Clin. exp. Immunol. (1985) **60**, 559–564.

Dynamics of T cells of L3T4 and Ly 2 phenotype within granulomas in murine listeriosis

H. NÄHER, U. SPERLING, L. TAKACS & H. HAHN *Institut für Medizinische Mikrobiologie, Freie Universität, Berlin, FRG*

(Accepted for publication 7 January 1985)

SUMMARY

Monoclonal antibodies anti-Ly 1, anti-Ly 2 and GK1.5 were applied to determine phenotypes of T cells within granulomas formed as a result of infection of mice with the facultative intracellular bacterium *Listeria monocytogenes*. Early in granuloma formation, equal numbers of Ly 1^+, Ly 2^+ and L3T4$^+$ cells were found, T cells of different phenotypes being evenly distributed over the lesions. In mature granulomas, numbers of Ly 1^+ and L3T4$^+$ cells about doubled as compared to incipient granulomas, Ly 2^+ cells, however, remained constant. Whereas Ly 1^+ and L3T4$^+$ cells within mature granulomas still were evenly distributed, Ly 2^+ cells were predominantly localized in the periphery of the lesions. The data indicate that both, specific Ly 2^+ and L3T4$^+$ T cells, display characteristic dynamics within granulomas: Ly 2^+ T cells which most likely mature from Ly 1^+2^+ T cells over time locate to the periphery. Concomitantly, L3T4$^+$ T cells are enriched maintaining their distribution all over the lesions.

Keywords immunochemistry granuloma formation T cell subset monoclonal antibodies

INTRODUCTION

Protective immunity in infections with facultative intracellular bacteria is based on macrophage activation (Mackaness, 1968) and formation of granulomatous lesions (North, 1970) in infected tissues. Recently, we demonstrated (Näher, Sperling & Hahn, 1985a) in the murine listeriosis model that two T cell populations are involved in these phenomena. The T cells can be distinguished with respect to phenotype, genetic restriction, and antigenic requirements: macrophage activation is a function of H-2 I-A restricted Ly 1^+2^- T cells which secrete macrophage activation factor (MAF) (Farr *et al.*, 1979; Sperling, Kaufmann & Hahn, 1984). Granuloma formation, on the other hand, critically depends on H-2 K restricted Ly 1^+2^+ T cells. Whereas expression of granuloma formation requires living bacteria, heat killed bacteria suffice for the stimulation of T cells which function to activate macrophages.

Macrophage activation is most marked within granulomas (Dannenberg *et al.*, 1968) indicating that macrophage activation is a necessary element in the function of granulomas. The question arises therefore whether in murine listeriosis T cells of both phenotypes are represented within granulomatous lesions, as recently shown for T4$^+$ and T8$^+$ T cells in leprosy (Van Voorhis *et al.*, 1982; Modlin *et al.*, 1983).

We have studied the phenotypes of T cells in granulomatous lesions formed as a result of infection of mice with *Listeria monocytogenes*. In incipient and mature lesions, the following

Correspondence: Dr Helmut Näher, Institut für Medizinische Mikrobiologie, Freie Universität Berlin, Hindenburgdamm 27, D-1000 Berlin 45, FRG.

markers were looked for: Ly 1, Ly 2, and the recently identified marker L3T4 which is exclusively expressed on H-2 I-A restricted T cells (Dialynas *et al.*, 1983a). It was found that T cells of different phenotypes all were present in granulomas. Over time, $Ly2^+$ T cells changed their distribution pattern, whereas $L3T4^+$ and Ly 1^+ T cells increased in numbers.

MATERIALS AND METHODS

Mice. C57Bl/6, 6–8 weeks old, were obtained from Bomholtgard, Ry, Denmark.

Bacteria. L. monocytogenes, strain EGD (LD_{50}: 2×10^5/mouse) was kept virulent by continuous mouse passage. Suspensions of bacteria were prepared from 18 h trypticase–soy broth cultures of homogenates from infected mouse spleens. For injection, bacteria were diluted in 0·15 M NaCl to appropriate concentrations, and numbers of bacteria injected confirmed by plate counts, according to (Mackaness, 1962).

Collection of peritoneal exudate cells and preparation of peritoneal exudate T lymphocyte enriched cells (PETLE). Mice were injected i.v with 5×10^3 *L. monocytogenes* in volume of 0·2 ml. On day 7, peritoneal exudates were induced with 1·5 ml protease peptone (DIFCO, Detroit, Michigan, USA) injected i.p. and 3 days later peritoneal exudate cells were collected (North & Spitalny, 1974). PETLE were obtained after incubation of peritoneal exudate cells on nylon wool columns, according to the method of (Julius, Simpson & Herzenberg, 1973).

Adoptive transfer of granuloma formation. Recipient mice were injected i.v. with 5×10^6 immune PETLE together with 5×10^4 *L. monocytogenes* in a final volume of 0·2 ml. Sixty hours or 96 h later, respectively, livers were removed for immunohistochemical staining procedures.

Preparation and of staining tissue sections. Frozen sections (7–9 μm) of liver tissue were prepared in a cryostat and stored at $-70°C$. After air drying, sections were acetone fixed for 10 min, washed three times in phosphate-buffered saline (PBS) at pH 7·4, and overlaid with 50 μl of an appropriate dilution of rat IgG monoclonal antibody (MoAb) for 30 min. Slides were then washed three times, incubated with biotinylated rabbit anti-rat IgG antibody (Vector Laboratories, Burlingame, Virginia, USA) for 30 min and again washed three times with PBS. Afterwards an avidin–biotinylated horseradish peroxidase complex was applied to the sections for 30 min, washed three times and peroxidase reaction was developed with 2 mg/ml diaminobenzidine (Sigma, München, FRG) solution containing 0·01% H_2O_2 in PBS, for 7 min in the dark. After a final PBS wash, slides were counterstained with methylene blue.

Standarization of immunohistochemical reagents and staining controls. The appropriate dilutions of the monoclonal reagents were determined as well as controls were carried out using the biotin–avidin labelled peroxidase method described above on frozen sections of murine thymus. The results of these studies were as follows. (a) Tissue sections treated with the amplification and development system but without previous application of the different MoAb were not stained. (b) Rat anti-Ly 1 IgG, rat anti-Ly 2 IgG MoAb (Becton–Dickinson, Mechelen, Belgium), and rat GK1.5 MoAb (kindly provided by Dr M. Simon with the permission of Dr F. Fitch) were used at dilutions staining equally well sections of thymus obtained from C56Bl/6 mice.

RESULTS

Presence of Ly 1^+, $L3T4^+$ and Ly 2^+ T cells within granulomas. Five million immune PETLE were injected i.v. together with 5×10^4 *L. monocytogenes* into syngeneic mice. Sixty hours later, livers were removed and cryostat sections stained by use of either anti-Ly 1, anti Ly-2 or GK1.5 MoAb. Ly 1^+, Ly 2^+ as well as $L3T4^+$ T cells were found within granulomatous lesions (Fig. 1, left panels). Their relative numbers were in a similar range for each subset (Table 1). T cells of all phenotypes were evenly distributed within granulomas.

Increase of $L3T4^+$ and Ly 1^+ T cells over Ly 2^+ T cells in mature granulomas

When liver sections were stained 96 h after adoptive immunization with immune PETLE and *L.*

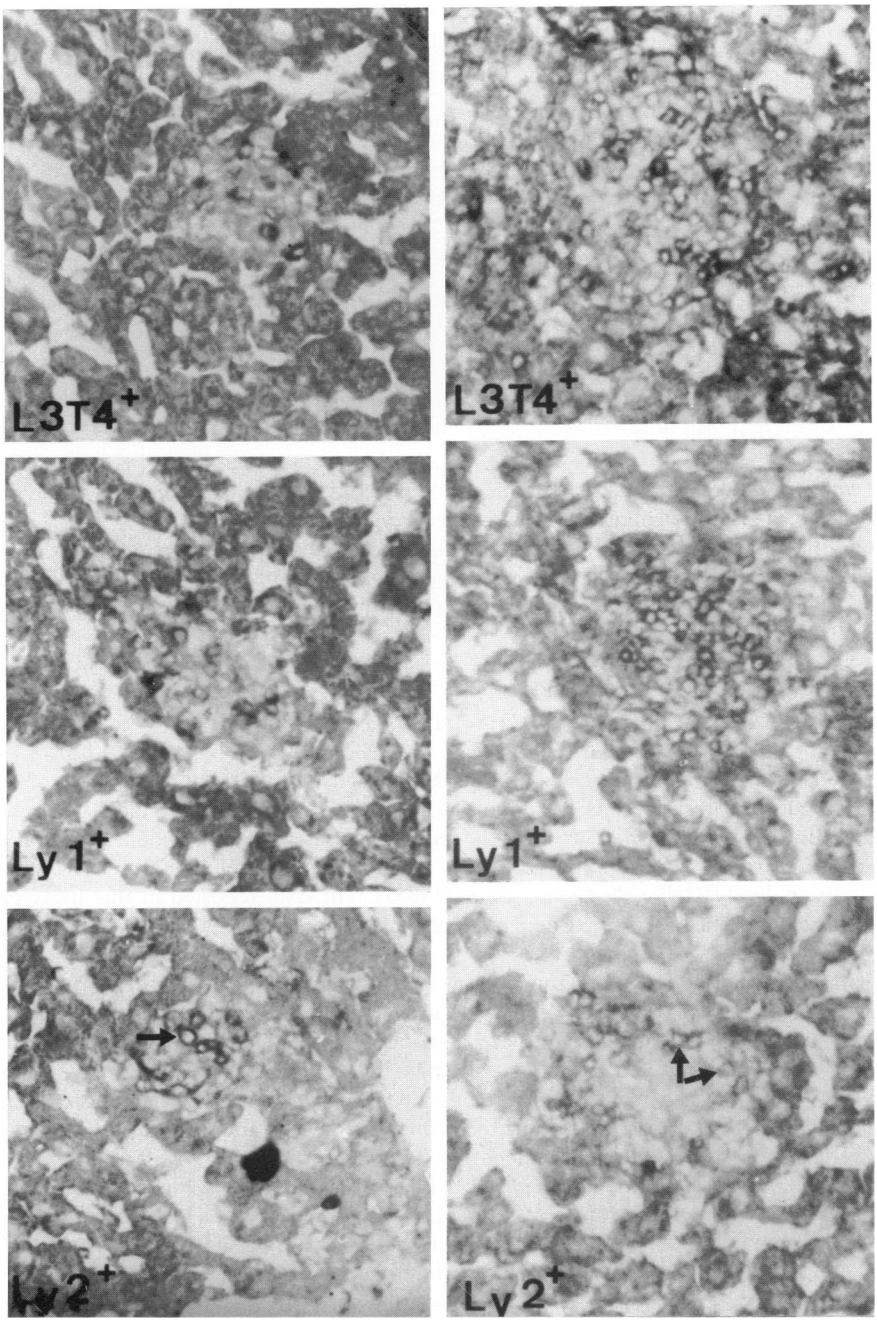

Fig. 1. Immunohistochemical staining of granulomas. Cryostat sections prepared from livers 60 h (left panels) or 96 h (right panels) after adoptive immunization with 5×10^6 *L. monocytogenes* immune PETLE and 5×10^4 *L. monocytogenes* were stained with either anti-Ly 1, GK1.5 or anti-Ly 2 MoAb. The arrows illustrate where Ly 2^+ T cells are located inside the granulomatous lesion at different stages of granuloma formation.

Table 1. Proportion of cells within incipient and mature granulomas reactive with a given MoAb

Surface antigens	% reactive cells within granulomas	
	Incipient*	Mature*
Ly 1	22·3±3·7†	41·0±3·7
L3T4	19·3±2·2	39·1±4·3
Ly 2	24·6±3·2	20·2±3·1

* Sixty or 96 h, respectively, after adoptive transfer of 5×10^6 *L. monocytogenes* immune PETLE and 5×10^4 *L. monocytogenes*.

† Mean ± s.d. of five granulomas.

monocytogenes, different results were obtained: Whereas Ly 2^+ T cells remained constant, L3T4$^+$ and Ly 1^+ T cells increased in relative proportion to Ly 2^+ T cells. Ratios of L3T4$^+$ to Ly 2^+ T cells about doubled in mature granulomas (Table 1). Also, the distribution pattern of T cells within granulomas changed: Ly 2^+ T cells were now predominantly located at the periphery of the lesion, whereas L3T4$^+$ T cells and Ly 1^+ T cells still were evenly distributed over the lesions (Fig. 1, right panels).

DISCUSSION

In infections with facultative intracellular bacteria, both activation of mononuclear phagocytes and their accumulation resulting in granuloma formation depends on specific T lymphocytes (Mackaness, 1968; Näher *et al.*, 1985b). In the murine listeriosis model macrophage activation is a function of Ly 1^+2^- T helper cells (Sperling *et al.*, 1984), whereas granuloma formation results from the activity of Ly 1^+2^+ T cells (Näher *et al.*, 1985a). The data reported in this study provide morphological evidence for the participation of both L3T4$^+$ and Ly 2^+ T cells in granuloma formation.

Phenotypes of T cells were determined in granulomas formed after transfer of immune cells (Näher *et al.*, 1985b). This excludes possible effects by non-immune mechanisms which could result in granuloma like lesions, such as have been described (Heymer *et al.*,1976).

Both T cells of the cytotoxic/suppressor cell phenotype (Ly 2^+) and of the helper/inducer cell phenoytpe (L3T4$^+$) were detected within granulomas. Ly 1^+ cells within the lesions are assumed to be identical with the L3T4$^+$ T cells. For, the expression of Ly 2 and L3T4 antigens is mutually exclusive, whereas the Ly 1 antigen, albeit constantly present on L3T4$^+$ T cells, can also be expressed on Ly 2^+ T cells (Ledbetter *et al.*, 1980; Dialynas *et al.*, 1983b). Our conclusion of identity of Ly 1^+ and L3T4$^+$ T cells is further supported by the finding that at a later stage of granuloma formation the number of L3T4$^+$ and Ly 1^+ cells increased in parallel, whereas the number of Ly 2^+ T cells remained constant. In addition, distribution of L3T4$^+$ cells and Ly 1^+ cells within granulomas showed an identical pattern which was different from that of Ly 2^+ cells.

In adoptive granuloma formation, Ly 1^+2^+ T cells are critically needed (Näher *et al.*, 1985a). From the conclusion that Ly 2^+ T cells within granulomas do not express the Ly 1 marker follows that Ly 1^+2^+ T cells after transfer must have matured to Ly 2^+ cells. A similar maturation process of Ly 2^+ T cells has been shown by others, too (Simon *et al.*, 1981). However, the possibility cannot be excluded that the Ly 1 marker was only seemingly lost due to less expression of Ly 1 antigen on Ly 1^+2^+ cells and less sensitivity of the immunohistochemical method as compared to the depletion method (Ledbetter *et al.*, 1980) used in adoptive transfer studies.

Numbers of L3T4$^+$ and Ly 1$^+$ T cells increased over time in granulomas. The underlying mechanism appears to be either continued recruitment and/or proliferation. Recently, increasing protective capacity of Ly 1$^+$ T cells over time in inflammatory peritoneal exudates was reported by us (Näher, Sperling & Hahn, 1984), making it likely that increase of T cells at infective foci is of functional significance.

Ly 2$^+$ cells could play a role in recruitment of L3T4$^+$ T cells into granulomatous lesions. Recruitment of T4$^+$ cells by chemotactic activities of T 8$^+$ cells has recently been demonstrated (Van Epps, Durant & Potter, 1983). Further support comes from our findings (Näher *et al.*, 1984) that under conditions where homing of T cells was crucial, protection depended on Ly1$^+$2$^+$ and Ly 1$^+$ T cells. In contrast, when injected at the site of bacterial implantation, Ly 1$^+$ T cells sufficed for mediating protection. Therefore, with respect to the functional interplay of L3T4$^+$ and Ly 2$^+$ cells within granulomas, we should like to suggest that Ly 2$^+$ T cells are predominantly responsible for the focusing, whereas L3T4$^+$ T cells function to activate macrophages.

Distribution of Ly 2$^+$ and L3T4$^+$ T cells in incipient granulomas in murine listeriosis resembles distribution of T8$^+$ and T4$^+$ T cells in granulomas of lepromatous leprosy, whereas mature granulomas were similar to those of tuberculoid leprosy (Van Voorhis *et al.*, 1982; Modlin *et al.*, 1983). This suggests that the development of granulomas in lepromatous leprosy is arrested at an early stage and is best explained by assuming impaired recruitment and/or proliferation of helper/inducer T cells. In keeping with this notion is the lack of circulating *Mycobacterium leprae* specific lymphocytes (Godal *et al.*, 1971) and unresponsiveness of T cells (Haregewoin *et al.*, 1982) in lepromatous leprosy.

In analogy to granuloma formation in listeriosis it is furthermore suggested that in leprosy T8$^+$T cells, similar to *L. monocytogenes* specific Ly 2 bearing T cells, function to activate granuloma formation (Näher *et al.*, 1985a). This does, however, not exclude that part of T8$^+$ T cells in leprosy, especially in the lepromatous form of the disease, could represent suppressor cells, too, as suggested (Van Voorhis *et al.*, 1982).

Our results provide some insight into the dynamics of L3T4$^+$ T cells in granuloma formation. Further studies will have to define the underlying functional interplay of both T cell populations with each other and cells of the mononuclear phagocyte system.

The authors are indebted to Dr F. Fitch for permit to use mc A GK1.5, and thank Dr M. Simon for providing us with supernatant and hybridoma cells. The excellent technical assistance of Mrs D. Oberbeck-Müller is gratefully acknowledged, and we thank Mrs A. Häusler for typing the manuscript.

LT is on leave of absence from the Department of Anatomy, Semmelweis University, Budapest, Hungary. This work was supported by DFG grant N. 163/1-1 to HN and US.

REFERENCES

DANNENBERG A.M., MEYER, O.T., ESTERLY, J.R. & KAMBARA, T. (1968) The local nature of immunity in tuberculosis, illustrated histochemically in the dermal BCG lesions. *J. Immunol.* **100,** 931.

DIALYNAS, D.P., QUAN, Z.S., WALL, K.A., PIERRES, A., QUINTANS, J., LOKEN, M.R., PIERRES, M. & FITCH, F.W. (1983a) Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human LEU-3/T4 molecule. *J. Immunol.* **131,** 2445.

DIALYNAS, D.P., WILDE, D.B., MARRACK, P., PIERRES, A., WALL, K.A., HAVRAN W., OTTEN, G., LOKEN, M.R., PIERRES, M., KAPPLER, J. & FITCH, F.W. (1983b) Characterization of the murine antigenic determinant, designated L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen reactivity. *Immunol. Rev.* **74,** 29.

FARR, A.G., WECHTER, W.J., KIELY, J.-M. & UNANUE, E.R. (1979) Induction of cytocidal macrophages after *in vitro* interactions between Listeria-immune T cells and macrophages—role of H-2. *J. Immunol.* **122,** 2405.

GODAL, T., MYKLESTAD, B., SAMUEL, D.R. & MYRVANG, B. (1971) Characterization of the cellular immune defect in lepromatous leprosy: a specific lack of circulating *Mycobacterium leprae* reactive lymphocytes. *Clin. exp. Immunol.* **9,** 821.

HAREGEWOIN, A., GODAL, T., MUSTAFA, A.S., BELEHU, A. & YEMANEBERHAN, T. (1982) T-cell conditioned media reverse T-cell unresponsiveness in lepromatous leprosy. *Nature*, **303,** 342.

HEYMER, B., HOF, H., EMMERLING, P. & FINGER, H. (1976) Morphology and time course of experimental listeriosis in nude mice. *Infect. Immun.* **14,** 832.

JULIUS, N.D., SIMPSON, E. & HERZENBERG, A. (1973)

A rapid method for the isolation of functional thymus derived murine lymphocytes. *Eur. J. Immunol.* **3,** 645.

KAUFMANN, S.H.E., SIMON, M.M. & HAHN, H. (1979) Specific Lyt 123 T cells are involved in protection against *Listeria monocytogenes* and in the delayed-type hypersensitivity to listerial antigens. *J. exp. Med.* **150,** 1033.

LEDBETTER, J.A., ROUSE R.V., MICKLEM, H.S. & HERZENBERG, I.A. (1980) T cell subsets defined by expression of Lyt-1,2,3 and Thy-1 antigens. *J. exp. Med.* **152,** 280.

MACKANESS, G.B. (1962) Cellular resistance of infection *J. exp. Med.* **116,** 381.

MACKANESS, G.B. (1968) The influence of immunologically committed lymphoid cells on macrophage activity. *J. exp. Med.* **129,** 973.

MODLIN, R.L., GEBHARD, J.F., TAYLOR, C.R. & REA, T.H. (1963) *In situ* characterisation of T. lymphocyte subsets in the reactional states of leprosy. *Clin. exp. Immunol.* **53,** 17.

NÄHER, H., SPERLING, U. & HAHN, H. (1984) Developmental interrelationship of specific Lyt 123 and Lyt 1 cell sets in expression of antibacterial immunity to Listeria monocytogenes. *Infect. Immun.* **44,** 252.

NÄHER, H., SPERLING, U. & HAHN, H. (1985a) H-2 K restricted granuloma formation by Ly 2^+ T cells in antibacterial protection to facultative inracellular bacteria. *J. Immunol.* **134,** 569.

NÄHER, H., SPERLING, U., SKUPIN-SCHÜSSLER, H. & HAHN, H. 1985b) An adoptive transfer system for the investigation of granuloma formation in murine listeriosis. *Med. Microbiol. Immunol.* (In press.)

NORTH, R.J. (1970) The relative importance of blood monocytes and fixed macrophages to the expression of cell-mediated immunity to infection. *J. exp. Med.* **132,** 521.

NORTH, R.J. & SPITALNY, G. (1974) Inflammatory lymphocytes in cell-mediated antibacterial immunity: factors governing the accumulation of mediator T cells in peritoneal exudates. *Infect. Immun.* **10,** 489.

SIMON, M.M., EDWARDS, J., HÄMMERLING, U. MCKENZIE, F.C., EICHMANN, K. & SIMPSON, E. (1981) Generation of effector cells from T cell subsets. III. Synergy between Lyt-1 and Lyt-123/23 lymphocytes in the generation of H-2-restricted and alloreactive cytotoxic T cells. *Eur. J. Immunol.* **11,** 246.

SPERLING, U., KAUFMANN, S.H.E. & HAHN, H. (1984) Production of macrophage-activating and migration inhibition factors *in vitro* by serologically selected and cloned Listeria-monocytogenes-specific T cells of the Lyt 1^+2^- phenotype. *Infect. Immun.* **46,** 111.

VAN EPPS, D.E., DURANT D.A. & POTTER, J.W. (1983) Migration of human helper/inducer T cells in response to supernatants from Con A-stimulated suppressor/cytotoxic T cells. *J. Immunol.* **131,** 697.

VAN VOORHIS, W.C., KAPLAN, G., SARNO, E., NUNES, HORWITZ, M.A., STEINMAN, R.M., LEVIS, W.R., NOGUERIA, N., HAIR, L.S., ROCHA, G., ARRICK, B.A. & COHN, Z.A. (1982) The cutaneous infiltrates of leprosy. Cellular characteristics and the predominant T-cell phenotypes. *N. Engl. J. Med.* **307,** 1593.