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CELLULAR AND HUMORAL IMMUNE RESPONSES TO *Corynebacterium pseudotuberculosis* INFECTION IN GOATS

By

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THESIS

submitted in partial fulfilment of
the requirement for the degree

Doctor of Philosophy

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

Department of Microbiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
Mannuthy - Trichur

1986

DECLARATION

I hereby declare that this thesis entitled "CELLULAR AND HUMORAL IMMUNE RESPONSES TO *Corynebacterium pseudotuberculosis* INFECTION IN GOATS" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship, or other similar title, of any other University or Society.

(V. JAYAPRAKASAN)

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CERTIFICATE

Certified that this thesis, entitled "CELLULAR AND HUMORAL IMMUNE RESPONSES TO *Corynebacterium pseudotuberculosis* INFECTION IN GOATS" is a record of research work done independently by Sri. V. Jayaprakasan under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship, or associateship to him.

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Introduction

INTRODUCTION

Augmentation of the total output of animal products, quantitatively and qualitatively can contribute a lot in bettering the living conditions of small and marginal livestock farmers, in rural areas. One of the main impediments that interferes with animal production is the loss due to various microbial infections. A thorough and proper health coverage of the animals has to be provided for reducing the economic loss due to infectious diseases. The basic information regarding the mode of perpetuation of the disease, pathogenic propensity of the infective agent, the relative susceptibility of the spectrum of hosts, the immune status - naturally existing or artificially induced - and the possibility of developing a suitable vaccine are all helpful in formulating suitable control measures.

Among the umpteen number of infectious agents causing diseases in divergent species of livestock, Corynebacterial infections pose great problems for the farmers rearing goat and sheep (Nairn and Robertson, 1974; Campbell et al., 1982). Since Corynebacterium pseudotuberculosis (Corynebacterium ovis) is ubiquitous in distribution, infection due to this bacteria, commonly known as caseous lymphadenitis is world wide. Though this infection runs a chronic progressive course in the affected herd, the cumulative total loss resulting from poor reproductive efficiency, lowered growth rate due to stunting and emaciation,

condemnation of carcasses and inferior quality of the hide is considerably high (Stoops et al., 1984). As the disease is chronic and progressive, involving mainly lymphnodes, and to a lesser extent spleen and other visceral organs causing partial or total damage of the affected tissue, there is every likelihood of reduction in the immune status of the animal. More over this organism, during multiplication, releases fairly potent toxin in the system which keeps the animal under constant stress leading to the reduction in general resistance to several other infections.

A review of literature reveals that several classic research works have been carried out on caseous lymphadenitis with sheep as the experimental animal model for the disease. But, well documented epizootiologic informations on morbidity, mortality, influence of age on the incidence, mode of transmission, pathogenesis and immune response in caprine species against C. pseudotuberculosis infection are scanty (Ayers, 1977; Nairn et al., 1982). The prevalence of this disease in goats of Kerala was reported by Venugopal et al. (1981). It has been observed that certain basic differences exist between the disease in sheep and goats with regard to lesions, disease transmission, pathogenicity and immunity. Nature and mechanism of immunity to C. pseudotuberculosis infection in sheep have been debated very extensively but, still it requires clarifications as to whether the immunity is cellular, humoral or both. There is dearth of information regarding the

pathogenesis and immune response to C. pseudotuberculosis infection in caprine species. Clinical evidence suggests that goats do not develop strong natural immunity. In view of the above facts, the immune response elicited by C. pseudotuberculosis infection in goats has been chosen as the subject for the present study.

Adult goats were experimentally infected with C. pseudotuberculosis and the development of humoral and cellular immune response were assessed employing the following parameters:

a) Total serum protein
b) Quantitative change in different fractions of serum protein
c) Qualitative change in the specific antibody activity of the serum
d) Change in the absolute leukocyte count
e) Delineation of peripheral blood lymphocytes:
    i) T-lymphocytes
    ii) B-lymphocytes
f) Alteration in the migration property of leukocytes
g) Delayed hypersensitivity reaction

The pathogenic effects of the organism in experimentally infected animals were also studied by observing clinicopathological alterations manifested at varying periods of infection.

Review of Literature

REVIEW OF LITERATURE

Ever since the isolation and elucidation of the identity of the etiological agent of caseous lymphadenitis (CLA) made for the first time independently by Nocard (1889) and Preisz (1894) a lot of information on its various aspects has accumulated. Corynebacterium pseudotuberculosis (C. pseudotuberculosis) is accepted as the principal causative agent for CLA in a wide spectrum of animal hosts (Jubb and Kennedy, 1970; Bruner and Gillespie, 1973; Jones and Hunt, 1983). Many specific names have been used in the past to designate this organism. Of these various synonyms, Corynebacterium ovis (C. ovis) is the one most accepted and frequently used (Buchanan and Gibbons, 1974). CLA is considered as a primary disease of sheep and goats all over the world, characterised by suppurative infection of lymphnodes and less frequently other organs of the body (Maddy, 1953; Jensen, 1974; Blood et al., 1979).

The first record of C. pseudotuberculosis infection of sheep suffering from renal abscess was made by Preisz and Guinard (1891). Then onwards different forms of this infection generally caseous lymphadenitis and caseous bronchopneumonia were reported from all over the world.

The morphology, staining property, cultural characters, biochemical and biological properties inclusive of toxigenicity of C. ovis were reviewed by Benham et al. (1962). Carne et al. (1956) demonstrated a toxic surface lipid in this organism and

further demonstrated that removal of this lipid by ether extraction did not affect the viability of the organism. Quantitative preponderance of this toxic principle, more in virulent strains was documented by Jolly (1966) and Muckle and Gyles (1983), corroborated the view that this toxic principle confers the pathogenic propensity. Hard (1969b) demonstrated a thick lipid layer as an electron dense flocculer layer exterior to cell wall of virulent C. ovis by electron microgrophy. The surface lipids impeded with the lytic function of phagocytes (Hard, 1972).

Transformation of beta haemolytic C. ovis to alpha haemolytic type with changes in colony morphology and biochemical characters, consequent to repeat subculturing, was reported by Barakat et al. (1970). In the year 1974 Shigidi reported similarity of antigenic configuration among the various isolates of C. pseudotuberculosis from different sources. Based on immunoprecipitation, nitrate reduction and animal pathogenicity, Barakat et al. (1984) differentiated strains of C. pseudotuberculosis into two serotypes I and II. The infection by the former type which was nitrate negative culminated in the death of guinea pigs in 15 days time with the production of local and generalised abscesses, whereas the latter type which was nitrate positive caused mortality in shorter time i.e., 5 days with tissue necrosis but without formation of abscesses.

Cultivation

C. pseudotuberculosis grew poorly on ordinary nutrient media, but growth was often improved by the addition of blood or serum (Carne, 1939; Benham et al., 1962). The peptic digest broth prepared by Carne (1940) supported profuse growth of the organism and the medium was reported suitable for production of toxin (Doty et al., 1964; Jolly, 1965a; Lovell and Zaki, 1966a&b; Shigidi, 1978). Cameron and Swart (1965) used a nutrient broth without incorporating blood or serum but supplemented with yeast extract and lactalbumin hydrolysate, for high yield and toxin production from C. ovis. Maskintepe (1976) further modified the medium by incorporating protease peptone and glucose. Protease peptone broth containing yeast extract and glucose enriched with sheep serum was reported to be a superior medium for production of exotoxin from C. ovis (Burrell, 1979; Burrell, 1980).

Toxin

C. pseudotuberculosis was known to produce a powerful exotoxin which accounted for its virulence and pathogenic propensities. The toxin was endowed with several biologic properties such as lysis of red cells (Zaki, 1965; Burrell, 1979), dermonecrotoxicity (Carne, 1940; Doty et al., 1964; Lovell and Zaki, 1966b), inhibition of staphylococcus beta haemolysin (Hartwigk, 1963b; Fraser, 1964), potentiation of staphylococcus epsilon haemolysin (Fraser, 1964), enzymic action of phospholipase-D (Soucek et al., 1971) and toxaemic death of

experimentally injected laboratory animals (Jolly, 1965a; Lovell and Zaki, 1966a).

There was disagreement with regard to the haemolytic property of the toxin (Carne, 1939; Fraser, 1964), but results of the work of Zaki (1965) and Burrell (1980) gave conclusive evidences that the toxin had lytic action on ox, sheep and horse erythrocytes. Benham et al. (1962) reported about the prevalence of non-toxigenic strains of C. ovis. Eventhough Rottgardt (1930) reported loss of toxigenicity of one of the strains studied, several other workers have shown toxin production as one of the distinct character of C. ovis (Desaaseaux, 1929; Carne, 1940; Lovell and Zaki, 1966a; Burrell, 1980).

Delaunay (1943) observed delayed leukocytic chemotaxis followed by inhibition of leukocytic migration when severe intoxication was induced. C. ovis stained with methyl violet failed to attract leukocytes on subcutaneous injection but abscess formation resulted once the dye had diffused away from bacteria (Bull and Dickinson, 1935). Maddy (1953) reported that the leukocidin, which killed the leukocytes and deposited in situ was responsible for abscess formation by C. ovis

Optimum toxin production was reported when the organisms were incubated at 37°C (Rottgardt, 1930; Lovell and Zaki, 1966a; Burrell, 1979) aerobically (Carne, 1940; Lovell and Zaki, 1966a), though Fraser (1961) and Burrell (1979) recorded an atmosphere containing increased carbondioxide as superior.

Filtration of toxin through earthen ware, asbestos and glass filters resulted in loss or reduction in toxicity (Bull and Dickinson, 1935; Awad, 1960; Lovell and Zaki, 1966b; Burrell, 1979; Burrell, 1980) but filtration through cellulose filter did not reduce toxicity (Burrell, 1980).

The exotoxin of C. *ovis* was demonstrated to be thermolabile at 55°C in 30 minutes time (Zaki, 1965; Burrell, 1979). Potency of the toxin remained unaltered at 4°C (Rotigardt, 1930; Lovell and Zaki, 1966b; Burrell, 1980c); at -20°C (Cameron and Smit, 1970; Burrell, 1981) or when dialysed against distilled water and lyophilised (Cameron and Smit, 1970).

Afnan (1969) reported that few strains of C. *ovis* produced wide zone of alpha haemolysis while majority of the strains produced beta haemolysis around the colony when grown on sheep, rabbit, horse or bovine blood agar. The haemolytic activity of the toxin was shown to be influenced by pH of the medium (Burrell, 1979). Though the toxin at acidic pH (pH 5 to 5.5) caused haemolysis both in aerobic and anaerobic conditions, the extent of haemolysis was more at anaerobic conditions indicating that gaseous pressure was also one of the factor influencing the haemolytic pattern.

Dermonecrotoxicity of exotoxin from C. *ovis* on guinea pig or rabbit skin was used as criterion to measure the potency of the toxin (Carne, 1940; Doty *et al*., 1964; Lovell and Zaki, 1966b; Burrell, 1980; Garg and Chandiramani, 1983). Doty *et al*. (1964) observed antigenic similarity between isolates of C. *ovis*

from divergent species of animals through cross neutralisation of dermonecrotoxicity of toxins in guinea pig and rabbit skin. Jolly (1965a&b) through a series of experiments had shown the ability of crude extract of exotoxin to increase the vascular permeability. He also showed that the antitoxin retarded the dissemination of C. ovis to the local lymph nodes or prevented multiplication in situ or both.

Employing a modified CAMP technique Hartwigk (1963b) demonstrated the ability of C. ovis to produce antihaemolysin against beta haemolytic staphylococci. Fraser (1964) demonstrated that the metabolic products of C. ovis and C.haemolyticum inhibited the haemolytic property of both alpha and beta toxins of staphylococci on sheep, ox or goat red cells, while the haemolytic effect of epsilon toxin was potentiated. Lovell and Zaki (1966a) using the mouse protection test showed that toxins obtained from sheep, horse and buffalo strains were related to each other.

Synergestic haemolytic effect produced by the mixed cultures of C. ovis and C. equi on sheep, goat, ox and rabbit red cells was demonstrated by Fraser (1961). He also observed that the diffusible substance produced by C. ovis was inactive against horse red cells but the activity could be potentiated with 10 per cent carbondioxide.

C. pseudotuberculosis possessed a heat labile toxic component and a heat stable pyogenic factor and both were

proved to be lethal in action independently (Petrie and McClean, 1934; Bull and Dickenson, 1935). Cameron (1964) established that apart from toxic lipid, C.ovis cells contained a pyogenic factor as well as protoplasmic toxins. Blood tryptose agar supported high production of endotoxin and pyogenic factor. Both endotoxin and pyogenic factor were heat labile, the former was destroyed at 60°C within an hour and the latter by autoclaving.

The nature of protoplasmic toxins and its relationship to the exotoxin was studied by Cameron and Smit (1970). The leukocidal activity and dermonecrotoxicity of the crude protoplasmic toxins, fractionated protoplasmic toxins and exotoxin were assessed. The results indicated that exotoxin preparation contained antigens derived from products of bacterial lysis. Crude exotoxin was purified and concentrated by fractional precipitation with ammonium sulphate and dialysation against distilled water (Goel and Singh, 1972; Garg and Chandiramani, 1983).

Soucek et al. (1971) demonstrated an enzyme which belonged to the group of phospholipase-D in C. pseudotuberculosis. This enzyme could split sphingomyelin to N-acetyl spingosyl phosphates and choline and lysophosphatedyl choline to lysophosphatidic acids and choline. The presence of enzyme phospholipase-D with exotoxin of C. ovis was attributed to be one of the reasons for its toxic action on blood capillaries leading to increased permeability and extravasation (Carne and Onon, 1978).

Caseous lymphadenitis in sheep

Caseous lymphadenitis is an infection seen in a wide spectrum of animal hosts but the records of incidence in ovine species rank first. Since the report of C. pseudotuberculosis infection in sheep appeared in the year 1891 by Preiss and Guinard, incidence of this disease was frequently reported (Benham et al., 1962; Ayers, 1977; Stoops et al., 1984).

CLA often occurs as an inapparent infection in sheep and goat but occasionally it causes overt disease and rarely death. Specific clinical symptoms are not exhibited by this disease except for the enlargement of superficial lymphnodes and subsequent development of abscesses. Lesions are generally produced in lymphnodes, occasionally in lungs and less frequently in kidney and other visceral organs (Jones and Hunt, 1983). The results of the survey made by Nadim et al. (1966) indicated that non-palpable lymph nodes of sheep were more frequently affected than superficial palpable lymph nodes. CLA was considered to cause little effect on the general health of the animal unless the disease become generalised (Blood and Henderson, 1974). The disease insidiously occurs with high incidence causing extensive waste and economic loss to sheep industry (Jensen and Swift, 1982; Stoops et al., 1984).

Cases of C. ovis infection involving tissues and sites other than lymph nodes are also on the record. Marsh (1958) reported the isolation of C. pseudotuberculosis from inflammed joints and bursae of young lambs. An outbreak of mastitis in

ewes in France was reported by Grobet (1958). Abortion in sheep in the later half of pregnancy and reproductive failure due to C. ovis infection were reported (Szemardi and Temeri, 1960; Dennis and Bamford, 1966; Mostafa et al., 1973; Gates et al., 1977; Renshaw et al., 1979). Orchitis and epididymitis in rams were reported and semen from such infected animals was shown to be of low quality (Khalimbekov et al., 1961; Galloway, 1966; Shegidevich, 1969). Williamson and Nairn (1980) reported palpable lesions due to C. ovis infection within the scrotum without involvement of testes or epididymus, in which case the semen quality was not affected.

Fatal C. ovis infection in adult sheep was considered rare and the infection usually ended in complete recovery when the pus escaped from the abscess (Zaki, 1966). But Kalinski (1962) observed 20% mortality in an infected flock of sheep with involvement of lung, pleura and skin. Perinatal lamb mortality was reported by Dennis and Bamford (1966) due to C. ovis infection with involvement of brain, abdmasum and lymph nodes. Shegidevich (1969) reported the association of C. pseudotuberculosis with Pasteurella multocida and Mycoplasma species in purulent necrosis of lymph node, pleuropneumonia, arthritis, mastitis, orchitis, epididymitis and subcutaneous abscesses.

Non-palpable deep seated lymph nodes, thoracic and abdominal viscera were reported to be frequently affected by C. ovis (Nadim et al., 1966; Seddik et al., 1983; Stoops et al., 1984). But according to several other workers superficial

lymph nodes were the principal sites affected in CLA (Aktas, 1971; Awad et al., 1979). A chronic debilitating condition in ewes generally known as "thin ewe syndrome" was reported to be due to the involvement of visceral organs with lesions produced by C. ovis along with several other bacteria (Renshaw et al., 1979).

Caseous lymphadenitis in goats

Caseous lymphadenitis is considered as an important disease in goats but the gravity of its prevalence, epizootiology, pathogenicity and immunity have not been studied in detail (Ayers, 1977). Only scanty reports have appeared on CLA in goats (Shaw and Seghetti, 1939; Stafseth et al., 1945; Purchase, 1944; Gallow and Morris, 1965; Addo and Eid, 1978; Hein and Cargill, 1981; Burrell, 1981). The early record of CLA in goats in India was about an outbreak of this disease in Uttar Pradesh by Dhanda and Singh (1955). Since then few more reports have appeared about CLA in goats from India (Sarkar and Bhattacharya, 1975; Natarajan and Nilakantan, 1975; Lalkrishna et al., 1977; Venugopal et al., 1981; Garg and Chandiramani, 1984).

Though there is an overall similarity between clinical picture of CLA of sheep and goats, there exist differences in distribution of affected lymph nodes, morphological appearance of the lesions and frequency and severity of the visceral form of infection. The distribution of abscess was more in the head and neck lymph nodes of goats infected with C. ovis (Ayers, 1977;

Campbell et al., 1982). The involved lymph node had no recognisable parenchyma remaining by the time the abscess ruptured spontaneously (Ayers, 1977; Burrell, 1981; Nairn et al., 1982; Campbell et al., 1982). Progressive emaciation and sporadic death in goats were attributed to the development of internal abscess by C. ovis (Guss, 1977).

Thoracic form of the disease with lung involvement was found to be frequent in goats and this resulted in bronchopneumonia and death in badly affected goats (Sarkar and Bhattacharya, 1975; Sharma and Dwivedi, 1977; Nairn et al., 1982). Incidence of CLA in goats was found to be increased as the age advanced which indicated the deficiency of natural acquired immunity (Ashfaq and Campbell, 1979; Campbell et al., 1982). Muckle and Gyles (1982) reported that strains of C. pseudotuberculosis isolated from lesions of CLA in goats were found to be uniform in cultural characters, biochemical reactions and susceptibility to antimicrobial agents.

Caseous lymphadenitis in other animals

Several animal species including ruminants and non-ruminants suffer from CLA caused by C. ovis and the clinical manifestations are more or less similar to what has been described in members of capridae. C. pseudotuberculosis has been reported to be associated with disease condition of the following animal species: Swine (Naglie et al., 1978) ant bear (Roth and Vickers, 1966), hedge hog (McAllister and Kealey, 1971), cheetah (Boomker and Henton, 1980) and primates (Holt and Goffe, 1961).

Cattle occasionally suffer from C. pseudotuberculosis infection and only a few cases have so far been reported (Benham et al., 1962). Cattle developed pathological syndrome which resembled that of sheep but under natural conditions only localised infection was common, and the lesion confined to one or two regional lymph nodes, draining an infected surface wound (Jubb and Kennedy, 1970). Rising and Hesselholt (1973) reported isolation of C.ovis from abscessed lymph nodes of cattle. Acute mastitis due to this bacterial infection was also reported by Adakeye et al. (1980). Karinki and Poulton (1982) observed that C. ovis played a major role in producing skin lesions of cattle, either alone or in association with other microbial agents.

Infection with C. ovis in horse was shown to produce a clinical picture of ulcerative lymphangitis or local abscessation (Benham et al., 1962). Hughes et al. (1962) reported cases of generalised infection in mares with large retroperitoneal abscesses involving kidney, cervix, abdomen, muscle and popliteal lymph nodes. Acute preumbilical, pectoral and abdominal wall abscesses were also reported in equines due to C. ovis infection (Wisecup et al., 1964; Mayfield et al., 1979; Moirs and Ley, 1980). Serological evidence for CLA in equines was reported by Knight (1970). Zaki et al. (1980) reported isolation of C. pseudotuberculosis from two aborted equine foetuses and this formed the first record about the association of C. pseudotuberculosis with abortion in mares.

Transmission of caseous lymphadenitis

Under natural condition, the mode of transmission of this disease was observed to be mainly through contamination of skin wounds (Jensen and Swift, 1982). The role played by the contaminated dipping fluid in the transmission of CLA in sheep was established (Nairn and Robertson, 1974; Nairn et al., 1982). Nagy (1976) observed a definite relation between the route of infection and the lymph node abscessed. Burrell (1981) pointed out that ingestion of pus containing C. ovis in large quantity accounted for the development of many abscesses in the lymph nodes at the head and neck region of goats. C. pseudotuberculosis was not voided in the faeces or nasal secretion of infected sheep and goats (Ashfaq and Campbell, 1980; Chandiramani and Garg, 1982).

Though Seddon (1929) demonstrated the possible role of ticks in the transmission of CLA in sheep, Nagy (1971) however could not establish any relationship between tick infestation and incidence of CLA in sheep. Mechanical transmission of C. pseudotuberculosis by house fly feeding on oozing lesions of ulcerative lymphangitis in horse was reported by Addo (1983). Nairn and Robertson (1974) have shown the possibility of infection through intact skin also, when sheep were dipped in contaminated dipping fluid. Hein and Cargill (1981) reported frequent occurrence of lesions in the thoracic lymph nodes and pulmonary paranchyma in goats which suggested that inhalation of C. ovis may also result in infection in goats.

Experimental infection

Experimentally laboratory animals as well as domestic animals were shown to be susceptible to C. pseudotuberculosis infection. Though infection could be readily reproduced in sheep and goats, laboratory animals like mice, guinea pig and rabbit were commonly used as experimental animal models for pathogenicity and immunity studies considering economy and managemental conveniences (Jolly, 1965a&b; Cameron,1972).

As early as 1930 Cesari, reported experimental infection of guinea pigs with C. pseudotuberculosis which resulted in local abscess formation. Among other laboratory animals, guinea pigs were frequently used for assessing virulence and pathogenicity of C. ovis isolates and its toxin, since this species exhibited a high susceptibility (Holt and Goffa, 1961; Cameron, 1964; Barakat and Sayour, 1967; Nadim and Farid, 1973; Khater et al., 1978). Injection of virulent bacteria or toxin often caused death in guinea pigs which made the species unsuitable for immunity studies (Lovell and Zaki, 1966a; Hard, 1969a).

Since rabbits were extremely susceptible to C. pseudotuberculosis infection or to its toxins, this species was extensively utilised for testing the dermonecrotoxicity of the exotoxin (Doty et al., 1964; Cameron, 1964; Burrell, 1980a&b).

Certain doubts existed about the susceptibility of mouse to C. pseudotuberculosis infection and the lethal effect of exotoxin (Guillot, 1934). The classic experiments reported

by Jolly (1965b) and Lovell and Zaki (1966a) established the usefulness of this species as an experimental model for caseous lymphadenitis in animals. The experimental infection in mouse simulated natural infection of CLA in sheep (Jolly, 1965b). Besides CLA, osteomyelitis and arthritis were induced in mice by intravenous administration of C. ovis (Zaki, 1966).

Mice were extensively used for immunization trials with various antigens from C. pseudotuberculosis (Cameron and Minnar, 1969; Hard, 1969a; Hard, 1970; Cameron and Smit, 1970; Cameron and Purdom, 1971; Cameron, 1982; Brodgen et al., 1985). Zaki et al. (1980) reported that mice were superior to guinea pigs and golden hamsters for determining pathogenicity of C. ovis.

Experimental infection in sheep

The experimental inoculation of C. pseudotuberculosis to sheep did not produce infection similar to natural disease. Massive dose of bacteria often caused death in sheep due to acute toxicity (Cameron et al., 1972; Gamsel and Tartour, 1974; Garg and Chandiramani, 1984). Percutaneous injection of bacteria invariably produced local abscess while intravenous injection culminated in death consequent to abscessation of lungs and kidneys (Cameron et al., 1972).

Nairn and Robertson (1974) produced experimental infection in sheep by directly smearing C. pseudotuberculosis broth culture over intact skin and also mixing the culture in dipping fluid. They observed that sheep developed lesions of lymphadenitis

starting from seven days post inoculation. Since lesion developed also in visceral organs of some experimentally infected sheep, they suggested a possible haematogenous dissemination.

Pregnant ewes aborted on experimental infection with C. ovis during the second half of pregnancy (Addo, 1979). Local and lung abscesses suggestive of CLA were reported to be produced with subcutaneous injection of C. ovis and simultaneous infection with Trichostrongylus colubriformis (Bergstrom et al., 1980). Garg and Chandiramani (1984) showed that the clinical manifestations in experimentally infected sheep had direct correlation with the dose of bacteria administered. They observed that subcutaneous injection of massive dose of C. ovis produced acute intoxication while smaller doses developed subacute to chronic form of infection with lesions in superficial lymph nodes.

Jolly (1965c) could successfully establish infection in sheep, using single cell suspension of C. ovis prepared in bile salt saline. Cameron et al. (1972) reported the suitability of C. pseudotuberculosis grown in shake culture for experimental infection of sheep by intravenous route. But Cameron (1982) claimed the superiority of surface pellicle in broth culture over the shake culture for inducing experimental infection. Seddik et al. (1983) failed to produce infection in sheep through skin scarification but succeeded in establishing infection by intravenous injection of 3.2×10^6 colony forming

units of C. ovis. Sheep thus experimentally infected developed multiple abscess in lungs and lymph nodes 28 days post-injection.

Successful attempts to study the pathogenesis and immune response were made by Husband and Watson (1977) and Burrell (1978) by injecting live/killed bacterial suspension of C. ovis through intralymphatic route and collecting the efferent lymph to monitor the immunological events ensued.

Experimental infection in goats

Information on pathogenesis and immunity in caprines with natural or experimental C. ovis infection is scanty as is seen on perusal of available literature (Ayers, 1977; Campbell et al., 1982).

Abdel Hamid (1973) had injected subcutaneously varying doses of C. pseudotuberculosis broth culture to study the clinical manifestations of the disease in goats. He reported that a two millilitre dose of culture had produced severe intoxication in goats while 0.01 to 0.25 ml culture produced subacute to chronic form of infection characterised with lesions in regional lymph nodes and at the site of injection or scarification. Hamid and Zaki (1973) have shown prevalence of specific antibody in the serum of artificially infected goats detectable at 4 to 8 weeks post infection.

According to Ashfaq and Campbell (1980) 1×10^6 single cell suspension of C. ovis injected intradermally, subcutaneously

or submucosally could produce pathognomonic lymphadenitis in regional lymph nodes of goats with an average incubation period of 95.2 days. They observed that a large majority of animals infected in this procedure did not produce generalised lesions in thoracic or abdominal viscera. C. ovis was shown not to be excreted through nasal discharge or faeces of clinically infected goats.

Intradermal inoculation of 0.5×10^6 colony forming units of C. pseudotuberculosis at the flank region of kids produced multiple abscesses both peripherally and in visceral locations (Brown et al., 1983). Intranasal inoculation of bacteria failed to produce infection which indicated that respiratory tract would not be a portal of entry for C. pseudotuberculosis. Infected goats mounted specific antibody response detected by synergestic haemolysis inhibition test within one month post-infection.

Experimental infection of buffalo steers and cattle steers with C. ovis had been reported (Hassan et al., 1983; Khater et al., 1983a&b). Leukocytosis, decrease in red cell count, haemoglobin content and packed cell volume have been observed in infected animals. The pathological changes induced in these species were of powerful cytotoxic nature, both in lymph nodes and visceral organs.

Haematology

Goats

Normal blood values of goats have been reported but the observations are few in numbers and not adequate for statistical presentation and generalisation of values (Schalm, 1970). The tentative cell counts of goats as reported by Schalm (1970) were RBC 13 x 10^6, leukocytes 9 x 10^3, Neutrophils 3.25 x 10^3, lymphocytes 5 x 10^3, monocytes 250, eosinophils 450, basophils 0 to 50 per cubic millimetre of peripheral blood. The percentage distribution of leukocytes were reported as neutrophils 36%, lymphocytes 56%, monocytes 2.5%, eosinophils 5% and basophils 0 to 0.5%. The plasma protein concentration was estimated to be 6 to 7.5 g percentage. Haemoglobin content is estimated to be 11 g percentage and the packed cell volume as 35 mm in normal goats.

Several reports from India (Verma, 1947; Vaidya et al., 1970; Ramakrishna Pillai, 1972; Bhargava, 1980; Ghosh et al., 1981) and from abroad (Holman and Dew, 1963; Lewis, 1976; Edjtehadi, 1978; Earl and Carranza, 1980) presented haematological values of normal goats belonging to different breed, sex and age. Vaidya et al. (1970) observed that the lymphocyte percentage was significantly ($P< 0.01$) more in kids compared to adults. Edjtehadi (1978) also reported the age associated changes in the haematological values of goats and further observed that the changes in the neutrophil counts were inversely related to changes observed in lymphocyte counts.

Quantitation of lymphocyte subpopulations

The T-lymphocytes and B-lymphocytes denote the functional entities of the two major classes of immunocompetent cells. Identification of lymphocyte subpopulation is of great importance in understanding their functions in health and disease. Enumeration of T and B cells in peripheral blood and tissues have wide spread applications in both diagnosis and understanding of pathophysiologic mechanisms of many disease states especially immunodeficiency, malignancy of lymphoid cells, autoimmune diseases, alterations in immune competency of cancer patients and several infectious diseases (Wybran and Fudenberg, 1974; Winchester and Ross, 1976).

To delineate the various subpopulations of lymphocytes several methodology and protocols are in vogue.

Mammalian T-lymphocytes are routinely characterised and/or quantitated through receptors for heterologous erythrocytes (E-rosette receptor) (Winchester and Ross, 1976), receptors for Fc portion of immunoglobulins (Grewal et al., 1978) reactivity with heterologous antibrain or antithymocyte serum (Brown et al., 1974), ability to react with blood group A reactive haemagglutinin from Helix pomatia (Hammerstrom et al., 1973; Morein et al., 1979; Broetrom et al., 1985) non-specific enzyme alpha naphthyl acetate esterase activity (Mueller et al., 1975; Ranki et al., 1976) purine nucleoside phosphorylase activity (Bergers et al., 1977; Bergers and Thone, 1978) lymphocyte specific cell surface antigens such as T-cell surface antigen of OKT series

Shima et al., 1976; Yang et al., 1977; Prabhu and Reddy, 1983). E-rosette technique had been employed to enumerate T-cells from several animal species viz. bovines (Grewal et al., 1976; Wardley, 1977; Higgins and Stack, 1977; Wilkie et al., 1979; Grewal and Babuik, 1978; Paul et al., 1979b; Kushroo et al., 1981; Belden et al., 1981; Outteridge and Dufty, 1981) Ovines (Heron et al., 1978; Binns, 1978; Outteridge et al., 1981) caprine (Yang and Shein, 1980; Shein and Wang, 1982; Sulochana et al., 1982) equines (Tarr et al., 1977; Mayur and Schlegen, 1978) swine (Escajadillo and Binns, 1975a and b; Shimuzu et al., 1976) canines (Bowles et al., 1975; Miller et al., 1978) and felines (Tayler et al., 1975).

Results obtained by several workers indicate that the heterologous red cells are to be chosen depending on species of animals whose lymphocytes are subjected to E-rosette formation. T-lymphocytes from human beings formed maximum rosettes with sheep erythrocytes (Jondal et al., 1973; Bach, 1973). Taylor et al. (1975) showed that rodent erythrocytes formed highest rosettes with feline T-cells. According to several workers, sheep red cells were the choice for E-rosette formation with bovine T cells (Grewal et al., 1976; Higgins and Stack, 1977; Kaura et al., 1979). Equine (Tarr et al., 1977) rabbit (Wilson and Coombs, 1973) and canine (Bowles et al., 1975) T-cells formed maximum rosette with guinea pig erythrocytes.

E-rosette formation by T-lymphocytes was reported to be affected by many variables such as incubation time, temperature,

serum concentration in the medium and the proportion of population density of erythrocytes and lymphocytes (Mendes et al., 1974; Tarr et al., 1977; Woody, 1975; Grewal et al., 1976). Treatment of erythrocytes with neuraminidase (Weiner et al., 1973; Tarr et al., 1977; Grewal and Babuik, 1978; Grewal et al., 1978; Wilkie et al., 1979), trypsin (Chapel, 1973), papain (Escajadillo and Binns, 1975b), AET (Kaplan and Clark, 1974; Paul et al., 1979a&b; Grewal and Babuik, 1978), dextran (Brown et al., 1975; Paul et al., 1979a&b) and bromelin (Florey and Poelsom, 1976) have shown to enhance rosette formation by T-cells. Higher rosette forming ability was reported with T-cells when suspending medium was incorporated with foetal calf serum (Higgin and Stack, 1977; Kuchroo et al., 1981) or bovine serum albumin (Higgin and Stack, 1977) or Ficoll (Outteridge and Dufty, 1981; Outteridge et al., 1981).

Wybran and Fudenberg (1973) had recognised two subpopulation of T-lymphocytes as active E-rosette forming cells and total E-rosette forming cells. The former group required shorter duration of incubation with erythrocytes while the latter required longer incubation. The proportion of these two populations of cells was reported to vary in clinical condition and the active rosette forming cell numbers reflected better T-cell competence (Wybran and Fudenberg, 1974).

Alpha naphthyl acetate esterase (ANAE) activity

The earliest records about the non-specific alpha naphthyl acetate esterase activity as a marker for identification of

mouse T-lymphocytes were made by Mueller et al. (1975) and Ranki et al. (1976). Since then this marker had been reported to be efficient in delineation of T-lymphocytes from other cells (Totterman et al., 1977; Kulenkampff et al., 1977; Pangalis et al., 1978; Pinkus et al., 1979). In cryostatic sections of lymphoid tissue Knowles and Hock (1978) demonstrated characteristic monocyte and T-lymphocyte staining pattern of ANAE reaction. They considered cytochemical and histochemical demonstration of ANAE activity as a useful marker for T-lymphocytes in tissues as well as in peripheral blood.

Knowles et al. (1978) compared the efficiency of histochemical demonstration of ANAE activity with that of E-rosette assay as markers for T-cells in human peripheral blood and lymphoid organs of normal and lymphocytic leukaemic patients. The results obtained by them showed that the percentages of E-rosette and ANAE positive lymphocytes were always comparable. They further described the ANAE staining pattern, characteristic for T-cells, as single prominent cytoplasmic red brown nodular reaction product seen adjacent to cell membrane (T-cell pattern) while in monocytes as diffuse cytoplasmic red brown reaction product (M pattern).

Knowles et al. (1979) evaluated ANAE activity as a T-cell marker in human lymphoid malignancies comparing with several other markers such as E-rosette formation, presence of Ia antigens and surface immunoglobulin. The percentages of E-rosette and ANAE positive cells from patients with T-cell

malignancy were strikingly similar which established ANAE activity as a good T cell marker. The neoplastic cells from patients with B-cell malignancy, acute lymphoblastic leukaemia and null cell malignancy failed to exhibit ANAE activity.

T-lymphoblasts obtained by stimulating T-cells with mitogens such as phytohaemagglutinin, poke weed mitogen or alloantigen were able to retain the E-rosette formation property but lost their ANAE activity (Knowles et al., 1978; Totterman et al., 1977). The ANAE negative blast cells regained ANAE activity on revision to lymphocytes (Totterman et al., 1977).

Grossi et al. (1979) had shown that T-cell subpopulation bearing receptor for Fc portion of IgM only presented ANAE activity of T pattern while cells bearing receptors for Fc portion IgG were devoid of this property.

Giorno and Beverly (1981) developed a rapid method for demonstration of ANAE activity in lymphocytes, which required incubation of lymphocytes in the reaction mixture for one hour as against overnight incubation required with the procedure described by Knowles et al. (1979). They have also observed that smears fixed in acetone-sodium citrate fixative could be dried and stored for relatively longer time before staining for ANAE activity.

Reddy et al. (1980) showed that ANAE activity was a useful marker to distinguish T-lymphocytes from other blood cells when bovine blood smears were directly stained. Rajan et al. (1982)

and Visalakshan et al.(1984) had used ANAE activity as a marker to enumerate T-cells of pigs. Valsala et al. (1981) had demonstrated ANAE activity in the peripheral blood lymphocytes of ducks and the percentage of positive lymphocytes ranged from 12 to 17 with an average of 15.5.

Erythrocyte emboconter complement (EAC) rosette

Bianco et al.(1970) were the first to demonstrate a rosette system employing erythrocyte sensitised with complement fixing antibody to detect complement receptors on lymphocytes. The complement components for which receptors demonstrated on lymphocytes included several degradation products in complement activation process but the receptors for C_3 was the one most commonly detected (McConnell and Lachmann, 1977). Sidman and Unanue (1975) reported the appearance of complement receptors at a fairly late stage of postnatal development while Hammerling et al. (1976) observed that their appearance on B-cells followed the appearance of surface membrane immunoglobulins and Ia antigen during ontogeny.

Higgins and Stack (1978) described EAC rosette formation employing mouse serum as the source of complement and used this property as a marker for B-cells enumeration from peripheral blood of cattle. Grewal et al. (1978) characterised surface receptors on bovine leukocytes employing five surface markers viz., surface immunoglobulin, sheep erythrocyte receptor, complement receptor and Fc receptors to both IgG and IgM. To further ascertain the value of surface property

as a cell type marker, they performed double marker techniques. The results obtained by them indicated that cells do bear more than one marker. They demonstrated that though Fc receptors are commonly shared by T as well as B-cell subpopulations, occurrence of combination of more than one marker distinct to each subpopulation had been observed as exemplified by surface immunoglobulin and complement receptors by B-cells and absence of E-receptor characteristic for T-cells.

EAC rosette formation was commonly applied for quantitation of B-cells from several animal species (Shimazu et al., 1976; Wilkie et al., 1979; Kaura et al., 1979; Outteridge et al., 1981; Outteridge and Dufty, 1981; Sulochana et al., 1982). Though sheep erythrocytes sensitised with haemolysin and complement are regularly used for EAC rosette formation, the successful use of bovine erythrocytes sensitised with haemolysin and mouse complement (Outteridge and Dufty, 1981; Outteridge et al., 1981) or chicken red cells coated with naturally occurring porcine antibody against chicken red cells and mouse complement (Shimazu et al., 1976) was also reported. Stites (1980) suggested that though sheep red cells could be used for EAC rosette assay, ox red cells which do not spontaneously bind to lymphocytes would be preferred to avoid confusion with T-cell rosettes.

Lymphocyte subpopulations in goats

On perusal of the available literature it could be seen that reports on characterisation and enumeration of subpopulations of lymphocytes in goats are only few.

Yang and Shain (1980) employing E-rosette, EAC rosette and surface immunoglobulin fluorescent tests identified T and B-cells and determined their relative and absolute values in peripheral blood of normal Taiwan goats. When erythrocytes from eleven heterologous animal species were tried, fowl erythrocytes were found most suitable for E-rosette. The relative values of each type of cells obtained were E-rosette positive cells (T-cells) 9.59 ± 2.06% (range 6.19-13.5%), EAC positive cells (B-cells) 35.12 ± 7.02% (range 23.84-47.70%) and surface immunoglobulin positive cells (B-cells) 86.22 ± 0.90% (range 85.0-87.25%). The mean and range absolute values were found to be respectively 674.9 ± 185.6 (486.1-1094.4) for T-cells, $2.49 \times 10^3 \pm 0.74 \times 10^3$ (1.42×10^3-4.34×10^3) for B-cells by EAC method and $7.10^3 \pm 2 \times 10^3$ (4.97×10^3-9.59×10^3) for B-cells bearing surface immunoglobulin per microlitre of blood.

Banks and Greenlee (1982) have studied the lymphocyte subpopulations in peripheral blood, thymus, spleen and lymph nodes of goats. Density gradient centrifugation using Ficoll-Hypaque was employed for separation of lymphocytes from peripheral blood and the separated lymphocytes were further fractionated and concentrated to T-cells by nylon wool seiving and to B-cells by complement binding method. Unfractionated lymphocytes, and enriched T and B-cells were further characterised for nonspecific esterase activity, presence of surface immunoglobulins, receptors for complement, and immunoglobulin and for

peanut agglutinin binding. Majority of the thymocytes and nylon wool purified blood T-cells (76 ± 13% and 91 ± 4% respectively) bound peanut agglutinin. More than 85% of B-cells concentrated by complement binding showed surface immunoglobulin and complement receptors while a smaller portion (14±6%) still bound to peanut agglutinin. In peripheral blood lymphocytes the mean percentage of cells positive for non-specific esterase, surface immunoglobulin, complement receptor, Ig receptor and peanut agglutinin binding cells were 4 ± 4, 19 ± 9, 17 ± 7, 14 ± 12 and 69 ± 11 respectively. The thymocytes lacked surface immunoglobulin, receptor for complement and immunoglobulin. A 40 to 50% of lymphocytes from spleen and lymphnode failed to reveal surface markers distinct for either T or B cells.

The usefulness of E-rosette and EAC rosette techniques for enumeration of T and B lymphocytes has been shown by Shein and Wang (1982) in an experiment wherein immunosuppressive effect of Trypanosoma evansi infection in goat was studied.

Identification and enumeration of T and B-cells in peripheral blood of normal, Corynebacterium pseudotuberculosis infected and ethmoid tumour bearing goats, employing E-rosette, EAC rosette and ANAE activity were reported by Sulochana et al. (1982). When erythrocytes from sheep, cattle and chicken were tried, only sheep erythrocytes formed rosette with goat lymphocytes. In normal goats the percentage of E-rosette ranged from 15 to 41.7 (mean 26.57 ± 2.05) and EAC rosette from 14.29 to

39.87 (mean 22.2 ± 0.83). Goats with C. pseudotuberculosis infection had only 4.4 to 14.5 (mean 10.29 ± 0.67) percentage of E-rosette positive T cells. A mean percentage of 24.35 ± 0.43, 19.19 ± 1.37 and 36.46 ± 1.75 cells were positive for E-rosette, EAC rosette and null cells respectively in goats with malignancy. The mean percentage of ANAE positive cells were 26.84 ± 3.56, 11.58 ± 1.36 and 22.91 ± 1.63 in normal, infected with C. pseudotuberculosis and tumour bearing goats, respectively. It was also shown that E-rosette formation was inhibited by antigoat thymus serum without complement but not the EAC rosette formation.

DeMartini et al. (1983) studied the abnormalities in number and function of peripheral blood lymphocytes of goats suffering from chronic caprine arthritic encephalitis (CAE). Characterisation and quantitation of lymphocyte subpopulations were done based on presence of complement receptor, Ig receptor, surface immunoglobulin, PHA binding and mitogen induced blastogenesis. In normal goats they recorded an average of 17 ± 2, 57.4 ± 4, 10 ± 3 and 16 ± 4 percentages of B-cells, T-cells, monocyte and null cells respectively. Out of the nine infected goats, seven had the same proportion of lymphocyte values as did controls. It was also shown that concanavalin-A induced more division in T-lymphocytes of infected goats than the lymphocytes of non-infected goats, while reaction to PHA, PWM and bacterial lipopolysaccharide were not different.

Haematology of goats with caseous lymphadenitis

Nadim et al. (1966) observed significant reduction in erythrocyte count, haemoglobin content and marked leukocytosis with noticeable monocytosis in sheep suffering from CLA but the lymphocyte-monocyte ratio was found to be relatively constant. Gameel and Tartour (1974) reported varying degrees of haemolytic anaemia characterised by reduction in RBC count, haemoglobin content and packed cell volume, when sheep were infected with large dose of C. pseudotuberculosis. Though leukocytosis with rise in neutrophils with corresponding decrease in lymphocyte count was noted, monocytosis was not a unique feature of CLA.

Total protein and albumin increased in animals which suffered septicaemia and death while a substantial decrease was noted in animals with subacute to chronic form of infection. Sheep suffering from CLA had a reduction in total serum protein, albumin and calcium as reported by El Abdin et al. (1977). A substantive increase in gammaglobulin content with associated decrease in beta globulin was reported by Mottelib et al. (1979) in sheep with chronic form of CLA. Similarly Desiderio et al. (1979) observed a significant increase in gamma globulin content associated with a compensatory decrease in alpha-2 and beta globulins in serum of goats suffering CLA. Significant differences were also noted with alpha-1, alpha-2, gamma globulin and total serum protein content between young and adult healthy goats.

Brown et al. (1985) did not observe any difference in packed cell volume and plasma protein values between goats infected with C. pseudotuberculosis and controls. All infected animals displayed slight persistent leukocytosis with an increase in fibrinogen content of about 2 to 5 times than that of controls.

Serum proteins - goat

The total protein content and protein electrophoretic pattern of serum of goats have been documented only in few reports. Lewis (1976) estimated total serum protein, albumin, alphaglobulin, alpha-2 globulin, beta globulin and gammaglobulin by electrophoresis and the values obtained were 8.25, 4.02, 0.19, 0.69, 0.56 and 2.04 grams per 100 ml of serum, respectively. The micro kjeldahl method for estimation of total serum protein and paper electrophoresis (Whatman 3 mm filter paper, barbital buffer pH 8.6 with ionic strength 0.075) for fractionation and quantitation of normal serum protein of goats were employed by Castro et al. (1977). The values obtained were total serum protein 7.3 ± 0.7, albumin 3.2 ± 0.8, alpha-1 globulin 0.6 ± 0.1, alpha-2 globulin 1.0 ± 0.3, beta globulin 0.5 ± 0.1, globulin 4.5 ± 0.6 and albumin globulin ratio 0.8 ± 0.3 mg per dl. They also observed that albumin and albumin-globulin ratio showed significantly different values due to sex difference.

Desiderio et al. (1979) used cellulose acetate membrane electrophoresis to quantitate serum protein fractions of normal as well as sheep affected with CLA. Using pevicon block

electrophoresis Wang and Shein (1980) separated goat serum into five zone viz., gamma, beta, alpha-2, alpha-1 globulins and albumin. Further sub fractionation and purification of beta and gamma globulins were done by column chromatography and the purified subfractions, IgM(A) and IgG were utilised to raise specific antisera in rabbits.

Pathogenesis

C. pseudotuberculosis is a known facultative intracellular bacteria (Jolly, 1965a&b; Hard, 1969b; Campbell et al., 1982) producing a powerful exotoxin in vitro and in vivo (Smith, 1966; Zaki, 1968; Burrell, 1979). This bacteria produce chronic disease in animals especially in sheep and goats, characterised by suppurative lesions either in lymph node alone or both in lymph nodes and visceral organs. The pathogenesis is apparently related to the organism's ability to produce exotoxin (Lovell and Zaki, 1966a&b; Jolly, 1965c; Zaki, 1976; Burrell, 1978) pyogenic factors (Zaki, 1976) and the ability to cause cell aggregation imposing local physical hindrance to host's defence mechanism (Jubb and Kennedy, 1970; Burrell, 1978; Gillespie and Timoney, 1981). In vitro studies demonstrated that the tough outer lipid coat of the organism resisted digestion by phagocytes and remained as a facultative intracellular parasite (Hard, 1972; Tashjian and Campbell, 1983) and ultimately caused destruction of the host cell (Hard, 1972).

Jolly (1965c) demonstrated that the exotoxin facilitated the spread of bacteria to regional lymph nodes by increasing

vascular permeability. The potent exotoxin which was lytic for endothelial cells due to the phagolipase enzyme activity, promoted local spreading of bacteria (Soucek et al., 1971; Carne and Onon, 1978). Sheep experimentally exposed to high doses of exotoxin either by way of infection or by injection of toxin developed icterus, haemoglobinuria and death (Cameron et al., 1972; Cameron and Fuls, 1973; Gamael and Tartour, 1974; Garg and Chandiramani, 1984). The leukocidin which was shown responsible for the progressive necrosis that caused persistent lesion in sheep (Marsh, 1938) was later identified as nothing but exotoxin (Jubb and Kennedy, 1970; Jensen, 1974).

The presence of antitoxin in the serum of naturally infected sheep indicating exotoxin production in vivo has been demonstrated by several workers (Zaki, 1968; Zaki and Abdul Hamid, 1971; Zaki and Abdul Hamid, 1974; Barrell, 1981). Though acute intoxication and death were not a feature of natural disease in domestic animals, the exotoxin was shown to be produced and liberated in the body in small amounts not sufficient to kill the animal but sufficient to elicit the formation of antitoxin (Zaki, 1976) but Cameron and Minnar (1969) and Cameron and Smit (1970) were of the opinion that the exotoxin was of little importance in the pathogenesis of chronic lesions of CLA in natural infection.

Caseous lymphadenitis is characterised by abscessation of peripheral lymph nodes especially prescapular and prefemoral in sheep (Marsh, 1965; Jubb and Kennedy, 1970; Jensen, 1974)

submandibular and parotid in goats (Ayers, 1977; Ashfaq and Campbell, 1980; Burrell, 1981; Campbell et al., 1982) with thick and dry greenish white purulent exudate. Occasionally the disease become generalised and abscess develop in many abdominal and thoracic viscera, including internal lymph nodes, skin and subcutaneous tissue, brain and spinal cord (Marsh, 1965; Jubb and Kennedy, 1970; Jensen, 1974). Progressive emaciation and occasional death in sheep and goats have been reported to occur due to the internal abscesses developed (Dhanda and Singh, 1955; Marsh, 1965; Guss,1977). When massive dose of virulent C. *ovis* cells were inoculated to the popliteal lymph node, sheep exhibited haemoglobinuria and icterus, two days post-infection until death (Burrell, 1981).

On intralymphatic inoculation of viable C. *ovis* cells, Husband and Watson (1977) noted development of patent abscesses with greyish yellow pus in hypertrophied, congested and inflammed popliteal lymph node of sheep within three days post inoculation. Oedema and slight degree of haemorrhage were the macroscopic lesions in the lymph node at early stage (Burrell, 1978). The chronic abscess of lymph node was covered with a thick fibrous capsule and was often adherent to adjacent muscle fascia (Burrell, 1978; Campbell et al., 1982). Distinctive laminated appearance was observed in lesions of advanced cases of CLA in sheep due to the enlargement of lesions through progressive necrosis and reformation of the capsule (Jones and Hunt, 1983).

In goats, severely involved lymph nodes had no recognisable parenchyma remaining by the time they ruptured spontaneously (Ayers, 1977). The natural abscess was found to contain thick pasty purulent material within a thick fibrous capsule (Ashfaq and Campbell, 1980). The reason for this contrast was suggested to be due to the difference in the nature of enzymes in the phagocyte cells of goats from that of sheep.

Sarkar and Bhattacharya (1975) reported acute bronchopneumonia due to C. *ovis* infection in goats which was fatal in majority of cases. Gross multiple abscesses of varying sizes in lungs with adhesion of pleura and thickened interlobular septa were noted in badly affected goats. Infected lungs of sheep and goats showed discrete and extensive area of consolidation with slightly raised subpleural or deep seated round circumscribed greenish yellow caseo purulent/caseocalcified encapsulated nodules embedded mostly in the anterior lobe and in few cases in the diaphragmatic lobes (Sharma and Dwivedi, 1977). Pleural adhesion was also noted in some cases.

Histopathology

Congestion, oedema, hyperplasia, cellular infiltration, necrosis and calcification were reported to be the sequential events taking place in the lymph node affected with C. *pseudotuberculosis* (Zaki, 1976; Gameel and Tartour, 1974; Sharma and Dwivedi, 1977; Husband and Watson, 1977; Burrell, 1978; Ashfaq and Campbell, 1980; Stoops *et al*., 1984;

Brown et al., 1985). Caseated necrotic mass surrounded with epithelioid cells admixed with lymphocytes peripherally reinforced with layers of fibrous connective tissue were described to be typical for lesions of CLA in lymph nodes. Calcification of the necrotic mass may occur in advanced lesions. Concentrically laminated appearance for the lesions may be produced by the successive necrosis of cells external to the fibrous connective tissue layers (Jones and Hunt, 1983).

Burrell (1978) demonstrated clumps of bacteria surrounded by nuclear debris from cells which had undergone lysis in the popliteal lymph node of sheep infected through intralymphatic inoculation of C. ovis cells. He had observed that lymphocytes were only randomly distributed inside the layer of fibrous tissue but numerous eosinophils and scanty neutrophils were noted at the border of the caseous necrotic material. Plasma cells at different stages of development and mononuclear macrophages were the predominant cells at the periphery of the lesions. The infiltrating cells were superimposed in the degenerating fibrous tissue and the lesions were walled off with a zone of intact fibrous tissue. Occasionally degenerating mast cells were also encountered.

Lymph node parenchyma which had not been obliterated by development of lesions had undergone hyperplasia. Such lymph nodes showed several active cortical follicles and inter follicular areas and the medullary cords were densely lined with plasma cells at various stages of development (Burrell, 1978).

Brown et al. (1985) observed that goats which were inoculated with C. ovis intradermally developed moderately severe multifocal acute suppurative lymphadenitis. Histologically the paranchyma of the affected lymph nodes was pushed aside by the expanding abscess which comprised amorphous central eosinophilic mass of dead macrophage surrounded by a rim of degenerating neutrophils and peripheral to this a band of giant cells and fibrous tissue. Bacterial mass were also seen dispersed through the central necrotic area.

Histopathological changes observed in lungs of sheep with abscess were similar to changes described in lymph nodes except for infiltration of polymorpho nuclear leukocytes (Gameel and Tartour, 1974). Giant cells were demonstrated in tissue sections of lung lesions of CLA along with macrophage and lymphocyte infiltrations (Sharma and Dwivedi, 1977). Stoops et al. (1984) observed prominent plasma cell infiltrations in the fibrous capsule of advanced lesions of CLA in sheep lungs. Firm red to grey zones varying from a few millimeters to several centimeters in thickness were noted in the lung parenchyma adjacent to abscesses. The grey zones revealed interstitial fibrosis and compression of lung parenchyma. Peribronchiolar lymphoid infiltration, bronchiectasis, bronchiolar epithelial cell hyperplasia and peribronchialar fibrosis were the changes associated with in the air ways.

Lovell and Zaki (1966a) observed that mice injected with lethal dose of C. ovis exotoxin produced symptoms of acute

intoxication and death. Histopathological changes observed in such animals were congestion in the stomach, and small intestine degenerative changes in the liver cells and the epithelial cells of the convoluted tubules of the kidney. In few animals lungs and adrenals were also congested but testes were found unaffected.

Zaki (1966) experimentally produced osteomyelitis and arthritis in mice by injecting C. ovis cells intravenously. The earliest detectable lesions noted were congestion and haemorrhage in the marrow cavity of long bone metaphyses and in the intramuscular and intraarticular spaces.

Gameel and Tartour (1974) observed cloudy swelling in the hepatic cells of sheep and the nuclei of hepatic cells were hyperchromic. The parenchyma was partially degenerated and infiltrated with round cells. Eosinophils were also observed in most of the liver sections. Catarrhal enteritis and increased numbers of goblet cells were noted in intestine. Reticular and lymphoid hyperplasia were observed in spleen and lymph nodes.

Abortion in ewes when experimentally infected with C. pseudotuberculosis was reported by Addo (1979). Gross lesions included reddish brown coloured placenta enlarged oedematous and autolytic cotyledons. Histopathological changes observed were hyperaemia of septal vessels, vasculitis cellular infiltration in the hilar central and basal zones and necrosis of the chorionic villi and maternal septa.

Toxic hepatitis and nephrosis were observed to occur along with abscessation of skin and lymph nodes of buffalo calves and cattle experimentally infected intradermally with C. ovis (Khater et al., 1983a&b).

Diagnosis

Animal affected with CLA due to C. ovis infection exhibits no specific clinical signs other than the occasional presence of detectable superficial abscesses. Although there are no pathognomonic symptoms indicative of CLA in animals suffering from the visceral form of the disease, lesions in lungs may produce signs of respiratory tract infection (Marsh, 1965). For diagnosis of CLA in animals, palpation of lymph nodes is unreliable since the detection of early as well as deep seated abscesses is not possible. The lack of distinct clinical features warrants the need for specific and sensitive immunological methods for diagnosis (Ayers, 1977; Shigidi, 1979). Several laboratory tests were reported for the diagnosis of this infection but none was found to be completely satisfactory in terms of simplicity, accuracy, sensitivity and economy (Shigidi, 1979; Shen et al., 1982).

Agglutination test

Awad (1960) used agglutination test for the first time to detect serum antibody against C. ovis in sheep. Barring autoagglutination exhibited by several isolates of C. ovis used as antigen in agglutination test, this test was reported

to be convenient and sensitive for diagnosis of CLA in sheep. Cameron et al. (1972) used agglutination test to monitor antibody response to various inactivated C. ovis vaccines in sheep. Stable agglutinating antigen from C. ovis culture was prepared by shaking in ethanol or by subjecting to frequent freezing and thawing (Shigidi, 1974). He also reported instances where in known infected animals and animals without detectable infection revealed low titres when their sera were subjected to agglutination test.

Keskintepe (1976) reported that spontaneous agglutination of antigen prepared from C. pseudotuberculosis could be prevented by suspending them in saline containing magnesium chloride supplemented with 1% tween-80. Non-immunoglobulin mediated agglutination reaction was observed by Burrell (1978) when C. ovis cells were incubated with precolostral and young lamb sera. He suggested that agglutination test cannot be reliably used as a diagnostic test for CLA in young sheep. Lund et al. (1982a) used both agglutination and haemolysis inhibition test for detection of antibodies to C. pseudotuberculosis in naturally infected adult goats.

Shigidi (1979) compared the efficacy of five serological tests viz., tube agglutination, complement fixation, gel diffusion, antihaemolysin inhibition and indirect haemagglutination tests for diagnosis of experimental C. ovis infection in sheep. He observed that tube agglutination test was of value for diagnosis of CLA between three and eighteen weeks

of infection. After 18 weeks of infection all the infected animal remained seronegative with tube agglutination test while they were positive with other tests.

In India, Chandiramani and Garg (1984) employed agglutination test for field survey of CLA in sheep and goats. Out of 1302 sheep sera, 61 (4.6%) samples and 48 (10.2%) samples out of 467 goat sera were positive by the test.

Anti-haemolysin inhibition test

Hartwigk (1963a) demonstrated that C. ovis produced filterable anti-haemolysin against beta haemolytic Staphylococcus by modified CAMP technique. The neutralisation of the haemolytic effect of Staphylococcus on sheep red cells by the antihaemolytic factor differentiated C. ovis from C. pyogenes, C. renale, Listeria monocytogenes and Erysipelothrix insidiosa (Hartwigk, 1963b). Fraser (1964) and Zaki (1965) showed that a filterable product from C. ovis inhibited the haemolytic effect of alpha and beta toxins of Staphylococcus on sheep or goat red cells while delta lysin remained unaffected and the action of epsilon toxin was enhanced. The diffusible nature of the antihaemolysin was confirmed by Lovell and Zaki (1966b) and a quantitative tube test was recommended for the assay. They further showed that the antihaemolysin property of the toxin was inhibited by antiserum.

The inhibition of the antihaemolytic property of the C. ovis toxin by its specific antiserum was successfully

utilised for the development of a serological test viz., antihaemolysin inhibition test (AHIT) for the diagnosis of CLA in animals by Zaki (1968). Serum antibody response in goats after experimental infection with C. pseudotuberculosis was detected, commencing 4 to 8 weeks of infection using AHIT (Hamid and Zaki, 1973). Zaki and Abdel-Hamid (1974) evaluated and compared AHIT with that of in vivo mouse protection test and observed that AHIT was easier, cheaper, rapid and less liable to experimental error than mouse protection test. Though comparatively high percentage of false negative results were recorded with AHIT, this test was recommended as a screening test for CLA in animals. The AHIT was reported to be inferior to indirect haemagglutination test for detection of serum antibody against C. ovis in sheep (Shigidi, 1978; Shigidi, 1979). The AHIT failed to detect antitoxin in a high proportion of infected sheep while no false positive results were recorded (Nairn and Robertson, 1974).

Indirect haemagglutination test

Indirect haemagglutination test (IHAT) using erythrocytes sensitised with exotoxin of C. ovis for the serodiagnosis of CLA in sheep was developed and described by Shigidi (1978). Purified C. ovis toxin was used to sensitise formalinised sheep erythrocytes treated with tris-diazobenzidine. IHAT was found to give a high percentage of false positive reaction when compared to AHIT. Shigidi (1979) evaluated and compared

serological tests for the detection of CLA in animals and reported that IHAT was reliable and could detect antitoxin for longer periods in the serum of infected animals.

Mouse protection test

In an experiment designed to detect the relationship of toxins of representative strains of C. ovis to that of C. diphtheriae employing mouse protection test (MPT), Lovell and Zaki (1966a) could not establish any relationship with diphtheria. They also observed that C. ovis antibacterial serum protected mice against the lethal toxin, provided the serum contained sufficient level of antibody to neutralise the toxin.

MPT was successfully used by Hamid and Zaki (1973) to detect serum antitoxin in experimentally infected goats. According to Zaki and Abdul Hamid (1974) MPT was more valuable than AHIT for detecting antibodies to C. ovis. Abdul Hamid (1975) reported a high sensitivity of the test when one millilitre of the serum was used to neutralise 2 MLD of C. ovis toxin.

Rabbit skin test

Doty et al. (1964) developed a diagnostic skin test in rabbits for CLA in animals. In the test, the ability of antitoxin to abrogate the dermal reaction produced by the toxin of C. ovis in rabbit skin was assessed. The rabbit skin test was also found suitable to study the antigenic relationship

between strains of C. ovis. Awad et al. (1979) reported that the test was useful in detecting CLA in animals though a few false positive reactions were noted.

Synergestic haemolysis inhibition test

Synergestic haemolysis inhibition test (SHIT) was a serological method developed by Knight (1978) for detection of C. pseudotuberculosis infection in horses. The test was based on the principle that antiserum to C. ovis inhibited the synergestic haemolytic effect of C. ovis toxin to the erythrocytes sensitised with sterile filtrate of C. equi culture.

Haemolysis inhibition test

Haemolysis inhibition test (HIT) described by Burrell (1980) was based on the principle that antiserum to C. ovis inhibited the haemolysis by exotoxin. He showed that the test was efficient in diagnosis of CLA in naturally infected animals, for detection of passively transferred maternal antibody in lamb serum and to monitor the antibody response in vaccinated animals. Burrell (1981) surveyed two dairy goat herds for C. ovis infection by clinical examination, cultural recovery, haemolytic inhibition and double immunodiffusion tests. He found double immunodiffusion as a better field diagnostic test. Lund et al. (1982a) recommended haemolysis inhibition and agglutination tests for seroepidemiological investigation of CLA in goats. Lund et al. (1982b) opined that the level of maternal antibody against C. ovis in kids could be demonstrated by HIT.

Immunodiffusion test

Specific precipitin formation in gels on reaction between purified fractions of C. *pyogenes* culture supernatant and antiserum was first noted by Cameron and Smit (1970). Later in 1974, Shigidi detected antibody to C. *pyogenes* in the serum of CLA affected sheep using immunodiffusion technique. The antigenic relationship between isolates of C. *pyogenes* was also conveniently evaluated by this test (Awad *et al.*, 1979). Shigidi (1979) reported that gel diffusion test gave a high percentage of false positive reaction when compared to other serological tests used for diagnosis of CLA in experimentally infected sheep.

Burrell (1980) modified the gel immunodiffusion test by employing concentrated supernatant of C. *pyogenes* culture as antigens and reducing the concentration of agar to 0.5% for gel preparation. He had observed that culture supernatant of C. *pyogenes* having haemolytic titre of 1 in 16,384 or more produced readily detectable precipitin lines with specific antitoxin in the range of concentrations commonly found in sera of naturally infected animals. The test provided an extremely economical and convenient method for screening large number of animals with CLA within 24 hours of setting the test.

Field survey for C. *pyogenes* infection in animals was undertaken using this test (Burrell, 1981; Nairn *et al.*, 1984) and the test was found reliable since there was no false positive reaction.

Immunofluorescence test

Addo (1978) used fluorescent antibody technique (FAT) to identify C. ovis in pus smear prepared from cases of CLA. Hyperimmune serum prepared in rabbits labelled with fluorescein isothiocyanate was used for the purpose and the method allowed rapid identification of organism in cases of CLA of horses. Direct and indirect methods of FAT were used to identify C. ovis in the semen from cases of ovine epididymitis (Ajai et al., 1980).

Enzyme linked Immunosorbent Assay (ELISA)

The application of ELISA for the detection of antibody against C. pseudotuberculosis in goats was first reported by Shen et al. (1982). The test was performed with cell wall antigen to coat a solid phase and alkaline phosphatase to conjugate antigoat immunoglobulin. The cell wall antigen produced only specific reaction since it did not cross react with antibody to C. pyogenes. ELISA was considered to be specific, sensitive, simple to perform, readily reproducible and economical for epizootiological studies.

Garg and Chandiramani (1984) reported the use of soluble antigen prepared from sonicated C. ovis cells. The toxin and cell wall antigens were independently tried as antigen in ELISA to assess antibody level in sera of lambs experimentally infected with C. ovis by Maki et al., 1985. The results obtained by them indicated that toxin was better for the

purpose. Hsu (1984) reported that sonicated cells and cell wall antigens were unsuitable to be employed in ELISA for assessing antibody to C. *ovis* in goats.

ELISA was reported to be comparatively superior to synergestic haemolysis inhibition test (Shen *et al*., 1982) agglutination test (Gary and Chandiramani, 1984) and anti-haemolysin inhibition test (Maki *et al*., 1985) for the detection of serum antibody to C. *ovis*.

Hypersensitivity reaction

Carne (1932) was the first person to show that intradermal inoculation of allergenic agent prepared from C. *ovis* in a similar manner to tuberculin preparation, induced local delayed reaction in animals suffering from CLA. Cameron and McOmie (1940) described the preparation of antigen from the so called 'S' strain of C. *ovis*. Hypersensitive reaction produced in sheep and cattle on intradermal inoculation of this antigen indicated that the method was of value in detecting CLA in these animals. Farid and Mahmoud (1961) used filtrate of heat killed broth culture of C. *ovis* for intradermal injection to diagnose CLA in sheep. They observed that in known positive animals there was an increase in the thickness of the skin ranging from 1 to 8 mm after 48 hours of infection while in known negative animals there was no such increase in thickness. Renshaw *et al*. (1979) used a skin testing reagent prepared by sonicating C. *pseudotuberculosis* to detect internal abscess due

to C. pseudotuberculosis infection in sheep suffering from 'thin ewe syndrome'. The results indicated that the intradermal test was of limited value since the test produced several false positive and negative reactions.

Leukocyte migration inhibition test

Leukocyte migration inhibition test (LMIT) was used as an in vitro measure of cell-mediated immune response (Buening, 1972; Carson et al., 1977a&b; Myindo et al., 1980; Asadegan et al., 1981). Antigen dependent inhibition of leukocyte migration was suggested as an in vitro correlate of delayed type hypersensitivity (George and Vaughan, 1962; Rosenberg and David, 1970; Timms, 1979; Chambers and Kleaius, 1984). Peritoneal exudate cells and peripheral blood leukocytes were used in both capillary tube method (Buening, 1973; Woldehiwit and Scott, 1982) and agarose plate method (Carson et al., 1977a&b; Nagaraja et al., 1982; Bendixen, 1977; Asadegan et al., 1981) of LMIT. Direct migration inhibition assay under agarose was proved to be convenient, rapid and easily reproducible method for leukocyte migration inhibition assay (Clausen, 1971; Bendixen, 1977; Asadegan et al., 1981). LMIT was successfully employed for assessing CMI and for the diagnosis of many bacterial diseases like tuberculosis (Little and Naylor, 1977), Johne's disease (Bendixen, 1977; Timms, 1979) and Brucellosis (Asadegan et al., 1981).

According to the available literature, there was only a solitary reference about the use of LMIT to demonstrate CMI

in animals affected with CLA. Chandiramani and Garg (1982) performed capillary tube method of LMT using peripheral blood leukocytes of infected/immunised sheep and sonicated C. ovis cell extract as antigen. Results obtained by them indicated that there was gradual rise in the mean percentage of migration inhibition in infected/vaccinated sheep. Depending on the type, route, dose and periodicity of vaccine administered for immunization, the percentage of migration inhibition ranged from a maximum of 61.7 to a minimum of 11.5 over a period of 25 weeks.

Immunity

The exact mechanism conferring immunity to animals against C. pseudotuberculosis is not yet completely understood. Whether the immunity conferred by humoral mechanism or cell-mediated events or a coupled action of both is yet to be clarified (Ayers, 1977; Cameron, 1982; Cameron and Bester, 1984).

Studies on immunity to CLA were earlier carried out by several workers using different vaccines. Cabess and Alvares (1954) used an autogenous vaccine treated with penicillin to immunise horses. Preventive vaccination with formalinised culture was tried at the face of an outbreak of CLA in sheep (Van Dorssen, 1952). Quevedo et al. (1954) reported success in curtailing C. pseudotuberculosis infection in sheep with the use of aluminium hydroxide/potassium alum adsorbed vaccines. Antitoxin protected experimental animals against lethal doses of exotoxin

infection in animals. Jelly (1965c) postulated the functioning of cellular immunity to this infection in immunised mouse. As proof for this, he showed that elimination of infection and resolution of lesion in immunised mouse were associated with local development of mature and specialised macrophages with enhanced bactericidal activity. In vitro cultures of macrophages from C. pseudotuberculosis infected mouse showed strong bactericidal activity compared to that of control cells.

The morphological changes caused to mouse peritoneal macrophages following C. ovis infection were suggestive of strong cellular immune response against the bacteria (Hard, 1969a). Immunized mice with attenuated strains of C. ovis resisted challenge with virulent organism (Hard, 1969b). Morphological changes associated with increased bactericidal property of macrophages included, increased lysosomes, polyribosomes and hypertrophied golgi apparatus (Hard, 1969a&b). The immunity thus produced was of nonspecific in nature, since the immunised mice afforded protection against Listeria monocytogenes also. Hard (1972) demonstrated that increased lysosomes of activated macrophage broke down the lipid layer of the bacterium and lyse them.

Barakat (1979) reported low incidence of CLA in sheep which were inoculated with the non-specific antigen - Bacillus Calmette Guerin (BCG) within one month after birth.

Humoral immunity

Sheep which were repeatedly injected with toxoid and crude toxin (culture filtrate) of C. pseudotuberculosis found to develop acquired immunity mediated by antitoxin (Jolly, 1965a). He opined that antitoxin neutralised the vascular permeability increasing power of the toxin, thus reduced the spread of bacteria from local site in immunised animal on challenge infection. Cameron and Buchan (1966) demonstrated that the toxic factors and immunising antigens of C. pseudotuberculosis were distinct. Cameron and Minnar (1969) immunised mice with formalinised C. pseudotuberculosis cells to which various adjuvants were added. Immunity obtained even with washed whole cells alone, indicated that antitoxin played little role in conferring immunity to this infection.

Purified cell walls of C. pseudotuberculosis was successfully used to immunize mice by Cameron et al. (1969) and the immunogenicity of the cell wall was found unaffected by purification procedures. This observation suggested that the antigen which conferred immunity was an integral part of the cell wall itself. Cameron and Smit (1970) were of opinion that the exotoxin preparation obtained from culture filtrate ought to contain antigens derived from bacterial cells which would induce immunity in animals. These workers fractionated the protoplasm of C.ovis into two toxic fractions, A and B. Fraction B was shown to be identical to the exotoxin by neutralisation test.

The cell walls and protoplasm were shown to possess common antigens (Cameron and Purdon, 1971). They observed that no single cell wall or other antigen was solely responsible for inducing protective immunity but the intact complex configuration of the cell wall or a combination of more than one antigen was required to induce effective immunity. A substantive evidence for humoral immunity to C. pseudotuberculosis was reported by Cameron and Engelbrecht (1971). They could passively protect mice with hyperimmune rabbit serum prepared against killed bacterial cells. Attempts were also made to passively transfer immunity with transfer of peritoneal macrophages but without success. The protective effect of serum was not due to its antitoxic activity or its ability to promote phagocytosis but was due to the retarded secondary multiplication of bacteria.

Cameron et al. (1972) reported that in sheep, immunized against CLA with formalin inactivated, alum precipitated whole culture vaccine, the level of immunity produced was not absolute and incorporation of adjuvants or repeated dose of vaccine did not accelerate the level of immunity. Attempts were also made by Cameron and Fula (1973) to enhance the level of immune response in mice, guinea pig and sheep to C. ovis vaccines. In sheep, concentrated inactivated vaccine was found to accelerate antibody response and gave comparatively better protection, while use of live attenuated bacteria did not have any beneficial effect.

Husband and Watson (1977) studied the immunological events following injection of live/killed C. pseudotuberculosis into the afferent lymphatics of popliteal lymph node of sheep. A high lymphocyte output with increased blast cells and immunoglobulin bearing cells were noted in the efferent lymph. A moderate increase in the antibody titre was also observed in the lymph and serum.

Passive transfer of specific antibody to C. pseudotuberculosis from infected mother to kids through colostrum was recorded by Lund et al. (1982b). Andersson and Nairn (1984) observed that the passive immunity acquired by the kids through colostrum accounted for the increased resistance of the kids to CLA infection.

A combination of aluminium hydroxide and saponin as adjuvant in vaccine was found to produce fairly good immunity in sheep against CLA (Cameron and Baxter, 1984). Brogden et al. (1985) compared protection induced in lambs by C. ovis whole cell and cell wall vaccine and reported that none of these vaccines stimulated absolute immunity. Among the two vaccines the cell wall vaccine with adjuvant was found to evoke superior serological response in mouse.

Materials and Methods

MATERIALS AND METHODS

MATERIALS

Bacteria.

Corynebacterium pseudotuberculosis obtained from American Type Culture Collection, Maryland, USA (ATCC 19410) was used throughout the study.

Animals.

Twenty-four clinically healthy Malabari cross-bred goats (20 males and 4 females) of 8 to 12 months age, procured from household herds were used as experimental animals. Before procurement, these goats were screened for serum antitoxin of *C. pseudotuberculosis* and only those which were found negative were purchased. These goats were dewormed by giving 'Thiabendasole' and were observed for serological or clinical evidence of CLA for a period of one month before the start of the experiments. The goats were randomly grouped into three groups. Group I comprising eleven goats (8 males and 3 females), Group II six goats (all males) and Group III seven goats (six males and one female) and individual animals were identified assigning english alphabets (Table 1). All goats were put on same feeding schedule which included pelletted concentrate, leaves/grass and water.

Inoculum.

Homogenous single cell suspension of *C. pseudotuberculosis* was prepared following the method described by Jolly (1965).

Moderately heavy growth of bacteria on blood agar slope was washed with 15 ml of chilled bile salt solution containing 0.1% sodium chloride and 0.01% sodium deoxycholate, pH adjusted to 8.9 with 5% sodium carbonate. This bacterial suspension was shaken with sterile glass beads on a 'vortex cyclomixer' for 20 minutes and then centrifuged for 15 minutes at 1200 x g. The supernatant was discarded and the sediment was resuspended again in chilled bile salt solution, shaken for another 20 minutes, followed by centrifugation for five minutes at 1000 x g. After centrifugation, the cloudy supernatant was filtered through sterile Whatman No.1 filter paper. The bacterial count in the filtrate was taken by using Petroff-Hausen counting chamber under phase contrast microscope at 500 x magnification. The bacterial concentration in the filtrate was adjusted to 1 x 10^7 per ml using chilled bile salt solution as diluent and used as inoculum.

Exotoxin from C. Pseudotuberculosis.

Culture supernatant which had haemolytic property to sheep red cells and dermonecrotoxicity to rabbit skin was used as the source of exotoxin from C. pseudotuberculosis. The method followed by Burrell (1979) was adopted with slight modifications for the preparation of toxic culture supernatant.

C. pseudotuberculosis was grown in Lemeo protease broth containing 10% sheep serum, aerobically incubating at 37°C for 72 hours. The culture was then centrifuged under sterile conditions at 4°C for 45 minutes at 1500 x g. Decanted

supernatant was again centrifuged repeatedly under the above conditions and bacterial load in the supernatant was determined by culturing it on blood agar. The culture supernatant which was made free of bacteria was tested for its dermonecrotoxicity to rabbit skin and haemolytic effect to sheep red cells. The toxic culture supernatant distributed in small quantities was stored at -18°C until used and the haemolytic titre of the stored toxin was periodically checked.

Mononuclear cells from peripheral blood.

Mononuclear cells were separated from heparinised blood by density gradient centrifugation using Ficoll-paque (Pharmacia, Uppsala, Sweden) following the method reported by Sulochana et al. (1982) with certain modifications.

Three millilitre of whole blood was carefully layered over 1.5 ml of Ficoll-paque and centrifuged at 4°C for 45 minutes at 720 x g. The cell layer on top of the Ficoll-paque was collected together with little cloudy Ficoll-paque and washed in sterile phosphate buffered saline (pH 7.4) at 4°C for 15 minutes at 200 x g to avoid platelets in the cellular deposit. When there was contamination with red cells, the suspension of mononuclear cells in phosphate buffer saline was treated with 0.87% ammonium chloride for 1 to 2 minutes. The cells were then washed two times in tissue culture medium, RPMI-1640 (Hi-media, Bombay) and the cell pellet was finally resuspended with same medium containing heat inactivated calf

serum (20% final concentration), penicillin (200 iu per ml) and streptomycin (200 micrograms per ml). Cell counts in the suspension was made using Petroff Hausen counting chamber and concentration of cells in the medium was finally adjusted 5×10^6 cells per ml.

The viability of cells was assessed by trypan blue dye exclusion test by mixing one drop of cell suspension with one drop of trypan blue (0.05%) in Hank's Balanced Salt Solution (HBSS) and cell types were determined by Giemsa's staining method.

Sheep erythrocytes (SRBC).

The source of sheep erythrocytes was from one and the same animal throughout the experiment. Blood was collected in Alsever's solution, the proportion being equal and stored at 4°C until used.

SRBC for E-rosette

Sheep erythrocytes kept in Alsever's solution for about 10 days at 4°C was washed four times with sterile normal saline and the washed erythrocytes were made up as a 2% suspension in RPMI-1640 medium (Hi-Media, Bombay).

SRBC for Haemolysis Inhibition Test.

Sheep erythrocytes stored in Alsever's solution upto two weeks was washed three times with normal saline and resuspended in normal saline to make a 5% suspension.

Antiserum to bovine erythrocytes.

This was prepared in rabbit following the method described by Garvey et al. (1977).

Bovine erythrocytes (BRBC) collected in Alsever's solution were washed five times in normal saline and 10% red cell suspension was injected intravenously to healthy rabbit at a dose of one ml per kilogram body weight. The injections were repeated on alternate days for 12 days and a booster dose on the 20th day. Test bleeding of rabbit was done on the 7th day after the booster dose and agglutination titre in the serum was determined. When serum showed sufficient agglutinating antibody titre, animal was bled to death, serum separated and inactivated at 56°C for 30 minutes. The inactivated serum was distributed in small quantities and stored at -18°C until used.

Complement.

Fresh mouse serum was used as the source of complement.

Sensitised BRBC for complement rosette (EAC rosette).

The method described by Cutteridge and Dufty (1981) was followed for the preparation of sensitised BRBC.

Antiserum to BRBC was diluted to subagglutinating level using normal saline and then mixed with equal volume of packed BRBC for one hour at 37°C with occasional shaking. The BRBCs were then washed five times in sterile normal saline, resuspended in 1 in 20 dilution of fresh mouse serum as a source

of complement and incubated for 30 minutes at 37°C. After incubation the cells were washed three times with sterile normal saline and were resuspended in medium RPMI-1640 to give a 2% suspension.

Alphanaphthyl acetate esterase (ANAE) activity.

Fixative (Giorno and Beverly, 1981).

The fixative for ANAE activity was prepared by mixing acetone and sodium citrate (0.038 M) in the proportion 6:4 and the pH was adjusted to 5.4 with 0.01 N Hydrochloric acid.

Phosphate buffer (0.067 M, pH 5.2).

Solution A.

$Na_2HPO_4 2H_2O$ - 1.192 g

Distilled water - 100 ml

Solution B.

KH_2PO_4 - 0.911 g

Distilled water - 100 ml

1.25 ml of solution A was mixed with 48.75 ml of solution B and pH adjusted to 5.2 with solution B.

Hexazotised pararosaniline.

Solution A.

Freshly prepared 4% sodium nitrite solution in distilled water.

Solution B.

One gram pararosaniline was dissolved in 20 ml distilled water and to which 5 ml 12 N Hydrochloric acid was added.

Equal volumes of solution A and B was mixed and allowed to stand for 60 seconds.

Reaction mixture.

Reaction mixture was prepared only just before use. To 40 ml of phosphate buffer (0.067 M, pH 5.2) 2.4 ml hexazotised pararosaniline and 10 mg of alphanaphthyl acetate previously dissolved in 0.4 ml acetone were added. The pH of the reaction mixture was adjusted to 5.8 with 2 N sodium hydroxide and filtered before use.

Leukocyte Migration Inhibition Test (LMIT).

Leukocytes from peripheral blood.

Twenty-five millilitres of venous blood were collected into sterile bottle containing 50 mg of Ethylene diamine tetra acetic acid (disodium salt) (EDTA) as anticoagulant and mixed with 45 ml of sterile tripple glass distilled water for 20 seconds. Isotonicity of the solution was rapidly restored by the addition of 5 ml physiological saline 10 x. The leukocytes were sedimented from the haemolysed blood by centrifugation at 750 x g for 30 minutes at 4°C and the cells were further washed three times with sterile normal saline. The washed leukocytes were resuspended in normal saline to contain approximately 1.5×10^8 cells per ml.

Antigen for LMIT.

Toxin prepared as culture supernatant of C. pseudotuberculosis having haemolytic titre 1:16, pH adjusted to 7.2 with 0.1 N sodium hydroxide was used as antigen in LMIT.

Agarose medium.

The agarose medium was prepared fresh on the day of use. Two per cent agarose (Sisco, Bombay) in distilled water was boiled and cooled to 45°C to which equal quantity of double concentration Hank's balanced salt solution (HBSS 2 x), pH 7.2 containing 1% lactalbumin hydrolysate, 0.4% yeast extract, 20% calf serum, streptomycin (200 mcg per ml) and penicillin (200 iu per ml) was added. This agarose medium kept at 45°C was poured into even bottomed Petri-dishes to get 3 mm thickness when solidified. The plates were then incubated at 37°C for one hour prior to use.

Fixative.

The fixative was prepared by mixing 7 parts methanol, 1 part acetone and 2 parts distilled water.

Electrophoresis.

Tris-barbital buffer.

Barbitone sodium	- 9.9 g
Tris (hydroxy methyl) amino methane	- 17.7 g
Sodium azide	- 0.3 g
Distilled water	- 2000 ml

pH adjusted to 8.6 with 1 N Hydrochloric acid.

Agar coated slides.

Clean microscope slides (2.5 x 7.5 cm) were dipped in 0.5% melted agar in distilled water and dried in air by

keeping the slides horizontally over glass rods. Dried slides were stored at room temperature until used.

Buffered agar.

One gram Japanese agar was boiled in 50 ml distilled water until the agar dissolved completely and then mixed with equal quantity of hot tris-barbital buffer. This buffered agar was stored at 4°C until used.

Preparation of agar gel on slides.

Agar coated slides were placed on a perfectly horizontal surface and 3 ml of melted buffered agar was poured on each slide and allowed to form gel at room temperature.

Fixative.

The fixative was prepared by mixing acetic acid, methanol and distilled water in the proportion 1:7:2 respectively.

Stain for electrophoretogram.

Amido black 10 B	- 1 g
Sodium acetate-acetic acid buffer 0.2 M pH 3.6	- 1000 ml

Decolourising solutions.

Solution I:

Methyl alcohol	- 45 vol
Glacial acetic acid	- 10 vol
Distilled water	- 50 vol

Solution II:

Ethyl alcohol	- 40 vol
Glacial acetic acid	- 10 vol
Distilled water	- 50 vol

Lemco proteose broth with sheep serum (LPBS).

Composition.

Proteose peptone	- 10 g
Sodium chloride	- 5 g
Meat extract (Lab Lemco)	- 5 g
Yeast extract	- 1 g
Distilled water	- 1000 ml

The ingredients were dissolved in distilled water by boiling and pH adjusted to 7.5 with 1 N sodium hydroxide. The medium was sterilised at 121°C for 15 minutes and filtered. Then 1.5 g glucose was added to the medium and sterilised again at 110°C for 20 minutes. Sterile sheep serum was then added to the medium to a final concentration of 10% (v/v).

Phosphate buffered saline (PBS) (pH 7.2, 0.15 M).

Composition.

Sodium chloride	- 8.00 g
Potassium chloride	- 0.20 g
$Na_2H\ PO_4$	- 1.15 g
KH_2PO_4	- 0.20 g
Distilled water	- 1000 ml

Hank's balanced salt solution (2 x) (HBSS 2 x).

Solution A.

Calcium chloride	- 0.28 g
Distilled water	- 200 ml

The solution was sterilised at 110°C for 30 minutes and cooled to atmospheric temperature.

Solution B.

Glucose	- 2 g
NaCl	- 16 g
KCl	- 0.8 g
$MgSO_4$ 7 H_2O	- 0.4 g
KH_2PO_4	- 0.12 g
Na_2H PO_4 12 H_2O	- 0.24 g
Phenol red	- 0.04 g
Distilled water	- 800 ml

The ingredients were dissolved in distilled water by boiling and sterilised at 110°C for 30 minutes and cooled to atmospheric temperature. Then the solution A was slowly added to solution B with continuous mixing. The medium was stored at 4°C until used.

METHODS

Toxin from C. pseudotuberculosis

Titration of haemolysin.

Haemolytic titre of the toxin was determined following the procedure described by Burrell (1979).

A 0.5 ml quantity of double fold dilutions of culture supernatant having lytic property to red cells (haemolysin) was prepared in normal saline and to each of the dilutions 0.05 ml of sheep red cell suspension (5% SRBC) was added. After thorough mixing of the contents, the tubes were incubated for 18 hours at 37°C and examined for haemolysis.

The reciprocal of the highest dilution of toxin which showed complete haemolysis was taken as the haemolytic titre of the toxin.

Dermonecrotoxicity

Rabbits were employed for assessing dermonecrotic activity of the toxin. The flank region was chosen as the site of test which was shaved clean before injection. Intradermal injection of 0.1 ml of toxin was given at several sites and the dermonecrotic changes were observed after 48 hours based on the development of palpable dermal plaque with inflammed centre overlaid by a white pustular exudate.

Immune Response Studies

Experimental infection.

The animals were experimentally infected with C. pseudotuberculosis following the procedure described by Ashfaq and Campbell (1980).

Group I goats (A, B, D, F, N, H, Q, R, X, Y and Z) were inoculated at both sides of the body with 2×10^6 bacteria (0.2 ml of inoculum) per site of injection. The details of

inoculations were as follows: all the eleven goats were given submucosal injection at the ventral side of the lower jaw in the mouth cranial to the incisor teeth, six of them (A, D, N, R, X and Y) were given intradermal injection at the scapular region cranial to the superficial cervical lymph node and subcutaneous injection at flank region dorsal to the subiliac lymph node and the remaining five goats (B, F, H, Q and Z) were given intradermal injection at the flank region and subcutaneous injection at the scapular region (Table 2). Uninoculated group III goats (O, M, C, T, K, S and J) served as controls. Experimental and control group of goats were housed seperately in different sheds and precautions were taken to prevent direct or indirect contact between the groups.

Clinical observations.

Rectal temperature of all goats were recorded daily for a period of 7 days post-inoculation. The animals were observed for any clinical symptoms enlargement and abscess formation of palpable superficial lymph nodes suggestive of caseous lymphadenitis, for a period of three months.

Goats (Group I and III) were sacrificed at the end of the experiment. Detailed autopsy was conducted on the carcasses and gross abnormalities were recorded.

Sampling procedure.

Blood samples were collected from all 18 goats (Group I and III) prior to experimental infection to determine the base

data. After experimental infection blood was collected at weekly interval from all the goats (Group I and III) to determine total leukocyte count, differential leukocyte count, total serum protein, changes in the serum protein fractions, specific antibody activity in the serum and T and B-lymphocyte counts for over a period of 91 days. For leukocyte migration inhibition test, blood was collected first on the 15th day and subsequently four more collections were made at an interval of 15 days.

Aliquot volume of blood was collected by jugular vein puncture. One portion of blood was anticoagulated with heparin (10 iu per ml) for separation of mononuclear cells; another portion was mixed with EDTA (2 mg per ml) for total leukocyte count and/or leukocyte migration inhibition test and the remaining portion was used for separation of serum. Blood smears were directly prepared from the needle tip after the vein puncture for differential leukocyte count.

Estimation of total serum protein.

The total protein content in the blood serum was estimated by following the Biuret method described by Inchiosa (1964).

Serum Electrophoresis.

The proportions of serum protein fractions were studied employing agar gel electrophoresis procedure described by Hudson and Hay (1980).

Electrophoresis was carried out on 1% agar gel taken on glass slides using Tris barbital buffer having pH 8.6 with ionic strength of 0.075, in an electrophoresis chamber (Toshniwal). The strength of current was adjusted to 3 milliampere per slide and the running time was 140 minutes. For each sample, 2 µl of serum was used for charging the gel. After the run, the agar matrix was fixed in fixative (methanol-acetic acid-distilled water mixture) for 30 minutes. The fixed slides were then transferred to solution containing 90 ml acetone and 10 ml distilled water and kept for 4 h and later dried and stained by flooding with amidoblack 10 B for 45 minutes. The stained preparation were cleared by changing over to decolourising solution I and II and subjected to densitometer readings.

Haemolysis inhibition test.

The method described by Burrell (1980) was adopted for performing this test.

A 0.5 ml quantity of doubling dilutions of serum in normal saline were mixed with an equal quantity of haemolysin (culture supernatant) having a titre of 1 in 8 and kept for two hours at 37°C. Following incubation, 0.05 ml of 5% suspension of sheep red cells (SRBC 5%) was added to each tube and mixed well. This mixture was further incubated at 37°C for 18 hours and examined for inhibition of haemolysis.

Negative controls were set by adding 0.05 ml of 5% SRBC suspension to one ml of normal saline and positive controls by mixing 0.5 ml of haemolysin, 0.5 ml of normal saline and 0.05 ml of 5% SRBC suspension.

Haemolysis inhibition titre was recorded as the reciprocal of the highest dilution of serum showing specific inhibition of haemolysis.

Total and differential leukocyte count.

Enumeration of total and differential leukocyte counts of the peripheral blood was done as per the methods described by Schalm (1970).

Enumeration of B-lymphocytes.

B-lymphocytes were enumerated based on its erythrocyte-amboceptor-complement rosette (EAC rosette) forming property, following the method described by Cunteridge and Dufty (1981).

EAC Rosette technique.

A 0.2 ml of mononuclear cell suspension obtained from peripheral blood was mixed with 0.4 ml of sensitised bovine red cells (2% suspension of SRBC to give lymphocyte-red cell ratio approximately 1:50) and incubated at 37°C for 15 minutes. The cell mixture was then centrifuged at 200 x g for 5 minutes at 4°C and left at 4°C for overnight. The cell pellet was then gently resuspended by blowing air with a wide mouthed Pasteur pipette. A drop of this suspension was placed on a clean glass slide and covered with a cover slip. Rosettes were counted

in these slide preparation under the high power (400 X) of a light microscope. Each lymphocyte binding three or more red cells was counted as a rosette. A total of 200 lymphocytes were counted and the number of rosette forming cells were indicated as percentage of total cells counted.

Enumeration of T-lymphocytes.

For enumeration of T-lymphocytes, two markers viz., ANAE activity and E-rosette formation were employed.

Erythrocyte rosette (ER) technique.

Total E-rosette forming cells were determined employing the technique described by Jondal et al. (1973).

Of the mononuclear cell suspension prepared from peripheral blood, 0.2 ml was mixed with 0.4 ml of 2% SRBC suspension (lymphocyte-red cell ratio approximately 1:50) and incubated at 37°C for 15 minutes. Subsequent steps were carried out as were done for EAC rosette technique.

ANAE activity.

Smears prepared from mononuclear cells separated from peripheral blood were used to stain for ANAE activity of lymphocytes.

Smears were fixed following the method reported by Corino and Beverly (1981). Before the smears were dry, they were dipped in fixative (acetone-sodium citrate mixture) for 30 seconds and immediately rinsed in distilled water, dried and stored at room temperature until stained.

The fixed smears were stained following the method of Knowles et al. (1978). The fixed smears were incubated for 18 to 21 hours at room temperature in the reaction mixture containing phosphate buffer, hexazotised pararosaniline and alphanaphthyl acetate, pH adjusted to 5.8. Following incubation, the slides were washed with distilled water and counter stained with 1% aquous toludine blue for 60 minutes. The slides were then rinsed in distilled water, dehydrated in ascending concentrations of isopropyl alcohol, cleared in xylol and mounted with DPX.

The slide preparations were examined for ANAE activity in cells using 1000 x magnification. To work out the percentage of ANAE positive T-lymphocytes, a total of 200 cells per slide were examined and counts were made.

Leukocyte migration inhibition test (LMIT).

The test was carried out according to the method described by Bendixen (1977) with minor modifications.

The leukocyte suspension prepared from the peripheral blood (1.5×10^8 cells per ml) was divided into two equal parts and to one portion was added 0.1 ml of antigen and to the other portion 0.1 ml of sterile normal saline. Contents of each tube was thoroughly mixed and then incubated for one hour at 37°C with occasional shaking to avoid cell clumping. The contents of each tube was filled in six wells of 3 mm diameter cut 8 mm apart in the agarose gel. The charged plates were incubated at 37°C in a humid chamber for 20 hours. At the end of

incubation period, the cells were fixed to the glass surface by flooding the plates with methanol acetic acid fixative for 15 minutes. The agar gel in the plates was then partially dried to facilitate peeling off agar gel from the plates.

Migration area of leukocytes was measured by taking average diameter of the opaque zone around the wells. The migration index was calculated as the average area of migration of cells treated with antigen divided by the average area of migration of cells treated with normal saline. Leukocyte migration index less than 0.8 was considered as a positive reaction.

Skin hypersensitivity test.

C. pseudotuberculosis toxin prepared as culture supernatant having haemolytic titre 1 in 64 and mammalian tuberculin (IVRI, Izatnagar) were used as the test materials for the skin test. A dose of 0.2 ml of each of the antigens (test materials) was given intradermally using a tuberculin syringe and needle on the neck region (toxin at the left side and tuberculin at the right side) in experimental as well as control animals (Group I and III) after shaving and disinfecting the area. Measurements of skin thickness at the site of injections were made using Vernier callipers before injection and 24 h, 48 h and 72 h after injection. From some animals under each group, skin biopsy from the site of injection were collected at 48 h post-injection and preserved in 10% formol saline. Sections of these tissues were prepared by paraffin embedding and stained by hematoxylin and eosin to study the tissue reactions.

Pathogenesis studies

Experimental infection.

Group II goats (P, I, K, E, W and U) were inoculated as described for the experimental infection of goats for immune response study except that all animals were injected submucosally in the mouth, intradermally at the scapular region and subcutaneously at the flank region. Group II goats were also housed away from group III goats to prevent cross infection.

Necropsy of animal for pathological studies.

One animal each, from group II goats was sacrificed by exsanguination on every 15th day post-infection. Detailed autopsy was conducted on carcasses and gross abnormalities were recorded.

Immediately after the slaughter of goats from group II, lymphnodes (both peripheral and deep) were dissected out and examined for gross evidence of CLA lesions. Portions of lymphnodes and other tissues were collected in 10% formol-saline for histopathological examination. Tissues were processed by routine paraffin embedding technique. Paraffin sections cut at 5 to 6 microns thickness were stained routinely with Harris haematoxylin and eosin as described by Drury and Wallington (1967).

Analysis of data.

Statistical analysis of the data was done as per the methods of Snedecor and Cochran (1967).

Table 1. Details of animals used for experimental studies

Group No.	Number of goats	Age (in months)	Sex of the animals		Identity of goats
			Male	Female	
I	11	8-12	8	3	A, B, D, F, N*, H, Q, R, X*, Y, Z*
II	6	8-12	7	-	P, I, K, E, W, U
III	7	8-12	6	1	O, M, C, T, K, S, J*

* - Female goat

Table 2. Routes of inoculation adopted for experimental infection of Group I goats

Identity of goat	Routes of inoculations				
	Submucosal injection at buccal cavity	I/D at scapular region	s/c at flank region	I/D at flank region	s/c at scapular region
A	+	+	+		
D	+	+	+		
N	+	+	+		
R	+	+	+		
X	+	+	+		
Y	+	+	+		
B	+			+	+
F	+			+	+
H	+			+	+
O	+			+	+
Z	+			+	+

+ - indicate injection at the site

Results

RESULTS

Inoculum

Uniform suspension of evenly distributed individual bacterial cells was obtained by repeated shaking, centrifugation and resuspension of C. pseudotuberculosis in bile salt-sodium chloride solution. Very rarely small bacterial clumps were also observed but for the purpose of enumeration, they were considered as single cell units. C. pseudotuberculosis suspended in chilled bile salt-sodium chloride solution (4°C) retained viability for more than six hours as evidenced by the comparable growth obtained on blood agar, soon after the preparation of bacterial suspension and six hours later.

Exotoxin from C. pseudotuberculosis

Lemco protease broth containing 0.15% glucose and 10% sheep serum was found to support good growth of C. pseudotuberculosis under aerobic conditions at 37°C. The growth in the broth was characterised by granular deposits in the bottom with apparently clear fluid column on top. The granular deposit gradually increased and produced maximum deposit by the end of 72 h of incubation. Repeated centrifugation of the culture supernatant at this stage at 1500 x g for 45 minutes under sterile conditions made the supernatant free of bacteria as evidenced by the sterility tests done by seeding the culture supernatant on blood agar which failed to give any bacterial growth.

It was also observed that there was considerable reduction in the pH of the medium after 72 hours of incubation. The final pH of the toxic culture supernatant was found to be 5.00 ± 0.2 as against the initial pH of the medium, 7.5.

Titration of Haemolysin.

Undiluted culture supernatant of C. pseudotuberculosis grown in Lemco proteose broth was haemolytic to sheep red cells. The haemolysin titre of the culture supernatant was periodically tested and a maximum titre of 1:256 was obtained after 72 hours of incubation of culture. The potency of the toxin, when stored at -18°C was without any reduction for more than one year as proven in subsequent experiments in which the same toxin stored at -18°C was used.

Dermonecrotoxicity.

Intradermal inoculation of toxic culture supernatant of C. pseudotuberculosis which had 1:256 haemolysin titre produced characteristic dermonecrotic lesions in the rabbit skin. Hyperaemic skin over an area of three centimeter square with indications for abscess formation at the site of injection were the features at 24 h post-injection. By 48 h, the thickness of the skin increased, three fold and the hyperaemia diffused over a wider area of 4 to 5 cm square with the production of definite necrosis at the point of injection. A small area of the necrotic skin at the site of injection was seen to contain pus (Fig.1). The skin at the point of injection ruptured releasing the seropurulent fluid which accumulated underneath

by 72 h. The necrotoxic reaction at the site of injection lasted for about a week by which time the pustular area healed up with formation of scab.

Immune Response study

Clinical observations.

All goats in group I which were inoculated with single cell suspension of C. pseudotuberculosis at the rate of 2×10^6 bacteria per site of injection developed symptoms and lesions characteristic for caseous lymphadenitis in local and regional lymphnodes. All the goats in group III were normal and without any evidence for CLA throughout the period of study.

Within 24 hours all experimentally inoculated goats showed an increase in body temperature, which lasted for 72 to 96 h. During the first three days these animals exhibited general weakness, lethargy, rough coat, and impaired appetite. The sites of injections showed varying degree of inflammatory reactions. All goats had oedematous lower lips (Fig.2) consequent to the submucosal injections in the oral cavity and the area was found to be highly painful when touched.

The intradermal injection sites showed marked inflammatory reaction characterized by painful, hyperaemic skin which was thickened and having signs of necrosis and pustular lesions at the points of injection (Fig.3). The reaction subsided by 10-14 days post-inoculation.

The sites which received subcutaneous injections were

Fig.2. Goat: Oedema developed in lower lip after submucosal injection with C. pseudotuberculosis in the buccal cavity

Fig.3. Goat skin: Necrotic lesion at the site of intradermal injection of C. pseudotuberculosis

found to be highly painful and the skin over the area showed increased thickness and was hard to touch. Local subcutaneous abscesses developed in some goats (goat B and N) at the site, about 14 to 20 days following injection (Fig.4).

All experimentally inoculated goats developed enlargement of one or more regional/local lymph node (scapular, mandibular or femoral) within 21 days (Fig.5 and 6) irrespective of the routes of inoculation except the goat 'N' which showed involvement of lymphnodes only by 35 days. The enlarged lymphnodes later showed caseation, and abscessation. The abscessed lymphnodes opened spontaneously and discharged thick white creamy pus.

C. pseudotuberculosis was regularly recovered from the pus discharged from the abscessed lymphnode and also from the cutaneous abscesses developed at the site of inoculation.

Necropsy findings.

At the end of the 13th week post-infection, all goats of group I (except goat 'N' and 'Y') and III were necropsied and detailed post-mortem examination was conducted. All goats in group I had revealed lesions suggestive of caseous lymphadenitis in superficial local/regional lymphnodes(Table 3). In few goats (goats B, F and O) the afferent lymphatic channel at the dorsal side of the mandible was seen with nodules suggestive of lymphadenitis and few nodules were found even abscessed. The pus contained in the affected lymphnodes and nodules was culturally positive for C. pseudotuberculosis. No gross

Fig.4. Goat: Local abscess developed after subcutaneous injection of C. pseudotuberculosis

Fig.5. Goat: Lymphadenitis in the prefemoral lymph-node after experimental infection with C. pseudotuberculosis

Fig.6. Goat: Lymphadenitis in mandibular and scapular lymphnodes after experimental infection with C. [redacted]

Table 3. Distribution of lesion in the lymphnodes of experimentally infected and control goats

Animal's identity	Retropharyngeal		Parotid		Submaxillary		Prescapular		Prefemoral		Lymphatic channel at the dorsal region of mandible
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	
A	+	+	+	NL	+	+	+	+	+	+	NL
B	+	+	NL	NL	+	+	+	+	+	NL	+
D	NL	NL	+	NL	+	+	+	+	NL	+	NL
F	NL	+	NL	+	+	+	+	+	+	+	+
H	+	+	NL	NL	NL	+	+	+	+	+	NL
Q	NL	NL	NL	NL	+	+	+	NL	+	+	+
R	NL	NL	+	+	NL	+	NL	NL	+	+	NL
X	NL	NL	+	+	+	+	+	+	+	+	NL
Z	NL	NL	+	+	+	+	+	NL	+	+	NL

+ - Specific lesions for CLA

NL - No lesions detected
All control animals were negative for specific lesions of CLA

abnormality was noted in any of the visceral organs or tissues of goats which received experimental infection. Control goats (Group III) did not show any lesions on necropsy examination.

Total serum protein.

The range and mean values of protein concentration of experimentally infected and control goats and the t-values are given in table 4. The '0' week values represent the serum protein concentration before the commencement of the experiment and the rest of the values represent the concentrations at weekly intervals. The mean values are graphically presented in fig.7.

At '0' week the serum protein concentration ranged from 7.187 to 9.500 gram percentage in group I goats with a mean value of 8.579 $\pm$ 0.243 while it ranged 8.395 to 9.750 with a mean of 9.071 $\pm$ 0.205 in group III control goats and between the groups the serum concentration was not significantly different.

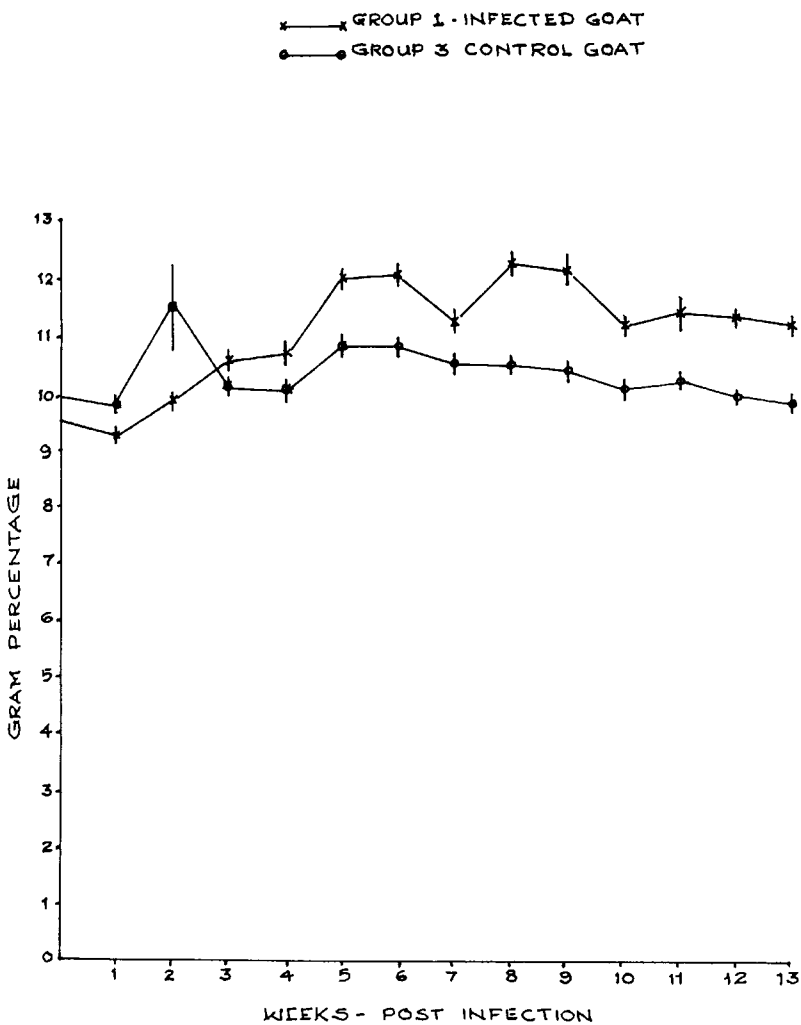
The concentration of protein in the serum of group I and III goats was not significantly different till 4th week post-infection. Significantly higher ($P < 0.05$) serum protein concentration in group I goats were recorded during 5th, 6th, 8th, 9th, 10th and 12th week post infection compared to uninfected controls (group III). The mean concentrations during the above periods were 11.113 $\pm$ 0.228, 11.102 $\pm$ 0.316, 11.346 $\pm$ 0.355, 11.289 $\pm$ 0.477, 10.386 $\pm$ 0.256 and 10.414 $\pm$ 0.305 g %

Table 4. Mean value of total serum protein (g %) of experimental and control animals at weekly intervals

Week		Range	Mean	SE	t-value	Significance
0	E	7.187-9.500	8.579	0.243	1.4154	NS
	C	8.375-9.750	9.071	0.203		
1	E	6.562-9.750	8.357	0.321	1.0754	NS
	C	7.937-9.937	8.874	0.324		
2	E	6.062-10.187	8.995	0.352	1.2346	NS
	C	8.312-10.875	10.615	1.576		
3	E	8.187-11.812	9.607	0.337	0.6670	NS
	C	8.625-10.000	9.294	0.245		
4	E	8.000-13.562	9.789	0.448	0.9695	NS
	C	8.187-10.437	9.169	0.372		
5	E	9.500-12.437	11.115	0.286	2.9063	*
	C	9.250-10.750	9.937	0.222		
6	E	9.500-12.437	11.102	0.316	2.6726	*
	C	9.250-10.625	9.964	0.181		
7	E	8.437-11.625	10.374	0.395	1.4369	NS
	C	8.437-10.312	9.629	0.246		
8	E	9.875-13.312	11.346	0.355	3.3398	**
	C	8.187-10.250	9.669	0.281		
9	E	9.062-14.375	11.289	0.477	2.7037	*
	C	8.625-10.625	9.580	0.232		
10	E	9.250-11.812	10.386	0.250	3.3630	**
	C	8.250-9.812	9.187	0.207		
11	E	8.250-13.000	10.518	0.506	1.8559	NS
	C	8.625-9.937	9.348	0.186		
12	E	8.500-12.187	10.414	0.305	3.4376	**
	C	8.750-9.625	9.044	0.117		
13	E	8.125-10.312	9.363	0.237	0.9171	NS
	C	8.187-10.562	8.999	0.329		

E - Experimental animals
C - Control animals
* Significant ($P < 0.05$)
** Significant ($P < 0.01$)
NS Not significant

FIG. 7 - MEAN VALUE OF TOTAL SERUM PROTEIN (g%)



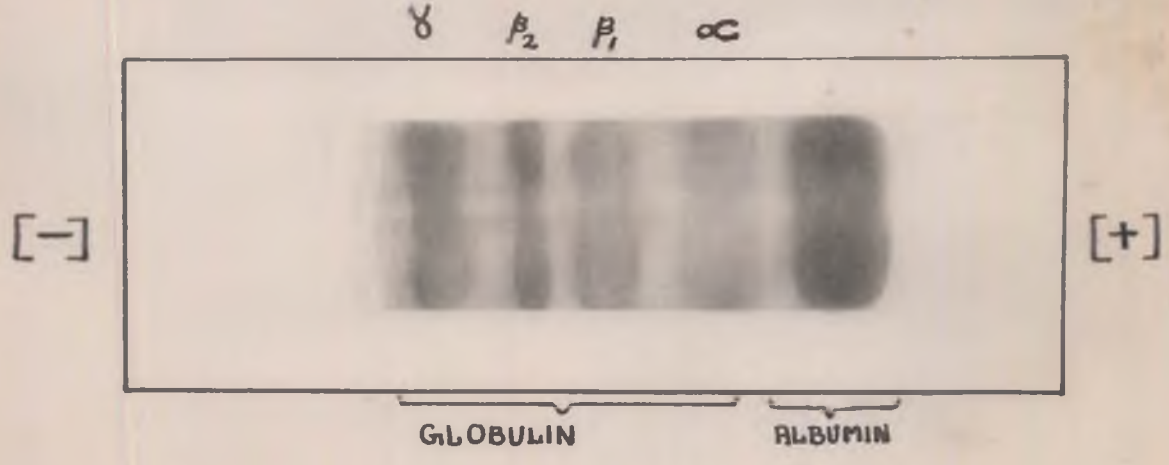
respectively for group I goats and 9.937 ± 0.222, 9.964 ± 0.181, 9.669 ± 0.281, 9.580 ± 0.232, 9.187 ± 0.207 and 9.044 ± 0.117 respectively for group III. The differences in serum protein concentrations between the two groups at 9th, 10th and 12th week post-infection were highly significant ($P < 0.01$).

Though higher serum protein concentrations were recorded in group I goats during 7th, 11th and 13th week post-infection (10.374 ± 0.395, 11.518 ± 0.506 and 9.363 ± 0.237 g % respectively) compared to group III goats (9.629 ± 0.246, 9.348 ± 0.186 and 8.999 ± 0.329 g % respectively), the differences between the groups were not statistically significant.

Serum electrophoresis.

Serum proteins were separated into several fractions of globulins and a single fraction of albumin by agar gel electrophoresis. The different fractions of globulins, which could be arbitrarily identified were alpha globulin, beta globulin and gamma globulin (Fig.8). In certain electrophoretograms, there was overlapping between the subfractions of globulins and hence quantification of all serum protein fractions could not be arrived at based on total protein concentration and proportion of different fractions. The protein fractions which were identified as globulin was compared to that of albumin to find out the quantitative shift in the proportion between albumin and globulin (albumin-globulin ratio). The mean albumin-globulin ratio (A/G ratio) estimated for

Fig.8. Electrophoretogram of goat serum showing different zones of protein fractions


γ
β2
β1
∝
[−]
[+]
GLOBULIN
ALBUMIN

group I and III goats are presented in table 5. The '0' week value represents the A/G ratio estimated for both groups before the start of the experiment and then at weekly intervals after experimental inoculation of group I goats with C. pseudotuberculosis. The mean A/G ratio of infected and control goats are graphically represented in fig. 9.

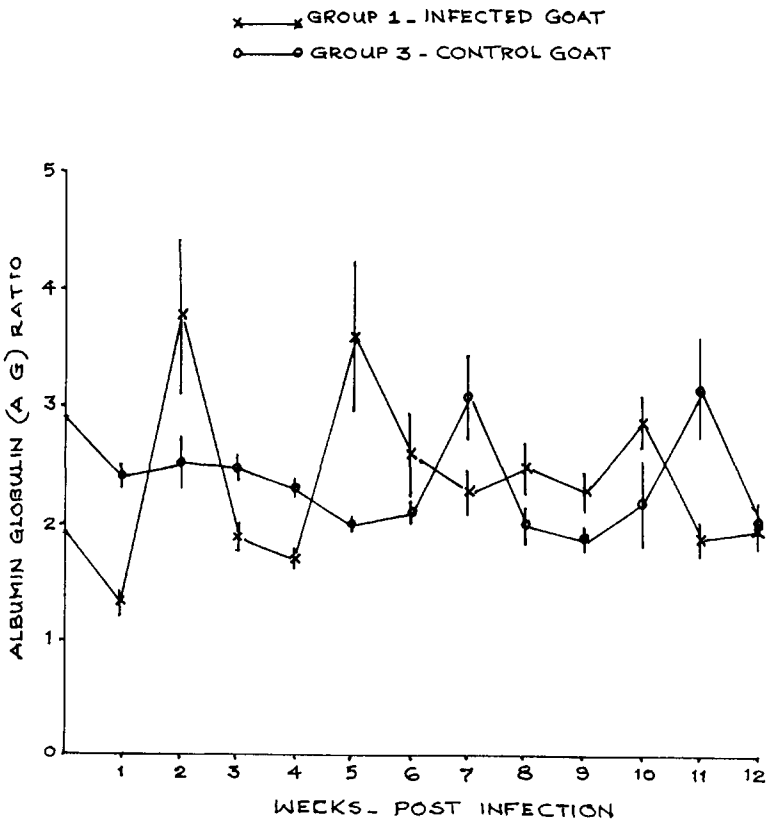
The mean A/G ratio estimated with group I and III goats before the start of the experiment were 1.94 ± 0.26 and 2.92 ± 0.25 respectively and the difference in values between the two groups was not significant. During the first week of inoculation the A/G ratio were 1.39 ± 0.13 for group I and 2.45 ± 0.18 for group III goats and the values were significantly different ($P < 0.05$). The decrease in A/G ratio in group I inoculated goats indicated an increase in the proportion of globulin in serum. During the post-infection period, from the 2nd week onwards the A/G ratio in group I goats, recorded high values till the end of the observation period except on the 4th week. The results thus indicated that during the post-inoculation periods, the proportion of globulin in the serum of infected animal was uniformly low. On the other hand A/G ratios estimated in control goats were uniformly low during the entire period of observation except on two samples at 7th and 11th week. The decrease in A/G ratio in turn indicated the proportionate rise in globulin fraction in serum. Though there were apparent difference in A/G ratio between group I and III goats, the difference was not significant from week 2 through week 12.

Table 5. Range and mean value of Albumin-globulin ratio of experimental and control animals

Week		Range	Mean	SE	t-value	Significance
0	E	1.17-2.84	1.94	0.26	2.361	NS
	C	2.44-3.29	2.92	0.25		
1	E	1.10-1.89	1.39	0.13	4.565	*
	C	3.09-2.70	2.45	0.18		
2	E	0.78-9.55	3.83	1.35	0.655	NS
	C	1.55-3.15	2.52	0.49		
3	E	1.26-2.67	1.97	0.28	1.279	NS
	C	2.10-2.80	2.49	0.20		
4	E	1.29-2.15	1.76	0.14	2.327	NS
	C	2.26-2.39	2.32	0.06		
5	E	1.34-8.62	3.69	1.28	0.975	NS
	C	1.98-2.05	2.01	0.02		
6	E	1.27-5.96	2.67	0.74	0.505	NS
	C	1.76-2.31	2.12	0.18		
7	E	1.41-3.63	2.37	0.36	1.234	NS
	C	2.00-4.68	3.28	0.77		
8	E	1.33-4.16	2.59	0.47	0.746	NS
	C	1.33-2.44	2.05	0.36		
9	E	1.63-3.13	2.35	0.28	0.965	NS
	C	1.41-2.36	1.91	0.27		
10	E	0.91-3.68	2.96	0.41	0.888	NS
	C	1.18-3.69	2.26	0.74		
11	E	0.84-2.52	1.92	0.24	2.008	NS
	C	2.18-4.93	3.24	0.85		
12	E	1.50-3.01	2.02	0.22	0.150	NS
	C	1.73-2.18	2.06	0.09		

E - Experimental animals
C - Control animals
* - Significant ($P < 0.05$)
NS - Not significant

FIG. 9 MEAN VALUE OF ALBUMIN GLOBULIN RATIO



Haemolysis inhibition titre in serum.

Haemolysis inhibition test was found to detect specifically the serum antibody produced against C. pseudotuberculosis in infected goats. All the control animals were uniformly negative for this antibody in their sera. The serum samples collected from group I goats after experimental infection had specific haemolysis inhibition titre which ranged from 1:8 to 1:256 during the 13 week observation period while sera from group III goats were negative as the titre was uniformly less than 1:4 except with two samples whose titre was 1:8.

The geometric mean of haemolysis inhibition titre recorded at weekly intervals in pre and post-infection serum samples from group I and III goats are presented in table 6. The mean titre of group I and III goats are also graphically presented in fig. 10.

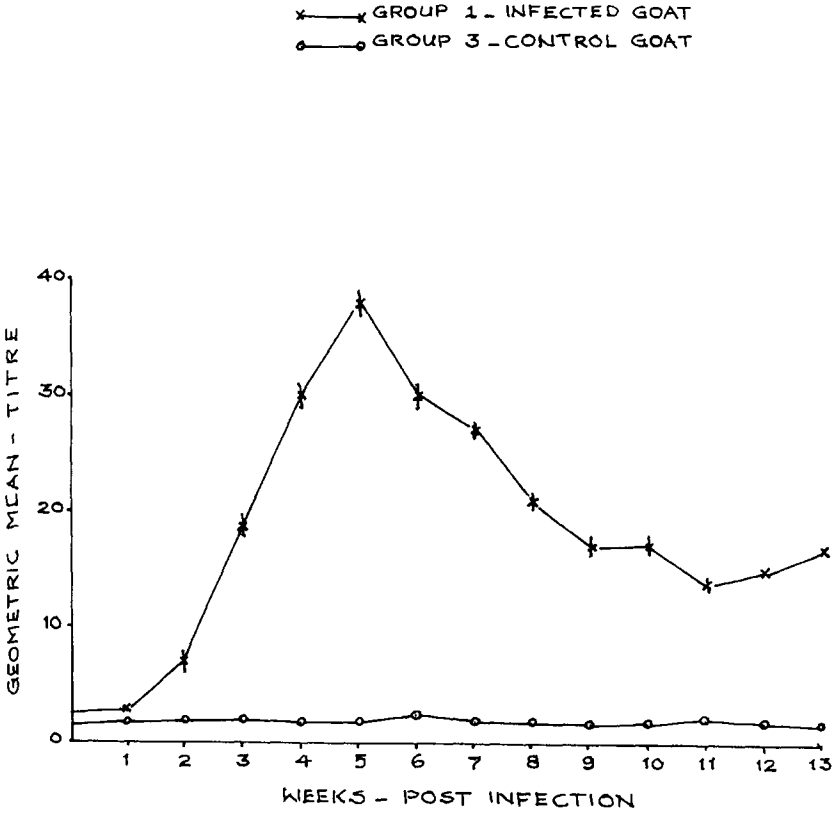
The pre-infection sera of group I goats had a mean titre of 2.42 while group III goats had 2.24 and thus all the animals used in these experiments were found negative for HIT. There was an increase in the mean titre (2.92) of serum from group I goats from the 2nd week of infection but the increase was not significant. From this week onwards sera from group I had shown marked increase in the titre and the maximum titre (38.60) was reached by the end of the 5th week. The serum titre recorded for group I goats collected from 2nd week to 13th week post infection were significantly higher ($P < 0.01$) from that of group III goats. Throughout the observation period serum

Table 6. Geometric mean value of serum antibody (Haemolysis inhibition) titre of experimental and control goats at weekly intervals

Week		Range	Geometric mean	SE	t-value	Significance
0	E	2.0-4.0	2.41	0.10	0.616	NS
	C	2.0-4.0	2.10	0.11		
1	E	2.0-4.0	2.91	0.11	1.759	NS
	C	2.0-4.0	2.10	0.11		
2	E	2.0-16.0	7.04	1.10	4.755	**
	C	2.0-4.0	2.10	0.11		
3	E	3.9-63.9	19.33	1.25	7.170	**
	C	2.0-4.0	2.10	0.11		
4	E	7.9-128	30.29	1.30	7.521	**
	C	2.0-2.0	2.00	0.00		
5	E	16.0-128	38.66	1.25	10.127	**
	C	2.0-2.0	2.00	0.00		
6	E	8.0-256	30.03	1.35	5.802	**
	C	2.0-8.0	2.69	0.12		
7	E	8.0-64	27.86	1.25	8.888	**
	C	2.0-4.0	2.20	0.11		
8	E	8.0-64	21.93	1.18	9.948	**
	C	2.0-4.0	2.20	0.11		
9	E	8.0-64	17.04	1.18	8.973	**
	C	2.0-2.0	2.00	0.00		
10	E	8.0-64	17.76	1.25	6.592	**
	C	2.0-4.0	2.20	0.11		
11	E	8.0-16.0	14.09	0.10	9.309	**
	C	2.0-8.0	2.43	0.12		
12	E	8.0-32.0	15.02	0.11	8.621	**
	C	2.0-4.0	2.43	0.11		
13	E	8.0-32.0	17.03	0.11	10.156	**
	C	2.0-4.0	2.20	0.11		

E - Experimental goats
C - Control goats
** - Significant ($P < 0.01$)
NS - Not significant

FIG-10 GEOMETRIC MEAN VALUE OF SERUM ANTIBODY (HAEMOLYSIS INHIBITION) TITRE



samples from the control goats were negative with HIT.

In group I goats Haemolysis inhibition titre showed a sharp increase during the 2nd, 3rd, 4th and 5th week post-infection and the mean titres were 7.06, 19.13, 30.29 and 38.66 respectively. From the 6th week onwards the titres gradually declined and the mean titres recorded were 30.03, 27.86, 21.93, 17.04, 17.76 and 14.09 with serum samples collected at 6th, 7th, 8th, 9th, 10th and 13th week respectively. A marginal increase in the titre was then noted during the 12th and 13th week of infection and the titres recorded were 15.02 and 17.03 respectively.

Total leukocyte count.

Total leukocyte count of each goat from group I and III was made before the experimental infection and then at weekly intervals upto 13 weeks post-infection. The range, mean values and the results of t-test are presented in table 7. The mean values are graphically represented in fig. 11.

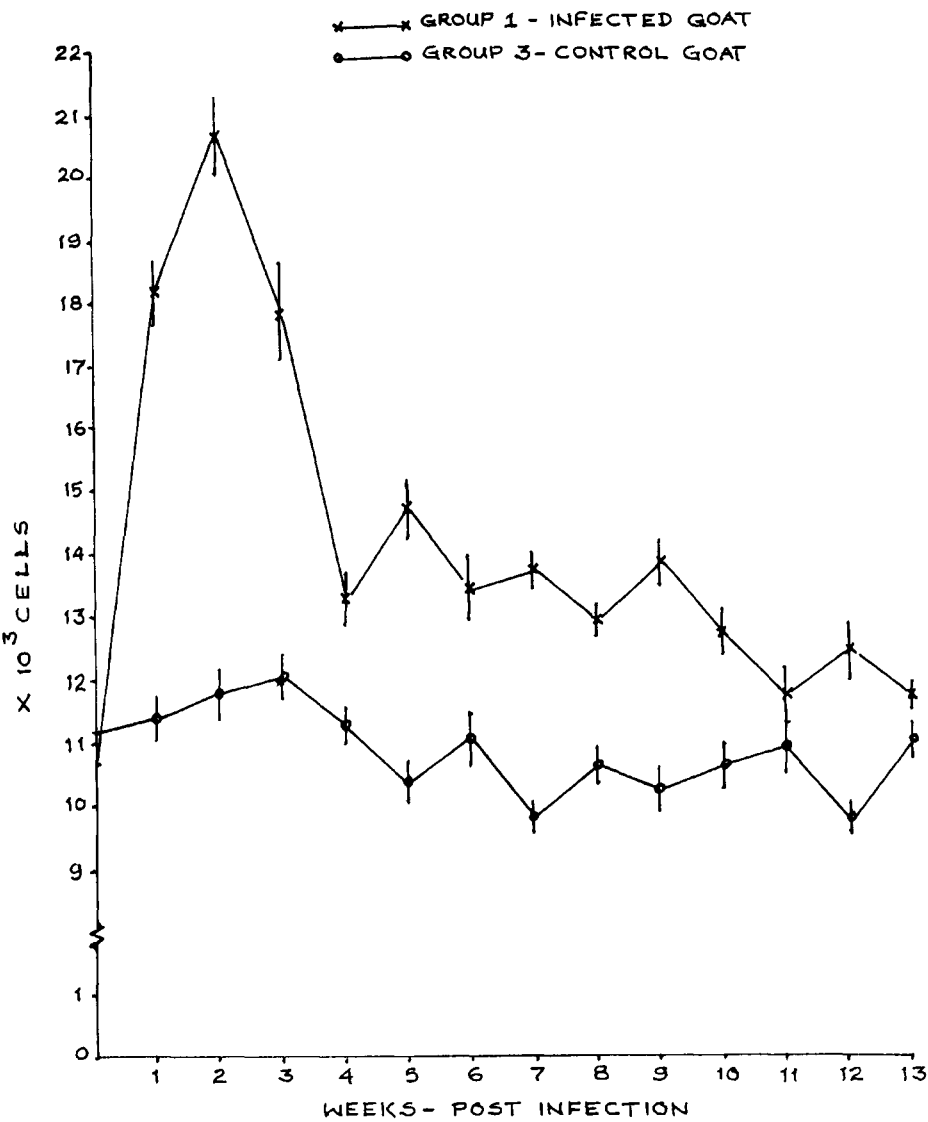
Before the experimental inoculation, group I goats had a mean leukocyte count of 10718 $\pm$ 627 per mm^3 (range 8250-14400) while the counts were 11242 $\pm$ 630 (range 8900-13400) for group III goats. The difference in leukocyte counts between the groups was not significant. Throughout the period of observation total leukocyte count in group I goats were uniformly high after the experimental inoculation with C. pseudotuberculosis when compared to Group III control goats.

Table 7. Mean value of total leukocyte count (number/mm^3) at weekly intervals

Week		Range	Mean	SE	t-value	Significance
0	E	8250-14400	10718.18	626.87	0.5606	NS
	C	8900-13400	11242.86	630.89		
1	E	14200-25300	18266.18	1071.62	4.5768	**
	C	8500-13900	11457.14	775.39		
2	E	14650-27700	20790.91	1255.78	5.1852	**
	C	8350-14000	11857.14	841.01		
3	E	10750-28800	17881.82	1715.28	2.5452	*
	C	9900-15050	12157.14	733.74		
4	E	10450-17950	13368.18	805.30	1.8068	NS
	C	9600-13750	11300.00	664.39		
5	E	11000-18800	14822.73	767.41	4.0460	**
	C	9200-13900	10450.00	600.79		
6	E	10850-21600	13518.18	923.98	1.7288	NS
	C	7950-14100	11178.57	865.12		
7	E	10850-16400	13800.00	541.92	4.9153	**
	C	8300-11900	9971.42	468.57		
8	E	11600-16950	13054.55	497.92	3.0454	**
	C	9750-13000	10792.86	499.45		
9	E	10150-19050	13922.73	784.69	3.2842	**
	C	8950-12100	10378.57	531.82		
10	E	9750-17050	12640.91	688.63	2.0876	NS
	C	8750-14200	10714.29	670.08		
11	E	9000-17950	11868.18	803.49	0.6584	NS
	C	8400-14900	11071.43	833.86		
12	E	10200-12750	13531.82	969.85	2.2524	*
	C	8350-11200	9964.28	368.34		
13	E	9800-14500	11890.91	448.06	0.9072	NS
	C	8500-13400	11235.71	569.32		

E - Experimental animals
C - Control animals
* - Significant ($P < 0.05$)
** - Significant ($P < 0.01$)
NS - Not significant

FIG-11 MEAN VALUE OF TOTAL LEUKOCYTE COUNT (CELLS/mm^3)



The difference in counts between experimentally infected and control goats was significant at one per cent level at first, second, fifth, seventh, eighth and ninth weeks; at five per cent level, at third and twelfth week and not significant at fourth, tenth, eleventh and thirteenth week post-infection. The highest mean leukocyte count (20790 ± 1255) was recorded by second week of infection in group I goats as compared to the near normal values (11857 ± 841) in group III goats. Though leukocytosis in experimentally infected animals was a constant feature, it showed varying degree of fluctuation.

Differential count.

The differential leukocyte counts for group I and III goats was done before experimental inoculation with C. pseudotuberculosis and at weekly intervals upto 13 weeks post-inoculation. The results obtained as mean percentage distribution of leukocytes for group I and III goats are presented in table 8.

Lymphocytes.

The mean percentage distribution of lymphocyte in group I and III goats estimated before and after infection is presented in table 9 and figure 12. Mean percentages of lymphocytes estimated for group I and III goats (58.36 ± 3.13 and 58.14 ± 2.49 respectively) at pre-infection period were almost the same and the difference in values was not significant. During the first three weeks following experimental inoculation, there was significant reduction ($P < 0.01$) in the percentage

Table 8. Mean differential count (%) at weekly intervals

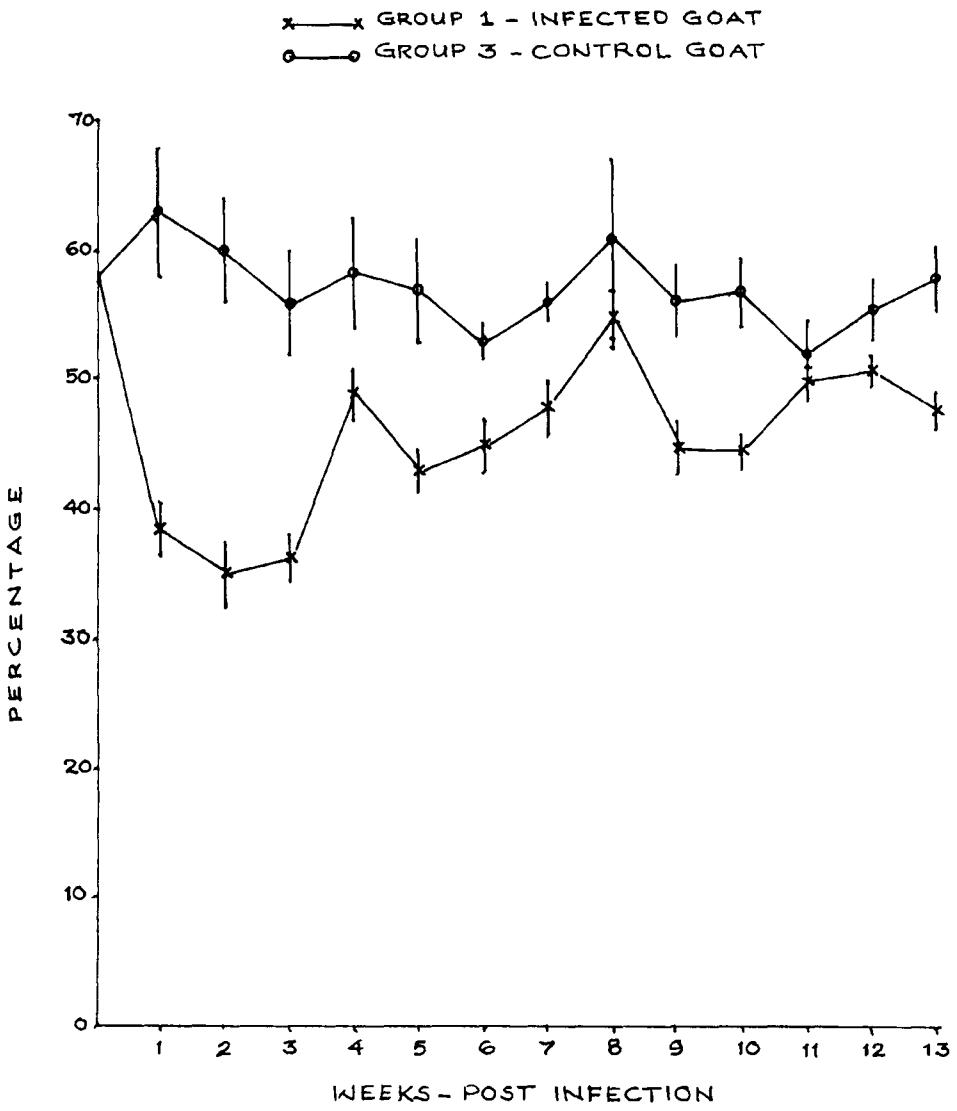
Weeks		Lympho-cyte	Neutro-phil	Eosino-phils	Mono-cytes	Baso-bils
0	E	58.36	39.18	1.72	0.72	-
	C	58.14	37.57	3.43	0.72	0.14
1	E	38.90	58.81	1.20	1.09	-
	C	63.00	33.28	3.00	0.57	0.15
2	E	35.81	61.90	1.56	0.64	0.09
	C	60.00	36.28	2.58	1.14	-
3	E	36.30	61.20	1.60	0.60	0.10
	C	56.00	40.00	3.43	0.57	-
4	E	49.36	49.00	1.18	0.46	-
	C	58.85	38.57	2.43	0.15	-
5	E	43.18	54.27	1.92	0.63	-
	C	57.14	37.85	4.29	0.72	-
6	E	45.72	50.18	1.90	1.73	0.27
	C	53.57	40.42	4.16	1.85	-
7	E	48.27	49.27	1.83	0.63	-
	C	56.14	39.85	2.76	1.25	-
8	E	55.28	42.85	1.45	0.42	-
	C	61.00	37.00	1.66	0.34	-
9	E	45.18	51.63	1.90	1.29	-
	C	56.57	39.14	2.85	1.44	-
10	E	45.45	52.45	1.65	0.45	-
	C	57.60	38.20	3.29	0.91	-
11	E	50.00	48.00	1.45	0.55	-
	C	52.57	44.85	2.29	0.29	-
12	E	51.45	46.00	2.00	0.55	-
	C	56.71	38.57	3.28	1.44	-
13	E	48.00	49.36	1.90	0.74	-
	C	58.42	38.71	2.15	0.72	-

Table 9. Mean lymphocyte percentage at weekly intervals

Period in weeks		Range	Mean	SE	t-value	Significance
0	E	40-74	58.36	3.18	0.0457	NS
	C	45-71	58.14	3.49		
1	E	28-70	38.90	4.29	3.5151	**
	C	40-76	63.00	3.30		
2	E	18-70	35.81	4.95	3.3326	**
	C	40-76	60.00	4.63		
3	E	25-54	36.50	3.17	3.7473	**
	C	41-71	56.00	4.27		
4	E	31-70	49.36	3.00	1.8853	NS
	C	44-75	58.85	4.21		
5	E	31-62	43.18	3.04	2.6893	*
	C	41-76	57.14	4.43		
6	E	20-76	45.72	4.53	1.3291	NS
	C	49-60	53.57	1.67		
7	E	33-74	48.27	3.79	1.5350	NS
	C	47-68	56.14	2.24		
8	E	36-65	55.28	3.98	0.7522	NS
	C	52-75	61.00	7.09		
9	E	24-66	45.18	3.41	2.2323	*
	C	45-69	56.57	3.43		
10	E	34-65	45.48	2.87	2.4868	*
	C	49-64	57.60	3.44		
11	E	37-60	50.00	2.20	0.6490	NS
	C	38-68	52.57	3.62		
12	E	37-65	51.45	2.30	1.3440	NS
	C	49-68	56.71	2.92		
13	E	31-63	48.00	2.78	2.5269	*
	C	48-69	58.42	2.72		

E - Experimental animals
C - Control animals
* - Significant ($P < 0.05$)
** - Significant ($P < 0.01$)
NS - Not significant

FIG-12 MEAN PERCENTAGE OF LYMPHOCYTE IN PERIPHERAL BLOOD



distribution of lymphocytes in the peripheral blood. During the 1st, 2nd, and 3rd week of infection, mean percentage of lymphocytes recorded were 38.9 ± 4.20, 35.81 ± 4.95 and 36.5 ± 3.17 respectively for group I and 63.00 ± 3.00, 60.00 ± 4.63 and 56.00 ± 4.27 respectively for group III goats. It was further observed that reduction in the percentage distribution of lymphocytes was a feature in group I goats during the entire period of observation but significant reductions ($P < 0.05$) were noticed only during the 5th, 9th, 10th and 13th week.

The mean values of absolute lymphocyte counts for group I and III goats and the results of t-test are given in table 10 and figure 13. Mean values of absolute lymphocyte counts obtained at pre-infection period were 6301 ± 572 for the experimental and 6589 ± 614 for the control goats and the difference between the groups was not significant. Similarly after infection also the absolute lymphocyte count at weekly intervals for the experimental and control goats did not show any significant reduction or increase.

Neutrophils.

Mean percentage distribution of neutrophils in group I and III estimated at weekly intervals is presented in table 11 and figure 14. During the pre-infection period, percentage distribution of neutrophils in peripheral blood of group I goats was 39.18 ± 3.14 and in group III 37.57 ± 3.95. The difference between the groups was was not significant. During

Table 10. Mean absolute count of lymphocytes (number/mm^3) at weekly intervals

Weeks		Range	Mean	SE	t-value	Significance
0	E	3827-10323	6301.36	372.28	0.3313	NS
	C	4183-8384	6589.85	614.18		
1	E	3822-12040	6929.27	669.70	0.0737	NS
	C	3980-9538	7007.42	809.49		
2	E	4113-12760	7184.00	885.09	0.0260	NS
	C	4342-10602	7150.42	810.94		
3	E	3870-9660	6127.90	621.81	0.7275	NS
	C	4407-9940	6826.00	698.36		
4	E	4807-9240	6475.18	403.56	0.3118	NS
	C	4180-9262	6706.57	585.92		
5	E	3875-10540	6377.65	554.21	0.5139	NS
	C	3936-7562	5965.00	498.23		
6	E	3537-10368	6162.81	683.01	0.1673	NS
	C	4054-8178	6002.28	526.80		
7	E	3797-10952	6790.72	729.62	1.1489	NS
	C	4559-7259	5684.14	342.62		
8	E	4338-10848	7393.00	753.98	0.9369	NS
	C	5148-7425	6216.32	661.04		
9	E	4320-9372	6164.00	459.24	0.3001	NS
	C	4387-8280	5938.28	607.37		
10	E	4059-8056	5898.72	354.26	0.2013	NS
	C	4550-7026	5776.80	437.93		
11	E	3300-8580	5922.27	448.35	0.1827	NS
	C	4009-8344	5792.14	547.17		
12	E	4717-11284	6459.00	592.89	0.9972	NS
	C	4340-6784	5654.00	373.88		
13	E	4138-8875	5716.00	437.98	1.2913	NS
	C	4420-8308	6586.00	467.03		

E - Experimental animals

C - Control animals

NS - Not significant

FIG-13 MEAN VALUE OF ABSOLUTE LYMPHOCYTE COUNT (No OF CELLS/mm^3) IN PERIPHERAL BLOOD

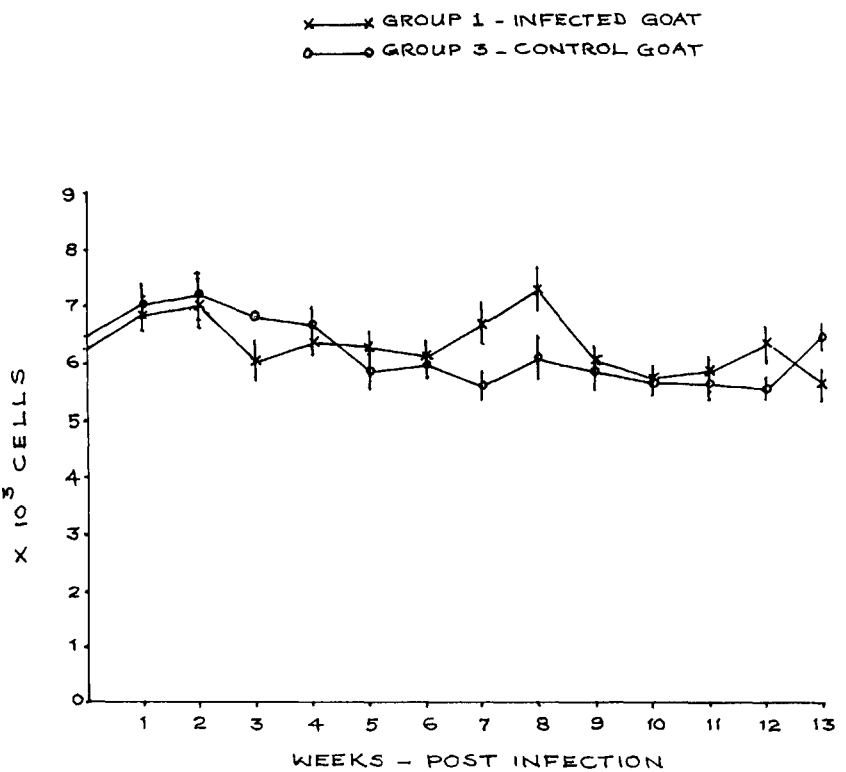
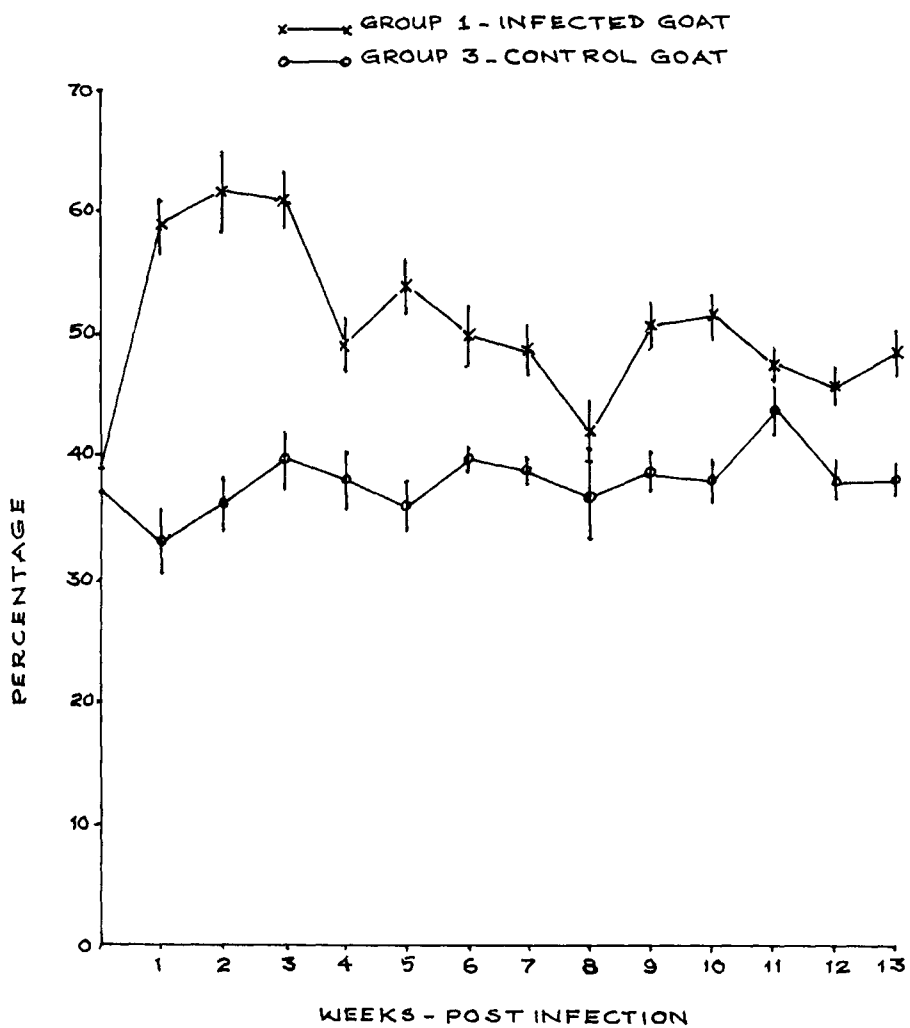


Table 11. Mean neutrophil percentage at weekly intervals

Period in weeks		Range	Mean	SE	t-value	Significance
0	E	23-59	39.18	3.14	0.3190	NS
	C	25-52	37.57	3.95		
1	E	28-73	50.81	4.26	3.7968	**
	C	20-55	33.28	5.11		
2	E	28-80	61.90	4.97	3.5922	**
	C	22-54	36.28	6.26		
3	E	42-74	61.20	3.23	3.9969	**
	C	23-56	40.00	4.34		
4	E	30-68	49.00	2.97	2.062	NS
	C	24-56	38.57	4.30		
5	E	37-68	54.27	3.31	3.338	**
	C	20-55	36.42	4.21		
6	E	24-80	50.18	4.70	1.5838	NS
	C	35-47	40.42	1.95		
7	E	25-65	49.27	2.67	1.9135	NS
	C	30-47	39.85	2.02		
8	E	33-63	42.85	4.15	0.7513	NS
	C	23-45	37.00	7.02		
9	E	30-75	51.63	3.47	2.5342	*
	C	29-49	39.14	2.82		
10	E	38-65	52.45	2.91	2.9115	*
	C	31-48	38.20	3.29		
11	E	38-57	48.00	2.22	0.7829	NS
	C	29-60	44.85	3.64		
12	E	33-61	46.00	2.64	1.8349	NS
	C	27-48	38.57	3.32		
12	E	33-68	49.36	2.98	2.4416	*
	C	28-51	38.71	2.77		

E - Experimental animals
C - Control animals
* - Significant ($P < 0.05$)
** - Significant ($P < 0.01$)
NS - Not significant

FIG. 14 MEAN PERCENTAGE OF NEUTROPHILS IN PERIPHERAL BLOOD



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post-inoculation, group I animals consistently showed higher percentage distribution of neutrophils in the peripheral blood but significant difference from that of the controls were recorded, only during the 1st, 2nd, 3rd, 5th, 9th, 10th and 13th week of observation. Post inoculation mean percentage distribution of neutrophils in group I goats ranged from 42.85 ± 4.15 to 61.90 ± 4.97 while in group III controls it ranged from 33.28 ± 5.11 to 44.85 ± 3.64.

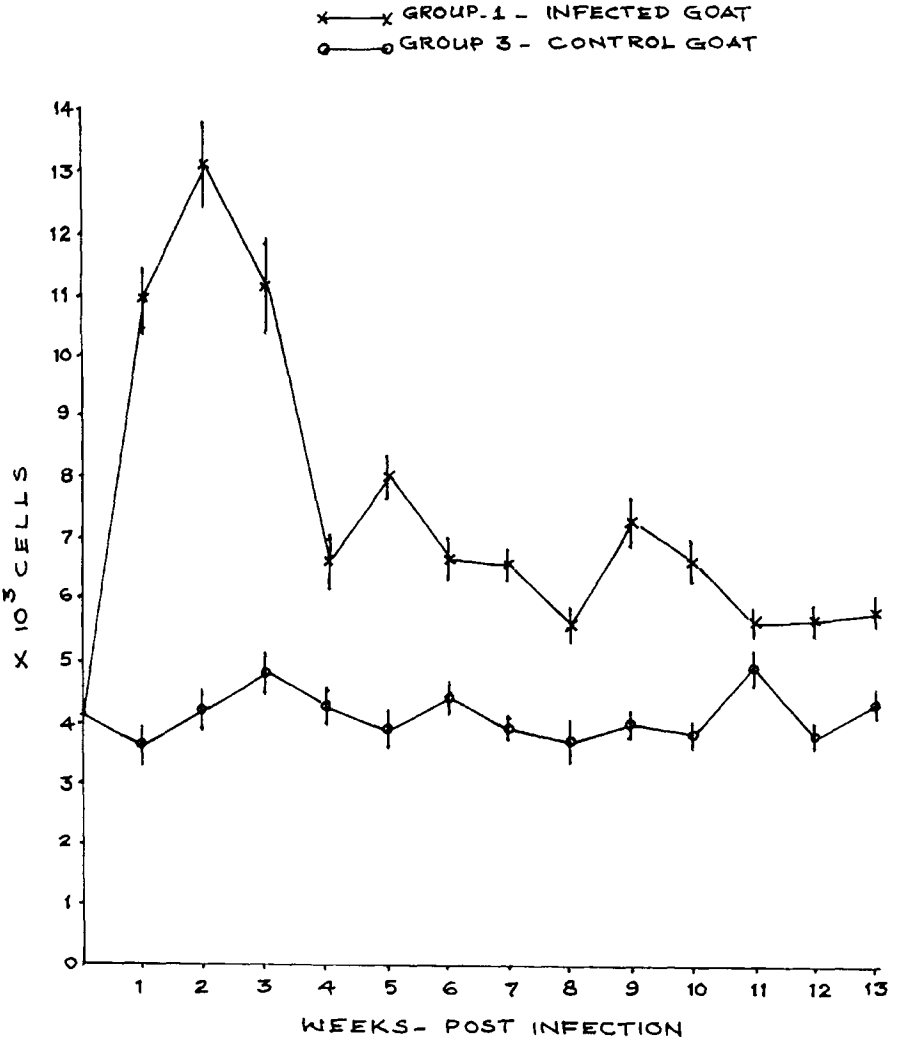
Range and mean values obtained as absolute neutrophil counts at weekly intervals from experimentally infected group I and uninfected control group III goats are presented in table 12 and figure 15. The absolute mean values of neutrophils for group I and Group III goats were almost the same (4154.18 ± 355.49 and 4154.85 ± 370.43 respectively) before the commencement of experiment. Following experimental infection, group I goats consistently showed high absolute neutrophil count compared to group III control goats throughout the observation period of 13 weeks. The counts with infected goats were significantly high ($P < 0.01$) compared to controls at the 1st, 2nd, 3rd, 5th, 7th and 9th week post-infection. The mean counts obtained during the above periods were 10928.18 ± 1191.28, 13120.36 ± 1487.07, 11157.10 ± 1355.57, 8068.90 ± 706.13, 6660.90 ± 392.95 and 7319.81 ± 848.00 respectively for group I goats and 3624.42 ± 512.44, 4267.28 ± 553.32, 4817.14 ± 585.97, 3956.85 ± 559.22, 3986.28 ± 299.77 and 4046.42 ± 284.88 respectively for group III control goats. The differences in

Table 12. Mean absolute count of neutrophils (number/mm^3) at weekly intervals

Period in weeks		Range	Mean	SE	t-value	Significance
0	E	2228-6401	4154.18	355.49	0.0012	NS
	C	2625-5564	4154.85	370.43		
1	E	4816-16008	10928.18	1191.28	4.6725	**
	C	1700-5335	3626.42	512.64		
2	E	4102-17366	13120.36	1494.07	4.5854	**
	C	2579-6453	4267.28	553.32		
3	E	6333-20736	11157.10	1555.57	3.2697	**
	C	3220-7826	4817.14	585.97		
4	E	3960-12204	6664.18	735.80	2.3299	*
	C	2964-6737	4306.57	500.96		
5	E	5124-12596	8068.90	706.13	4.1259	**
	C	1990-6116	3956.85	559.22		
6	E	2804-9432	6760.00	670.41	2.5475	*
	C	3456-5699	4485.57	349.01		
7	E	3700-8287	6660.90	392.95	4.8544	**
	C	2700-5240	3986.28	299.77		
8	E	4077-7592	5652.14	534.31	1.9296	NS
	C	2277-4666	3799.33	763.59		
9	E	4260-13500	7319.81	848.00	2.9813	**
	C	3262-4977	4046.42	284.88		
10	E	3217-11082	6738.18	645.17	2.8910	*
	C	2712-5040	3833.60	373.45		
11	E	3960-9514	5703.09	479.52	0.9438	NS
	C	2856-6575	4995.00	561.18		
12	E	4284-8463	5757.72	478.82	2.8321	*
	C	2790-5374	3848.00	384.72		
13	E	4108-9656	5861.81	444.81	2.4053	*
	C	3010-5890	4340.71	365.36		

E = Experimental animals
C = Control animals
* = Significant ($P < 0.05$)
** = Significant ($P < 0.01$)
NS = Not significant

FIG - 15 MEAN VALUE OF ABSOLUTE NEUTROPHIL COUNT (CELLS/mm^3) IN PERIPHERAL BLOOD



the mean absolute counts of infected and control goats were significant only at five per cent level ($P<0.05$) during the 4th, 6th, 10th, 12th and 13th week and not significant at 8th and 11th week post-infection.

Eosinophils, Monocytes and Basophils.

The percentage distribution and absolute counts of eosinophils, monocytes and basophils in group I goats during pre and post-inoculation periods did not differ significantly from group III control goats.

Mononuclear cells from peripheral blood.

Mononuclear cells and platelets were segregated to form a band between the plasma and Ficoll-paque layers, when peripheral blood of goat was subjected to density gradient centrifugation using Ficoll-paque at 720 x g for 45 mts at 4°C. Mononuclear cells were further separated from platelets and obtained in pure form, by subjecting the cells collected from the band to low speed centrifugation at 200 x g for 15 mts in phosphate buffer saline. The smear made from the pure mononuclear cell suspension on staining by Giemsa's method revealed on an average 91.72% lymphocyte (range 88-95%) and 8.27% monocytes (range 5.0 to 12%). Viability of the cells thus separated was checked by dye exclusion test using trypan blue and on an average 91.22% (range 87.5 to 95.5%) of the cells were found viable (Table 13).

Table 12. Mean values of lymphocytes and monocytes in the mononuclear cells separated by density gradient centrifugation and their viability

	Lymphocyte (%)	Monocyte (%)	Viability (%)
Experimental animals:			
A	88.0	12.0	90.5
B	90.0	10.0	91.5
D	94.0	6.0	87.5
F	91.5	8.5	95.5
N	92.5	7.5	90.5
H	94.5	5.5	92.0
Q	91.5	8.5	91.5
R	91.5	8.5	93.0
X	91.0	9.0	92.0
Y	94.5	5.5	94.0
Z	92.5	7.5	91.5
Control animals:			
O	95.0	5.0	91.0
M	94.5	5.5	90.0
C	88.0	12.0	91.0
T	88.5	11.5	91.5
K	88.0	12.0	90.0
S	94.0	8.0	91.5
J	91.5	8.5	89.5

Overall average : Lymphocytes - 91.72%
Monocytes - 8.2%
Viability - 91.22%

Enumeration of B-lymphocytes.

EAC rosette.

Bovine red cells sensitised with subagglutinating level of haemolysin and mouse complement (EAC) formed rosettes with goat lymphocytes. The lymphocytes which had shown adherence of three or more red cells were considered as EAC rosette positive B cells and their percentage distribution in peripheral blood mononuclears was enumerated. The range and mean percentage of EAC rosette positive B-cells from group I infected and group III control goats and the results of t-test are given in table 14. Fig. 16 depicts the mean percentage of B-cells from infected and control groups.

At the pre-infection period, the mean percentage of EAC rosette positive lymphocytes in group I goats was 11.08 $\pm$ 0.79 whereas in group III it was 9.17 $\pm$ 0.57 and the difference between the groups was not significant. During the first week of inoculation, the mean percentage of EAC rosette with group I was higher (12.56 $\pm$ 1.31) in comparison in group III controls (10.42 $\pm$ 0.94), but there again the difference was not significant.

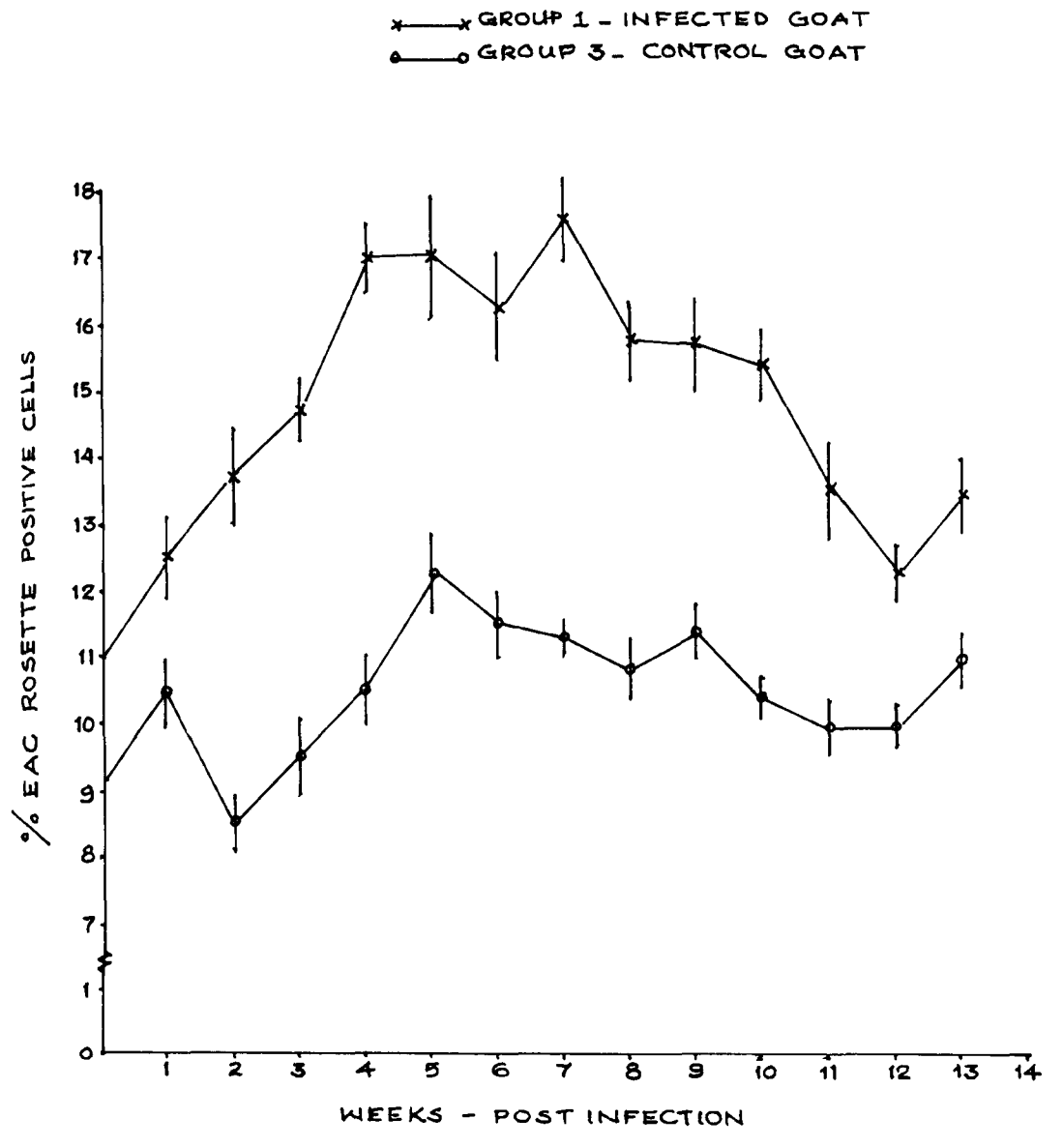
Significantly high percentage of EAC rosette positive B-cells was recorded in group I goats from 2nd to 10th week post-inoculation, except in 5th week, in comparison to the corresponding values obtained with group III goats. The mean percentage of B-cells recorded in case of group I goats were 13.79 $\pm$ 1.48, 14.79 $\pm$ 0.98, 17.05 $\pm$ 1.08, 16.35 $\pm$ 1.67,

Table 14. Mean percentage of EAC rosette forming cells at weekly intervals

Week		Range	Mean	SE	t-value	Significance
0	E	8.00-15.74	11.08	0.79	1.7408	NS
	C	6.90-11.53	9.17	0.57		
1	E	7.00-19.67	12.56	1.31	1.1768	NS
	C	7.45-14.14	10.43	0.94		
2	E	7.45-24.77	13.79	1.48	3.6547	*
	C	5.00-11.53	8.56	0.75		
3	E	8.00-20.00	14.79	0.95	3.4376	**
	C	7.00-13.61	9.58	1.07		
4	E	10.30-23.38	17.05	1.08	4.0982	**
	C	5.71-14.35	10.54	1.02		
5	E	5.82-32.77	17.03	1.96	1.8003	NS
	C	8.73-15.00	12.30	1.09		
6	E	9.64-26.54	16.35	1.67	2.1914	*
	C	8.63-15.32	11.58	0.98		
7	E	10.61-23.07	17.63	1.20	3.9895	**
	C	9.80-13.25	11.36	0.48		
8	E	10.00-35.00	15.74	1.24	2.7617	*
	C	7.48-13.68	10.86	1.00		
9	E	10.50-33.33	15.71	1.46	3.2380	*
	C	9.50-16.39	11.42	0.89		
10	E	11.42-21.42	15.42	1.08	3.4410	**
	C	8.60-12.38	10.44	0.98		
11	E	6.31-23.62	13.56	1.62	1.8566	NS
	C	6.50-12.50	10.01	0.80		
12	E	9.09-18.40	12.30	0.85	1.8870	NS
	C	7.62-13.00	10.05	0.62		
13	E	8.33-19.17	13.52	1.13	1.6070	NS
	C	7.60-14.15	11.05	0.84		

E - Experimental animals
C - Control animals
* - Significant at ($P < 0.05$)
** - Significant at ($P < 0.01$)
NS - Not significant

FIG-16 MEAN PERCENTAGE OF EAC ROSETTE FORMING CELLS IN PERIPHERAL BLOOD



17.63 ± 1.20, 15.74 ± 1.24, 15.71 ± 1.46 and 15.42 ± 1.08 respectively at 2nd, 3rd, 4th, 6th, 7th, 8th, 9th and 10th week post-infection as compared to the corresponding values of 8.56 ± 0.75, 9.58 ± 1.07, 10.34 ± 1.02, 11.58 ± 0.98, 11.36 ± 0.48, 10.86 ± 1.00, 11.42 ± 0.89 and 10.44 ± 0.58 obtained with group III goats.

Though the mean percentage of EAC rosette positive B-lymphocyte recorded with group I goats at 5th, 11th, 12th and 13th week post-infection (17.03 ± 1.96, 13.56 ± 1.42, 12.30 ± 0.85 and 13.52 ± 1.13 respectively) were high in comparison to the percentages obtained with group III goats (12.30 ± 1.09, 10.01 ± 0.80, 10.05 ± 0.62 and 11.05 ± 0.84 respectively) the differences were not significant.

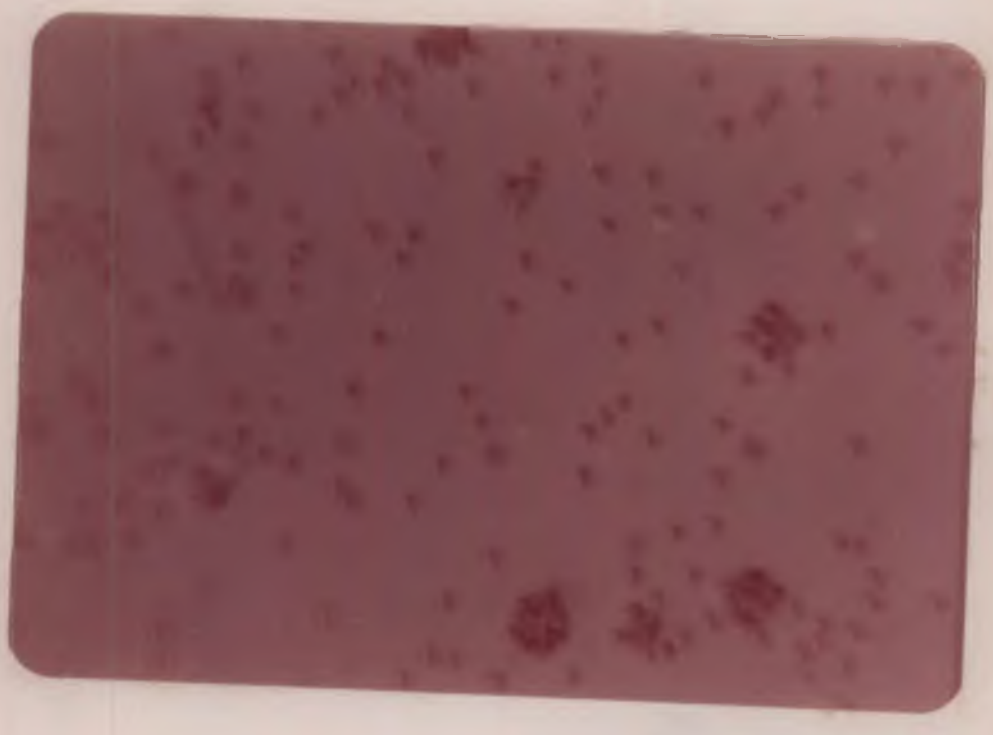
During the post-infection period the highest percentage of EAC positive B-cells (17.63 ± 1.20) was recorded at the 7th week while the least percentage (12.30 ± 0.85) was recorded at the 12th week.

Enumeration of T-lymphocytes.

E-rosette.

Sheep erythrocytes formed spontaneous rosettes with goat peripheral blood lymphocytes(E-rosette) and in majority of such rosettes, the red cells were surrounding the entire periphery of lymphocytes (Fig.17). Lymphocytes which formed E-rosettes with SRBC (at 4°C incubated for 18 hrs) were considered as T-lymphocytes and their percentage in peripheral

Fig. 17. Goat lymphocytes which formed erythrocyte rosettes (200 X)



blood of group I and III were enumerated. The mean percentages of E-rosette positive cells obtained with each group and the results of t-test are presented in table 15. Fig.18 depicts the mean percentage of T-cells enumerated with group I infected and group III control goats.

Mean percentage of 20.74 ± 1.34 (range 18.26-33.46) E-rosette positive cells was recorded for group I goats during pre-inoculation period as compared to 24.55 ± 3.06 with group III goats and the values did not differ significantly between the groups. During the first week of infection significant reduction($P < 0.05$) in E-rosette positive lymphocytes was noticed with group I goats (mean 18.44 ± 1.40%, range 12.5 to 25.55%). Such a reduction was not seen with group III animals and in them the values were almost similar to that of '0' week (mean % 24.83 ± 2.53, range 14.29-33.17%). Though the percentage of E-rosette forming cells remained low (mean 31.13 ± 1.82, range 15.09-33.18) in the infected group during the 2nd week also, this difference was not statistically significant when compared to group III animals (mean 25.92 ± 3.14 range 16.09-36.28).

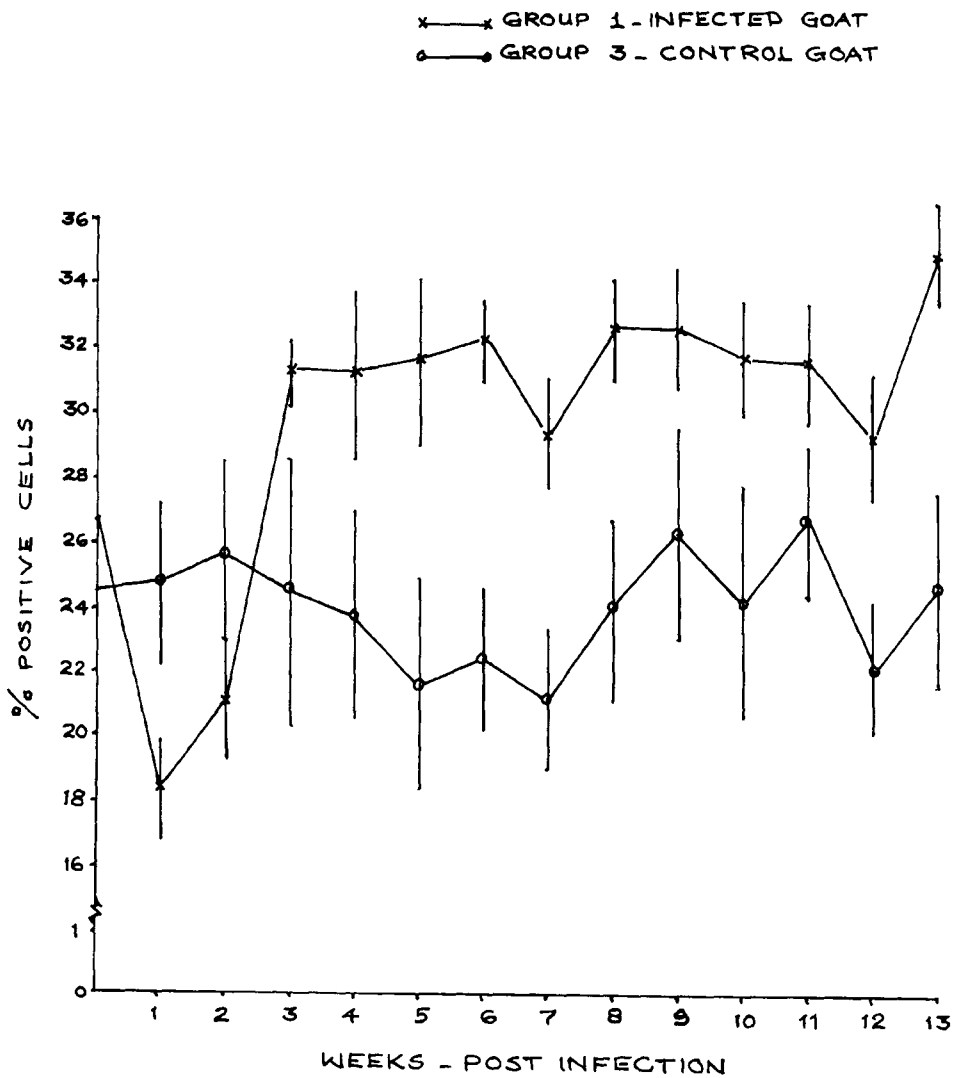
During the 3rd and 4th week of experimental infection, the mean percentage of E-rosette positive cells showed a further increase (mean 31.23 ± 1.06 and 31.24 ± 3.69 respectively) with group I goats, but not statistically significant from that of the group III control animals (24.61 ± 4.09 and 33.78 ± 3.13). However a significantly higher percentages of E-rosette

Table 15. Range and mean percentage of E-rosette forming cells at weekly intervals

Week		Range	Mean	SE	t-value	Significance
0	E	18.28-33.46	26.74	1.34	0.7479	NS
	C	14.54-35.85	24.58	2.06		
1	E	12.50-25.55	18.44	1.40	2.4026	*
	C	14.29-33.17	24.83	2.52		
2	E	15.09-33.18	21.13	1.82	1.4211	NS
	C	16.09-36.28	25.92	3.14		
3	E	25.69-36.38	31.23	1.06	2.0160	NS
	C	12.00-40.86	24.61	4.09		
4	E	9.09-40.97	31.24	2.69	1.7785	NS
	C	15.68-31.37	23.78	3.12		
5	E	17.64-42.86	31.71	2.56	2.5061	*
	C	11.29-32.37	21.68	3.09		
6	E	26.66-36.39	32.39	1.24	4.0699	**
	C	14.21-31.55	22.98	2.29		
7	E	18.26-38.31	29.31	1.71	2.9753	**
	C	14.67-28.62	21.19	2.11		
8	E	22.76-42.64	32.77	1.51	3.0051	**
	C	14.06-31.90	24.09	2.76		
9	E	23.16-41.83	32.64	1.80	1.7980	NS
	C	16.38-36.32	26.40	3.37		
10	E	19.28-39.56	31.08	1.83	2.1122	NS
	C	13.18-36.02	24.24	3.48		
11	E	21.60-40.12	31.73	1.87	1.6350	NS
	C	17.32-33.72	26.86	2.30		
12	E	18.69-39.01	29.38	1.79	2.5606	*
	C	17.39-32.52	22.28	2.04		
13	E	27.38-43.12	35.24	1.58	3.4686	**
	C	18.36-36.58	24.69	2.94		

E - Experimental animals
C - Control animals
* - Significant ($P < 0.05$)
** - Significant ($P < 0.01$)
NS - Not significant

FIG-18 MEAN PERCENTAGE OF E-ROSETTE FORMING CELLS IN PERIPHERAL BLOOD



positive cells were recorded in group I goats during 5th, 6th, 7th and 8th weeks of infection (31.72 ± 2.56, 32.39 ± 1.24, 29.31 ± 1.71 and 32.77 ± 1.51 respectively) when compared to group III goats (21.68 ± 3.09, 22.58 ± 3.29, 21.19 ± 2.11 and 24.09 ± 2.76 respectively). Though there was no significant difference between the percentages of E-rosette positive cells obtained with group I and III goats during 9th, 10th and 11th week of observation, the percentage values obtained with group I goats (32.64 ± 1.80, 31.88 ± 1.83 and 31.73 ± 1.87 respectively) appeared high to group III animals (26.40 ± 3.37, 24.24 ± 3.48 and 26.84 ± 2.30 respectively).

Towards the end of the observation period (at 12th and 13th week) the percentage of E-rosette increased with group I goats and the values were significantly higher ($P<0.05$) than the corresponding values obtained with group III goats. During the 12th week, the mean percentage of E-rosette positive lymphocyte in group I and III goats were 29.38 ± 1.79 and 22.28 ± 2.04 respectively and at 13th week the counts were 25.24 ± 1.58 and 24.69 ± 2.94 respectively.

ANAE activity.

ANAE activity was demonstrated in the mononuclear cells from the peripheral blood of goats.

The smears prepared from the mononuclear cells separated from the peripheral blood were fixed with acetone-citric acid solution and it was found that such fixed smears could be

stored in dry state without interference to the demonstration of ANAE activity for varying periods, facilitating batch staining.

Majority of goat lymphocyte which revealed ANAE positive reaction was with one or two localised nodular pink to red coloured reaction product in the cytoplasm adjacent to the cell membrane (Fig.19) while few cells were with more than two scattered punctate nodular reaction product of comparatively small size. Both type of cells were identified as T-lymphocytes. The monocytes presented diffuse reaction product in the cytoplasm which could be easily distinguished from lymphocytes.

The mean percentage of ANAE positive lymphocytes in the peripheral blood from group I and III goats obtained at weekly intervals are presented in table 16. Fig.20 depicts the graphical representation of mean value of ANAE positive cells.

Mean percentage of ANAE positive lymphocytes estimated in group I goats before experimental infection was 28.09 $\pm$ 1.52 (range 15.31-32.59) as against 35.86 $\pm$ 4.87 (range 18.22-38.65) for group III goats. The difference in values between the two groups was not significant. It was further observed that the lymphocytes examined for ANAE activity at weekly intervals from infected and control goats did not show any statistically significant difference in the percentage distribution in the peripheral blood throughout the post-infection observation period, eventhough the numerical values showed a tendency towards marginal increase of ANAE positive lymphocytes in experimentally infected goats.

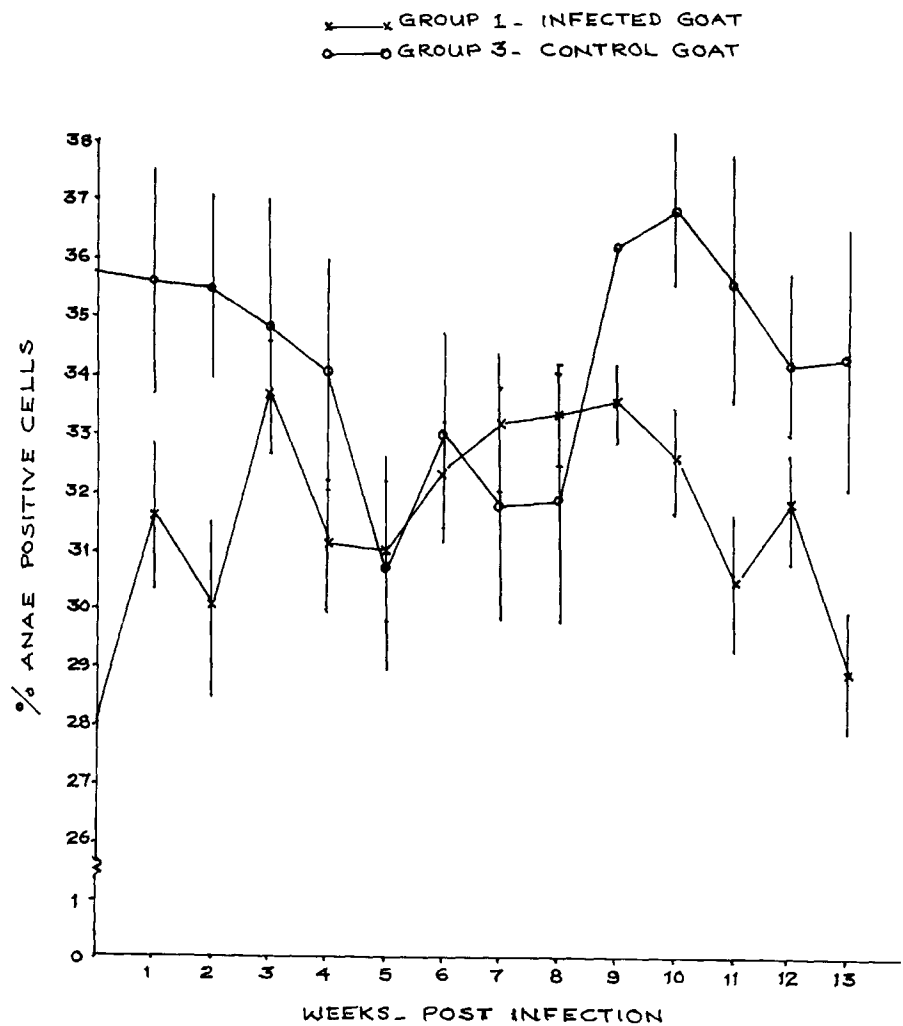
Fig. 19. Goat lymphocytes stained for NSE activity. Red spot in the cytoplasm indicates positive reaction (1000 X)

Table 16. Range and mean percentage of ANAE positive cells at weekly intervals

Week		Range	Mean	SE	t-value	Significance
0	E	15.31-32.59	28.09	1.52	1.8162	NS
	C	18.22-58.65	35.86	4.87		
1	E	18.13-44.00	31.67	2.53	0.8914	NS
	C	18.84-50.64	35.62	3.90		
2	E	17.85-50.90	30.44	3.01	1.1180	NS
	C	21.18-44.85	35.50	3.12		
3	E	18.93-43.13	33.78	1.99	0.2526	NS
	C	17.47-47.00	34.81	4.16		
4	E	16.35-40.00	31.40	2.29	0.6245	NS
	C	18.42-52.50	34.04	3.93		
5	E	21.07-47.11	31.00	2.42	0.1094	NS
	C	18.22-43.66	30.83	3.50		
6	E	23.87-45.32	32.38	1.79	0.1970	NS
	C	20.09-49.00	33.09	3.54		
7	E	19.21-47.50	33.23	2.39	0.3314	NS
	C	18.81-49.50	31.79	4.00		
8	E	23.78-42.64	33.34	1.68	0.3411	NS
	C	18.39-54.00	31.96	4.39		
9	E	27.26-36.70	33.63	1.37	1.0099	NS
	C	24.40-47.50	36.35	2.56		
10	E	21.39-39.93	33.64	1.81	1.1702	NS
	C	25.25-52.50	36.91	3.61		
11	E	23.00-40.77	30.55	2.10	1.2014	NS
	C	20.09-56.32	35.68	4.15		
12	E	22.50-43.13	31.94	2.12	0.6149	NS
	C	21.15-45.63	34.20	3.18		
13	E	20.00-40.38	28.91	2.06	1.2210	NS
	C	16.17-49.50	34.38	4.90		

E - Experimental animals
C - Control animals
NS - Not significant

FIG-20 MEAN PERCENTAGE OF ANAE POSITIVE CELLS IN PERIPHERAL BLOOD



Leukocyte migration inhibition test.

Leukocytes were easily separated from the peripheral blood of goats by the method of RBC lysis using distilled water. The leukocytes thus separated was without any red cell contamination and on an average 90% cells were found viable by dye exclusion method. The leukocyte suspension prepared at a concentration of 1.5×10^8 cells/ml did not show clumping or reduction in viability after incubation with the toxic culture supernatant of C. pseudotuberculosis which had haemolytic titre 1:16 and the pH adjusted 7.2.

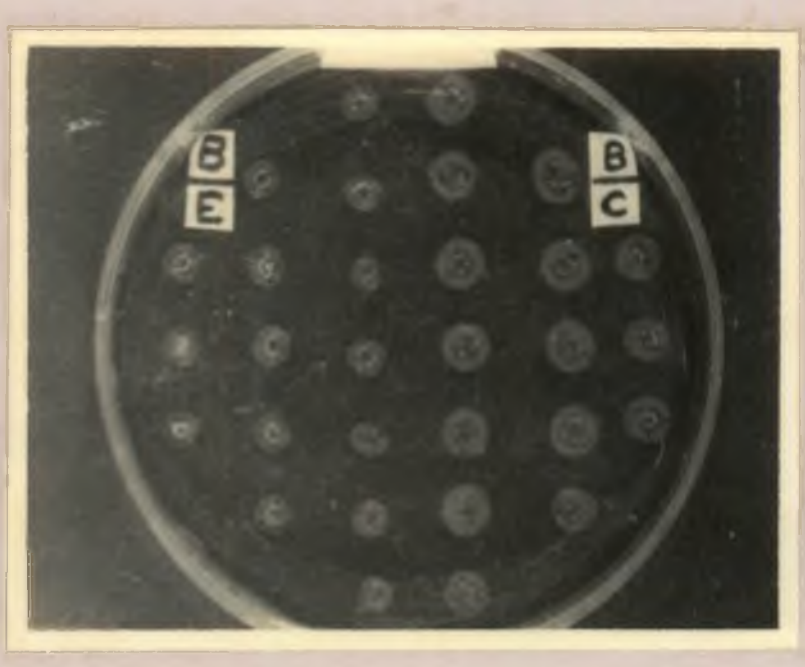
Leukocytes from experimentally infected and control goats did show migration on the glass surface and the zone of migration was well discernible. The zone of migration of cells appeared as circular opaque area around the wells and the diameter of the area was measured.

The LMIT was performed with leukocytes collected from group I and III goats before the start of the experiment and thereafter at 15 days intervals post-infection upto 75th day.

The results of the LMIT indicated that there was significant reduction in the migration property of leukocytes obtained from infected animals when treated with toxic culture supernatant of C. pseudotuberculosis (Fig.21), while the migration of leukocytes from control goats was not affected. Leukocytes from infected as well as control goats did show normal comparable zone of migration in the agarose medium when suspended in physiological saline without any antigen treatment.

Fig. 21. Leukocyte migration agarose test at 20 h of incubation

LE - Leukocytes with antigen added
LC - Leukocytes without antigen added


B
E
B
C

LMI index less than 0.8 was considered as positive reaction with LMIT.

The mean values of LMI index obtained with group I and III goats and their comparative differences are given in table 17. Fig.22 depicts the mean LMI index obtained for group I and III goats.

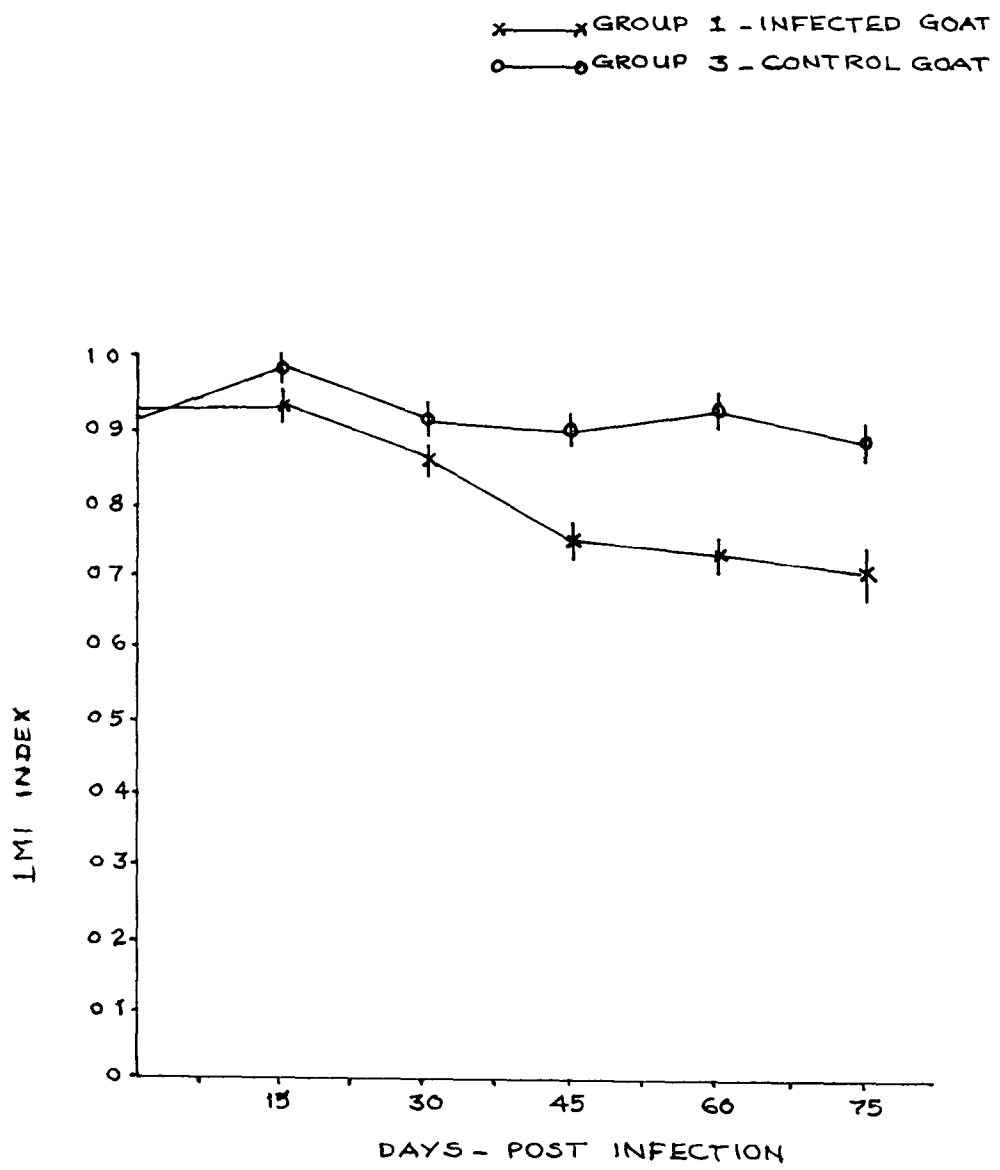
The mean LMI index obtained with goats from group I and III before the start of the experiment were 0.93 ± 0.02 and 0.94 ± 0.01 respectively and the difference was not significant between the groups. With the post-inoculation samples from group I animals taken at 15th and 30th day, the mean LMI index calculated were 0.94 ± 0.02 and 0.87 ± 0.03 respectively, while the corresponding values with control animals were 0.98 ± 0.04 and 0.91 ± 0.03 and here again the values were not significantly different between the two groups. By 45th day onwards there was significant reduction ($P < 0.05$) in the migration property of leukocytes from group I goats while leukocytes from control goats continued to show normal migration profile. It was further observed that by the 60th day post-infection, the LMI index was recorded minimum in case of group I goats (significantly low, $P < 0.01$). The mean LMI index recorded with group I goats by 45th, 60th and 75th day post-infection were 0.75 ± 0.03, 0.74 ± 0.03 and 0.71 ± 0.04 respectively while the values obtained with group III goats were 0.90 ± 0.03, 0.93 ± 0.03 and 0.89 ± 0.02.

Table 17. Mean leukocyte Migration Index calculated for infected and control goats

Period in days		Range	Mean	SE	t-value	Significance
0	E	0.80-1.06	0.93	0.02	0.8251	NS
	C	0.88-1.00	0.94	0.01		
15	E	0.87-1.13	0.94	0.02	0.8939	NS
	C	0.76-1.10	0.98	0.04		
30	E	0.71-1.06	0.87	0.03	0.745	NS
	C	0.80-1.07	0.91	0.03		
45	E	0.65-0.94	0.75	0.03	2.837	*
	C	0.82-1.00	0.90	0.03		
60	E	0.56-0.97	0.74	0.03	3.507	**
	C	0.82-1.01	0.93	0.03		
75	E	0.56-0.96	0.71	0.04	2.594	*
	C	0.82-0.97	0.89	0.02		

E - Experimental goats
C - Control goats
* - Significant ($P < 0.05$)
** - Significant ($P < 0.01$)
NS - Not significant

FIG-22 MEAN LMI INDEX



Skin hypersensitivity.

C. pseudotuberculosis toxin.

Group I goats were intradermally injected at the neck region with toxic culture supernatant of C. pseudotuberculosis at 13th week post-infection. The results obtained showed that injection of toxic preparation in small quantities (0.2 ml) to an infected animal developed typical delayed hypersensitive skin reaction (Fig.23). Control goats which were negative for C. pseudotuberculosis infection (Group III goats) were also similarly injected and the results were negative.

The range and mean skin thickness of group I and III goats before intradermal injection and after 24, 48 and 72 h post-injection are presented in table 18. The mean thickness of skin in group I goats was 4.09 $\pm$ 0.28 mm before injection (normal thickness of skin) while it was 4.28 $\pm$ 0.42 mm in group III goats and the values were not significantly different between the groups. By 24 h post-injection significant increase ($P < 0.01$) in skin thickness was recorded in group I animals (8.36 $\pm$ 0.53 mm) compared to group III goats (5.92 $\pm$ 0.22 mm). The swelling at the site of injection in case of goats from group I was highly painful and hard on palpation while reactions with group III goats were at minimum. The inflammatory reactions suggestive of delayed hypersensitivity was at maximum by 48 h with the known infected animals and the mean skin thickness recorded with the group was 9.31 $\pm$ 0.62 mm, while in uninfected control animals by 48 hrs whatever little

Fig. 23. (cont: Delayed skin hypersensitivity reaction at 48 h post-injection of C. pseudo-tuberculosis toxin

Table 18. Results of Delayed Type Hypersensitivity test in *C. pseudotuberculosis* infected and control goats: *C. pseudotuberculosis* exotoxin as antigen

Period in hours		Skin thickness in mm				Significance
		Range	Mean	SE	t-value	
0	E	3-6	4.09	0.29	0.3987	NS
	C	3-6	4.28	0.42		
24	E	6.5-12.0	8.36	0.53	3.441	**
	C	5.0-7.0	5.92	0.22		
48	E	6.0-14.0	9.31	0.62	4.752	**
	C	4.5-6.0	5.42	0.22		
72	E	7.0-12.5	8.91	0.82	4.064	**
	C	4-6	5.16	0.42		

E - Experimentally infected goats
C - Control goats
** - Significant ($P < 0.01$)
NS - Not significant

inflammatory reactions exhibited at 24 h had subsided and a reduction in skin thickness was noted (5.42 ± 0.22 mm). The intensity of inflammatory reaction at the site of injection showed a reduction by 72 h and even then the skin thickness recorded was 8.91 ± 0.82 mm with Group I goats, which was significantly higher compared to the skin thickness of group III goats (5.16 ± 0.42 mm).

Skin biopsy.

Skin biopsy taken at 48 h post-injection from group I goats showed characteristic histopathological changes suggestive of hypersensitive reactions of type IV. Perifollicular and periglandular infiltrations of lymphocytes, macrophages and sparse neutrophils were the features. Cystic glandular dilatation and mononuclear infiltration in cystic space and also in glands was also noted. Blood vessels were congested and there was perivascular infiltration of lymphocytes and macrophages. Dermal oedema and myositis were also the features in the skin (Fig.24).

The skin biopsy taken from group III goats at 48 h post-injection was histologically near normal except for the presence of sparse lymphocytes and neutrophils at the perivascular space.

Tuberculin.

Intradermal injection of tuberculin in group I and III goats did not produce any reaction suggestive of hypersensitivity till 72 h post-injection. Mean skin thickness of

Fig. 24. Goat skin: Perifolliculer and perigiandular infiltrations of lymphocytes and macrophages due to hypersensitive reaction. H & E 200 X

group I and III goats before and after injection with tuberculin are presented in table 19. Skin biopsy taken from both group I and III goats was histologically near normal barring slight oedema and perivascular infiltration of few mononuclear cells.

Pathogenicity

Group II goats which were inoculated with C. pseudotuberculosis at a dose rate of 2×10^6 bacteria per site of injection also developed general symptoms and inflammatory reactions similar to what was described for group I goats, infected for immune response studies. All the six goats had exhibited rise in body temperature which lasted for three to four days along with general weakness, lethargy and impaired appetite. The intradermal injection sites in all the goats developed marked inflammatory reaction characterised by development of painful hyperaemic skin thickening and the site of injection showed necrotic and pustular lesions. The injection sites at the flank region (s/c injection) showed thickening of skin which was painful to touch. Barring the local lesions lasted beyond one week, all goats were near normal in habits and behaviour by one week post-inoculation.

Necropsy findings

Gross lesions.

Each of the six goats which was sacrificed at 15 days interval did not reveal any lesion suggestive for CLA in any of the internal deep seated lymphnodes and visceral organs,

Table 19. Results of Delayed Type Hypersensitivity test in *C. pseudotuberculosis* infected and control goats: Tuberculin as antigen

Period in hours		Skin thickness in mm				Significance
		Range	Mean	SE	t-value	
0	E	3-6	4.60	0.30	0.5379	NS
	C	4-6	5.00	0.40		
24	E	4-6	5.10	0.33	0.3444	NS
	C	5-6	5.25	0.25		
48	E	3-6	4.60	0.50	0.6918	NS
	C	5-5	5.00	0		
72	E	3-5	4.25	0.47	0.6599	NS
	C	4-5	4.66	0.33		

E - Experimentally infected goats

C - Control goats

NS - Not significant

while regional/local superficial lymphnodes were invariably affected with varying degree of involvement.

By the 15th day through 90 days the goats necropsied showed involvement of prefemoral, prescapular submaxillary and parotid lymphnodes with lesions suggestive of CLA. In severely affected lymphnodes there was enormous enlargement (several folds) with total damage of parenchyma and accumulation of caseated pus (Fig.25). In few goats, the abscess developed in the lymphnodes had opened spontaneously and had evacuated the creamy pus. The skin wound thus produced later healed with scab formation. In less severely affected instances, the lymphnodes were seen enlarged in size two to three folds. When such lymphnodes were cut, it contained necrotic area in the parenchyma and the size of the lesions varied depending the severity of affection. In certain animals the lymphatic channel at the dorsal side of the mandible was seen affected with development of nodular lesions suggestive of CLA. The popliteal, mediastinal, internal iliac, mesenteric, renal and other deep seated lymphnodes were normal and none of the visceral organs showed any lesions suggestive of CLA.

Pus collected from gross lesions was positive for C. pseudotuberculosis by direct staining and for cultural recovery.

Histopathology

Histopathological findings pertaining to the peripheral and deep seated lymphnodes and visceral organs collected from

the macropaied animals at various intervals revealed varied degree of inflammatory reactions as described below.

Lymphnodes.

15th day post-infection.

Depletion of lymphocytes from the cortex and medulla was a feature on the 15th day in the precapular lymphnode. Active germinal centres were absent in the follicles and some of the lymphatics and blood vessels were thrombosed. Focal areas showed histiocyte proliferation and reticular hyperplasia (Fig. 26).

Prefemoral lymphnode also revealed depletion of lymphocytes from the cortex and medulla. An encapsulated caseating abscess consisting chiefly of neutrophils was seen with a festooning of plasma cells at the periphery (Fig. 27).

In the submaxillary lymphnode there was depletion of lymphocytes from the cortical region and there was no clear differentiation between the cortex and medulla. Subcapsular and cortical oedema, congestion of blood vessels, haemorrhage and infiltration of mononuclear cells in the lymphatics and blood vessels were evident. Accumulation of macrophages and plasma cells in the medullary region and dilatation of sinusoids were characteristic features (Fig. 28).

Parotid lymphnode showed lymphoid depletion and accumulation of histiocytes in the cortex. Dilatation of lymphatics and blood vessels and accumulation of macrophages in the

Fig. 25. Enlarged predesmeral lymphnode showing caseated pus

Fig. 26. Lymphnode: Depletion of lymphocyte from the cortex, absence of active follicles, histiocytic proliferation and reticular hyperplasia. H & E x 400

H
PF

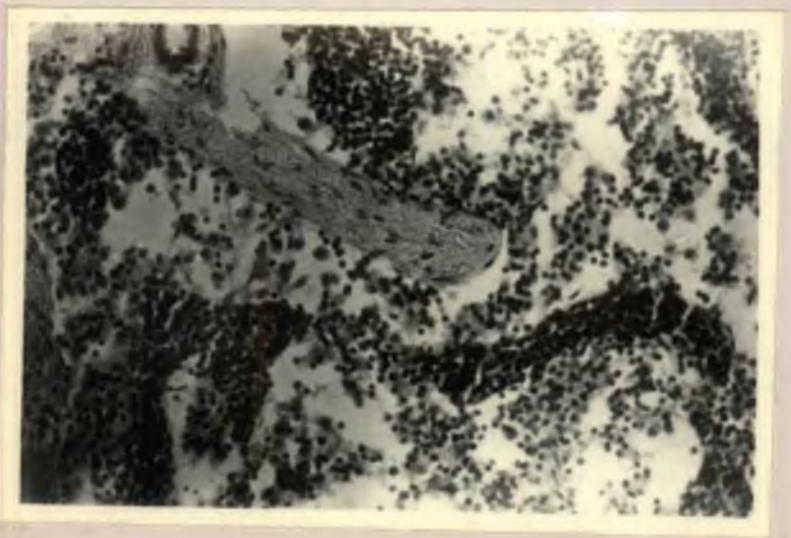
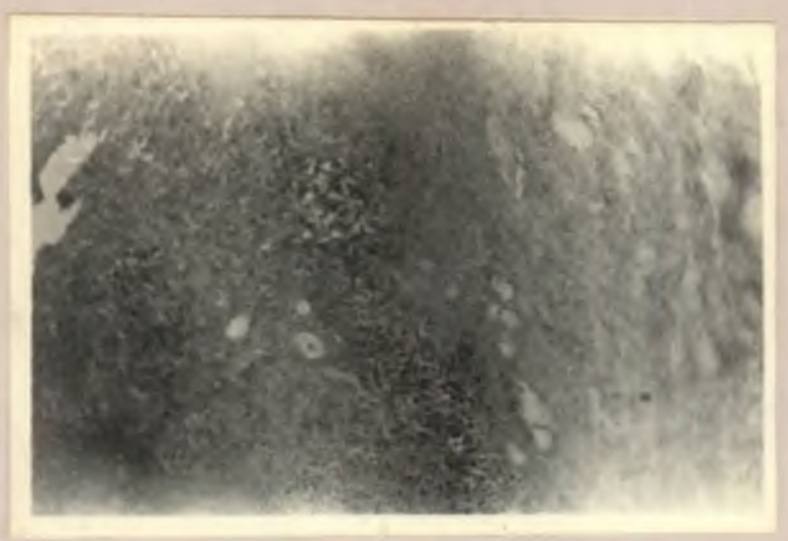
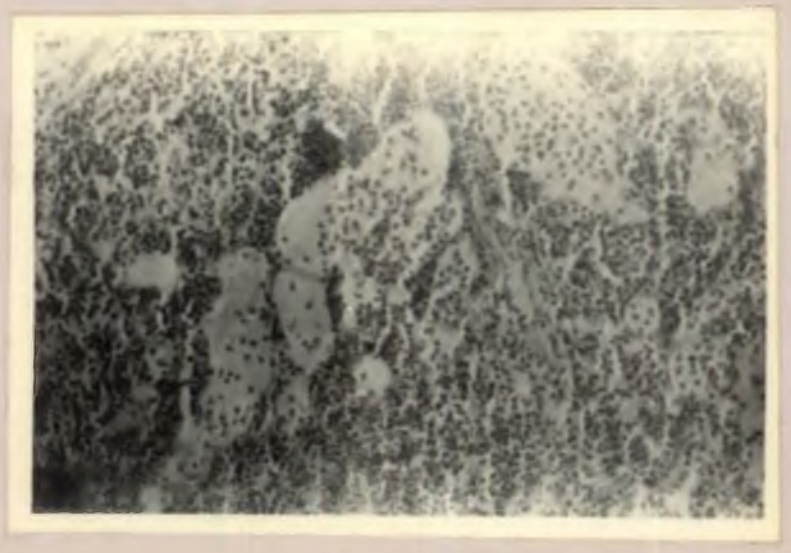


Fig. 27. Lymphnode: Caseated material surrounded by inflammatory cells. H & E x 250

Fig. 28. Lymphnode: Oedema, dilatation of sinusoids and accumulation of macrophages and plasma cells in the medullary region. H & E x 400





lymphatics and blood vessels was seen (Fig. 29). The sinusoids were engorged. Focal collections of macrophages were seen in the medullary region.

Mediastinal lymphnode was oedematous and there was depletion of lymphocytes from the cortical region and dilatation of sinusoids and capillary sclerosis. Extensive infiltration of plasma cells in the medulla and marked sinus histiocytosis were noted (Fig. 30).

Tracheal, mesenteric, popliteal and renal lymphnodes presented a picture of reactive response characterised by hypertrophied follicles. Sinus histiocytosis was also noted in the tracheal and popliteal lymphnodes.

30th day post-infection.

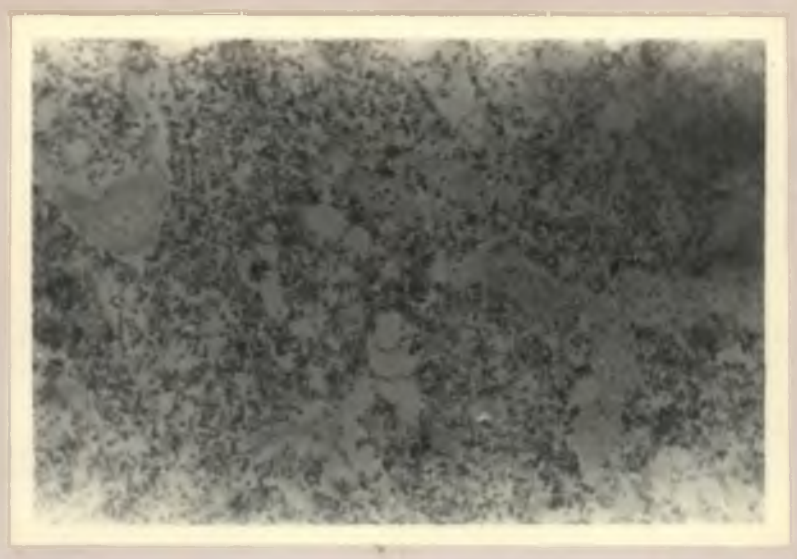
Prescapular lymphnode was seen highly reactive and mature lymphocytes were seen in the cortex with hyperplastic follicles (Fig. 31). There was accumulation of lymphocytes and moderate degree of sinus histiocytosis in the medullary region.

Prefemoral lymphnode appeared to be a caseated mass enclosed by fibrous tissue. The capsule showed hyalinisation of the muscle fibres and collections of lymphocytes were seen in the fibrous capsule (Fig. 32).

In the submaxillary lymphnode, the follicles were hyperplastic. Fibrous tissue proliferation was a feature. Many hypertrophic follicles with well stimulated large and small

Fig. 29. Lymphnode: Depletion of cells from the cortical and paracortical region. Dilatation of lymphatics is also evident.
H & E x 220

Fig. 30. Lymphnode: Severe plasma cell infiltration in the medulla and sinus histiocytosis.
H & E x 400



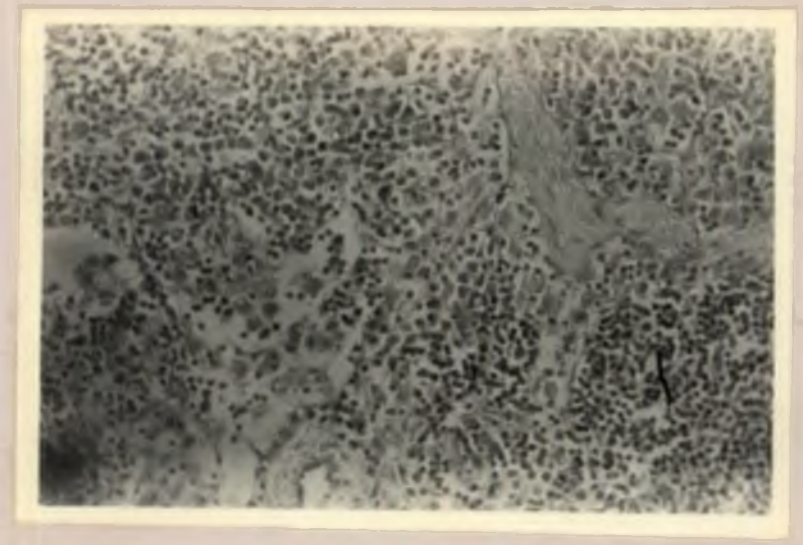
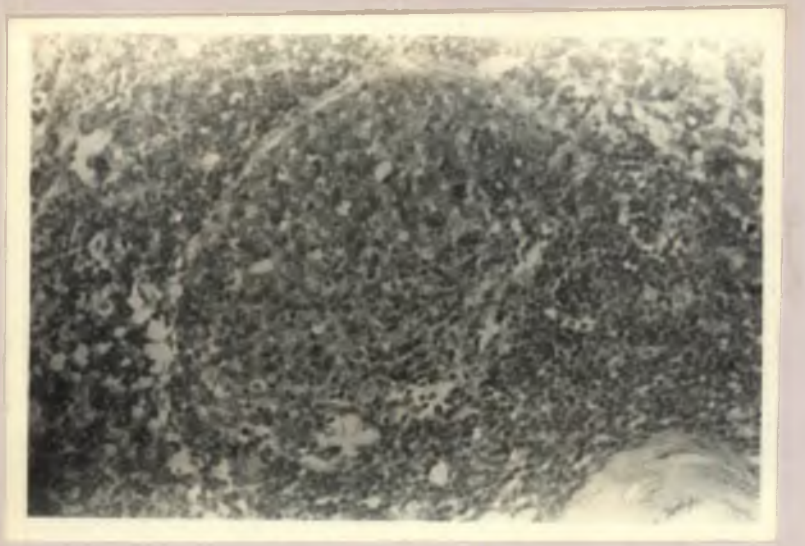
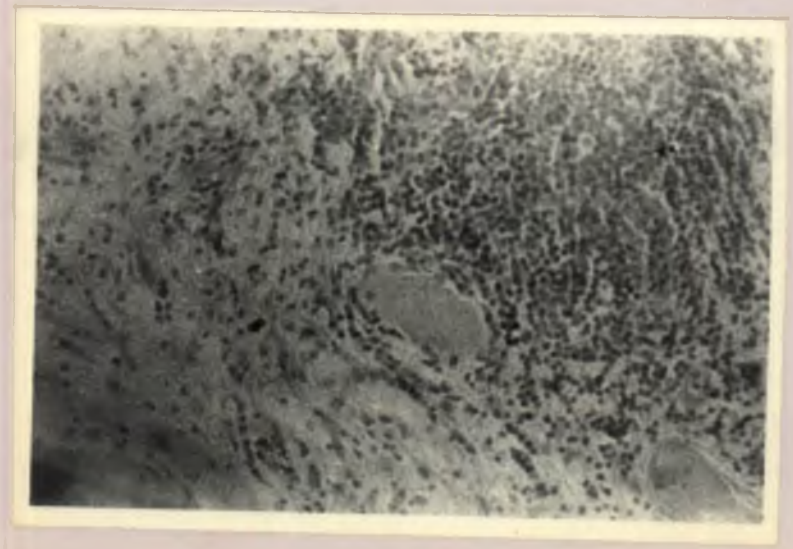


Fig. 31. Lymphnode: A reactive hyperplastic follicle surrounded by mature lymphocytes. H & E x 400

Fig. 32. Lymphnode abscess: Capsule showing hyalinisation. H & E x 400





germinal centres were seen in the cortex (Fig. 33). Focal depletion of lymphocytes from the cortical region and sinus histiocytosis in the medulla were evident.

An organised chronic suppurative focus was evident in the parotid lymphnode. Surrounding the focus, sinus histiocytosis and foreign body giant cell reaction were seen. There was also accumulation of lymphocytes and macrophages in the sinuses.

In the mediastinal lymphnode, medullary caseation was seen and the area of caseation was surrounded by large number of macrophages, histiocytes and few giant cells (Fig. 34).

The histological picture of the tracheal, mesenteric, popliteal and renal lymphnodes was not much different from what was observed on the 15th day.

45th day post-infection.

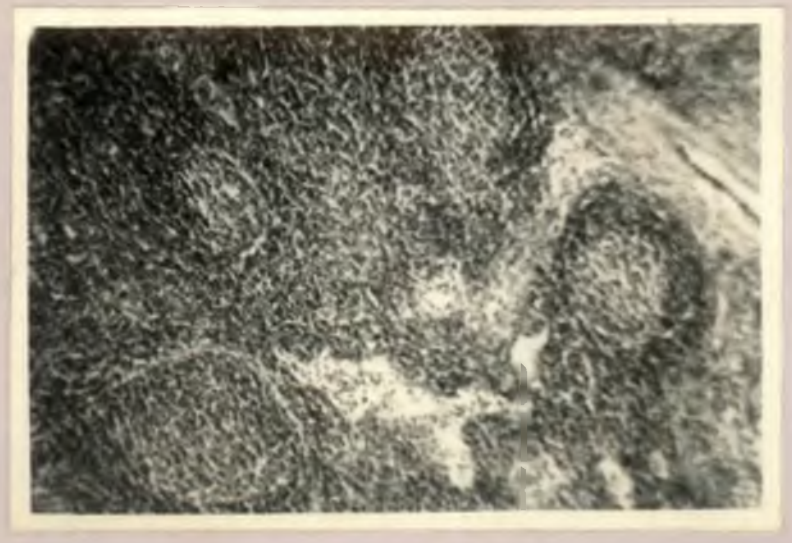
Prescapular lymphnode presented hypertrophied follicles in the cortical region. Scattered areas of caseation necrosis were seen in the paracortex and the medulla contained only few lymphoid cells.

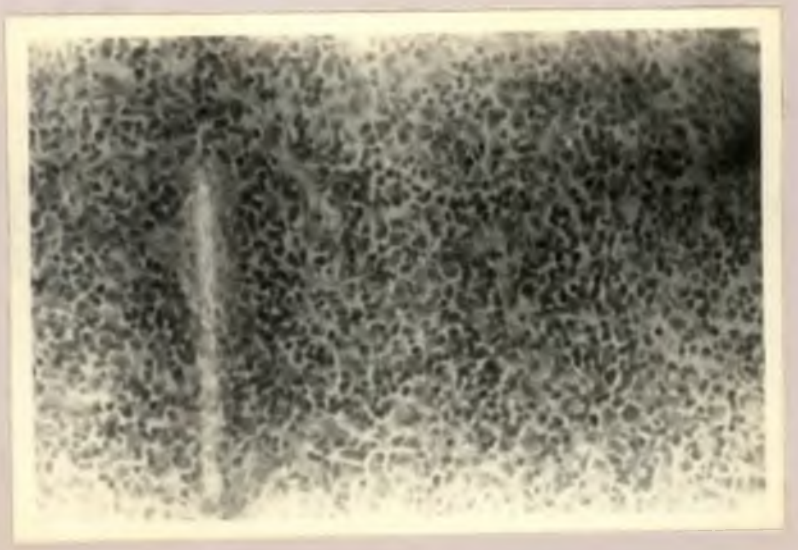
Caseated mass replaced the lymphnode parenchyma and pronounced fibrosis was seen in the prefemoral lymphnode.

Submaxillary lymphnode was seen to contain stimulated hypertrophic follicles with large and small germinal centres. Focal depletion of lymphocytes from the cortical region and sinus histiocytosis in the medulla were evident.

Fig. 33. Lymphnode cortex: Several hyperplastic follicles with active germinal centre. H & E x 250

Fig. 34. Lymphnode: Medulla showing accumulation of macrophages, histiocytes and giant cells. H & E x 250





In the parotid lymphnodes, the histological changes were almost similar to that seen on the 30th day.

The changes in the mediastinal tracheal, mesenteric, popliteal and renal lymphnodes were not much different from what was observed on the 30th day.

60th day post-infection.

Prescapular lymphnode showed reactive response of the lymphocytes and follicular hyperplasia was the characteristic feature.

In the prefemoral lymphnode, the lymphoid follicles at the cortex showed hyperplasia. The caseating foci were extensive and calcification was evident. This was associated with fibrous tissue proliferation around the foci and at the medullary region. The granulomatous reaction was accompanied by the accumulation of mature lymphocytes at the periphery of the lesion (Fig. 35).

Lymphoid hyperplasia and accumulation of lymphocytes in the medulla and sinus histiocytosis were seen in the submaxillary lymphnode. The sinuses were filled with lymphocytes. Fibrous tissue encapsulated focal areas of calcification and caseation surrounded by layers of macrophages and lymphocytes were seen.

Histological picture of parotid, mediastinal, tracheal, mesenteric, popliteal and renal lymphnode was not much different from that recorded on the 45th day.

75th day post-infection.

Caseation, calcification and fibrous tissue proliferation

were observed in the medullary region of the prescapular lymphnode. Groups of large lymphocytes were seen infiltrated into the fibrous capsule which surrounded the lesion.

Prefemoral lymphnode showed follicular hyperplasia and sinus histiocytosis.

Submaxillary lymphnodes showed hyperplastic follicles with well developed germinal centres.

Focal cessation, depletion of lymphocytes and accumulation of histiocytes were the features seen in the parotid lymphnode. Sinus histiocytosis, accumulation of macrophages, epitheloid cells and giant cells were also noted in the medulla (Fig.36).

Histologically, the mediastinal, tracheal, mesenteric, popliteal and renal lymphnodes were reactive with hyperplastic follicles. Tracheal lymphnode showed capsular sclerosis, engorgement of venules and infiltration of mononuclear cells.

90th day post-infection.

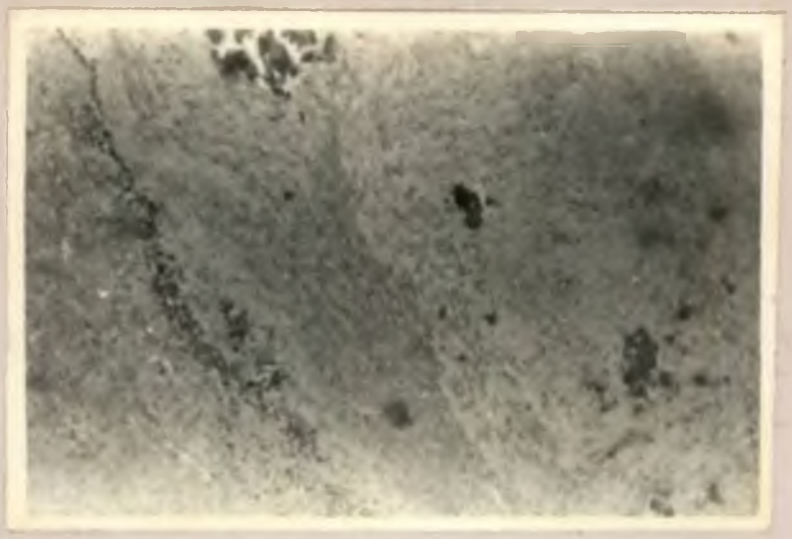
Prescapular lymphnode showed focal suppurative foci in the cortical region.

The paracortex of the prefemoral lymphnode presented hypertrophic follicles and proliferative lymphocytes. There was lymphoid depletion from the germinal centres of the cortical lymphoid nodules.

Submaxillary lymphnode was oedematous and there was no differentiation into the cortex and medulla. There was degenerative and necrotic changes of lymphocytes in the cortex and

Fig. 35. Lymphnode: Area of coagulation and calcification surrounded by fibrous tissue reaction. H & E x 250

Fig. 36. Lymphnode: Histiocyte, macrophage and giant cell reaction. H & E x 250



medulla. Depletion of lymphocytes from focal areas of cortex was seen. Besides macrophages, giant cells were also observed in the medulla.

The parotid lymphnode contained foci of caseation, calcification and these were surrounded by a layer of proliferating fibrous tissue. Foreign body giant cells and epithelioid cells were seen at the periphery of the suppurative lesion.

Mediastinal and mesenteric lymphnodes presented a picture of reactive lymphnode with more pronounced fibrosis.

Tracheal lymphnode was with capsular sclerosis, engorgement of venules with infiltration of mononuclear cells.

Popliteal and renal lymphnodes showed focal caseation at the medulla with sinus histiocytosis and plasma cell reaction.

Liver.

There was no tissue changes on the 15th day. However, by the 30th day there was granular degeneration and by the 60th day focal areas of fatty changes and necrosis were seen. The central vein and sinusoids were engorged. There were mild to moderate necrobiotic changes on the 75th and 90th day of infection.

Kidney.

There was mild granular degenerative changes in the tubules of the kidney from day 30 onwards. However, by day

75 and 90, there were foci of necrosis in the tubules and haemorrhagic foci were seen in the medulla.

Spleen and thymus.

Spleen and thymus did not show any histological changes.

Discussion

DISCUSSION

Caseous lymphadenitis caused by C. pseudotuberculosis in caprines is now recognised as a world wide problem (Ashfaq and Campbell, 1980; Burrell, 1981) and is one of the major causes of economic loss to the goat industry. Though the importance of the disease in goats is undoubtedly felt, reports giving information on mode of transmission and perpetuation, pathogenesis, immune response, morbidity, mortality and other epizootiological factors are few. The present study reports, the results on immune responses and pathogenicity of C. pseudotuberculosis experimental infection in goats.

Experimental infection of animals with C. pseudotuberculosis was previously attempted with several forms of inocula such as suspension of evenly distributed individual cells, bacteria grown as pellicles and broth culture. In the present study C. pseudotuberculosis suspended in chilled sodium chloride bile salt solution subjected to repeated shaking and centrifugation was quite useful for the preparation of a stable suspension of evenly distributed single cells which could be employed for experimental infection of goats. The bacterial cells remained in even suspension with uniform distribution for fairly long time and the viability of the cells did not decrease even after six hours of storage of suspension under chilled conditions. Barring the presence of stray numbers of small clumps which comprised 3 to 4 bacteria the suspension was uniform with single cells and the bacterial concentration

was enumerable by counting in Petroff Hausen counting chamber.

It was Jolly (1965a), who first reported the preparation of single cell suspension of C. pseudotuberculosis in chilled bile salt-saline solution during his studies on the pathogenic action of the bacterium on the vascular bed. He had observed that the method was successful in obtaining single cell suspension most of the time but occasionally a few clumps were encountered. The same method was subsequently adopted by Hard (1969a) for the inoculum preparation for experimental infection of mouse.

Cameron (1972) observed that bacteria derived from pellicles in static culture were superior to dispersed bacteria derived from shake culture for intravenous injection of sheep for the establishment of discrete abscess in visceral organs.

Several methods like, resuspending bacteria in buffer solution (0.02 M pH 7.2-7.4) containing 5% lactose and 1% peptone (Cameron and Minnar, 1969; Cameron et al., 1972); use of supernatant of heavy bacterial suspension in saline which was allowed for gravitational settling for one hour at room temperature (Zaki, 1966) and growing bacteria in dialysis bag containing phosphate buffered saline, suspended in nutrient medium (Ashfaq and Campbell, 1980) were advocated for the preparation of bacterial suspension to be employed for experimental infection. Few other workers were of the opinion that

mechanical agitation of C. pseudotuberculosis growth in saline would produce only suspension of bacteria with clumps and single cells (Burrell, 1978; Brown et al., 1985).

The results of the present experiment showed that the suspension of single cell bacteria, though contained a few clumps also, did not evoke severe local reaction or chronic abscess formation at the site of injections. Instead the preparation used was found to be able to initiate chronic lymphadenitis. Thus the present methodology adopted for the preparation of single cell suspension was satisfactory to experimentally establish chronic caseous lymphadenitis in goats.

Goats which were injected with 2×10^6 bacteria per site of injection had developed specific lesions and abscess in adjacent lymphnodes. The number of bacteria used for experimental infection could produce chronic lymphadenitis in goats as the dose did not produce fatal infection or acute illness. During the first week of infection, all the goats had shown febrile response with inflammatory reaction at the site of injection followed by general weakness, lethargy and inappetance. These initial symptoms were attributable to the reaction of the animals to the massive dose of bacteria and their toxic products received at different sites in the body.

Similar results were observed by Ashfaq and Campbell (1980) when they had used 1×10^6 CFU of C. pseudotuberculosis for experimental infection of goats by different routes.

Brown et al. (1985) reported that a dose of 0.5×10^6 CFU of caprine strain of C. pseudotuberculosis was sufficient to produce chronic abscessation of lymphnodes in goats. According to Gameel and Tarteur (1974) subcutaneous injection of 3.8×10^5 bacteria could produce acute severe haemolytic anaemia and death in sheep within four days of inoculation and 1.9×10^5 to 0.76×10^5 bacteria could produce only subacute to chronic infection. Cameron (1972) had also observed that severity of infection in sheep was directly related to the dose of bacteria administered and his view was supported by Brodgen et al.(1985).

The results obtained in the present study evidenced the comparative resistance of caprines to C. pseudotuberculosis infection as all the goats resisted fatal infection even after inoculation with a massive total dose of 1.2×10^7 bacteria per animal. Comparison of the results obtained in the present study and those reported by other workers suggests that goats are more resistant than sheep. Moreover it could also be inferred that a relatively greater number of bacteria could be inoculated into goats without causing acute or fatal form of CLA.

Natural infection of animals with CLA was shown to be by several routes viz., skin contamination and wound infection (Nairn and Robertson, 1974; Nagy, 1976) inhalation and ingestion (Carne, 1932; Maddy, 1953) and transmitted buccal mucosa (Burrell, 1981; Campbell et al., 1982). Experimental infection of animals with C. pseudotuberculosis had been successful

with several routes viz., intravenous (Cameron et al., 1972; Bredgan et al., 1986) intralymphatic (Husband and Watson, 1977; Burrell, 1978) subcutaneous (Gansel and Tartour, 1974) and skin scarification (Nairn and Robertson, 1974).

In the present study goats were inoculated with live C. pseudotuberculosis suspension by three routes - submucosal, subcutaneous and intradermal. All the three routes of inoculation produced lesions suggestive of CLA in lymphnodes at the immediate vicinity. No absolute preference was noted with any of the routes for the ability to set lesions in the lymphnodes. The incubation period taken for the development of detectable lesions in lymphnodes also did not show any preference as the regional lymphnodes were uniformly affected within 14 to 21 days post-inoculation, irrespective of route employed. Necropsy findings indicated that these routes of inoculations were generally incapable of setting up generalised infection in deep seated lymphnodes or visceral organs within 13 weeks of observation period except for the involvement of mediastinal lymphnode in two animals under group II. This finding was in close agreement with the results reported by Ashfaq and Campbell (1980) who employed 14 goats for experimental infection, out of which only one goat developed focal abscess in lung parenchyma and mediastinal lymphnode. Brown et al.(1985) also reported abscessation of mediastinal lymphnode of goats which received C. pseudotuberculosis at the flank region by intradermal route. But several others (Sarkar and Bhattacharya,

1975; Sharma and Dwivedi, 1977; Nairn et al., 1982) were of the opinion that goats are more susceptible for thoracic form of CLA in case of natural infection. The results of the present study indicate that the goats when experimentally infected evinced thoracic form of CLA only in isolated cases.

C. pseudotuberculosis is well known for its ability to produce potent exotoxin when grown in vivo or in vitro (Wilson and Miles, 1980).

In the present study, growth of C. pseudotuberculosis in Lemco proteose broth containing sheep serum had produced maximum toxin in the culture supernatant by 72 h of aerobic incubation at 37°C. The pH of the culture was found to get lowered from 7.5 to 5.00 ± 0.2 as the toxin accumulated in the medium.

Similar procedure was reported by Burrell (1979) for the preparation of toxin from several strains of C. pseudotuberculosis and he observed that an atmosphere containing increased CO_2 was superior for maximum toxin production.

The toxin content of the culture supernatant as assayed by dermonecrotoxic action on rabbit skin and haemolytic action on sheep red cells, attained the peak level at 72 hours of incubation, following which there was gradual reduction in the toxin level. The culture supernatant was found to contain a maximum haemolysin titre of 1:256 at 72 hours of growth. Intradermal inoculation of this toxic culture supernatant produced characteristic dermonecrotic reaction in rabbit skin. The inflammatory and necrotic reactions were maximum by

48th hours. The point of injection was seen exudating sero-purulent fluid by 72 hour post-injection. The reactions subsided and the area healed up within a weeks time.

Among the various biological properties of the toxin from C. pseudotuberculosis, dermonecrotoxicity to rabbit skin is most commonly used for assaying its potency (Doty et al., 1964; Burrell, 1979). When Lemco protease broth was used for toxin production from several strains of C. pseudotuberculosis, Burrell (1979) reported a maximum titre of 1:32 assayed by rabbit skin test. Burrell (1980a) observed a very close comparison between haemolysin and rabbit dermonecrotoxin titre. High yield of C. ovis exotoxin (haemolysin titre 1:32768) was reported to be produced by Burrell (1981) when Lemco protease broth was periodically supplemented with dextrose under controlled pH.

Several other media and methods for the production of exotoxin from C. pseudotuberculosis have been employed with fairly good results (Carne, 1940; Cameron and Swart, 1965; Keskintepe, 1976). Cameron and Smit (1970) showed that exotoxin preparations from C. ovis contained antigens derived from products of bacterial lysis.

The medium used and gaseous and temperature requirement provided for the growth were found to favour the production of sufficient quantity of toxin by C. pseudotuberculosis - ATCC 19410, which could be detected by haemolytic and dermonecrotoxic property. The results also indicated the ability

of the strain used to produce detectable quantity of toxin by 24 hours which increased to reach the peak level by 72 hours and then declined gradually thereafter. These observations deviate from the findings of Burrell (1979) who reported toxin production only after 48 hours, attaining the peak titre at 72 hours and persisted at the same level till 120 hours. This difference in results could probably be attributed to the difference in the strains used.

Serum protein concentration is known to be lowered by several factors such as blood loss, hepatopathy, malnutrition and heavy parasitism, while it is increased as age of the animal advanced with a decrease in albumin and increase in globulin fractions. Though plasma proteins fall within normal range in many disease conditions, elevated concentration occurs in many chronic diseases. The reason for this increased plasma concentration is understood to be either due to dehydration or increase in gamma globulin synthesis or both.

Goats which were used in the present study had normal serum protein concentration which ranged from 7.187 to 9.730 gm%. Before the start of the experiment, group I goats had a serum protein concentration of 8.579 gm % while group III had 9.071 and the difference in values between the groups was not statistically significant. Group I goats showed a significant increase in serum protein content from the 5th week of infection onwards.

After experimental infection the serum protein concentration in group I goats showed an initial decline but from the

2nd week onwards there was an increase, the maximum level being 11.346 gm % at the 8th week. Throughout the period of observation, the Group III control goats presented only minimum variation in serum protein content and it ranged between 8.874 and 10.615 gm %.

The mean concentration of serum protein at the preinfection period was more with group III goats as compared to group I goats. The situation was reversed as infection advanced in group I goats. Though there was increase in serum protein, significant difference from that of control was observed only during the 5th, 6th, 8th, 9th, 10th and 12th week post-infection.

The results presented here showed a higher serum protein concentration in normal cross-bred Malabari goats as compared to normal values published by Schalm (1970). Castro et al. (1977) estimated normal serum protein concentration for pygmy goats as 7.3 ± 0.7 g % by microkjeldahl method and Lewis (1976) reported as 8.35 g % by electrophoresis.

The quantitative or qualitative change in the serum protein profile of C. pseudotuberculosis infected animals was not reported by many workers. Gamal and Tartour (1974) observed that sheep which developed acute disease had high serum protein content while it was reduced when animal suffered subacute to chronic form of infection. The rise in protein value was considered due to the presence of free haemoglobin in plasma as a result of in vivo haemolysis while the decrease was considered as an effect of stress and inappetance which supervened after infection.

The present result indicated that as corynebacterial infection progressed in goats, the serum protein concentration increased, while there was no substantial change in control goats as the age of the animals advanced. These observations are in close agreement with the results reported by Mottalib et al. (1979) and Desiderio et al. (1979) with respect to the serum proteins of sheep with CLA. According to Schalm (1970), in chronic form of diseases, especially in those involving antigen antibody reaction, the plasma protein was found significantly increased above normal. As a controversy to these observations, there are reports of decrease in serum protein (El-Abdin et al., 1977) or no change (Brown et al., 1985) in goats affected with CLA.

Electrophoresis is considered as a valuable technique for evaluating the quantitative and qualitative distribution of serum protein fractions. Agar gel electrophoresis method which was followed in the present study was able to separate distinct protein fractions of serum of goat. The electrophoretogram clearly distinguished albumin, alpha globulin, beta globulin and gamma globulin fractions of serum while subfractions of globulin could not be uniformly discerned in all cases. Separation of protein fractions of serum into albumin, alpha-1, alpha 2, beta and gamma globulins by paper electrophoresis was reported by Lewis (1976) and Castro et al. (1977).

With several samples the protein phases were seen overlapped in electrophoretograms and hence quantitation of

individual globulin fractions could not be attempted. Osbaldiston (1972) had quantitated normal serum protein fractions of domestic animals using electrophoresis on cellulose acetate medium. He too had observed that several of the serum samples did not provide distinct separation into protein containing zones in the electrophoretograms.

With the present study the albumin-globulin ratio was easily obtained by densitometer tracing of the electrophoretograms and this ratio was used to work out the quantitative shift in the major serum protein fractions of goats infected with C. pseudotuberculosis.

During the post-infection period, group I goats showed an increase in globulin content only during the 1st and 4th week while with all other samples the globulin content was found to be decreased. The globulin content was found to be uniformly increased with serum samples collected from control goats throughout the period of observation except at the 7th and 11th week. Though there were some apparent difference with A:G ratio between infected and control animals, significant difference was recorded only during the 1st week of infection.

Castro et al. (1977) reported the normal A:G ratio of Pygmy goats as 0.8 ± 0.3. Gamael and Tartour (1974) reported a definite decrease in serum gamma globulin in sheep suffering from subacute form of CLA, while El-Abdin et al. (1977) and Brown et al. (1985) observed no change.

The present results showed that with infected goats, there was significant increase in total serum protein while the concentration of globulin fraction relatively decreased. The increase in total serum protein might be due to the free haemoglobin in the serum consequent to the haemolytic action of toxin from C. pseudotuberculosis as reported by Gansel and Tartour (1974). Brown et al. (1983) observed that there was a drastic increase in fibrinogen content in the serum of goats immediately after experimental infection with C. pseudotuberculosis, which would also account for the increased total serum protein. In an immune stimulated animal, an increase in protein content need not be due to the increase in gamma globulin fraction alone. The immune complexes formed and resorption of the pus might also result in increase of total serum protein (Schalm, 1970).

The globulin content in sera of control goats did show slight increase and this might probably indicate that the control animals had reacted normally to the extraneous agents to which they might have got exposed. On the other hand, globulin fraction of infected goats was found relatively decreased and this probably indicates the possibility of suppressed globulin production, in general by C. pseudotuberculosis infection, even though it elicited specific antibody production.

Humoral antibody response to C. pseudotuberculosis infection in animals was assessed by several serological methods and each method was reported to have advantages and

disadvantages (Shigidi, 1979). Haemolysis inhibition test was shown to be highly efficient in detecting antibody to C. cruzi exotoxin in naturally/artificially infected animals (Burrell, 1980a) and in actively/passively immunised animals (Burrell, 1981; Lund et al., 1982a and b). Due to the simplicity of the procedure and the high efficiency of the test reported, HIT was employed in the present study to monitor the antibody response.

Specific antibody activity in the serum of infected goats was seen with the increase in haemolysis inhibition titre. The control animals remained seronegative with HIT, throughout the observation period. In the present study, the group I goats which were infected experimentally, showed detectable level of specific antibody from the 3rd week post-inoculation. The peak antibody level was achieved by the 5th week of infection and thereafter the titre was found to dwindle gradually till the 11th week. Shen et al. (1982) recorded similar results when antibody response was monitored in goats employing ELISA technique.

During the later part of the observation period (12th and 13th week) a marginal increase in the antibody titre was noted and this could possibly indicate secondary immune response by the animals which were continuously exposed to the tsetse. Evidences for secondary response during the later period of infection have been put forward by Husband and Watson (1977).

or its products was considered as an indispensable mechanism by which the immunity is mediated in animals. The results obtained in the present experiment also indicated that during in vivo multiplication of bacteria, it liberated sufficient antigenic toxin which induced humoral antitoxin production and diagnostic level of antitoxin in serum was detectable in infected animal by HIT from the 4th week onwards.

The leukocyte counts obtained before the start of the experiment with goats from group I and III (10718 ± 627 and 11242 ± 630 respectively) were in close agreement with the normal values for the species described by Schalm (1970). The present data thus indicated that there were not much difference in the leukocyte counts of Malabari cross-bred goats from that of other breeds. The total leukocyte counts recorded for group III control goats were again within the normal range (9964 ± 368 to 12157 ± 733) during the entire observation period.

On infection of group I goats with C. pseudotuberculosis, the animals presented marked leukocytosis and the degree of leukocytosis seemed to fluctuate as days passed by. The periodical leukocytosis might indicate the recurrent flare up of bacterial cells in tissues. It was worth to note that the peak leukocytosis in group I goats was during the 2nd week of infection, probably the time at which massive number of virulent bacteria attack the host system and to which the phagocytic system reacts drastically with the resultant leukocytosis.

The absolute lymphocyte counts obtained both at pre and post infection periods of group I and III goats did not show significant differences and the values were comparable to the normal counts reported for the species by Schalm (1970). Percentage distribution of lymphocytes in differential counts showed a substantial reduction during the first three weeks, and moderate reductions at 5th, 9th and 13th weeks of infection. It was worth to note that throughout the period of observation, the infected animals showed numerically low lymphocyte percentage compared to control goats, but absolute count remained unaffected. This indicated that the infection did not detrimentally influence the lymphocyte populations but the percentage distribution of these cells was kept low in the peripheral blood consequent to the increase of other leukocytes.

Consequent to infection, the absolute neutrophil counts recorded for group I goats were consistently high throughout the observation period, compared to control goats. The increase in neutrophil counts was significant except during the 8th and 11th week of observation.

The percentage distribution of neutrophils recorded in differential counts was uniformly high with group I infected goats when compared to control goats but the differences between the group values were significant only during 1st, 2nd, 3rd, 5th, 9th, 10th and 13th week of observation.

Similar results were reported earlier in sheep infected with C. pseudotuberculosis by Gumsel and Tartour (1974) and

they observed absolute leukocytosis with a hike in neutrophil number and a drop in lymphocyte percentage in differential count. Bacterial infection with localisation and pus formation stimulates marked neutrophilia. Considering the slow and progressive nature of the C. pseudotuberculosis infection, it would be appropriate to consider that the period of neutrophilia to coincide the time of multiplication and spread of bacteria in the body.

Maddy (1953) reported that the leukocidin which killed leukocytes and deposited in situ was responsible for abscess formation by C. ovis. Cameron and Smit (1970) demonstrated the in vitro leukocidal activity of the protoplasmic toxin of C. ovis and opined that leukocidal action of toxin contributed the necrotising property of the bacteria.

The results obtained in the present study did not evidence any in vivo leukocidal property of the toxin, as there was no reduction in absolute leukocyte count in the peripheral blood, even after the infected animals had developed abscess in lymphnodes.

The absolute count or the percentage distribution in differential count with regard to eosinophil, monocyte and basophil did not differ between the infected and control goats, which indicated the noninvolvement of these cell types in the pathogenesis or immune response mounted against C. pseudotuberculosis infection in goats.

Quantification of cellular changes brought out by the

immune response in the system would be possible only by separating the effector cells (lymphocytes) and their characterisation. Assays of T and B-lymphocytes in the peripheral blood are currently in wide use in clinical immunology for understanding immunity to infectious diseases (Stites, 1980).

Separation of the peripheral blood mononuclear cells from several species of animals has been successfully done by density gradient centrifugation (Boyum, 1968; Bowles et al., 1975; Cutteridge et al., 1981; Sulochana et al., 1982; James, 1986). Banks and Greenlee (1982) reported the requirement of a higher density and gravity (1.079 to 1.082 g/ml centrifuged at 1200 x g) for better separation of goat mononuclears, but the present study had proved a low density and gravity (1.077 g/ml centrifuged at 720 x g) would be sufficient for the separation of goat mononuclears. Sulochana et al. (1982) also successfully employed similar methodology as adopted in the present study.

The separated mononuclear cells were found to contain on an average 91.8 % lymphocytes and 8.2% monocytes with an average viability of 91.2%. Almost similar values were reported by Banks and Greenlee (1982) when mononuclears of goat, separated by isopycnic centrifugation.

Assessment of lymphocyte populations and their characterisation would provide evidences of alteration in the immune system due to disease, malignancy or any other abnormality. The lymphocytes are distinguished into two major subpopulations -

T and B-lymphocytes - based on distinct surface markers and their functions.

Characterisation of lymphocyte subpopulations and changes in the B and T-cell ratio might provide an insight into the effectiveness of the immune response or about the aberrant nature of the infection. Since the lymphocytes in the peripheral blood are considered as a crosscut representation of the total lymphocyte population in the body, assessment and characterisation of peripheral blood lymphocytes would tell upon the general changes in the immune system of the body.

The B and T-lymphocyte subpopulations of goats have not yet been studied by many workers.

B-lymphocytes of human and animal origin are readily distinguished from other subpopulations based on the presence of membrane Ig and receptors for complement and Fc region of IgG (Winchester and Ross, 1976). Cells bearing surface Ig are quantitated by staining with fluorescinated anti Ig, whereas cells possessing receptors for Fc and C3 are enumerated by EA and EAC rosette assays respectively.

EAC rosette assay is widely used to distinguish B-cells (Bianco et al., 1970; Ehlenberger and Nussenzweig, 1976). EAC rosette assay method employing bovine red cells was considered superior over methods employed with sheep red cells, since bovine red cells do not form spontaneous rosette with lymphocytes (Stites, 1980). B-cells enumerated by EAC rosette assay from bovines ranged 12-22% (Wilkie et al., 1979;

Kaura et al., 1979) equine 21% (Nayar and Schlegar, 1978) porcine 16.6-19.2% (Shimmon et al., 1976; James, 1986) and ovines 25.8% (Cutteridge et al., 1981).

During the present study, the normal percentage of B-cells in the peripheral blood of goats was estimated to range between 8.56 and 12.3 by EAC rosette assay. More or less similar percentage of B-cells in the peripheral blood of goat was reported (Sulochana et al., 1982; Banks and Greenlee, 1982; De Martini et al., 1983) while higher percentage (35.12 ± 7.02) was reported by Yang and Shien (1980).

When B-cells were quantitated at weekly intervals, there was significant increase in the percentage of cells with group I goats by the 2nd week of infection compared to group III goats. This increase was recorded till the 10th week of infection except at the 5th week, during which period the increase was not statistically significant. During the post-infection period B-cell percentage ranged 12.3 ± 0.85 - 17.62 ± 1.2 with group I goats while in control goats it ranged 8.56 ± 0.75 - 12.3 ± 1.09. The results thus presented here indicated that C. pseudotuberculosis infection in goats had produced uniformly high B-cell count in peripheral blood, compared to control goats.

It would be worth while to note that the specific antibody activity in the serum of infected goats was also found to be increasing from the 2nd week and it attained the peak level by the 5th week of infection. The increase in the antibody

titre in the serum seemed to be well corresponding with the increase in the B-cell population in the peripheral blood (see table 9 and 19). This observation shows a positive correlation of antibody titre and B-cell counts.

Since exotoxin was the antigen in the HIT, the test detected only the antitoxin in the serum of the animal. But the sustained increase in the B-cell count in the infected animal, would probably indicate the humoral antibody response to other antigens of the bacteria as goats were inoculated with live virulent C. pseudotuberculosis. Burrell (1981) observed multiple precipitin lines with exotoxin preparation and serum from CLA affected sheep. Though development of antibody against toxin, cell wall, protoplasm and whole cell antigens had been observed, absolute immunity to C. pseudotuberculosis could not be established in either of the above responses (Jolly, 1965a; Cameron and Minnar, 1969; Cameron and Purdon, 1971).

Brodgen et al. (1985) showed that cell wall antigens were superior over whole cell antigen for antibody production in lambs and peak level of antibody attained by 41st day post-injection. Husband and Watson (1977) had observed only poor antibody response in the serum and lymph of sheep, following injection of live/killed C. ovis into the afferent popliteal lymphatic duct. They demonstrated the increased output of blast cells having surface IgM (indicative of primary response) and IgG (long standing secondary response) from the infected lymphnode as direct evidence for humoral immune response.

This immune response was pronounced and prolonged with live bacteria when compared to killed C. ovis and effect could be due to the continued liberation of the antigenic substances from multiplying bacteria.

T-lymphocytes of vertebrates have been identified and enumerated employing several markers. Out of the many assaying techniques described, demonstration of lymphocyte specific surface antigens by using monoclonal antibody (Khan et al., 1980; Reinhers et al., 1981), non-immune rosette (E-rosette) formation with heterologous erythrocytes (Jondal et al., 1972; Collins et al., 1976) and demonstration of non-specific enzymic alphanaphthyl acetate esterase (ANAE) activity (Knowles et al., 1978; Reddy et al., 1980) are routinely employed to identify T-cells.

In the present study also T-lymphocytes of goats were successfully identified and enumerated by E-rosette assay and ANAE activity.

Sheep erythrocytes formed spontaneous rosettes with goat peripheral blood lymphocytes when suspended overnight in tissue culture medium supplemented with 20% calf serum. Majority of such rosettes presented erythrocytes at the entire periphery of lymphocytes which indicated the presence of several receptors to sheep red cells on the surface of goat lymphocytes.

Lymphocytes from several species of animals were shown to vary in their ability to form rosette with heterologous erythrocytes (Jondal et al., 1972; Taylor et al., 1975; Bowles et al.,

1975; Higgins and Stack, 1977; Kaura et al., 1979). According to Yang and Shain (1980) among the eleven heterologous erythrocytes which they tried, fowl erythrocytes were found to form maximum rosette with lymphocytes from peripheral blood of goat. But Sulochana et al. (1982) reported that only sheep erythrocyte formed E-rosette with goat lymphocytes, when they had employed, sheep, cattle and chicken red cells for comparison. The present study proves that sheep erythrocytes could very well be used for delineation of T-cells by E-rosette assay as observed by Sulochana et al. (1982).

In the present experiment the mean percentage of E-rosette positive lymphocytes ranged 24.55 ± 3.66 - 26.74 ± 1.34 in normal goats and almost similar value (26.51 ± 2.05) was reported earlier by Sulochana et al. (1982) for the same breed of goat. Yang and Shain (1980) reported that only 9.59 ± 2.06% of peripheral blood lymphocyte of goat were positive for E-rosette. On the other hand Banks and Greenlee (1982) had reported that 69 ± 11% of goat lymphocytes were T-cells when estimated by peanut agglutinin binding property and De Martin et al. (1983) recorded 57.4 ± 4% T-cells with the same technique. The difference in breeds of goats tested and sensitivity of each of the assaying system employed would explain the marked variations in values of T-cells of goats reported from this laboratory and from that of others.

E-rosette formation by T-cells was reported to be affected by many variables such as incubation time, temperature, serum concentration and the proportion of erythrocytes to lymphocytes

(Mendes et al., 1974; Woody, 1975; Grewal et al., 1976; Tarr et al., 1977). Treatment of erythrocytes with chemicals or enzymes has been shown to enhance rosette formation (Chapel, 1973; Escajadillo and Binns, 1975a; Paul et al., 1979a). In the present study the variables affecting E-rosette formation with goat lymphocytes have not been studied.

The enumerated level of E-rosette positive T-lymphocytes in controls did not show much variation over a period of 13 weeks (21.19 ± 2.11 - 26.86 ± 2.30). This observation substantiated the point that sheep erythrocyte rosette formation by T-cells of goat had not been influenced by unknown variables and the present method could detect T-cells of goats easily.

Cellular immune response is understood to play very vital role in conferring resistance against several facultative intracellular bacteria (Youmans, 1975; Collins and Campbell, 1982). Since T-cells are the pivot cells which mediate cellular immune response, estimation of circulating T-cells in the peripheral blood is an accepted method for determining the level of CMI in disease conditions (Campbell, 1976). T-cells are best enumerated by E-rosette assays either as active or total E-rosette forming cells. Active rosette forming cells are subpopulation of total rosette forming cells and the proportion between these subsets varies in different disease conditions (Yu, 1975; Wybran and Fudenberg, 1974). It has been further reported that number of active rosette forming cells would reflect better the T-cells competence as

prognostic aid in diagnosis of diseases (Wybran and Fudenberg, 1974; Prabhu and Reddy, 1983).

On experimental infection, group I goats showed a significant reduction in T-cell percentage (18.44 ± 1.40) compared to controls (24.83 ± 2.53) at the first week. By the second week also the E-rosette positive T-cells were found to be low (21.13 ± 1.82) but the reduction was not significant. When T-cells were enumerated based on ANAE activity, the concentration of these cells was not found to be changed significantly from that of controls during the entire observation period. Though a reduction in E-rosette positive T-cells was recorded, the absolute lymphocyte count during the period was not affected.

Though T-cells are identified and enumerated based on E-rosette assay and ANAE activity, it is reported that estimation of active rosette forming T-cells would be of more value for determining T-cell competence (Wybran and Fudenberg, 1974; Prabhu and Reddy, 1983). Moreover the stimulatory and functional alterations brought about in the lymphocytes were also found to influence the E-rosette formation and ANAE activity of the cells (Knowles *et al*., 1978).

Reduction of E-rosette forming cells in the peripheral blood of infected goats would probably indicate the transient functional alteration caused to this subpopulation of cells. Patients with acute bacterial and viral diseases have been shown to have low percentage of T-cells when assayed for total

E-rosettes and anti T-serum markers (Niklasson and Williams, 1974). Wyhran and Fudenberg (1973) reported that active E-rosette percentage was found decreased in viral diseases but remained normal in bacterial infections. In the present experiment also the goats developed transient acute symptoms of bacterial infection following inoculation of C. pseudotuberculosis and the period at which reduction in E-rosette positive cells observed, coincided with the acute stage of the initial bacterial infection.

Yet another possibility for the initial reduction of T-cells could be the temporary arrest of T-cell circulation from the lymphnode. The total lymphocyte number was found maintained either due to the blast cell response from the B-cell area or due to the active mobility of B-cells from secondary lymphoid organs as observed by Husband and Watson (1977). Zatz (1976) also observed that lymphocytes were found to be trapped within the draining lymphnodes of actively infected animals.

From the 3rd week of infection onwards, increase in the E-rosette forming T-cell percentage was recorded and significant increase was noted by the 5th, 6th, 7th, 8th, 12th and 13th week of infection, the maximum was recorded at the 13th week (35.24 $\pm$ 1.58%). During the above periods the T-cell percentage in the control goats did not show any substantial change as it ranged between 21.68 $\pm$ 3.09 - 25.52 $\pm$ 3.14. Even at the 9th, 10th and 11th week of infection, E-rosette positive

cells were numerically high but the difference from that of controls was not significant.

There are no published literature available on T and B-lymphocyte profile of animals affected with CLA except the report by Sulochana et al. (1982). They observed that compared to normal, CLA affected goats had only lower percentage of T-cells estimated by E-rosette assay. In the above mentioned study, the goats used were older (1 to 3 years old) as compared to younger goats (8 to 12 month old) employed in the present study. The stage of the infection in the goats which were employed for the study by Sulochana et al. (1982) was not assessed. Since older animals with natural infection were employed in their study, it would be appropriate to presume that the animals were suffering from advanced chronic form of CLA, wherein reduction of T-cells could be expected.

On the contrary, the results obtained in the present study showed a definite increase in E-rosette positive T-cells in the peripheral blood of goats, experimentally infected with C. pseudotuberculosis, from the 2nd week of infection onwards.

Several previous workers had pointed out the significance of cell-mediated immune response for conferring immunity to CLA (Jolly, 1965b; Hard, 1969a; Hard, 1970; Husband and Watson, 1977).

Cameron (1972) was of the opinion that neither the cellular immune response nor the humoral antibody response against the bacteria independently confer absolute immunity to C. ovis

but the participation of both the mechanism would be required.

Hard (1970) demonstrated adoptive transfer of immunity to C. cuniculus when peritoneal cells were transfered from immune mice to recipient mice. He had further observed that lymphocyte like cells and mononuclear phagocytes were apparently equally capable of inducing immunity in mice. The association of mature immune macrophages was postulated for the functioning of cellular immunity to C. pseudotuberculosis by Jolly (1965c) and Hard (1972).

Husband and Watson (1977) by demonstrating an increase in the number of lymphoblast output from infected lymphnodes demonstrated that immunity to C. pseudotuberculosis was cell-mediated.

The results presented in this study supported the theory of cell-mediated immune response to C. pseudotuberculosis since infected goats presented substantial increase of T-cells in the peripheral blood. The hike in E-rosette positive T-cells was not continuous and this could be anticipated since the immune response is mediated by a population of short lived lymphocytes which increase sharply in numbers following infection with most of the facultative intracellular bacteria, which decline after a remarkably short period of time as reported by North (1973).

From the clinical picture of the animals it was observed that all the lymphnodes were not simultaneously affected but there existed some variation in periods at which different

nodes were affected. The fluctuation in the E-rosette positive cells would probably correspond to the stage of infection of lymphnodes in the animals. Beh and Lascelles (1974) suggested a temporary cessation of recirculation of T-lymphocytes during the early phase of response in the lymphnode. Zatz (1976) demonstrated trapping of lymphocytes in the draining lymphnodes of tuberculous animals when BCG was injected.

Demonstration of ANAE activity is yet another marker considered for enumeration of T-cells. Considering the efficacy and easiness of the method, demonstration of ANAE activity is widely used for identifying T-cells in many species of animals (Knowles et al., 1978; Reddy et al., 1980; Rajan et al., 1982; Sulochana et al., 1982).

In the present study, T-cells of goat were identified and enumerated based on demonstration of ANAE activity. Goat lymphocytes which presented ANAE activity were with one or two localised nodular pink to red coloured reaction products in the cytoplasm adjacent to the cell membrane and it similated the T-pattern of ANAE activity described for human T-lymphocytes by Knowles et al. (1978). The reaction pattern obtained with lymphocytes of goat was similar to what had been described earlier for other species (Reddy et al., 1980; Valsala et al., 1981; Rajan et al., 1982).

Fixing of smears of mononuclear cells with acetone-citric acid solution had made possible to keep the fixed smears in dry state, which facilitated batch staining. Giorno and Beverly

(1981) had reported earlier, the usefulness of acetone-citric acid for fixing the mononuclear cells for the demonstration of ANAE activity and such fixing was shown not to interfere with the enzymic activity of the cell even when the fixed smears were dried and stored for longer periods. The results obtained in the present study also indicated the usefulness of acetone-citric acid solution for fixing of smears for ANAE staining. Unlike the procedure followed by Knowles et al. (1979) wherein the fixed smears need to be kept in wet state throughout the fixing and staining operations, the method of fixation followed in the present study, retained ANAE activity of cells even when the cells were dried after fixation.

Before the start of the experiment the mean percentage of ANAE positive cells in group I goats was 28.09 ± 1.51 while it was 35.80 ± 4.86 for group III control goats. The difference in values between the two groups was not significant. The mean percentage of ANAE positive T-cells ranged between 30.83 ± 3.5 - 36.91 ± 3.61 during the 13 weeks observation period in group III control goats, while it ranged 28.91 ± 2.06-33.78 ± 1.99 for group I infected goats. Though a slight numerical increase in the ANAE positive T-cells was observed in infected goats, compared to their preinfection normal values, it did not show any significant difference from that of the controls.

The mean percentage of T-cells estimated by E-rosette assay for group I and group III goats (26.74 ± 1.34 and 24.55 ± 3.04 respectively) was less compared to T-cells enumerated by

ANAE activity (28.09 ± 1.52 and 35.86 ± 4.8 respectively) during the preinfection period. Significant hike in E-rosette positive lymphocytes concentration was recorded in group I goats after infection while such change could not be observed with ANAE positive lymphocytes.

Earlier Sulochana et al. (1982) recorded ANAE positive lymphocytes from normal goats as 26.89 ± 2.56 % in the peripheral blood cells which is near to the values obtained in the present study. The normal level of ANAE positive lymphocytes in the peripheral blood was reported to vary according to the species of animals tested but remained at more or less constant level within the species (Reddy et al., 1980; Rajan et al., 1982).

Knowles et al. (1978) and Knowles et al. (1979) employed E-rosette assay and ANAE activity simultaneously for evaluating the lymphocytes of man and reported that the percentage of E-rosette forming cells was nearly comparable to the ANAE positive cells in the peripheral blood. Totterman et al. (1977) and Knowles et al. (1978) had further observed that lymphoblasts obtained by stimulating T-cells with mitogen were able to retain the E-rosette formation property but lost their ANAE activity. It was also demonstrated that ANAE negative blast cells could regain ANAE activity on reversion to lymphocytes.

In the present study, there was substantial increase in E-rosette forming cells in the peripheral blood of infected goats while such change was not noted with ANAE positive cells.

eventhough both the assays were intended to identify the T-lymphocytes. When goats were infected with C. pseudotuberculosis, the bacterial antigens would necessarily stimulate the specific clones of lymphocytes which in turn get transformed to lymphoblasts. The mature T-lymphocytes and lymphoblasts from such animals were expected to form E-rosettes, which might have accounted for the increase in total E-rosette forming cells. Since lymphoblasts were known to be devoid of ANAE activity, corresponding increase in ANAE positive cells could not be recorded in infected animals as against E-rosette forming cells. This observation supports the view that estimation of active rosette forming cells reflects the T-cell competence in disease conditions (Wybran and Fudenberg, 1974; Prabhu and Reddy, 1983).

Antigen dependent inhibition of leukocyte migration was considered as a reliable measurement of cell mediated immunity and the test was often used as an *in vitro* method to assess cell-mediated immunity in many infectious diseases. The results obtained in the present study indicated positive leukocyte migration inhibition in all goats experimentally infected with C. pseudotuberculosis which also pointed the role of cell-mediated immune response in this infection.

In the present method leukocytes from peripheral blood was collected by flash lysis of RBC using distilled water and subsequent centrifugation. High percentage of viability (average 90%) for such separated cells was observed and the

method was found easy and reliable for separation of leukocytes from peripheral blood. The same method was previously employed by several other workers (Bendixen, 1977; Dorsey and Doyce, 1982; Sulochana et al., 1983). Leukocytes from blood was separated by several other methods viz., osmotic shock with 0.83% ammonium chloride solution (Waldshimet and Scott, 1982; Chambers and Klesius, 1984), Ficoll-Hypaque technique (Asadegan et al., 1981) and sedimentation for buffy coat collection (Rosenberg and David, 1970; Timms, 1979).

A population density of 1.5×10^6 cells/ml was found suitable for LMIT with goat leukocytes. Asadegan et al. (1981) had observed that as the concentration of cells increased, the migration zone was also increased.

The culture supernatant having exotoxin activity (where pH adjusted to 7.2) prepared from C. pseudotuberculosis could be successfully used as antigen in the test. The toxin concentration was uniform to contain a haemolysin titre of 1:16 and at this concentration and pH, it was found non-toxic to leukocytes as evidenced with the normal migration of cells from control animals. Chandiramani and Garg (1982) reported the use of sonicated C. ovis cell extract as antigen in LMIT.

The direct LMI assay under agarose was considered a simple and rapid in vitro technique, but the results have been critically affected by a number of factors such as cell concentration, pH, length of incubation and endotoxin action (Chambers and Klesius, 1984). In the present study variables

affecting the test were not studied as it was not the primary objective.

Before the start of the experiment the average migration index recorded for both group I and III goats was 0.93 ± 0.02 and 0.94 ± 0.01 respectively, and the difference between the groups was not significant. After infection of group I goats, the migration index recorded at 15 days intervals showed reduction. Significant reduction of migration index was noted by the 45th day post-infection and the maximum reduction recorded by the 60th day (0.56 ± 0.97). It was observed that leukocytes from all experimentally infected goats produced migration index less than 0.8 during the post-infection period (75 days observation) while leukocytes from control goats exhibited migration index of above 0.8.

Migration inhibition of leukocytes was considered as a measure of CMI response and used to diagnose microbial infections (Buening, 1972; Bendixen, 1977; Timms, 1979; Asadegan et al., 1981; Dorsey and Dayoe, 1982). It was reported that cellular immunity required the involvement of living cells and was dependent on the presence of antigen in the host. Even then the cell-mediated immune response was found to be of short duration especially against intracellular bacteria (Collins and Campbell, 1982). However, Collins (1969) observed that eventhough CMI protective level precedes peak humoral response, it is not of much significance for protection against primary infection.

Chandiramani and Gary (1982) made a solitary report about the use of LMIT to monitor CMI in sheep infected/vaccinated with C. ovis. In the present study also the data obtained presented significant difference between the migration of leukocytes from infected and non-infected goats. Since LMIT was one of the methods followed to assess the cell-mediated immune response, the results presented here indicated the participation of cell-mediated immune response in goats infected with C. pseudotuberculosis.

Results of the skin hypersensitivity reaction in goats experimentally infected with C. pseudotuberculosis (group I goats) indicated that delayed type of hypersensitivity was elicited following intradermal administration of toxic culture supernatant prepared from the bacteria. The control animals (group III) remained negative for hypersensitive reactions when injected with toxic culture supernatant. In the infected animals, delayed hypersensitivity was noticed maximally at 48 h post-sensitisation as indicated by significant increase in skin thickness which was painful and hard on palpation.

Skin biopsy at 48 h revealed significant infiltration of lymphocytes and macrophages in the perifollicular and periglandular area in contrast to the skin biopsy from control animals. Cystic glandular dilatation with mononuclear infiltration in cystic space, congestion of blood vessels with perivascular infiltration of lymphocytes and macrophages and dermal oedema were also the features of the reaction in the

skin from infected animals. Histologically the skin biopsy from control animals was near normal except for the presence of sparse lymphocytes and neutrophils at the perivascular space.

Tuberculin which was employed as a non-specific antigen to elicit hypersensitive reaction failed to produce positive test either in the infected or control animals. Skin biopsy from both the group of animals was also histologically normal.

Delayed hypersensitive reaction (Type IV hypersensitivity) is known to be evoked by several infectious agents which mount cell-mediated immune response. This reaction measures the cellular response of the individuals to an allergen injected subsequently to a sensitised animal. The test is considered of high value for diagnosis of infections due to several of the facultative intracellular bacteria (Collins and Campbell, 1982) and fungus (Kaufman, 1976). Existence of delayed type hypersensitivity is considered as one of the characteristics of acquired cellular resistance (Woolcock, 1973).

Delayed hypersensitive reaction in animals suffering from CLA had been observed in few studies reported earlier. Allergenic materials prepared from C. pseudotuberculosis in the same manner as tuberculin preparation (Carne, 1932) antigen from 'S' strain of C. ovis (Cameron and McOmie, 1940) filtrate of heat killed broth culture of C. ovis (Farid and Mahmoud, 1961) and sonicated C. ovis cells (Renshaw et al., 1979; Chandiramani and Garg, 1982) were the sensitising antigens employed for the test previously.

Farid and Mahmoud (1961) and Chandiramani and Garg (1982) were of the opinion that delayed hypersensitive reaction produced in infected animal was diagnostic and indicative of strong cellular immune response evoked by the bacteria. Renshaw et al. (1979) observed that this test was of little use in the diagnosis of CLA in sheep as only 50% of the known infected animals reacted positive to the test.

The result obtained in the present study evidenced the usefulness of toxic culture supernatant of C. pseudotuberculosis as sensitizing antigen for inducing hypersensitive reaction, as all infected animals reacted positive to the test. The histological picture observed in the skin was similar to the characteristic tissue changes described for tuberculin hypersensitivity (Tizard, 1982).

The present results showed the inability of the non-specific antigen, tuberculin, to evoke hypersensitive reaction in CLA affected goats. Barakat (1979) reported successful vaccination of sheep against CLA with viable BCG. The results obtained in the present study did not support the observation of Barakat (1979) since tuberculin could not elicit any hypersensitive reaction in known CLA affected goats.

The results of the pathogenicity studies indicated that irrespective of the route of infections viz., submucosal, intradermal and subcutaneous, there was an initial local lesion at the site of infection and this was followed later by the development of abscess in the local/regional lymphnodes. At

necropsy, gross lesions were evident in the superficial lymph-nodes on the 15th day post-inoculation itself; the time at which the first goat was necropsied. From the results it was observed that certain lymphnodes were severely affected with total destruction of the parenchyma and the lymphnodes were seen as a mass of caseating pus encapsulated by thick fibrous capsule. These abscesses in the lymphnodes matured and opened spontaneously discharging the pus and the sinus tract healed up when the pus was completely discharged. Other than the superficial lymphnodes none of the deep seated lymphnodes or the visceral organs revealed any gross lesions of lymphadenitis.

Ayers (1977) and Ashfaq and Campbell (1980) also reported gross lesions of CLA in goats only in the superficial lymph-nodes. Sarkar and Bhattacharya (1975) and Sharma and Dwivedi (1977) reported that goats were more prone to thoracic form of infection with C. pseudotuberculosis. Results obtained in the present study did not support the above view as the thoracic viscera of all experimentally infected goats were free of gross lesions even after 90 days of observation period.

Histopathological changes in the lymphnodes of goats necropsied at varying intervals differed depending on the degree of infection caused by C. pseudotuberculosis. The observations made in the present study demonstrated that there was no correlation between the extent of histopathological changes and the duration of infection. This was perhaps due to the fact that all the lymphnodes of every experimental goat

did not get infected simultaneously; instead, they showed a randomised involvement at varying periods. The possible reason for this could be the accidental trapping of bacteria into the lymphatics which directly led to the infection of lymphnodes in certain animals. In certain others the organism failed to get trapped in the lymphnodes and infection did not occur. Husband and Watson (1977) observed that intralymphatic inoculation of live C. pseudotuberculosis could produce patent abscess in lymphnodes of sheep within three days. It would appear that the involvement of lymphnode in the infection is not stereotype but it is often chance factor.

Histopathological examination of the superficial lymphnodes viz., prescapular, prefemoral, submaxillary and parotid, which were close to the site of inoculation showed lymphadenitis. The histological changes in the lymphnode were characterised by hyperplastic response of the lymphoid follicles, histiocytic reaction and formation of a caseating granuloma with calcification. These histological features observed in the present study were similar to those reported earlier as characteristic features of caseous lymphadenitis in sheep and goats (Gamsel and Tartour, 1974; Sharma and Dwivedi, 1977; Husband and Watson, 1977; Burnell, 1978; Stoops et al., 1984; Brown et al., 1985).

The changes observed in lymphnodes were basically of two types; hyperplastic stimulatory reaction and degenerative changes. The hyperplastic stimulatory reactions were

characterised by the presence of several active follicles in the cortical region with well formed germinal centres, distinct medullary cords densely lined with plasma cells and sinus histiocytosis. These changes are reflections of the initial antigenic stimuli induced in the lymphnode by the antigens of the invading bacteria. The presence of several active follicles in the cortex with well distinguished medullary cords which are lined by plasma cells indicates the operation of humoral immune response in the lymphnodes of affected animals. Similar observations were reported earlier by Burrell (1978) when he studied the pathogenesis of C. ovis in the popliteal lymphnode of sheep.

The degenerative and necrotic changes noted in the affected lymphnode were characteristic of caseous lymphadenitis. These changes were seen both in the cortical and medullary regions. The changes are perforce the effect of the toxic degenerative action of the toxin and other products of the invading bacteria in the system. The in vivo 'leukocidin' property of the exotoxin from C. pseudotuberculosis was demonstrated by Cameron and Smit (1970) and the continued production of this toxin by the bacteria was considered responsible for the pathological changes in the tissues (Jolly, 1965a; Jubb and Kennedy, 1970; Zaki, 1976).

Summary

SUMMARY

The immune responses and pathological features in Corynebacterium pseudotuberculosis infection were studied by experimental infection of cross-bred Malabari goats of 8-12 months of age. Single cell bacterial suspension in chilled sodium chloride bile salt solution was used for this purpose. Goats were inoculated at both sides of the body by three routes viz., intradermal, subcutaneous and submucosal, with 2 x 10^6 bacteria per site of injection. The experimentally infected and control goats were observed for clinical manifestations of caseous lymphadenitis for a period of 13 weeks.

The development of immune response in experimentally infected goats was assessed by comparing the data with those of the controls with respect to total serum protein, serum protein fractions, antibody activity of the serum, leukocyte counts, counts of lymphocyte subpopulations, leukocyte migration inhibition index and skin hypersensitivity reaction.

Gross and histopathological changes in the lymphnodes and other tissues of necropsied goats were studied at 15 days interval for a period of 90 days.

All experimentally infected goats exhibited rise in temperature, general weakness, lethargy and impaired appetite which lasted for 72 to 96 h. The sites of inoculations showed varying degree of inflammatory reaction during the first two

to three weeks of infection. All experimentally inoculated goats except one developed lesions typical of caseous lymphadenitis in regional/local lymphnodes within 21 days post-inoculation. Route of infection did not influence the ability to set up lesions in lymphnodes. Although massive dose of bacteria (1.2×10^7) was administered, none of the goats had fatal infection indicating that goats are relatively resistant to this infection. Majority of goats did not develop generalised form of caseous lymphadenitis as there was no lesions in visceral/deep seated lymphnodes or organs.

The normal serum protein concentration of cross-bred Malabari goats was estimated to range from 7.187 to 9.750 g %. Consequent to experimental infection, serum protein concentration was increased and recorded significant rise from the 5th week onwards reaching the peak value by the 8th week - 11.346 g%.

Estimation of quantitative distribution of serum protein fractions was done by agar gel electrophoresis and densitometer tracing of electrophoretogram. Though there was initial increase in globulin content in infected animal followed by a decrease, no significant alteration in the albumin-globulin ratio (A:G ratio) was noted compared to the control group.

C. pseudotuberculosis was cultivated in Lemco proteose broth containing sheep serum and incubated aerobically at 37°C for 72 h. Supernatant obtained from the above culture, having maximum haemolysin titre and dermonecrotoxicity was used as the toxin of the bacterium in the present studies.

The haemolysin content of the culture supernatant was estimated by the haemolysis test using sheep red cells. A maximum titre of 1:256 was found in the culture aged 72 h. The dermonecrotoxicity of the toxic culture supernatant was tested by intradermal inoculation into the rabbit skin. The inflammatory and necrotic reactions were maximum by 48 h post-injection.

Specific antibody activity against toxin of C. pseudotuberculosis in the serum was monitored by haemolysis inhibition test and the test was adjudged as a useful test for detecting humoral immune response to C. pseudotuberculosis infection in goats. In infected goat from the 3rd week of infection onwards HIT was positive while it was negative in control goats during the period of 13 weeks of observation. The peak antibody level was achieved by the 5th week of infection and thereafter the titre was found to dwindle gradually till the 11th week. Towards the end of the observation period (12th week) there was a marginal increase in the antibody titre, which would be considered as secondary immune response against the toxin of the multiplying bacteria.

Infected goats showed leukocytosis during the entire period of observation and maximum leukocytosis was observed during the 2nd week of infection. The periodical fluctuation in leukocytosis indicated the recurrent flare up of bacterial invasion in the body.

The absolute lymphocyte count obtained both at pre and post-infection periods with experimentally infected goats did

not show any change which indicated no deleterious effect on peripheral blood lymphocytes. Throughout the period of observation infected animals showed numerically low lymphocyte percentage in differential counts and with several samples the percentage distribution was significantly low.

Absolute counts of neutrophils were consistently high in experimental goats when compared to those of controls and the same was reflected in differential count also. The other blood cells were absolutely without any change in infected as well as control goats.

Density gradient centrifugation using Ficoll-paque (1.077 g/ml, centrifuged at 720 x g) was found quite useful for separation of mononuclear leukocytes from the whole blood of goats. Such separated mononuclear cells were found to contain on an average 91.80% lymphocytes and 8.2% monocytes with an average viability of 91.2%.

Peripheral blood B-lymphocytes of goats were successfully enumerated by EAC rosette assay employing bovine red cells. The normal percentage of B-cells was estimated to range 8.56 and 12.3. Significantly high percentage of B-cells was recorded in infected animal from the 2nd to 10th week post-infection except at the 5th week. B-cell percentage in infected goats ranged between 12.3 $\pm$ 0.85-17.63 $\pm$ 1.2 while it was 8.56 $\pm$ 0.75 to 12.3 $\pm$ 1.09 in control goats indicating the operation of humoral immune response to concurrently boost the specific antibody activity in the serum.

T-lymphocytes of goats were identified and enumerated by E-rosette assay and ANAE activity. Goat lymphocytes presented several receptors to sheep red cells, as majority of rosettes presented erythrocytes at the entire periphery of lymphocytes.

The mean percentage of E-rosette positive lymphocytes in the peripheral blood of control goats ranged from 24.55 ± 3.66 to 26.74 ± 1.34 during 13 weeks of observation. The E-rosette technique employed in the present study was assumed unaffected by unknown variables as the data recorded in the control goats remained near normal throughout the observation period.

During the first two weeks of infection E-rosette positive lymphocyte count was found numerically decreased and the reduction was significant at the first week (18.44 ± 1.40). From the third week onwards an increase in the E-rosette forming cells was observed and significant increase was noted during the 9th, 6th, 7th, 8th, 12th and 13th week of infection, the maximum being at the 13th week (35.24 ± 1.58).

T-cells were also identified and enumerated based on the demonstration of ANAE activity. Fixing of mononuclear cells in acetone-citric acid solution enabled the fixed smears to be stored in dry state without any interference to the enzymic activity for longer periods. T-lymphocytes presented one or two localised red coloured reaction product in the cytoplasm adjacent to the cell membrane.

Mean percentage of ANAE positive cells in experimental goats was 28.09 ± 1.51 while it was 35.80 ± 4.86 for control goats when estimated before the start of the experiment. During infection, the count of ANAE positive cells in the peripheral blood did not show any change as the mean percentage ranged between 28.9 ± 2.06-33.78 ± 1.99 as against the corresponding values (30.83 ± 3.8-36.91 ± 3.81) in controls.

In infected animals significant hike in E-rosette positive lymphocyte counts was recorded while such a change could not be observed with ANAE positive lymphocytes. Thus the results of T-cell estimation by E-rosette assay and ANAE demonstration indicated that estimation of total rosette forming cells could reflect better the T-cell competence.

Cell mediated immune response to C. pseudotuberculosis infection in goats was demonstrated by leukocyte migration inhibition test under agarose. A population density of 1.5×10^8 leukocytes/ml was found suitable for LMIT. Toxic culture supernatant having haemolysin titre 1:18 whose pH adjusted to 7.2 could be successfully used as antigen in the test. In experimentally infected goats leukocyte migration index was less than 0.8 during post-infection period while with control goats it was above 0.8. Significant reduction in LMI index was noted by 45th day of infection through 75 days showing maximum reduction by the 60th day.

Intradermal injection of toxic culture supernatant elicited characteristic delayed type skin hypersensitivity reaction

in all experimentally infected goats, while a negative reaction in controls. Skin hypersensitivity reaction was found to be maximum by 48 h post-injection.

Histopathology of skin biopsy taken from the site of inoculation revealed infiltration of lymphocytes, and macrophages at perifollicular and periglandular areas, congestion of blood vessels with perivascular infiltration of lymphocytes and macrophages and dermal oedema.

Tuberculin failed to produce a positive skin hypersensitive reaction in C. pseudotuberculosis infected or control goats.

From 15th day onwards, experimentally infected goats which were necropsied presented gross lesions typical of caseous lymphadenitis in lymphnodes. The lesions were found to confine to superficial lymphnodes adjacent to the site of inoculations.

The histological changes observed in lymphnodes were basically of two types: hyperplastic stimulatory reaction and degenerative changes. The changes were hyperplastic reactive follicles with well distinguished germinal centre, accumulation of lymphocytes and varying degrees of sinus histiocytosis in medullary region, dense lining of medullary cords with plasma cells, depletion of lymphocytes from the cortical area; subcapsular and cortical oedema, congestion of blood vessels,

haemorrhage, infiltration of mononuclear cells in lymphatics and blood vessels, accumulation of macrophages and plasma cells in the medulla, dilatation of sinusoids, fibrous tissue proliferation, degenerative and necrotic changes of lymphocytes in the cortex and medulla, fibrous tissue encapsulated focal areas of caseation and calcification surrounded by lymphocytes, macrophages and giant cells and finally conversion of parenchyma to a caseated mass enclosed in fibrous tissue capsule.

In brief, the results obtained from the present study revealed the operation of both cell-mediated and humoral immune responses in goats against C. pseudotuberculosis infection. Of the various methods employed to monitor the immune responses, leukocyte migration inhibition and delayed skin hypersensitivity tests were suitable for ascertaining the cell-mediated immune response and haemolysis inhibition test for humoral immune response. Leukocyte migration inhibition test and haemolysis inhibition test can be successfully employed for the early diagnosis of C. pseudotuberculosis infection in goats.

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CELLULAR AND HUMORAL IMMUNE RESPONSES TO *Corynebacterium pseudotuberculosis* INFECTION IN GOATS

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ABSTRACT OF A THESIS

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ABSTRACT

Caseous lymphadenitis was experimentally produced in cross-bred Malabari goats aged 8 to 12 months by bilateral inoculation of 1 x 10^6 viable C. pseudotuberculosis (ATCC 19410) through intradermal, subcutaneous and submucosal routes. The clinical picture, immune response and pathological features were studied upto a period of 13 weeks. The development of immune response in experimentally infected goats was assessed, by comparing the data with those of the controls, with respect to total serum protein, serum protein fractions, antibody activity of the serum, leukocyte count, counts of lymphocyte subpopulations, leukocyte migration inhibition index and skin hypersensitivity reaction. Pathological features in the lymphnodes and other tissues of infected goats necropsied at 15 days interval were also studied.

Initial febrile reaction which lasted for 72 to 96 h, local inflammatory changes caused at the site of inoculations during the first two to three weeks of infection and the development of lesions typical of caseous lymphadenitis in local/regional lymphnodes within 21 days post-inoculation were the main features of clinical manifestations of the disease. As a result of infection, neutrophilic leukocytosis was maximum during the 2nd week of infection. No appreciable change in counts of other blood cells in terms of their absolute numbers was noted during the entire period of observation.

The humoral immune response in infected goats was indicated by the rise in serum protein, antitoxic antibody and B-lymphocyte count in the peripheral blood. The serum protein concentration increased to significant levels from the 5th week onwards and it reached the peak value (11.346 g%) by the 8th week. From the 3rd week onwards haemolysis inhibition test, which detected antitoxic antibody in the serum, was positive in infected goats and persisted till the end of the observation period. The peak antibody level was recorded by the 5th week of infection and thereafter there was gradual reduction in the titre. Significantly high percentage of B-lymphocyte was recorded in infected animals from the 2nd to 10th week, except at the 5th week. The percentage of B-cells in infected goats ranged between 12.3 ± 0.85-17.63 ± 1.2 while it was 8.56 ± 0.75-12.3 ± 1.09 in control goats. This was considered as an indication of stimulation of humoral immune response.

The cell-mediated immune response was evidenced by the increased T-lymphocyte count in the peripheral blood, inhibition of leukocyte migration and the development of delayed skin hypersensitivity. The mean percentage of T-lymphocytes in the peripheral blood of infected goats by E-rosette assay recorded an initial reduction at the first week (18.44 ± 1.4) followed by an increase which was significant during the 3th, 6th, 7th, 8th, 12th and 13th week of infection. The maximum value was recorded (25.24 ± 1.58) at the 13th week. In the

case of control goats the percentage values ranged from 24.55 ± 3.66 to 26.74 ± 1.36. The T-lymphocyte count in the peripheral blood enumerated by ANAE method did not show any significant change even after infection. In the experimentally infected goats, leukocyte migration inhibition index was less than 0.8 during post-infection period while the control goats had the index value above 0.8. Significant reduction in the migration index was noted by 45th day of infection and the maximum reduction was on the 60th day. Intradermal injection of the toxic supernatant of the culture elicited characteristic delayed skin hypersensitivity reaction in all the experimentally infected goats while there was no reaction in the controls. The positive reaction was found to be maximum by the 48th hour post-injection .

The pathological changes were characterised by an initial stimulatory hyperplastic reaction in the lymphnodes and this was accompanied by necrobiotic changes typical of caseous lymphadenitis. The hyperplastic stimulatory reactions were characterised by the presence of several active follicles with well developed germinal centres in the cortex, distinct medullary cords densely lined with plasma cells and sinus histiocytosis indicating the early elicitation of humoral immune response to the bacterium or to its *in vivo* products.

The results obtained from the present study revealed the operation of both cell-mediated and humoral immune responses in goats against *C. pseudotuberculosis* infection. Of the

various methods employed to monitor the immune response, leukocyte migration inhibition and delayed skin hypersensitivity tests were found to be of value in assessing the cell-mediated immune response and haemolysis inhibition test for humoral immune response. Leukocyte migration inhibition test and haemolysis inhibition test could be employed for early diagnosis of C. pseudotuberculosis infection in goats.