INFECTION AND IMMUNITY, July 1996, p. 2821–2823 Vol. 64, No. 7
0019-9567/96/$04.00+0

T- and B-Lymphocyte-Independent Formation of Alveolar Macrophage-Derived Multinucleated Giant Cells in Murine *Pneumocystis carinii* Pneumonia

RALPH HANANO,[1] KURT REIFENBERG,[2] AND STEFAN H. E. KAUFMANN[1,3]*

Department of Immunology[1] and Central Animal Facilities[2] of the University of Ulm, Ulm, and Max-Planck-Institute for Infection Biology, Berlin,[3] Germany

Received 13 November 1995/Returned for modification 22 December 1995/Accepted 11 April 1996

Multinucleated giant cells developed in *Pneumocystis carinii*-diseased gene disruption mutant mice deficient in major histocompatibility complex class II molecules, T-cell receptor αβ cells, or all mature T and B lymphocytes. These findings demonstrate lymphocyte-independent fusion of alveolar macrophages under morbid conditions. Pulmonary parasite burden seems to be a decisive factor in multinucleation.

Multinucleated giant cells (MGC) were assigned a role in microbial sequestration within tuberculous granulomas more than a century ago (6, 11). These cells are now believed to be a common feature of chronic infectious diseases, including tuberculosis, leprosy, and syphilis, as well as of granulomas induced by biologically inert particles, such as silica, asbestos, iron particles, and plastic beads (21). More recently, MGC have been reported to occur in the central nervous systems of AIDS patients (3). MGC derive from cells of the monocytic lineage by cell fusion (13, 16, 19) and usually develop in macrophage-rich tissues in which a persistent inflammatory agent resides (2). Fusing cells are believed to be in an activated and nonproliferative stage (1, 9, 16). Although T-cell-derived factors are considered responsible for initiation of macrophage fusion, the precise nature of these factors remains a matter of great controversy. Also, the immunological significance of MGC in antimicrobial protection has yet to be resolved.

A variety of T-cell-derived cytokines, of which interleukin-4 (IL-4) and gamma interferon (IFN-γ) are considered the most important, have been implicated in MGC formation from in vitro studies (10, 12, 22). Induction of monocyte/macrophage fusion by IL-4 alone or in combination with IL-3 was reported by some investigators (7, 10) but refuted by others (12, 14, 20). Similarly, evidence for fusogenic activities of IFN-γ has been obtained by demonstrating that monoclonal antibodies directed against this cytokine abrogate multinucleation in human peripheral blood monocytes (12), but contrasting data were obtained in microglia fusion assays (7) and also with human peripheral blood monocytes (20).

(This study is part of the Ph.D. thesis of R. Hanano.)

During our investigation of naturally acquired *Pneumocystis carinii* pneumonia in H-2I-Aβ$^{-/-}$ (Aβ$^{-/-}$) (deficient in major histocompatibility complex class II and thus devoid of conventional CD4$^+$ T cells), T-cell receptor β$^{-/-}$ (TCRβ$^{-/-}$) (deficient in TCRαβ cells), and recombinase activating gene 1$^{-/-}$ (RAG-1$^{-/-}$) (deficient in all mature T and B lymphocytes) gene disruption mutant mice, we consistently observed MGC in the lungs of all diseased mutants. To further examine this phenomenon, cytospins of bronchoalveolar lavage (BAL) cells were prepared. Mice were euthanatized by exposure to CO_2, and their lungs were exsanguinated by perfusion with sterile phosphate-buffered saline. BAL was performed as described recently (4) by using Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (PAA Labor- und Forschungsgesellschaft, Linz, Austria). Aliquots of 50 μl were used to prepare cytospins (Cytospin 3; Shandon Scientific Ltd., Runcorn, England). Giemsa (Merck, Darmstadt, Germany) staining was employed to characterize cell morphology as well as nucleated *P. carinii* organisms, encompassing intracystic bodies and trophozoites. The total numbers of parasites isolated by BAL were quantified by standard procedures (8, 17). Briefly, pulmonary cells were largely eliminated from 1-ml BAL fluid aliquots by filtration through 10-μm-pore-size membranes (Millipore, Eschborn, Germany). Triplicates of 3-μl filtrate portions were spotted onto glass slides, dried, and stained with Giemsa; *P. carinii* nuclei were determined as described (8, 17). BAL cell cultures were performed for 24 h (10^5 cells per well) in flat-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark), and supernatants were analyzed for IL-4 (monoclonal antibody BVD4-1D11 [Dianova, Hamburg, Germany] and BVD6-24G2 [DNAX, Palo Alto, Calif.]) and IFN-γ (monoclonal antibodies R4-6A2 and AN18-17.24, kindly provided by J. Langhorne after subcloning) by standard sandwich enzyme-linked immunoabsorbent assay (ELISA) procedures, as described previously (18). Cytokine concentrations were assessed with ELISA LITE software (Meddata Inc., New York, N.Y.).

Examination of cytospins revealed large numbers of pulmonary phagocytes in all diseased mutant strains (Fig. 1). Phagocytes encompassed both alveolar macrophages and granulocytes. MGC with three or more nuclei were consistently identified in BAL fluids from all three diseased mutant strains but never in heterozygous or healthy homozygous mice. Although these cells were encountered only occasionally in TCRβ$^{-/-}$ and Aβ$^{-/-}$ mutants (2 to 4 MGC per cytospin with an average of four nuclei), they occurred particularly prominently in both number (10 to 14 MGC per cytospin with an average of nine nuclei) and size (MGC from TCRβ$^{-/-}$ and Aβ$^{-/-}$ mutants attained an average diameter of 37 μm and MGC from RAG-1$^{-/-}$ mice attained an average diameter of 48 μm as measured with a micrometer scale) in morbid RAG-1$^{-/-}$ mice (Fig. 1). To our knowledge, this finding for the first time demonstrates unequivocally lymphocyte-independent MGC generation. MGC have been detected in athymic nude

* Corresponding author. Mailing address: Department of Immunology, University of Ulm, D-89081 Ulm, Germany. Phone: 0731/502-3361. Fax: 0731/502-3367. Electronic mail address: Stefan.Kaufmann@Medizin.Uni-Ulm.DE.

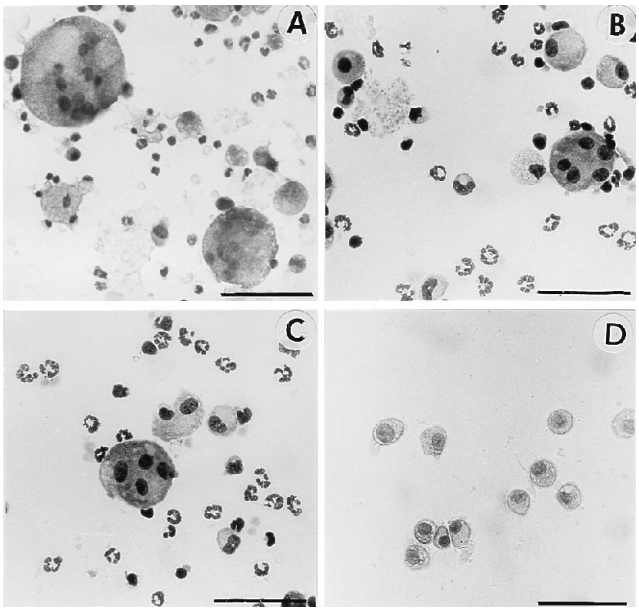

FIG. 1. Cytospins of BAL fluids. BAL fluids from morbid RAG-1$^{-/-}$ mutants (A) contained large MGC of foreign body type with abundant nuclei, while in BAL fluids from TCR$\beta^{-/-}$ (B) and A$\beta^{-/-}$ (C) mutants these cells were smaller and had fewer nuclei. Pulmonary cells isolated from healthy homozygous mutants consisted almost exclusively of alveolar macrophages (D). Giemsa staining was used. Bar, 50 μm.

mice following implantation of glass coverslips (15), but a T-cell influence cannot be ruled out because of the existence of residual T-lymphocyte populations in these animals (5).

No IL-4 was ever detected in any BAL cell cultures (Table 1). Thus, we consider it unlikely that this cytokine has an appreciable impact on multinucleation. In contrast, IFN-γ levels were elevated in all *P. carinii*-infested mutant lungs compared with their healthy counterparts (Table 1). However, the following reasons argue against a major role of IFN-γ in MGC formation. (i) Although diseased TCR$\beta^{-/-}$ and RAG-1$^{-/-}$ lungs displayed similar IFN-γ levels, MGC frequency and size differed markedly between these two mutants. (ii) Morbid TCR$\beta^{-/-}$ and A$\beta^{-/-}$ mutants exhibited similarly low frequencies of MGC, even though TCR$\beta^{-/-}$ mice expressed higher IFN-γ levels than A$\beta^{-/-}$ mutants. (iii) IFN-γ levels differed considerably between diseased animals of the same mutant strain, although MGC appeared with similar frequencies within each group.

TABLE 1. Isolated nucleated *P. carinii* organisms and cytokine production by BAL cells[a]

Mutant	Total no. of *P. carinii* organisms in BAL fluid (10^6)[b]		IFN-γ (U/ml)[c]	
	Healthy	Diseased	Healthy	Diseased
RAG-1$^{-/-}$	ND[d]	6.8 ± 0.4[e]	ND	20.0 ± 16.0
TCR$\beta^{-/-}$	ND	5.9 ± 0.5	ND	15.3 ± 14.7
A$\beta^{-/-}$	ND	5.7 ± 0.4	ND	2.9 ± 2.2

[a] IL-4 not detected.
[b] Means ± standard deviations of at least three mice per group.
[c] Means ± standard deviations of at least five mice per group (threshold values for IL-4 = 1 pg/ml; threshold values for IFN-γ = 0.1 U/ml).
[d] ND, not detectable.
[e] RAG-1$^{-/-}$ versus TCR$\beta^{-/-}$, $P = 0.05$. RAG-1$^{-/-}$ versus A$\beta^{-/-}$, $P = 0.02$ (Student's *t* test).

Our observation that A$\beta^{-/-}$ mutants exhibited MGC emphasizes that surface expression of major histocompatibility complex class II molecules by fusion partners is not a prerequisite for MGC formation, as proposed recently (14). Interestingly, BAL fluids of parasitized RAG-1$^{-/-}$ mutants liberated significantly larger numbers of nucleated *P. carinii* organisms than did BAL fluids from the other two mutant strains (Table 1). We therefore assume that differential parasite loads among these mouse strains are a driving force in multinucleation. In support of this assumption, we observed that MGC occurred almost exclusively in the form of the foreign body type, which is favored over the Langhans type in the presence of particulate matter (9).

In conclusion, the findings presented here obtained with *P. carinii* pneumonia-diseased gene disruption mutant mice (i) prove the T- and B-lymphocyte independence of MGC formation, (ii) exclude an essential role of major histocompatibility class II molecules, (iii) argue against IL-4 and are not consistent with IFN-γ as crucial mediators of multinucleation, and (iv) suggest that the parasite load induces fusogenic mechanisms in murine alveolar macrophages.

We thank S. Tonegawa, P. Mombaerts, and D. Mathis for providing breeding pairs of mutant mice. J. Langhorne, G. Adolf, E. A. Havell, S. Landolfo, and R. L. Coffman kindly provided helpful hybridomas and reagents.

Financial support was obtained from the SFB 322 "Lymphohämopoese" and the Graduiertenkolleg "Biomolekulare Medizin." Further support through the Interdisciplinary Centre of Clinical Research is acknowledged.

REFERENCES

1. **Abe, E., Y. Ishimi, H. Tanaka, C. Miyaura, H. Nagasawa, T. Hayashi, and T. Suda.** 1986. The relationship between fusion and proliferation in mouse alveolar macrophages. Endocrinology **121:**271–277.
2. **Adams, D. O.** 1976. The granulomatous inflammatory response. Am. J. Pathol. **84:**164–191.
3. **Budka, H.** 1989. Human immunodeficiency virus (HIV)-induced disease of the central nervous system: pathology and implications for pathogenesis. Acta Neuropathol. **77:**225–236.
4. **Hanano, R., and S. H. E. Kaufmann.** 1995. Nitric oxide production and mycobacterial growth inhibition by murine alveolar macrophages: the sequence of rIFN-γ stimulation and *Mycobacterium bovis* BCG infection determines macrophage activation. Immunol. Lett. **45:**23–27.
5. **Kennedy, J. D., C. W. Pierce, and J. P. Lake.** 1992. Extrathymic T cell maturation. Phenotypic analysis of T cell subsets in nude mice as a function of age. J. Immunol. **148:**1620–1629.
6. **Langhans, T.** 1968. Über Riesenzellen mit wandständigen Kernen in Tuberkeln und die fribröse Form des Tuberkels. Virchows Arch. Pathol. Anat. Physiol. Klin. Med. **49:**382–404.
7. **Lee, T. T., F. C. Martin, and J. E. Merrill.** 1993. Lymphokine induction of rat microglia multinucleated giant cell formation. Glia **8:**51–61.
8. **Limper, A. H., and W. J. Martin II.** 1990. *Pneumocystis carinii*: inhibition of lung cell growth mediated by parasite attachment. J. Clin. Invest. **85:**391–396.
9. **Mariano, M., and W. G. Spector.** 1973. The formation and properties of macrophage polycaryons (inflammatory giant cells). J. Pathol. **113:**1–19.
10. **McInnes, A., and D. M. Rennick.** 1988. Interleukin 4 induces cultured monocytes/macrophages to form giant multinucleated cells. J. Exp. Med. **167:**598–611.
11. **Metchnikoff, E.** 1988. Über die phagocytäre Rolle der Tuberkelriesenzellen. Virchows Arch. Pathol. Anat. Physiol. Klin. Med. **113:**63–94.
12. **Möst, J., H. P. Neumayer, and M. P. Dierich.** 1990. Cytokine-induced generation of multinucleated giant cells *in vitro* requires interferon-γ and expression of LFA-1. Eur. J. Immunol. **20:**1661–1667.
13. **Murch, A. R., M. D. Grounds, C. A. Marshall, and J. M. Papadimitriou.** 1982. Direct evidence that inflammatory multinucleate giant cells form by fusion. J. Pathol. **137:**177–180.
14. **Orentas, R. J., L. Reinlib, and J. E. K. Hildreth.** 1992. Anti-class II MHC antibody induces multinucleated giant cell formation from peripheral blood monocytes. J. Leukocyte Biol. **51:**199–209.
15. **Papadimitriou, J. M.** 1976. The influence of the thymus on multinucleate giant cell formation. J. Pathol. **118:**153–156.

16. **Postlethwaite, A. E., B. K. Jackson, E. H. Beachey, and A. H. Kang.** 1982. Formation of multinucleated giant cells from human monocyte precursors. Mediation by a soluble protein from antigen- and mitogen-stimulated lymphocytes. J. Exp. Med. **155:**168–178.
17. **Reddy, L. V., C. Zammit, P. Schumann, and L. R. Crane.** 1992. Detection of *Pneumocystis carinii* in a rat model of infection by polymerase chain reaction. Mol. Cell. Probes **6:**137–143.
18. **Slade, S. J., and J. Langhorne.** 1989. Production of interferon-gamma during infection of mice with *Plasmodium chabaudi chabaudi*. Immunobiology **179:** 353–365.
19. **Sone, S., C. Bucana, L. C. Hoyer, and I. J. Fidler.** 1981. Kinetics and ultrastructural studies of the induction of rat alveolar macrophage fusion by mediators released from mitogen-stimulated lymphocytes. Am. J. Pathol. **103:**234–246.
20. **Takashima, T., K. Ohnishi, I. Tsujuguchi, and S. Kishimoto.** 1993. Differential regulation of formation of multinucleated giant cells from concanavalin A-stimulated human blood monocytes by IFN-gamma and IL-4. J. Immunol. **150:**3002–3010.
21. **Turk, J. L.** 1980. The role of delayed hypersensitivity in granuloma formation. Res. Monogr. Immunol. **1:**275–283.
22. **Weinberg, J. B., M. M. Hobbs, and M. A. Misukonis.** 1984. Recombinant human gamma-interferon induces human monocyte polykaryon formation. Proc. Natl. Acad. Sci. USA **81:**4554–4557.

Editor: V. A. Fischetti