DISCUSSION
PART I

Development of cell mediated immunity during experimental infection of guineapigs with live N.asteroides

The results presented here indicate that guineapigs developed CMI during the course of experimental nocardia infection. This appeared in 6-7 weeks. In the early period, although skin hypersensitivity was present as indicated by positive skin test, there was no evidence of increased CMI as shown by increased KMI and Mic-A. Relationship between delayed hypersensitivity and immunity remains controversial and this has been extensively studied in tuberculous infection as has been extensively dealt with by Salvin and Mota (1975), the characteristics of delayed skin hypersensitivity (DH) are

(1) development of skin response to specific antigen
(2) mononuclear cell infiltration at the skin test site
(3) adoptive transfer with lymphocytes to normal recipients
(4) production of lymphokines. Cell mediated immunity (CMI) has been referred to as resistance mediated by cellular mechanisms rather than humoral antibody. CMI is generally associated with intracellular pathogens and is characterised by (1) a capacity for adoptive transfer with sensitized lymphocytes and not with serum (2) an increase in the antibacterial activity of host macrophages which is induced with the specific antigen but the action is nonspecific in that it is also directed against other organisms. Production of migration inhibitory factor (MIF) has long been described as an invitro correlate of DH (George et al, 1962). Though, there is an
association between MIF production and DH reaction (Ferraresi et al., 1969) in that both reactions achieve completion within 24-48 h, the speed with which MIF is produced suggest that it might well be a mediator of those antibacterial immune responses that are generated very quickly, such as the adoptive immune response to L.monocytogenes (Mackaness, 1969).

Macrophages incubated with MIF rich fraction showed a number of morphologic, metabolic and functional changes after 3 days. These include enhanced ability to adhere to culture dish and increased ruffle membrane motility and spread. Enhanced phagocytosis both in rate and extent of dead mycobacteria also was noticed (Nathan et al., 1971). This factor which activates the macrophages is recovered after isopynic centrifugation in CSS1 in a band corresponding to that of MIF. It indicates that with the present techniques, in all probability this factor is the same as MIF (Nathan et al., 1973; David, 1975). These macrophages could be activated to kill L.monocytogenes in 4 h if they are incubated in presence of lymphocytes and specific antigen (Simon and Sheagren, 1971). Thus it is felt that a better index for CMI in the animals was the extent of MIF and MIF-1. We have also observed that there was a dissociation between DH and MLI in the guineapigs. There was no correlation between the presence of DH and MLI as seen in guineapigs 5 and 113 where the DH was negative and the MLI was 62% and 17% with M.PPD and 68% and 20% with M.PP antigens. In 8-10 weeks after inoculation with N.aстероидes, there was a
marked increase in the MMI percentage in all the guineapigs, even though some of the guineapigs (GP 8, 50, 116) did not show any DH. Such a dissociation between skin hypersensitivity and GMH has also been observed in other infections like listeriosis (Osebold et al, 1974) and tuberculosis (Reggiardo and Midelbrook, 1974). It was also suggested that DH and GMH are mediated by different population of T cells (Osebold, 1974) and such dissociation of these T cells that mediate DH and GMH may be possible (Lefford, 1975).

We also found that there was no significant GMH but DH during the period when an active infection was present (4-5 weeks). From 6-7 weeks onwards there was a gradual increase in TMI and Mic-A. This increase generally coincided with the onset of healing of ulcers. Thus there was correlation between TMI and Mic-A with immunity rather than with DH. Similar findings were also reported by Arekar et al (1969) in mice which were protected against aerosgenic tuberculosis, the DH did not correlate with immunity, but that the MMI test did.

An attempt was made in our experiments to find out the effect of DH alone in some of the animals. The results of spleen cell transfer from donors with positive DH but little or no CMI (4-5 weeks, Table-6) did not show any protection in these animals. These guineapigs also did not survive longer than the control animals. There was also no change in the duration and appearance of subcutaneous lesions. In fact, the subcutaneous mycetoma like lesions appeared
earlier than that in normal guineapigs. There are some reports which show that Schistosoma mansonii eggs induce accelerated pulmonary granulomas in mice that are presensitized to that antigen and this response is specific for the inducing antigen (Warren, 1967). Other reports show that the production of these granulomas are dependent upon DH (Dormingo et al, 1967, 1963). Moore, Myrvik and Leake (1973) have shown that pulmonary granulomas induced by BCG are allergic in nature and are dependent upon the induction of local DH reactions. Similarly in the experiment described here there is an acceleration in the development of granuloma in presence of LH.

The characteristic feature of LH is that it can be transferred to normal animals by mononuclear cells from sensitized and not by serum. It was first shown by Landsteiner in Jewiss (1944) and Chase (1945) that contact and tuberculin hypersensitivity could be transferred to normal guineapigs with viable mononuclear cells. Metaxas and Metaxas-Buchler (1955) showed that LH to tuberculin was transferred to normal recipients by sensitized cells injected intravenously without a latent period and the intensity of reactions decreased with the number of skin sites tested. PEC or lymph node cells or spleen cells are capable of transferring DH and CMI to normal animals (Chase, 1960; Paas et al., 1961). In the present experiments it was observed that DH and CMI could be transferred by viable spleen cells from sensitive donor without a latent period.
Skin reactivity could be elicited within 24 h of spleen cell transfer. Paa, et al (1961) and Maclusky, et al (1963) have shown that when the lymphoid cells were transferred to the normal animals, the state of hypersensitivity was transferred to the recipients lymphocytes in that these recipients' cells infiltrate the skin test site. Here in the transfer experiments it was noticed that when the spleen cells were passed second time i.e. recipient guinea pig acts as donor to another guinea pig the Ld response was lost but MHI and Mic-A response persisted. The loss of Ld was not due to repeated skin tests since the animal was skin tested only once before the second transfer of spleen cells. After the transfer of these cells, the sensitivity could persist in the recipients for about 3 weeks (Chase, 1960) to 6 months (Paa, 1961) in outbred guinea pigs and for 24 months in inbred guinea pigs (Chase, 1963).

Since, second transfer and challenge experiments were completed within 10 days of spleen cell transfer. As has been pointed out by Salvin and Beta (1975), transfer of lymphoid cells from sensitized donors confers both Ld and MHI on normal recipients. These lymphoid cell populations, depending on their source contain various percentages and types of T cells. These differences in cell populations may therefore account for the variations in the observed responses.

When high CMI, as indicated by increased MHI and Mic-A was transferred to normal animals and the recipients challenged intravenously with N. asteroides, these animals survived for a longer time than the control unimmunized animals. Control.
guineapigs died in 15 days with extensive lesions in various organs like lungs, heart and kidneys. Virulent strains of N. asteroides induced a granulomatous response in rabbits (Horrol and Hestinstall, 1954), when saline suspensions were injected either intraperitoneally or intravenously in mice progressive metastatic lesions were formed in lungs, heart and kidneys that led to the death of the animal. These lesions were either abscesses containing predominantly polymorphonuclear neutrophils and macrophages or occasionally granulomas consisting of macrophages and lymphocytes. In the present work also it was seen that the N. asteroides strain induced granulomas in guineapigs when injected subcutaneously. These granulomas contained macrophages and lymphocytes along with the organisms. When injected intravenously it produced fatal infections in unimmunized guineapigs. The lesions were predominantly micro-abscesses in lungs, heart and kidneys. Immune animals, however, survived for a longer time than the controls and the organs were free of any lesions and organisms after 10 days. Beaman (1974) has shown that the lesions produced in mice by virulent and avirulent N. asteroides were different depending on the virulence of the organisms used. Virulent organisms were not affected by host defense mechanisms and there was no chemical and physical changes in the cell envelope of the organisms. Whereas in avirulent organisms there was significant structural
changes in the cell wall leading to the elimination of organisms from the tissues. It has also been shown that virulent organisms multiplied intracellularly in macrophages but avirulent ones were rapidly killed by these macrophages (Seaman and Smathers, 1976). In the present experiments, the protection seen in these immune guineapigs was due to CMI and not because the strain used was less virulent. Initially the strain was passaged 6 times in guineapigs to adapt and increase its virulence. Upon intravenous injection it invariably produced progressive lesion in internal organs of normal guineapigs.
PART - II

Development of cell mediated immunity after immunization with ribonucleic acid protein fraction of N.asteroides and protection afforded by it:

The results presented here showed that guineapigs immunized with N-PRNA developed high CMI as indicated by increased MI and Mic-A. The immunized animals however, did not show dermal reactivity (DH) when tested with N-PRD, N-PR and N-PRNA antigens. Similar findings were reported by Youmans and Youmans (1969) using RNA fractions prepared from M.tuberculosis. They prepared ribosomal and RNA vaccines from mechanically broken viable cells of M.tuberculosis and vaccinated guineapigs subcutaneously with this material. When these animals were skin tested 6-12 weeks later with mycobacterial ATP, they showed negative DH response. In the same way, mice vaccinated subcutaneously or intraperitoneally with the fraction gave negative DH reactions. But this fraction induced high degree of immunity to challenge with virulent tubercle bacilli in these animals (Youmans and Youmans, 1965). Such dissociation between DH and antimicrobial resistance has been reported by Osbold et al. (1974) in L.mono-cytogenes infection. It was also observed in our earlier experiments (Part - I) i.e. dissociation between, DH, MMI and Mic-A after immunization with live N.asteroides. The results of our experiments also lend support to the view that there is a dissociation between these two phenomenon.
RNA fraction from M. tuberculosis was highly immunogenic only when it was incorporated into Freund's incomplete adjuvant (Younana and Younana, 1966), in that the adjuvant may protect and prevent the RNA being rapidly destroyed by host ribonuclease immediately after injection. Sued (1965) showed that ribonucleic acid is rapidly degraded after injection into mice. Hence in the present experiment N-PRNA was injected along with IGRA.

Guinea pigs immunized with N-PRNA fraction of M.asteroides developed GMI in 14 days. This immunity appeared earlier than that induced during the course of experimental nocardia infection where considerable degree of GMI appeared in 6-7 weeks. A dose containing 6 μg RNA induced high GMI. Similar finding was also reported by Venneman and Berry (1971) in which they produced highest possible level of immunity against S.typhimurium in mice with RNA fraction from the same organisms in doses of 10 μg or less.

This immunity which was induced by N-PRNA was protective and the immunized animals survived much longer after intravenous challenge with M.asteroides. These animals did not fall sick at least for 56 days. After administration of immunogenic fraction of N-PRNA, the animals not only survived longer but the course of disease was restricted as the gross lesions on different organs like spleen, liver, kidneys, lungs and heart became less and finally no gross nocardial lesions were present. Similarly, the viable count of nocardia in various tissues were
much less than in control animals after challenge and finally there was no living inocardia in the tissues. Misfeldt and Johnson (1976) have produced high level of immunity in mice with RNA fraction from S. typhimurium. When these animals were challenged with live organisms, there was an enhanced clearance of the bacteria from the tissues and the mice were protected from death.

Normal recipients which received spleen cells from PRNA immunized donors on intravenous challenge were protected and the organisms were cleared off from the tissues. The successful passive transfer of CMI with spleen cells from animals immunized with inocardial PRNA indicated, the observed protection afforded were due to cell mediated immune response.

RNA fractions from many bacteria like salmonella, streptococci etc have been shown to provide significant levels of immunity (Kurland et al, 1971; Johnson, 1972; Schalla and Johnson, 1975). But the mechanisms by which they stimulate resis. tance was not clearly understood. Vanneman and Berry (1971) and Smith and Bigley (1972) have suggested that ribosomal RNA may stimulate CMI. In the present work it is clearly shown by using various parameters of CMI like MLI, MA and Mic-A that bacterial fraction induces CMI. Increased MLI and Mic-A have also been reported by Agarwal and Sundararaj (1976,1977) in rabbits immunized with PRNA extracted from V. cholerae L formolysates. Thus the PRNA fraction extracted from N. asteroides induced CMI in guineapigs which protected them upon challenge with live organisms.
Role of macrophages in experimental *N. asteroides* infection

(1) In vivo killing of *N. asteroides* in macrophages in actively and passively immunised guinea pigs and

(4) Effect of antimacrophage serum

Recently Beaman and Smathers (1976) and Beaman (1977) have shown multiplication of *N. asteroides* inside nonimmune alveolar macrophages from rabbits. Virulent *N. asteroides* multiplied rapidly inside the macrophages whereas avirulent organisms are destroyed by these macrophages. The multiplication of *N. asteroides* inside macrophages corresponded to the multiplication of other facultative intracellular bacteria like *M. tuberculosis* and *L. monocyctogenes*. Intracellular multiplication of nocardia has also been demonstrated in human infection (Ahsh et al., 1961). It has been established that acquired resistance to infections with such bacteria rests firmly on cell mediated form of immunity (Mackaness, 1971). Studies have shown that when sensitive lymphocytes are stimulated with specific antigens they liberate several molecular mediators resulting in the activation of macrophages which ultimately inhibit the intracellular multiplication of these bacteria and eliminate them. The present experiment was designed to find out the role of these macrophages in affording protection in *N. asteroides* infection.
Guineapigs were immunized with N-PRNA fraction of N.asteroides in ICFA. When they developed high CMI they were challenged intraperitoneally with N.asteroides. Another group of guineapigs was immunized with live N.asteroides. When they developed high CMI spleen cells were transferred to normal recipients and the recipients challenged intraperitoneally with N.asteroides. Studies in vivo have established that resistance to intracellular parasites is transferable to normal recipients by lymphocytes if the cells are also stimulated with specific antigen to which they are sensitized (Mackaness, 1969). Hence in all transfer experiments spleen cells were transferred along with N-PRL antigen.

As has been shown earlier (Part-II), there was an increase in M-I and M-A 25-40 days after immunization with BHI. At 60 days, though there was a decrease in MMI percentage the invitro Mio-A activity against L.monocytogenes was still present. At the time of transfer of spleen cells, donors actively immunized with N.asteroides also possessed increased M-I (Table-14). Before the intraperitoneal challenge with N.asteroides peritoneal exudate cells were induced in guineapigs with BHI containing 3% peptone. Large quantity of PEC are obtainable upto 96 h of peritoneal stimulation. Twenty four hour after PEC induction with BHI as above, guineapigs were challenged intraperitoneally with N.asteroides. Viable counts were done 2 h and 72 h post
challenge. The results showed that the macrophages from these immune guineapigs exhibited marked invivo intracellular killing of N.asteroides, which was reflected in the decrease in the intracellular viable count between 2 h and 72 counts. This activity was high during 25-40 days post immunization and existed upto 60 days. To exclude the possibility that the decrease may be due to the presence of extracellular bacteria, cultures were put up from the supernatant after depositing the PEC by centrifugation. The culture yielded no extracellular organisms. The intracellular viable counts were done i.e. 2 h and 72 h, well before the macrophages disappear from the peritoneal cavity. Moreover, fixed number of PEC (1 x 10^6 cells) were taken lysed and counts put up. Thus it clearly showed that the activated macrophages exhibited a specific invivo killing of N.asteroides.

To exclude the possibility of humoral immunity playing a role in the elimination of these bacteria by the macrophages, the spleen cells from N-PHMA immune guineapigs were transferred to normal recipients. The results clearly showed that these recipients also exhibited increased killing of N.asteroides indicated by the decrease in the intracellular viable count (Table-13) CMI was passively transferred from animals actively immunized with live N.asteroides. In these recipients also there was a decrease in the intracellular viable count of
N. asteroides (Table-14). Thus the CMI plays an important role in the activation of these macrophages which ultimately eliminate the bacteria.

To find out the role of these macrophages in affording protection, the effect of AMS was studied. It has been shown earlier (Part-I, Table-7) that when normal unimmunised guinea pigs challenged intraperidially with N. asteroides, they became sick in 5-7 days and died in 15 days with gross lesions in lungs, heart and kidneys. The viable count of N. asteroides in organs such as lungs, heart, kidneys, liver and spleen varied from 1500-15000 x 10²; 400-2000 x 10²; 1000-5000 x 10²; 50-150 x 10² and 3-750 x 10² respectively. But the normal unimmunized animals treated with AMS became sick in 3 days and died in 5 days time with similar high count in various organs (Table-15). Thus in these animals the organisms multiplied freely in various organs reaching a high count which was fatal in five days. Similarly in the earlier investigations (Part I and II) it was shown that protection was afforded by CMI induced by live N. asteroides and N-PRNA fraction derived from it. In these immune guinea pigs there was a marked decrease in the viable count in various organs and the animals survived for a longer time. In contrast, such immune animals when treated with AMS, became sick in 3 days and died in 5 days time with a high count of N.asteroides in various organs (Table 15,16,17). The bacteria multiplied freely in various organs as they did in unimmunized
animals. There was no protection in these animals. The protection afforded by AMS was lost as the result of treatment with it. Macrophages are considered to be the cells ultimately responsible for disposing of most foreign materials (Macanesss, 1964, 1969). They are also known to be host cells for a variety of obligatory and facultative intracellular parasites like M. tuberculosis, L. monocytogenes and S. typhimurium (Collins, 1974; Macanesss, 1964). Recently Beaman et al. (1976, 177) have shown the multiplication of N. asteroides inside alveolar macrophages from rabbits. It is well known that activated macrophages effectively eliminate many intracellular bacteria (Macanesss, 1969). In the present study it is shown that there is a marked reduction in the number of nocardia in these immune macrophages, indicating the need for the macrophages in eliminating these bacteria. In the AMS treated animals with these macrophages are destroyed or incapacitated or it is activation has not taken place. It is well known that lymphocytes are required for the activation of these macrophages—a contained very little of antilymphocytic activity (3) at which level the damage to all lymphocytes resulting in nonactivation of these macrophages was highly improbable. It has been shown by Middlebrook et al. (1974) that these specifically activated macrophages produce a factor which has Listeria sterilizing activity. Such factor, which may also kill N. asteroides, may not have been produced as the result of AMS treatment. Thus the present study indicates that increased microbicidal activity of macrophages plays an important role in the effective elimination of N. asteroides, restricts the nocardial infection and affords protection against it.