known for their role in inflammation. It is also important to mention the ability of IL-6 to stimulate the release of the glucocorticoid hormones which, in turn, inhibit the secretion of IL-1, TNF and IL-6. Injecting meningococcal LPS or viable meningococci into rabbits resulted in the sequential release of TNF, IL-1 and then IL-6 (Waage *et al*, 1989). Together with the fact that serum IL-6 is detected after both TNF and IL-1, the previous observations, suggest that a negative feedback is provided by IL-6 and the glucocorticoid hormones in inflammation. It was of considerable interest, therefore, to investigate whether the administration of CVF induces high circulating serum levels of IL-6 and if these high levels are involved in the mediation of the complement depletion-associated protection in challenged animals. The detection of significant levels of this mediator in the serum of

CVF-treated animals, compared to the control group, before being prepared for a Shwartzman reaction could be an indication of such a role for IL-6. In spite of the difference in the extent of tissue damage produced in the two groups, both have presented the same level of cytokine following both preparation and provocation. Both groups have also demonstrated high levels after single preparatory or provocatory injections. A possible protective role for IL-6 is further investigated and discussed in the following chapter.

In summary, this chapter shows that this new model of LSR involves a cascade of events including C`, derivatives of membrane phospholipids and cytokines.

CHAPTER SIX

The modulation of the sensitivity of a prepared site to the TNF-induced tissue damage.

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6.1. INTRODUCTION

Endotoxin (lipopolysaccharide or LPS) is a major component of the cell wall of gram-negative bacteria and has been shown to play a major role in the pathogenesis of infections caused by these bacteria (Morrison, 1983). The *in vivo* administration of LPS results in the production of various cytokines such as TNF, IL-1, granulocyte-macrophage colony stimulating factor and interferon followed by the acute phase proteins. TNF and IL-1 were demonstrated by several groups to mediate both the pathological (fever, shock and death) and the beneficial biological effects (resistance to lethal irradiation, non-specific resistance to infection, the anti-inflammatory effects and/or tolerance) associated with the inflammatory response to LPS (Rosenbaum *et al*, 1983; Vogel *et al*, 1988; Neta *et al*, 1991). Therefore, various groups have established different experimental models to investigate the mechanisms involved in both of these effects and to test potential therapeutic agents. The ability of an initial sublethal exposure to LPS to render experimental animals refractory to a subsequent challenge with LPS is known as the early phase tolerance (Vogel *et al*, 1988). Recombinant preparations of both TNF and IL-1 were shown to synergize *in vivo* to induce such tolerance. The induction of the LPS-mediated tolerance in nude, B cell deficient and splenectomized mice suggested that spleen cells are not required for the production of the state of hypo-responsiveness (Madonna & Vogel, 1986). The non-specific resistance to infection (or tolerance) induced by LPS have been also described previously by Zehavi-Willner *et al* (1991). They demonstrated the ability of LPS pretreatment to protect mice from *Pseudomonas aeruginosa* exotoxin-induced lethality. In

addition, C3H/HeJ mice (LPS resistant) were shown to be more susceptible to the lethal infection with *Escherichia coli* than the C3H/HeN mice (LPS sensitive) and that the pretreatment of C3H/HeJ mice with both IL-1 and TNF can protect them from the infection (Cross *et al*, 1989). Results described in the first section of this chapter demonstrate the induction of tolerance to LSR by a sublethal dose of LPS and investigate the possible role of TNF in the induction of such tolerance.

In the second section of this chapter I investigate the possible regulatory role of heat shock proteins in LSR. Heat shock (or stress) proteins are highly conserved proteins that exist in cells at low to moderate levels under normal growth conditions but are produced massively in response to stresses such as a rise in temperature, oxygen radicals or infection (Young and Elliot, 1989). They were grouped into various families (hsp90, hsp70, hsp60, etc.) according to their molecular weight. Stress proteins have been described as immunodominant targets in both bacterial and parasitic infections. Recently, pretreatment with the mycobacterial heat shock 65 KDa (hsp65) protein has been shown to block the arthritis induced in various experimental models by Freund·s complete adjuvant (Van Eden *et al*, 1988), the streptoccocal cell wall (Van den Broek *et al*, 1989) and pristane (Thompson *et al*, 1990) and to prevent the development of insulin-dependent diabetes mellitus in NOD/Lt mice (Elias *et al*, 1990). In these models most of the chronic tissue damage is cytokine induced and IL-6 is one of the cytokines involved. This leads to the possibility that the heat shock proteins can regulate either the release of cytokines or the sensitivity of a T cell-dependent inflammatory site to cytokines.

In this chapter I demonstrate that it is possible to immunise mice with mycobacterial antigens so as to prime them for delayed type hypersensitivity (DTH) responses which are sensitive to subsequent injection of TNF (+ve LSR) or for DTH responses which are unaffected by TNF (-ve LSR). In view of the immunomodulatory roles of mycobacterial hsp outlined above, I have tested the possible role of hsp in the control of this TNF sensitivity and correlated these results with the serum IL-6 levels.

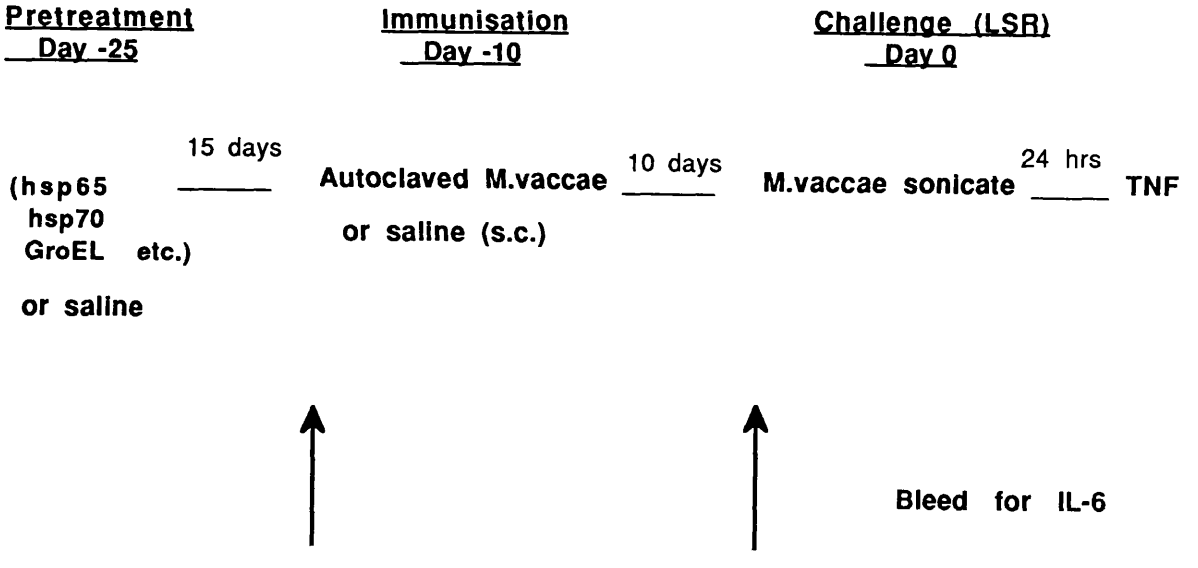
6.2.1. The effect of pretreatment with LPS on the generation of LSR :

C57/Bl/T mice were given, intraperitoneally, 25 ug of LPS at -5 hours (before the preparatory injection of *M.vaccae* sonicate), at 19 hours (before the provacative injection of TNF) or at both -5 and 19 hours. The effect of such pretreatment on LSR induced by *M.vaccae* sonicate was then investigated. Furthermore, the role of TNF in the induction of LPS-mediated tolerance was examined by pretreating test animals, intravenously, with rabbit anti-murine TNF (kindly provided by G.E. Grau, Switzerland) at 1 mg/mouse 4 hours prior to the administration of the tolerizing dose.

6.2.2. The *in vivo* experimental model :

Test animals were pretreated with the following antigens: *M.vaccae* sonicate, recombinant *M.bovis* heat shock proteins: the 65-KDa (hsp65, a kind gift from Drs. J.D.A. van Embden and R. van der Zee, Bilthoven, The Netherlands) and the 70-KDa (hsp70, a generous gift from van der Zee), recombinant *E.coli* heat shock protein 65-KDa (GroEL, kindly donated by Dr. A. Mehlert, University of Dundee, G.B.), the mycobacterial BCG85 complex antigen purified from 3 weeks old culture filtrate of *M.tuberculosis* as described in chapter two, and Keyhole limpet haemocyanin (KLH, Sigma, H-2133). These antigens were given at 50 ug in 0.2 ml Freund·s Incomplete Adjuvant (FIA), injected intraperitoneally (i.p.) on day - 25. Then as shown in Fig.6.1, on day - 10 mice were given (s.c.) either autoclaved *M.vaccae* (10^9) or saline. On day 0, the foot-pads were prepared for a Shwartzman reaction with *M.vaccae* sonicate (17 ug) and challenged, 24 hours later, with TNF (1 ug)(protocol is shown

Fig.6.1. The protocol for the in vivo experiments.



in Fig.6.1.). The difference in the thickness of the foot-pads and the haemorrhage was measured 16 and 20 hours later, respectively. Sera were collected from test animals on day -25 and day -10 (before and 15 days after their pre-treatment) and on day 0 (just before challenging them for LSR) (Fig.6.1). To test the involvement of TNF in mediating the inhibition of LSR induced by pretreatment with antigen/FIA, the challenged animals were given, i.v., an injection of rabbit anti-murine TNF (1 mg/mouse). One group of animals was given the antibody simultaneosly with the pretreatment and was also given another injection of the antibody seven days later (the antibody disappears from the circulation in one week). Another group, however, received one injection of the antibody four hours before the preparatory injection (*M.vaccae* sonicate) of LSR.

6.2.3. IL-6 assay :

IL-6 bioassay was conducted as detailed in the previous chapter.

6.3. RESULTS

6.3.1. The role of LPS as an anti-inflammatory agent :

LPS at 25 ug injected intraperitoneally 5 hours prior to challenging them with the preparatory injection significantly reduced the LSR, both the swelling ($p<0.005$) and haemorrhage ($p<0.05$)(Fig.6.2.). In contrast, the same treatment given at 19 hours produced a significant inhibitory effect on the swelling ($p<0.05$) but not the hamorrhagic component of the reaction. LPS administered at both -5 and 19 hours completely abolished the LSR. To investigate whether TNF is participating in the production of the LPS-mediated tolerance, rabbit anti-murine TNF was given 4 hours before injecting LPS (given at 25 ug/mouse, 5 hours before the preparatory injection). This did not reverse the tolerance (Fig.6.3). In addition, the anti-murine TNF polyclonal antibody, on its own, did not have any effect on the LSR induced by recombinant murine TNF.

6.3.2. The effect of pretreatment with various antigens/FIA on TNF-induced local tissue damage :

Test animals were pretreated, intraperitoneally, with 100 ug of *M.vaccae* sonicate mixed up with FCA and then challenged, 12 days later, for a LSR using *M.vaccae* sonicate for preparation and TNF for provocation. These animals were compared with those injected, s.c., with autoclaved *M.vaccae* bacilli (10^9/mouse). Control animals were pretreared with FCA or saline. The sonicate of *M.vaccae* in FCA induced a DTH reaction similar to that induced by the autoclaved mycobacterial bacilli. Although the autoclaved bacilli enhanced significantly both the swelling and the haemorrhage in the challenged foot-pads, the inclusion of the sonicated antigen in FCA had an inhibitory effect on the haemorrhagic component of the

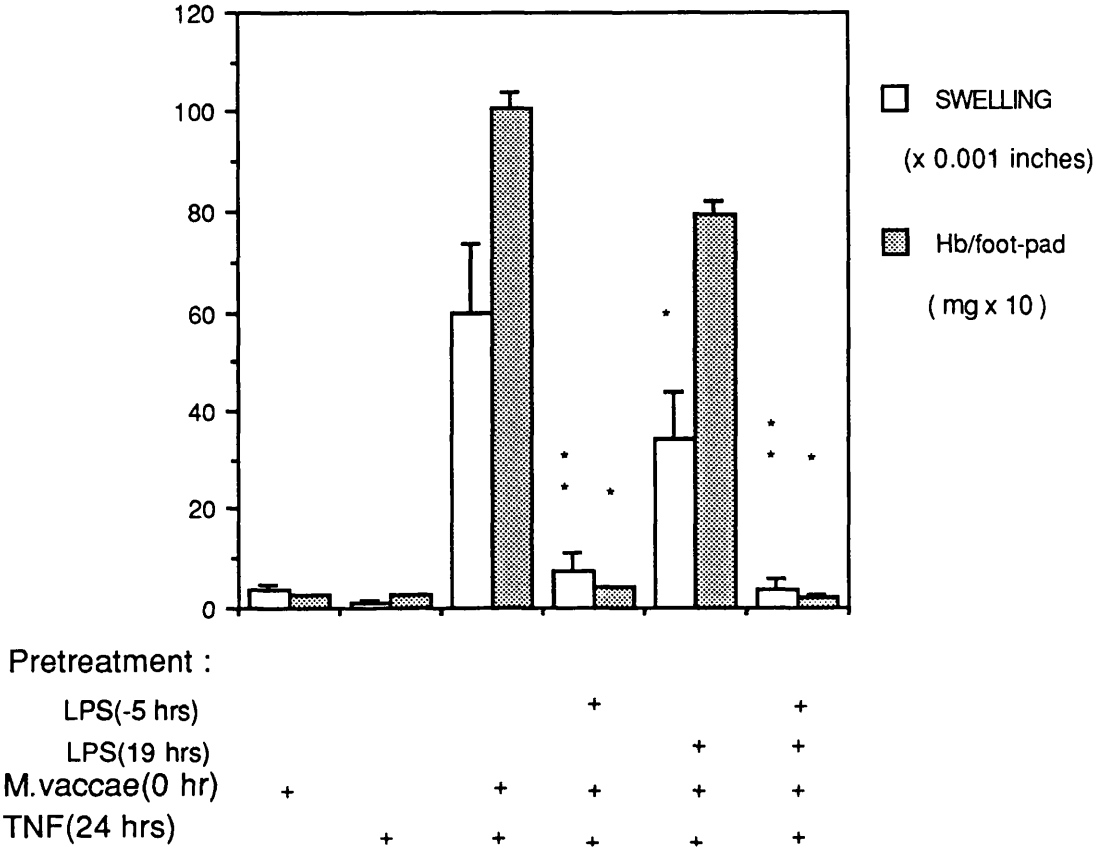


Fig.6.2. Tolerance to LSR induced by LPS

* p<0.05 (Student t-test)
* * p<0.005 (Student t-test)

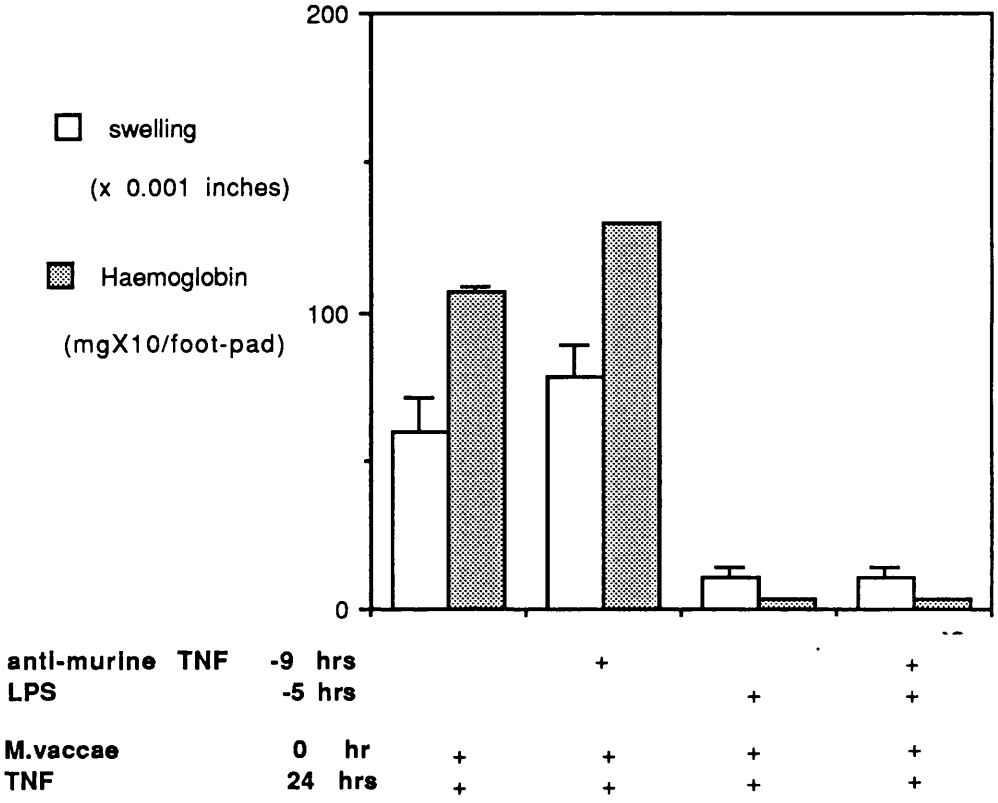


Fig.6.3. The effect of anti-murine TNF on the LPS-induced tolerance to LSR.

reaction (Fig.6.4). Moreover, FIA not containing *M.vaccae* was almost as effective as autoclaved *M.vaccae* at enhancing the susceptibility to LSR.

To investigate the role of heat shock proteins in mediating this phenomenon, mice were injected, i.p., with 50 ug of various bacterial heat shock proteins, the mycobacterial hsp65, recombinant *M.bovis* 70 KDa (hsp70) or the *E.coli* 65 KDa (GroEL) mixed with FIA (in 0.2 ml). Control animals were given saline or FIA. Mice were then injected, s.c., with autoclaved *M.vaccae* bacilli (10^9) 12-15 days following pretreatment and 10 days prior to their challenge for a LSR. This immunisation schedule was chosen because, as shown in the previous figure, without pretreatment it results in priming for DTH responses which are very sensitive to TNF. Compared to the saline pretreated group, all treatments were effective in inhibiting LSR (both the swelling and the haemorrhage) when challenged with *M.vaccae* sonicate and then TNF (Fig.6.5). The serum interleukin-6 level was measured using the IL-6 dependent B9 cell line. The cytokine level was significantly raised 15 days after pretreatment with FIA and the hsp65 (Fig.6.6). However, such levels were reduced at the time of challenge with *M.vaccae* sonicate except for the FIA injected group. The saline group, however, showed a slight increase in their serum IL-6 level after the injection of *M.vaccae* bacilli.

In an attempt to investigate whether the inhibition of LSR is produced specifically by heat shock proteins, we pretreated the mice with a non-heat shock purified mycobacterial protein, BCG85, or an unrelated non-mycobacterial antigen, KLH. All of these antigens inhibit, to a variable extent, the LSR (Fig.6.7). These results

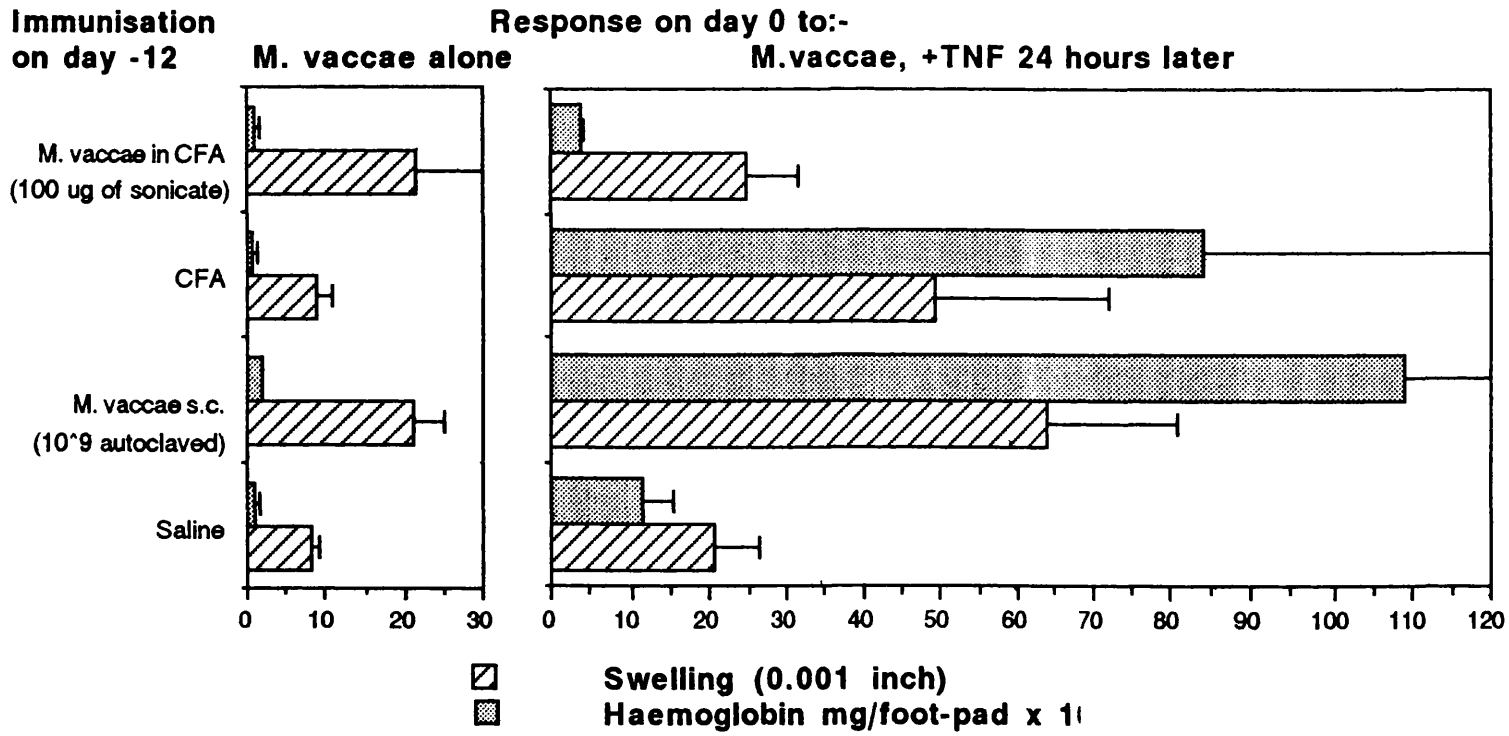
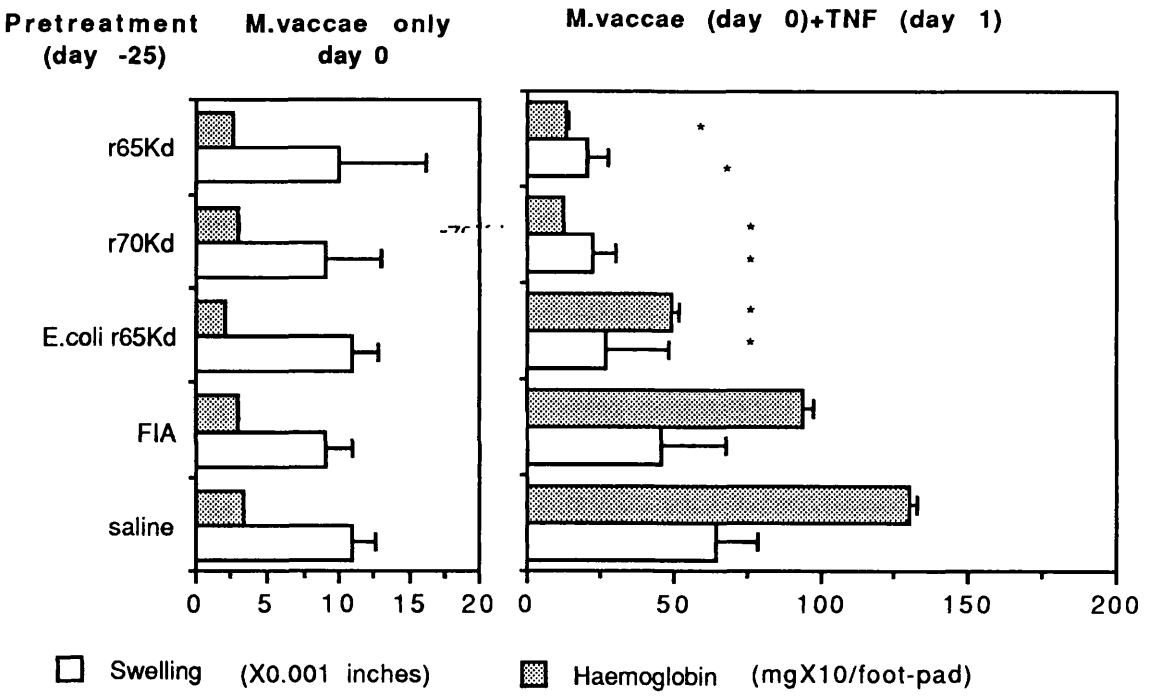


Fig.6.4. The modulation of the sensitivity of DTH sites to the TNF-induced tissue damage.



All mice received 10^9 M.vaccae on day -10

Fig.6.5. The effect of pretreatment with various heat shock proteins on the TNF-induced local tissue damage.

*** p<0.05 (Mann-Whitney U test)**

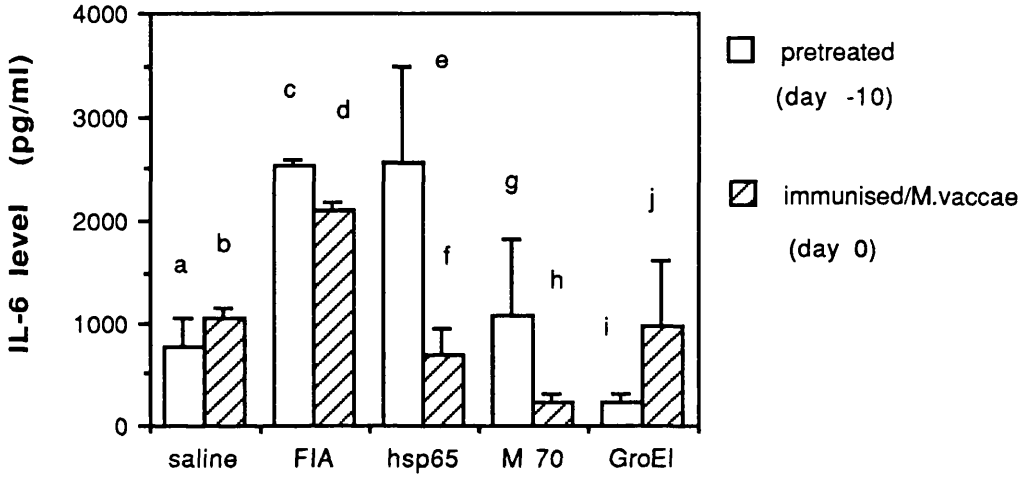
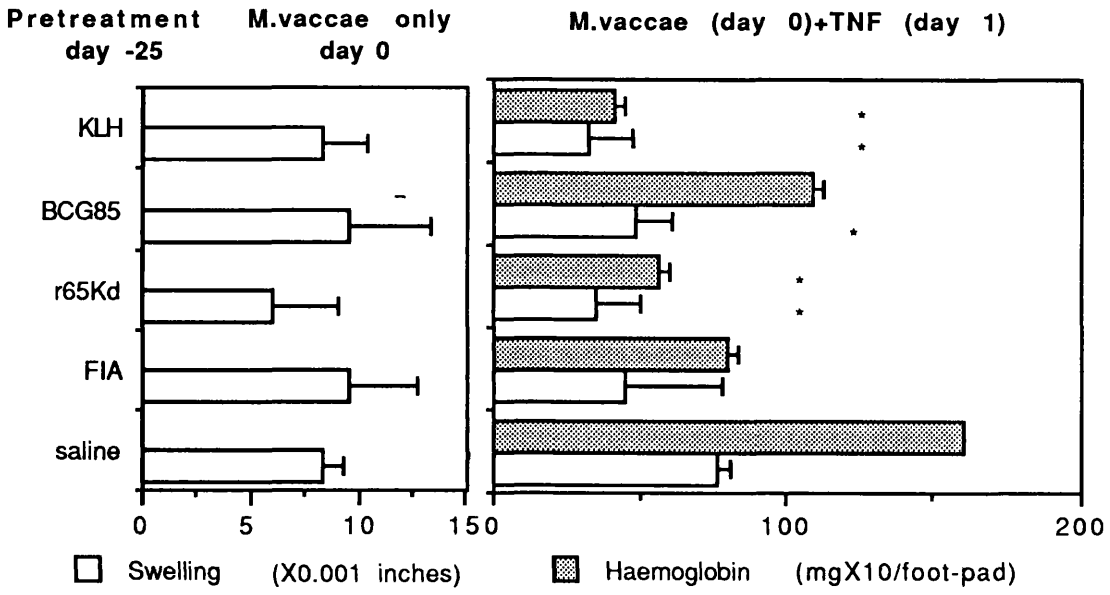


Fig.6.6. IL-6 level in the serum of animals pretreated with various heat shock proteins mixed with FIA.

c vs a	**p<0.025**	**(Students t-test)**
e vs a	**p<0.025**	**"**
d vs b	**p<0.025**	**"**
h vs b	**p<0.025**	**"**



All mice received 10^9 M.vaccae on day -10

Fig.6.7. The effect of pretreatment with various antigens mixed with FIA on the TNF-induced local tissue damage.

*** $p<0.05$ (Mann-Whitney U test)**

indicate that pretreatment of mice with various antigens in FIA renders them hyporesponsive to the TNF-mediated tissue necrosis at the previously prepared sites. Similar to our previous findings, the IL-6 level was increased in both the hsp65 and FIA-treated groups at the time of immunisation (Fig.6.8) on day -10, but the level of the cytokine, at the time of the challenge for a LSR (day 0), was significantly reduced in the hsp65-injected animals. However, there was no obvious correlation between IL-6 levels at the time of immunisation or challenge, and the subsequent diminution of the LSR.

All these experiments used the protocol in which the size of the LSR is enhanced 10 days before challenge. This type of LSR is susceptible to inhibition bt anti-CD4 (chapter 4) whereas the weaker LSR seen in animals which have not been pre-immunised was susceptible to suppression by anti-CD8. Therefore, it seemed possible that different regulatory pathways are involved. To check this point, further experiments were planned in which the effects of pretreatment on day - 25 with FIA, with or without the protein antigen, was tested in animals which were not subsequently immunised on day - 10.

To investigate whether the s.c. injection of *M.vaccae* following the pretreatment had a role in the modulation of the sensitivity of DTH reactions to TNF, mice were pretreated with hsp65 or KLH in FIA, FIA or saline followed 15 days later by a subcutaneous injection of either the autoclaved *M.vaccae* bacilli or saline. These test animals were challenged 10 days later for their ability to produce a LSR. The various groups which were given saline 15 days following pretreatment with FIA, with or without antigen, all showed less haemorrhage in their challenged feet

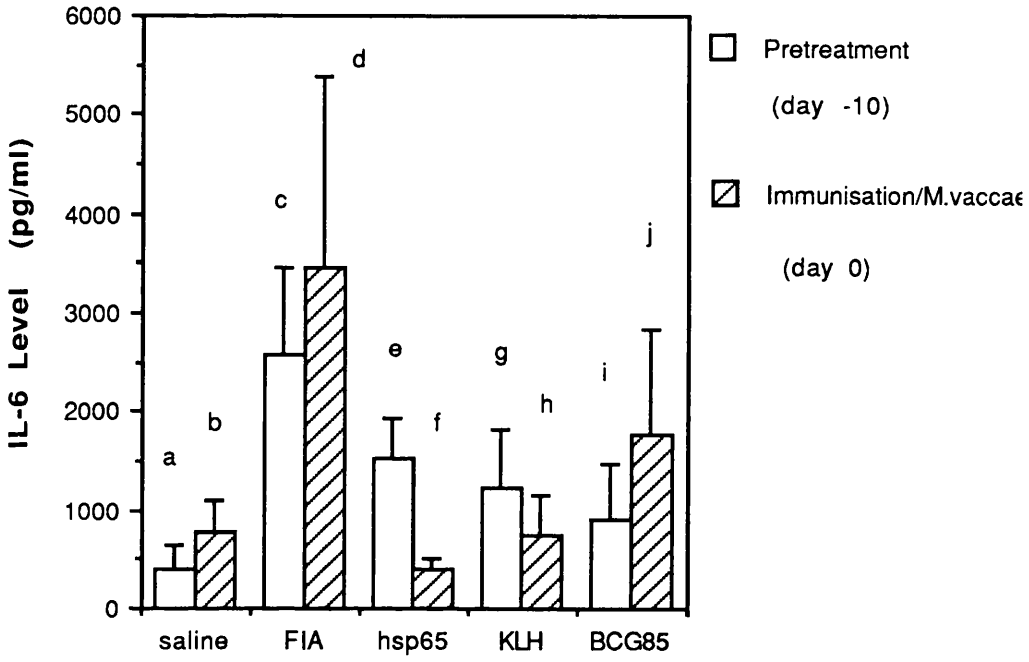
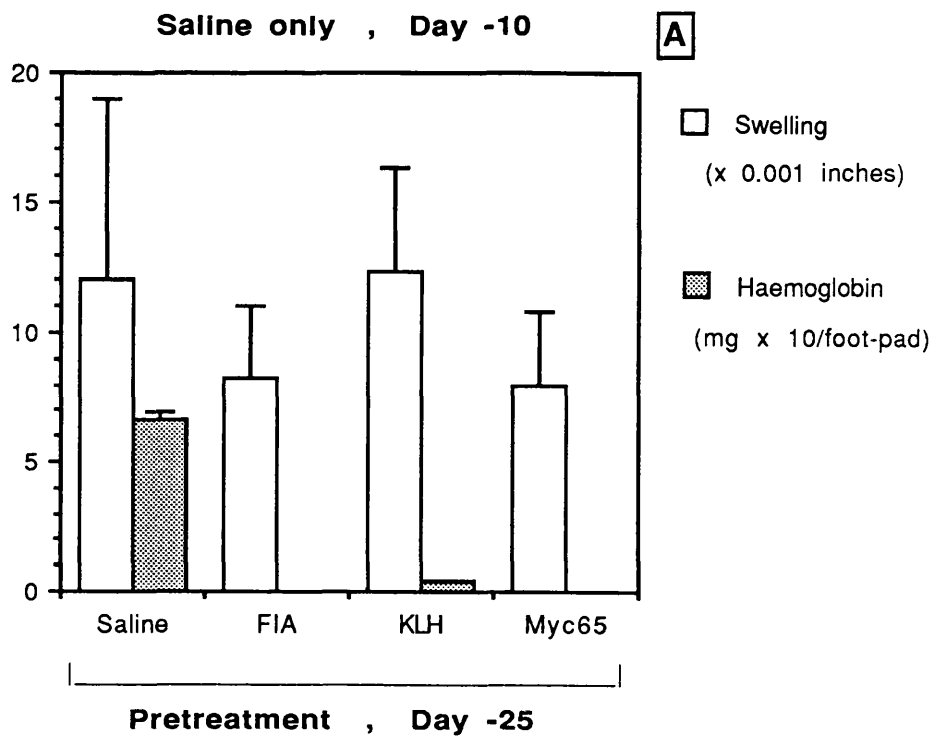


Fig.6.8. IL-6 level in the sera of mice pre-treated with various antigens mixed with FIA.

c vs a p<0.025 (Students t-test)
e vs a p<0.005 "



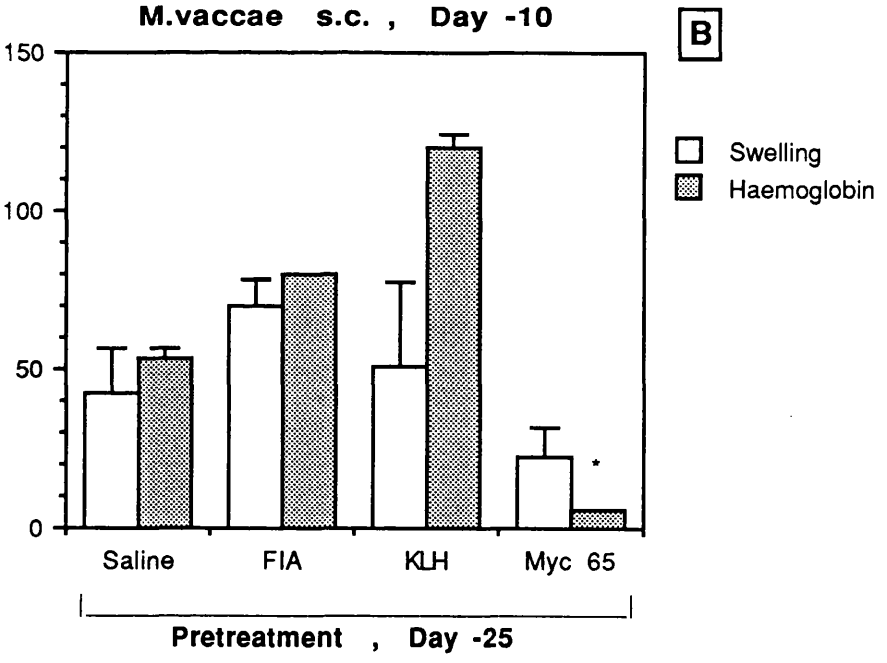


Fig.6.9. The role of immunisation with autoclaved M.vaccae in inducing a state of tolerance to the TNF-induced LSR.

*** p<0.05 (Mann-Whitney U test)**

(Fig.6.9). This indicated that the modulation of the sensitivity to TNF-induced local damage does not depend on the inclusion of an immunisation step. However, the mice which were not immunised with *M.vaccae* on day -15 showed very weak LSR, of which the haemorrhagic component was eliminated by any pretreatment with FIA on day -25, with or without antigen. In contrast, the strong LSR seen in *M.vaccae* -immunised mice was in this experiment, diminished only by the pretreament with FIA containing hsp65. Similar to the previous findings, IL-6 levels increased significantly after pretreatment with various antigens (hsp65, KLH and FIA) and decreased extensively with time whether they were given, prior to their challenge for a LSR, a s.c. injection of *M.vaccae* or saline (Fig.6.10). On examination of the correlation between the Hb concentration in the challenged feet and the serum IL-6 levels measured in animals just before immunisation with *M.vaccae* (in the 3 previously discussed experiments, Fig.6.11), two of the experiments showed a negative correlation (one of them has a weak correlation) while the third showed a very weak positive correlation. Similarly, the level of serum IL-6 at time of challenge correlates negatively with the concentration of Hb in the LSR lesions in two experiments and positively in one experiment (Fig.6.12). Although the present results suggest that serum IL-6 level in the pretreated animals is very high and it decreases with time, the pathological or protective role of IL-6 in such a reaction needs to be further examined.

Furthermore, we tested the hypothesis that the pretreatment with an antigen/FIA stimulates the release of a number of cytokines in the circulation and hence tolerizes the animals to a subsequent challenge that involves cytokine release and interaction. Rabbit anti-murine TNF was given either simultaneously with the

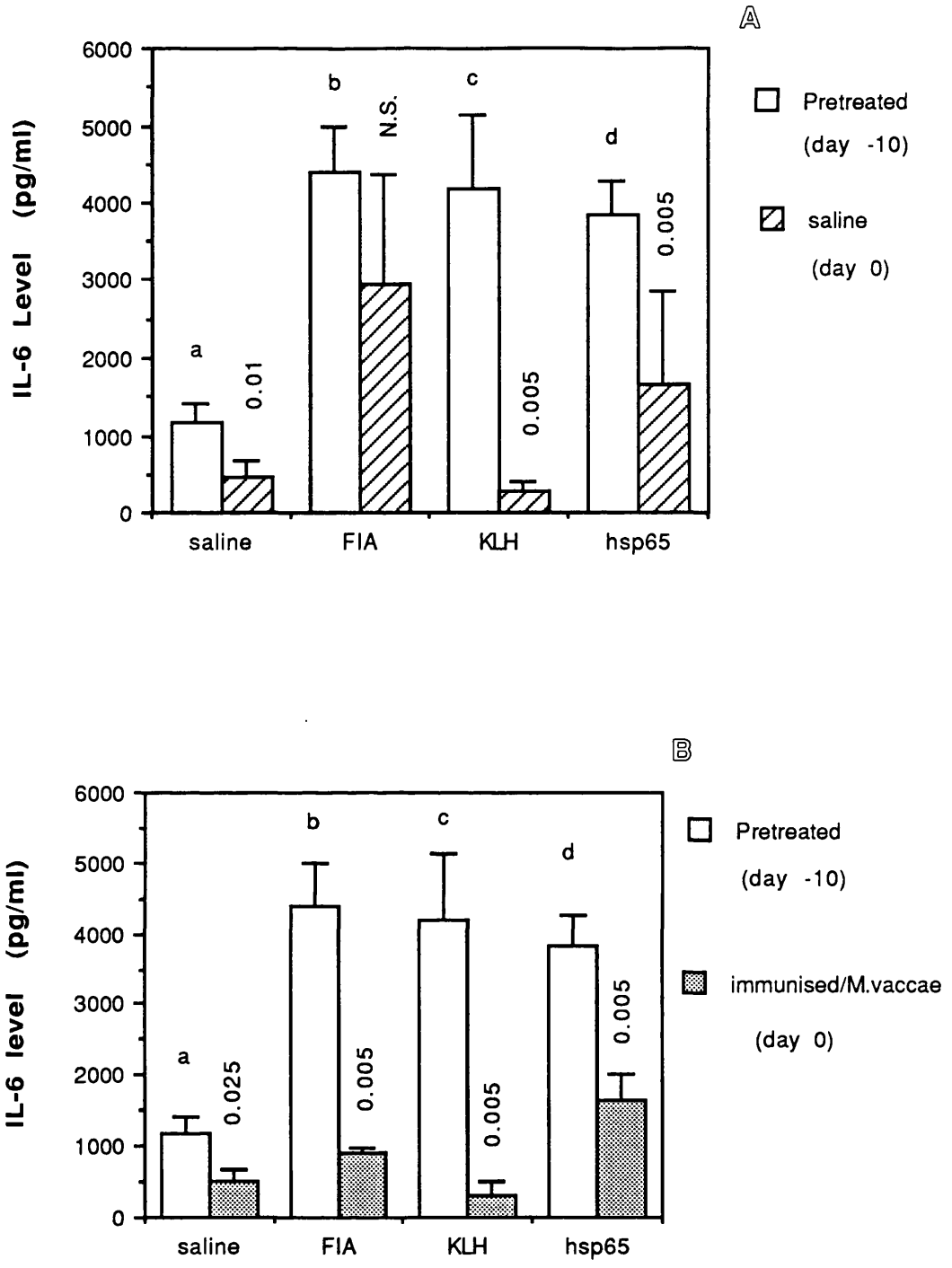
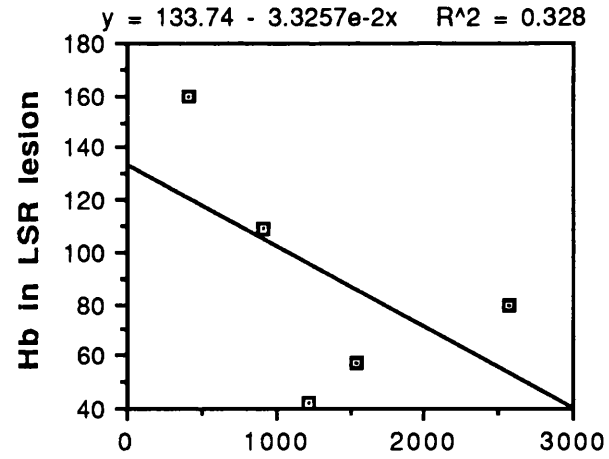
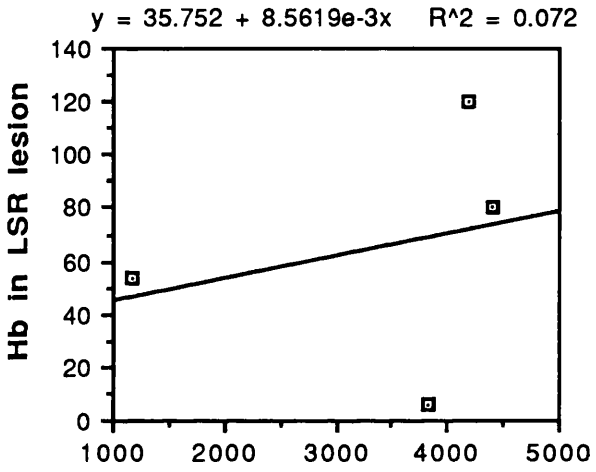


Fig.6.10 . IL-6 level in the sera of mice pre-treated with antigens in FIA and then immunised with M.vaccae (B) or given saline (A).

(each group was compared for its IL-6 level before and after saline or M.vaccae injection, significance is shown on the figure)

b vs a	**p<0.005**	**(Students t-test)**
c vs a	**p<0.005**	**"**
d vs a	**p<0.005**	**"**





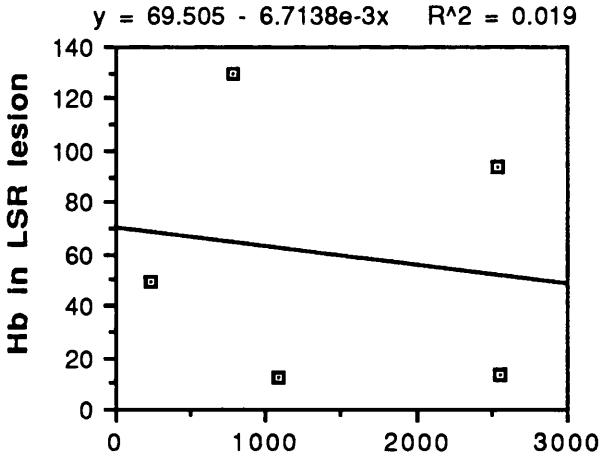
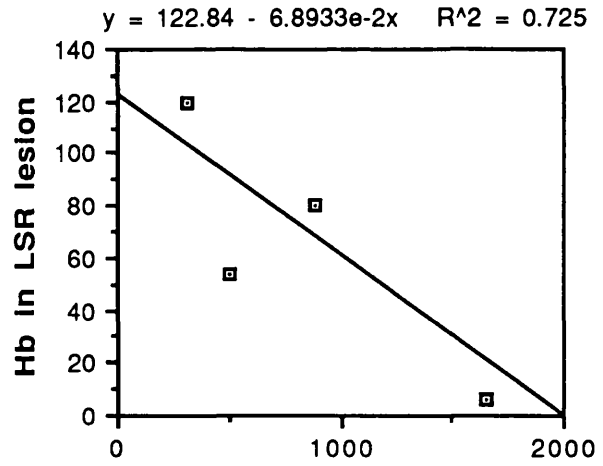
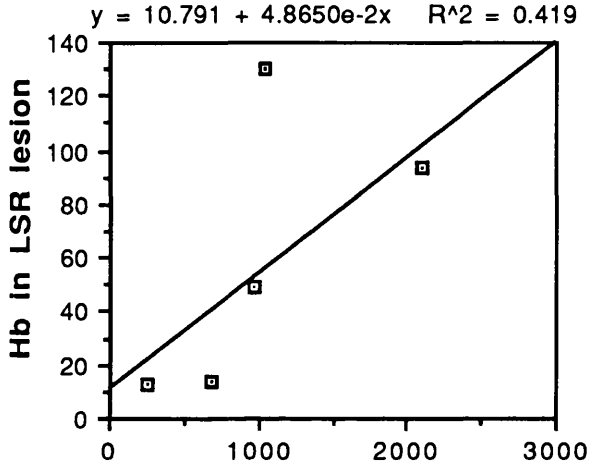


Fig.6.11. The correlation between the level of serum IL-6 and the concentration of Hb in mice feet at the time of immunisation with M.vaccae (in 3 separate experiments).





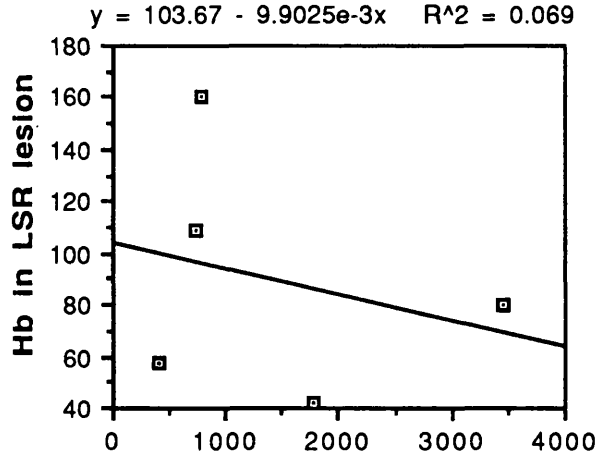


Fig6.12. The correlation between the level of serum IL-6 and the concentration of Hb in mice feet at time of LSR challenge (in 3 separate experiments).

pretreatment (one injection was given on day -15 and another one was given to the same group a week later) or on the day of challenge (day 0, given 4 hrs before preparation). In both cases, the neutralizing antibody had no effect on the inhibition of the LSR produced by the pretreatment, *M.vaccae* sonicate/FIA (Fig.6.13).

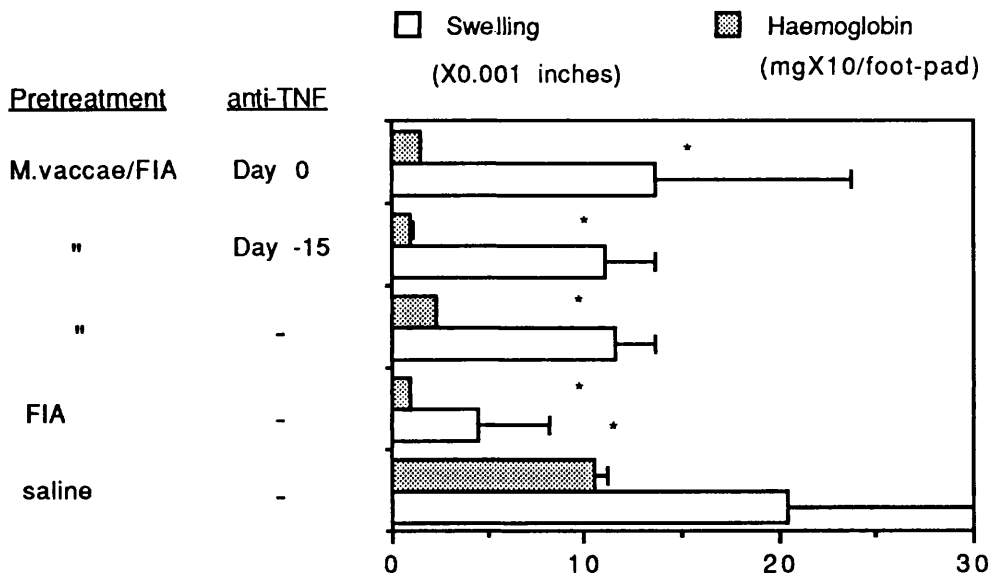


Fig.6.13 . The role of TNF in mediating the immunomodulation of LSR induced by M.vaccae mixed with FIA.

*** $p<0.05$ vs saline group (Mann-Whitney test)**

6.4. DISCUSSION

6.4.1. Suppression of the LSR by pretreatment with LPS :

Bacterial lipopolysaccaride (LPS or endotoxin) is a major cell wall component of both pathogenic and non-pathogenic bacteria (Morrison, 1983). LPS has been previously shown to play an important role in the production of the Shwartzman reaction, septic shock and in the pathogenesis of infections caused by gram-negative bacteria (dicussed in detail in chapters 1 & 3). Other researchers, however, have demonstrated that LPS can provide a non-specific protection against these infections. Zehavi-Willner *et al* (1991) have shown that the simultaneous administration of LPS and *Pseudomonas aeruginosa* exotoxin A (PA) to mice enhances the lethal effect of PA whereas protection was afforded to the animals exposed to PA after they had been pretreated with LPS. In addition, increase in vascular permeability and polymorphonuclear cell (PMN) exudation were detected in rabbit skin that had been injected with zymosan and in reversed passive arthus reactions (RPA) in which anti-ovalbumin serum is injected intradermally followed immediately by the i.v. administration of ovalbumin. These inflammatory changes were inhibited if the test animals were pretreated with LPS 24 hours previously (Rosenbaum *et al*, 1983). Results of experiments described in this chapter demonstrate that the pretreatment with a sub-lethal dose of LPS significantly inhibits both components of the LSR (swelling and haemorrhage). Various researcher groups developed different experimental models in order to investigate the mechanisms of both the pathophysiological and the so-called anti-inflammatory biological effects. One of these models examines the D-galactosamine-induced sensitization to the lethal effects of LPS (Galanos *et al*, 1979). This group has shown that pretreatment of

mice, rabbits and rats with D-galactosamine renders them several thousand fold more susceptible to the LPS-induced lethality. In addition, the C3H/HeJ mice (known for their resistance to LPS effects) were rendered sensitive to the toxic effects of D-galactosamine+LPS treatment when they received macrophages obtained from C3H/HeN mice (LPS responsive). The hyper-responsivness induced by D-galactosamine was found to be due to the formation of UDP-galactosamine in the liver resulting in the depletion of the hepatic UTP (uridyltriphosphate) required for the biosynthesis of macromolecules (RNA, proteins, glycogen, etc). Furthermore, D-galactosamine is hepatotoxic but not lethal unless combined with LPS. D-galactosamine also increased the sensitivity of mice to the toxic effects of TNF, a cytokine that has been described as being a major mediator of the LPS-induced lethality (Lehmann *et al*, 1987). Interestingly, The administration of UDP can reverse this hypersensitization. Recently, Freudenberg and Galanos (1988) found that pretreatment of mice with LPS 1-48 hours previously renders them tolerant to the lethal effect of D-galactosamine+LPS. Moreover, they provided evidence that macrophages are required for the induction of both the lethality and tolerance detected in this experimental model. Vogel *et al* (1988) have also been interested in exploring the mechanisms of the various biological activities of LPS and have demonstrated that IL-1 and TNF synergize *in vivo* to mimic both the toxicity of LPS (including weight loss, colony stimulating factor induction and lethality) and the tolerance which can be induced by LPS at sublethal doses. This was seen when both cytokines were used at sublethal doses. Since LPS was found to induce the secretion of colony stimulating factor (CSF) among other cytokines, the measurement of its level in the serum was used as an indicator

of the *in vivo* LPS activity. In a recent report, Henricson *et al* (1991) have shown that the administration of recombinant IL-1 receptor antagonist to mice could partially inhibit both the induction of LPS-induced CSF production (or toxicity) and the induction of LPS tolerance confirming the previous finding that IL-1 plays an important role in mediating both activities of LPS. Based on these previously described observations, we hypothesised that high levels of various cytokines, specifically IL-1 and TNF are released in the circulation of mice pretreated with LPS and that these cytokines are responsible for the elimination of LSR detected in those LPS-pretreated animals. However, injecting these mice with a neutralizing antibody specific to murine TNF failed to reverse the inhibitory effect of LPS. This could be explained by the fact that other cytokines such as IL-1 and IL-6 contribute to the production of such tolerance. There are several possible explanations for the LPS-induced tolerance, these will be discussed later in the following sections.

6.4.2. Changes in the functions of phagocytic cells :

Injecting LPS, IL-1 or TNF, i.v., into rats was shown to induce peripheral lymphopenia and initial neutropenia followed by neutrophilia (Ulich *et al*, 1989). Although transient, the neutropenia could mean that there are less cells available, in the circulation, to migrate to inflammatory sites. It has been recently found that neutrophils from the peripheral blood of rabbits pretreated with TNF show a reduced ability to migrate towards chemoattractants *in vitro* (Otsuka *et al*, 1990). This group has also demonstrated that the systemic administration of TNF inhibits the local accumulation of leukocytes in mouse skin that was injected previously with zymosan-activated plasma. The effect of TNF lasted

for about 48 hours. Since the neutrophilia, induced by LPS, IL-1 and TNF, is mainly produced by immature neutrophils recruited from the bone marrow, these cells might have impaired ability to respond to chemotactic stimuli. LPS and the two cytokines can also stimulate the secretion of granulocyte-macrophage colony stimulating factor (GM-CSF) that is known to activate the various effector functions of mature phagocytes. On the other hand, a recent report demonstrated that continuous infusion of recombinant human GM-CSF into patients with malignant diseases reduces the ability of their neutrophils to migrate to sites of inflammation (Addison *et al*, 1989). Another interesting observation, made by Holtmann and Wallach (1987) is that the pretreatment of TNF-sensitive cells with small doses of either TNF or IL-1 renders these cells resistant to the cytotoxic effect of TNF combined with cycloheximide, a protein synthesis inhibitor. The reduction in the number of TNF-cell surface receptors induced by the pretreatment could not explain the acquired state of resistence since the TNF receptors were fully recovered within few hours while the cells were still tolerant to the cytotoxic effects of TNF. Several other mechanisms which are likely to be important in the modulation of the LSR response by LPS are discussed later in this section.

6.4.3. TNF sensitive and TNF insensitive DTH sites, suppression of the LSR by pretreatment with heat shock proteins and other antigens:

The present results also describe the ability of the soluble antigens of sonicated mycobacteria made up in oil (FIA), and given intraperitoneally, to inhibit the TNF-mediated local tissue damage, mainly the haemorrhagic component of the reaction. This effect is in contrast with the significant enhancement of the LSR induced by the subcutaneous injection of the autoclaved mycobacterial bacilli.

Therefore, we describe in this context the remarkable finding that two differently prepared antigenic preparations of the same mycobacterial species induce two types of DTH reactions that differ in their sensitivity to TNF-mediated damage.

Based on the fact that the mycobacterial sonicate is rich in the hsp65 KDa, an immunodominant antigen that has been recently found to block the arthritis induction in several animal models where cytokines cause tissue damage in T cell-dependent inflammatory sites, an attempt was made to investigate the possible role of this mycobacterial protein in the *M.vaccae*-mediated inhibition of LSR. Animals pretreated with the different heat shock proteins, the hsp65, hsp70 and GroEL were tolerant to the subsequent challenge for the induction of the haemorrhagic component of LSR. Hsp65 was most reproducibly effective but KLH and even FIA alone could also suppress. Possible explanations are outlined below.

6.4.4. Suppressor T cell :

It is possible that a specific immune response mounted to these antigens was responsible for the suupression of LSR. An injection of the mycobacterial hsp65 KDa in FIA into rats was shown to block the streptococcal cell wall-induced arthritis (van Den Broek *et al*, 1989). The protective effect was transferred to naive animals by spleen cells. Interestingly, in animals pretreated with the hsp65 and then immunised with ovalbumin in FIA there is no interference with the delayed type hypersensitivity (DTH) reaction in response to ovalbumin. Conversely, the pretreatment with hsp65 does inhibit the DTH reaction to ovalbumin if the animals were immunised with a mixture of ovalbumin and streptococcal cell wall (which contains hsp65) in FIA. This indicated the induction, by the pretreatment with hsp65, of suppressor mechanisms that are specific to the cross-

reactive hsp but exerted on other antigens subsequently injected with the hsp.

However, this explanation is not appropriate to my results. First, suppressed LSR was seen in animals with enhanced DTH responses. Secondly, similar, though sometimes weaker inhibition of LSR was seen when the antigen used for pretreatment was not an hsp, or not even mycobacterial (KLH). The latter observation suggest one of the possible explanations outlined below.

6.4.5. Immunosuppressive effects of granulomata :

Oil is known for its adjuvant properties. It induces the formation of granulomas which may provide an enhanced presentation of the antigen injected with the oil. Kobayashi *et al* (1985) had demonstrated that mice immunized with methylated bovine serum albumin (MBSA) in Freund·s complete adjuvant develop pulmonary granulomas following the intra-tracheal administration of plain agarose beads or beads conjugated with the specific antigen (MBSA). Granuloma formation in the immunised animals was associated with a reduced delayed type hypersensitivity (DTH) reaction to both the specific antigen (MBSA) and non-specific antigens (purified protein derivative, PPD and phyto-haemagglutinin, PHA). Furthermore, lymph node cells of these animals exhibited suppressed *in vitro* proliferation in response to both the specific and non-specific antigens and that was associated with reduced interleukin-2 production. Granulomas caused by the subcutaneous injection of talc powder suspension into rats were also associated with immunosuppression (Radic *et al*, 1988). These rats showed a significant delay in the first-set and second-set allograft rejection. However, this type of suppression also fails to explain

my data since the DTH responses were not usually diminished and there was low susceptiblity to LSR even when the DTH reaction was enhanced.

6.4.6. Cytokine release :

Granuloma formation is also associated with high circulating levels of cytokines which could, in turn, tolerize (or desensitize) the mice and hence result in the suppression of LSR seen in our model. Although the correlation between the serum IL-6 level and the concentration of Hb in the LSR lesions was variable in three expariments presented in this chapter, it is clear from the results presented here that all animals pretreated with an antigen in FIA had vast amounts of circulating IL-6 which was reduced slowly with time. It was suggested by Kishimoto (1989) that the intraperitoneal injection of mineral oil into mice induces IL-6 secretion and this explains the priming of animals with pristane before injecting them with hybridoma cell lines. It is also possible that these mice had high levels of other cytokines such as IL-1, TNF, GM-CSF and interferon in their circulation. Thus, the release of a mixture of cytokines into the circulation may induce a state of tolerance even in the presence of neutralizing anti-TNF antibodies. Patients undergoing bone marrow transplantation (BMT) showed less BMT-related complications if they had chronic TNF production (Holler *et al*, 1991). However, patients with rising TNF levels following the BMT revealed endothelial complications and acute graft-vs-host disease. A previous report has shown that the continuous infusion of murine recombinant IL-1 into rats using implanted osmotic pumps resulted, within 3 days, in reduction in body weight and liver enzymes such as serum glutamic pyruvic transaminase and sorbitol dehydrogenase (used as a measure of liver function) accompanied by peripheral

leukocytosis and granuloma formation locally where the IL-1 was released (Otterness *et al*, 1989). However, there was a complete loss of the IL-1-induced changes, except for the granuloma formation, within 10 days. A reduction in the acute response induced by IL-1 was noticed even if these animals were challenged with a new IL-1-releasing pupms. The authors confirmed that the tolerance to IL-1 effects is responsible for the restoration of normal physiology.

6.4.7. Steroid induction by cytokines :

Recently, glucocorticoids have been implicated in the anti-inflammatory pathways that operate during various pathological events. A previous report has demonstrated the ability of dexamethazone to block the LPS-induced biosynthesis of TNF not only by inhibiting the TNF mRNA accumulation but also by reducing its translation (Han *et al*, 1990). An intraperitoneal injection, into mice, of a sublethal dose of LPS results in a maximal release of serum TNF within 1 hour and then it disappears from the circulation in 3-4 hours (Zuckerman *et al*, 1989). The decrease in TNF levels *in vivo* correlated with the appearance of significant amounts of endogenous corticosterone by 3 hours. This was confirmed by the fact that TNF levels remained elevated in adrenalectomized or hypophysectomised mice. In contrast, IL-1 production was maximum by 4 hours and remained elevated at 24 hours. This could be explained by the observation that dexamethazone inhibited *in vitro* the monocyte-released IL-1 but not the endothelial cell-released cytokine. Therefore, it was suggested that a regulatory mechanism involving the hypothalmus, the pituitary and the adrenal glands operates to control the endogenous release of IL-1 and TNF. Moreover, a recent study demonstrated that hydrazine sulfate pre-

treatment protected mice against the lethal effects of endotoxin, and of the combination of endotoxin and D-galactosamine (Silverstein *et al*, 1991). Hydrazine sulfate also protected the animals from the lethal effects of TNF+D-galactosamine. They have provided the evidence that such protection involved the participation of the pituitary, the adrenal glands and the liver. Furthermore, an injection of LPS was found to induce haemorrhagic necrosis of most murine tumors (North & Havell, 1989). The association of the LPS effect on the tumors with the intra-tumor synthesis of TNF explains the ability of the glucocorticoid treatment to abolish the LPS-mediated tumor necrosis and regression. It is possible that this pathway is involved in the suppression of the LSR (our model) since the inflammatory cytokines are known to induce the production of the glucocorticoids, a major inducer of acute phase reactants. It is also important to note that the administration, to mice, of polyclonal anti-murine TNF antibody eliminated both the TNF and CSF induced by LPS (Vogel & Havell, 1990). In contrast, the antibody treatment did not affect the production of interferon, corticosterone and hypoglycemia induced by LPS. The administration of anti-TNF in our model affected neither the LPS-induced tolerance nor the pretreatment-mediated loss of sensitivity to TNF. This could be explained by the previously mentioned work, that glucocorticoid production was not inhibited by the abrogation of TNF production. However, only in the last experiment (Fig.6.9 and Fig.6.10) was there a significant negative correlation between IL-6 levels at the time the LSR challenge was given, and the quantity of Hb found in the LSR site.

6.4.8. Other possible mechanisms for the pretreatment-mediated inhibition of LSR :

The tolerance induction could also involve the release of the soluble cytokine inhibitors that can regulate the cytokines harmful biological effects, the production of IL-10, a product of T helper 2 (T_H2) cells and capable of inhibiting the synthesis of cytokines produced by T_H1 cells (Mosmann & Moore, 1991) and the expression of two types of TNF receptors that may mediate different modes of signalling and function (Dembic *et al*, 1990). It is also possible that a blend of all the previously described mechanisms operates to control the pathology associated with inflammation.

6.5. CONCLUSION

1. LSR, like the endotoxic shock, can be blocked by the pretreatment with sublethal doses of LPS.

2. Both TNF-sensitive and TNF-insensitive DTH sites exist.

3. Pretreatment with an antigen in FIA inhibits the haemorrhagic component of LSR.

CHAPTER SEVEN

In a model of murine tuberculosis, Does tumor necrosis factor play a protective or a pathogenic role.

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7.1. INTRODUCTION

The *in vivo* effects of TNF can be correlated, in different infectious or pathological events, with either protection or pathology depending on its concentration, the timing of its production and the presence of other mediators in the cellular environment (Tracey *et al*, 1989; Rook *et al*, 1991c).

In *M.bovis* BCG-infected mice, TNF was shown to play an important role in the organogenesis occuring in adulthood life, by inducing the formation of well developed granulomas as a part of cell-mediated immunity (Kindler *et al*, 1989). It was demonstrated that the BCG-infected mice treated with control rabbit IgG have large granulomas in the liver, containing acid phosphatase positive macrophages and epitheloid cells. In addition, TNF protein accumulates locally around the activated macrophages and then declines in parallel with the development and disappearance of granulomas. Mycobacteria were also eliminated efficiently from these mice. However, the anti-TNF treated infected animals showed very small ill-defined granulomas with acid phosphatase negative macrophages. In addition, there were neither epitheloid cells nor TNF in their granulomas. This histopathological picture was associated with the persistence of both the macrophages and the acid fast bacilli for a longer period of time. The conclusion from that study was that TNF is produced by activated macrophages (during a cellular immune response) in the microenvironment of developing granulomas leading to a process of autoamplification. Therefore, the anti-TNF antibody inhibited the TNF synthesis, the formation and persistence of granulomas and the effective elimination of mycobacteria. Moreover, it has been recently suggested that the

bactericidal effect of macrophages activated by TNF and IFN-gamma can be attibuted to the production of nitric oxide (Dr. B.Bloom, personal communication to Dr. G. Rook).

It was previously suggested that the immunopathology (necrotic tissue damage) detected in an infection with *M.tuberculosis* is associated with dysregulation in the production of cytokines, particularly TNF (Rook & Al Attiyah, 1991a). There are several *in vitro* and *in vivo* pieces of evidence for the production of TNF in Tb patients. First, although TNF has never been demonstrated in the serum samples of tuberculosis patients, high levels of circulating TNF inhibitor were detected (Foley *et al*, 1990). Second, alveolar macrophages in the lavage fluid collected from Tb patients, compared to those of the normal donors cells, release TNF spontaneously (Rook *et al*, 1991b). Third, the detection in Tb patients sera of a high proportion of agalactosyl IgG, which clinically seems to be associated with T cell dependent inflammation and cytokine production. Fourth, the clinical picture of the disease, namely fever and weight loss which have been considered to result from the *in vivo* release of this cytokine. Furthermore. macrophages primed *in vitro* with IFN-gamma and Vitamin D_3 secrete TNF in response to *M.tuberculosis* bacilli (Rook *et al*, 1987). Evidence for a major role of TNF in the production of necrosis also exists. Fibroblasts infected *in vitro* with *M.tuberculosis* are rendered more sensitive to the cytotoxic effects of TNF (Filley *et al*, 1991). *In vivo* , TNF causes necrosis locally at skin sites of mice prepared with soluble antigenic preparations of mycobacteria (Chapter 3). There is accumulating evidence that activated T lymphocytes provide protection in tuberculosis. However, it is not known whether well-developed granuloma formation, induced by activated T lymphocytes,

can efficiently eliminate tuberculosis bacilli and therefore provide protection. Therefore, we attempted to examine the effect of the neutralisation of TNF in a murine model of tuberculosis. We also studied the development and organisation of granulomas, the production of necrotic tissue damage and the rate of mycobacterial growth in infected organs.

7.2. MATERIALS AND METHODS

7.2.1. Animals :

Female CBA/Ca mice were bred and maintained in our animal house at the School of Pathology and they were used at 8-10 weeks of age.

7.2.2. Tolerance induction :

Rabbit IgG was purified from normal rabbit serum using protein A sepharose chromatography (Sigma, P-5906). To remove aggregates, this antibody preparation was ultracentrifuged for 150 minutes at 40,000 rpm at 4°C using Beckman L-75 ultracentrifuge. After measuring the protein concentration, mice received intraperitoneally a tolerogenic dose of 2 mg of the deaggregated normal rabbit IgG (Izui and Masuda, 1984).

7.2.3. Infection with *M.tuberculosis* :

M.tuberculosis H37Rv used in this study was a gift from J. Grosset (Paris, France). The organisms used were passaged for several years in mice and then subcultured from the organs of infected animals on Lowenstein-Jensen medium. Then they were harvested into a culture medium, collected again and prepared as a single organism suspension. This suspension was then diluted to $2X8^{8}$ bacilli/ml and stored in liquid nitrogen as 1 ml aliquots.

The optimal dose of *M.tuberculosis* ($5X10^{6}$ bacilli) used for infecting the mice was found in a previous study (Leveton *et al*, 1989). Higher doses led to death within few days. Lower doses led to very prolonged survival. However, the intermediate dose range used here caused a reproducible disease, which was sensitive to immunological manipulation. Therefore, an aliquot of the frozen bacteria was thawed rapidly in RPMI 1640 (Gibco laboratories, Grand

Island, N.Y.) and then mashed into a single organism suspension. Mice were infected intravenously (via the tail vein) with $5X10^6$ bacilli/mouse ten days after receiving the tolerogenic dosage of rabbit IgG. Simultaneously, the infected tolerant mice were divided into 2 groups, one of which received a weekly intravenous injection (the first injection was given at the time of infection) of 1 mg/mouse of rabbit anti-recombinant mouse TNF IgG (donated generously by G. Grau, Geneve, Switzerland), purified on a protein A column and deaggregated by ultracentrifugation. The other group received weekly injections of 1 mg purified deaggregated normal rabbit IgG. These two groups were compared to saline-treated infected mice. Groups of mice were bled and then killed after 2, 4 and 6 weeks of infection. Various organs (spleen, liver and lung) were collected and fixed in 10% Formalin in PBS for at least one week.

7.2.4. TNF bioassay :

Levels of TNF in the saline-treated infected mice were determined by its cytolytic activity against murine L929 cells. Cells were plated in 96-flat bottomed microtiter wells (Nunc) at $2.5X10^4$ cell/well in 100 ul of RPMI 1640 (GIBCO laboratories) supplemented with 5% FCS, penicillin (100 units/ml) and streptomycin (100 ug/ml). After overnight incubation, the supernatants were removed from the plate. Serially diluted serum samples were added to the wells in 100 ul of medium containing 1 ug/ml emetine and then incubated at 37°C in an atmosphere of 5% CO_2. A series of doubling dilutions of recombinant murine TNF (a gift from Genetech) were included in the assay as a standard. Twenty one hours later, the supernatants were poured off and the cells were fixed with methanol for 1 minute. The monolayer was then stained with 0.75% crystal violet for 5 minutes and washed

with water. Acetic acid (33%, 50 ul) was added to the wells to dissolve the dye and the optical density was then determined at 570 nm using MicroElisa AutoReader (Dynatech).

7.2.5. Bacterial counting :

Organs fixed in Formalin were homogenised in saline and then 2 and 4ul were spread on a known area of a glass slide. These were then fixed, stained with auramine and destained with potassium permanganate. The bacteria were counted using a fluorescence microscope and the counts per organ (liver, lung or spleen) were calculated according to this formula:

The number of acid fast bacilli(AFB)/ml=

$$\frac{\text{area over which bacilli were spread}}{\text{area of the field}} \times \text{dilution factor} \times \text{AFB/field} \times (\text{the volume spread (ml)})^{-1}$$

7.2.6. Enzyme-Linked Immunosorbent Assay (ELISA) :

ELISA assay was used to check the state of tolerance in the CBA/Ca mice. The assay was conducted as previously described in chapter 2. In brief, plates (Nunc) were coated with the rabbit anti-murine TNF or the normal rabbit IgG at 10 ug/ml. After blocking the plate with PBS containing Tween 20 and BSA (1%, W/V), mice sera were added at a dilution of 1:250 and incubated at 37°C for 1 hour. Finally, HRP conjugated rabbit anti-mouse immunoglobulins (Dako P260) was added at 1:1000 and then the reaction was developed using ABTS.

The persistence of the rabbit anti-mouse TNF antibody in the circulation of mice 7 days after injecting it was also detected by ELISA. Therefore, plates were coated with TNF at a concentration of 3 ug/ml. Plates were then blocked and incubated with mouse sera at a

dilution of 1:500 or rabbit anti-murine TNF antibody at 10 ug/ml(as a positive control) at 37°C for 1 hour. This was followed by the addition of HRP conjugated swine anti-rabbit immunoglobulins (Dako P217) and then the development of the reaction using ABTS.

Furthermore, we attempted to investigate whether the neutralisation of TNF in tuberculosis infection has any effect on the immune response mounted to mycobacterial antigens. Therefore, plates were coated with Tb culture filtrate (1 ug/ml), BCG85B antigen (5 ug/ml), LAM (5 ug/ml) (a kind gift from Dr. C. Moreno) or hsp65 (2 ug/ml) (a generous gift from Drs. J.D.A. van Embden and R. van der Zee, The Netherlands) and incubated overnight. The plates were then washed and blocked with PBS containing Tween 20 (0.05%) and BSA (1%). Mice sera of the three groups were added to the coated wells at 1:250 and incubated at 37°C for 1 hour. HRP-conjugated rabbit anti-mouse immunoglobulins (Dako P260) was added to the plates at 1:1000 followed by the addition of ABTS.

7.3.1. TNF level in the sera of tuberculosis mice :

CBA/Ca mice infected with *M.tuberculosis* H37Rv intravenously were bled 2, 4 and 6 weeks after infection. Using a sensitive bioassay (with a minimum detection limit of 3 pg/ml), TNF was undetectable in the sera of these animals at all the time points tested.

7.3.2. The effect of TNF neutralisation *in vivo* on the growth of *M.tuberculosis* :

Tolerised CBA/Ca mice were infected via the tail vein, with $5X10^6$ pathogenic *M.tuberculosis* H37Rv. Simulataneously, they were injected intravenously with 1 mg/ml of rabbit anti-murine TNF IgG or a control rabbit IgG and they received further identical injections weekly until the end of the experiment. Groups of 2-4 mice were killed after 2, 4 and 6 weeks of infection. The number of mycobacteria was counted in the Formalin-fixed liver, spleen and lung. There was no diffenence in the number of bacilli or the weight of organs between the saline or normal rabbit IgG-treated mice (Figs.7.1, 7.2 and 7.3). Two weeks after infection, there was no difference, between the three groups, in the number of mycobacteria in the liver and the spleen and they were undetectable in the lungs. However, the anti-TNF IgG-treated mice demonstrated an enhancement in the growth of mycobacteria and hence much higher numbers of bacilli, compared to the control groups, in their organs at 4 ($p<0.05$ for the liver; $p<0.05$ for the lung; $p<0.05$ for the spleen, vs. the saline group) and 6 ($p<0.005$ for the liver; $p<0.01$ for the lungs; $p<0.025$ for the spleen, vs. the saline group) weeks of infection. The control groups showed detectable bacilli in their lungs only after 6 weeks of infection. The growth of bacilli seemed

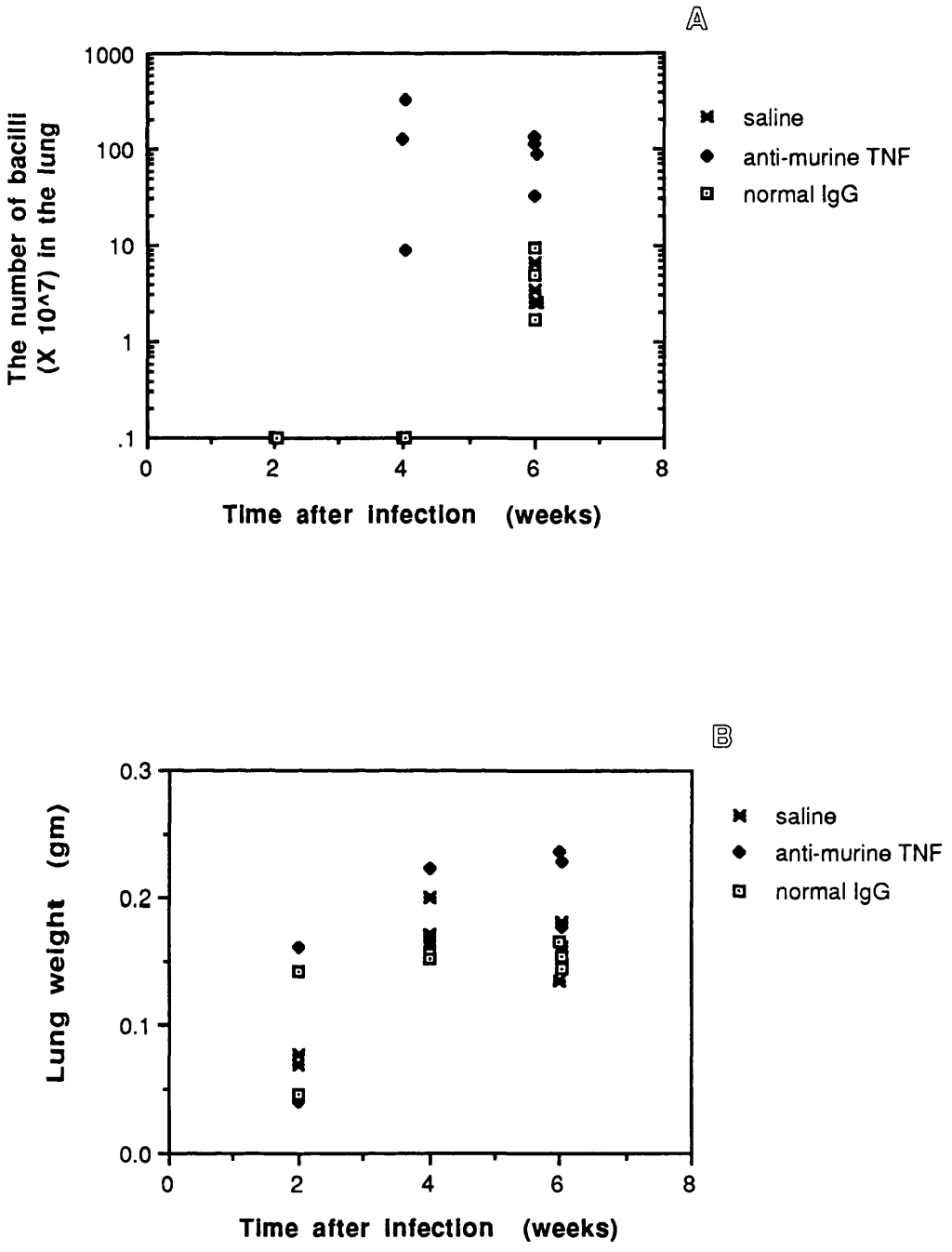

Fig.7.1. The number of Tb bacilli recovered from (A) and the weight of (B) the lungs of anti-TNF treated animals compared to the saline- and the normal IgG-treated animals.

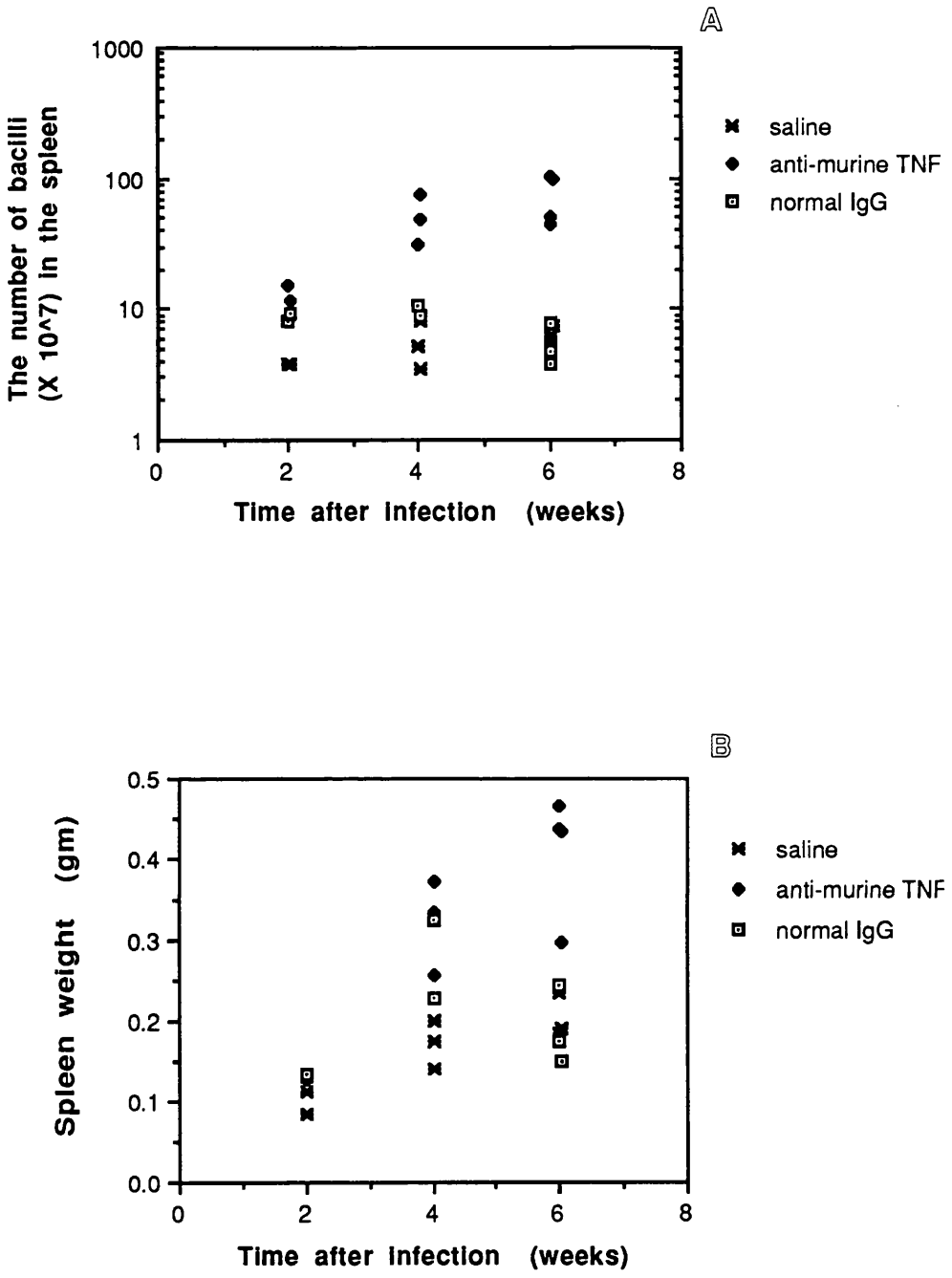

Fig.7.2. The number of Tb bacilli recovered from (A) and the weight of (B) the spleen of anti-TNF treated animals, compared to those treated with saline or normal rabbit IgG.

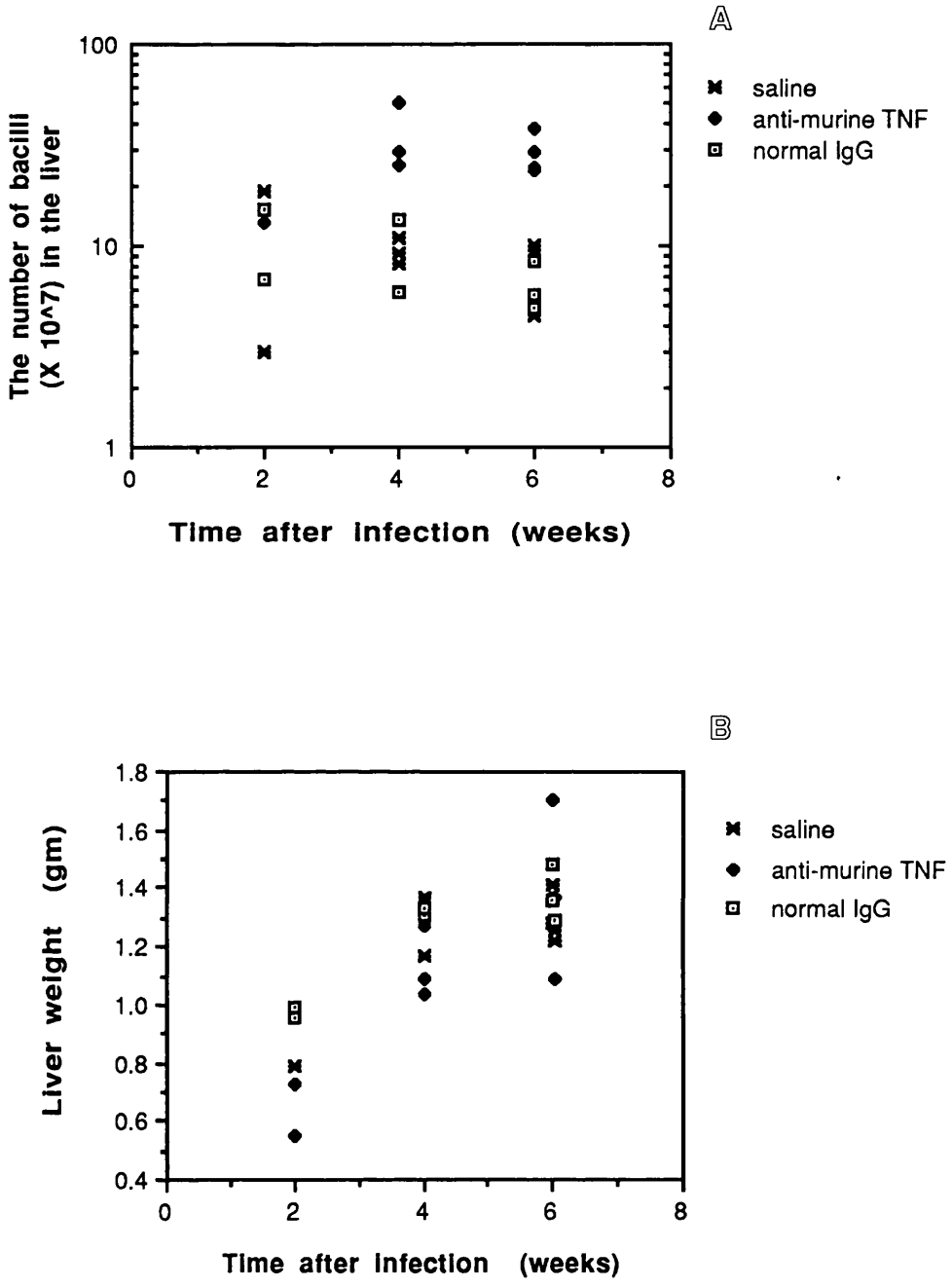

Fig.7.3. The number of Tb bacilli recovered from (A) and the weight of (B) the liver of anti-TNF treated animals compared to those treated with saline or normal rabbit IgG.

to plateau from 4 weeks in all groups, and no further increase in the number of bacilli was detected at 6 weeks after infection.

All the goups also demonstrated an increase over time in the weight of their organs. However, the weight of the lungs and spleens of anti-TNF IgG treated mice was larger than those of the control groups. In addition, tolerised mice still showed detectable levels of the injected rabbit antibodies, in their circualtion, 7 days later with very weak antibody response to the rabbit IgG developing after 6 weeks, as measured by ELISA (data not shown).

7.3.3. The detection of antibody responses to mycobacterial antigens in infected animals:

Animals infected with tuberculosis showed an antibody response to both the Tb culture filtrate and the purified BCG85B secreted antigen as early as 2 weeks after infection (Fig.7.4). However, there was no significant difference in the response of the three groups indicating that the neutralisation of TNF does not have any infleunce on the development of the humoral immune response to mycobacteria. Furthermore, the infected animals did not show any response to the mycobacterial LAM and hsp65 antigens (data not shown).

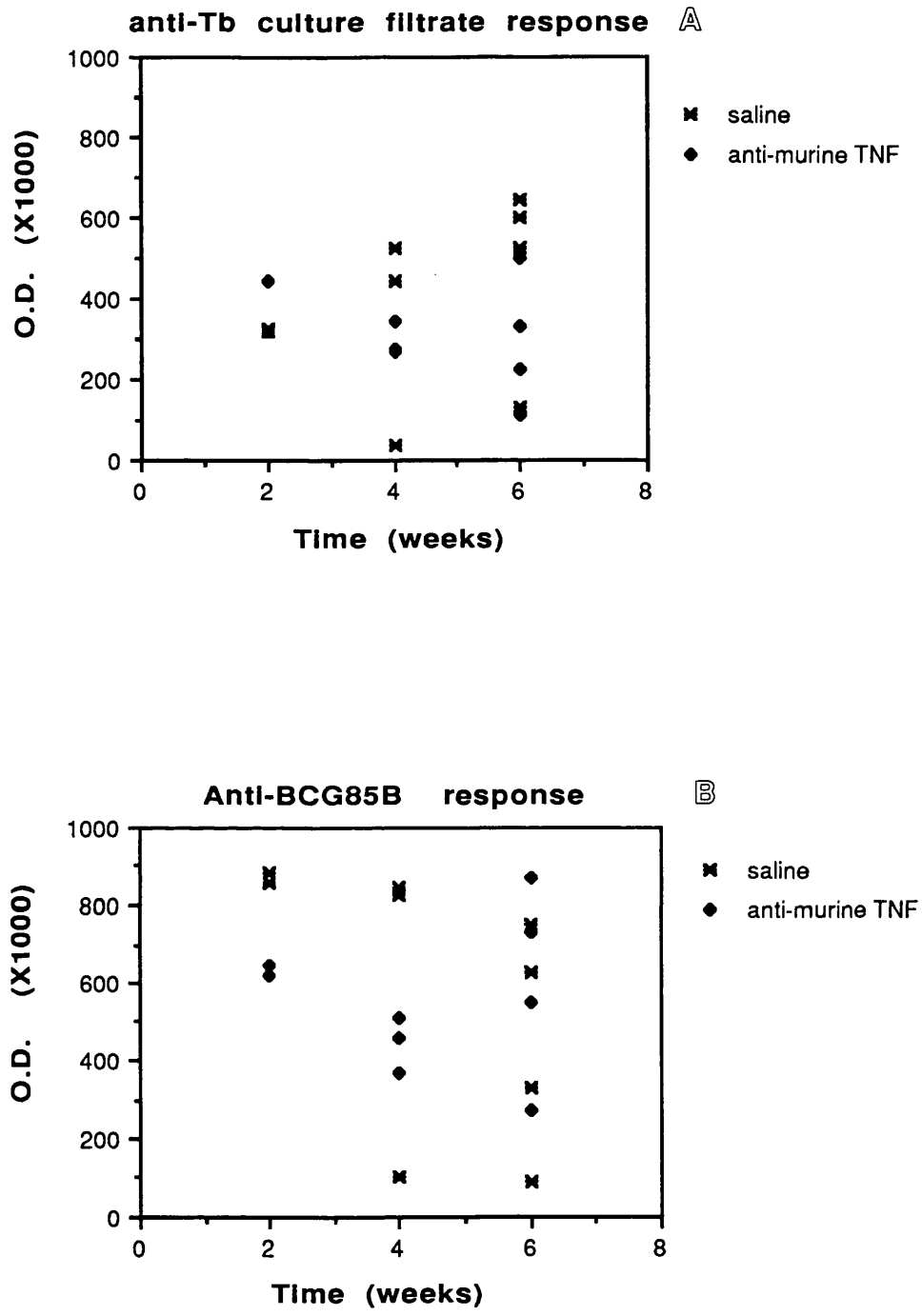

Fig.7.4. The antibody reponse to the Tb culture filtrate (A) and the BCG85B (B) in the control and the anti-TNF treated infected groups.

7.4. DISCUSSION

The present study demonstrated that TNF can not be detected in the serum of CBA/Ca mice infected with tuberculosis. However, pre-treating infected animals with rabbit anti-murine TNF accelerated the infection leading to a higher bacterial load in their livers, lungs and spleens, as compared to the numbers of bacteria in the organs of the control groups. Furthermore, as early as 2 weeks following infection, the infected animals showed an antibody response to the culture supernatant of *M.tuberculosis* and the BCG85B secreted antigen but not to the mycobacterial cell wall component, LAM or the cytoplasmic hsp65 antigen. Although it may appear that the neutralisation of TNF in infected animals did not interfere, when compared to the control groups, with the development of a normal humoral immune response to the mycobacterial antigens, the level of the antibody response produced does not correlate with the bacterial load detected in these animals.

It has been previously found in various experimental infectious diseases such as *M.bovis* BCG and *Listeria monocytogenes* that TNF is undetectable in the serum of infected animals (Nakane *et al*, 1988; Kindler *et al*, 1989). Vast amounts of the cytokine can be systemically detected only when these animals were challenged with LPS. However, the production of TNF and the role it played during these infections were proved by the exacerbation of the lethal effects of these pathogens following the administration of neutralising anti-TNF serum. In addition, TNF mRNA and protein were detected in the activated macrophages and their microenvironment in the fully developed granulomas formed in the livers of mice infected with BCG. Therefore, although TNF has been always associated with

pathological events such as septic shock and various infectious diseases such as tuberculosis, these events may be due to the exaggerated systemic release of the cytokine while its local release or transmembrane expression is more correlated with beneficial biological effects and protection. This study presents only preliminary results since the continuous administration of the anti-TNF antibody until the end of the experiment does not allow for the analysis of the specific effect of TNF at various stages of tuberculosis infection.

TNF exerts various biological effects that may account for its protective role in tuberculosis. Previous reports revealed that TNF is released early in *L.monocytogenes* infection, since a single injection of the anti-TNF antibody given to mice on the first day of infection could suppress their anti-listerial resistance (Nakane *et al*, 1988). In aggreement with the previous findings, TNF was suggested to act as a first line defence by activating the macrophages and the natural killer cell activity which act in a non specific manner, before the production of a specific immune response (Bancroft *et al*, 1989). In addition, the production of specific T lymphocytes shown to provide protection in tuberculosis infection also leads to the secretion of TNF. Interferon-gamma released by activated T lymphocytes can induce via the IFN-gamma/calcitriol pathway, anti-tuberculosis activity in human monocytes (Rook *et al*, 1986a). The IFN-gamma/calcitriol combination can also prime the macrophages for an enhanced release, in response to *M.tuberculosis* bacilli, of TNF that can further activate the macrophages (Rook *et al*, 1987). Moreover, IFN-gamma increases the cell surface expression of TNF receptors (Aggarwal *et al*, 1985).

TNF secreted during this infection can attract phagocytic cells to sites of inflammation by inducing the production of IL-8 (NAF, neutrophil activating factor), MCP (monocyte chemoattractant peptide), GM-CSF and IL-1 (Libby *et al*, 1986; Strieter *et al*, 1989; Rollins *et al*, 1990). Moreover, it enhances the transmigration of leukocytes through the blood vessels by acting on both the leukocytes and the endothelial cells (discussed in detail in Chapter 1 & 4). IFN-gamma synergises with TNF to activate the cytotoxic properties of macrophages such as the reactive oxygen intermediates, lysosomal enzymes and more importantly, the nitric oxide which was shown to exert effective cytolytic effects on various parasitic and bacterial pathogens (Johnston, 1988; Liew & Cox, 1991). T lymphocytes express HLA-DR antigens, high affinity IL-2 receptors and enhanced proliferation in response to TNF (Scheurich *et al*, 1987). Finally, although the increased sensitivity of host cells infected with mycobacterial bacilli to the cytotoxic effects of TNF (Filley & Rook, 1991) may result in pathological tissue damage and haemorrhagic necrosis usually seen in mycobacterial lesions but this effect may also limit the growth and the dissimination of mycobacterial bacilli. This can be achieved by destroying the heavily infected cells and therefore releasing the bacilli to be phagocytosed and killed by activated macrophages. More knowledge can be gained by investigating where and when TNF is produced in the course of infection and by examining the pathological changes in the the infected organs of both the test and control groups (work is in

progress). Thus, we attempt to microscopically examine the organs (liver, spleen and lung) of the infected animals to investigate :

1) the effect of the neutralisation of TNF on the formation of granulomas.
2) whether the Tb bacilli grow inside or outside the granulomas.
3) whether there is an increase or a decrease in the tissue damage following the inhibition of TNF synthesis.

CHAPTER EIGHT

GENERAL DISCUSSION

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8.1. Introduction

Most research on mycobacterial disease at present concentrates on defining the epitopes evoking T cell responses. There are two reasons for this. First, the techniques required for this type of analysis, such as recombinant DNA technology and peptide sequencing have become available, and are fashionable. Secondly, there has been a tendency to assume that susceptibility to tuberculosis must depend on the failure to respond to some unidentified "protective" antigen, or even to a critical epitope within such an antigen. This view derives from past experience with viruses and toxigenic organisms, of which the pathogenicity sometimes depends on part of a single molecule. However after several years of effort there is still no evidence that this is true for the mycobacterial infections. Moreover, the relatively weak associations with HLA phenotype in these diseases make it unlikely that such a critical antigen will be found. Meanwhile the work of Mosmann and his colleagues, when applied to *Leishmania* models in the mouse, has made it clear that the nature of the response, rather than its fine specificity, can be the critical question in relation to an intracellular parasite (Mosmann and Moore, 1991).

Without knowing the cellular or molecular basis for it, the group at the Middlesex has for several years believed that a similar division between appropriate protective (non-necrotising) and non-protective (Koch phenomenon) types of response is at the heart of the problem of immunity to tuberculosis. For the reasons outlined in the introduction to chapter 2, several preliminary trials of immunotherapy with *M. vaccae* have been undertaken. The most recent clinical results using optimised doses of *M. vaccae* given early after diagnosis, are exciting. Patients with multiply drug-

resistant tuberculosis in the U.K. and Iran (J. Stanford. personal communication), or patients in Nigeria where drugs were not available (P. Onyebujoh, personal communication) have shown dramatic improvement of wellbeing, weight gain, and clearance of organisms from the sputum. Clearly the efficacy of this therapy could depend on either changes in specificity of the response, or on changes in its nature.

This thesis aimed to study the possible mechanism of this immunotherapy in two ways. First, we wanted to study changes taking place in recipients of immunotherapy in Kuwait. For logistical and political reasons this part of the study was limited to serological studies. Secondly, we wanted to find a model enabling us to explore the mechanism of the necrotising response to mycobacterial antigens, and to find a model for the modulation of this necrosis by immunotherapy with mycobacterial preparations.

8.2. The effects of immunotherapy with *M. vaccae* in tuberculosis.

8.2.1. Antibody to secreted proteins.

One aim of the present study was to seek possible qualitative or quantitative changes in the antibody responses of patients undergoing immunotherapy, since these could indicate changes in either the nature or the specificity of the response. These patients were given various preparations of autoclaved or irradiated *M.vaccae* mixed with tuberculin, murabutide or both, in conjunction with chemotherapy, one month after diagnosis. When compared to the sex and age matched saline-treated group, the immunotherapy-treated patients were previously shown to demonstrate significant improvement in their T lymphocyte and antibody responses to sonicated antigens of various mycobacterial species

(Bahr *et al*, 1990). Examination of the clinical, bacteriological and immunological parameters used indicated that the formulations of irradiated *M.vaccae* + Tuberculin and murabutide, or autoclaved *M.vaccae* alone were the most effective. Thus in agreement with the previous findings, we found that groups of patients injected with either of these two immunotherapeutic formulations mentioned before have a significant increase in their antibody response to the secreted BCG85 antigen. If immunotherapy causes enhanced recognition and destruction of the live bacilli, which are the only source of this fully secreted antigen, this rise in antibody is surprising. However it is possible that the rise would have been short-lived, and followed by a rapid fall as the load of 30kDa antigen fell, but later bleeds were not available. Such a fall in antibody to the 30kDa antigen has been seen after 6 months in leprsoy patients given this therapy (Filley and Waters, personal communication). Chemotherapy induced a smaller increase in the anti-BCG85 response in the saline treated patients. In addition, those who received irradiated *M.vaccae* + Tuberculin and murabutide also showed an enhanced response to the secreted 23 kDa antigen.

Is this rise in antibody merely an indicator of other immunological events, such as death of organisms, or increased helper activity, or are these antibodies useful to the host? Both the BCG85 and the 23 kDa antigens may be virulence factors of mycobacteria and thus an immune response to these antigens could be important for protection. Although in general antibody has been thought to play no role in immunity mycobacteria, little attention has been directed towards the possibility that antibody able to neutralise certain pathogenetically important functional components of mycobacteria might play a supplementary protective role. A

previous report demonstrated that BCG85 complex antigen has a high affinity to fibronectin, a glycoprotein found in both the serum and in the extracellular matrix and it bears binding sites to collagen, gelatin and heparin (Abou-Zeid *et al*, 1988). Interestingly, the addition of the culture fluid of BCG, the purified BCG85A antigen or the anti-BCG85 serum was shown to inhibit the binding of mycobacteria to fibronectin coated surfaces (Ratliff *et al*, 1988). Therefore, this antigen may play a major role in the adherence and invasion of pathogenic bacteria into their host cells. The 23 kDa antigen is secreted by *M.tuberculosis* massively in the first few days of culture and was shown to function as a superoxide dismutase enzyme (Andersen *et al*, 1991; Zhang *et al*, 1991). Therefore pathogenic mycobacteria may surround themselves with this enzyme as protection from the toxic effects of oxygen radicals released by activated phagocytes. Indeed the suggestion at present is that mycobacteria are controlled by the Nitric oxide pathway, rather than by oxygen radicals (B. Bloom, personal communication to G. Rook).

8.2.2. Decreased antibody to the hsp65

In contrast to what was seen with the secreted 30kDa antigen, the best immunotherapeutic preparations caused a fall in antibody to the 65kDa hsp. Since this antigen is only released after organisms have been killed, this finding is the reverse of what was expected. On possible explanation is that this protein, or human hsp which are highly homologous to it, may play an immunoregulatory role. We know that antibody to it is raised in rheumatoid arthritis (Tsoulfa *et al*, 1989) and in prediabetic twins (P. Lydyard, personal communication. Moreover in animal models and RA, antibody to it shows some correlation with the %agalactosyl IgG, which is also regarded as a marker of a type of dysregulation (Rook *et al*,

1991). Therefore the fall in antibody level seen here is potentially interesting and deserves further study in future immunotherapy trials.

8.2.3. The fall in % agalactosyl IgG

It has been sugested that the pathological changes detected in tuberculosis infections are associated with the dysregulation of cytokine production (Rook *et al*, 1991a). One correlate of the abnormal release of cytokines, particularly of IL-6, is the increased proportion of serum agalactosyl IgG (Gal(0)) in Tb patients which correlates with T cell-dependent inflammation and an acute phase reaction (Rook, 1988c). (Agalactosyl IgG is also raised in mice transgenic for the human IL-6 gene (Rook et al, 1991)). Patients given the most successful immunotherapeutic preparations showed significant reduction in their Gal(0) levels after therapy (chapter 2). Further studies will be needed to determine whether this test can be used as a reliable marker for monitoring the clinical improvement in these patients, and the efficacy of immunotherapy. Serum collections from trials in Gambia (where immunotherapy very significantly reduced the death rate in a group of tuberculosis patients; Corrah *et al.*, in preparation), and from Nigeria, are now being studied. In view of the known circumstantial associations between hsp65 and agalactosyl IgG in human and rodent arthritis (Rook *et al*, 1991), antibodies to the hsp65 should also be monitored in these patients.

8.3. The necrotising response to mycobacterial antigens

8.3.1. A new model for TNF-mediated tissue damage

In comparison to healthy subjects and tuberculoid leprosy patients, only tuberculosis patients are known to have necrotic

lesions and present necrotic skin test reactions to non-toxic preparations of Tuberculin despite the fact that the former groups have cellular immune responses to mycobacterial antigens and hence respond with non-necrotic DTH reactions. This observation suggested that the necrotic component of the Tb patient's reaction is not a requirement for a protective cellular immune response. In addition, there are various types of experimental evidence suggesting a possible homology between the Koch phenomenon and the Shwartzman reaction (discussed in Chapter 1). Therefore, we tested this hypothesis using a murine model. In agreement with the previous reports, we found that mice do not develop a classical Shwartzman reaction (chapter 3). However, Rothstein and Schreiber (1988) developed a murine model for a local Shwartzman-like reaction in which they prepared the skin of the back of the animals with LPS or killed bacteria followed 24 hours later by a subcutaneous injection of recombinant TNF. There were two problems with this model. First, the necrotic lesions were evaluated by measuring their diameters. Second, high concentrations were used for both the preparatory (LPS, 100 ug) and the provocative (TNF, 10 ug) injections. Thus, one of our preliminary aims was to find sensitive quantitative measures for the pathological damage produced in LSR. Monastral Blue dye, previously used by some investigators to label areas of increased vascular permeability, failed because of the complete destruction of the outer layers of the skin (data not shown). Therefore, we used the foot-pad skin as an alternative target. Interestingly, we found that although the challenged foot-pads did not macroscopically show necrotic skin damage, there was marked necrotic tissue damage when the histology of the skin sections was examined. Furthermore, the challenged feet became swollen and haemorrhagic reflecting the increased

extravasation of fluid, inflammatory cells and red blood cells. The two markers we used for measuring the damage produced in LSR were sensitive enough for us to reduce the amount of LPS (preparatory) and TNF (provocative) used 10 fold. In the subsequent experiments, we adressed the question whether the sonicated antigens of mycobacteria can prepare for a TNF-mediated LSR. We found that both pathogenic (*M.tuberculosis* and *M.leprae*) and non-pathogenic (*M.vaccae*) mycobacteria can equally prepare for a LSR though this depends on the immunisation schedule used. It may be therefore that although mycobacterial antigenic preparations of various species are capable of preparing for a necrotic skin reaction, this reaction can be negatively regulated to prevent the production of tissue damage in normal individuals. *In vivo* this regulation could operate at the level of local TNF release into the lesion though we have not tested this possibility. The work presented here, involving an external source of TNF, shows that it can also operate at the level of the intrinsic sensitivity of the DTH site to TNF. These experiments are discussed later.

In addition, lipoarabinomannan (LAM), a component of the mycobacterial cell wall and a TNF inducer (Moreno *et al*, 1989) was found to participate in the preparation of a local site for the LSR and more significantly, in the provocation of the reaction.

It is also important to mention that the murine local Shwartzman-like reaction is subject to seasonal variation in its intensity (chapter 3). This phenomenon could be attributed to the use of pathogen-free mice which were exposed to the environmental mycobacteria in their food and drinking water and this correlates with the requirement of T cells for the production of the reaction (chapter 4). This would not, however, explain the seasonal variation in the LPS-primed LSR.

8.3.2. Mechanism of the mycobacterium-prepared LSR

Our next aim was to examine the various cellular and humoral mediators participating in the production of LSR. Previous reports suggested that the complexity of such reactions is due to the participation of numerous biological mediators. It was suggested that TNF, in combination with IL-1 and IFN-gamma, are major initiators of the reaction. The the participation of inflammatory cells and other biological mediators leads to the amplification of the reaction. Liposomes containing dichloromethylene diphosphonate, toxic to the phagocytic cells, revealed the involvement of these cells in the LSR (chapter 4). Previous studies showed that lysosomal lysates, oxygen intermediates and other unidentified factors released by activated phagocytes can be cytotoxic to endothelial cells (Peri *et al*, 1990). In addition, the phagocytic cells required their cell surface receptors to adhere to the endothelium and then to transmigrate to the site of inflammation. Although there are various receptors expressed on the surface of both activated inflammatory and endothelial cells, the blockage of one of these receptors, the CR3 molecule, can reverse the tissue damage indicating that cell-to-cell adhesion is a cooperative process. Monoclonal antibodies to CR3 were shown previously to effectively prevent the tissue damage in other pathological experimental events such as diabetes mellitus (Hutchings *et al*, 1990). T lymphocytes ($CD4^+$ and $CD8^+$) also seem to play a role in the production of the haemorrhagic tissue damage in this reaction.

Histological examination showed that foot-pads injected with *M.vaccae* or TNF alone have a small increase in the thickness of the dermis layer accompanied with moderate inflammatory cell (including macrophages and neutrophils) infiltration (chapter 4).

However, those injected with both *M.vaccae* and TNF sequentially showed not only a larger increase in the thickness of the dermis layer but also massive cellular infiltration (with perivascular cuffing) and haemorrhagic necrosis leading to the destruction of the epidermis, the muscular and connective tissue in the dermis layer.

Using the foot-pad model allowed us to both evaluate the LSR reaction by measuring two different sensitive indicators of the pathological changes and to test for the possibility of producing a non-haemorrhagic swelling. Thus, in order to explore this possibility, we attempted to test the effect of various therapeutic drugs. Both WEB 2170, a platelet activating factor (PAF) antagonist, and misoprostol, a prostaglandin E_1 analogue, were found to reduce the haemorrhage, with no major effect on the swelling seen in the challenged foot-pads even if they were administered to the challenged animals almost simulataneosly with the provocative injection (chapter 5). PAF was previously shown to mediate some of the pathological changes such as the hypotension, lesions in the intestines and mortality seen following the administration of LPS or TNF. Furthermore, the feet of animals depleted of their complement, by the intravenous administration of cobra venom factor, were completely protected from the TNF-mediated local tissue damage. Thus, when compared to the feet of normal mice, the foot-pads of decomplemeted animals injected with *M.vaccae* +TNF had almost similar thickness of the dermis layer and a similar number of inflammatory cells in their skin. However, the protected animals had neither necrosis nor haemorrhage in their feet. Such an effect could be partially attributed to the depletion of the animals of their C3a and C5a which play a major role as chemotactic factors for leukocytes. Activated components of the complement

pathway do also participate in the induction of the coagulation system which results in the deposition of fibrin thrombi in the blood vessels.

The major problem with patients undergoing septic shock is that most of the pathological changes are irreversible and even the injection of anti-TNF antibodies given when the first symptoms appear does not seem to offer much help. However, the administration of the anti-CR3 monoclonal antibody, WEB 2170 or misoprostol almost simultaneously with the provocative injection of LSR reduced the haemorrhage. Therefore, since there is no effective treatment or prophylaxis for the occurrence of shock, it would be of considerable interest to further investigate the therapeutic effectiveness of these agents.

8.3.3. Inhibition of the LSR by LPS

We also demonstrate, in this study, the ability of small non-toxic doses of LPS to tolerise the animals and therefore, protect them from the TNF-mediated local tissue damage by eliminating both components of the reaction (swelling and haemorrhage) (chapter 6). LPS-induced tolerance was found by previous investigators to protect against the lethal effects of LPS and bacterial infections (Freudenberg and Galanos, 1988; Vogel *et al*, 1988; Zehavi-Willner *et al*, 1991). This protective effect could be partially reduced by the administration of recombinant IL-1 receptor antagonist (Henricson *et al*, 1991). In addition, the tolerising dose of LPS can be replaced by a combined preparation of IL-1 and TNF (Vogel *et al*, 1988). However, our attempts to abrogate the protective effect of the sub-lethal tolerising dose of LPS by pretreating the test animals with polyclonal anti-TNF serum failed. These results could be explained by the notion that administration of anti-murine TNF inhibits the production of LPS-induced TNF and colony stimulating

factor but not the release of interferons and glucocorticoids which may exert a protective effect (Vogel and Havell, 1990).

8.3.4. Inhibition of the LSR by preimmunisation with mycobacterial antigens

As mentioned previously, when tested with tuberculin, patients with past or present Tb infection show a necrotic DTH reaction while the healthy donors show a non-necrotic induration. We have shown using anti-CD4 and anti-CD8 antibodies that T cells are involved in the preparation for the LSR. An interesting finding of the present study is the fact that one can experimentally induce DTH sites that can be either sensitive or insensitive to the TNF-mediated local tissue damage (chapter 6). We have shown that although the subcutaneous injection of animals with killed *M.vaccae* profoundly enhanced both the swelling and the haemorrhagic components of the reaction, the inclusion of *M.vaccae* in FIA or FCA, before injecting it intraperitoneally rendered the animals hyporesponsive to the TNF-mediated necrotic damage at the local DTH site. The pretreatment of animals with various antigens such as heat shock proteins, BCG85 and KLH mixed with FIA also significantly inhibited the haemorrhagic component of the LSR. This effect was stronger and more reproducible when the mixture hsp65/FIA was used. Possible explanations for the loss of the sensitivity to the TNF-mediated local necrosis were discussed in detail in chapter 6. A particularly topical possibility is a change in the balance of TH1 to TH2 helper T cells.

8.3.5. Does IL-6 play a protective or a pathogenic role in the production of LSR

Attempts to investigate the role of IL-6 in the production or the prevention of the local tissue damage associated with LSR led

to obscure results. The cytokine was detected in the sera of animals injected with either *M.vaccae* or TNF locally. Higher concentrations of IL-6 were detected in those chellenged with both *M.vaccae* and TNF (chapter 5). Moreover, mice pretreated with an antigen mixed with FIA also had massive serum levels of the cytokine which diminished with time. However, high levels of IL-6 detected at the time of either immunisation (day -10) or challenge (day 0) did not correlate with either the pathology or protection. Nevertheless, both the CVF-treated animals and those pretreated with antigen/FIA gaved non-necrotic positive DTH reactions and both groups had vast amounts of IL-6 in their sera before they were challenged for a LSR. Therefore, it would be interesting to test the effect on LSR of pretreatment with a recombinant preparation of IL-6 or a neutralising serum to this cytokine.

8.3.6. Relevance of this new model of LSR

At present we cannot prove that this new model of TNF-mediated tissue damage in mycobacterium-prepared sites is a true model of what happens in human lesions, but we feel that the eveidence is compatible with this view, and the fact that it can be modulated by prior exposure to mycobacterial antigens may parallel the effects of immunotherapy and contact with environmental mycobacteria, on the response to human pathogens.

8.4. The role of TNF in experimental tuberculosis.

We next wished to assess the effcet of the neutralisation of TNF in experimental murine tuberculosis. Compared to infected mice injected with only saline or normal rabbit IgG, test animals treated with polyclonal anti-murine TNF serum had significantly greater bacterial load in the liver, lung and spleen, 4 weeks after infection (chapter 7). Although TNF was undetectable in the

serm of Tb infected animals, TNF expressed on the surface of phagocytes or secreted by activated macrophages seems to play a major protective role. In addition, despite the enhanced mycobacterial growth in the organs of anti-TNF treated mice, they showed an antibody response, to both the culture filtrate of *M.tuberculosis* and the BCG85 antigen, that is equivalent to that of the control groups. It is not known yet whether the anti-TNF antibody administration had any effect on the development of granulomas (work is in progress), as it did in the *M.bovis* BCG murine infection or on the haemorrhagic necrosis. Thus it is possible that TNF has both protective and pathogenic effects in tuberculosis depending on its serum concentration, the timing of its release and the release of other serum biological mediators. Since BCG growth is inhibited by granuloma formation in mice, TNF neutralisation resulted in the uncontrolled growth of BCG. However, Tb grows in both extracellular sites and in the granulomas causing haemorrhagic necrosis, presumably by rendering the TNF more toxic to the host tissues. Thus, it would be interesting to examine the effect of the neutralisation of TNF on the formation of granuloma in tuberculous mice and hence on the growth of the Tb bacilli and the degree of tissue damage produced.

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